Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Medicine and Health Sciences
Department of Circulation and Medical Imaging

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High-intensity exercise training exacerbates amyloid pathology and impairs memory function in a transgenic rat model of Alzheimer's disease

Master's thesis in Exercise Physiology
Supervisor: Ulrik Wisløff
June 2020

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Kunnskap for en bedre verden

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Abstract

Background:

The rising prevalence of Alzheimer's disease calls for effective strategies of prevention and treatment. Observational human studies have shown higher levels of physical activity and higher cardiorespiratory fitness are associated with reduced risk of Alzheimer's disease and dementia, and studies in transgenic animal models of Alzheimer's disease have further suggested exercise diminishes progression of amyloid pathology and cognitive decline at least when initiated before the onset of cognitive impairment and amyloid plaque pathology. However, later interventions have been less studied and have shown more limited effects.

Purpose:

The aim of this study was to provide first insight into the effects of chronic exercise on cognitive function and amyloid pathology when initiated pre- and post-cognitive impairment in the transgenic McGill-R-Thy1-APP rat model of Alzheimer's disease.

Methods:

Seven 2-month-old and seven 5-month-old McGill-R-Thy1-APP rats were subjected to one hour of high-intensity interval training five times a week for four weeks. The effects of the exercise intervention in comparison to remaining sedentary were assessed on amyloid pathology, recognition memory, and fear-associative learning and memory.

Results:

Four weeks of high-intensity interval training improved physical capacity regardless of the age of the rats but showed limited effects on cognitive function. Results from novel object recognition testing and fear conditioning testing showed non-significant tendency of exercise to improve recognition memory but not auditory fear conditioning, contextual recall, or cued recall in the younger rats. In the older rats, exercise impaired recognition memory ($P=0.0253$) and produced almost significant impairment of contextual memory ($P=0.0519$). Exercise did not seem to significantly affect intracellular accumulation of amyloid-β in the younger rats but exacerbated amyloid-β accumulation and plaque deposition in the older rats when compared to sedentary rats.

Discussion:

The effects of high-intensity exercise training on the progression of amyloid pathology and cognitive decline may be greatly different depending on the timing of exercise. Earlier interventions may have the best potential to attenuate the progression of Alzheimer's disease and later interventions may be detrimental.

Sammendrag

Bakgrunn:

Den økende prevalensen av Alzheimers sykdom gir behov for effektive strategier for forebygging og behandling. Observasjonsstudier på mennesker har vist at økt nivåer av fysisk aktivitet og forbedret kondisjon er assosiert med redusert risiko for Alzheimers sykdom og demens. Videre tyder studier på transgene dyremodeller for Alzheimers sykdom på at trening reduserer utviklingen av amyloid patologi og svekkelse av kognitiv funksjon dersom treningen initieres før kognitiv svikt og amyloid plakkpatologi inntreffer. Det er derimot gjort få intervensjonsstudier, og de som er gjort har vist begrensede effekter, flere med svakheter i valg av metode.

Formål:

Målet med denne studien er å gi innsikt i effektene av langvarig trening på kognitiv funksjon og amyloid plakkpatologi ved å gjennomføre et høyintensiv treningsprogram i en transgen rottemodell for Alzheimers sykdom (McGill-R-Thy1-APP), før og etter etablert reduksjon i kognitiv funksjon.

Metoder:

Syv 2 måneder gamle og syv 5 måneder gamle McGill-R-Thy1-APP-rotter gjennomgikk én times høyintensiv intervalltrening fem ganger i uken i fire uker. Effektene av treningsintervensjonen i Alzheimers sykdom rottene sammenlignet med Alzheimers sykdom kontrollrotter som ikke trente ble vurdert på grunnlag av amyloid patologi, gjenkjennelsesminne og fryktassosiert læring og hukommelse.

Resultater:

Fire uker med høyintensiv intervalltrening forbedret fysisk kapasitet uavhengig av rottenes alder, men hadde begrenset effekt på kognitiv funksjon. Resultater fra hukommelsestester (Novel Object Recognition og Fear Conditioning) viste en ikke-signifikant tendens til at trening forbedret gjenkjennelsesminnet, men ikke de andre typene hukommelse som ble testet (Auditory fear conditioning, Contextual recall eller Cued Recall) hos de yngre rottene. Hos de eldre rottene forverret trening gjenkjennelsesminnet ($P=0.0253$) og ga nesten signifikant svekkelse av kontekstuelt minne ($P=0.0519$). Trening så ikke ut til å påvirke intracellulær akkumulering av amyloid-ß i de yngre rottene, men forverret amyloid-β-akkumulering og plakkavsetning hos de eldre rottene, sammenlignet med kontrollrotter som ikke trente.

Diskusjon:

Effekten av høyintensiv trening på utviklingen av amyloid plakkpatologi og kognitiv svekkelse i denne rottemodellen varierer veldig avhengig av når i forløpet av sykdommen treningen gjennomføres. Tidlige intervensjoner virker å ha størst potensial til å hemme progresjon av sykdommen, mens senere intervensjoner virker og til og med være skadelige.

Preface

The present Master's thesis is the final part of an international master's degree programme in Exercise Physiology. It was conducted at the Department of Circulation and Medical imaging, Faculty of Medicine and Health Sciences at the Norwegian University of Science and Technology under supervision of Ulrik Wisløff, Atefe Rafiee Tari, and Nathan Scrimgeour. The primary aim of the thesis was to investigate the effects of chronic exercise on amyloid pathology and cognitive function in the McGill-R-Thy1-APP transgenic rat model of Alzheimer's disease when applied at two different stages of disease progression.

Thank you Ulrik and Atefe for all your support, particularly during the last weeks of my studies. Thank you Nathan for your help with technical issues and exercise testing of animals, guidance with the writing process, and overall support throughout the process. Thank you Ragnhild Røsbjørgen for your cheerful presence and all the guidance and help with the use of different laboratories, equipment, and methods. A special thank you to Cecilie Skarstad Norevik. I really appreciate your contribution, guidance, and support with the behavioural testing, injections, laboratory booking, and immunostaining. To all of you I worked with, thank you for always being ready to help whatever the situation.

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List of Abbreviations

Aβ	Amyloid-β
AD	Alzheimer's disease
ANOVA	Analysis of variance
APOE	Apolipoprotein E
APP	Amyloid precursor protein
BDNF	Brain-derived neurotrophic factor
BrdU	5-bromo-2'-deoxyuridine
CRF	Cardiorespiratory fitness
DAB	3,3'-Diaminobenzidine
DI	Discrimination index
FC	Fear conditioning
IGF1	Insulin-like growth factor 1
MCI	Mild cognitive impairment
NFT	Neurofibrillary tangle
NORT	Novel object recognition test
PA	Physical activity
PB	Phosphate buffer
SD	Standard deviation
SEM	Standard error of the mean
TBS-Tx	Triton X-100 in tris-buffered saline
VEGF	Vascular endothelial growth factor
VO_2 max	Maximal oxygen consumption
VO_2 rest	Resting oxygen consumption

1 Introduction

1.1 Alzheimer's disease and dementia

Dementia is an overall term used to describe a condition of impaired cognition and functional abilities which may be caused by various neurological disorders [1]. By far, the most common cause of dementia is Alzheimer's disease (AD), which accounts for up to 70% of all dementia cases [1]. AD is a slowly progressive neurodegenerative disease in which the pathological changes in the brain may begin and progress even decades before the onset of symptoms and diagnosis of dementia [2-4]. The clinical progression of AD may be viewed as a continuum of three phases graded by the level of cognitive impairment [5-7]. The first is a preclinical phase, where clinical symptoms are not yet evident or they are very mild despite progressive pathological changes in the brain [5]. The second phase is mild cognitive impairment (MCI), where cognitive symptoms such as memory deficit are evident, but functional independence is not yet compromised [6]. The last stage of the AD continuum is clinically diagnosable dementia, where the cognitive, behavioural and functional symptoms interfere with the ability of an individual to perform activities of daily living independently [7]. The total duration of AD has been estimated to be 24 to 15 years for a 60- to 80-year-old individual with asymptomatic AD pathology, the estimate respectively changing with age [4]. In these estimates, the dementia phase would last 7 to 4 years before the inevitable death [4].

1.1.1 Increasing burden of dementia calls for action

Age is the greatest risk factor of AD and dementia overall, and as the human life expectancy keeps increasing, the number of people with dementia is expected to increase as well [1, 8]. With nearly 10 million new diagnoses made every year, the number of people with dementia has been estimated to triple from the current 50 million to 152 million patients worldwide in just 30 years [8, 9]. Concomitantly, the total annual costs of dementia have been estimated to at least double from the current 1 trillion US dollars by 2030 [9, 10], and reach over 9 trillion US dollars by 2050 [10]. Up to 85% of the total costs are related to family and social care [11]. Thus, the increasing dementia burden presents a worldwide challenge that affects not only the people with dementia and their families and friends, but also the society through a rising need for health and social care [1, 11].

1.2 AD pathology

The AD pathogenesis is very complex and the full picture of processes causing and driving the neurodegeneration, or the loss of neurons and synapses, and subsequent decline in cognitive and physical function is unclear [12]. Nevertheless, accumulation of amyloid-β (Aβ) peptides and tau protein and their subsequent aggregation into amyloid plaques and neurofibrillary tangles (NFTs), respectively, are the two hallmarks distinguishing AD from other causes of dementia, and well-established to represent central processes in AD pathogenesis [12, 13]. An important aspect of AD pathology is also neuroinflammation, which is thought to play both protective and exacerbating effects in AD pathology depending on the stage of the disease [14].

1.2.1 Amyloid pathology

1.2.1.1 Amyloid-β accumulates early in AD

Accumulation and aggregation of amyloid-β (Aβ) peptides into extracellular plaques in the brain is a major hallmark of the AD pathology [12, 13]. Intracellular accumulation of Aβ is thought to be a key early event in AD pathogenesis, and the amyloid cascade hypothesis proposes Aβ deposition as an initiating factor that triggers subsequent pathological changes such as the formation of NFTs and loss of synapses and neurons [15, 16]. This hypothesis was mainly based on the findings from studies of rare genetically-determined AD showing genetic mutations related to increased Aβ production [15]. Supporting this proposal, several studies have shown that the accumulation of Aβ may begin decades before the onset of symptoms [4, 17, 18], and may be the first biomarker of AD to show abnormality [13, 19]. Furthermore, several longitudinal studies have reported faster cognitive decline and greater risk of progression to MCI and dementia in asymptomatic individuals with elevated Aβ burden detected by neuroimaging methods or indirectly through cerebrospinal fluid biomarkers [20-26].

Accumulation of Aβ begins in the precuneus and posterior cingulate regions of the brain [19, 27, 28]. These anatomically connected areas contribute to a functionally connected default mode network and increasing Aβ burden seems to be accompanied by decreases in the functional connectivity within this network, which could indicate increasing neuronal dysfunction [19, 29]. Accordingly, impaired glucose metabolism and synaptic dysfunction have been reported accompanying early Aβ deposition in preclinical individuals [20, 30, 31], and brain regions functionally connected to the regions with Aβ deposition may be particularly vulnerable to hypometabolism [31]. Furthermore, progressive decreases in functional connectivity within several brain networks correlated with cognitive decline in subjects from preclinical stage to AD dementia [32]. However, even though Aβ can exert toxic effects on synaptic and neuronal function [33], the degree of amyloid pathology alone does not correlate well with the degree of cognitive impairment [34]. Instead, these findings may partly reflect progression of tau pathology [32]. Indeed, accumulation of Aβ seems to precede, and may be essential to promote, tau pathology which correlates better with cognitive decline [12, 34]. Overall, all these findings support the view that accumulation of Aβ does play an important role early in AD [15, 16].

1.2.1.2 Enzymatic processing of an amyloid precursor protein generates Aβ

The accumulation of Aβ supposedly results from a disrupted balance between Aβ production and clearance [35]. Aβ peptides are produced through a proteolytic cleavage of an amyloid precursor protein (APP) by two secretase enzymes [36, 37]. The APP is a transmembrane protein that is synthesized in the endoplasmic reticulum and post-translationally modified into its mature form before its integration into the cell membrane [36, 37]. It is found not only in neurons, microglia and astrocytes in the brain, but also in several peripheral tissues [38]. Its physiological roles are not clear [37].

There are two distinct pathways of APP processing called the amyloidogenic and non-amyloidogenic pathways, and the post-translational modifications of the APP may play a role determining which pathway it is processed through [37]. The amyloidogenic pathway results in the formation of Aβ peptides [36, 37]. In this pathway, the APP may be first endocytosed from the cell membrane into early endosomes, before its cleavage into a soluble APP fragment and a C-terminal fragment by β-secretase enzyme [37]. Subsequently, the C-terminal fragment is further cleaved by γ-secretase complex, which

results in Aβ peptides and other C-terminal fragments of different sizes depending on the specific cleavage site [36, 37]. Eventually, predominantly two isoforms of Aβ, the 40- and 42-amino-acid long Aβ40 and Aβ42, are generated and may be secreted into the extracellular space where they may accumulate and aggregate [36, 37].

1.2.1.3 Specific forms of Aβ may be particularly neurotoxic

The Aβ peptides can form aggregates of different sizes and composition, including oligomers, protofibrils, fibrils, and plaques [33, 39]. While Aβ40 may mainly remain in monomeric form or assemble small oligomers of two to four peptides, Aβ42 seems more predisposed to aggregation into larger oligomers of six or ten peptides [39]. The oligomers can further aggregate into shorter protofibrils or longer fibrils that eventually build up into the plaques [33].

The oligomeric forms of Aβ may be particularly neurotoxic [33]. Studies where either synthetic Aβ oligomers or those extracted from human AD brain tissue have been injected in the brain of wild-type animals have demonstrated detrimental effects of Aβ oligomers on memory and learning [40]. In turn, antibodies specifically targeting oligomeric Aβ species have shown ameliorating effects on cognitive performance in transgenic animal models of AD [40]. At a cellular level, oligomeric Aβ species may promote tau pathology [41, 42], calcium dyshomeostasis [42, 43], and neuroinflammation [44, 45], as well as impair synaptic integrity and plasticity [45, 46]. Similar findings were recently made in a novel transgenic mouse model of AD that expresses a fusion protein of green fluorescent protein and human Aβ42 in neurons of predominantly the hippocampus and cerebral cortex [47]. These mice showed intracellular accumulation of Aβ oligomers and deficits in synaptic morphology and plasticity with functional impairment of recognition memory [47].

1.2.1.4 Aβ can be cleared by various mechanisms

In the majority of AD cases, accumulation of Aβ may be predominantly driven by impaired clearance of Aβ instead of increased production [48]. In healthy brain, Aβ can be cleared in several ways [35]. For example, glial cells such as microglia and astrocytes can phagocytose extracellular Aβ and degrade it in lysosomes, while in neurons Aβ can be degraded through lysosomal pathways, a ubiquitin-proteasome pathway, and soluble degrading enzymes [35]. Extracellular Aβ can also be transported into the blood, either across the blood-brain barrier or via cerebrospinal fluid through various pathways, where specific soluble enzymes may degrade them or they can be phagocytosed and degraded by blood cells [35]. Some of the Aβ in blood can be transported into peripheral tissues where they can be degraded by macrophages or excreted in bile and urine by the liver and kidneys [35].

1.2.2 Tau pathology

Tau is a soluble protein associated with axonal microtubules that can phosphorylate and aggregate into intracellular NFTs [49]. Hyperphosphorylation of tau may make it more prone to aggregation into NFTs and disassociation from microtubules, which in turn may cause synaptic dysfunction [49, 50]. The degree of NFT pathology also correlates with neuronal loss and cognitive function in preclinical and symptomatic AD [51-55]. In line with the amyloid cascade hypothesis, human studies suggest accumulation of Aβ to precede tau pathology in preclinical AD [29, 56]. Furthermore, studies *in vitro* and *in vivo* in animals have shown that Aβ, both as oligomers and plaques, may promote tau aggregation and formation of NFTs [41, 57, 58].

1.2.3 Neuroinflammation

In healthy brain conditions and during very early AD, glial cells microglia and astrocytes are important in maintaining synaptic function and overall neuronal homeostasis [59]. For example, these cells are thought to mediate important neuroprotective functions such as anti-inflammatory signaling and clearance of Aβ, phosphorylated tau, and cellular debris [35, 60, 61]. However, accumulation of soluble Aβ peptides and oligomers within neurons early in AD pathogenesis may trigger neuronal release of proinflammatory signaling molecules and aberrant activation of microglia [44, 62]. Subsequent alterations in microglia may in turn result in chronically increased production of proinflammatory mediators and impaired ability to phagocytose and degrade Aβ, hence further exacerbating neuroinflammation and Aβ accumulation [60, 61]. Furthermore, the microglial activation and release of proinflammatory cytokines may also drastically change astrocytic functions from neuroprotective to neurotoxic [63], promote tau hyperphosphorylation [64], and cause mitochondrial dysfunction [65], which may further promote inflammatory responses and neurodegeneration.

1.3 Risk factors for AD

1.3.1 Age and sex

The greatest risk factor for AD is age, and in up to 95% of all AD cases the stage of dementia is not reached before the age of 65 years [66]. These cases are commonly referred to as late-onset AD, while the rest of the cases, where dementia symptoms occur before the age of 65 years, are called early-onset AD [66]. In terms of pathological changes in the brain, the late-onset and early-onset AD are much like each other [67].

Biological sex of an individual also affects the risk of AD such that the risk is greater in women than in men [68, 69]. For instance, a population-based cohort study following 7901 individuals showed that the risk of developing AD after the age of 45 was 20% for women and 10% for men [69].

1.3.2 Genetic factors in early-onset AD

Almost all of the early-onset cases are attributable to genetic factors, mostly to autosomal recessive mutations [70]. Only about every tenth of the early-onset AD cases are caused by autosomal dominant mutations in one of the three genes known as *APP*, *PSEN1*, and *PSEN2* [67, 70]. The *APP* gene encodes the APP and the *PSEN1* and *PSEN2* genes encode presenilin proteins 1 and 2, which are constituents of the γ-secretase complex [67]. In addition to the genetic mutations in these genes, another significant risk factor for AD is an extra copy of the *APP* gene as commonly seen in Down syndrome due to an extra copy of chromosome 21 where the *APP* gene locates [71]. Notably, all the three genes are involved in the amyloidogenic pathway and the mutations causing AD are thought to increase production of either Aβ overall, or specifically the Aβ42, hence providing support to the amyloid cascade hypothesis and a key early role of Aβ in AD overall [15, 16, 67]. Furthermore, a rare protective mutation in the *APP* gene has also been identified and is associated with reduced Aβ production [71].

1.3.2.1 The Swedish and Indiana mutations of the APP gene

Several different pathologic mutations have been identified for each of the aforementioned genes [67], but only two *APP* mutations are presented here in more detail because of their expression in the McGill-R-Thy1-APP rat model of AD used in this study. The Swedish mutation (KM670/671NL) named after its discovery in two Swedish

families is a double point mutation located next to the β-secretase cleaving site in the exon 16 of the *APP* [72]. This mutation causes the substitution of amino acids lysine (K, amino acid 670) and methionine (M, 671) by asparagine (N) and leucine (L), and due to consequent changes in the processing of the APP, results in increased levels of both Aβ40 and Aβ42 [71-74]. The so-called Indiana mutation (V717F) is a missense point mutation in the Aβ-coding exon 17 of the *APP* gene [75]. It causes the substitution of valine (V, 717) by phenylalanine (F), and similar to other *APP* mutations near the γ-secretase cleavage site, it specifically increases the production of Aβ42 [71, 76].

1.3.3 Genetic factors in late-onset AD

While only playing a minor role, several genetic risk factors have been identified for late-onset AD as well [77]. Particularly the different variants, or alleles, of the *APOE* gene are known to affect the risk of developing AD [78-80]. The apolipoprotein E encoded by this gene is an important mediator in both peripheral and central lipid transportation and metabolism [81]. There are three major alleles of the *APOE* gene, namely ε2, ε3, and ε4, which are differently associated with the risk of AD [81]. Compared to people with the most common *APOE* ε3ε3-genotype, individuals with one ε4 allele have approximately 3 to 4 times higher risk of developing AD dementia, depending on the other allele, while having two ε4 alleles increases the risk to 9- or up to 15-fold [78-80]. In contrast, having a genotype with at least one ε2 allele decreases the risk of AD relative to the *APOE* ε3ε3-genotype [78-80].

Several human imaging studies have shown the genotypes with ε4 allele to be associated with greater Aβ burden [82-84], which may result from less efficient Aβ clearance mechanisms that have been linked to the *APOE* ε4-encoded isoform of apolipoprotein E [81, 85]. Thus, an increased predisposition to exacerbated amyloid pathology is thought to at least partly explain why the ε4 allele associates with an increased risk of AD [81]. However, the *APOE* ε4 has also been linked to several other pathological findings in AD, such as tau pathology, neuroinflammation, and impaired synaptic function [77, 81].

1.4 Modifiable risk factors

As there is still no cure for AD despite years of huge efforts in drug development, and all promising treatments have failed to show disease-modifying effects in mild-to-moderate-stage patients, it seems probable that after the disease has progressed to a clinically diagnosable stage, it cannot be stopped or reversed [12, 86]. Due to the difficulties in the development of pharmaceutical therapies, alternative ways to meet the challenge of increasing prevalence of AD are needed [1]. It is thought that earlier approaches may be particularly effective, and hence, establishing early preventive strategies targeting modifiable AD risk factors or the AD pathology already in the preclinical stage of AD is highly relevant [1, 2, 11]. Importantly, preventing or delaying the progression of early AD to dementia could not only remarkably reduce the overall prevalence and costs of dementia, but also improve the patients' quality of life through reduced length of functional dependency and compromised cognition [1, 11].

An estimated 30% of all AD cases may be preventable through potentially modifiable risk factors of AD [11, 87]. Remarkably, it has been estimated that the total prevalence of AD in 2050 could be reduced by 8-15% if the prevalence of the following seven risk factors was reduced by 10-20% per decade: physical inactivity, low education, depression, diabetes, hypertension, obesity, and smoking [87].

1.4.1 Physical activity, cardiorespiratory fitness, and risk of AD

Physical inactivity has been estimated to contribute to almost 18% of all AD cases worldwide and to 31 and 32.5% of AD cases in Europe and the United States of America, respectively [87]. There is considerable evidence that greater physical activity (PA) at midlife decreases the risk of developing AD or dementia of any cause [88-95], and a recent meta-analysis of prospective cohort studies suggested such an inverse dose-response relationship between leisure-time PA and the risk of AD where each additional 10 metabolic equivalent task-hours of PA per week decreased the risk of AD by 13 % compared to not being physically active [96].

While PA as a term comprises any daily activities with skeletal muscle work and increased energy expenditure, exercise describes more specifically activity that aims to maintain or improve physical fitness [97]. One measure of physical fitness is cardiorespiratory fitness (CRF), which refers to the capacity of the cardiovascular and respiratory systems to provide oxygen to working skeletal muscles and the ability of the muscles to utilize the oxygen [98]. CRF can be improved over time with regular exercise of high enough volume and intensity [99], and higher CRF in midlife has been shown to be associated with reduced risk of AD and dementia of any cause [100-102]. A recent prospective cohort study by Tari e*t al.* also demonstrated the relevance of improving CRF over time for reduced risk of dementia and dementia-related mortality [103]. In this study, those individuals whose estimated CRF had increased during a median follow-up time of 7.6 years had 48% reduced risk of incident dementia compared to those whose CRF remained low throughout the follow-up period [103]. Overall, these findings from observational studies suggest that PA and exercise have beneficial effects on the brain that provide protection against AD and dementia.

1.5 Aerobic exercise, cognitive function and AD

1.5.1 Exercise and cognition

Consistent with the evidence that PA and CRF associate with the risk of developing dementia, greater levels of PA and higher CRF have been found associated with better preserved cognitive function over time [104-108]. A meta-analysis of 15 longitudinal cohort studies in cognitively healthy individuals found that when compared to sedentary individuals, those reporting either low-to-moderate or high levels of PA had approximately 35 and 38% reduced risk of future cognitive decline, respectively [104]. Higher CRF in 349 healthy older adults was associated with better executive functions and global cognition 6 years later [106], and in another cohort of 421 older adults with an increased risk of dementia, higher CRF was associated with improved executive function, processing speed and overall cognitive function two years later [108]. Also, in a larger cohort of adults aged 19 to 95 years, higher CRF was associated with less memory decline over a follow up time of up to 18 years [107].

Providing further evidence of a causal relationship between exercise and cognitive function, randomized controlled trials (RCTs) have shown positive effects of aerobic exercise interventions on cognitive function both in healthy older adults and older adults with cognitive impairment [109-112]. A meta-analysis of 23 RCTs cognitively healthy individuals found positive effects of exercise on executive function and memory [111]. A meta-analysis of 11 RCTs assessing the effects of aerobic exercise on cognition in individuals with mild cognitive impairment found significant beneficial effects of exercise on global cognitive function and memory [112]. In line with these results, a recent meta-

analysis of 36 RCTs also concluded that there were beneficial effects of aerobic exercise and several other modes of physical exercise on cognitive function in older adults independent of cognitive status [109]. Similarly, other recent meta-analyses have found positive effects of exercise on cognitive function in dementia patients [113], as well as more specifically in AD patients [113-115]. One meta-analysis of 18 RCTs found positive effects of exercise on cognitive function both in AD and non-AD dementia patients, but positive effects were only found from exercise interventions including aerobic exercise and not from those with only non-aerobic exercise [113]. Interestingly, it was also found that interventions with low frequency of exercise had greater effect on cognitive function than those with high frequency [113], and similar findings were made in a meta-analysis of 13 RCTs with only AD patients [115]. It is still notable that most of the studies in patients have included rather small number of subjects and several recent, larger RCTs found no beneficial effects of exercise on cognition [116-120].

While aerobic exercise seems to have potential to improve cognitive functions and slow down cognitive decline with aging, high enough volume and intensity of exercise may be required for such benefit to be realized [105, 109, 121-123]. A systematic review of 98 RCTs assessing the effects of exercise on cognition in healthy or cognitively impaired individuals concluded that in order to observe improvements in cognitive function from an exercise intervention, a total of at least 52 hours of exercise may needed [121]. Also, low-intensity exercise may not be enough to improve cognition [105, 109], and some studies have suggested greater effects with greater intensities [122, 123]. For instance, a recent RCT in 64 older adults showed superior effect from 12 weeks of high-intensity interval training on memory compared to moderate-intensity continuous training or stretching [123]. Interestingly, some studies have found correlation between changes in cognitive test performance and changes in CRF [108, 123-125], which, in line with the recent evidence of improved CRF predicting reduced risk of dementia [103], suggests that exercise prescription aiming at improving CRF may be beneficial.

1.5.2 Exercise and amyloid pathology

Some observational evidence from human studies suggests that in addition to improving cognitive function, PA may reduce the risk of dementia due to AD through reduced amyloid pathology [126-130]. Studies in cognitively healthy older adults found lower Aβ burden in those individuals who reported highest levels of PA compared to those reporting least PA [126, 127]. Similarly, in cognitively normal older adults, greater volumes of objectively measured moderate-intensity PA were found to be associated with higher levels of Aβ42 in the cerebrospinal fluid, indicative of less amyloid plaque burden [129]. Also, within a cohort of 40 to 65-year-old cognitively healthy adults, self-reported physical activity reduced the effect of increasing age on Aβ burden [128]. Furthermore, a recent longitudinal study in older adults reported an association between higher objectively measured levels of PA at baseline and slower Aβ-related cognitive decline within a median follow-up time of 6 years [130]. However, interventional studies with exercise interventions of 4 to 6 months in AD patients have not detected significant changes in soluble plasma or cerebrospinal fluid Aβ levels [131, 132], or cortical Aβ plaque burden [133].

Overall, while the above discussed evidence suggests that PA may improve cognitive function and reduce amyloid pathology and the risk of AD in cognitively healthy individuals, it is less well-known whether PA can enhance cognition or modify amyloid pathology in individuals with symptomatic AD. Furthermore, while some evidence suggests that aerobic exercise of high enough volume and intensity may be particularly

beneficial for cognitive function, the optimal volume, intensity and timing of exercise for improving cognitive function or reducing brain amyloid burden are not known. Animal models of AD can be particularly helpful when studying these aspects further.

1.5.3 Transgenic rodent models of AD and exercise

Several transgenic animal models have been created to express various aspects of AD pathology, especially the amyloid deposition, and are widely used in AD research [134]. Usually the transgenic models of AD express genetic mutations underlying human autosomal dominant AD, such as mutations in the *APP* or *PSEN1* genes that cause increased Aβ production and subsequent plaque deposition [134]. Some transgenic models have also been generated that express tau pathology, or both amyloid and tau pathologies [134]. Due to easier production of transgenic mice than rats [135], most of the present AD models are mice [134]. However, whereas the brain anatomy is identical in mice and rats, only rats express six isoforms of the tau protein similar to humans, which may make them more suitable for studies of AD pathogenesis [135].

1.5.3.1 Transgenic McGill-R-Thy1-APP rat model of AD

The McGill-R-Thy1-APP rat model of AD expresses the human Aβ precursor protein gene (hAPP751) with the double Swedish (K670N and M671L) and Indiana (V717F) mutations under the transcriptional control of the murine Thy1.2 promoter [136]. Rats homozygous for this gene show both intracellular Aβ accumulation and extracellular plaque deposition, thus providing a good model of AD-like amyloid pathology [136-138]. Intracellular Aβ appears in the cortical and hippocampal neurons as early as the age of one week [136]. Specifically, the 42-amino-acid long isoform of Aβ (Aβ42) and Aβ oligomerization show progressive age-dependent accumulation [137, 139], which is in line with findings from studies in the human brain [140, 141]. The first amyloid plaques become observable in the subiculum of the hippocampus at the age of 6 to 9 months [136, 138], and subsequent plaque deposition covers the hippocampal region beginning in the CA1 and spreading gradually to the CA2, CA3 and dentate gyrus [138]. First cortical plaques appear at the age of 9 to 12 months [138], while at the age of 18-21 months, plaques appear almost throughout the brain with the greatest load present in the hippocampus and the entorhinal and parietal cortices [136, 142].

Cognitive deficits are displayed at the age of 3 months, thus preceding the occurrence of earliest amyloid plaques and suggesting the contribution of intracellular Aβ to cognitive impairment [136, 137]. More specifically, impaired test performance in Morris water maze, fear conditioning (FC) test, novel object recognition test (NORT), and novel object location test have been reported, indicative of deficits in forms of learning and memory [136, 137]. These studies also showed that the severity of cognitive impairment may correlate with the levels of soluble Aβ42 and Aβ trimers [136, 137], which is in line with other literature suggesting neurotoxic effects of soluble Aβ42 and Aβ oligomers [33]. Accompanying the AD-like amyloid pathology, the McGill-R-Thy1-APP rats also show progressive neuroinflammation [62]. Increases in inflammatory markers were found preceding the formation of amyloid plaques in the hippocampal and cortical areas [62]. These areas of early intracellular Aβ accumulation also showed increased microglial and astrocytic cell densities [62], supporting findings of other studies suggesting intracellular Aβ, particularly oligomeric forms of Aβ, as a key driver of neuroinflammation [44]. Early deficits in BDNF expression and loss of presynaptic boutons [142], as well as impaired synaptic plasticity have also been shown in rats of this transgenic line [143, 144]. Overall, even though it does not develop NFTs, the McGill-R-Thy1-APP rats express a

wide range of AD-like pathology and cognitive decline and can hence be considered a good model for studies of early AD pathology [136]. Only the effects of acute exercise on recognition memory in NORT have been investigated in this rat model before, and no beneficial effects were found [145].

1.5.3.2 Use of transgenic models of AD in exercise studies

Studying the effects of exercise on cognition and AD pathology in animals has several advantages [135, 146-148]. For example, variation in factors such as living conditions, diet, social activity, and environmental cognitive stimulation within a study population may affect the results of human studies but can be unified to a great extent in animals [146]. Similarly, the volume, intensity and mode of PA and exercise can be better controlled in animals to provide information about the optimal prescription of exercise for improved cognition, reduced pathology, and brain health overall [147]. Importantly, anatomy of the brain in humans and rodents is similar [135], and studies in different transgenic models of AD can provide unique information about the mechanisms underlying the effects of exercise on specified aspects of AD pathology and brain function [148]. Also, in AD models with an established time-course of disease progression, the effects of exercise can be assessed at different stages of pathology and cognitive impairment [148].

In animal studies, exercise is generally applied as either voluntary or forced exercise, practically meaning that the animals are either given free access to a running wheel *ad libitum* or for a specific duration, or they are exercised on a treadmill using mild electric shocks or gentle touch for encouragement [146, 149]. Both types of exercise have some strengths and weaknesses [149]. In voluntary exercise without external encouragement, the urge to exercise is natural and the exercise is not likely to induce adverse effects [149, 150]. However, when voluntary, the intensity and volume of exercise may not reach high enough level to produce beneficial effects on the brain, similar to low intensity and level of PA in humans [105, 109, 149]. In contrast, with treadmill exercise the parameters of exercise can be adjusted to desired level, but when the motivation to run is not purely inherent, the exercise may also induce stress and anxiety in the animals [149, 150].

1.5.4 Exercise and amyloid pathology in transgenic AD models

Several studies in transgenic AD mice have reported reduced levels of hippocampal and/or cortical Aβ40 and Aβ42 [151-153], amyloid plaques [154-156], or both soluble Aβ and plaque load with 3 to 12 months of treadmill exercise training when initiated before onset of cognitive impairment and plaque pathology [154, 157-162]. Similarly, in transgenic TgF344 rats exhibiting amyloid pathology from the age of six months, 8 months of treadmill exercise training initiated at the age of two months significantly reduced cortical and hippocampal amyloid plaque number when compared with remaining sedentary [163]. However, some studies have not found differences in soluble Aβ levels [164], or plaque load between sedentary and exercised mice regardless of early implementation of exercise [165]. It may be that the slightly lower running speed used in these studies compared to others may have been too low to significantly modify amyloid metabolism [164, 165].

Emphasizing the significance of exercise intensity, a study in Tg2576 mice which express progressive amyloid pathology with first plaques emerging after the age of 9 months found that one hour of high-intensity exercise 5 times a week from the age of 3 months to 6 months resulted in significantly lower levels of Aβ40 in the cortex and Aβ42 in both

the cortex and the hippocampus when compared to an equal volume of low-intensity exercise [152]. Similarly, low-intensity exercise significantly reduced these Aβ levels compared to remaining sedentary, hence suggesting an intensity-dependent effect of exercise on soluble Aβ [152]. A recent study with a longer, 12-month intervention but otherwise the same study setting, extended these findings by showing that the intensity of exercise training also similarly affected long-term amyloid deposition, determining the level of cortical and hippocampal amyloid plaque load in the aged mice [154]. Another recent study in Tg2576 mice where groups of older mice were given different duration access to running wheels for 4 months also found that when the running patterns of the mice were assessed, higher speed and density (wheel rotations per hour), but not daily volume of exercise, were associated with lower Aβ42 levels [166].

A few studies have also included groups of older mice with progressed pathology and cognitive impairment in addition to young mice to investigate both the preventive and therapeutic effects of treadmill exercise on amyloid pathology [158, 164, 165]. In the study by Cho *et al.*, 3xTg mice that express human mutations in the *APP* and *PSEN1* genes along with a mutation causing tau pathology performed treadmill exercise for a total of 30 minutes a day, five times a week, for a total of 5 months [164]. This exercise intervention was found to reduce hippocampal and cortical Aβ42 but not Aβ40 in the old mice compared to remaining sedentary [164]. The young exercised and sedentary mice had similar levels of soluble Aβ and plaque number, but these did not significantly differ from those in non-transgenic control either [164]. The other two studies used APP/PS1 mice that also express mutations of the *APP* and *PSEN1* genes [158, 165]. In the study by Ke *et al.* 7- to 8-month-old and 24-month-old mice exercised 5 times per week for 5 weeks, and the volume of exercise was gradually increased during the second week of exercise from an initial 10 minutes to reach 60 minutes per day for the last 3 weeks [165]. Exercise was found to reduce hippocampal Aβ40 and Aβ42 in adult but not in old mice, whereas no differences in plaque burden were found at either age [165]. In the study by Zhao *et al.* the mice were younger, 3-month-old and 12-month-old, and they exercised 5 days a week for 30 minutes per day [158]. While the exercise training was found to reduce the hippocampal levels of Aβ40 and Aβ42 in both adult and old mice, number and area of amyloid plaques were reduced only in the younger [158].

Results from studies where transgenic AD mice disease were given voluntary access to a running wheel may also suggest reduced efficacy of exercise to attenuate brain amyloid plaque load at an advanced stage of pathology. While voluntary running reduced levels of Aβ peptides, oligomers, and plaques when initiated at a pre-plaque stage [167, 168], beginning exercise at an advanced stage reduced soluble Aβ [166, 169], but not plaques [170]. Collectively these findings suggest that the ameliorating effect of exercise on amyloid pathology may be greater when initiated at earlier stages of the pathology development.

1.5.4.1 Effects of exercise on Aβ production and clearance

Several studies have assessed the effect of exercise on expression levels of enzymes involved in Aβ peptide production [151, 157, 159, 160, 162, 164, 167, 171]. While many of these studies suggested reduced amyloidogenic APP processing to account for reduced Aβ levels with exercise by showing lower expression levels of β-secretase and/or presenilin 1 in exercised animals compared with sedentary controls [151, 159, 160, 162, 171], not all studies found support for such a mechanism [157, 164, 167]. One study that found evidence of reduced Aβ production with exercise, reported reduced expression

of proteins involved in Aβ degradation and transportation through the blood-brain barrier, suggesting reduced clearance of Aβ in response to its reduced production [159].

Some studies have also provided evidence of upregulated Aβ clearance with exercise training [152, 153]. Suggesting enhanced clearance of Aβ to underlie reduced levels of soluble Aβ with exercise training in young Tg2576 mice, exercise was found to increase expression of various proteins associated with enzymatic degradation and blood-brain barrier -mediated clearance of Aβ in a similar intensity-dependent manner as for Aβ levels [152]. Furthermore, levels of a heat-shock protein 70, which has been found to be upregulated also in some exercise studies with old AD mice [172, 173], changed accordingly [152]. This protein has been shown to inhibit aggregation of Aβ *in vitro*, as well as to reduce hippocampal and cortical plaque burden in AD mice when administered exogenously [174, 175]. Thus, these results suggest that exercise training may reduce amyloid plaque deposition through enhanced clearance and inhibited aggregation of Aβ in an intensity-dependent manner [152]. Collectively studies suggest that exercise may attenuate the progression of amyloid pathology through both reduced Aβ production and clearance, and at least the degree of clearance might be intensity dependent.

1.5.5 Exercise and cognitive function in transgenic AD models

Considerable evidence suggests beneficial effects of voluntary and forced exercise on hippocampus-dependent spatial learning and memory in several transgenic AD models both when exercise is initiated at an early stage [154, 156, 158, 161, 164, 165, 167, 171, 176, 177], or at an advanced stage of AD [151, 158, 164, 172, 173, 178-180]. Nevertheless, not all studies found such benefit; not when mice were given long-term access to a running wheel at a very early stage of AD with no cognitive impairment [181], or when given the access at the age characterized with cognitive deficits and onset of plaque pathology [170]. Effects of exercise on non-spatial forms of memory have been less studied in transgenic AD models. Some studies reported improved performance in tests of recognition memory when exercise was initiated before cognitive impairment [154, 155, 163], whereas later exercise did not show beneficial effects [166, 170]. Some studies also reported improved conditioned fear learning and memory as assessed in a passive avoidance test [163, 176], and improved contextual and cued fear learning and memory in a conditioning test [153], all when exercise was initiated before cognitive impairment. In contrast, one study found no effect of exercise on aversive learning or memory with early [156], and another with either early or late start [165].

Where most studies have only investigated the effects of rather low or moderate intensity exercise on cognition in transgenic AD models, a couple recent studies including higher-intensity exercise have provided some evidence to suggest a threshold intensity of exercise for improved cognitive function [154, 162], similar to what could be concluded based on the findings from human exercise intervention studies [109]. A study in APP/PS1 mice reported similar beneficial effects of 12 weeks of high-intensity interval training and moderate-intensity continuous training on spatial learning and memory [162], whereas in Tg2576 mice, 12 months of high-intensity exercise training resulted in significantly better recognition memory and spatial learning and memory when compared to same volume of low-intensity exercise training or remaining sedentary [154]. Furthermore, the low-intensity training provided only slight improvement in spatial memory and no benefit in recognition memory compared to not exercising at all [154].

Collectively, the findings from exercise studies in transgenic AD models suggest that sustained, regular exercise training of high enough intensity may alleviate brain amyloid

burden and preserve aspects of cognitive functions, at least when initiated before plaque pathology and cognitive deficits have become apparent.

1.5.5.1 Potential mechanisms underlying improved cognitive functions

Both studies in humans and in animals have suggested potential mechanisms underlying the beneficial effects of exercise on cognitive performance. Where studies in humans have shown exercise and improved CRF associated with greater volume of the hippocampus, a key region of the brain in learning and memory [182-185], animal studies have further demonstrated that this volume increase could result from increased production of new hippocampal neurons and blood vessels, or neurogenesis and angiogenesis, respectively [156, 168, 186-193]. Suggesting neurogenesis as beneficial for cognitive functions, promoted neurogenesis has been widely reported with accompanying improvements in learning and memory following exercise in wild-type animals [186, 189-191], as well as in transgenic AD animals [156, 168, 188, 191]. The increases in angiogenesis may in turn represent a supportive mechanism to ensure adequate supply of trophic factors to new-born cells [194]. Some studies have also reported enhanced synaptic plasticity as a potential mechanism underlying improved memory both in wild-type and transgenic AD animals [158, 177, 190, 195].

Several exercise-induced factors have been suggested to mediate the beneficial effects of exercise on cognition [196]. One of such factors is brain-derived neurotrophic factor (BDNF), whose levels in blood may increase not only acutely but also chronically with exercise [197, 198]. In the brain, BDNF may mediate the improvements in learning and memory through its promoting effects on neurogenesis and synaptic plasticity [164, 172, 177, 190, 199]. Insulin-like growth factor 1 (IGF1) and vascular endothelial growth factor (VEGF) that are important mediators of angiogenesis have also been shown potentially essential to exercise-induced neurogenesis and improved memory [189, 192, 193, 200-202]. Potentially showing some indication why the beneficial effects of exercise on the brain may be intensity-dependent, increased levels of lactate from high-intensity exercise have been recently reported to correlate with BDNF and VEGF expression in mice [192, 203], and with BDNF, IGF1, and VEGF expression in humans [204].

2 Purpose and aim

Greater PA engagement and higher CRF have been shown to be positively associated with cognitive function and inversely associated with the risk of AD in healthy humans [88-95, 104-108]. Exercise interventions in cognitively unimpaired individuals and those with MCI have also shown positive effects of exercise on cognitive test performance [109-112]. However, results from RCTs assessing the effects of exercise on cognition and brain pathology in AD patients have been more inconsistent and some larger RCTs have failed to show any beneficial effects [116-120]. Thus, it seems probable that PA and exercise can improve cognition and reduce the risk of AD in healthy individuals, but that exercise may have limited therapeutic effects in AD patients. Similarly, studies in animal models of AD have shown mainly beneficial effects of exercise training when initiated before clear cognitive deficits [157, 160, 163, 167], whereas later interventions have shown contradictory results [169, 170, 180]. Only few studies have investigated the effects of the same exercise training intervention at a pre-symptomatic and a post-symptomatic stage of AD [158, 164, 165], and only one study has utilized high-intensity interval training as the method of training for a transgenic AD model [162]. Furthermore, no study has investigated the effects of chronic exercise in the McGill-R-Thy1-APP transgenic rat model of AD before.

To address the gap in literature and to provide valuable information on whether the timing of exercise in relation to the AD progression state is of importance, this study aimed to assess the effects of four weeks of high-intensity interval training when initiated at a pre-symptomatic and a post-symptomatic stage in the McGill-R-Thy1-APP transgenic rat model of AD. As this rat model is a good model of early AD amyloid pathology and shows progressive cognitive decline from 3 months of age, it was of interest to study the effects of exercise specifically on Aβ deposition and test performance in behavioural tests indicative of learning and memory. Thus, the overall purpose of this study was to investigate whether a high-intensity exercise intervention has preventive or therapeutic effects on AD pathology and cognitive decline.

It was hypothesized that four weeks of high-intensity interval training would, in comparison to remaining sedentary, reduce amyloid pathology and improve cognitive function in the McGill-R-Thy1-APP rats. It was further hypothesized that the beneficial effects of exercise on cognitive function and amyloid pathology would be more pronounced with the earlier initiation at a pre-symptomatic stage than with the later initiation at a symptomatic stage.

3 Methods

3.1 Animals

All the rats used in this study were homozygotic male rats of the transgenic McGill-R-Thy1-APP rat model of AD which expresses the human APP (isoform APP751) gene with the double Swedish and Indiana mutations under the control of the murine Thy1.2 promoter [136]. Rats were housed in ventilated cages at 23 °C and 70% humidity with two or three rats per cage. The rats had access to standard rodent chow and water *ad libitum*. The cages were enriched with a wooden block for chewing, a plastic house for sheltering, and paper for nesting material. A reversed 12-hour light cycle was used so that all the exercise sessions, behavioural testing, and other procedures took place during the dark phase of the circadian cycle.

3.2 Ethical statement

All experimental procedures of the present study were approved by the Norwegian Food Safety Authority (FOTS ID: 11740), and were in accordance with the Norwegian Animal Welfare Act §§ 1-28, Norwegian Regulations on Animal Research §§ 1-26, and the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes.

3.3 Study design

The rats (n=25) were allocated into four groups based on age and applied treatment. Two groups of rats performed four weeks of exercise training beginning either at the age of 2 months (n=7) or 5 months (n=7). These exercised groups of rats are later referred to as Ex3 and Ex6 respective to their age at the beginning of post-exercise testing. Both exercising groups performed exercise testing before and after the 4-week training period. In addition, Ex6 rats performed behavioural testing both before and after exercise training, and Ex3 performed the same tests only after exercise training. Other two groups of rats served as control groups for the exercise groups and did not do any exercise training but performed same testing at corresponding ages; 3 months old (n=6, Sed3) and 6 months old (n=5, Sed6). Timeline diagrams for exercise trained rats are presented in figure 1.

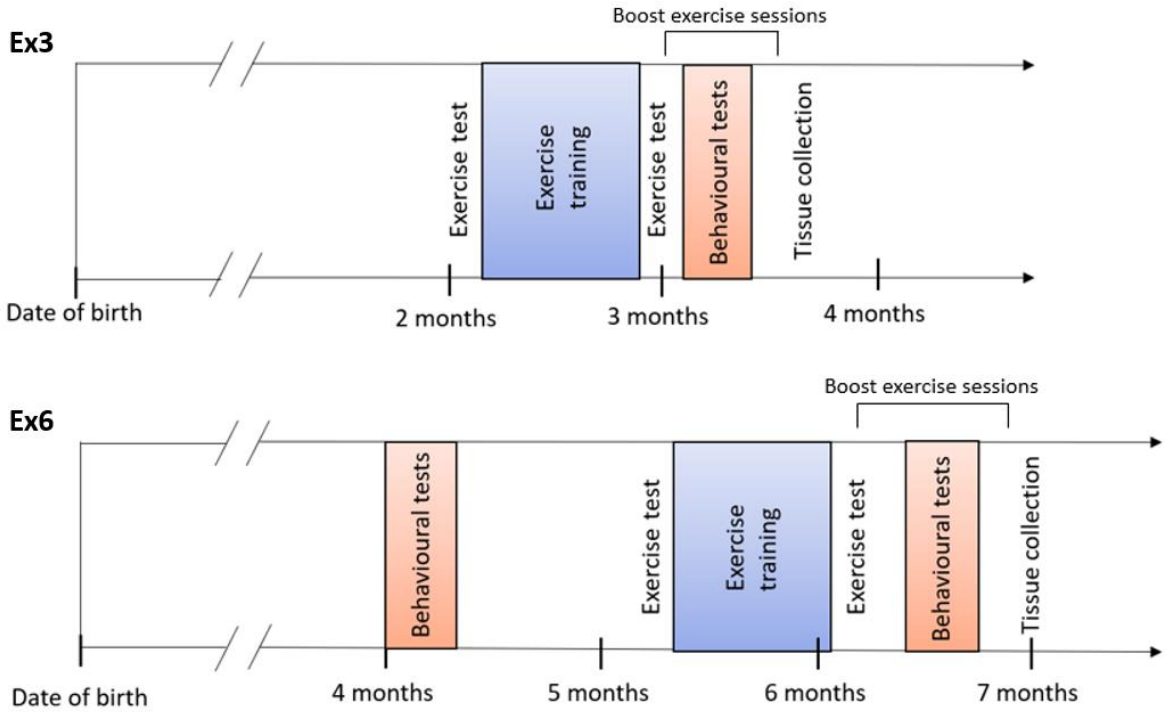

Figure 1. Timeline diagrams for the exercise-trained rats. The upper diagram presents the timeline for the Ex3 rats and the lower diagram for the Ex6 rats. Both the Ex3 and Ex6 rats performed high-intensity exercise training 5 times a week for 4 weeks. A graded exercise test until exhaustion was applied before and after the 4-week training to determine the efficacy of exercise on improving fitness. Behavioural tests were conducted after the primary training period for both groups of rats, and control groups performed same tests at corresponding ages. The NORT always took place before the FC. The Ex6 rats performed the behavioural tests also before exercise training to verify and determine the degree of cognitive impairment in these rats before exercise training, as well as to be able to assess the effect of exercise training on the performance in these tests.

In addition to the treatments described above, all the rats were given intraperitoneal injections of either saline or 5-bromo-2'-deoxyuridine (BrdU) under anaesthesia induced with isoflurane two to three times a week for future assessment of adult hippocampal neurogenesis. A total number of injections received by a rat was 14.68±1.282 (mean ± standard deviation). The Sed3 rats served as controls also for another study and were additionally given intravenous injections of saline in tail vein under the same periods of anaesthesia.

3.4 Exercise testing

A graded maximal exercise protocol in an enclosed treadmill (Columbus Instruments, Columbus, OH, USA) with an indirect open circuit calorimeter system (Oxymax, Columbus Instruments) was used to determine the maximal oxygen consumption (VO_2 max) of the rats. All the tested rats were habituated to the treadmill a day before the testing. The incline of the treadmill was kept at 25° for both the habituation and the entire test protocol. The habituation started with 5 minutes on a still treadmill, after which the speed of the treadmill was set to 8.0 meters per minute for the last 10 minutes. After the first 5 minutes of this exercising phase, electricity was turned on to the electrical grid at the bottom of the treadmill for the rest of the habituation.

On the testing day, a calibration of the testing apparatus was first performed. The rat being tested was first weighed and then put in the treadmill chamber to measure resting oxygen consumption (VO_2 rest). The test was started with 10 minutes of warm-up at 8

m/min, after which the speed was increased by 1.8 m/min every 2 minutes. The test was terminated when the rat touched the electrical grid three times within five seconds or was in contact with it for 3 seconds.

The validity of some of the (the post-exercise training values for older rats and some for younger rats) oxygen consumption measures was questioned due to great intraindividual changes and variability in both resting and peak values. A technical problem was located to an air filter and the VO_2 rest and VO_2 max measurements were all decided to be excluded from statistical analysis. Instead, speeds at exhaustion (maximal speeds) were used for result analysis and, as the changes in running speed do not take into account the change in weight of the rats, estimates of running power at the peak speed were additionally used to determine the efficacy of the exercise training on physical fitness.

The running power estimates were calculated using the following equation,

$$P = (i \times v_{max} \times m) \times c \,,$$

where *i* is the incline (25°) of the treadmill converted into a percentage slope (in decimal form, ≈0,4663), v_{max} is the maximal speed obtained by the rat, *m* is the mass of the rat immediately before the test, and *c* is the value for conversion into watts (i.e. 0,1635, as 1 kg·m/min ≈ 0,1635 W).

3.5 Exercise procedure

Rats were exercised on two treadmills with an incline of 25° (Columbus Instruments) in a room with dim lighting. The treadmills were divided into two lanes by a separator wall so that while three to four rats ran on the same treadmill, only one to two rats ran on each lane. When there was an odd number of rats to be exercised, the rat running alone was changed for each session to minimize any confounding effect from running alone versus running with another rat.

The high-intensity interval exercise sessions always started with a 10-minute warm-up at 6.0 m/min, which was immediately followed by 10 high-intensity intervals separated with 2-minute active resting periods at 6.0 m/min. The speed for the first sessions was approximately 80-90 % of the rats' maximum speed at initial testing. At the bottom of the treadmill, there was an electric grid, but electricity was not kept constantly on. Instead, if a rat was touching the grid, it was encouraged to get further on the treadmill by a noise created with a tap on the treadmill wall or lid, or by giving the rat a little push. In rare cases where a rat kept in touch with the grid despite these actions, a brief electric shock was introduced. The interval speed for the sessions was increased every time it seemed that the rats could complete the intervals at a faster speed without a need for use of electric shocks.

3.6 Behavioural testing

All behavioural testing was performed for each rat individually and before any behavioural tests, rats were habituated to the experimenter by having them on the experimenter's lap for 30 minutes.

3.6.1 Novel Object Recognition Test

Novel object recognition test (NORT), which relies on the natural tendency of animals to explore novelty [205], was used to assess recognition memory of the rats. Recognition memory and performance in the NORT is dependent on the integrity of hippocampal and parahippocampal regions (including perirhinal and entorhinal cortices) of the brain [205].

In the NORT each rat was tested individually in a circular arena with a white distal cue and a camera set directly above. The arena was cleaned with 10% ethanol between each rat. On the first day of the NORT, rats were allowed to explore the arena for 15 minutes. The next three days (for familiarization), rats were placed in the center of the same arena where two identical objects (dark mugs) had now been placed. Rats were allowed to explore the arena and objects for 10 minutes each day. On the fifth day, to test recognition memory, one of the mugs was replaced by a novel object (a green "tower" built of Lego-blocks), and rats were again placed in the center of the arena. This time rats were allowed to explore the objects for 4 minutes. Time spent by each rat exploring the objects was manually measured from a recorded video using stopwatches.

Based on the time spent by a rat investigating each object, a discrimination index (DI) was calculated separately for the first two minutes and for the whole period of four minutes as

$$DI = \frac{(T_{novel} - T_{familiar})}{(T_{novel} + T_{familiar})},$$

where T_{novel} and $T_{familiar}$ are the times spent by the rat investigating the novel and familiar object, respectively. Thus, positive values indicate preference for the novel object and negative for the familiar object. Full NORT protocol is shown in Appendix 1.

3.6.2 Fear Conditioning Test

Fear conditioning (FC) test was used to assess associative fear learning and memory, which are dependent on the amygdalar and hippocampal regions of the brain [206]. Each rat was tested individually in a four-day protocol utilizing a combination of a tone as a conditioned stimulus and an electric shock as an unconditioned stimulus. On the first day, rats were allowed to freely explore a rectangular arena for 5 minutes for habituation. On the second day, rats were allowed to explore the same arena for the first 120 seconds (baseline), after which a tone (70 dB, 2kHz) lasting for 30 seconds and a co-terminating 2-second foot shock (0.5 mA) were introduced. After a 120-second stimulus-free period, the tone and foot shock (tone-shock) were reintroduced as earlier for auditory conditioning. Rats were given a 180-second recovery period (post-shock) before taking them back to their home cage. On the third day, to test contextual recall, freezing behaviour was measured while rats were placed in the familiar arena for 8 minutes before taking them back to their home cage. On the fourth day, to test cued recall, rats were carried straight from their home cages into the testing room and to a new circular arena with new distal cue and scent. After 120 seconds (baseline), three cued 30-second tones (tone;70 dB, 2 kHz) were introduced with a 30-second pause in between to test cued recall. After termination of the last tone, rats were allowed to recover for 210 seconds (post-tone). Freezing behaviour of each rat during each phase was tracked using ANY-maze video tracking system and software (ANY-Maze, Stoelting Co.). Threshold for minimum freeze was set at 500 ms. The full FC test protocol is shown in Appendix 2.

3.7 Tissue collection

Rats previously given intraperitoneal injections of BrdU were anaesthesized with 5% isoflurane in a chamber, weighed, given intraperitoneal injection of pentobarbital (approximately 0.2 ml/100 g), and ensured to be deeply anaesthesized before performing transcardial perfusion with Ringer's solution (0.85% NaCl, 0.025% KCl, 0.02% $NaHCO_3$; pH 6.9) and 4% paraformaldehyde. Solutions for perfusion are described in Appendix 4. Once perfused, the brains were collected into tubes with 4% paraformaldehyde and let to post-fixate for 24 hours at +4°C. When fixed, the brains were transferred into tubes with 2% dimethyl sulfoxide and stored at +4°C until

sectioning. Rats given intraperitoneal injections of saline were similarly anaesthesized with 5% isoflurane in chamber and weighed before collecting and snap freezing the following tissues for future analyses: blood, heart, brain, soleus muscle, gastrocnemius muscle.

3.8 Brain tissue sectioning

The paraformaldehyde-fixed brains were sectioned serially at 40 µm in a coronal plane using a freezing microtome (Microm HM430, Thermo Fisher Scientific). Five series were stored in tubes with 2% dimethyl sulfoxide in 0.1 M phosphate buffer (PB) with 20% glycerol, and one series was mounted on Superfrost glass slides and left to dry overnight.

3.9 Immunostaining

One series of sections from the paraformaldehyde-fixed brains were stained in a free-floating immunohistochemistry procedure utilizing a highly specific anti-human Aβ antibody McSA1 that detects both intracellular Aβ and amyloid plaques [136, 138]. For unmasking, the sections were incubated in +60 °C PB (0.125 M, pH 7.4) for 2 hours, followed by two rinses with PB and one with 0.5% Triton X-100 in Tris-buffered saline (TBS-Tx; 50 mM Tris, 150 mM NaCl, pH 8.0) for cell permeabilization. The sections were then incubated with 10% goat serum in TBS-Tx in order to block non-specific antibody binding, before incubating the sections with the McSA1 primary antibody (MédiMabs, MM-0015-P; 1:4000 in TBS-Tx) overnight at +4 °C.

The following day, the sections were rinsed with TBS-Tx three times before incubating them with a biotinylated goat anti-mouse secondary antibody (1:200 in TBS-Tx) for 90 minutes. The sections were then rinsed with TBS-Tx three times, followed by incubation with the avidin-biotin complex (Vectastain ABC kit, Vector Laboratories) for 90 minutes in room temperature. Then the sections were rinsed three times with TBS-Tx and twice with Tris-HCl before incubating with 3,3'-Diaminobenzidine (DAB) and hydrogen peroxide in Tris-HCl (pH 7.6) for 2 minutes. Finally, sections were rinsed twice with Tris-HCl and mounted on Superfrost glass slides or stored in Tris-HCl overnight at +4 °C before mounting. After mounting, sections were left to dry for at least one night before clearing the slides with Xylene and coverslipping with Entellan. The full protocol and list of solutions used are shown in Appendix 3 and Appendix 4, respectively.

3.10 Detection

The mounted sections were imaged using Invitrogen EVOS FL Auto 2 Imaging System (Thermo Fisher Scientific) and the brain atlas *Brain maps 4.0: structure of the rat brain* was used as a reference for neuroanatomical description of amyloid pathology [207].

3.11 Statistical analysis

All results are presented as mean ± standard error of the mean (SEM) in figures and as mean ± standard deviation (SD) in tables. Statistical analysis was performed using GraphPad Prism 4.01 (GraphPad Software). Paired t-tests were performed for comparison of pre- and post-training data in the Ex3 and Ex6 rats. Unpaired t-tests were performed for comparison of independent data from the exercise-trained rats and age-matched sedentary rats. One-way analysis of variance (ANOVA) was used for comparison of means of three groups and Bonferroni's multiple comparison test was applied for pair-wise comparison. Two-way ANOVA with Bonferroni post-tests was used for analysis of freezing behaviour in different phases of the FC test. P-values <0.05 (95% confidence interval) were considered significant for all analysis.

4 Results

4.1 Graded exercise test until exhaustion

A graded maximal exercise test was used to assess the effect of four weeks of high-intensity interval training on physical fitness in the rat model of AD. Characteristics and test results of the exercised rats before and after the exercise training are shown in Table 1.

Table 1 Characteristics of the rats at exercise testing pre- and post-training

	Ex3 (n=7)		Ex6 (n=7)	
	Pre-training	Post-training	Pre-training	Post-training
Age (days)	59.9±1.1	87.3±1.6	159±0	187±0
Weight (g)	254±34.8	299±37.7	349±19.9	343±22.0
Maximal speed (m/min)	14.69±1.361	18.54±1.242	10.83±1.757	17.77±2.913
Power (W)	0.282±0.0303	0.423±0.0651	0.288±0.0497	0.465±0.0836

All data are presented as mean ± standard deviation.

After four weeks of high-intensity interval training, maximal speed obtained during the graded exercise test increased by 26.2% in the Ex3 rats (P=0.0018; Table 1, Figure 2a) and by 64.1% in the Ex6 rats (P=0.0012; Table 1, Figure 2b). The maximal speeds of Ex3 and Ex6 rats after exercise training also differed significantly from their age-matched controls; Ex3 compared with Sed3 (P=0.0099), and Ex6 compared with Sed6 (P=0.0020). The weight of the Ex3 rats increased significantly over the period of exercise training (P<0.0001, Table 1), while there was no significant difference in the weight of the Ex6 rats at the pre-training test and the post-training test (P=0.5296, Table 1). Change in maximal speed as an indicator of increased physical fitness does not consider changes in weight over time, and therefore running power at maximal speed was calculated. Running power at maximal speed increased by 49.9% (P=0.0004; Table 1, Figure 2c) and 61.4% (P=0.0012; Table 1, Figure 2d) in the Ex3 and Ex6 rats, respectively. Running power was also significantly different between the groups of exercised and sedentary rats; Ex3 compared with Sed3 (P=0.0036), and Ex6 compared with Sed6 (P=0.0234). These results suggest improved physical fitness in the exercise-trained rats.

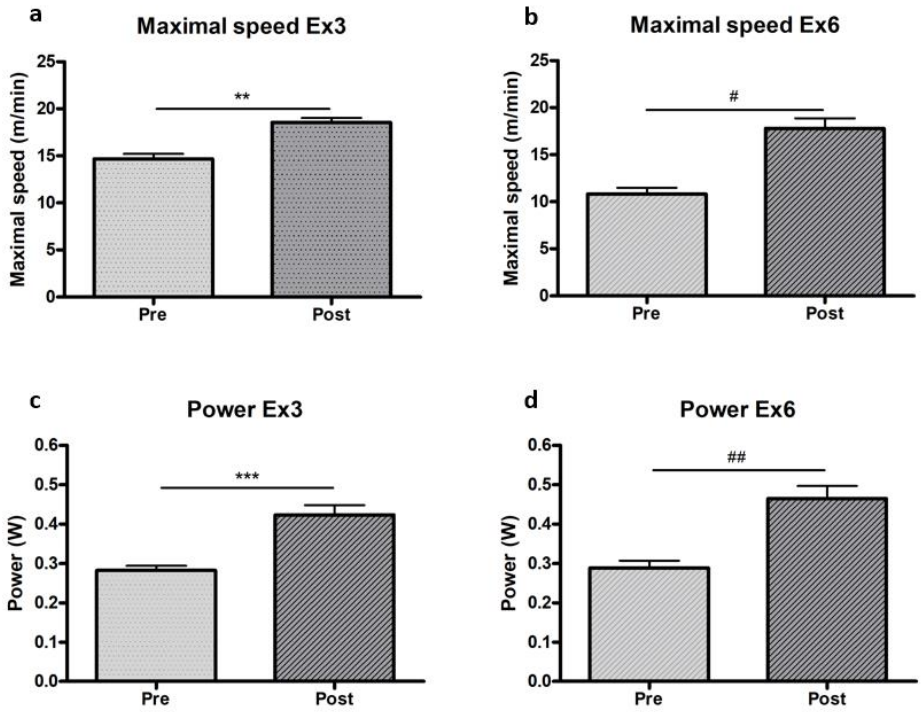

Figure 2. Results from graded exercise test until exhaustion. Maximal speeds in meters per minute and calculated power at maximal speed in watts of the Ex3 (**a** and **c**, n=7) and Ex6 (**b** and **d**, n=7) rats before and after four weeks of high-intensity exercise training. All data are presented as mean ± SEM. Paired t-test, **P=0.0018, $^{\#}$P=0.0012, ***P=0.0004, $^{\#\#}$P=0.0012.

4.2 Behavioural characterization of aging in sedentary rats

In order to determine the effects of sedentary aging and AD progression in the McGill-R-Thy1-APP rats on recognition memory and associative fear learning and memory, the Sed3 and Sed6 rats performed NORT and FC test at the age of three months and six months, respectively. The Ex6 rats also performed the same tests at the age of 4 months, one month before exercise training, and were included in the analyses.

4.2.1 NORT

In the NORT, all groups of rats showed preference for the novel object over the familiar object in the NORT, as indicated by positive DI values (Figure 3). The 4-month-old Ex6 rats showed significantly greater DI compared to the Sed3 rats when calculated for the first two minutes of the test (P<0.05, Figure 3a), but not when considering the whole four-minute test period. There were no statistical differences between the DIs of Sed3 and Sed6 rats, or the Ex6 and Sed6 rats, neither during the first two minutes of the test nor the whole four minutes (Figure 3a and b). These results suggest no age-related decline up to six months of age in recognition memory in the McGill-R-Thy1-APP rats.

4.2.2 FC test

In the FC test, there were no significant differences in the baseline freezing behaviour between any of the groups when initially placed in the FC arena (Figure 3c, baseline). During the auditory FC pairing tone with a foot shock, the Sed3 and Sed6 rats showed significantly greater freezing behaviour compared to the 4-month-old Ex6 rats (P<0.001 and P<0.05, respectively; Figure 3c, tone-shock). After the tone-shock presentations, Sed3 showed greater freezing compared to both the Ex6 and Sed6 rats (P<0.001 and

P<0.01, respectively; Figure 3c, post-shock), while there was no difference in the freezing behaviour between the Ex6 and Sed6 rats.

In the contextual recall test where the rats were placed in the same arena without tone or shock presentations, Sed3 showed significantly greater freezing behaviour than the Ex6 rats (P<0.01, Figure 3d), indicating better contextual recall in the 3-month-old rats compared to the 4-month-old rats. However, there was no significant difference in the freezing behaviour between the Sed3 rats and the Sed6 rats, or between the Ex6 and Sed6 rats.

In the cued recall test, where the rats were placed in a new arena, the Sed3 rats showed significantly greater freezing behaviour compared to Ex6 rats (P<0.05; Figure 3c, baseline), while there were no significant differences in the baseline freezing behaviour between the Sed3 and Sed6 or the Ex6 and Sed6 rats. During the familiar tone presentations in the new environment, the Sed3 rats showed significantly greater freezing behaviour compared to the Ex6 and Sed6 rats (P<0.001 and P<0.01, respectively; Figure 3e, tone). Similarly, the Sed3 showed greater freezing behaviour after the tone presentations compared to the Ex6 and Sed6 rats (P<0.001 and P<0.05, respectively; Figure 3e, post-tone,). These findings suggest declined cued recall in the 4-month-old and 6-month-old rats compared to the 3-month-old rats, but no further decline from the age of 4-months to 6-months.

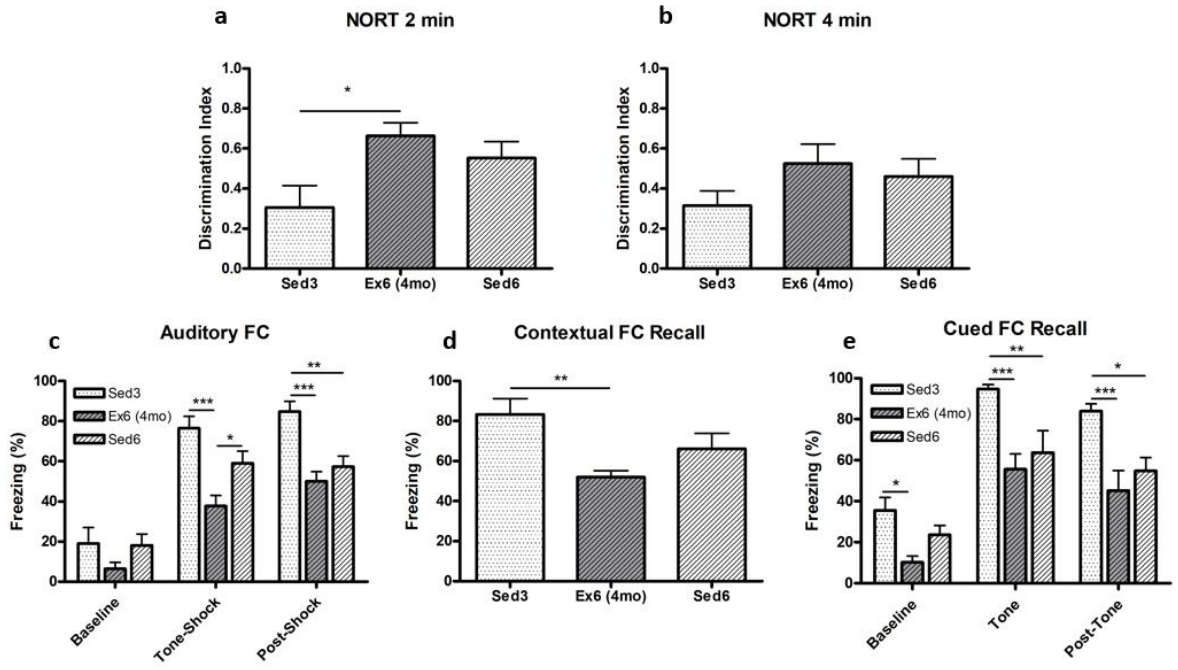

Figure 3. Changes in behaviour with sedentary aging in McGill-R-Thy1-APP rats. Results from NORT and FC test in the Sed3 (n=4) and Sed6 (n=5), as well as the Ex6 rats (n=7) pre-training at the age of 4 months. DI values from the NORT when calculated for the first two minutes of the test (**a**) and for the whole test period of four minutes (**b**). Percentage freezing time during each test phase of the FC; auditory conditioning phase (**c**), contextual recall test (**d**), and cued recall test (**e**). One rat from both the Sed3 and Ex6 groups were excluded from cued recall test analysis due to lacking data. All data are presented as mean ± SEM. One-way ANOVA with Bonferroni's multiple comparison test (**a**, **b**, and **d**) and two-way ANOVA with Bonferroni post-tests (**c** and **e**), *P<0.05, **P<0.01, ***P<0.001.

One rat each from both the Sed3 and Ex6 groups jumped out of the testing arena during the cued FC recall test and were hence excluded from the statistical analysis of cued FC recall (Figure 3e).

4.3 Recognition memory in the exercise-trained rats

To study the effects of 4 weeks of high-intensity exercise training on recognition memory in 3- and 6-month-old McGill-R-Thy1-APP rats, NORT was performed post-training in the Ex3 and Ex6 rats. Results from these tests were compared with the test performance in the age-matched sedentary groups of Sed3 and Sed6. All groups showed preference for the novel object over the familiar object (Figure 4). There were no statistically significant differences in DI between the exercised Ex3 rats and their sedentary counterparts, neither when DI was calculated based on the first two minutes of the test (Figure 4a), nor when based on the entire four minutes (Figure 4b). In contrast, the exercised Ex6 rats had significantly lower DI compared to the Sed6 rats when calculated for the first two minutes (P=0.0253, Figure 4c), even though there was no significant difference when considering the entire test duration (Figure 4d). These results suggest no significant effect from four weeks of high-intensity exercise training on recognition memory in McGill-R-Thy1-APP rats when initiated at a very early cognitively unimpaired, pre-plaque stage, but an impairing effect when initiated at a more progressed stage of pathology.

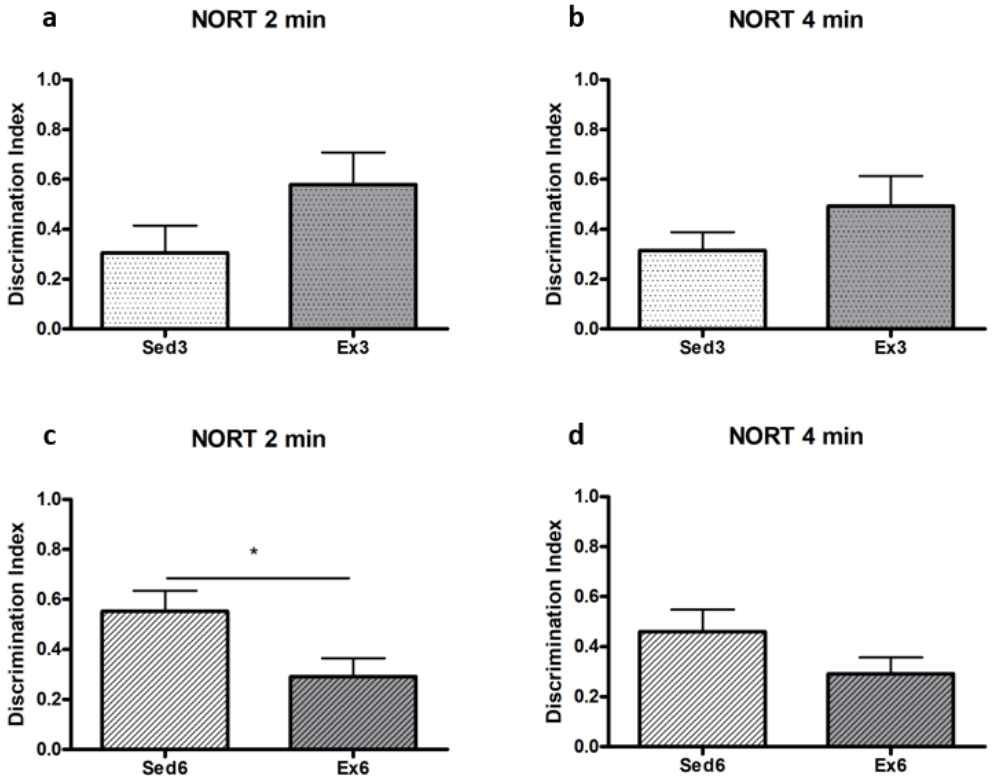

Figure 4. Effects of high-intensity exercise training on performance in the NORT. DI values for the Ex3 (n=7, **a** and **b**) and the Ex6 rats (n=7, **c** and **d**) post-training compared with their age-matched sedentary controls (Sed3, n=4, and Sed6, n=5, respectively) calculated for the first two minutes of the test (**a** and **c**) and for the whole test period of four minutes (**b** and **d**). All data are presented as mean ± SEM. Unpaired t-test, *P=0.0253.

4.4 Fear learning and memory in the exercise-trained rats

To further study the effects of 4 weeks of high-intensity exercise training on recognition memory in 3- and 6-month-old McGill-R-Thy1-APP rats, FC test was performed post-training in the Ex3 and Ex6 rats. There was a significant difference in freezing behaviour between the exercised Ex3 and the sedentary Sed3 rats only during the tone and shock presentations in the auditory conditioning phase of FC ($P<0.01$, Figure 5a), and no significant differences in baseline or post-shock freezing behaviour. There were no significant differences in freezing behaviour when the rats were placed in the familiar arena with no tone or shock presentation to test contextual FC recall (Figure 5b). There were also no significant differences in freezing behaviour in a novel environment testing cued FC recall either; not at baseline, during tone presentations, or after them (Figure 5c). These results suggest no beneficial effects of high-intensity exercise training on contextual or cued aversive memory recall in young McGill-R-Thy1-APP rats.

There were no significant differences in freezing behaviour between the Ex6 and Sed6 rats at the baseline, during the tone-shock presentations, or following them in the auditory conditioning phase (Figure 5d, e, and f). Similarly, there were no significant differences in freezing behaviour between the Ex6 and Sed6 rats when placed again in the familiar environment ($P=0.0519$, Figure 5e) or when exposed to the novel environment, presenting familiar tone, or after the tone presentations (Figure 5f). These results suggest no significant effects of chronic high-intensity exercise on auditory FC or cued fear memory recall, but a potentially impairing effect on contextual recall, in McGill-R-Thy1-APP rats when applied at an age characterized with cognitive deficits.

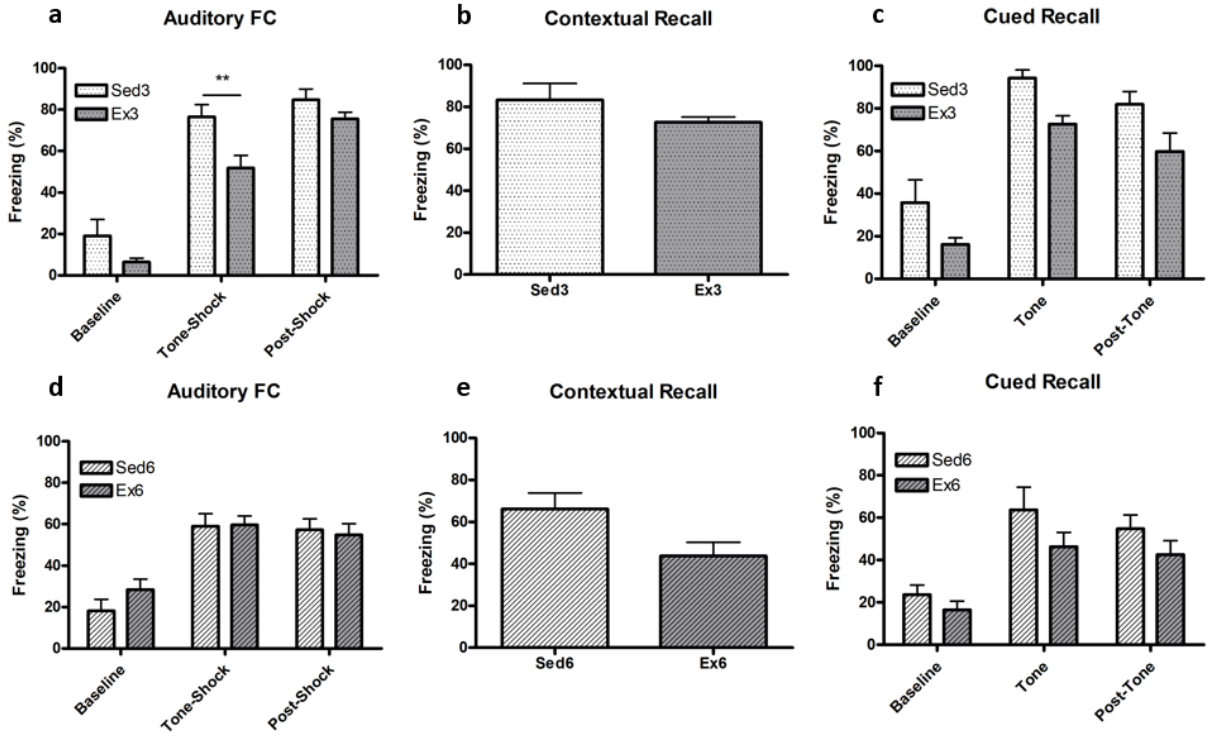

Figure 5. Effects of high-intensity exercise training on freezing behaviour in the FC test. Percentage freezing time of the exercised Ex3 (n=7) and Ex6 (n=7) rats with the age-matched sedentary groups Sed3 (n=4) and Sed6 (n=5) during each test phase; auditory conditioning phase (**a** and **d**), contextual recall test (**b** and **e**), cued recall test (**c** and **f**). One of the Sed3 rats was excluded from the cued recall test analysis due to lacking data. All data are presented as mean ± SEM. Unpaired t-test (**b** and **e**) and two-way ANOVA with Bonferroni post-tests (**a**, **c**, **d**, **f**), **P<0.01.

4.5 Amyloid pathology

To determine the effect of high-intensity exercise training on Aβ pathology, intracellular Aβ and amyloid plaques were visualized in an immunohistochemical procedure utilizing highly Aβ-specific antibody McSA1 [136, 138]. As visually estimated, the 3-month-old Sed3 (n=3) and Ex3 (n=2) rats had similarly substantial intracellular accumulation of Aβ in the neocortex and hippocampal region, but no amyloid plaques were found in the brain of rats from either group (Table 2; Figure 6a and b). In contrast, both groups of 6-month-old rats showed early plaque pathology accompanying the intracellular accumulation of Aβ that was visible almost throughout the neocortex and the hippocampal region (Table 2; Figure 6c-f). The greatest difference in intracellular Aβ burden between the Sed3 and Sed6, as well as the Ex3 and Ex6 rats, was seen within the upper layers of neocortex where the 6-month-old rats had more intense staining. Less difference was found in hippocampal areas. Interestingly, Aβ staining was more intense in the Ex6 (n=3) rats compared to Sed6 (n=2) and where the Sed6 rats only had few plaques present in the subiculum area of the hippocampal formation, the Ex6 rats showed more widespread plaque pathology with some plaques present not only in the subiculum but also in the neocortex, CA1, dentate gyrus, and in one case in the CA3 as well (Table 2; Figure 6d, e, and f). These findings suggest that when initiated at a stage of AD characterized by extensive intracellular Aβ and cognitive impairment, intense high-intensity interval training may promote AD pathology and Aβ deposition into extracellular plaques.

Table 2 The degree of amyloid plaque pathology in different areas of the brain

Group	Neocortex	Subiculum	CA1	CA3	Dentate Gyrus
Sed3	-	-	-	-	-
Sed3	-	-	-	-	-
Sed3	-	-	-	-	-
Ex3	-	-	-	-	-
Ex3	-	-	-	-	-
Sed6	-	+	-	-	-
Sed6	-	+	-	-	-
Ex6	++++	+++++	++	+	+
Ex6	++++	++++	+	-	+
Ex6	++	++	+	-	+

Semi-quantitative analysis of amyloid plaque pathology. Only the areas of the brain displaying plaques are shown in the table. More plus (+) signs indicate greater number of plaques.

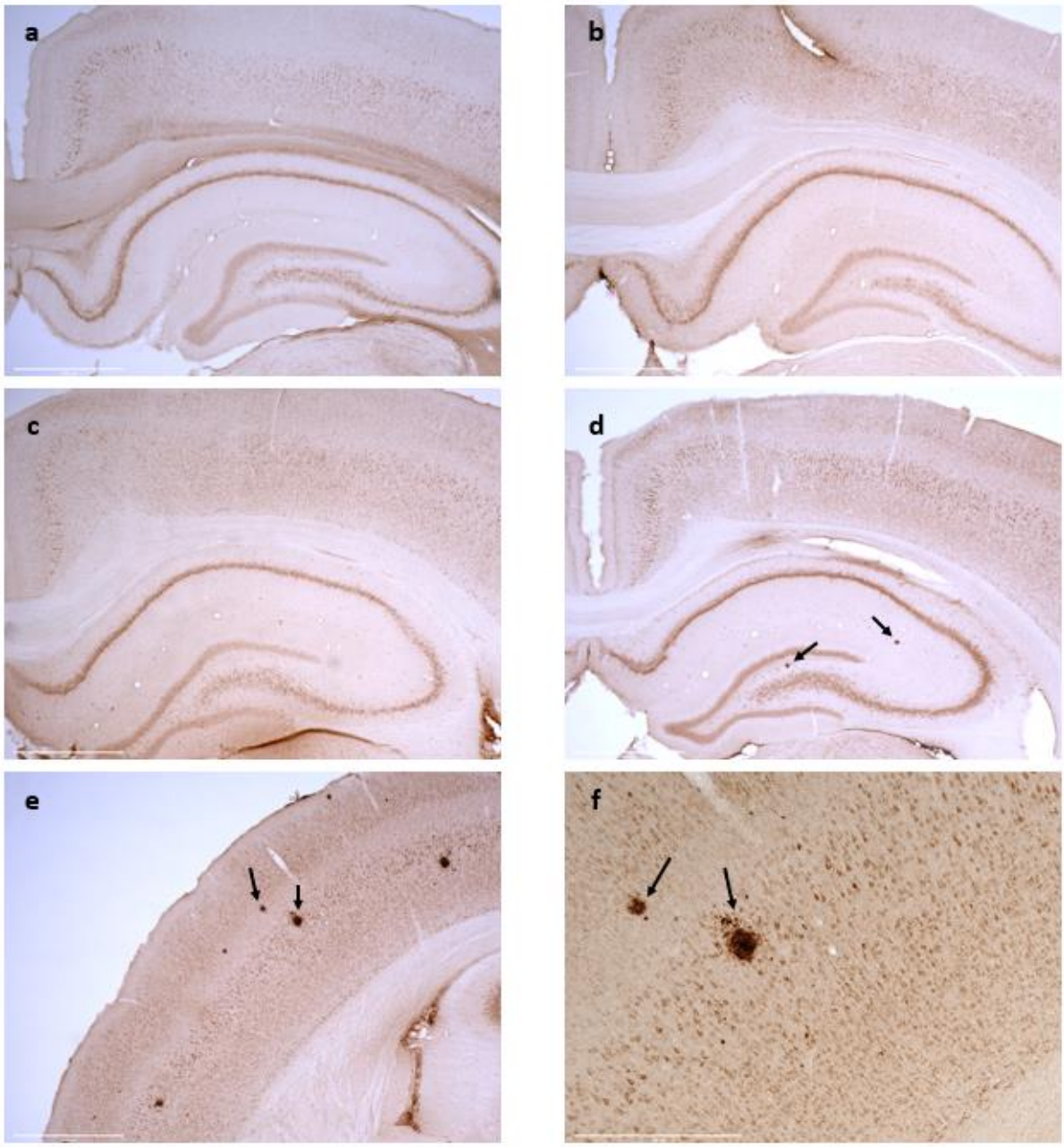

Figure 6. Aβ pathology in the sedentary and exercised McGill-R-Thy1-APP rats. The 3-month-old Sed3 (**a**) and Ex3 (**b**) rats showed substantial intracellular Aβ accumulation, but no extracellular plaque deposition. The 6-month-old Sed6 rats (**c**) had almost no plaques at all, whereas the exercised 6-month-old Ex6 rats also had plaque pathology accompanying intracellular Aβ accumulation (**d**, **e**, and **f**). Extracellular amyloid deposition in the dentate gyrus and CA1 of the hippocampal formation (**d**), as well as in the neocortex (**e**, magnified in **f**) in the brain of an Ex6 rat. Arrows point at examples of plaques. Scale bar is equal to 1000 μm (**a**-**e**) or 500 μm (**f**).

5 Discussion

The present study is the first to have investigated effects of chronic exercise in the McGill-R-Thy1-APP transgenic model of AD. Only effects of acute exercise on spatial and recognition memory have been investigated in McGill-R-Thy1-APP rats before, and no beneficial effects on cognition were found in that study [145]. The main overall findings of this study were that four weeks of high-intensity interval training produced no significant effects on intracellular Aβ burden, recognition memory, or fear-associated learning or memory when initiated before the onset of cognitive symptoms, but contrary to what was hypothesized, promoted amyloid plaque deposition and deteriorated recognition memory when initiated after the onset of characterized cognitive symptoms and preceding the first plaque deposition.

5.1 Behavioural characterization of sedentary aging

McGill-R-Thy1-APP rats have been earlier characterized to show impaired performance in various behavioral tests of cognitive functions from the age of three months when compared to wild-type rats [136, 137]. Factors potentially contributing to the cognitive deficits include the progression of amyloid pathology and neuroinflammation, reduced expression of BDNF, and impaired synaptic plasticity [44, 62, 137, 142-144]. In the present study, the effects of sedentary aging and AD progression on performance in the NORT and FC test were assessed expecting a progressive decline in performance with increasing age.

5.1.1 Recognition memory

By relying on the natural exploratory behaviour of animals where they tend to explore a novel object over a familiar object, the NORT can be used to assess the recognition memory of an animal by comparing the time spent exploring the novel object and the familiar object [205]. Longer time spent exploring the novel object, translating here into a greater DI, indicates better recognition memory. Four minutes was previously found to be an optimal test duration in this rat model, because with a longer exploration time the effect of novelty could be lost [145]. Therefore, in the present study the rats could explore the objects for four minutes and the DI values were calculated for both the first two minutes and the whole four-minute period.

Although intracellular Aβ and particularly the oligomeric forms of Aβ have been shown to promote neuroinflammation and impair synaptic function [44, 46], resulting in impaired recognition memory [47], there was no decline in recognition memory with increasing age of the McGill-R-Thy1-APP rats in the present study, and the 4-month-old rats even showed significantly higher preference for the novel object during the first two minutes of the NORT compared to 3-month-old rats (Figure 3a and b). These findings are, however, in line with a previous investigation of NORT performance in this AD model where 3-month-old, but not 6-month-old or 12-month-old transgenic rats showed significantly lower DI values compared to wild-type rats of the same age [145]. Differences in the handling and treatment of the rats preceding the test might have contributed to the lower DI in the Sed3 rats compared to the 4-month-old Ex6 rats, because repeated anaesthesia may alter AD pathology and cognitive function [208, 209]. Where the Sed3

rats received intravenous and intraperitoneal injections two to three times a week for four weeks before the NORT, the Ex6 rats were not yet anaesthetized or injected at all before the test at the age of four months. It may also be that due to the very early onset and rapid progression of intracellular Aβ accumulation in this rat model [136], the levels of Aβ may already be so high at the age of 3- to 4-months that further progression with the next 2- to 3-months of sedentary aging is not great enough to cause significant decline in recognition memory. Indeed, intracellular Aβ burden was found similarly extensive within the hippocampal area in the Sed3 and Sed6 rats (Figure 6).

5.1.2 Fear learning and memory

Associative learning and memory function of the rats were assessed by measuring the freezing behaviour of the rats in response to the auditory conditioning, associated context, and the conditioned auditory stimulus. The results of the FC test suggested impaired auditory FC, or associative learning, and contextual and cued fear recall in the 4- and 6-month-old McGill-R-Thy1-APP rats compared to the 3-month-old, but no significant change in behaviour from 4- to 6-months of age (Figure 3). Still, it is notable that the mean percentage of freezing behaviour of the 3-month-old rats was also greater at the baseline both in the first arena and the novel arena compared to that of the older groups, although only reaching statistical significance when compared to the 4-month-old in the novel arena. The greater baseline freezing may indicate greater anxiety in the youngest rats, and it might have contributed to the results universally [210]. Similarly to the NORT, these results may reflect the differences in the treatment of the rats during the four weeks preceding the tests.

5.2 The effects of exercise training on exercise capacity

Literature suggests PA to improve cognition and reduce the risk of AD [96, 109, 114], and improving or maintaining high level of CRF may play a significant role underlying the risk-reduction [103]. High-intensity interval training has earlier been shown to be superior for improving CRF compared to moderate-intensity exercise training in healthy humans [211], patients with cardiometabolic lifestyle diseases [212], as well as in rats [213], and it was also chosen as the method of training for the rats in the present study. To assess the effects of the high-intensity training on CRF, the gold-standard method of measuring VO_2 max in a graded maximal exercise test was used [214]. However, the validity of the VO_2 measurements were questioned due to a technical issue and had to be excluded from the analysis. Instead, measured maximal speeds and calculated estimates of running power at the maximal speeds were used to determine the efficacy of the exercise training on improving exercise capacity and reflect changes in fitness, even though not taking into account changes in running economy [215, 216]. As expected, the four weeks of high-intensity interval training significantly increased running speed and power in the McGill-R-Thy1-APP rats regardless of their age or the stage of pathology, hence suggesting improved CRF [216].

5.3 The effects of exercise training on cognitive functions

To investigate the effects of high-intensity interval training on cognitive functions, the Ex3 and Ex6 rats performed the same behavioural tests as the age-matched sedentary rats. Considering the substantial literature showing improved spatial learning and memory in various transgenic AD models both when exercise is initiated at an early stage [154, 156, 158, 161, 164, 165, 167, 171, 176, 177], or at an advanced stage of AD [151, 158, 164, 172, 173, 178-180], and several studies reporting beneficial effects of

early exercise also on performance in tests of non-spatial cognition [153-155, 163, 176], the high-intensity exercise training was hypothesized to improve NORT and FC test performance in both age groups.

5.3.1 Recognition memory

Even though the comparison of DI values from the NORT did not reveal significant differences between the exercised Ex3 and sedentary Sed3 rats, there was tendency for higher DI in the Ex3 compared to Sed3 (Figure 4), which may suggest slight beneficial effect of the four-week high-intensity exercise training on recognition memory when initiated at an early stage of AD. Previous studies in transgenic AD mice have reported improved performance in NORT when voluntary or high-intensity treadmill exercise was initiated before cognitive impairment [154, 155]. However, the mice in these studies exercised five times a week for 4 to 12 months [154, 155], and it may hence be that a longer training period covering a greater range of pathology and allowing more time for exercise to mediate pathology progression would be needed to detect significant differences between sedentary and exercised animals. Indeed, in contrast to the present study where the four-week-long exercise intervention did not visibly reduce intracellular accumulation of Aβ either (Figure 6), the previously studied interventions extended over the stage of amyloid pathology where plaques form and found reduced amyloid pathology accompanying the improved recognition memory [154, 155].

Interestingly, the Ex6 rats showed significantly lower DI values compared to Sed6 rats when considering the first two minutes of the NORT test, and a similar tendency when also taking into account the last two minutes of the test (Figure 4), hence suggesting a worsening effect of the four-week intensive exercise intervention on recognition memory. Therefore, the effects of the exercise training in McGill-R-Thy1-APP rats might be even opposite depending on the stage of AD when the training is initiated. No earlier studies in transgenic AD models have applied exercise training at two different stages of AD pathology and investigated its effect on recognition memory, but studies with only symptomatic AD mice reported neither significant beneficial nor deteriorating effects of exercise on performance in NORT [166, 170]. Hence, the effects of later onset exercise on recognition memory may be limited or even deleterious. However, as the Ex6 rats were tested both at the age of 4 and 6 months, the possibility that the rats would have remembered the test environment and the objects from the first test to the second cannot be excluded, and it might have confounded these results.

5.3.2 Fear learning and memory

Contrary to the majority of previous studies reporting improved fear learning and memory after exercise training [153, 163, 176], the Sed3 rats showed greater freezing behaviour throughout the different phases of the FC test when compared to the Ex3 rats, although the difference reached statistical significance only during the tone-shock presentations in the conditioning phase (Figure 5a). The greater baseline freezing of the Sed3 rats compared to the Ex3 rats, although not significant, may reflect greater stress and anxiety in these rats which might have resulted in increased freezing behaviour throughout the test [210]. Greater anxiety could originate from differences in handling time and time under anaesthesia; where both the Ex3 and Sed3 rats were handled due to injections two to three times a week, the Ex3 rats were additionally handled five times a week for the four weeks of exercise training preceding the FC test. Also, the Sed3 rats received intravenous injections of saline in tail, and while these additional injections did

not add the number of times anaesthetized, they undoubtedly increased the time spent under anaesthesia.

The Ex6 and Sed6 rats showed no significant differences in freezing behaviour during any phase of the FC test, although they almost reached statistical significance in the test of contextual fear recall (P=0.0519, Figure 5d, e, and f). Therefore, the high-intensity exercise training may also have an impairing effect on contextual recall when initiated at a post-symptomatic stage of AD. Although utilizing a passive avoidance test instead of FC test, another study also reported no effects of exercise on fear learning or memory when initiated after the onset of cognitive symptoms [165]. As the Ex6 rats were tested also at the age of 4 months, the notion that they would have remembered the arena and context until the post-training test cannot be excluded. Indeed, these rats showed rather high freezing behaviour during the first 120 seconds of the post-test (Baseline, Figure 5d), which might reflect retained memory of the environment and the associated aversive event.

5.4 The effects of exercise training on amyloid pathology

The McGill-R-Thy1-APP rats show progressive intracellular accumulation of Aβ from as early as one week of age with first plaque pathology displayed from the age of 6 months [136]. In the present study, the effects of high-intensity interval training on amyloid pathology were investigated by staining coronal brain sections utilizing Aβ-specific antibody McSA1 [136, 138]. The form of intracellular Aβ was not determined, but earlier investigations of this transgenic model have shown that a considerable portion of the Aβ in this rat AD model is oligomeric [136].

In line with previous findings [136], the 3-month-old Sed3 rats showed substantial intracellular accumulation of Aβ within the cortical and hippocampal regions of the brain (Figure 6a), and the 6-month-old Sed6 rats showed further progressed intracellular accumulation accompanied by first extracellular deposits within the subiculum (Figure 6c). It was not possible to conclude significant differences in the level of intracellular Aβ accumulation between the Sed3 and Ex3 rats (Figure 6 a and b), suggesting minor effects at most of exercise on soluble Aβ when initiated months before the appearance of plaque pathology. Other studies have commonly reported reduced hippocampal and/or cortical Aβ following exercise initiated before cognitive impairment [152, 153, 157, 158, 161-165, 167, 176]. However, the duration of the exercise period in these studies varied from 9 weeks to 12 months and was hence significantly longer than the four weeks in the present study. The findings of the present study are also limited by the visual estimation of the degree of intracellular Aβ accumulation which might have been too insensitive to reveal smaller and potentially significant differences between the Sed3 and Ex3 rats.

Interestingly, the Ex6 rats showed more progressed amyloid pathology, with regard to both intracellular accumulation and plaque deposition, compared to the Sed6 rats (Table 2, Figure 6c and d). In the brain of the Sed6 rats only a few plaques were detected in the subiculum, whereas the Ex6 rats also displayed plaque pathology in several other areas of the hippocampal formation, including CA1, CA3, and dentate gyrus, as well as in the neocortex (Table 2). In regards to the progression of amyloid pathology, these findings are well in line with the previous characterization of amyloid pathology in this AD model describing subiculum as the region commonly displaying the earliest plaque deposition and subsequent deposition in other hippocampal areas and the neocortex [136]. However, these findings are contrary to what has been reported in transgenic AD models following exercise initiated at a post-symptomatic stage; several studies found reduced

hippocampal and/or cortical Aβ levels with either voluntary wheel running or treadmill exercise training [158, 164, 165, 169, 172, 173], and some also found significantly reduced plaque load [166, 180]. Still, similar to present findings, studies with younger and older groups of AD mice found reduced effect of later exercise training on amyloid pathology as demonstrated by reduced soluble Aβ or plaque load in younger, but not in older mice [158, 165]. Furthermore, one study where AD mice were given access to a running wheel after plaque deposition already occurred found the volume of running positively correlated with the number and area of amyloid plaques [170]. The voluntary nature of running in that study does, however, make it impossible to conclude that a greater volume of exercise would have resulted in a greater plaque deposition, and the causality might even be the opposite [170]. The low number of rats from each group and the visual estimation-based analysis were limitations of the amyloid pathology assessments in the present study. However, the brain sections from the rats of same groups showed similar Aβ immunoreactivity and plaque numbers (Table 2; Figure 6), suggesting no significant effect of exercise on amyloid pathology in the younger rats but an accelerating effect in the older rats. These results could be verified by performing later stereological quantification of plaques using remaining histological series.

Previous studies in McGill-R-Thy1-APP rats have shown the progression of amyloid pathology to be accompanied by inflammatory responses [44, 62, 136]. Specifically, increased levels of a proinflammatory marker interleukin-6 were found within the subiculum already at the age of 2 months [44], and several proinflammatory markers were upregulated within the hippocampal and cortical areas at the age of 4 months with further increases with aging [62]. Furthermore, these studies found evidence of hippocampal microglial activation in 5-month-old [44], and both microglial and astrocytic activation in 6-month-old rats [62]. Therefore, changes in glial cell function from anti-inflammatory to proinflammatory in the Ex6 rats might explain the differences in the effects of high-intensity exercise on amyloid pathology and cognitive functions in the Ex3 and Ex6 rats. The highly intensive exercise training and possible stress from frequent handling and anaesthesia in combination with altered glial function might have exacerbated oxidative stress and inflammation [217-219], hence promoting amyloid pathology (Figure 6), impairing hippocampal neurogenesis and synaptic plasticity [219-221], and ultimately manifesting as deterioration of memory function (Figures 4 and 5) in the Ex6 rats. In contrast, in the Ex3 rats with supposedly unaltered glial function and better anti-inflammatory capacity, the same exercise intervention might have reduced inflammation as commonly found [222], eventually resulting in tendency to improve recognition memory (Figure 4). However, none of these potential underlying mechanisms were investigated in the present study and should be assessed in future investigations instead to explain why later initiation of intensive exercise training may be detrimental for AD pathogenesis.

6 Conclusion

In conclusion, the results of the present study showed that four weeks of high-intensity interval training initiated before cognitive impairment does not significantly attenuate intracellular Aβ accumulation or improve recognition memory or fear-associated learning and memory in transgenic McGill-R-Thy1-APP rat model of AD. Instead, when initiated at a more progressed stage characterized by cognitive impairment, similar long-term exercise may accelerate Aβ accumulation and plaque deposition, as well as impair recognition memory and fear-associated contextual memory. These findings may suggest that at least when highly intensive, exercise training might have adverse effects on the amyloid pathology and cognitive functions in early AD. However, the McGill-R-Thy1-APP rats do not express AD-like tau pathology and although a fraction of AD cases are caused by the same or similar mutations expressed in this rat model, most AD cases are thought to be of a multifactorial cause, which limits the translatability of these findings.

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Appendices

Appendix 1: Novel object recognition test protocol

Appendix 2: Fear conditioning test protocol

Appendix 3: Immunostaining protocol

Appendix 4: Solutions for perfusion and immunostaining

Appendix 1: Novel object recognition test protocol

Before starting:

- Make sure the light is set to approximately 40 lux and play calm classical music in the background.
- Make 10 % ethanol in a spray bottle to clean the apparatus with after every habituation/familiarization/test to remove olfactory cues between test phases.
- Always go in from different sides of the apparatus, to place the rat (each phase of the test is performed with rats entering from the same side; e.g. all rats from the north in familiarization 1, all rats from the west in familiarization 2, all rats from the south in test phase).
- Only one experimenter is present during testing. The experimenter must handle the rats with calm movements and be quiet during the tests. One rat is tested at a time.
- For habituation to the experimenter, let the rats sit in your lap for 30 minutes a day, for two days.

Day 1: Habituation phase

- Let the rat explore the empty apparatus for 15 minutes

Day 2, 3 and 4: Object familiarization phase

- Acclimate the rat in a cage in the testing room while cleaning the apparatus and getting ready (5 minutes).
- Place the 2 identical objects diagonally opposite each other x cm from the edge of the open field.
- Start the recording, place the rat in the box at the center of the apparatus so that its head points between the objects (not towards either of them), and let it explore the objects for 10 minutes.
- Repeat the familiarization 24 and 48 hours later.

Day 5: Test phase

- Exchange one of the objects with a novel one (new shape and color).
- Start the recording, place the rat in the box with at the center of the apparatus, and let it explore the objects for 4 minutes.

Appendix 2: Fear conditioning test protocol

Before starting:

- Scent with 1 % vanilla extract and clean with ethanol 10% between animals (day 1-3).
- To create a different environment for the fear memory retention test (day 4), use circular arena, change distal cue; scent with 1 % peppermint extract and clean with acetic acid 1% between animals.

Day 1: Habituation

- Let the rat explore the chamber for 5 min.

Day 2: Conditioning (total 8 min)

- Turn on speakers and shock box. Check that the current is set to 0.6 mA.
- Baseline - 120 sec phase of exploration.
- 30 sec tone (70 dB, 2 kHz) co-terminated with a 2 sec foot shock (0.5 mA).
- 120 second intertrial wait.
- 30 sec tone (70 dB, 2 kHz) co-terminated with a 2 sec foot shock (0.5 mA).
- Recover – 180 sec before returning to home cage

Day 3: Contextual fear conditioning

- Do not disconnect the shock box. It must be connected to run the test in Anymaze.
- Put the rat in the chamber for 8 min

Day 4: Conditioned fear response (new arena)

- Change to circular arena (painted bucket), remove the plate underneath the arena, change the distal cue (from white square to purple circle), wash with acetic acid and use peppermint extract.
- Turn on speakers and shock box.
- Have a transport cage in the testing room to put the rat in while you clean after test.
- Don’t take cage into room. Pick up rat in a towel and carry to testing room, straight into test.
- Baseline – 120 sec exploration
- Three consecutive tone presentations (30 sec, 70 dB, 2 kHz), each separated by a 30 sec pause.
- Recover – 8 minutes in total in arena.

Appendix 3: Immunostaining protocol

Day 1

1. Leave sections in 0,125M PB at 60°C for 2 hours.
2. Rinse sections 2 x 10 min in PB.
3. Rinse sections 1 x 10 min in TBS-Tx.
4. Incubate for 30 min with 10% goat serum in TBS-Tx.
5. Draw off excess solution (do not wash).
6. Incubate with primary antibody, McSA1, 1:4000 in TBS-Tx, overnight at +4° C

Day 2

1. Rinse 3 x 10 min in TBS-Tx. Mix secondary antibody during first wash and start dissolving DAB during second wash. Leave DAB on heated stirrer for 2 hours, then leave on bench.
2. Incubate with secondary antibody, biotinylated goat anti-mouse, 1:200 in TBS-Tx, 90 min in room temperature. Mix ABC right before next step.
3. Rinse sections 3 x 10 min in TBS-Tx.
4. Incubate with ABC for 90 min in room temperature.
5. Rinse 3 x 10 min in TBS-Tx.
6. Rinse 2 x 5 min in Tris-HCl.
7. Incubate with DAB for 2 minutes.
8. Rinse 2 x 5 min in Tris-HCl.
9. Mount sections in Tris-HCl on Superfrost slides and let them dry.
10. Coverslip with Toluene and Entellan.

ABC

From the ABC-kit, put 4 drop of solution A and 4 drop of solution B in 20 mL TBS-Tx. Mix well and leave on the bench for 30 min before use.

DAB

Dissolve 1 tablet (10 mg) in 15 mL Tris-HCl by leaving it on a stirrer with heat (max 50°C) for about 2 hours. Add 12 µL H_2O_2 just before use and filtrate. For dissolving the DAB use a disposable brain cup and for filtrating use a disposable syringe with filter. Remember that DAB and H_2O_2 are hazardous chemicals. Wear gloves, work in the hood, and put the DAB-solution and the first HCl-waste in the DAB waste-bottle.

Appendix 4: Solutions for perfusion and immunostaining

2% dimethyl sulfoxide

- For 100 ml: 31.25 ml 400 mM PB, 46.75 ml H2O, 20 ml glycerine, 2 ml dimethyl sulfoxide.

PB 0.4M pH 7.4

- A (acid): Sodium dihydrogen phosphate (NaH2PO4H2O) 27.6 g/500 ml H2O.
- B (base): Sodium hydrogen phosphate dihydrate (Na2HPO42H2O) 35.6 g/500 ml H2O.
- Make solutions A and B. Add solution A to solution B until the pH is 7.4. Store in the dark in room temperature for up to one month.

PB 0.125M pH 7.4

- Dilute 0.4M phosphate buffer. Store at 4°C for up to one week.
- For 500 ml: 146 ml 0.4M PB + 344 ml H2O.

Ringer solution

- 0,85 % NaCl (2,125 g/250 ml H2O).
- 0,025 % KCl (0,0625 g/250ml H2O).
- 0,02 % NaHCO3 (0,05 g/250 ml H2O).
- Filter with funnel and filter paper. Heat to about 40 °C before use.
- Set pH to 6,9 using Carbogen (95% O2, 5% CO2).

10 % Paraformaldehyde

- Heat 100 ml of H2O to 60°C in the microwave oven.
- In a ventilated hood, measure 10 g of paraformaldehyde and add to the water.
- Add a few drops of 10M NaOH and leave the solution on hot stirrer until solution is clear.

4% Paraformaldehyde

- In a ventilated hood, mix: 100 ml 10 % PFA, 78 ml 0,4 M phosphate buffer, and 72 ml H2O.
- Set the pH to 7,4 using HCl and filter using filter paper and funnel.

Tris-buffered saline pH 8.0

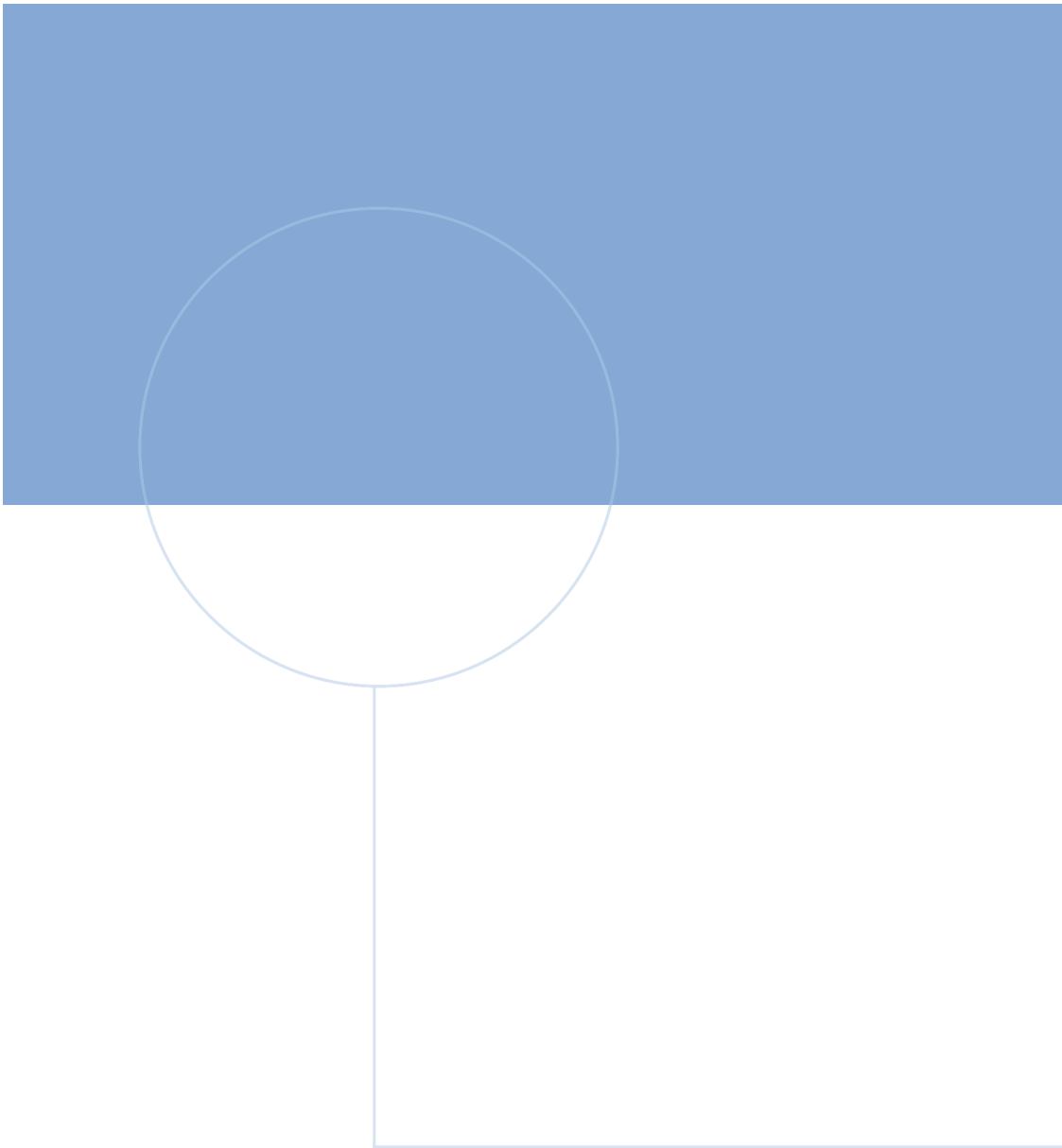
- 500 ml: 3.03 g Tris and 4.48 g NaCl in 500 ml H2O.
- Adjust pH to 8.0 with HCl. Store in refrigerator for up to one week.

TBS-Tx (0.5%) pH 8.0

- 500 ml: 3.03 g Tris and 4.48 g NaCl in 500 ml H2O.
- Use HCl to adjust the pH to 8.0. In a ventilated hood, add 2.5 ml Triton X-100 and mix well. Store in refrigerator for up to one week.

Tris-HCl pH 7.6

- 500 ml: 3.03 g Tris in 500 ml H2O
- Use HCl to adjust the pH to 7.6. Store in refrigerator for up to one week.



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