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Cognitive function during disease progression in a rat model of Alzheimer's disease

Effect of a single bout of exhaustive aerobic exercise

Master's thesis

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This master's thesis was carried out at the Cardiac Exercise Research Group (CERG), at Department of Circulation and Medical imaging, Faculty of Medicine and Health Sciences at The Norwegian University of Science and technology (NTNU), and completes the master's program in Molecular Medicine. The main purpose of the thesis was to characterize baseline cognitive function during disease progression and post exercise in a transgenic rat model of Alzheimer's disease. In addition, fitness level and presence of amyloid plaques in brain sections from the rats was also determined.

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Abstract

Alzheimer's disease (AD), the most prevalent form of dementia, is a devastating neurodegenerative disorder affecting over 35 million people worldwide. As age is the main risk factor for AD, and no current treatment options are effective, one expects a substantial increase in the prevalence of AD, alongside the rapidly aging population. However, promising studies suggest that exercise may ameliorate or prevent the progression of AD. Even a single bout of exercise can give acute beneficial neurological changes. It is hypothesized that exercise-induced blood borne factors can exert protective effects on neurons. Based on this, there is evidence to suggest that even one single bout of exercise could have a positive effect on short-term cognitive function. This project aimed to characterize cognitive function during disease progression and the potential effect of a single bout of exhaustive aerobic exercise in a transgenic rat model of AD, McGill-R-Thy1-App.

This project consisted of two studies. Study 1 was a characterization of cognitive function in the aging AD rat model at 3, 6 and 12 months of age, fitness level and presence of amyloid plaques in the brains of rats at 6 and 12 months of age. The aim was to determine cognitive function using the Novel Object Recognition test (NORT) and Novel Object Localization test (NOLT), fitness level measured as peak oxygen uptake and amyloid plaque load in coronal brain sections stained with 3,3'-Diaminobenzidine (DAB) and McSA1. It also aimed to refine the two cognitive tests used. Study 2 aimed to determine the effect of a single bout of exercise on cognitive function in the AD rat model at 6 and 12 months of age.

Cognitive impairment was evident from 3 months of age, however the cognitive test results did not show further impairment at 6 and 12 months, and rats aged 12 months were too sedentary for effective testing. The NORT engaged the rats to a greater extent than NOLT. Amyloid plaque pathology was present in brain sections from transgenic rats at 6 months of age, mainly in the subiculum, but also the dentate gyrus, CA1 and entorhinal cortex, while at 12 months they had large amounts of amyloid plaques in those areas, as well as in several other brain regions. The visually estimated amount of amyloid plaques found seemed to increase with decreasing cognitive function. One bout of aerobic exercise at 6 and 12 months of age was not sufficient to improve cognitive function with the current protocol and exercise program.

Abbreviations:

AD – Alzheimer's disease

APP – Amyloid precursor protein

Aβ – Amyloid beta

CVD – Cardiovascular disease

DAB - 3,3'-Diaminobenzidine

DI – Discrimination index

LTP – Long-term potentiation

NOLT – Novel Object Localization test

NORT - Novel Object Recognition test

PB – Phosphate buffer

VO_2 peak - Peak oxygen uptake

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1. Introduction

1.1 Alzheimer's disease

The neurodegenerative disorder Alzheimer's disease (AD) is the most prevalent form of dementia [1]. The disease starts in the lateral entorhinal cortex and hippocampus, located in the medial temporal lobe of the brain, before spreading to other areas of the brain as the disease advances. The lateral entorhinal cortex is considered a gateway to the hippocampus, which is important for short-term, long-term and spatial memory, learning and navigation [2, 3]. Over time AD spreads to other areas of the cerebral cortex, responsible for among other reasoning and language, particularly to the parietal cortex [4]. The disease progresses gradually, and in the final stage the whole brain is affected.

AD is a progressive disease that causes degeneration of the brain, resulting in neuronal death and cognitive decline [6]. Patients start experiencing changes in thinking and behaviour, and develop symptoms of memory loss that typically start mildly and gradually deteriorate until they are no longer able to take care of themselves [7]. Examples of these changes are problems with writing, speaking and processing visual images. The resulting complications from the disease are ultimately fatal. For instance, AD can eventually result in death by aspiration pneumonia due to inability to properly swallow food. The lack of movement can make patients bedridden and exposed to blood clot development, or malnutrition from weight loss can weaken the immune system and expose the patient to serious infections [8-10].

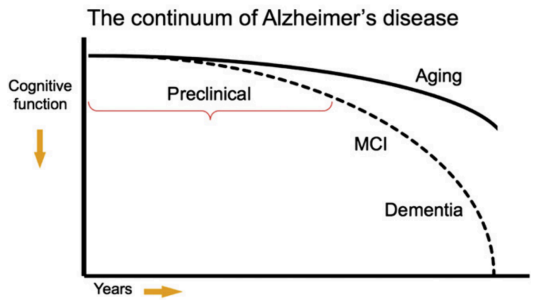

Figure 1.1: The impairment in cognitive function in dementia compared to normal aging [5].

As seen in figure 1.1, the decline in cognitive function due to dementia is much more rapid than in normal aging [11]. Before clinical manifestation and diagnosis, AD has a preclinical, non-symptomatic phase as long as 20-30 years [12]. The duration of the deterioration phase from diagnosis of AD to death varies depending on sex, ethnicity and other modifiable and non-modifiable factors described in sections 1.3 and 1.4. In general an AD patient diagnosed at age 65 or older will survive for about 4 to 8 years, even though some

can live 20 years after they are diagnosed [13-16].

Worldwide, an estimated 46.8 million people suffers from dementia, where AD accounts for 60-80 percent of these cases. In the United States, one person is diagnosed with AD every minute and the annual number of new cases is expected to double by 2050 [13, 18]. This increased AD incidence is mostly due to demographic aging with increased life expectancy and population growth, which together with living conditions is different for high-income countries and low/middle-income countries, resulting in the differences between the two seen in figure 1.2 [19].

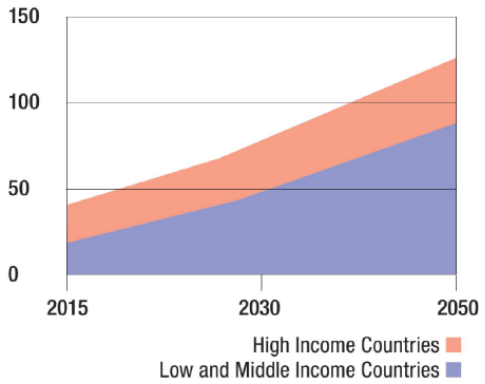

Figure 1.2: The number of people with dementia (millions) is expected to increase over the next years. Figure from the World Alzheimer report 2015 [17].

The economic impact of AD is huge. On average, a patient with dementia costs considerably more to take care of than patients with conditions as cancer, stroke and heart disease [6, 21]. The costs per patient are compared in figure 1.3, and the difference is mainly due to the fact that a dementia patient in general will need on-going close-care follow-up for a longer duration of time compared to the other diseases. In addition to the costs, patients with dementia in the US occupy up to 25 % of hospital beds at any time [22]. Considering this and the fact that age is the major non-modifiable risk factor for cognitive decline and development of AD, the world stands before huge public health challenges [18]. Therefore, optimized prevention, diagnostics and treatment of AD are much needed [23].

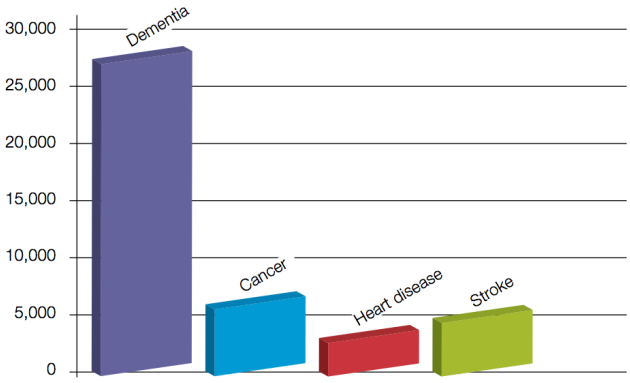

Figure 1.3: The annual costs of dementia in pounds (£) compared to other diseases for one patient [20].

1.2 AD etiology and neuropathology

1.2.1 Etiology and disease development

The etiology, and hallmarks, of AD are the presence of extracellular amyloid plaques and neurofibrillary tangles in the brain, leading to neurodegeneration [7]. Neurodegeneration

includes loss of function, or structure, and death of neurons, causing the brain to shrink, as shown in figure 1.4. In the AD brain, the folds on the surface are narrower and with wider gaps, than the healthy brain. This damage to the cortex impairs language, thinking and planning abilities. The ventricles, fluid filled spaces in the brain, become severely enlarged.

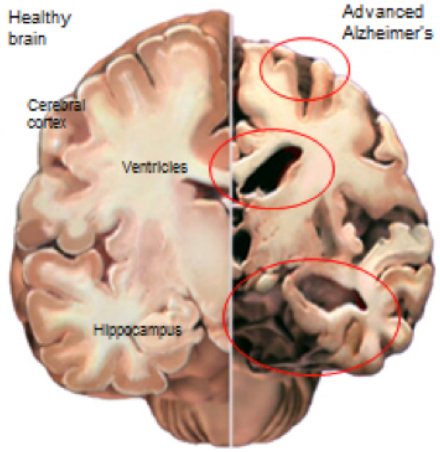

Figure 1.4: The Alzheimer brain shrinks in size due to the neuronal death, compared to a healthy brain. Figure modified from Alzheimer's Association [24].

The effect of AD on the hippocampus

As the disease starts in the lateral entorhinal cortex and hippocampus, the damage is most severe in this area [2]. In the hippocampus, these pathological features lead to impaired hippocampal neurogenesis [25], a reduction in hippocampal plasticity (the hippocampus' ability to grow and change phenotype in response to the environment) [26-29], and causes memory loss and disorientation. As the damage to the hippocampus continues, the damage to the brain spreads to other regions.

The effect of AD on other brain regions

For the cerebral cortex, damage of the frontal lobe causes psychological problems, making decision making and controlling impulses hard. The parietal, occipital and temporal lobe are also important brain regions damaged by AD. Furthermore, the corpus callosum, located below the cerebral cortex, allowing communication between the two brain hemispheres, is damaged by AD. So is the thalamus, located below the corpus callosum, which is important for relaying sensory signals to the cerebral cortex. [30-32].

When the disease reaches the hypothalamus, below the thalamus, the autonomic system with production of hormones that among other control sleep, metabolism and temperature regulation is affected. The perception of emotions is affected as the amygdala, in the temporal lobe, becomes damaged, and muscle movement, balance and speech are affected when the damage reaches the cerebellum [30-32].

Along the disease progress, neuronal connections within the brain are lost, causing the severe symptoms seen in the patients. In advanced cases of AD, the whole brain is affected, as illustrated in figure 1.4 [33].

The fundamental cause of AD is complex, multifactorial and not fully understood, but several hypotheses exist. None of these causes advanced AD on its own, as some might initiate the disease, some might be driving or exacerbating progression and others might just be a consequence, but combined they could explain the disease. The hypotheses are described in sections 1.2.2-1.2.6.

1.2.2 Amyloid cascade hypothesis

The amyloid cascade hypothesis has dominated AD research for the last 20 years, stating that amyloid β (Aβ) deposition and accumulation in the brain are central events in the development of AD [35]. Animal studies have shown the first amyloid deposition to occur in the subiculum in the hippocampus [36-38]. Aβ originates from APP, which is a single-pass transmembrane protein that is metabolized by a series of proteases. In the healthy brain, APP is related to synapse development and neuronal plasticity, although its specific function is unclear [39]. In the AD brain, extracellular amyloid plaques (figure 1.5) are formed as a result of a proteolytic cleavage of the APP into smaller fragments of Aβ [40]. Normally, the amyloid that is cleaved from APP is degraded and cleared via the blood brain barrier by degradation pathways [41], but in AD there is an imbalance between accumulation and degradation of Aβ. This leads to the formation of neurotoxic plaques causing neuronal death, that results in a progressive loss of function [42].

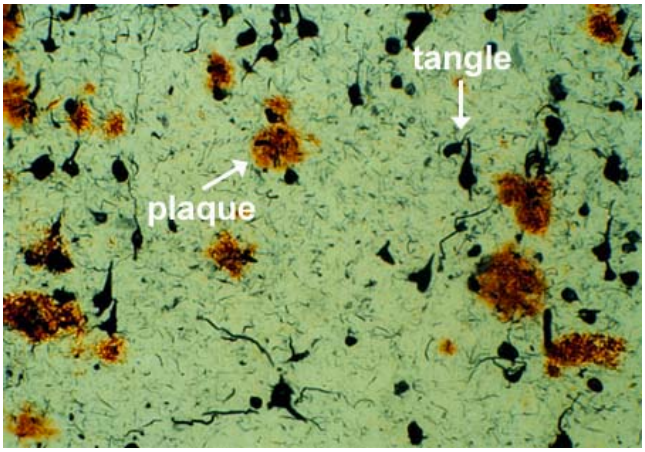

Figure 1.5: Brain section from an AD patient, showing amyloids plaques (extracellular) and hyperphosphorylated tau protein (tangles, intracellular) [34].

APP is cleaved by the proteolytic enzymes α-, β- and γ-secretase, and the two most common peptide isoforms formed are Aβ with 40 amino acids (Aβ40) and Aβ with 42 amino acids (Aβ42). The α- and β-secretase cleave APP in its N-terminal end, before the γ-secretase cleaves this product into the Aβ that is deposited in AD. β-secretase 1 (BACE1) is the transmembrane aspartyl protease responsible for the β-secretase processing [43]. Of the two peptides mentioned, Aβ40 is a much more prevalent form than the damaging Aβ42. In the majority of patients, Aβ42 is the peptide that aggregates and deposits in the brain. Low plasma Aβ42/Aβ40 ratio is associated with an increased cortical Aβ burden [44]. While the

molecular mechanism behind this phenomenon is unknown, studies performed on this ratio have shown that this is a promising biomarker for detection of AD [45, 46].

1.2.3 Tau protein hypothesis

The neurofibrillary tangles, shown in figure 1.5, are made up of tau, a microtubule-associated protein [42]. In AD, mutations in this protein can promote abnormal hyperphosphorylated tau protein [47], which can self-aggregate into neurofibrillary tangles within the neuron. The tangles inside the neuron destroy the cytoskeleton by disintegrating microtubules, and impair the axonal transport of nutrients and other necessary supplies [48]. Altered forms of the tau protein can also be neurotoxic [49]. These factors cause neuronal cell death and impair the communication between neurons in the hippocampus, causing the symptoms seen in AD [50]. The disease causes a disturbance in proteolytic and degradative pathways, impairing the degradation of tau protein by autophagy or proteosomes [49].

1.2.4 AD and chronic inflammation

AD is a chronic disease accompanied by chronic neuroinflammation [51], and in the cerebrospinal fluid of AD patients, elevated levels of pro-inflammatory cytokines such as IL-1ß, TNF, IL-6 and IL-12 are found [52]. In response to the dying neurons, the immune system utilizes resident immune cells called microglia (figure 1.6). Microglial cells are mononuclear phagocytes that are able to recognize foreign antibodies and activate T-cells. These cells are beneficial because they are capable of engulfing damaged neurons and Aβ [53], but they might also have a detrimental role as they can harm healthy cells [51]. Chronic inflammation may be more than a consequence of AD; it may initiate the disease. As suggested by Rogers et al (2007), inflammation may be sufficient to cause neurodegeneration itself [54]. For instance, serious head trauma like a concussive insult is often accompanied by persistent inflammation. This kind of injury has shown to increase the risk of development of AD [55, 56].

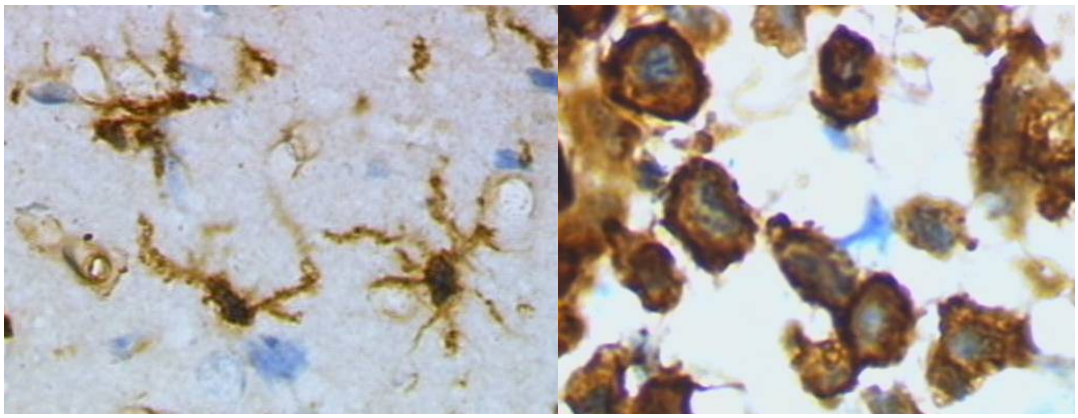

Figure 1.6: Microglial cells (brown) surrounding nerve fibers (blue) in the rat cortex. To the left are microglial cells in a resting state [57], and to the right are activated microglial cells after a traumatic brain injury engulfing damaged neurons [58].

1.2.5 Oxidative stress initiates neurodegeneration

Oxidative stress, an excess of free radicals and their products, is also implicated in AD although its mechanisms remain elusive. Reduction of oxygen causes formation of reactive oxygen species, an example of which is hydrogen peroxide. Reactive oxygen species can oxidise nucleic acids, proteins and lipids and change their function and structure. Neurodegeneration is both initiated and enhanced by oxidative stress [59]. The excessive reactive oxygen species may be generated from, for instance, mitochondrial dysfunction, causing a disruption in the redox balance. In AD, tau protein and the accumulation of Aβ promotes the redox imbalance that results in oxidative stress, and this in turn promotes aggregation of Aβ and the pathology of tau. The redox imbalance seems to be part of a vicious cycle in AD [60].

1.2.6 The vascular hypothesis

AD may be both a cause and a consequence of vascular and cerebral flow problems. Due to vascular disease and aging, cerebral hypoperfusion occurs, which can be an important cause of neuronal dysfunction [61]. The reduction in cerebral blood flow is not a result of reduced metabolic demand to the brain, but of inadequate blood supply, which is caused by impaired structure and function of the cerebral blood vessels. This impairment involves a reduced blood vessel caliber, increased blood vessel density and tortuosity, periods of obstructed blood flow and atrophy [62, 63]. These changes result in an accumulation of Aβ and generation of neurofibrillary tangles [64]. This is called the vascular hypothesis [65]. Vascular events that interrupt the blood flow to the brain can also cause a type of dementia, called

vascular dementia. A stroke is an example of such an event. Some of the symptoms in vascular dementia overlap with AD, but this is not a degenerative disease. [66]

1.3 Non-modifiable risk factors for AD

There are two main types of AD, early-onset AD and late-onset AD. Early-onset AD is a less prevalent form of AD defined as diagnosis of AD before the age of 65. Most of these patients are diagnosed at 40-50 years of age and the disease duration is normally 8-10 years after diagnosis. Early-onset AD accounts for less than 5 % of AD cases and the cause is strictly genetic involving mutations in the genes *amyloid precursor protein (APP), presenilin-1 (PS1)*, and *presenilin-2 (PS2)*. *De novo* (new) mutations in these genes are rare, making the disease inheritable. The mutations are inherited autosomal dominantly and have a high penetrance [35, 67]. Moreover, *APP* is located on chromosome 21, causing individuals with Down syndrome have a higher risk of developing AD, as they carry an extra copy of this chromosome.

The greatest risk factor for developing AD is age. The most prevalent form of AD is the late-onset AD, occurring after the age of 65 [68] (see table 1.1). For the late-onset AD the genetic risk factors do not guarantee the development of disease, unlike the *APP* mutations for early-onset AD. The biggest genetic risk factor for late onset AD is *apolipoprotein E (APOE)*, which is related to amyloid clearance [35]. *APOE* is responsible for regulation of cholesterol in the brain and the expression of this gene represents a major risk factor for the late-onset AD [69]. This gene has the alleles *ε2, ε3* and *ε4*, constituting six different genotypes. While the *ε3/ε3* genotype is the most common and not the related to AD risk, the *ε4* allele has been shown to cause disturbance of the clearance of amyloid from the brain [70]. Compared to those with two normal alleles consisting of *ε2 or ε3*, individuals that are heterozygous for *ε4* have a 20-30 % greater risk of developing AD, while homozygotes have a 50 % greater risk [71].

In addition to age, female sex is also a risk factor [72, 73] In the Framingham study the estimated lifetime risk for AD was 9 % for men and 17 % for women [74, 75].

Table 1.1: Non-modifiable risk factors for AD.

Risk factors	Explanation
Gene	*APP, PSEN1, PSEN2* [35] and *APOE - ε4* [70]
Age	Above the age of 65 [68]
Sex	The female sex is more exposed to developing AD [72, 73]
Down syndrome	Individuals with Down syndrome have an extra copy of chromosome 21, where the gene for APP is located [76].

1.4 Modifiable risk factors for AD

Various lifestyle factors can influence the likelihood of developing AD [77]. These factors, such as education, social interactions, depression, mental illness, brain injuries, physical activity and cardiovascular disease (CVD) are modifiable and therefore good targets for intervention to prevent or mitigate development of AD.

1.4.1 Low education and lack of social interactions

Higher education leads to a lower AD risk [78]. This is thought to be a result of the brain being more mentally stimulated, leading to increased connections between neurons and a cognitive reserve that can better compensate for changes that occur in the AD brain. These stimuli can for instance be educational or social interactions [78, 79]. A statistically significant relationship has been shown between lower cognitive reserve level and higher severity of dementia [80]. Furthermore, epidemiological data suggests that low educational attainment is the strongest modifiable population-attributable risk factor for developing the disease (odds ratio 19·1%, 95% CI 12·3-25·6) [81].

Social interactions are important to maintain good mental health. Social isolation might result in less cognitive activity, which can lead to a possibly increased AD risk or be a symptom of preclinical AD [82, 83]. Hearing loss can often result in social isolation as it can make it harder to connect with people and to participate in conversations. Moreover, depression has also been shown to be a risk factor for AD, although the mechanisms are unknown. Depression and can lead to social isolation, or the other way around [84, 85].

1.4.2 Unhealthy lifestyle and CVD

Cardiovascular disease (CVD), such as coronary heart disease, heart failure, stroke and atrial fibrillation is a risk factor for developing AD. Type 2 diabetes mellitus, hypertension, arterial stiffness, hypercholesterolemia, smoking and obesity are all CVD risk factors and are also associated with an increased AD risk. For instance, diabetes results in both vascular and oxidative stress, and smoking in oxidative stress, which is implicated in AD [85]. Interestingly, this means that both CVD and risk factors for CVD are associated with an increased risk of developing AD. This association could come from the shared risk factors or it could be a direct result of CVD as they, most importantly, cause hypoperfusion, oxidative stress and inflammation, a part of the AD etiology [73]. CVD and their risk factors can often be linked to an unhealthy and sedentary lifestyle, therefore, an unhealthy lifestyle has been associated with a greater risk of developing risk factors for CVD, and consequently also AD [73].

A longitudinal study on an aging population showed an association between smoking in mid-life and increased risk of AD, and the Rotterdam study also showed that the risk of AD is bigger in smokers than non-smokers [71, 86]. Furthermore, a healthy diet, like the Mediterranean diet, with foods such as fish, egg, vegetables and low intake of saturated fats is a beneficial approach to reduce several of the risk factors mentioned [87]. As an example, a healthy diet will reduce the risk for obesity, which also reduces the risk for CVD, which again reduces the risk for AD. Exercise can also beneficially affect the risk factors for CVD [88].

Furthermore, a poor diet can results in high LDL cholesterol, which in turn can cause cardiovascular diseases, through atherosclerosis [89]. There are two types of cholesterol; low-density lipoprotein (LDL) and high-density lipoprotein (HDL). A high serum LDL cholesterol level has been suggested to be an AD risk factor [90], by promoting APP endocytosis and cleavage into Aβ. The cholesterol causes APP and BACE1 to cluster in lipid rafts [91]. In a study on rabbits, a 2 % cholesterol-enriched diet for 2 months increased the number of Aβ immuno-positive cells significantly, showing that cholesterol induces amyloid deposits [92].

Table 1.2: Modifiable risk factors for late-onset AD.

Physical activity level	Reduces obesity and hypertension, and improves reduced endothelial function, all of which are risk factors for CVD [93]
CVD	Risk factors for CVD are risk factors for AD [73].
Low cognitive reserve	A less mentally stimulated brain due to social isolation and no or little education and cognitive training is associated with increased AD risk [85].
Depression	There is a possibility that previous depression is a risk factor for AD, but depression might also be an early prodrome of dementia [94]
Head injuries	Moderate or severe traumatic brain injuries can increase the risk of developing AD through chronic inflammation [13].
High serum LDL cholesterol	High serum LDL cholesterol promotes APP endocytosis and cleavage into Aβ, and causes APP and BACE1 to cluster in lipid rafts [91].

1.5 Role of physical exercise in AD prevention

1.5.1 Physical exercise

Physical activity (PA) is movement that is carried out by the skeletal muscles that requires energy, and can be anything from everyday activities to systematic exercise training. Exercise training is a type of PA that is planned, structured and repetitive [95], and can for instance be sports or training at a gym. Physical exercise exerts various positive effects on the body, as it is proven to counteract several diseases and health conditions, and help control weight and mood [96, 97]. PA can have a beneficial effect on mental health and symptoms of depression [98]. Leisure-time physical activity at midlife is associated with a decreased risk for AD [99]. Epidemiological studies show that the risk of developing AD in those with a high level of physical activity and/or high age-relative VO_2 peak compared with inactive/unfit counterparts is reduced by approximately 50 % [99-102].

1.5.2 AD and cardiorespiratory fitness level

A component of PA is cardiorespiratory fitness level, which is defined as "the ability of the circulatory and respiratory systems to supply fuel during sustained physical activity and to eliminate fatigue products after supplying fuel". Cardiorespiratory fitness is determined by maximal oxygen uptake (VO_2 max), where the ability to transport oxygen during exercise is measured. This is the point where the oxygen intake has reached its maximum, and no increase in effort can increase it [95]. Persons with a higher cardiorespiratory fitness have a lower risk of all-cause death and for developing CVD [103]. Fitness level is also related to the development of dementia. In a longitudinal population study in women, high cardiovascular fitness midlife, as assessed with VO_2 max, was associated with a decrease in development of dementia [104]. The same was also concluded in a similar study in men [105].

1.5.3 Exercise reduces the risk of CVD

As mentioned, PA and a high cardiovascular fitness level can reduce the risk of developing CVD, risk factors of CVD and therefore also the risk of AD via these pathways [73, 106, 107]. Most of the risk factors for CVD, for instance obesity and hypertension, can be beneficially affected by PA [88], and therefore decrease the risk of developing both CVD and AD [73]. Type 2 diabetes mellitus is a risk factor for AD [108], and in line with this, a study on humans showed that regular aerobic exercise over six months improved cognition in adults with glucose intolerance. In the study, improved cognition was measured as increased scores in memory and executive function tests [109]. In addition, they observed a decrease in plasma levels of amyloid (Aβ42), a hallmark of AD [110]. Another example of a risk factor for CVD, and therefore also AD, is hypertension. The fact that exercise can lower both diastolic and systolic blood pressure, has been widely accepted [111]. A precursor for hypertension is arterial stiffness. Arterial stiffness increases with age, but can be reduced by exercise. As an increased amount of exercise decreases the risk of CVD, this suggests that changes that occur in the cardiovascular system may affect neurons in the hippocampus [112-114].

1.5.4 Blood borne factors induced by exercise have neuroprotective effects

Physical exercise has been shown to be promising in slowing down both age-related cognitive decline and cognitive decline caused by dementia [109, 115]. It has also been associated with a reduced risk of developing dementia, including AD. Exercise can reduce the risk of developing AD up to 50 % [102]. Factors that can be beneficial for the brain, for instance vascular endothelial growth factor (VEGF), brain-derived neurotrophic factor (BDNF) and

insulin-like growth factor 1 (IGF-1), have been shown to increase in the blood following physical exercise [44, 45]. As an example, the lactate receptor hydroxycarboxylic acid receptor 1 (HCAR1) is activated in response to exercise and enhances VEGFA, which in turn stimulates angiogenesis [116]. These exercise-induced circulating factors can cross the blood-brain barrier and could potentially be protective against neurodegenerative diseases by exerting protective effects on neurons [117].

1.5.5 Exercise improves synaptic plasticity, including long-term potentiation

During learning or storage of memory in the brain, signal transmission neurons occur. With repeated stimulation of this communication, the synapse efficacy is strengthened. This process is known as long-term potentiation (LTP) and is an example of plasticity. As the brain ages, various synapses in the hippocampus show impaired LTP, which results in impaired synaptic plasticity [118]. Aβ has been shown to suppress induction and maintenance of LTP [119]. The proposed mechanism behind this is inhibition of NMDA receptor channels and disruption of protein synthesis. This can underlie the memory deficits implicated in AD [120]. Furthermore, exercise has been shown to enhance LTP in the mouse hippocampus [118].

The exercise-induced factors that can cross the blood-brain barrier may have the capacity to take part in the regulation of brain plasticity. The blood flow to the brain increases during exercise and more exercise-induced factors will cross the blood-brain barrier. For instance, in an intervention study on humans, physical exercise was shown to have several beneficial effects on the vascular hippocampal plasticity. The outcome was measured by hippocampal volume, cerebral blood flow and cerebral blood volume using magnetic resonance imaging (MRI) [110, 121].

1.5.6 Exercise increases neurogenesis and cerebral blood flow

In the adult brain, neurogenesis takes place in the subgranular zone in dentate gyrus in the hippocampus and in the subventrical zone of the lateral ventricles [122]. Neurogenesis can be affected by various factors, including age. As the brain ages, neurogenesis declines. Impaired neurogenesis takes place in AD, but it can also be enhanced as a compensatory mechanism that takes place when the brain repairs itself [25]. Exercise has a pro-neurogenic effect and increases neurogenesis. A study by Van Praag showed enhanced neurogenesis in mice following daily running during a 12-day period. Also, in a study on adult male Sprague-Dawley rats, the number of proliferating cells (using the marker Ki67) in the dentate gyrus of rats increased as a result of 3 days of physical exercise in the form of wheel running, although

increasing neurogenesis required more long term exercise, not presenting until after a minimum of 14 days [123].

As with decreased cerebral blood flow and neurogenesis in aging and AD, hippocampal dysfunction could be counteracted by protein factors with plasticity-promoting properties in young blood [3]. The neurogenic niche is a region where neurogenesis takes place and is concentrated around the brain blood vessels. This makes communication between the neurons and the systemic environment possible [112]. Aerobic exercise has been shown to improve cerebral blood flow to the neurogenic niche and to increase adult hippocampal neurogenesis [124]. The increased cerebral blood flow is related to the increased vascularisation that follows exercise.

Neuroinflammation is implicated in both aging and AD. Some studies show that long-term exercise has a role in reducing chronic inflammation, while other studies show no real effect. Since neuroinflammation is a negative modulator of neurogenesis, increased inflammation will be followed by decreased neurogenesis [125, 126].

1.5.7 Exercise reduces cognitive decline in AD

All of the conditions mentioned above*;* CVD, risk factors for CVD, increased inflammation and decreased synaptic plasticity, LTP and neurogenesis, are related to AD risk and AD pathology and can be improved by exercise [73, 106, 107, 110, 118, 121, 124-126]. In addition, exercise increases the level of various beneficial factors in the blood, as VEGF after a single bout of treadmill running at 15 and 30 m/min at 10 degrees incline for 1 hour (mice), BDNF after 10 weeks of aerobic exercise with 3 sessions per week (human), and IGF-1 after 1 hour treadmill running at 17 m/min per day for 15 days (mice) [127-129]. Also, in a study with 10 weeks of voluntary wheel running on mice Aβ burden and tau hyperphosphorylation was reduced [74]. Furthermore, depression and social isolation, which were mentioned as modifiable risk factors, can also improve with exercise. Based on this it is therefore reasonable to believe that exercise can have a positive effect on several aspects of the development AD and improve cognitive function.

Exercise is most beneficial if performed regularly over months or years, as chronic exercise may result in stronger beneficial effects than acute exercise. Nevertheless, even a single bout of exercise can give acute beneficial physiological changes, such as lowering of resting heart rate, improved vascular function and increasing BDNF levels and plasma catecholamines (the hormones epinephrine, norepinephrine and dopamine). These changes may affect the brain as

well, including cognitive function [130, 131]. Furthermore, one bout of exercise has been shown to lower 24-hour glucose level and reduce BACE1 content and activity [132, 133]. Also, evidence of regionalized brain effects and enhancement of episodic memory has been shown after one bout of exercise [134, 135]. Based on this, there is evidence to believe that even one single bout of exercise could have a positive effect on short-term cognitive function.

1.5.8 Exercise as AD treatment target

A more complete understanding of the effect of exercise on the brain could have the potential to provide treatment targets for neurodegenerative diseases such as AD. At some point, even clinical applications of exercised blood for neuroprotection can be implemented, by isolation of the beneficial neuroactive factor or factors from the blood of an exercised individual. A way of enhancing the effect of exercise pharmacologically could also be an option. Even if the result is not reversing the symptoms, just achieving maintenance or preservation of the neurons by modifying the risk factors would be a ground-breaking step [110]. Based on the aspects mentioned above, physical exercise appears to be promising in preventing AD.

1.6 Animal models for AD

1.6.1 About AD rat models

To study the pathology of AD, advance the understanding of the disease and to possibly find a treatment, genetically modified animal models of AD have been created. The most commonly used animal models are rats or mice, with different phenotypes and different ages of onset. Various models are used to display various characteristics of the disease, such as neuronal loss, plaques, tangles, gliosis (damage of glial cells), synaptic loss, changes in long-term potentiation, long-term depression and cognitive impairment. Limitations and challenges with using animal models are translational issues, as the physiology of the animal model organisms is not exactly the same as in humans. Also, it is not possible to recreate the identical disease in animal models, as the mechanisms behind AD are not fully known and it is difficult to recreate both amyloid plaque and tau pathology at once [136].

Even though no transgenic models can fully mimic early-onset AD, the rat model TGF344-AD has similar manifestations as in humans with early-onset familial AD, and displays amyloid and tau pathology, cognitive impairment and neuronal loss [137]. Likewise, the model McGill-R-Thy1-App is also similar to human pathology, and develops cognitive

impairment, inflammation, intracellular Aβ accumulation and amyloid plaques. A weakness in the McGill-R-Thy1-App model is that it does not display tau pathology or a substantial neuron loss. Still, it is a good AD model when it comes to Aβ pathology as many AD animal models do not display plaque pathology, but not in regards to neuron loss [36, 37]. Another advantage to this AD animal model it that it exhibits early signs of the disease, because it carries a double mutation (both Swedish and Indiana, explained below). This double mutation has been suggested to be beneficial for the rat model because the effect of amyloid precursor protein is dose-dependent. For instance TgAPPswe rats is a AD rat model that exhibit lower Aβ levels and no plaque pathology because they do not carry the *APP* Indiana mutation, only the Swedish. This suggests a threshold of Aβ for plaque formation and results in a milder phenotype for the rat model [138, 139].

1.6.2 The McGill-R-Thy1-App rat model

The McGill-R-Thy1-App transgenic rat model has mutations in a single transgene; cDNA encoding the human amyloid precursor protein 751 (hAPP751) (figure 1.7).

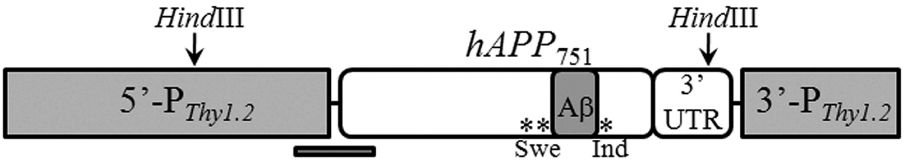

Figure 1.7: Human Amyloid Precursor Protein gene [37].

This protein is expressed in order to display AD-like-amyloid pathology. These rats carry both the double Swedish (KM670/671NL) and the Indiana (V717F) mutation, which are both under the control of the murine Thy1.2 promoter [37]. The levels of gene expression controlled by the Thy1.2 promoter are regulated by development and will increase as the brain matures, so that a neonatal rat brain will have lower levels of the promoter expressed compared to an adult brain [140]. In this rat model, the expressed levels of the *APP* transgene is high in the cortex, low in the cerebellum and not detectable in the liver, heart, kidney or thymus. The Swedish mutation is a double point mutation on exon 16, located just outside the N-terminus of the Aβ domain in *APP* (chr21:27269939 G>T, chr21:27269938 A>C), and results in β-secretase cleavage within the secretory pathway [141]. The amino acids lysine and methionine are changed to asparagine and leucine as a result of the mutation [142, 143]. The Indiana mutation is a missense point mutation, located on exon 17, 4-6 residues past the

wild-type *APP* (chr21:27264096 G>T). This mutation increases the Aβ42/Aβ40 ratio in blood plasma [144, 145].

This strain was generated at McGill University, by introducing the transgene in fertilized eggs by pronuclear injection of DNA [37]. It has an outbred genetic background (Wistar). The strain's phenotype is AD-like Aβ accumulation in the brain, which develops into plaques that can be detected in the hippocampus at 6 to 9 months in homozygous rats. In a study by Heggland et al. the first plaque pathology was detected in the subiculum at 9 months (figure 1.8) [36]. This display of the full AD-like-amyloid pathology results in inflammation and cognitive impairment, like learning and memory deficits, which can be presented from 3 months of age [37].

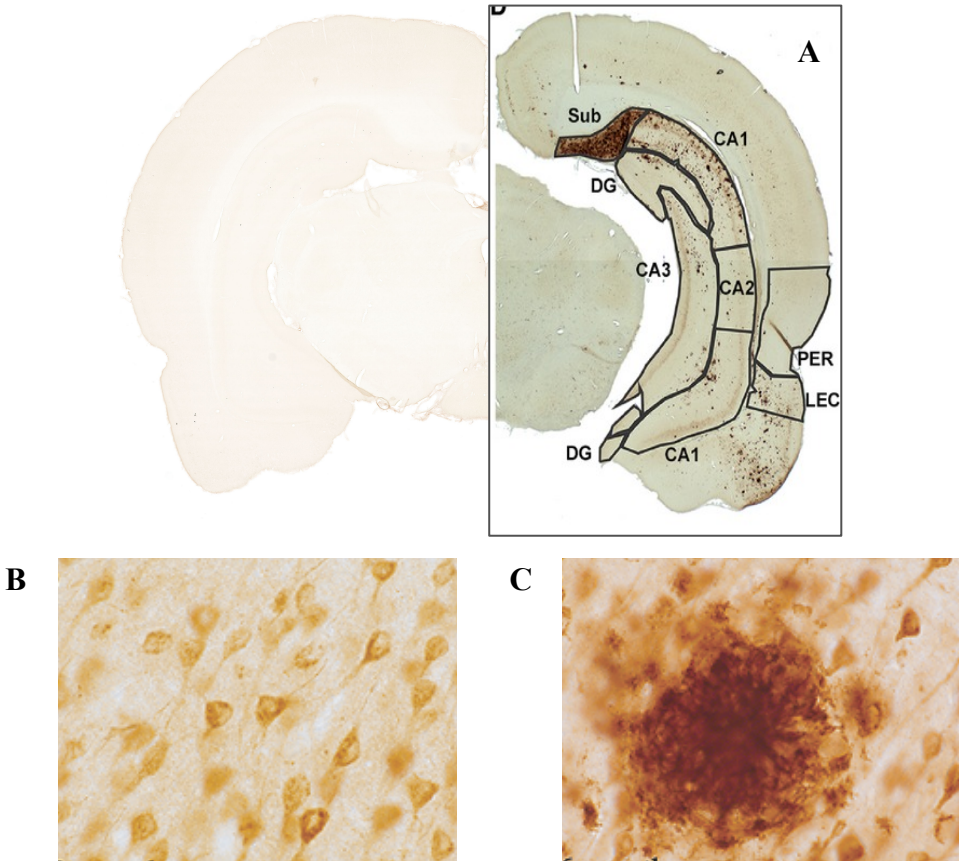

Figure 1.8: Coronal section of a McGill-R-Thy1-App rat. Figure A shows the main anatomical areas related to amyloid plaque formation in this model (Sub, subiculum; DG, dentate gyrus; CA, Cornu Ammonis; PER, perirhinal cortex; LEC, lateral entorhinal cortex). Figure adapted and modified from Heggland et al. [36]. Figure B shows an example of intracellular Aβ accumulation in the subiculum at 3 months. Figure C shows an example of extracellular Aβ plaque in the subiculum at 6 months. Figures B and C adapted from Leon et al. [37].

1.7 Cognitive function

1.7.1 Cognitive function in humans

Cognitive function is intellectual activity that involves mental processes such as memory, learning, moving and communication. Cognitive processes utilize existing knowledge to create new knowledge. As the body ages, cognitive function deteriorates naturally due to a decline in nerve cell function, while in AD the nerve cells eventually die. This is the main difference in cognitive impairment due to aging versus AD. There are several types of tests for measuring cognitive function, however there is no single behavioral marker that separates AD from other types of dementia [66].

1.7.2 Tests of cognitive function in rodents

The NORT and the NOLT are tasks used to assess recognition and spatial memory in rodents. Spatial and recognition memory are functions which depend on the medial temporal lobe structures [2]. To assess the rodent's recognition and spatial memory and thereby detect changes in cognitive function, the time it spent exploring either a novel object or a novel object location, compared to a familiar object or location respectively, is measured. Due to the natural curiosity of rodents, it is expected that rats with high functioning memory will exhibit a stronger preference for the novel object/location over a familiar object/location [147]. In the NORT a familiar object is switched with a novel object after a period of familiarization. This test is dependent on the perirhinal cortex, illustrated in figure 1.9, and measures non-spatial recognition memory [148]. In the NOLT, an object with a familiar location is switched to a new location after a period of familiarization, testing the spatial memory. This task is dependent on the hippocampus and measures spatial memory. Protocols for these cognitive tests varies greatly across studies, including the time and number of intervals for familiarization, type of object and fields used [147].

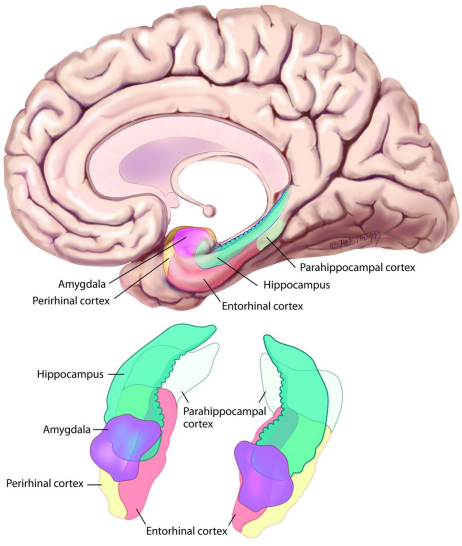

Figure 1.9: Location of the perirhinal cortex and hippocampus in the human brain [146].

The main region responsible for processing spatial information is the hippocampus, but its role in non-spatial recognition memory is controversial [149]. Studies on rats with neurotoxic

lesions can demonstrate the location of the structures responsible for the types of memory used in NORT and NOLT. Neurotoxic lesions in CA1 in the dorsal hippocampus disrupted spatial, but not object-recognition memory, showing that spatial memory is dependent on this structure [150]. Likewise, in another study, neurotoxic lesions in the fornix, a structure in and adjacent to the hippocampus, was impaired on all spatial tests, but left the recognition memory intact. Furthermore, lesions in the perirhinal cortex in the medial temporal lobe impaired recognition, but not spatial memory [148].

2. Aims

2.1 Underlying rationale for the project design

In a study by Leon et al., the McGill-R-Thy1-APP rat model's cognitive function was found to be impaired at 3 months of age [37]. In order to establish the use of this rat model for further studies in our laboratory, NORT and NOLT was used to validate and reproduce these findings. There are a variety of protocols for these tests, and this study aims to establish a valid protocol to determine cognitive function in our laboratory.

Several studies have been done on the effects on cognitive function of acute exercise on humans, but less has been done in rats and nothing in the McGill-R-Thy1-APP rat model.

2.2 Overall aims

In order to decide an appropriate age for initiating interventions in future studies, the projects **primary aim** was to reproduce findings by Leon et al. on cognitive decline in the McGill-R-Thy1-APP transgenic rat model of AD and determine whether the cognitive function status correlated with cardiorespiratory fitness level.

Secondary aims included:

- To refine protocols and method of analysis for the NORT and NOLT tests of cognitive function for future experiments in the laboratory.

- To determine the presence or absence of amyloid plaques and whether this correlated with cognitive function.

- To determine the effect of a single bout of exercise on cognitive function in McGill-R-Thy1-APP rats at 6 and 12 months.

The project consisted of two studies:

Study 1: Cognitive function during disease progression in an AD rat model, and its correlation with cardiovascular fitness level and amyloid plaque pathology.

Study 2: Effect of a single bout of exercise on cognitive function in AD rats.

2.3 Overall hypothesis

Study 1: Cognitive function, assessed by NORT and NOLT, is strongly correlated with cardiovascular fitness level assessed by VO_2 peak, and will decrease at a greater rate in transgenic McGill-R-Thy1-APP rats compared to non-transgenic control rats. The expected time for development of cognitive dysfunction is from 3 months of age. Amyloid plaque formation is expected at 6 months of age, and is expected to both spread and increase in numbers with decreased cognitive function and increased age.

Study 2: Recognition memory will improve after a single bout of exercise in both 6- and 12-month-old AD rats.

3. Materials and methods

3.1 Ethical statements

The study was carried out in accordance to the Norwegian regulation on animal experimentation. All experimental procedures were approved by the competent authority for animal research at the Norwegian Food Safety Authority (FOTS ID 11740/2017), and were in accordance with the Norwegian Animal Welfare Act §§ 1-28, Norwegian Regulations on Animal Research §§ 1-26 and European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes.

3.2 Animals

For the work in this thesis, the AD rat model McGill-R-Thy1-APP (mutations: hAPP751 Swe, Ind, Mouse Thy1.2 promoter) was used, in addition to non-transgenic littermates (wild type), provided by the laboratory of Professor Menno Witter at the Kavli Institute for Systems Neuroscience and Centre for Neural Computation at NTNU in Trondheim. The rats ranged from 3 to 12 months of age at the time of testing, weighed from 242 to 589 grams and were of both sexes. They were housed two or three together in individually ventilated cages and provided with standard rodent chow and water *ad libitum*, at 23 ˚C and 70 % humidity, on a 12-hour reversed light cycle. As environmental enrichment, the rats had access to a plastic opaque house and a wood stick. Before testing, the rats were acclimatized to the facility for two weeks. Before the cognitive tests they were habituated to the experimenter, the same person who performed all tests, through two rounds of 30 minutes handling.

3.3 Study design

In both studies, the same 46 rats were used, split in two cohorts. One cohort (n=5/0 +/+, n=4/2 +/-, n=4/2 -/-, M/F) was tested at 3 months and 6 month of age, and sacrificed following the conclusion of the tests at 6 months. The second cohort (n=5/4 +/+, n=5/5 +/-, n=4/6 -/-, M/F) was tested at 12 months of age and sacrificed following those tests.

For the detection of amyloid beta, the cohort consisted of 12 homozygous rats (both wild type and transgenic) at 6 and 12 months, divided in four groups based on age and genotype (n=3 per group, except for the 12-month-old homozygous transgenic rats where n=2). These rats were selected based on cognitive test results (DI). Rats with either a high or low DI value were selected for each group.

3.4 Methods

3.4.1 Cognitive function

To measure cognitive function, two tests where used; NORT and NOLT. Both tests were performed in an open field, called the apparatus, with two wooden objects of different sizes and colors depending on the test and test phase. After a set period, one of the objects was moved to a novel location and the time spent exploring the objects by the rat was measured. Both tests consisted of three phases:

1. **Habituation (2 x 25 min)** – to make the rat feel comfortable with being in an empty apparatus
2. **Familiarization (2 x 10 min)** – to make the rat familiar with the two objects placed in the apparatus
3. **Test (10 min)** – to test whether the rat spends more time exploring the NORT or NOLT

The first phase, habituation, was similar for both tests and was performed twice, by placing the rat in an empty apparatus for 25 minutes, with 24 hours in between habituations.

NORT: In the second phase, the two familiarizations for this test of recognition memory, two identical objects (A+A) were placed diagonally opposite each other to be explored by the rat, as shown in figure 3.1. The third and last phase was the test, where one object was switched with a novel object, and the time spent exploring the novel object by the rat gave an indication of its memory function. The natural tendency of a rodent with good memory function is to explore the novel object more than the familiar one [151].

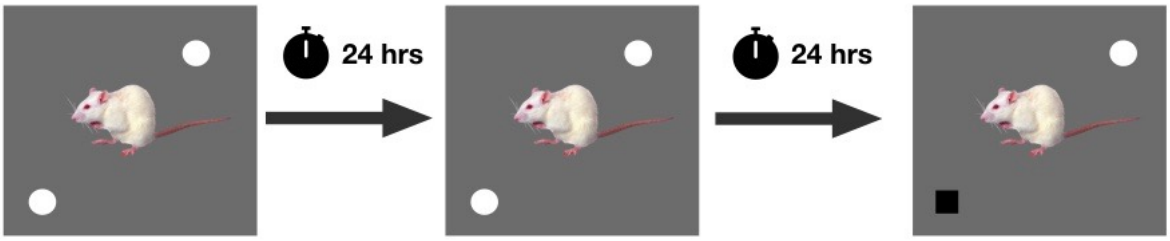

Figure 3.1: NORT, where two white sphered shaped objects (A) and a black square object (B) are used.

NOLT: For the two familiarization for the test of spatial memory, two identical objects were placed horizontally opposite. Instead of switching one of the identical objects in the test phase, as in the NORT, the location of one of the objects was changed (figure 3.2).

Figure 3.2: NOLT where two white sphered shaped objects (A) are used.

For both tests, the experimental arena was a plywood box, the apparatus, with black walls and a light brown floor. The arena and objects were cleaned with 10 % ethanol after each test in order to eliminate olfactory cues. The test room was an isolated room with no other than the experimenter and the rat in testing present, to reduce any disturbing noise. To reduce the noise coming from the experimenter (breathing, sitting down etc.), background music was played. This was calm, classical music without voices and was set to the same volume for every test. The same person they were habituated to always put the rats in the apparatus, from different sides each time. The experimenter was blinded to the age and genotype of the rats.

The apparatus was 100 x 100 centimeter with 50 centimeter tall walls, placed on top of a table with curtains around it to eliminate any outside visual stimuli. The lighting into the apparatus was approximately 60 lux. A blank A4 sheet of paper was attached to the curtain on one side of the apparatus as a distal cue. The recording camera was attached to the ceiling. Rats were tracked using the Any-maze software (Stoelting Europe, Dublin, Ireland), which is a video-

Figure 3.3: The apparatus set-up for cognitive tests, showing the recording camera, distal cue, curtains and the apparatus itself.

tracking system relying on contrast of the rat against a background. The experimental set-up is shown in figure 3.3.

Method of analysis

Zones were drawn around each object using the analysis software to discover which struck the best balance between accuracy and sensitivity, when measuring time spent by rats investigating the objects. Two types of zones were defined, a small zone and a large zone at 10 and 25 centimeters, respectively, with the object in center. The duration of each cognitive test was 10 minutes. For the purpose of the analysis the time each rat spent in the test apparatus was divided into 3 intervals; 0-2 minutes, 0-4 minutes and 0-10 minutes, to determine the appropriate time interval. To compare the results between rats and ages, discrimination index (DI) was calculated using the equation shown in figure 3.4. In the equation, T_{novel} is the time spent in the zone with the novel object and $T_{familiar}$ is the time spent in the zone with the familiar object. Rats that did not explore both objects for more than a second each after the first 4 minutes of the test, and 5 seconds after the whole duration of the test were excluded from analysis.

$$\text{DI (discrimination index)} = \frac{(T_{novel} - T_{familiar})}{(T_{novel} + Tf_{amiliar})}$$

Figure 3.4: Formula for calculation of the discrimination index.

All rats, aged 3, 6 and 12 months underwent both tests as a part of study 1, as cognitive impairment in this model has been seen from 3 months of age [37], and were randomized to perform either NORT or NOLT in week 1, and then the opposite test in week 2 (figure 3.5). In week 3, rats at 6 and 12 months were re-tested with NORT after exercise as a part of study 2. The NORT was chosen over NOLT chosen based on data obtained from the rats that had finished the two first weeks of cognitive testing. For this round of NORT, new objects were used, as shown in figure 3.6. The protocols are described in Appendix I.

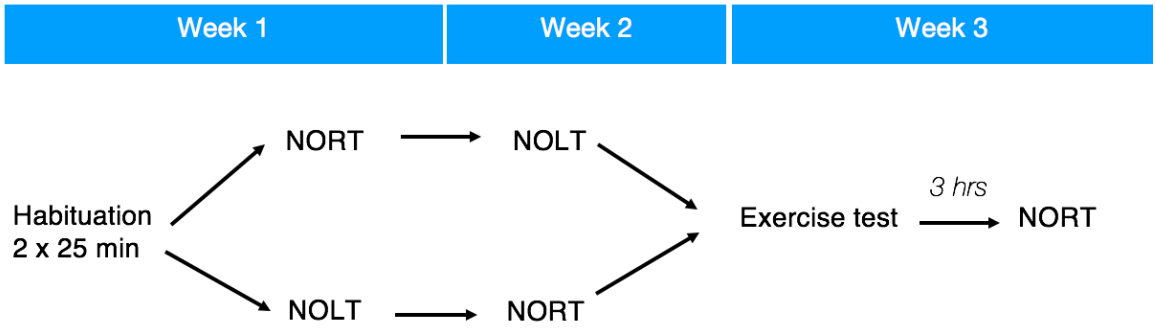

Figure 3.5: The course and order of tests for each rat in the experiment.

3.4.2 VO_2 peak test/one bout of exercise and cognitive function

The gold standard for cardiorespiratory fitness is VO_2 max, but this study utilized VO_2 peak. VO_2 peak is the highest reading achieved during a test, but without reaching the plateau where an increase in exercise does not increase VO_2. The VO_2 peak test and the bout of exercise were performed as one session, on an enclosed treadmill with 5 degree incline, and a shock grid at the bottom of the treadmill that kept the rat from sitting still. Gas in and out of the treadmill enclosure was analyzed using Columbus Oxymax hardware and software (Columbus Instruments, Massachusetts, USA). Before starting the test, rats were habituated to the apparatus 15 minutes each time, over the course of 3 days. Rats at 6 and 12 months performed the exercise and the following cognitive test (NORT).

In the test round the rats ran to exhaustion, to measure VO_2 peak. The form of exercise was chosen as it was equivalent to the exercise protocol performed in an *in vitro* study where serum from 3 hours post-exercise was found to be the best for HT22 cell survival (Tari & Scrimgeour, unpublished data). In this study, rats were acutely exercised to exhaustion, before blood was collected at different time points post-exercise. Serum from blood collected 3 hours

after the bout of exercise had the strongest effect on neural cell survival in vitro on HT22 cells, stronger than serum from blood collected before exercise. Based on those results, cognitive testing was performed 3 hours after exercise in this project.

Exhaustion for the VO_2 peak test was defined as the rat going down to the shock grid 3 times within 5 seconds or constantly staying on it. The test started with a 5-minute warm-up with starting pace 6.6 m/min for both sexes, ending at 7.4 m/min for males and 10.0 m/min for females, due to their lower workload. Thereafter, the pace was increased by 1.2 m/min every 5 min. This protocol was adapted from methods previously used in the same laboratory [152, 153]. The post-exercise cognitive testing took place in week 3 as shown in figure 3.5, and consisted of only the NORT, using two white squares for familiarization instead of white spheres, and a black sphere as the novel object (figure 3.6).

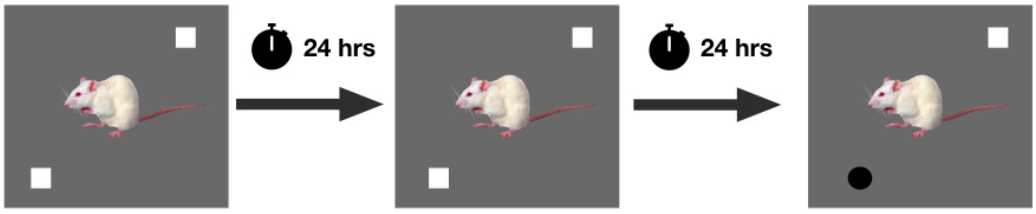

Figure 3.6: The objects used in the NORT performed 3 hours after exercise.

3.4.3 Transcardial perfusion and brain collection

Transcardial perfusions were performed to preserve the brains for future analysis. The rats were anesthetized in a chamber with 5 % isoflurane (Baxter, Puerto Rico, USA) for 2-4 minutes until unconsciousness, before receiving an intraperitoneal injection of an overdose of pentobarbital sodium. The animals were weighed in order to calculate dosage (approximately 0.2 mL/100 g). Subsequently, breathing and lack of reflexes and pain responses were checked before the procedure continued. The rat was placed back down on a perfusion grid in a fume hood, before it was cut open below the diaphragm and the rib cage to expose the heart. A needle was inserted into the left ventricle and the right atrium was cut to allow flow. The transcardial perfusions were conducted using a Unified Masterflex Drive, peristaltic pump (Thermo Fisher Scientific, Massachusetts, USA), first with Ringer's solution (145 mM NaCl, 3.35 mM KCl, 2.28 mM $NaHCO_3$) with pH adjusted to 6.9, to rinse out the blood, and then with 4 % paraformaldehyde (PFA, Merck kGaA, Darmstadt, Germany) in 125 mM phosphate buffer (PB) adjusted to pH 7.4 for fixation. The brains were dissected out of the scull and kept

in 4 % PFA for 24-48 hours, then transferred to 2% dimethyl sulfoxide (DMSO; Sigma-Aldrich, Missouri, USA) in 125 mM PB and 20% glycerol for cryoprotection in 4 °C until sectioning. See appendix III for solutions.

3.4.4 Sectioning of brain tissue

Brains were sectioned using a freezing microtome (Microm HM430, Thermo Fisher Scientific). Coronal sections were cut at 40 μm in 5 series, 1 mounted on Superfrost glass slides and the remaining put in tubes, before drying on a heating plate (37 °C) overnight.

3.4.5 Immunostaining of amyloid plaques with McSA1 (anti-Aβ)

The free-floating immunohistochemistry procedure was performed on the sections using the anti-Aβ antibody McSA1 (MédiMabs; MM-0015-P, Montreal, QC, Canada), specific for aggregates of the 39 to 43 amino acid long Aβ peptide. The sections were first unmasked in a 125 mM PB solution at 60°C to unmask the binding sites. They were then rinsed in a new round with PB for removing DMSO solution leftovers and in Tris-buffered saline with Triton-X-100 (TBS-Tx; 50 mM Tris, 150 mM NaCl, pH 8.0) for permeabilization. To block non-specific binding sites, the sections were next incubated in 10 % goat serum in TBS-Tx. Sections were then incubated with the primary antibody, McSA1, in a 1:4000 solution of TBS-Tx overnight.

The next day TBS-Tx was used for rinsing before and after incubating with secondary antibody, biotinylated goat anti-mouse that binds to the primary antibody, in a 1:200 solution of TBS-Tx. The avidin-biotin complex (ABC (Vectastain ABC kit, Vector Laboratories, California, USA) was then mixed and the sections incubated in this solution, before washing in TBS-Tx and Tris-HCl. The ABC was added to attach to the secondary antibody, giving peroxidase a binding site. The last step was incubation with 3,3'-Diaminobenzidine (DAB; 3.1 mM in Tris-HCl) added 12 μL H_2O_2 just before use, causing DAB to oxidise into brown polymers because of the cleaving of peroxidase into H_2O and O_2. The sections were then mounted on Superfrost glass in correct anatomical order and left to dry overnight on a heatingplate, before clearing with xylene, coverslipping with Toulene and Entellan. See full protocol in appendix II and solutions in appendix III.

3.4.6 Detection of amyloid plaques

Glass slides were digitalized using Slide Scanner Axio Scan.Z1 in brightfield mode with a Hitachi HV-F202SCL camera and a Plan-Apochromat 20x/0.8 objective, and processed using

the software Zen 2.3 (Carl Zeiss AG, Germany). The anatomical descriptions of amyloid plaques locations were based on the online atlases; Rat hippocampus atlas and Allen brain reference atlas [154, 155].

4. Results

4.1 Study 1: Aging characterization of AD rat model

4.1.1 Cognitive function test results

NORT

Cognitive function was impaired in homozygous transgenic rats compared to wild type rats at 3 months of age. The 3-month-old homozygous transgenic rats had a significantly lower DI values compared to wild type (one-way analysis of variance (ANOVA), $p < 0.05$), indicative of a pronounced preference for the novel object (figure 4.2C). The same tendency towards lower DI was also observed at 6 months, however this difference was not significant. As previously explained, the 12-month-old rats were from a different cohort than the 3- and 6-month-old rats. For these rats, DI values for the homozygous wild type was lower than for the transgenic rats from 4 minutes into the test. The hemizygous rats had more variable results, with both higher and lower DI values than the wild type rats, especially at 12 months (figures 4.1-4.3).

As described above, the open field apparatus was divided into a small and a large zone around each object. Results for the large zones were highly variable, suggesting that this zone was too large to specifically detect object investigation by the rats.

The optimal time interval for characterizing cognitive function in the rats was 0-4 minutes. From this point on the novelty of the object seemed to be lost and the results evened out. After 10 minutes the differences in DI in between the genotypes were less pronounced (figure 4.3). Furthermore, the results of the NORT were analyzed looking for differences between the sexes, but no differences were revealed (data not shown).

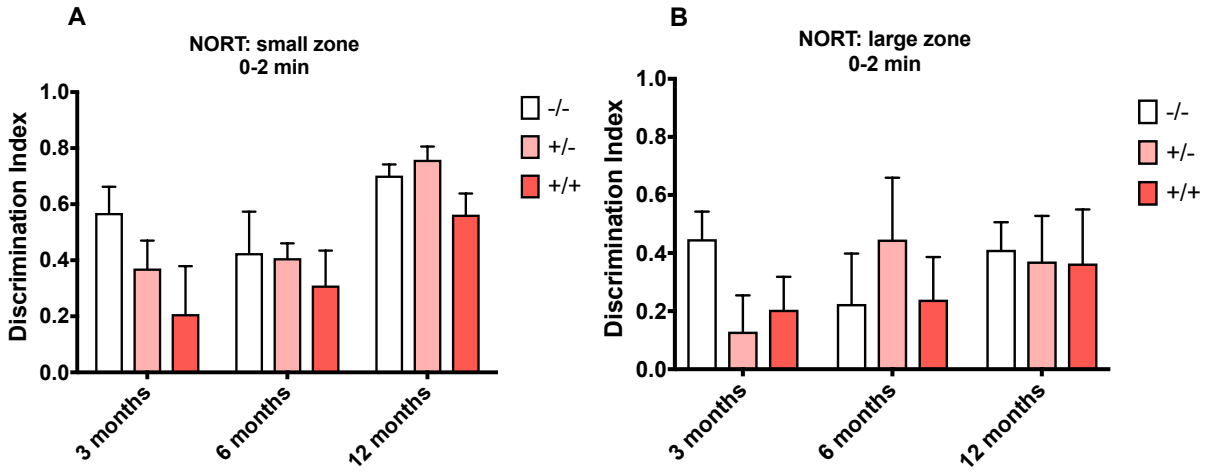

Figure 4.1: DI values for 3-, 6- and 12-month-old rats the first 2 minutes of the NORT, for small zones (10 cm) around the center of the two objects (A) and large zones (25 cm) (B). DI values from the small zone indicated a lower cognitive function in homozygous transgenic rats, compared to wild type, for all age groups. For the large zone the same pattern was seen at 3 and 12 months, but not 6 months. The cognitive test result from the small zone showed a small increase in DI value in homozygous transgenic animals with increasing age. DI values for the large zone showed the same pattern, although the increase was even smaller. For both zones, DI values for the hemizygous rats varied, from high to low, compared to the wild type.

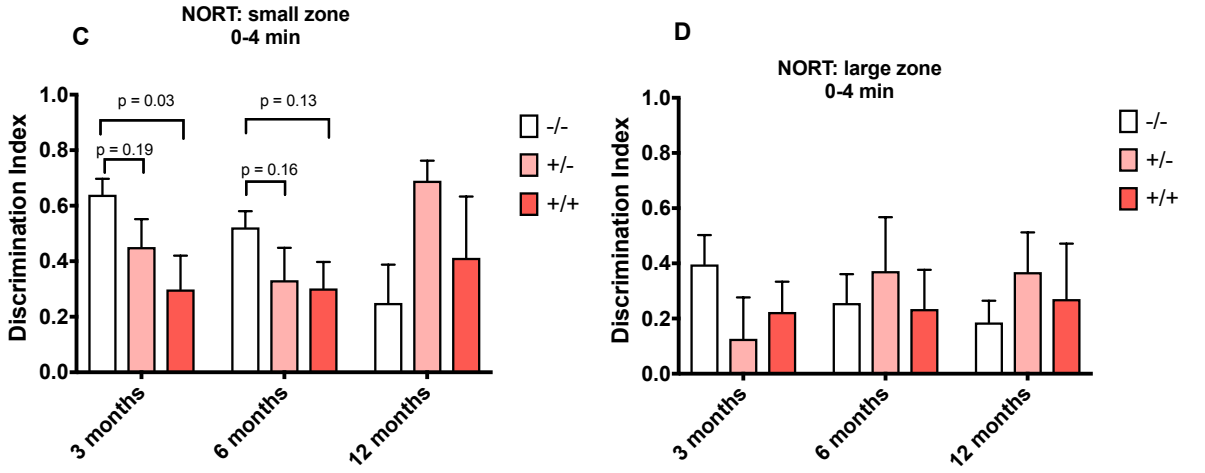

Figure 4.2: DI values for 3-, 6- and 12-month-old rats the first 4 minutes of the NORT, for small zones (10 cm) around the center of the two objects (C) and large zones (25 cm) (D). DI values from the small zone indicated a lower cognitive function in homozygous transgenic rats, compared to wild type at 3 and 6 months. Statistical analysis showed that this difference was only significant at 3 months. For the large zone, lower DI values in homozygous transgenic rats, compared to wild type genotypes are seen at 3 and 6 months, but not 12 months. The cognitive test results from the both zones showed a minor increase in DI value in homozygous transgenic rats with aging, although so minor that it was barely visible in the graphs. DI values for the hemizygous rats varied from high to low, compared to the wild type.

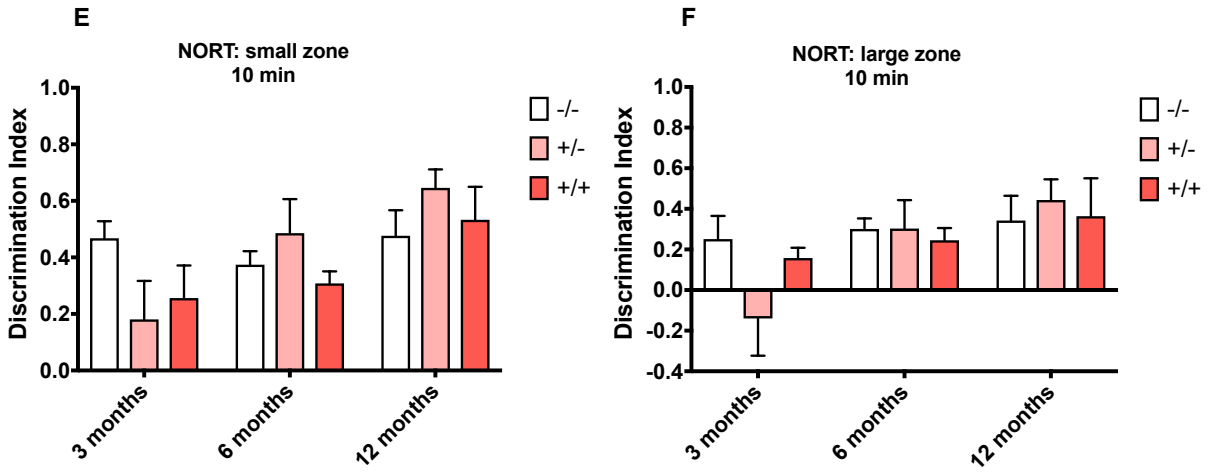

Figure 4.3: DI values for 3-, 6- and 12-month-old rats the whole 10 minutes of the NORT, for small zones (10 cm) around the center of the two objects (E) and large zones (25 cm) (F). DI values from the small zone indicated a lower cognitive function in homozygous transgenic rats, compared to wild type at 3 and 6 months, but not at 12 months. For the large zone the same pattern is seen. The cognitive test result from the both zones showed an increase in DI values for the homozygous transgenic rats with aging. DI values for the hemizygous rats varied from high to low, compared to the wild type.

NOLT

The results for the NOLT were less pronounced than for the NORT. Several DI values were around zero, for all three genotypes, showing that the rat did not show any signs of perceiving that the object location was novel, and did not prefer one object over the other (figures 4.4-4.6).

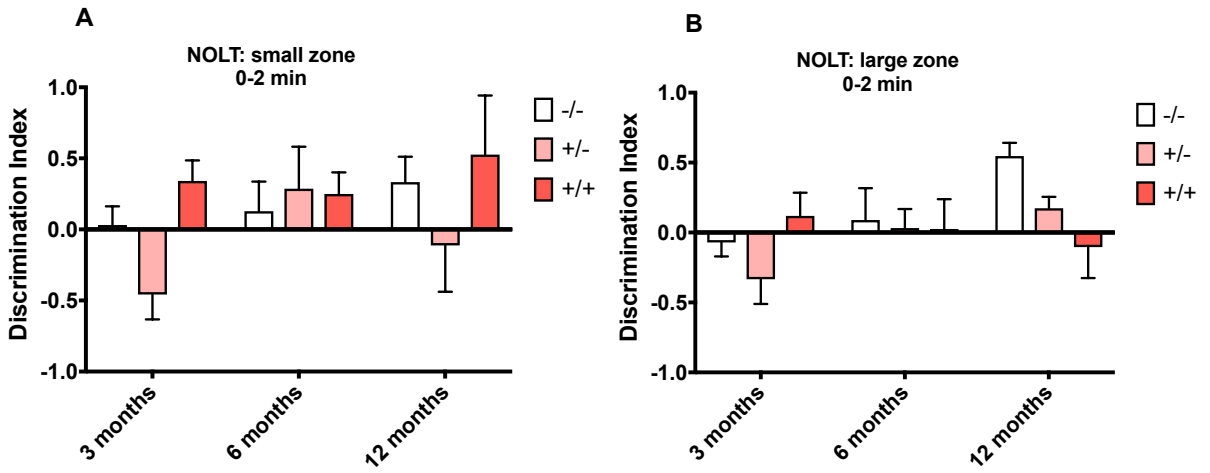

Figure 4.4: DI values for 3-, 6- and 12-month-old rats the first 2 minutes of the NOLT, for small zones (10 cm) around the center of the two objects (A) and large zones (25 cm) (B). The results were highly variable for both zones, but most showing little or no preference for the novel object. In the small zone, the homozygous transgenic rats had the highest DI values.

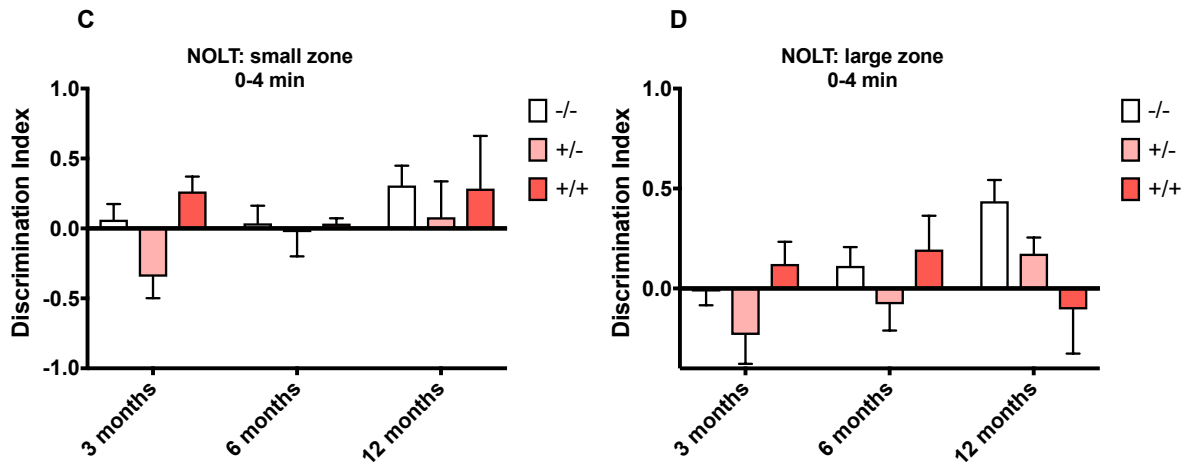

Figure 4.5: DI values for 3-, 6- and 12-month-old rats the first 4 minutes of the NOLT, for small zones (10 cm) around the center of the two objects (C) and large zones (25 cm) (D). The results were highly variable for both zones, but most showing little or no preference for the novel object. In the small zone, the homozygous transgenic rats had two out of the three highest DI values.

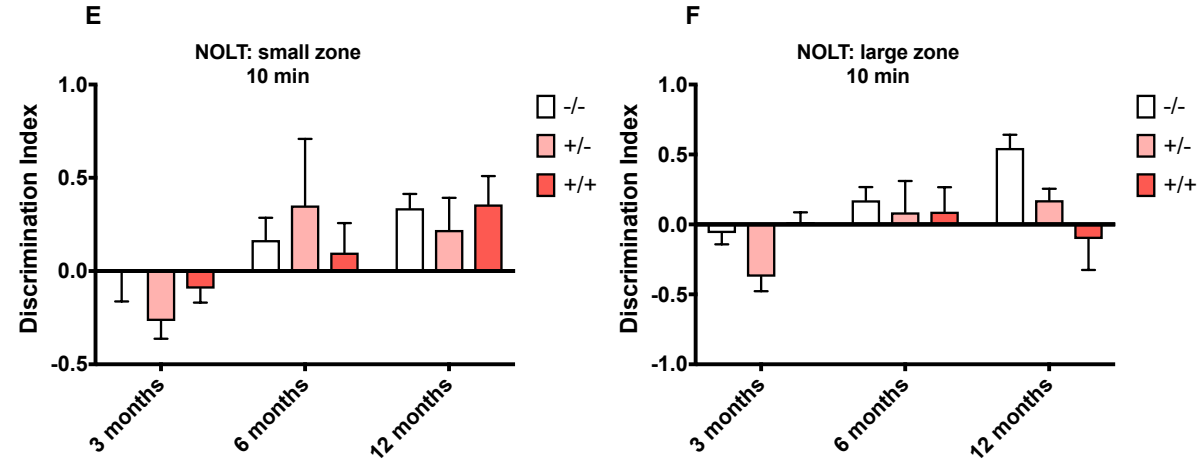

Figure 4.6: DI values for 3-, 6- and 12-month-old rats the whole 10 minutes of the NOLT, for small zones (10 cm) around the center of the two objects (E) and large zones (25 cm) (F). The results were highly variable for both zones, mostly showing little or no preference for the novel object. For the small zone they were slightly more pronounced at 6 and 12 months.

4.1.2 Further analysis

To investigate possible reasons for why almost no difference in the behavior of 12-month-old rats between genotypes was observed, and why the NORT gave more pronounced results than NOLT, further analyses were performed.

Total time spent in small zones

The NORT was found to be more suitable than the NOLT for characterizing cognitive function, as the objects in this test engaged the rats to a greater degree. Rats spent less time in the zones during the NOLT test, than the other two tests. The total amount of time spent in the zones was highest in the NORT for both the 3- and the 6-month-old rats (figure 4.7). This does not differ between the genotypes (figure 4.8). Also, the time spent in the zones decreases with age. The homozygous wild type rats spent the least time in the zones during NORT out of the genotypes with 27.0 seconds, and the hemizygous rats the most time with 29.8 seconds, only separated with 2.76 seconds. Our data demonstrated that the small zone was sufficient for cognitive testing in our rat model, and since the small zones are more precise than the large zones, they were preferred for analysis.

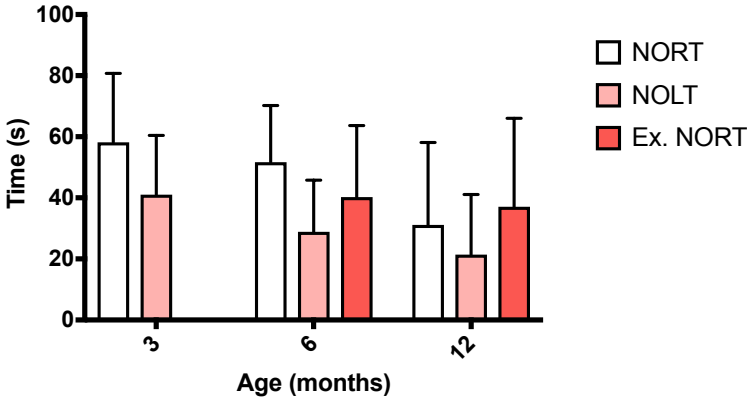

Figure 4.7: The total time spent in the two small zones for the 10-minute test, for each type of test (NORT, NOLT and NORT after exercise) and age group. The rats spent more time in the zones during the two NORT tests, than NOLT in all age groups.

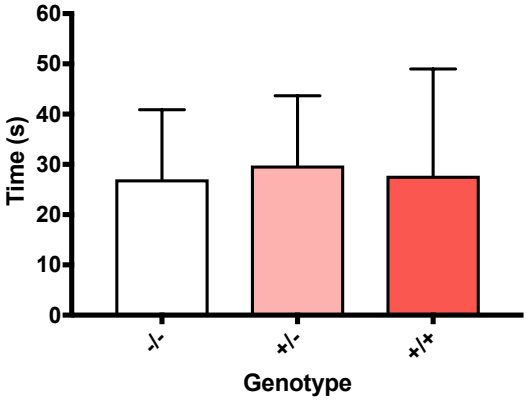

Figure 4.8: The total time spent in the two small zones for the 10-minute test during NORT, for all rats in all age groups. There was no significant difference between the genotypes.

Bodyweight

For 6-month-old males, the homozygous wildtype rats were approximately 10 % heavier than transgenic rats, although the difference was not significant (figure 4.9A). For the males at 12 months, the homozygous wild type rats where approximately 13 % heavier than transgenic rats (figure 4.9B). The difference in bodyweight between these two groups at 12 months was significant with a p-value of 0.02. No significant difference was found between genotypes among female rats.

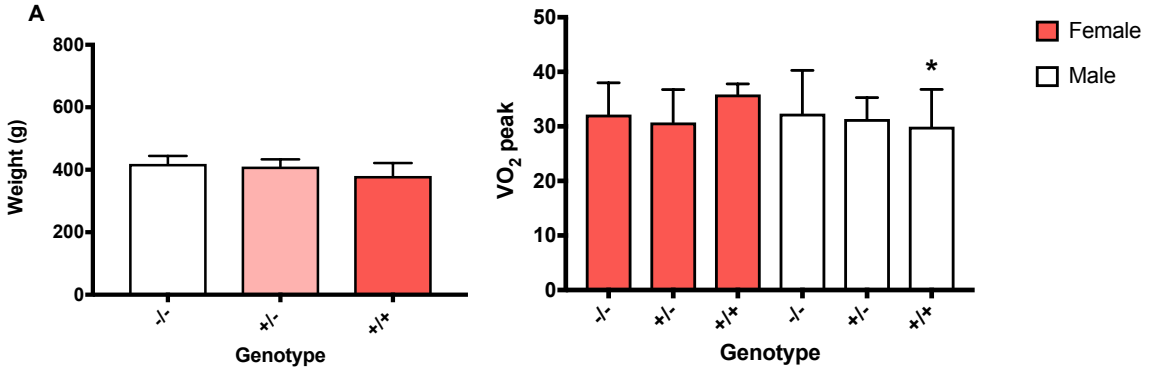

Figure 4.9: Bodyweight at 6 months distributed on genotype (A), showing no significant difference. Bodyweight at 12 months distributed on genotype and sex (B). For the females there were no significant difference, however the male homozygous transgenic rats weighed significantly less than the homozygous wild type. (*) $p < 0.05$ (one way ANOVA).

Total distance moved

Total distance moved decreased with age. The younger rats were more active than the older rats, moving a larger total distance during the test (figure 4.10A), as illustrated with the NORT, where the 3-month-old rats moved a mean of 43 meters, the 6-month-old 33 meters and the 12-month-old 19 meters during the 10 minutes the test lasted. This trend was apparent for all three cognitive tests. The 12-month-old rats lacked interest in the objects and were very sedentary, indicating that under the conditions used here, the rats of this age lack the eagerness to move and explore required for these tests. At 12 months, there was no significant difference in the total distance moved by the rats in the NORT, however there was a tendency that the homozygous transgenic rats moved the shortest distance during the test (figure 4.10B).

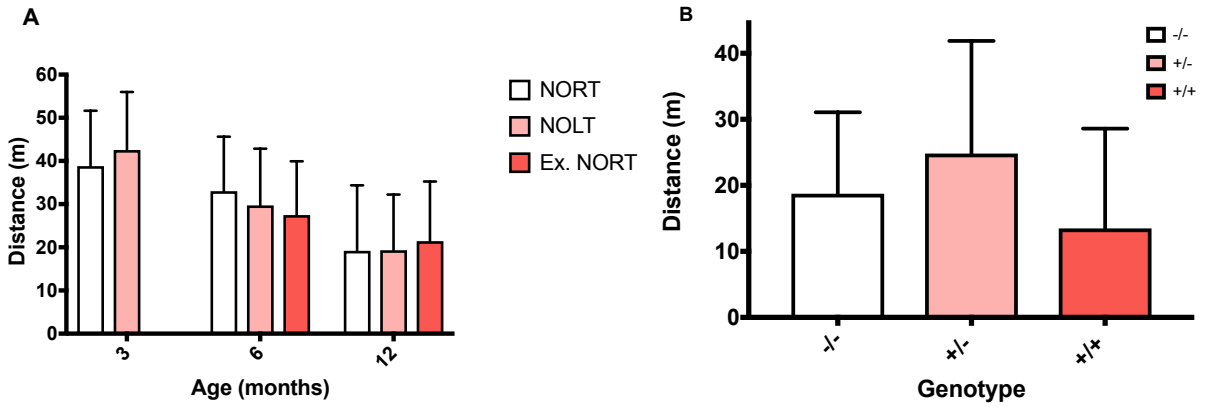

Figure 4.10: The total distance moved by the rats for the 10-minute test, for each type of test and age group. Which test the rats moved the largest total distance in, was different for each age group, however there is a decrease in distance moved with increasing age (A). Distance moved by 12-month-old rats in the NORT, for each genotype, showing that the homozygous transgenic rats moved more than the wild type rats. The hemizygous rats moved the longest distance (B).

4.1.3 VO_2 peak results

VO_2 peak results did not vary between genotypes and did not decrease with age in our AD rat model (figures 4.11 and 4.12). Therefore, there was no correlation between cardiovascular fitness and cognitive function. Only males at 6 months were included in figure 4.11, due to a low number of females. At 6 months of age, for the wild type, hemizygous and homozygous transgenic rats respectively, the mean VO_2 peak was 31.8, 31.5 and 32.7 mL/kg$^{0.75}$/ min. For the 12-month-old male rats the respective numbers were 32.2, 31.4 and 30.0 mL/kg$^{0.75}$/ min. The 12-month-old female rats had the highest VO_2 peaks with 32.2, 30.7 and 35.8 mL/kg$^{0.75}$/ min. The differences found were not significant ($p > 0.05$).

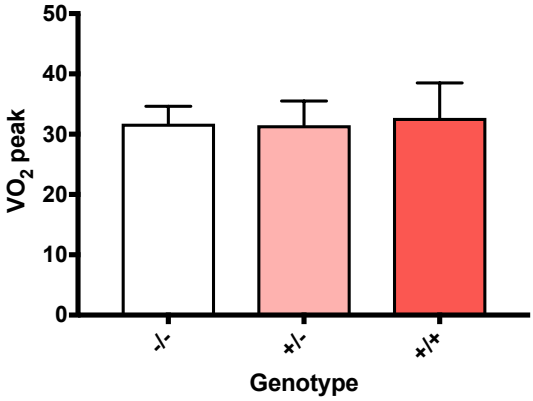

Figure 4.11: VO_2 peak for 6-month-old male rats sorted by genotype, measured in mL/kg$^{0.75}$/ min, showing no significant difference.

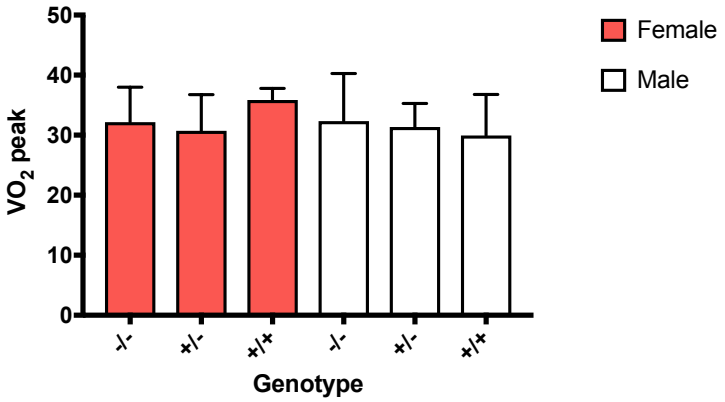

Figure 4.12: VO_2 peak for 12-month-old rats sorted by genotype and sex, measured in mL/kg$^{0.75}$/ min, showing significant differences.

4.1.4 Amyloid plaque quantification

Amyloid plaque pathology was present in brain sections stained with McSA1 and DAB (visual estimates) from transgenic rats at 6 and 12 months of age. The 6-month-old homozygous wild type showed no amyloid pathology (figure 4.13), wile transgenic rats had intracellular Aβ accumulation and some extracellular amyloid plaques (figure 4.14). The plaques from this age group were mainly located in the dorsal subiculum, but a small number was also observed in the dentate gyrus, CA1 and entorhinal cortex.

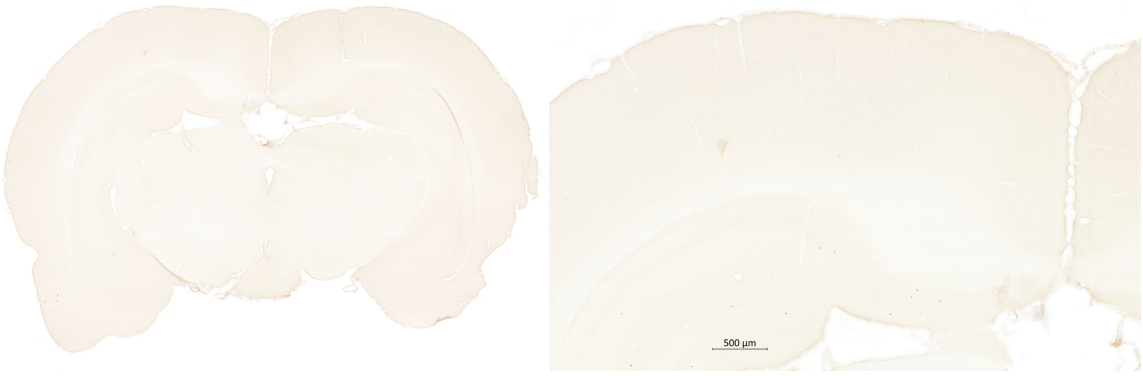

Figure 4.13: Male 6-month-old homozygous wild type rat with no evidence of amyloid pathology. The image to the right shows an enlarged photo of the dorsal subiculum. The scale bar is 500 µm for all figures 4.13-4.16.

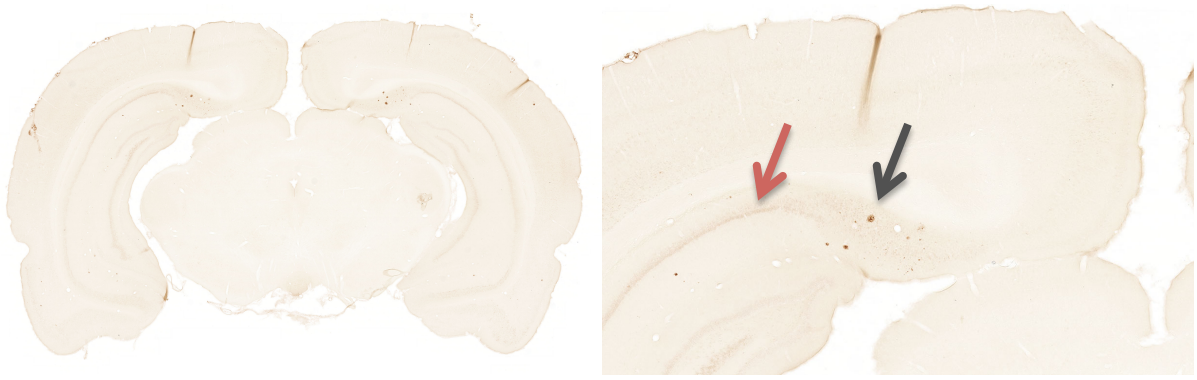

Figure 4.14: Male 6-month-old homozygous transgenic rat with intracellular Aβ accumulation (red arrow pointing at an example of this) and some amyloid plaques (black arrow pointing towards a plaque). The image to the right shows an enlarged photo of the dorsal subiculum. The largest amyloid plaques were observed in the subiculum, but there were also a small number in the dentate gyrus, CA1 and entorhinal cortex.

The 12-month-old rats had intracellular Aβ accumulation and large amounts of extracellular amyloid plaques. There was a clear increase in the amounts of amyloid plaques from 6 months to 12 months. The areas with the heaviest plaque pathology in the 12-month-old rats were observed in the same anatomical regions as the plaques in the 6-month-old rats (dorsal subiculum, CA1 and entorhinal cortex), however there was a considerable spread of the plaques in the 12-month-old rats, and the amyloid plaques were distributed over the entire cerebral cortex; the rest of the hippocampal formation, isocortex and olfactory area (figure 4.16). The homozygous wild type did not show any amyloid pathology (figure 4.15).

Figure 4.15: Male 12-month-old homozygous wild type rat with no evidence of amyloid pathology. The image to the right shows an enlarged photo of the dorsal subiculum.

Figure 4.16: Male 12-month-old homozygous transgenic rat with amyloid plaques in most parts of the cerebral cortex. The image to the right shows an enlarged photo of the region with the heaviest plaque load, the dorsal subiculum.

Cognitive test results for the cohort of the 12 rats stained with McSA1 loosely correlated with the amount of amyloid plaques. The homozygous transgenic group, the rats with low DI values, indicative low cognitive function, had more amyloid plaques present than those with higher DI values (table 3.1). Equally, the rats with high DI values had smaller amounts of amyloid plaques. The rat with the most plaques had a negative DI value, meaning that it had a preference for the familiar object.

Table 3.1: Correlation between age, DI value (from 0-4 minutes of the NORT) and amount of amyloid plaques for homozygous transgenic rat. The amount of plaques was an independent assessment based on this cohort.

Age	Genotype	DI value	Amount of amyloid plaques
6 months	+/+	0.5	+
6 months	+/+	0.2	++
6 months	+/+	0.1	+++
12 months	+/+	0.8	++++
12 months	+/+	-0.4	+++++

4.2 Study 2: Effect of a single bout of exercise on cognitive function in AD rats

Based on the results from the NORT after exercise, one single bout of exercise is not enough to have a positive effect on cognitive function (figure 4.17). The exercise did, however, influence test results, although it did not improve cognition as hypothesized. The DI values were negative after one bout of exercise, showing a preference for the familiar object after the

exercise training. That was not observed in the 12-month-old homozygous transgenic rats, which had a DI value around zero, indicating no preference for any object after exercise training.

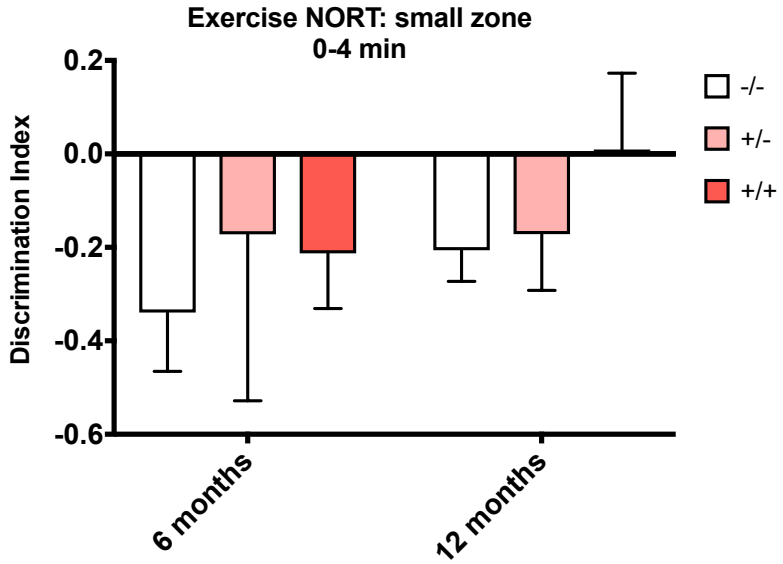

Figure 4.17: DI values for 6- and 12-month-old rats the first 4 minutes of the NORT after one bout of exercise, for small zones (10 cm). The DI values for all genotypes in both age groups indicate a small preference for the familiar object, except for the 12-month-old homozygous transgenic rats, which had no preference for any object.

5. Discussion

5.1 Summary of findings

5.1.1 Study 1: Characterization of cognitive function during disease progression in an AD rat model

This study aimed to characterize the change in cognitive function during disease progression and its correlation with cardiorespiratory fitness level and amyloid plaque pathology. We found that cognitive function was impaired in 3-month-old transgenic AD rats compared to the non-transgenic control, assessed by NORT. There was no difference in fitness level between genotypes, measured as VO_2 peak. Amyloid plaques were present at 6 months of age, observed mainly in the dorsal subiculum. The number of amyloid plaques increased considerably at 12 months, spreading to larger areas of the brain, such as the cerebral cortex and the remaining hippocampal formation. We found that rats with low DI values, indicative of low cognitive function, had higher presence of intracellular Aβ and amyloid plaques, and that rats with high DI values had less amyloid pathology.

The study also aimed to refine the NORT and NOLT protocols in order to determine appropriate test protocols and methods of analysis to be used in future experiments in our laboratory. We found that the type of tests chosen to measure cognitive function seemed suitable for 3- and 6-month old rats, but not for the 12-month-old rats (different cohort than the 3- and 6-month-old rats), as they were too sedentary. The total distance travelled in the open field apparatus during the test was found to decline with age. The NORT was more engaging than the NOLT, and for this protocol the 0-4 minutes interval was determined to be the optimal time interval for analysis. Interest in the objects was best measured using the strict small zone of 10 centimeter around the center of the object, compared to the large zone.

5.1.2 Study 2: Effect of a single bout of exercise on cognitive function in AD

Study 2 aimed to determine the effect of a single bout of exercise on cognitive function in the AD rat model. Cognition was found not to improve after one bout of exercise, however there were some changes in the test results from NORT and NOLT compared to the results obtained before exercise. The DI values were negative after exercise for the 3- and 6-month old rats, displaying that the rat had a preference for the familiar object, indicative of low

cognitive function, but not for the 12-month-old homozygous transgenic rats, where the DI values were close to zero, meaning no preference for any of the objects.

5.2 Cognitive tests

5.2.1 NORT and NOLT test results

An impairment in cognitive function (NORT results) in transgenic rats compared to wild type controls presented in 3-month-old animals, which is in line with the finding in a previous study [37]. However, the DI values were not indicative of cognitive decline during disease progression from 3 months to 6 months. On the contrary, the 6-month NORT results had a slight increase in DI values compared to 3 months. This might be related to the decrease in total distance travelled in the tests and the decreasing time spent in the small zones, with increasing age.

Older animals were more sedentary than the younger. Several of the 12-month-old males lacked interest in the objects and moved so little they had to be excluded from the test. This could possibly be related to their high bodyweight, making it more strenuous to move around and therefore affect their mobility and eagerness to move and explore. The homozygous transgenic rats were approximately 13 % lighter than the wild type rats. The reason for this is unclear, but since the transgenic rats were lighter than the wild type rats also at 6 months, it might be a result of the disease, as it is known that humans with AD have reduced appetite and bodyweight. However, this did not translate to any significant difference between genotypes in activity during tests, as all genotypes at 12 months were similarly active in the testing arena.

Analysis separating male and female rats to see which sex was best for cognitive testing was performed, but no differences were found. However, our experience in this study suggested using males is most suitable, as they were calmer while being handled and their behavior is not affected by an estrous cycle. The estrous cycle might alter the females behavior and cognitive test performance [156].

The test results for the hemizygous rats did not show a clear direction of change in cognitive function during disease progression. This group had variable results with large variations, ranging from DI values higher than the wild type rats to DI values lower than the transgenic rats. These variations are most likely due to true individual differences. Hemizygous animals

have only one copy of the mutated transgene, most likely no amyloid plaques, and only variable amounts of intracellular Aβ accumulation, as shown in previous studies [36, 37]. This may be an explanation for the individual variation observed in cognitive function. In further experiments, it would be of interest to determine whether the amyloid pathology in the hemizygous rat shows a middle ground between the homozygous transgenic and the wild type rats.

Rats younger than 3 months were not tested, as explained in the methods. Due to the fact that Leon et al. described cognitive impairment from 3 months of age [37], this age group was chosen as the youngest for reproducing the results in our laboratory, as there are many variables connected to both cognitive testing and complex behaviour of the rats. Investigations also had to be done to determine if whether NORT (and NOLT) were suitable cognitive tests. The decision was also based on practical considerations. The rats arrived our laboratory after being weaned and genotyped, by which time they were usually near 2 months of age. After habituation to facility, experimenter and cognitive testing apparatus, testing much earlier than 3 months would be challenging for this study. In future studies we will assess whether it is feasible to start testing earlier, as it would be interesting to determine how early cognitive impairment can be measured in this model, also in order to allow for primary intervention studies.

5.2.2 NOLT vs NORT

Out of the two tests performed for measuring cognitive function discussed, NORT engaged the rats more than NOLT, as seen in graphs of time spent in the zones (figure 4.7-4.8). The NOLT measures spatial memory, and our results correlate with the findings of Leon et al., who found that the spatial cognitive impairment did not become prominent before the age of 13 months [37]. A theory to explain the relationship between DI values within each age group is therefore that the rats in this study were not old enough to display this type of cognitive impairment. Our rats were 12 months old. Another theory to explain the low all-over scores could be that due to the fact that the novel object used in NORT was of an unfamiliar shape and color to the rat, it was more interesting to explore. As the NOLT ended up not giving much information about cognitive function, since not even the homozygous wild type showed novel object recognition, it is likely that this test will be left out in further studies.

A long-held view is that the dorsal subiculum is mainly involved in spatial memory, which was the main area for amyloid plaque pathology in this study. This would give reason to

believe that the amyloid plaques would have more effect on the transgenic rats in NOLT, than NORT. However, the dorsal subiculum is also mutually connected to the perirhinal and postrhinal cortices, areas which are important in recognition memory [157].

5.2.3 Experimental set up, exploratory behavior and effect of repeated exposure

The experimental setup included two consecutive cognitive tests at 3 months and three consecutive tests at 6 and 12 months, something which may have influenced the results. As the 3- and 6-month-old animals belonged to the same cohort, the rats had completed five cognitive tests by the time they were euthanized at 6 months. Even though they had a three-month-break from cognitive testing during aging from 3 to 6 months, this may have influenced the result, as repeated exposure to similar test setups may result in reduced exploratory behavior [158]. However, this was not observed in the final round of NORT (after exercise), where the rats were found to spend more time in the zones (figure 4.7-4.8). Discussed in section 5.3.2. In the future, if necessary, analysis of data from this study can be performed to compare results for NORT when it is performed as the only test, versus when it is performed after NOLT, using results from the rats that underwent testing with NORT in week 1 of study 1.

Furthermore, exploratory behavior has been shown to decrease with increasing age and weight. In a study on developmental risk taking behaviors in rats, male rats, adolescents were more reactive to novelty than adults [159]. This could have been improved by mild food deprivation, however that is a stressful treatment that should be avoided when possible. Another measure could be taken in switching the square apparatus with a circular one, as the rats tended to stay the corners in their inactive time, making such behavior impossible. Also, to enhance rat curiosity, bigger and more complex objects could have been used. In addition, more objects could have been used per test. Although our objects seemed to be slightly small, our objects, apparatus and zone sizes were in accordance with previous studies [137, 160, 161].

5.2.4 Refinement of test protocol

There is no uniform protocol for NORT and NOLT, and a number of alterations are possible for the protocol [137, 151]. For instance, less time between each familiarization and more familiarizations might make a difference by making the rats more known and secure in the setting. This way, the only investigations done by the rats will be of the objects, not of the apparatus itself. In a previous study where cognitive function of this rat model was examined,

the Morris Water Maze for spatial learning and memory test was used [37]. Here, each rat was tested 3 times per day for five consecutive days, with a memory recall 24 hours after completing the training phase. A possible measure for achieving better results using NORT and NOLT may therefore be to have more than the two familiarizations, as used in this study. Also, all the rats should have been put in the apparatus from the same side for a given phase of the test (first and second familiarization and the test phase), but in this study the rats were put in from different sides. This made the starting point, distance to the respective object, and orientation considering the distal cue slightly different for each rat and may have contributed to making the results less pronounced.

Out of the two tests of cognitive function discussed, NORT showed to be the most engaging test. This could be due to the novel object being of an unfamiliar shape and color to the rat, making it more interesting to explore. Another aspect is that the NOLT measures spatial memory, and Leon et al. found that the spatial cognitive impairment does not become prominent in rats before they are approximately 13 months [37]. One theory is therefore that the rats in this study were not old enough to display this type of cognitive impairment.

5.2.5 Discrimination index

When analyzing the calculated DI values, comparing the time spent with each object has to be considered. For instance, a rat with poor cognitive function can obtain a good score of close to +1 if the total time spent with the familiar object is less than half a second, but the time with the novel object is slightly more, without having explored the novel object sufficiently. In order to adjust for this, rats that did not explore both objects for more than 1 second each were excluded from analysis in the 0-2 and 0-4-minute time intervals and 5 seconds in the 10-minute time interval. These DI cut off values were determined based on the highest cut off value possible, that still maintained a sufficient number of animals per group (a minimal of n = 4).

5.2.6 Optimal test time interval

Analysis of the NORT and NOLT data showed that the optimal time interval for analyzing cognitive function in the rats was 0-4 minutes. The original testing interval of 10 minutes was found to be too long, as the rats did not spend more time with the novel objects in the final part of the test, as observed by the experimenter, suggesting that the novelty was lost. 0-2 minutes was also tested and found to be a too short time interval, as some rats were not done exploring both objects by that time.

5.2.7 Size of object zones

The DI values were calculated based on the two different zones with the object in the center; a small zone of 10 centimeter and a large zone of 25 centimeters. Originally, the small zone was set it was expected to give more accurate results, but as curious behavior, such as for instance turning towards the object from a distance was observed, the large zone was added. The large zone had increased sensitivity compared to the small zone, and it was hoped that it would allow examining whether some exploratory behavior was missed if only using the small zone. However, the large zone was not adequately specific and included time where the rat was not interested in the object, such as when only passing it randomly. Therefore, the small zone was found to be more suitable as it only recorded the time that the rat's muzzle was in the zone, and therefore turned towards the object.

In summary, the differences in cognitive function found in these tests were only significant at 3 months of age (p-value < 0.05), but followed trends observed in previous studies using this rat model [37, 162]. Also, it is plausible that with some adjustments and refinement to the protocol, significant differences may be attainable also at 6 months. These include more familiarizations before testing, entering the rats from the same side of the apparatus for the same phase of the test and using a circular apparatus and bigger and more complex objects.

5.3 One bout of exercise/VO_2 peak

5.3.1 Cardiorespiratory fitness level

As a low cardiorespiratory fitness level is associated with increased risk of AD development, this study looked in to whether there were any such differences to contribute to the cognitive phenotype. This was not the case as there were no differences in fitness level was observed, making it unlikely that any neurological differences in cognitive function were due to differences in cardiovascular fitness level. Echocardiograms were also performed on all rats to assess heart function, data of which is not yet analyzed. However, given the VO_2 peak test results it is unlikely that these will show any major differences.

An additional study performed during the work with this thesis, was the characterization of cognitive function in two strains with rats at 15 (n = 23) and 23 months (n = 21), with phenotypes for either high or low capacity for aerobic running, resulting in different risks of developing metabolic and cardiovascular diseases, including neurodegeneration [163]. The

high capacity runners have higher VO_2 max than the low capacity runner [164]. This study did not show any differences in cognitive function between phenotypes, indicating that the cardiovascular fitness level did not influence cognitive function (unpublished study, data not shown).

The VO_2 peak test had to be chosen instead of VO_2 max, due to ethical reasons as some of the rats refused to run further before the test protocol was finished. Therefore, the attained VO_2 during the test did not necessarily define the highest reading attainable for the rat, maybe explaining why there was no difference in cardiovascular fitness level between genotypes. However, a study on the relationship between VO_2 max and peak, indicated VO_2 peak to be a valid index of VO_2 max when the exercise effort is sufficient [165]. Moreover, the appropriate treadmill incline was determined to be 5 degrees. In the literature, the ideal incline for reaching VO_2 max in rats is stated to be 25 degrees, but this was found to be too demanding for the rats in this study, as they did not manage to run for a long enough time with such high incline [152]. After some attempts, 5 degrees was decided upon.

5.3.2 NORT following one bout of exercise

NORT was chosen for the post-exercise cognitive testing, based on the data obtained from the rats that had already finished both types of cognitive tests (NORT and NOLT) showing that this test engaged the rats to a greater extent than the NOLT. The NORT performed 3 hours after one bout of exercise showed decreased DI for all three genotypes in both age groups, i.e. either no preference for any object, or preference for the familiar object. Interestingly, the time spent in the small zones in this test did not differ much compared to NORT before exercise at 6 months, meaning that the rats still explored the object to almost the same extent. At 12 months the time spent in the zones increased, implying that the objects might have been explored to a greater extent. Therefore, they were not likely to be physically tired (figure 4.7), but one explanation could be mental tiredness. The VO_2 peak test/one bout of exercise could have stressed the rats despite three sessions of habituation or given the rats adequate mental stimulation to either lose some interest in the cognitive test, or possibly make them seek out the familiar and safe object. Furthermore, repeated testing can, as mentioned, result in reduced exploratory activity, which could have been the case since the NORT after exercise was the third test setting for the rats.

The homozygous transgenic rats had the highest mean DI value, indicating a stronger preference for the novel object. A theory to explain this could be that impaired cognition

made the rats forget having gone through this cognitive test twice before. The rats with the other two genotypes, with lower DI values compared to the homozygous, could have had decreased exploratory behavior because the objects, even though they were in a new shape and color, were not interesting enough after already having been through two tests.

Furthermore, the 3-hour time interval between exercise and cognitive testing might not be ideal and could be altered in future experiments. For instance, a study on the effects of acute aerobic exercise on cognitive functions in humans showed that vigorous acute aerobic exercise has beneficial effects on prefrontal cortex-dependent cognition, effects that can last for up to 2 hours after exercise. Acute exercise improved prefrontal cortex- but not hippocampal-dependent functioning [166]. Data from an earlier study in our lab on HT22 cell survival using serum from blood showed clearly that out of the time points for blood retrieval (pre-exercise and 1, 3, 6 and 24 hours post-exercise), serum from 3 hours post-exercise resulted in the highest cell survival (Tari & Scrimgeour, unpublished data). Due to this, 3 hours post-exercise was chosen over 1 hour, also to give the rats more time calm down before cognitive testing.

5.3.3 Types of exercise

It is not known what types or what dose of exercise is likely to have the most beneficial effect in AD rats. The beneficial effect of physical exercise depends on if the exercised-induced factor has an acute effect or if the effect comes over time, and also on the characteristics of the exercise regime, such as duration, frequency and intensity. In a cohort study, long-term adherence to a exercise regime appeared to be more important than the exercise dose for reduction of long-term mortality [167]. In addition, individual variations such as sex, age and genes will affect the benefits [168].

For this particular study, different exercise protocols are worth looking into in regards to intensity, duration and repetition (chronic exercise). Even though the exercise protocol used was chosen based on an earlier study on HT22 cells, another motive for choosing this exercise form was to refine the study design to minimize stress on the rats by only doing one round of treadmill exercise.

In hindsight, it would be interesting to see if anaerobic exercise could be a more beneficial for improving cognitive function after one bout of exercise. Exchanging the aerobic exercise with anaerobic exercise would cause the rats to produce more lactate, which is suggested to be an underlying part of the beneficial effects exercise had on the brain [169].

5.4 Immunological staining of amyloid plaques with McSA1 (anti-Aβ)

Based on findings in previous studies showing that amyloid pathology starts in the subiculum [36, 37], this area was used for comparison of amyloid pathology presence in the sections. The findings in this study correlated with the findings from Leon et al. regarding presence of intracellular Aβ accumulation at 6 months, extracellular amyloid plaques in the subiculum as well as occasionally in the entorhinal cortex. Our findings also correlated with their findings at 13 months, even though our rats were only 12 months at the time of testing. Our results show that amyloid plaques were present in most areas of the hippocampal formation with a spread to cortical areas. As the 3-month-old cohort of rats was continued until the rats were 6 months old for longitudinal testing, they were not included in this part of the study.

In the study by Heggland et al., extracellular amyloid plaques were not observed at 6 months, but at 9 months [36]. In this study, amyloid plaques were found in brain sections from all three 6-month-old transgenic rats in this cohort. Our results show, as mentioned, presence of plaques also in the 6-month-old animals. This indicates large individual differences in the McGill-R-Thy1-App rat model.

The amount of amyloid plaques found in the transgenic rats loosely correlated with the cognitive test score, in the way that rats with a small plaque load showed more preference for the novel object than those with a high plaque load, within the same age group. This indicates that rats with higher plaque loads have poorer cognitive function and vice versa. To obtain more accurate results of these findings, future studies quantifying the plaque load using stereological estimation would be very interesting.

5.5 The McGill-R-Thy1-APP rat model of Alzheimer's disease

5.5.1 Limitations of the model

To date, it is not possible to recreate AD in animal models to be identical to how it is in humans. In animal models it has been found to be challenging to design a model which displays the hallmark AD pathology, both amyloid and tau pathology, in addition to neuronal loss. This is because the mechanisms causing AD are not fully known. Due to this, most rodent models only display one of the two hallmark pathologies [136]. The McGill-R-Thy1-App rats used in this study do not display tau pathology or neuronal loss, however, the model has in a previous study been found to display neuronal loss in the subiculum after the age of

18 months [36]. This may limit the translational value of the findings in our study. Many AD treatments found promising when tested in animal studies have failed in clinical trials, maybe because the pathology simulated in the animal models is too different from the real disease pathology [170].

5.5.2 Choice of species

The rat is a suitable model organism for human disease, and has some advantages over for instance mice. They are larger animals, making it easier to study their physiology, giving less limitation on for instance volume of blood samples. Many studies on memory and learning have been performed in rats, and they are also more cognitively developed and intelligent than mice. The rat is also a better model organism than mice, considering the genetic closeness in relation to humans, making translation to humans from animal findings more reliable. In addition, their behaviour is easier to test as rats displays a richer spectrum, their social relations are more complex and their motor skills are finer than in mice [138].

The aims of the project cannot be achieved without the use of animals as the complexity of the brain and circulatory system cannot be replicated otherwise. In general, animal research can be justified because it has the potential to eventually improve or save human lives. Use of animals in research may raise ethical issues, as they are unable to consent to the testing. Some research may cause pain, suffering and discomfort in the animal and it is always imperative to consider the 3Rs, "Replacement, Reduction, Refinement", in able to reduce this.

In this study, a yearlong neuronal cell culture (HT22) study was performed in order to minimize the numbers of rats needed. Also, the same animals were used for testing at 3 and 6 months, further minimizing the number. The first study *in vivo* was a study for refining the further experiments. The cognitive testing may be viewed as an enrichment of their lives. The rats were housed together to avoid stress.

5.6 Translational value and future directions

This project will function as a pilot study for further studies (planned start up fall 2018), for which the characterisation of cognitive function and a valid method to test this needed to be established. Results from this project will be used as a baseline to determine the effect of the interventions on cognitive function. In the planned study, this AD rat model will undergo

primary and secondary interventions, comparing chronic exercise to injections of plasma from exercised rats before and after disease/pathology onset.

As described in the introduction, the risk of developing AD in individuals with a high level of physical activity and/or high age-relative VO_2 peak compared to inactive/unfit counterparts has been shown to be reduced by as much as 50%. AD drug trials have been shown to have a failure rate of 99,6%, higher than all other disease areas. In the last years, more and more research has shifted focus to prevention and early intervention, and at current, exercise seems to be the most promising AD "drug". It is well established that exercise is beneficial for the brain (increases neurogenesis, plasticity, cerebral blood flow), although the molecular mechanisms underlying this effect is not known. In our planned future studies, we hypothesize that factors released in the blood during exercise can be responsible for these neuroprotective effects. This hypothesis is also based on the knowledge that the beneficial factors mentioned in section 1.5.4, such as BDNF, IGF-1 and VEGF, are upregulated with exercise.

Because of the blood-brain barrier, it was traditionally thought that the beneficial effects of exercise on the brain could not be orchestrated through systemic changes in the periphery [171]. However, studies in both rodents and humans show that the effects of exercise on the brain are, in part, mediated by changes in the systemic environment [3, 172-174]. Recent studies have shown that blood from young mice injected into old mice resulted in counteracting age-related degeneration in various tissues, including the brain [114, 175], such as beneficial effects on hippocampal spine density, plasticity and learning. Inversely, old blood was found to impair neurogenesis and cognitive function in young mice, indicating that old blood contains age-promoting factors [114]. These findings indicate that soluble factors in the blood can have therapeutic effects and that there is communication between the blood and the hippocampus.

A more translational study shows that human plasma of an early developmental stage, namely umbilical cord plasma, enhances plasticity and improves neuronal function in the aged mouse brain and hippocampus. Injecting this into aged mice led to improvements in the rodents' learning, memory, and synaptic plasticity [3]. In humans, initial clinical trials (NCT02256306) report that plasma from young donors transfused to patients with early AD is safe (no adverse events) and possibly beneficial (statistically significant improvement in functional activity).

Future investigations are needed to look into whether beneficial effects of exercise is limited to the mechanisms behind aging, or if it could be extended to reversing or limiting the neurodegeneration with cognitive decline during dementia, such as in AD. Maybe as in the "young blood studies" [3, 114, 175], exercise could also provide a change in the systemic environment, by shifting it towards a more "youthful" state. If it is found to work in an animal model, plasma (or plasma factors) extracted from exercised humans may become a treatment option for AD patients.

6. Conclusion

Cognitive function was impaired in 3-month-old McGill-R-Thy1-APP rats compared to the non-transgenic control, as assessed by NORT. The cognitive test results did not show further impairment at 6 and 12 months, but with refinements to the tests, this may be obtainable. There was no significant difference in cardiovascular fitness level between genotypes measured by VO_2 peak, indicating no correlation between fitness level and cognitive function in the protocols used in this study. The NORT engaged the rats to a greater extent than NOLT. For improvement of this protocol, 0-4 minutes was determined to be the optimal test time interval, and measuring interest in the objects by using the strict small zone of 10 centimeters diameter. The total distance travelled in the apparatus during the cognitive test declined with age, and the 12-month-old rats were so sedentary that they were not suitable for testing under the conditions used.

Intracellular Aβ accumulation and amyloid plaques were present at 6 months of age, mainly in the subiculum, and were present in far greater numbers and brain areas in the 12-month-old rats. The areas with the highest plaque load were the subiculum, dentate gyrus, CA1 and entorhinal cortex. The visually estimated amount of amyloid plaques seemed to increase with decreasing measured cognitive function. Cognition did not improve after one bout of exercise, however the NOT changed compared to before exercise. Most DI values were negative, maybe indicating that the rats were mentally tired, seeking out the familiar object.

For further studies, quantification of the brain sections stained with McSA1 would be interesting to obtain an exact estimate of the correlation between cognitive test score and amyloid plaque load. Even though one bout of exercise did not improve cognitive function in this study, it could still have a positive effect on cognitive function with refinements to the cognitive test protocol and some alterations to the exercise program. If this positive effect on cognitive function is obtained, future research could look into whether plasma (or plasma factors) extracted from exercised rats could be used to treat a transgenic AD rat, with the goal of finding a treatment option for human AD patients.

7. References

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Appendix I A

Novel Object Localization Test (NOLT) Protocol

Before starting

- Open the Any-Maze software and fill in animal ID, treatment groups (blinded) and the correct settings for apparatus, tracking, zones, auto start, animal color, sensitivity etc.
- 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's phases
- Always go in from different sides of the apparatus, to place the rat
- The experimenter must handle the rats with calm movements and be quiet during the tests

1. Habituation phases (day 1 and 2)

- Let the rats sit in your lap for 30 minutes a day, for two days. Get as many rats at the same time as possible, so be more efficient. Change clothes when switching from one sex to the other.
- Let the rat explore the empty apparatus for 25 minutes
- Repeat after 24 hrs

2. Object familiarization phase (day 3 and 4)

- Acclimate rat in testing room while cleaning the apparatus and getting ready (5 minutes)
- Tape a piece of Velcro on the objects, as well as their localization in the apparatus, to make sure they stay in place. The objects should be placed parallel near the two top corners.
- Place the rat in the box at the center of the apparatus, with its back to the objects and let it explore the objects for 10 minutes
- Repeat the familiarization 24 hrs later

3. Test phase (day 5)

- Move one of the objects, so that they are placed diagonally opposite

- Place the rat in the box with at the center of the opposite wall with its back to the objects and let it explore the objects for 10 minutes

Appendix I B

Novel Object Recognition Test (NORT) Protocol

Before starting

- Open the Any-Maze software and fill in animal ID, treatment groups (blinded) and the correct settings for apparatus, tracking, zones, auto start, animal color, sensitivity etc.
- 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's phases
- Always go in from different sides of the apparatus, to place the rat
- The experimenter must handle the rats with calm movements and be quiet during the tests

1. Habituation phases (day 1 and 2)

- Let the rats sit in your lap for 30 minutes a day, for two days. Get as many rats at the same time as possible, so be more efficient. Change clothes when switching from one sex to the other.
- Let the rat explore the empty apparatus for 25 minutes
- Repeat after 24 hrs

2. Object familiarization phase (day 3 and 4)

- Acclimate rat in testing room while cleaning the apparatus and getting ready (5 minutes)
- Tape a piece of Velcro on the objects, as well as their localization in the apparatus, to make sure they stay in place. The objects should be placed diagonally opposite, near the corners.
- Place the rat in the box at the center of apparatus, with its back to the objects and let it explore the objects for 10 minutes
- Repeat the familiarization 24 hrs later

3. Test phase (day 5)

- Exchange one of the object with a novel one (new shape and color)

- Place the rat in the box with at the center of the opposite wall with its back to the objects and let it explore the objects for 10 minutes

Appendix II

Immunostaining McSA1 (anti-Aβ)
Peroxidase/DAB protocol

1. Leave sections in 0,125M phosphate buffer (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 in refrigerator (4° C)

7. 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.

8. Incubate with secondary antibody, biotinylated goat anti-mouse, 1:200 in TBS-Tx, 90 min in room temperature. Mix ABC right before next step.

9. Rinse sections 3 x 10 min in TBS-Tx

10. Incubate with ABC, 90 min in room temperature

11. Rinse 3 x 10 min in TBS-Tx

12. Rinse 2 x 5 min in Tris-HCl

13. Incubate with DAB for 30 minutes

14. Rinse 2 x 5 min in Tris-HCl

15. Mount sections in Gelatine on a heating plate (or Tris-HCl on Superfrost slides if doing a Nissl counterstain) and let them dry

16. Coverslip with Toluene and Entellan

ABC

From the ABC-kit, put 1 drop of solution A and 1 drop of solution B in 5 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 brain cup (or any other cup you can throw away)
For filtrating the DAB 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 III

Solutions for perfusion and staining with McSA1 and DAB

DMSO

100 ml: 31.25 ml 400 mM phosphate buffer, 46.75 ml H2O, 20 ml glycerine, 2 ml DMSO

Phosphate buffer (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.

Phosphate buffer 0.125M pH 7.4

Dilute 0.4M phosphate buffer. Store at 4˚C for up to one week.

500 ml: 146 ml 0.4M PB + 344 ml H2O

Paraformaldehyde (PFA) 10%

Heat 200 ml H_2O to 60˚C in the microwave oven. In a ventilated hood: Add 20 g of paraformaldehyde to the water, then add a few drops of NaOH and leave the solution on a hot stirrer until it is clear.

Paraformaldehyde (PFA) 4%

500 ml: 200 ml 10% paraformaldehyde, 156 0.4M PB, 144 ml H2O 90

In a ventilated hood: Adjust the pH to 7.4 using HCl and filtrate. Make new fixative for every perfusion.

Tris-buffered saline (TBS) 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.