Master's thesis

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

Kamilla Jacobsen

Effects of "exercised plasma" on adult neurogenesis in the hippocampus of a rat model of Alzheimer's disease

Master's thesis in Molecular Medicine
Supervisor: Ulrik Wisløff
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Abstract

The incidence rate of dementia is rising, following the rapidly aging population. Currently, there are no treatment options for the most prevalent cause of dementia, Alzheimer's Disease (AD). To impede the potential international crisis, WHO has listed efficient strategies to prevent and treat AD as a global health priority. Fortunately, promising findings in epidemiological studies indicate that exercise and high age-relative cardiorespiratory fitness appear to be the most promising preventive strategies against AD. Promising experimental studies indicate that beneficial blood factors induced by exercise can mitigate neuropathology. One emerging hypothesis is that systemically administered "exercised plasma" can cross the blood-brain barrier and modify neurodegeneration. Nonetheless, there is a knowledge gap in the literature describing the effects of exercise-induced blood-borne factors on the AD brain. This study aims to assess whether systemic plasma administration can transfer the beneficial effects of exercise on adult neurogenesis in the AD brain using a rat model of AD, and to examine the effects on neurogenic, inflammatory and angiogenic markers.

23 five-month-old McGill-R-1-Thy1-APP rats were investigated in response to injections with "exercised, young plasma", "sedentary, young plasma" or saline, or to exercise. Coronal brain sections were double-immunolabelled against BrdU and NeuN, targeting dividing- and neuronal cells, respectively. The effects of exercised plasma were evaluated based on the number of newborn neurons as a percentage of total neurons in the dorsal dentate gyrus of the hippocampus, and the change in hippocampal mRNA expression in markers of interest, assessed by RT-qPCR.

"Exercised, young plasma" significantly increased adult neurogenesis in AD rats ($p < 0.05$). The genetic analyses revealed a marker of interest; however, the underlying molecular mechanisms causing the increase are yet to be entirely deciphered. The present study was one of the first to demonstrate blood factors transferring therapeutic effects of exercise to the AD brain. The results of increased adult neurogenesis supported the hypothesis that the systemic environment may be targeted with exercised plasma as a strategy to modify the AD brain by increasing adult neurogenesis.

Sammendrag

Forekomsten av demens øker i sammenheng med den økende andelen eldre befolkning. I dag finnes det ingen behandlingsmuligheter for demenssykdommen med høyest prevalens, Alzheimers sykdom. For å hindre en potensiell internasjonal krise, har WHO listet effektive strategier for å motvirke og behandle Alzheimers sykdom som en helseprioritet verden over. Heldigvis finnes det epidemiologiske studier som indikerer at trening og høy aldersrelatert kardiorespiratorisk kondisjon er de mest effektive strategiene for å forebygge Alzheimers sykdom. Lovende eksperimentelle studier indikerer at det finnes gunstige faktorer i blodet indusert av trening som kan redusere nevropatologi. En ny og lovende hypotese foreslår at systemisk administrert «trent-plasma» krysser blod-hjernebarrieren og modifiserer nevrodegenerasjon. Det er manglende studier som beskriver effekten av trenings-induserte blod faktorer i en Alzheimer-hjerne. Denne studien har som mål å undersøke om systemisk administrering av plasma kan overføre de gunstige effektene fra trening på nevrogenese hos voksne med Alzheimers sykdom, ved å bruke en rottemodell for Alzheimers sykdom, i tillegg til å se etter endring i genuttrykk av markører for nevrogenese, inflammasjon og angiongenese.

23 fem måneder gamle McGill-R-1-Thy1-APP rotter injisert med «trent, ungt plasma», «stillesittende, ungt plasma» eller saltvann, i tillegg til en gruppe med trente rotter var inkludert i studien. Koronale hjernesnitt ble immunfarget med fluorescerende celle markører for å detektere BrdU og NeuN, som festes til delende celler og nevroner, respektivt. Effekten av de trengingsinduserte faktorene i blodet ble evaluert basert på kvantifisering av nydanna nevroner som prosent av det totale antallet nevroner i dorsal dentate gyrus av hippocampus, og på endring av genuttrykk av markørene av interesse.

«Trent, ungt plasma» øker nevrogenesen hos Alzheimer rotter ($p < 0.05$). De genetiske analysene avslørte én markør av spesiell interesse, men de underliggende molekylære mekanismene bak økt nevrogenese er fortsatt ikke fullstendig utarbeidet. Denne studien var en av de første til å vise terapeutisk effekt i en Alzheimer-hjerne grunnet treningsinduserte faktorer i blodet. Funnene av økt nevrogenese støttet hypotesen om systemisk behandling med trenings-induserte faktorer i blodet som en strategi for å modifisere Alzheimer hjernen ved å øke nevrogenese.

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Abbreviations

AD	Alzheimer's disease
Aβ	Amyloid beta
APP	Amyloid precursor protein
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BrdU	Bromodeoxyuridine
CNS	Central nervous system
CVD	Cardiovascular disease
DG	Dentate gyrus
EGR1	Early growth response 1
EYP	Exercised young plasma
FNDC5	Fibronectin type III domain-containing protein 5
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
NeuN	Neuronal nuclei
PLAU	Urokinase-type plasminogen
SGZ	Subgranular zone
Stat3	Signal transducer and activator of transcription 3
Shh	Sonic hedgehog
SYP	Sedentary young plasma
TNF	Tumour necrosis factor
VEGF	Vascular endothelial growth factor

1 Introduction

1.1 Alzheimer's Disease

As a result of increased life expectancy over the past decades, the world's population is rapidly aging. With increased longevity comes increased incidence and prevalence of dementia. Dementia poses a rising threat to public health worldwide (4, 5) and WHO have stated that dementia is now a global health priority (6). Dementia is a term covering symptoms of neurological disease affecting a person's ability to carry out everyday activities. The most common cause of dementia is Alzheimer's Disease (AD), which is a neurodegenerative disorder (7). AD patients experience a progressive cognitive decline as the disease primarily disables the hippocampus that is pivotal to learning and memory (7, 8). Of all dementia incidents, 60-80% are due to AD that at current is incurable and ultimately fatal (9).

The progression of AD is a continuum of three phases: 1) preclinical AD, 2) mild cognitive impairment and 3) dementia (9). Before clinical manifestation, preclinical AD with apparent molecular neuropathology has already been present for as long as 20 years or more (10, 11). In general, onset of the disease determines the duration; early-onset implies extended neurodegenerative disorder for up to 25 years (12). Hence preventative and disease-modifying measures in the early stages of AD are critical.

Cognitive disorders are a rising hurdle for both the global economy and quality of life of the patients and their next of kind. A report published by Menon Economics on behalf of Biogen assessed the socio-economic cost of dementia (predominantly AD) in Norway, and estimated a total societal cost of dementia per year of 96 billion NOK, of which AD was responsible for 62 billion NOK, more than the costs of heart failure and obesity combined (13). Undoubtedly, dementia carries enormous consequences to the individual patient, their relatives, the health care system and the society as a whole, but still, there is no cure. In correspondence with increased longevity in developing nations, the overall prevalence of AD has been expected to triple from today's 50 million to 152 million by 2050 (4). It has been estimated that postponing AD onset by five years before 2050 could potentially decrease the prevalence by 41%, and that even a modest delay of disease onset would lead to more years of life with good health for the patients and improve public healthcare and the economy significantly (14). At current, all available AD drugs are simply symptom-modifying, and AD-drug trials have the highest failure rate (99.6%) compared with any other disease area (15). At present; AD is a quandary, it is an incurable and terminal disease for which preventative measures are urgently needed (9, 16).

1.2 Risk factors for AD

Cognitive testing, lumbar puncture and MRI are commonly used as diagnostic approaches for AD, as less invasive strategies are currently lacking in clinical practise (9). Recent research indicates the possibility of identifying underlying pathology in asymptomatic individuals with specific biomarkers in blood (17). Although promising, these biomarkers' predictive value still needs to be clinically validated. One of the most promising biomarker candidates to date is plasma phospho-tau217, with the power to discriminate AD cases from other neurodegenerative diseases with significantly higher accuracy compared to other biomarkers for AD (18). Further research is needed for the validation and clinical relevance of phospho-tau217 (18). The enormous failure rate in AD drug trials indicates

that interventions after symptomatic onset might be too late (15). A need for early detection is therefore vital in order to be able to prevent or slow the progression of AD development (19). Addressing AD risk factors is a preventive measure that is currently plausibly more promising than approaching curative measures.

A 2017 Lancet Commission report addressed dementia prevention, intervention and care, where nine potentially modifiable risk factors for dementia were listed: hypertension, educational level, depression, obesity, diabetes, smoking, hearing impairment, little social interaction, and physical inactivity (20). In 2020, with convincing evidence, they expanded their list, adding air pollution, excessive alcohol intake and traumatic brain damage (4). All modifiable risk factors are potential targets for prevention and addressing them might delay the onset of dementia (4). Furthermore, some of these modifiable risk factors might also be potential targets for the future treatment of AD.

1.2.1 Nonmodifiable risk factors for AD

Epidemiological studies have assessed the prevalence and incidence of AD and strongly point to age as the most prominent risk factor for disease development (21). A prospective cohort study discovered a 20-fold increase in AD incidence rate in individuals from 65 to 69 years (2.8 per 1000 people) compared to individuals above the age of 90 (56.1 per 1000) (22). AD incidence increases exponentially with advancing age (23, 24).

Genetics is a nonmodifiable determinant of developing AD (25). One can separate AD into two subtypes: early-onset (before the age of 65 years) and late-onset (after the age of 65). The latter is the most common, whereas early-onset only accounts for 1-6% of AD cases (26). Familial AD occurs in both early- and late-onset AD. Studies have found that approximately 60% of early-onset cases are associated with a familial history of AD, most often with multiple susceptibility genes and environmental factors involved (27). Roughly 13% of early onset-AD cases are estimated to be inherited in an autosomal dominant manner (27), linked to Amyloid precursor protein gene (*APP*) and presenilin genes (*PSEN1* and *PSEN2*) with high penetrance (26-28). The *APP* gene encodes the precursor to amyloid-β (Aβ) -peptides, and mutations in the presenilin genes favour an overproduction of the neurotoxic $A\beta_{42}$ (29).

Literature supports late-onset AD as a multivariate disease, with a possibility of several susceptibility genes. Genome-wide association studies have identified genes modifying the susceptibility of late-onset AD, exceeding 20 risk loci (30). The strongest linked gene to AD predisposition is the gene that encodes apolipoprotein E, *APOE*, with the highest expression on glial cells within the brain. APOE is a vital ligand, mediating lipoprotein undergoing endocytosis (31), implicating neuroinflammation (32). The *APOE ε4* allele has been a well-established susceptibility factor for late-onset AD for decades (33). The most common genotype is *ε3ε3*, but individuals with either one or two *ε4* alleles expressed have an increased risk of developing AD by approximately three- and fifteen-fold, respectively (33). Although the predisposition of AD increases if one carries the *ε4* allele, it is neither required nor sufficient to develop the disease (34).

Contrary to the direct nonmodifiable risk factors such as age and genetics, indirect predisposing factors can be modified by behaviour, therapeutic interventions, and treatment. Targeting these modifiable risk factors could be a multivariate approach to

prevent or mitigate cognitive decline (35), and of high utility to elucidate pathogenic AD pathways.

1.2.2 Modifiable lifestyle risk factors for AD

A growing body of evidence suggests that lifestyle significantly impacts the risk of developing dementia. It has been estimated that 20% of AD cases globally are attributable to low education (36). Many factors associated with a higher risk of cardiovascular disease (CVD; e.g. high blood pressure, diabetes) as well as CVD itself (e.g. stroke, heart failure) are associated with a higher risk of developing AD (35). Hypertension is a commonly investigated risk factor for cerebrovascular disease and CVD; and extensive epidemiological studies have demonstrated a relationship between midlife hypertension and initiation of cognitive decline (37-39). Hypertension induces molecular alterations in brain vessels with a possible mechanistic link to dementia. Hypertension is destructive to the blood-brain barrier (BBB) and therefore harms Aβ clearance mediated by density lipoprotein receptor-related protein-1 lining the abluminal membrane of the BBB, and in turn this provokes a toxic milieu in the brain, and synaptic dysfunction (40). Undoubtedly, hypertension affects vascular health, representing a prospective preventative target in the development of cognitive decline (37). Diabetes is associated with an enhanced risk of developing AD and dementia. Recent reviews summarize a notable association between AD and diabetes, whereas patients with diabetes have been associated with having 60-70% increased risk of developing AD compared to individuals without diabetes (41, 42). A chronic exposure to glucose causes hyperglycemia, a condition that might increase extracellular Aβ (43), cause abnormal brain network structure (44), and neuroinflammation (45), all of which are associated with the pathophysiology of AD (29). Additionally, considering the global obesity pandemic (46), obesity is a growing concern as evidence demonstrates a strong association between midlife obesity and risk of developing AD (47).

Physical inactivity is one of the most significant modifiable risk factors to avoid developing chronic lifestyle diseases (48). In the USA, Europe and the UK, physical inactivity was attributable to the largest proportion of AD cases of all modifiable factors (36). Evidence points towards a direct risk reduction of AD by increasing physical activity, as inactivity is a risk factor for dementia. Additionally, increased activity seems to decrease the incidence of CVDs and CVD risk factors, possibly through secreted molecules which has been associated with reduced dementia risk, and thereby indirectly reducing the risk of AD development (49, 50).

A growing body of literature supports the efficacy of preventative strategies, suggesting multidomain approaches in the fight against cognitive impairment and dementia (51). The expected increase in AD cases needs prophylactic measures and are recognised as pivotal in halting the escalating pandemic crisis (52, 53).

1.3 Exercise and brain health

It is hypothesised that physical inactivity is a strong and independent risk factor for developing abdominal adiposity, and consequently activate inflammatory pathways, promoting insulin resistance, tumour growth, atherosclerosis, neurodegeneration. Lastly, physical inactivity leads to development of diseases belonging to "the diseasome of physical inactivity", of which dementia is included in (54). Fortunately, there is converging evidence that exercise delays age-related cognitive decline, alleviates neurodegeneration,

and improves memory and learning (55-57). Van Praag et al. (58) demonstrated counteractive effects against aging within an experimental group of aged mice housed with a running wheel. Increased physical activity was associated with improved learning, increased synaptic plasticity, and increased generation of new neurons in the dentate gyrus (DG; a neurogenic region within the hippocampus), compared to sedentary control mice (58). Furthermore, there is evidence that running induces a more pronounced effect on dorsal DG versus ventral DG (59, 60).

The advantageous effects of exercise have been linked directly to neurobiology, through positively influencing neurogenesis and maturation of newborn cells in the DG of the hippocampus (2, 61, 62), increasing synaptic plasticity (59, 62), and indirectly by reducing stress, improving sleep and decreasing the risk of cardiopathies, which positively impact brain health (63). Exercise has been found to alter the systemic environment (64), increasing blood flow due to capillary density and cerebral angiogenesis enhancement (55, 65-67).

1.4 The hippocampus

Deeply embedded inside the temporal lobe of the brain lies the hippocampal area (68). The hippocampus is the primary mediator of memory functions and consists of excitatory pathways through three subregions: the DG, cornu ammonis 1, 2 and 3 (CA1, CA2, CA3) (figure 1) and the subiculum. The subiculum connects the hippocampus with the entorhinal cortex (68). The afferent and efferent connectivity throughout the longitudinal axis of the hippocampus was found to change from one pole to the other, leading Moser and Moser in 1998 to propose that the dorsal and ventral part of the hippocampus have different and dissociable functions (69). Their theory suggested that cognitive processes like memory function are modulated explicitly by the dorsal pole of the hippocampus, while the ventral modulates affection and sensations (69). Even though most studies do not consider hemispheric differences of the left or right hippocampi, the lateralization is seemingly significant to consider and has been proposed to be critical for cognitive function (70). The entorhinal cortex is a crucial structure providing input to the hippocampal circuitry through its connections to the DG (71). The importance of projections from the entorhinal cortex to the hippocampal structures for spatial navigation and memory function has been demonstrated (72). Impairments in these entorhinal cortex related structures are associated with manifestations of mild AD, where memory impairment is the earliest symptom most frequently observed (73, 74).

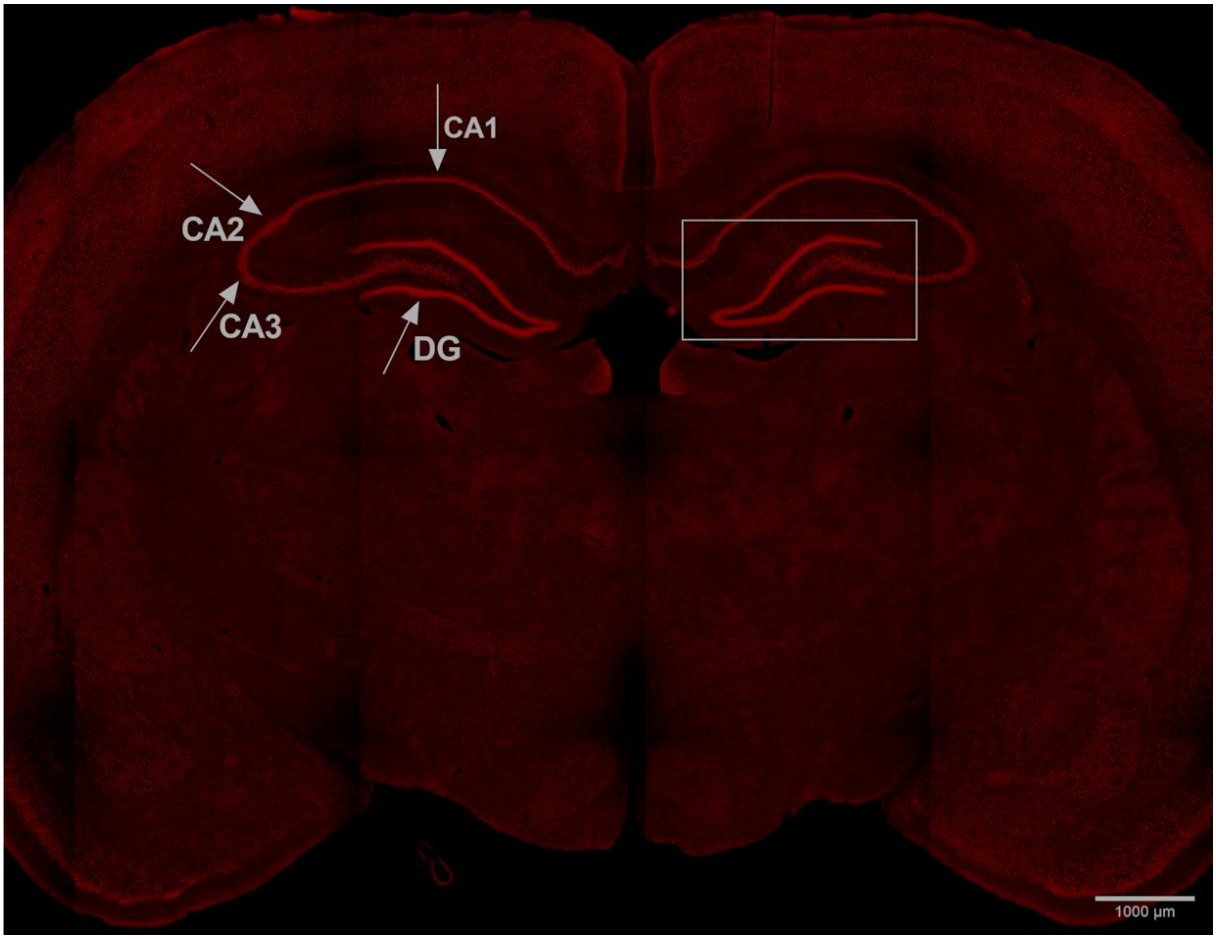


Figure 1: Overview of structures (DG, CA1, CA2, CA3) in a coronal section of the hippocampus, stained with anti-neuronal nuclei (NeuN), a neuronal marker. The DG is framed in the right hemisphere for visualization of the area of interest.

1.5 Adult neurogenesis

Previously, it was believed that new neurons do not arise in the adult brain, however in 1965 Altman and Das provided the first anatomical evidence of postnatal neurogenesis in the rat hippocampus (75). In 1998, Eriksson at al. (76) were the first to demonstrate the generation of new neurons in the DG of the hippocampus of an adult human brain. Neurogenesis is the development of new neurons, the endogenous process where neuronal stem cells go through phases of proliferation, differentiation and migration. This process is altered in neurodegenerative diseases (77). Adult neurogenesis arises within specific neurogenic regions; the subventricular zone of lateral ventricles and the subgranular zone (SGZ) of the DG (figure 2A) (78). The microvasculature is closely associated with cell division in the SGZ, and furthermore, some angiogenic factors are associated with increased adult neurogenesis (79). Newborn cells are highlighted with arrows in figure 2B.

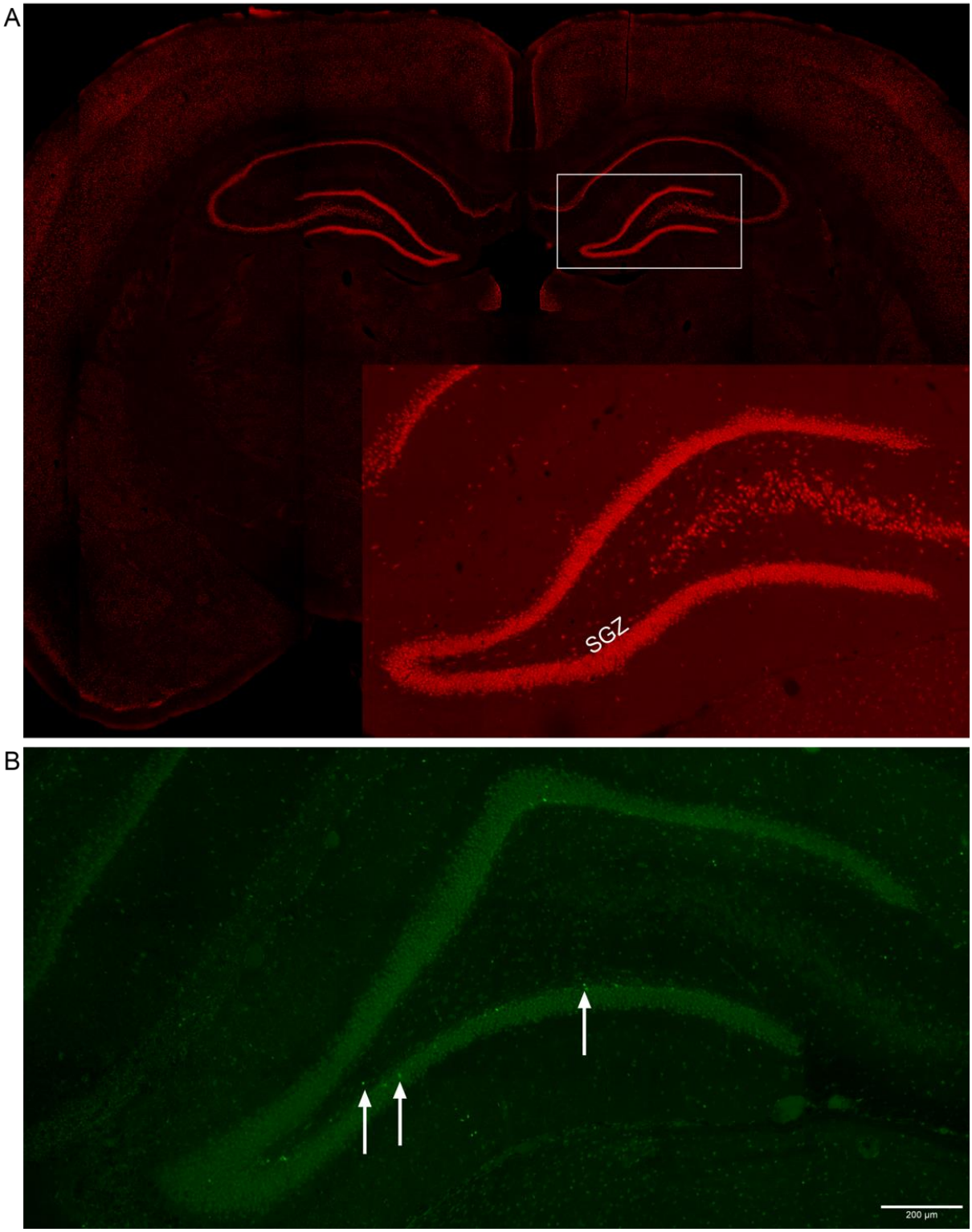


Figure 2: ***A****: Overview of the SGZ in the dorsal DG of the hippocampus.* ***B****: Adult neurogenesis in the dorsal DG of the hippocampus, stained with anti- bromodeoxyuridine (BrdU) to detect newly proliferated cells.*

In the SGZ of the DG, new neurons originate from neural progenitor cells, of which most of them differentiate to granule cells, and a minor proportion become glial cells (80). There are two types of adult neural progenitors in the SGZ, type I- and type II hippocampal progenitor cells. The microenvironment of the intertwined DG network circuitry in which these progenitor cells reside provide excitatory GABA and glutamate signals from the entorhinal cortex, and inhibitory GABAergic signals from the DG (1) (figure 3). New cells in the DG migrate and incorporate into the adjacent neuronal network (81). In young adult rats, approximately 9000 new cells are generated in the DG every day, a rate that becomes reduced with aging (82). Within three weeks, the main proportion of newborn neurons die (83), whereas the surviving cells of the adult DG mature into functional neurons (84). The distribution of newborn neurons in the DG is not even, and studies suggest that the quantity is higher in dorsal DG compared to ventral DG (85).

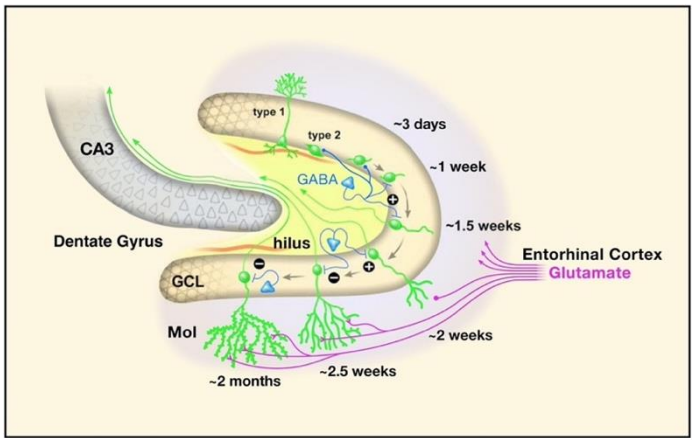


Figure 3: Schematic presentation of adult neurogenesis in the SGZ of the DG. The neural progenitor cells go through a maturation and differentiation phase, stimulated by inputs from the surroundings. The figure is adapted and modified from Zhao et al. (1).

It is assumed that cues within neurogenic niches where adult neurogenesis occurs initiate neurogenesis (84), and this directly affects cognitive processes such as learning and memory (81). Exercise and young plasma are two factors found to increase adult hippocampal neurogenesis in aging mice (61, 86). These studies in mice suggest that cognitive impairments during aging could be mitigated by changes of factors from the systemic environment, and that the underlying mechanisms are not well-characterised.

1.6 Neurobiology of AD

The hallmarks of AD are accumulation and aggregation of Aβ peptides into plaques and accumulation of neurofibrillary tangles, a phosphorylated tau protein in an abnormal form. Aβ plaques and neurofibrillary tangles occur extracellularly and intracellularly, respectively (figure 4) (9). It is likely that excess accumulation of Aβ peptides intracellularly leads to cellular autolysis, releasing the content in the extracellular matrix, and hence precedes the Aβ plaque formation (87, 88).

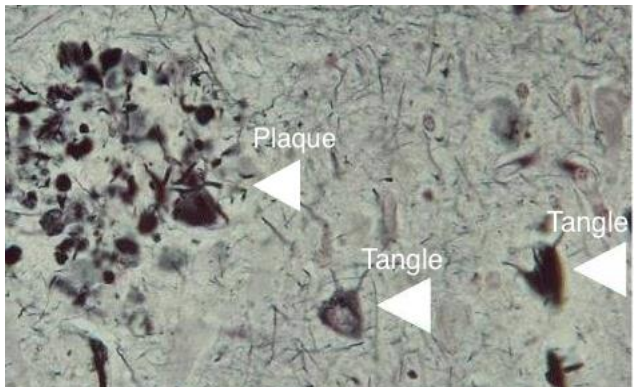


Figure 4: Neuritic plaques (extracellular) stained with Bielchowsky silver staining, which also labels neurofibrillary tangles (hyperphosphorylated tau, intracellular). The figure was adapted and modified from DeTure et al. (3).

Phosphorylated tau destroys microtubules and hinders essential conveyance inside neurons and the transport of molecules and nutrients (9). By interfering with the communication through synapses, Aβ plaques contribute to the damage and eventually death of 80% of neurons (16). The accumulation of Aβ plaques and phosphorylated tau increases as AD progresses, and is followed by depletion of synapses, in addition to

inflammation and atrophy of neurons (9, 16). The neuropathologic lesions are clinically alike among patients, however the cause of the disease is multifactorial and yet to be deciphered.

1.6.1 AD brain proteinopathies

In AD pathogenesis, one of the impaired essential mechanisms is the clearance of Aβ that leads to an imbalance in the generation and clearance ratio. A clearance abnormality will subsequently lead to the aggregation of Aβ peptides into plaques (89). The low-density lipoprotein receptor interacts with Aβ peptides and mediates clearance by transport through the BBB into cerebrospinal fluid or blood. In the AD brain the expression of lipoprotein receptor-related protein-1 is decreased (89, 90). Lower levels of lipoprotein receptor-related protein-1 have been found to contribute to the accumulation of Aβ in the AD brain (89, 91), additionally, creating a positive feedback amplification as pathological levels of Aβ are found to decrease the expression of lipoprotein receptor-related protein-1 further (92).

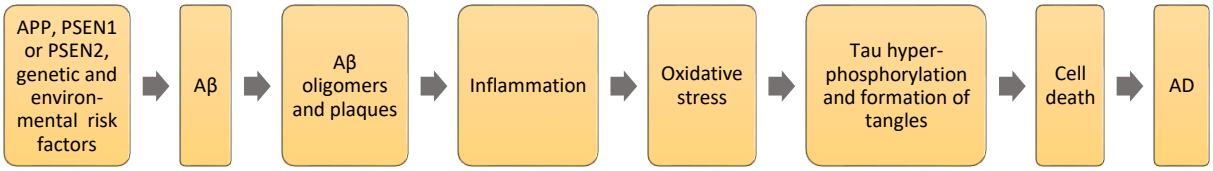


Figure 5: A schematic presentation of the amyloid cascade presented by Hardy and Higgins (93).

In 1992, Hardy and Higgins presented the amyloid cascade hypothesis as the prominent causative agent for the pathology of AD (figure 5) (93). The hypothesis was based on overproduction- and decreased degradation of Aβ with deposition of plaques as the initial event. As a result of this, numerous subsequent events occur, including chronic inflammation, oxidative stress, dysfunction of synapses, phosphorylation of tau and formation of tangles and neuronal loss. However, more recent evidence on the amyloid hypothesis points toward a multifaceted process where the initial linear cascade is no longer tenable (figure 6) (93, 94).

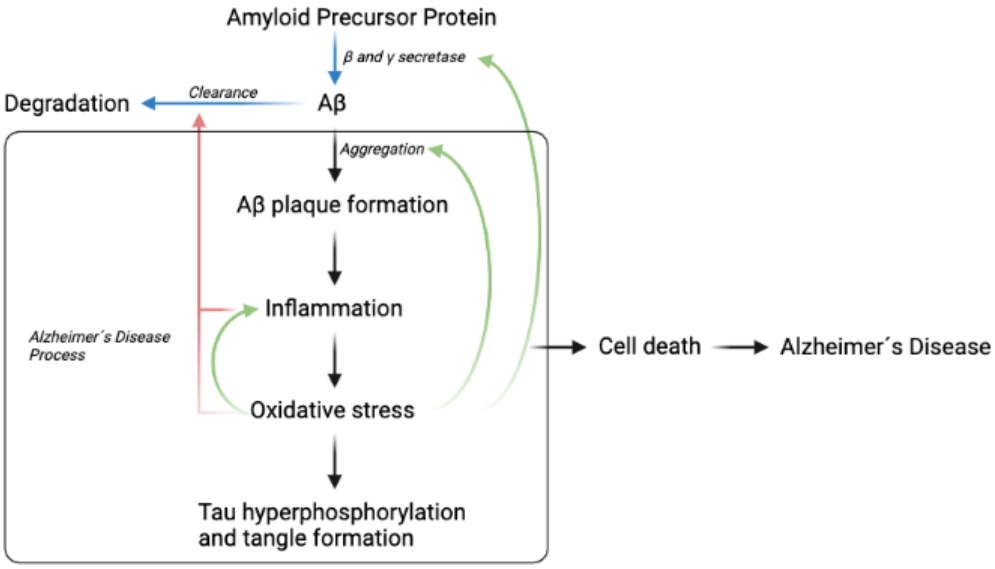


Figure 6: Presentation of feedback loops in the amyloid cascade. Blue arrows present pathways in healthy cells; black arrows present pathways in AD brain; green arrows present activated pathways in AD brain; red arrows present inhibited pathways in AD brain. The figure is adapted and modified from Doig et al. (94). Created with BioRender.com.

Neurofibrillary tangles are composed of aggregates of hyperphosphorylated tau-protein within neurons (11, 94). Tau dissociates from microtubules and forms tangles when hyperphosphorylated, shown downstream in the amyloid cascade in figure 5 and 6 (94). Neurons are the primary expressors of this microtubule-associated protein, which has been proposed to affect synaptic function (95), where the number of cells expressing neurofibrillary tangles has been associated with AD progression (96). The formation of neurofibrillary tangles as a mechanism in AD brain pathology leads to impaired functionality of the microtubule and compromises axoplasmic transport, in addition to causing neuronal and synaptic disabilities (figure 7) (11). Fortunately, AD proteinopathies seem to be modulated and mitigated due to physical activity and exercise, reviewed elsewhere (97).

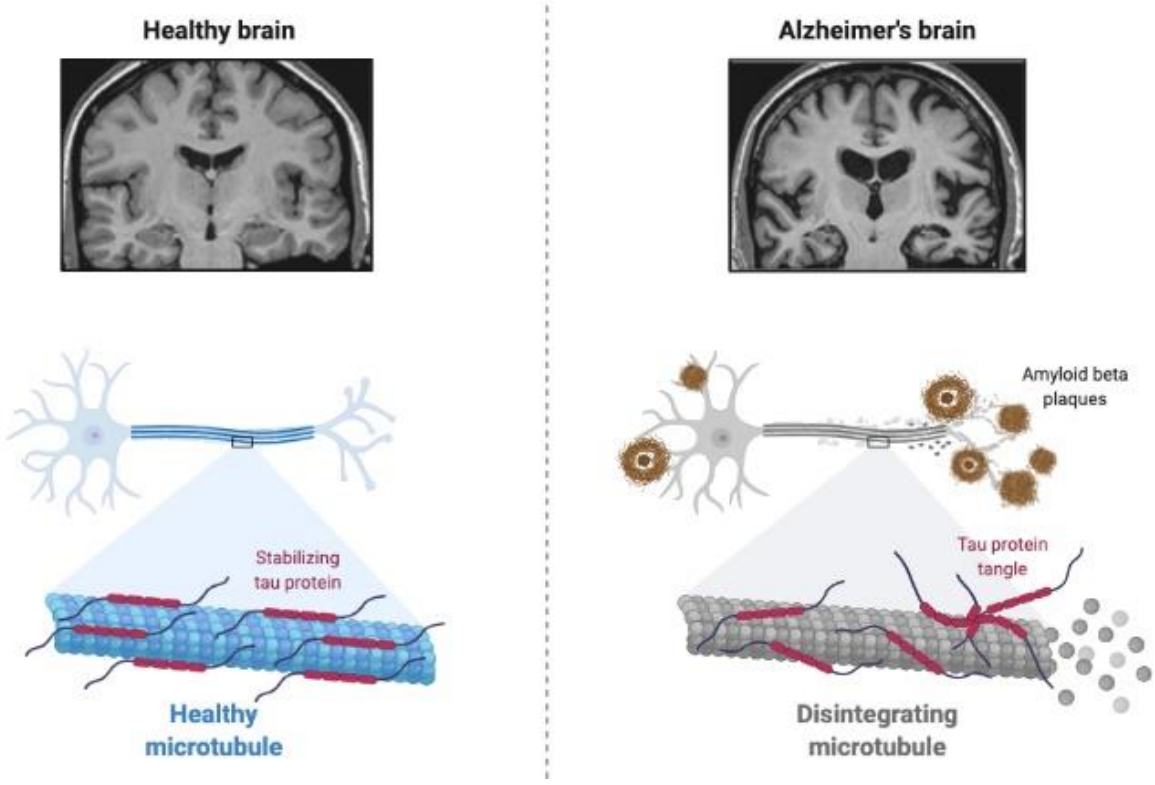


Figure 7: Comparison of features within a healthy brain and an AD brain. Adapted and modified from BioRender.com.

1.6.2 Chronic neuroinflammation in the AD brain

Persistent neuroinflammation is prominent in the AD brain (94). Inflammation and activation of complement are generally protective responses in response to cell and tissue injury. However, when this defence system is imbalanced, it can exert excessive damage primarily mediated by glial cells; microglia and astrocytes (98). Microglial cells are the most prominent innate immune cells in the central nervous system (CNS) (99). In the AD brain, microglia and astrocytes are activated in the presence of Aβ plaques and neurofibrillary tangles as an attempt to clear these toxic proteins. This activation generates a neuroinflammatory milieu and could resolve chronic inflammation if the immune cells are constantly activated and there is inadequate clearance (100). However, the activation of glial cells has been found both beneficial and damaging. Phagocytosis of Aβ by activated microglia may contribute to the clearance of neuropathological protein aggregates, but the activation is also neurodegenerative by recruiting astrocytes to release proinflammatory cytokines, such as interleukins and tumour necrosis factor (TNF) (98, 101, 102). There is

evidence of reduced pro-inflammatory marker levels and correspondingly enhanced anti-inflammatory marker levels as a response to exercise in the hippocampus of AD rodent models (103, 104).

1.6.3 Cerebral vascularization in the AD brain

The CNS is a complex system where vascularisation is vital for cells to function (105). High vascularization ensures sufficient oxygen and nutrition delivery to the CNS, on which glial cells and neuronal cells are dependent (106). A unique attribute of the blood vessel network in the CNS is the BBB (105). The cellular interactions in the BBB regulate vessel permeability between blood and brain by limiting molecular exchange intrinsically to protect the brain from toxins (106). Vascular abnormalities such as distortions in capillaries and small arterioles, and lowered vascular net density, particularly in the hippocampal area, are common pathological features in AD brains (107). To compensate for decreased tissue oxygenation, the physiological process of generating new blood vessels is the primary response (108). Administration of growth factors to restore pathological angiogenesis has been suggested a treatment to prevent cognitive decline (109). Additionally, exercise is demonstrated to increase the blood flow by upregulating certain angiogenic gene expressions; amongst these are the exercise-induced increase of *Vegf* mRNA in various tissues, such as rat skeletal muscle and brain tissue (110-113).

1.6.4 Neurogenesis in the AD brain

Concurrently with the progression and development of AD, deficits in the underlying mechanisms of adult neurogenesis occur, furthermore contributing to neuropathological conditions (77). In the AD brain, adult neurogenesis has been suggested to drop sharply compared to in nonpathological brains (114). Impaired adult neurogenesis in the AD brain might be due to disturbances in key pathways and signals necessary for hippocampal neurogenesis, perhaps involving tau, PSEN1 and APP, key signals found implicated in AD brains (78). Evidence suggests there could be therapeutic benefits from promoting adult neurogenesis to treat cognitive disorders, including AD (78). Brain-derived neurotrophic factor (BDNF) is a neurotrophic protein found to initiate the proliferation of neural stem cells. Additionally, BDNF induces the formation and promotes the survival of newborn neurons (115).

The advantageous effects of physical exercise on brain health are numerous; however, the underlying mechanism from the initial signal release until a response in the brain is reached, is yet to be established. Evidence points towards the importance of cerebral perfusion and increased vascular density in the brain in counteracting cognitive decline, by promoting neurogenesis, both during normal age-related decline as well as in neurodegenerative diseases (67). Recent discoveries have led to identification of direct effects of exercise on the CNS, and systemic mediators with positive effects on brain function (116). In line with this and the fact that adult neurogenesis arises in proximity to blood vessels and that newborn neurons proliferate in response to vascular growth factors (79), seemingly, the interaction between the systemic environment and neurogenic niches is improved and regulated by exercise. It still remains unknown how to fully take advantage of the beneficial traits of the systemic environment.

1.7 AD protective mechanisms

Numerous molecules are secreted by muscle during exercise. These exercise-induced myokines enable inter-organ communication. They are secreted from muscles into the

systemic circulation affecting distant organs, including the liver, adipose tissue and brain (116). One of these myokines secreted by skeletal muscle is Irisin, derived from its precursor membrane protein fibronectin type III domain-containing protein 5 (FNDC5), which is cleaved and sequentially secreted into the circulation. During exercise, Irisin has been shown to increase BDNF, an essential growth factor in the hippocampus, hence influencing neurogenesis, enhanced cognition and mood (117). Increased levels of cathepsin B in the systemic environment of mice induced by exercise has also been demonstrated (118). Cathepsin B is thought to stimulate neurogenesis by increasing the hippocampal mRNA expression of *Bdnf* (118). The brain and blood levels of BDNF is low in AD patients; hence physical activity and exercise has been suggested as a mechanism to mitigate symptoms of the disease (119).

1.7.1 Young plasma

Recent research has found the circulatory system as a route of communication from peripheral tissues to the brain (120). Systemic factors may cross the BBB and reach a cellular target within the parenchyma, to modulate neurons or glial cells. The systemic factors are then excreted back into the periphery (120). Systemic factors abundant in adolescence have been shown to increase adult neurogenesis in the DG of aged mice (121), and it was proposed that changes in the systemic environment could contribute to changes in neurogenesis and directly counteract age-related neurodegeneration (121). Increased hippocampal plasticity and dendritic spine density in aged mice receiving young plasma (122), have led to speculations of a therapeutic potential within systemic factors. Another study found improved vasculature, greater blood flow and activation of stem cell proliferation in the hippocampus (86). Few studies assess similar effects with young human plasma in aged mice. However, a study injected human plasma from the umbilical cord to old mice. They discovered a revitalised aged hippocampus function and proposed that the tissue inhibitor of metalloproteinases 2 was necessary to achieve the beneficial cognitive effects (123). Additionally, a small, randomized clinical trial found infusions of young plasma to patients with mild cognitive impairment- or early phase AD to be safe and possibly advantageous (124). Young plasma infusions were also associated with improved levels of activates of daily living in the patients (124). These studies indicate that young plasma is associated with rejuvenation of an aged brain. However, more research is needed on the mechanisms and the extent of the effect.

1.7.2 Exercised plasma

Evidence points towards an improved cognitive function due to exercise (125). Of high interest, similar effects have been found using systemically administered exercised plasma in aged mice (126). It is established that physical activity and high age relative cardiorespiratory fitness protects against neurodegenerative diseases and AD (127). A few studies are now investigating the possibility of conveying these beneficial effects via systemic factors, as observed in "young plasma-trials" (121). Injection of exercised plasma in mice have been shown to improve cognitive function and neurogenesis (128). These findings implicate a therapeutic potential from mechanisms induced by exercise, which could possibly be utilized in the development of future AD medication.

To date, few studies, and no clinical trials, have investigated the effects of exercised plasma on the AD brain. However, in 2020, a study assessed the effect of exercised plasma from mice infused into transgenic AD mice (129). The results indicated that cognitive impairment was improved after treatment, increased mitochondrial function and

hippocampal plasticity (129). The first study investigating the effect of exercised plasma in AD patients will be initiated by CERG/Nevro/Blodbanken St. Olav in the ExPlas trial from August 2021.

1.8 Candidate markers of importance

As reviewed above, systemic administration of beneficial factors seemingly contributes to brain rejuvenation (86, 122, 126, 128). Conversely, factors from an aged environment have been found to promote cognitive decline (121). The cause of the alterations seems to lie within the components of the plasma.

1.8.1 Neurogenic markers

Early growth response 1 (EGR1) regulates the transcription of neural cells (130). Silencing *Egr1* has been reported to lower tau phosphorylation, reduce Aβ morphology, and improve cognition (131), protective mechanisms against pathological hallmarks of AD. On the contrary, the Villeda study (2014) detected an increased expression of the EGR1 protein in brain sections of the heterochronic parabionts (aged-young) compared to isochronic parabionts (aged-aged), suggesting another preventative mechanism (122). Thus, the change of EGR1 expression might need to be interpreted in conjunction with other genes. FNDC5 is the precursor of Irisin and an essential regulator of metabolism. Irisin has been proven to have several beneficial physiological effects, particularly in reducing oxidative stress, anti-inflammatory effects, and neuroprotective effects through intracellular pathways (132). In response to exercise, FNDC5 is regulated through the receptor PGC-1α, modulating the significant beneficial effects of exercise on metabolism (117).

Another potential component of exercised plasma is BDNF, a neurotrophin and a fundamental regulator within neurobiological processes, induced as a response to endurance exercise (117, 133). The study on exercised plasma from mice infused into transgenic AD mice suggested that the protective effects were due to increased BDNF (129). BDNF binds selectively to a tyrosine-related kinase receptor, which in turn activates and increases the expression of PGC-1α, and hence increases FNDC5. Recent discoveries indicate PGC-1α/FNDC5/BDNF as an essential pathway for neuronal protection (133, 134). Another link is between Sonic hedgehog (Shh) and BDNF regulating neuroplasticity, where Shh increases the transcribed levels of BDNF in axons (135). The Shh pathway is necessary for signal transduction, and is known for its essential role as a morphogen in embryonic neurogenesis (136). Investigations in recent years have led to the identification of Shh signalling in the adult rodent brain, exerting effects on neural stem cells in the adult subventricular zone and SGZ of the DG (137), and astrocytes located in these neurogenic regions have been reported to promote neurogenesis by releasing Shh (138). Additionally, administration of Shh to mice has been demonstrated to increase adult neurogenesis by stimulating neural stem cell proliferation (138). Other studies present indirect protective effects of Shh on neurons, as cerebral angiogenesis may be stimulated by Shh signalling through interaction with the plasminogen system (139, 140). Hence there could be possible therapeutic links to Shh and diseases such as AD.

A member of the STAT-family, the signal transducer and activator of transcription 3 (Stat3) pathway is activated by pro-inflammatory cytokines, such as TNF and interleukin-1β, increasing the formation of reactive astrocytes found near Aβ-plaques (141), and conversely discovered to decrease neurogenesis (142). Protective attributes of genetic deletion of *Stat3* in a mouse model of AD has been demonstrated to induce several

beneficial effects, such as attenuation of neuroinflammation, reduction of soluble Aβ and amyloidosis, all attributes ameliorating AD pathology (141). Furthermore, evidence suggests that the suppression of *Stat3* promotes neurogenesis (142). A study demonstrated a link between the neurogenic markers BDNF and Stat3, however conversely, they found supressed Stat3 expression to attenuate the neurogenic effects of BDNF (143). Nevertheless, a change in the gene expression of neurogenic markers might indicate the occurrence of neurogenesis.

1.8.2 Inflammatory markers

Peripheral and central systemic factors are central modulators of cognitive function and overall brain health. If neurodegenerative pathology is present, the brain is compromised, and a common feature of the condition is systemic inflammation. CNS inflammation is exacerbated by systemic inflammation, and if not prevented, this milieu has been demonstrated to correlate with cognitive decline (55, 144). Exercise is found to counteract this "metabolic syndrome" by mediation of an anti-inflammatory environment (145), resulting in functional and cellular brain alterations in mice (126). TNF is demonstrated to be one of the pivotal proinflammatory cytokines involved in neurological disorders (146), and increased levels are found in AD patient serum (147). A decrease in the expression of the gene encoding inflammatory TNF could indicate mitigation of systemic inflammation.

1.8.3 Angiogenic markers

The CNS is a complex system where vascularisation is vital for cells to function. Glial cells and neuronal cells are dependent on a fresh supply of blood, and hence a well-vascularised CNS. A unique attribute of the blood vessel network in the CNS is the BBB (105). The BBB is built up of tight junctions of vascular endothelial cells in brain capillaries and astrocytes, restricting the passage of solutions from blood to the brain. This physiological barrier protects the nervous tissue from toxins, where alterations, such as Aβ plaques, might contribute to pathological hallmarks and the progression of neurodegenerative diseases (148). Physical exercise has been discovered to enhance capillary density due to angiogenesis and sprouting novel capillaries from vessels, an indirect mechanism of exercise that refines brain function (67).

Vascular endothelial growth factor (VEGF) has been established as an essential cytokine for vasculogenesis and angiogenesis in the CNS (105). *Vegf* expression in AD hippocampal arteries tends to be reduced compared to non-pathological brains (149). Lining the brain vasculature is the lactate receptor HCAR1, which enhances cerebral VEGFa when activated. Lactate has been found to induce regulation of angiogenesis in the CNS, and the downstream effect of HCAR1 activation was increased angiogenesis and VEGFa levels in the hippocampus, demonstrating a principal link between muscles, blood and brain health (67). These findings substantiated physical activity as a potential preventative measure for brain pathologies or lactate as an adjuvant for individuals further in the disease progression without the ability to be physically active (67). Following activation of VEGF, there are numerous downstream signalling cascades; amongst them is the urokinase-type plasminogen activator system encoded by urokinase-type plasminogen (*PLAU)*. PLAU is involved in degrading the build-up of aggregated Aβ and processing APP, preventing Aβ neurotoxicity (150), in addition to stimulating angiogenesis (139). An upregulated gene expression of angiogenic markers might imply amelioration of AD pathogenesis.

1.9 Animal model for AD – The McGill-R-Thy1-APP

Animal models cannot fully mimic the AD pathology in humans, but they aim to replicate symptoms, causes or lesions of the disease, in order to elucidate and understand aspects of the disease. For this cause, numerous genetically modified animal models have been adapted. (151). The morphologic signatures needed to diagnose AD, tau- and Aβ pathology are often replicated in AD models. In addition, neuronal loss, inflammation, gliosis and alterations in neuroplasticity are considered components of importance to mimic the aspects of the disease (152). The most common way to generate transgenic models of AD is to overexpress *App*. These models exhibit amplified levels of Aβ but do not demonstrate the hallmark tau-hyperphosphorylation and excessive neurodegeneration (2, 153, 154).

In the rat model of AD utilised for this study, McGill-R-Thy1-APP, two genes associated with early-onset AD are expressed: The Indiana- and the Swedish mutation, present in *App*. This transgene encodes the human APP 751 (hAPP751). The cDNA encoding hAPP751 controlled by the murine Thy1.2 promoter enables amyloid pathology, one of the hallmark molecular pathological changes in an AD brain. In the early 1990s, a double mutation in exon 16 in *APP* (chr21:27269939 G>T, chr21:27269938 A>C, KM670/671NL, (155)) was identified within two Swedish families, segregating with early-onset AD, hence its name "The Swedish mutation". The two base pair inversions on the N-terminus of β-amyloid of the transcript, close to the gamma-secretase cleavage site on the APP gene, is the probable cause of pathogenicity (156). This mutation causes an increase in the production of Aβ with a 6-8 fold compared to nonmutants, a direct relationship with the clinicopathological phenotype and the mutated genotype (157). In the same decade, another mutation linked to early-onset familial AD was identified, "the Indiana mutation". A missense mutation on exon 17 (chr21:27264096 G>T, V717F, (158)) increasing the Aβ42/Aβ40 ratio in blood plasma results in an increased accumulation of toxic $A\beta_{42}$ in the brain (159). This amino acid substitution, phenylalanine for valine, was found to be an essential pathogenesis contributor within familial related early-onset AD (160).

1.10 Purpose and aim

Adult neurogenesis decreases with age and possibly decreases at an even higher rate in the AD brain (114). Exercise has been shown to increase neurogenesis and the survival of newborn neurons (61). Exercise-induced blood borne factors have been shown to cross the BBB and affect the aging brain (126).

The overall aim of this thesis is to examine whether systemic factors can transfer the beneficial effects of exercise on neurogenesis to the AD brain. This is done by quantification of BrdU/NeuN -labelled neurons, and mRNA expression measurements of neurogenesis related markers.

Secondary aims are to examine the effects of exercised-blood factors on mRNA expression of inflammatory and angiogenic markers.

The hypothesis of this thesis is that adult neurogenesis in the hippocampus of the AD brain can be enhanced through systemic administration of exercise-induced blood factors.

2 Methodology

2.1 Ethical statement

The study was conducted in compliance with animal research regulations in Norway; European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, Norwegian Animal Welfare Act §§ 1-28 and Norwegian Regulations on Animal Research §§ 1-26. The experimental procedures involving rodents were approved by the Norwegian food and safety authority (FOTS ID: 11740). The experimental design was established based on the principles of the three Rs of animal research ethics; replacement, reduction and refinement.

2.2 Study design

At the age of two months, the donor rats were randomly divided into two subgroups; one group followed a six-week exercise protocol, and the other group were sedentary rats. Plasma was collected from the donor rats at the age of 3.5 months, pooled, snap frozen, and later thawed and injected intravenously into the tail of transgenic AD rats. Three- and six-month-old AD rats were included to assess potential effects before and after Aβ plaque formation. This thesis only assessed six-month-old due to the time limit. The AD rats, McGill-R-Thy1-APP, received 14 injections with 0.8 mL of three possible treatments: exercised young plasma (EYP), sedentary young plasma (SYP) or saline (control). Half of the rats received injections intraperitoneally with BrdU (5 mg BrdU / 100 g weight), while the remaining rats received saline. BrdU injections were given to rats that were to be fixed in 4% paraformaldehyde for 24 hours prior to immunohistochemistry, while saline injections were given to rats whose tissue was used fresh or snap frozen for molecular biological methods, such as RT-qPCR. In each group, there were three subgroups with three-four rats who received the same treatment. An additional experimental group composed of three AD rats subjected to high-intensity interval training was included in the mRNA analyses to assess the direct effect of exercise on the genetic markers of interest. A schematic overview of the study design is presented in figure 8.

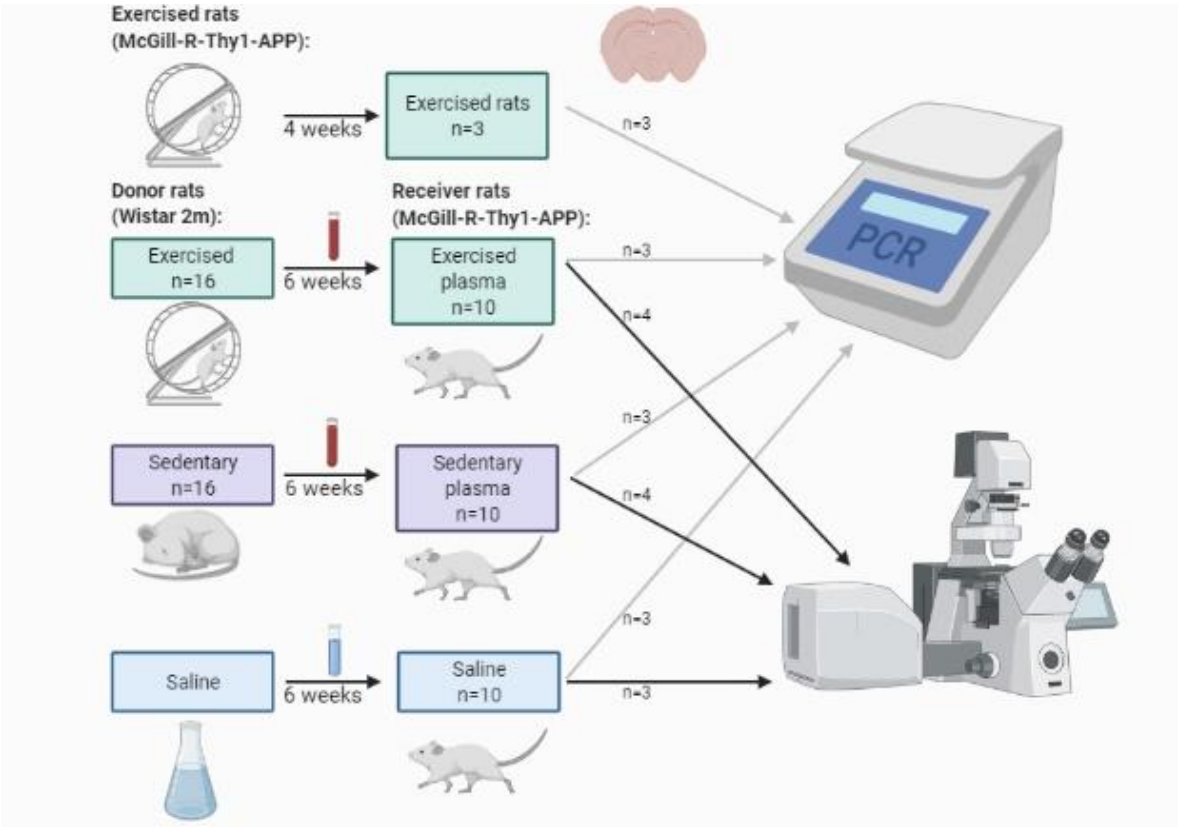


Figure 8: Experimental study design. For immunohistochemical analysis, three rats treated with saline, four rats treated with EYP, and four treated with SYP were chosen. Three rats from each of the treated groups and three exercised rats were chosen for the RT-qPCR analysis. Created with BioRender.com.

2.3 The McGill-R-Thy1-APP rat model

Male, wild type (non-transgenic) Wistar rats were used as plasma donors for the AD rats. The AD rats were homozygotic male transgenic McGill-R-Thy1-APP rats (provided by Professor Menno Witter at Kavli Institute for Systems Neuroscience at the Centre for Neuronal Computation at NTNU Trondheim) which expressed hAPP751, double Swedish and Indiana mutation, controlled by murine Thy1.2 promoter. The rats were housed on a 12-hour reversed light-dark cycle, two or three together in ventilated cages at 70% humidity, 23 °C and providing the rats access to water and standard rodent chow *ad libitum*. Experimental testing and procedures took place in the dark phase of the circadian cycle. All cages were environmentally enriched with wood stock for chewing and an opaque plastic house for shelter.

2.4 Brain tissue sectioning

For the immunohistochemistry procedures, fixated brains (24 h in 4% PFA, stored in DMSO till sectioning) were sectioned using a freezing microtome (HM430, Thermo Fisher Scientific). The brains were cut in 40 µm coronal sections in 6 series. Sections in series one were mounted on SuperFrost™ Plus Adhesion slides (Thermo Scientific), while the remaining sections were transferred in Eppendorf tubes containing DMSO storage buffer. In total, there were approximately 220 sections from each brain. The tissue collection occurred between 48-96 hours after the last injection of BrdU. Every sixth coronal

hemibrain section from each brain was stained with specific markers labelling neurons and BrdU expressing cells. All data correspond to every sixth coronal section.

2.5 Immunohistochemistry

BrdU, an analogue of thymidine, is an exogenous cell tracer known as the "gold standard" among cell proliferation research, including neurogenesis research (161). During DNA synthesis, the injected BrdU replaces the T in the genetic sequence, persists in the synthesised cells, and passes down to daughter cells (161). To enable the quantification of neurogenesis, segregating neuronal cells from the remaining cell types in a rat brain is vital (162). One can assume the presence of non-specific staining if BrdU is incorporated into every nucleus (163). NeuN is a phenotypic marker labelling the nuclei of mature neurons, exclusively expressed in the nervous tissue (161, 164). To ascertain the relative proportion of proliferating cells, counterstaining the tissue with NeuN combined with BrdU birth dating was performed. Quantification of neurogenesis utilising the expression of NeuN determines that the newborn cell is a neuron. In addition to identifying newborn neurons, decreased NeuN immunoreactivity could indicate neuronal damage, suggesting cell death (162).

2.5.1 Immunostaining protocol for BrdU and NeuN labelling

This was an indirect immunofluorescent protocol; specific monoclonal primary antibodies were detected by fluorescent conjugated secondary antibodies, that targeted and visualized BrdU and NeuN antigens in coronal brain sections. The Free-floating tissue sections were stained simultaneously with anti-BrdU (1:100 dilution, Invitrogen) and anti-NeuN (1:3000 dilution, Abcam). A detailed protocol is included in Appendix I. The positive BrdU control slides from rat intestines collected from one of the rats in this study were formalin-fixed and paraffin-embedded on glass slides and were first deparaffinised before following the protocol for the free-floating immunohistochemistry simultaneous with the brain sections of interest. Sections were incubated with sodium citrate buffer (10mM sodium citrate buffer, 0.05% Tween 20, pH 6.0) in a water bath at 60 °C for 30 minutes to unmask the binding sites. The sections were rinsed in Tris-buffered Saline (TBS, pH 8.0) to remove the DMSO buffer leftovers. For DNA denaturation, the sections were incubated for 30 minutes in HCl (1 M, pH 0-1) in a water bath at 45 °C, followed by rinsing and neutralizing for 10 minutes in borate buffer (0.1 M, pH 8.5). Three rinses in TBS were followed by incubation in 10% goat serum in TBS-Triton- X-100 (0.5% Tx, pH 8.0) to block non-specific binding sites for 60 minutes. Sections were incubated with primary antibodies; BrdU mouse monoclonal antibody (1:100 in TBS-Tx, Abcam) and NeuN rabbit monoclonal antibody (1:3000 in TBS-Tx, Abcam) at 4 °C overnight.

The following day the sections were rinsed in TBS-Tx before 60 minutes incubation with fluorescent conjugated secondary antibodies; Goat Anti-Mouse IgG H&L Alexa Flour 488 (1:100 in TBS, Abcam) and Goat anti-rabbit IgG H&L Alexa Flour 594 (1:100 in TBS, Abcam), followed by three rinses in TBS, and finally proceeding with mounting the sections on glass slides. See complete protocol and solutions in appendix IA and IB, respectively.

2.5.2 Detection and quantification of BrdU and NeuN labelled neurons

Z-stack images of the dorsal part of the DG from all sections, both left and right hemisphere, were imaged with Zeiss 880 Airyscan Confocal Microscope. A plan-apochromat 20x / 0.8 M27 objective lens was used. The fluorophores Alexa Fluor 488 (BrdU) and Alexa Fluor 594 (NeuN) were excited with the 488 nm and 561 nm laser, respectively. Z-stacks

were acquired at 3.98 µm intervals with a resolution of 1024 x 1024 pixels, and tile scanning made it possible to image the entire dorsal DG with a 10% spatial overlap. The Zen (Zeiss) image-acquisition software enabled a reusable imaging routine setup with the experiment designer module.

The images analyses were conducted utilizing Fiji (Fiji is just ImageJ) software for neuron quantification. A script was written to enable high throughput quantification of NeuN positive cells and cells positive for BrdU and NeuN. The script processed the raw images from the confocal microscope (Zeiss) by performing a user-defined background subtraction specific for each section. Subsequently, a predetermined intensity threshold was set to identify BrdU and NeuN positive regions, watershed segmentation separated densely packed NeuN positive bodies, and lastly, a colour intensity threshold was set to identify BrdU+ and NeuN+ colocalization (BrdU+/NeuN+). The script created three image files; one with both BrdU + and NeuN+ for colocalization (figure 9A); one to count all of the NeuN+ cells (figure 9B); and the last as a binary image of the colocalization used for quantification of newborn neurons (figure 9C). The region of interest was set before particle analysis for quantification (figure 9A). The script generated two separate files for the quantitative output; NeuN+ cells and BrdU+/NeuN+ cells. Fluorescent imaging, quantification and analysis of the results were performed while blinded to treatment.

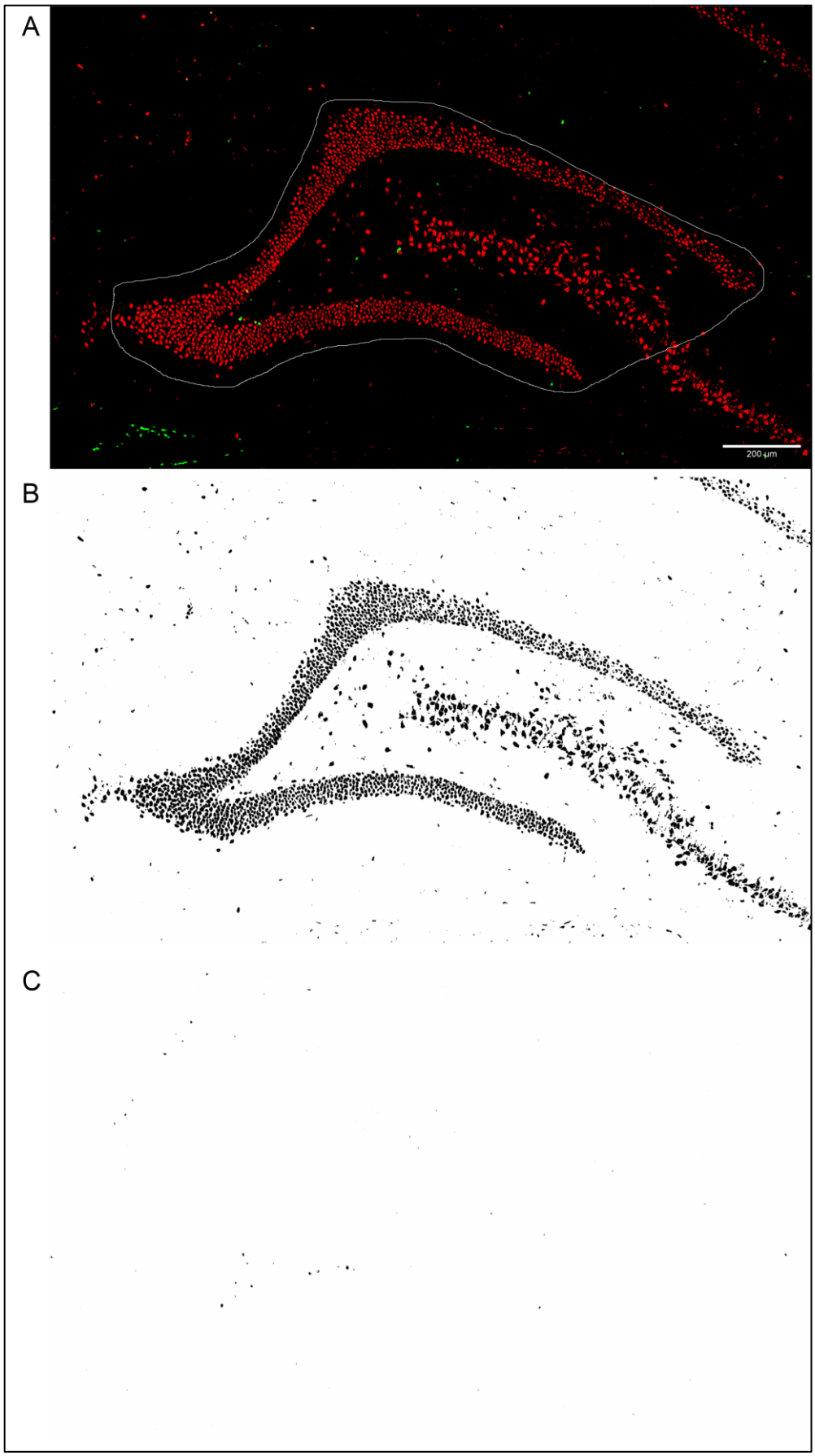


Figure 9: ***A****: Colocalization of BrdU + and NeuN+.* ***B****: Binary image of quantification of NeuN+ cells.* ***C****: Binary image of the colocalization used for quantification of newborn neurons.*

2.6 Quantitative reverse transcription-PCR

RNA isolation was performed using RNeasy mini kit (Qiagen) according to the manufacturer's instruction on all rat brains' right intermediate DG. The isolated RNA was quantified, and the purity assessed by ultraviolet-visible spectroscopy using a Thermo Scientific™ NanoDrop 2000 (Thermo Fisher). The total RNA generated from each sample (300 ng) served as the template for reverse transcription using QuantiTect Reverse Transcription Kit (Qiagen). The cDNA templates generated were stored at -20 °C until RT-qPCR was executed.

Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was the housekeeping gene chosen for normalisation for relative quantification. The primers targeting genes of interest were designed by using the "Basic Local Alignment Search Tool" (Primer-BLAST, National Centre for Biotechnology Information) (see appendix IIA, table 1 for base sequences). Validation of all primers against a standard serial dilution of rat cDNA was executed, assuring the adequate primer efficiency.

The RT-qPCR analyses were carried out on C1000™ Thermal Cycler, CFX96 ™ Real-Time System (Bio-rad). According to manufacturers' protocol, each validated primer was mixed with SYBR Green (Thermo Fisher) master mix in Eppendorf tubes. According to the plate layout, the reaction combinations were added to a 96-well plate, 20.0 µl in each reaction well. 5.0 µl of an 8-fold dilution of the previously transcribed cDNA was added in triplets to each reaction mix of the primers matching the genes of interest. A no-template control for every target gene was included to control for contamination of PCR mixes. The plate was sealed and centrifuged on ROTINA 420R centrifuge (Hettich) at 1000 rpm for 1 min to remove bubbles. See appendix IIB for the RT-qPCR cycling conditions. Melt curve analyses were performed to identify the presence of primer-dimers or nonspecific products.

Relative quantification by the comparative method ($2^{\Delta\Delta}$Ct) was used to analyse the RT-qPCR data. This method was formulated by Livak and Schmittgen and allows the assessment of the difference in gene expression of the gene of interest relative to the housekeeping gene (equation 1) (165).

$$\Delta\Delta CT = \Delta CT\,(target\ sample) - \Delta CT(reference\ sample)$$
$$= (CT\text{target gene} - CT\text{refernece gene}) - (CT\text{target gene} - CT\text{refernece gene})$$

Equation 1: Equation for fold change calculation. All fold change values were presented as $2^{-\Delta\Delta CT}$.

2.7 Statistical analyses

Statistical analyses were accomplished using GraphPad Prism 9.1.0 (GraphPad Software). A one-way ANOVA test was performed to analyze the variance between the groups for each of the experiments. If the overall variance test indicated significant variance between the treatments, it was followed by a Holm-Bonferroni post-hoc test. The Holm-Bonferroni test was applied to correct for multiple comparisons by adjusting the p-values and the rejection criteria: all p-values were sorted in increasing order. If the smallest p-value was greater than or equal to the set significance level ($\alpha = 0.05$) divided by number of comparisons (K), then no p-values were significant. If not, the next p-value was compared to $\alpha/(K-1)$. If greater or equal to this value, then no further p-values were significant, if not the next p-value was compared to $\alpha/(K-2)$, this continued until the p-value was greater or equal to the calculated threshold.

3 Results

3.1 Immunohistochemical analysis of neurons in the dorsal DG

AD rats injected with SYP (figure 10A) and EYP (figure 11A) had a greater number of BrdU+ cells than the control group (figure 12A). There was no difference in the total amount of neurons between the three groups (SYP: figure 10B, EYP: figure 11B, saline: figure 12B). The BrdU+/NeuN+ double labelled cells verified that the newborn cell was a neuron. These were primarily detected in the SGZ of the dorsal DG of the hippocampus, however also in other areas of the dorsal DG (SYP: figure 10C, EYP: figure 11C, saline: figure 12C).

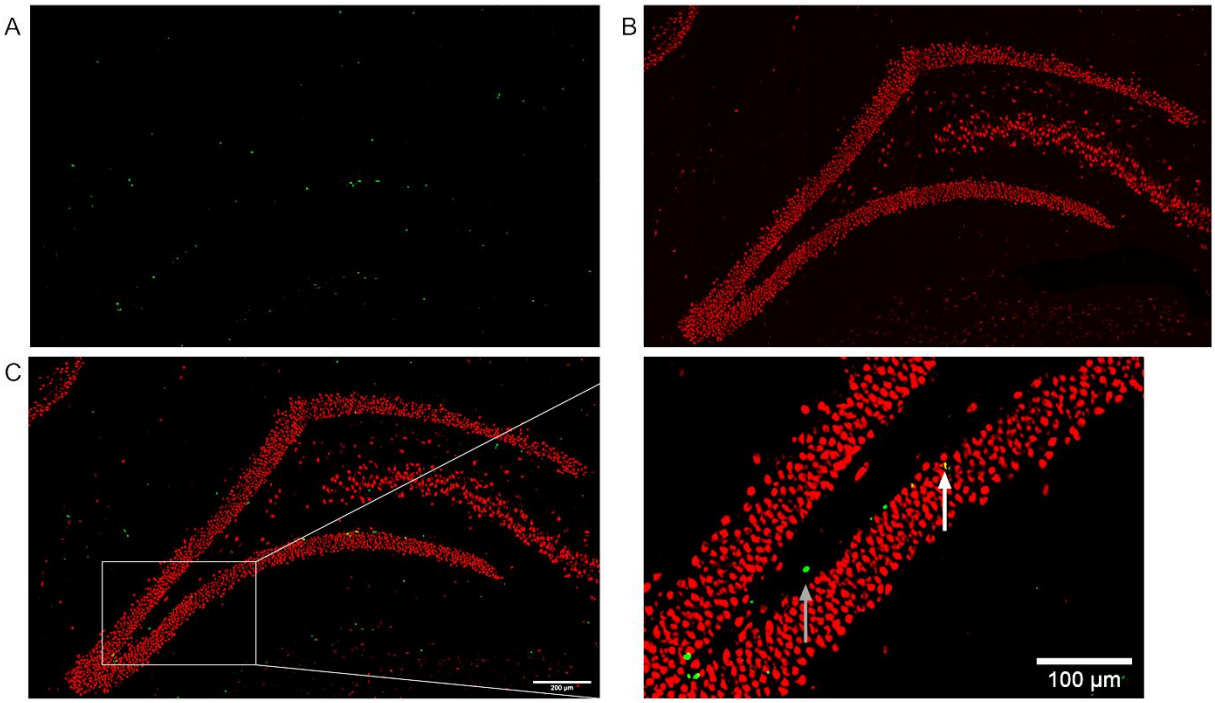


Figure 10: Example of images used for quantification from SYP treated rat. ***A****: A processed image of BrdU+ cells.* ***B****: A processed image of NeuN+ cells.* ***C****: A merged image of the two channels, where of double labelled cells are shown in yellow. The grey arrow points at a newborn cell that is not a neuron, while the white arrow points at a newborn neuron.*

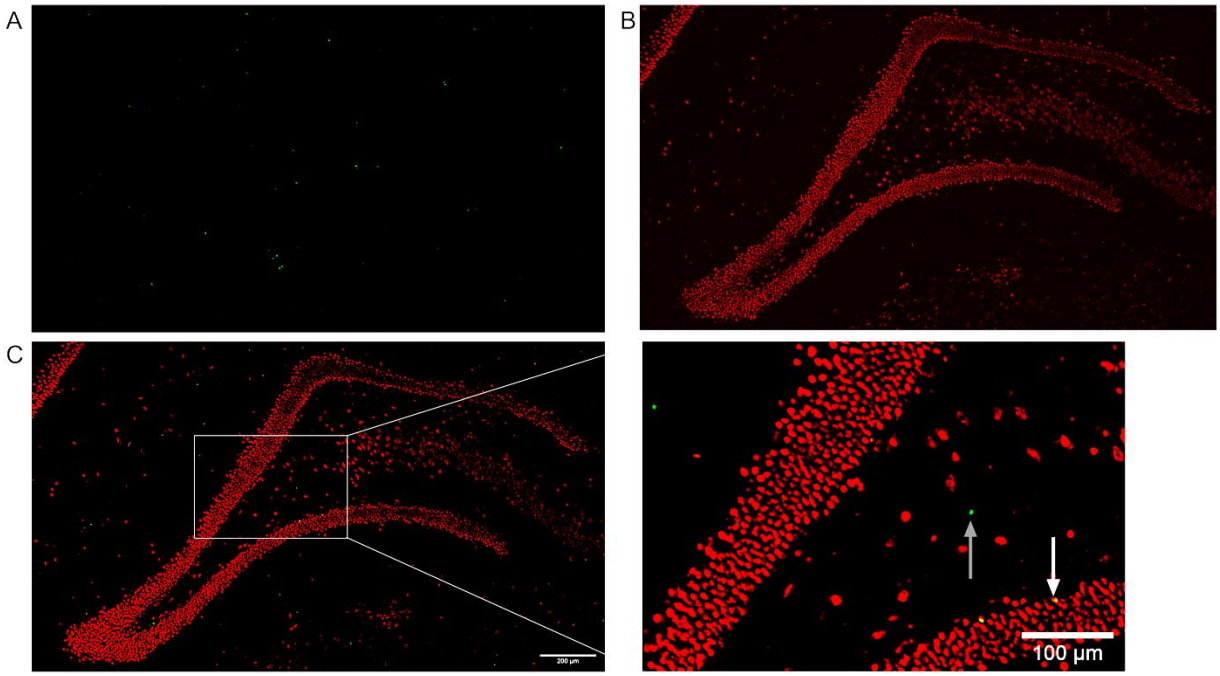


Figure 11: Example of images used for quantification from EYP treated rat. ***A****: A processed image of BrdU+ cells.* ***B****: A processed image of NeuN+ cells.* ***C****: A merged image of the two channels, where of double labelled cells are shown in yellow. The grey arrow points at a newborn cell that is not a neuron, while the white arrow points at a newborn neuron.*

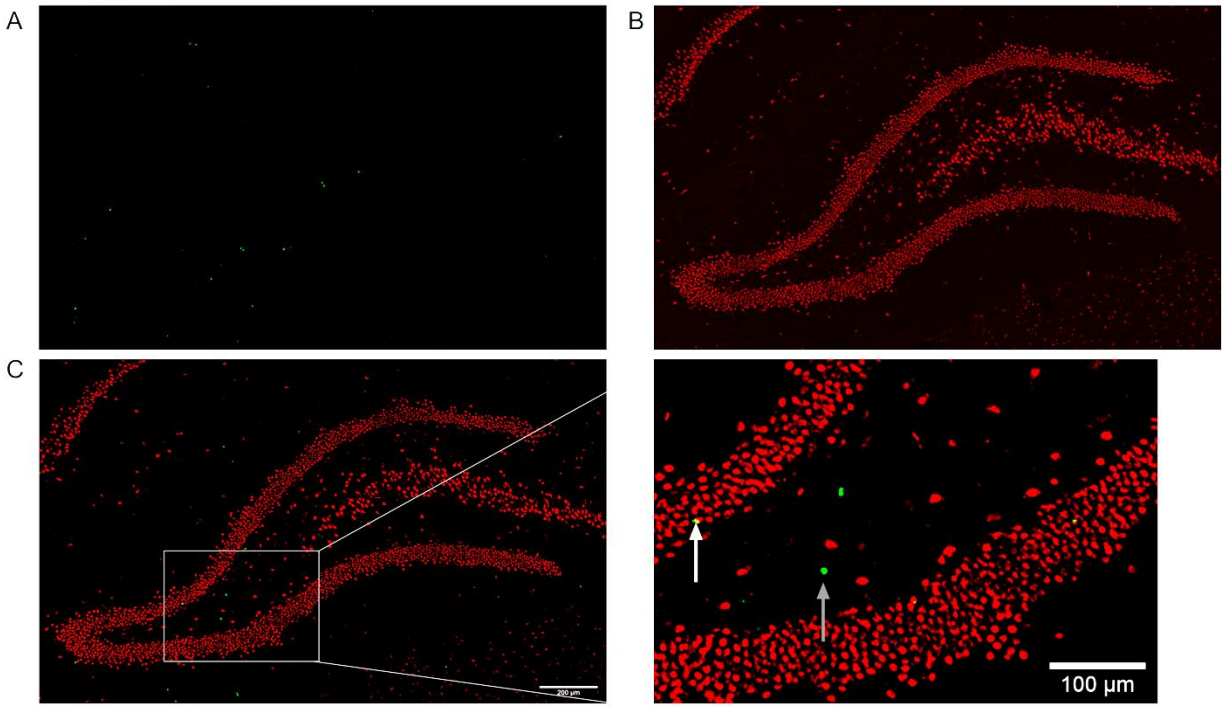


Figure 12: Example of images used for quantification from saline-treated rat. ***A:*** *A processed image of BrdU+ cells.* ***B:*** *A processed image of NeuN+ cells.* ***C:*** *A merged image of the two channels, where of double labelled cells are shown in yellow. The grey arrow points at a newborn cell that is not a neuron, while the white arrow points at a newborn neuron.*

Each set of BrdU stained sections was validated using an intestinal section as a positive control (figure 13A) and two negative controls without primary antibodies (figure 13B: BrdU-, figure 13C: NeuN-).

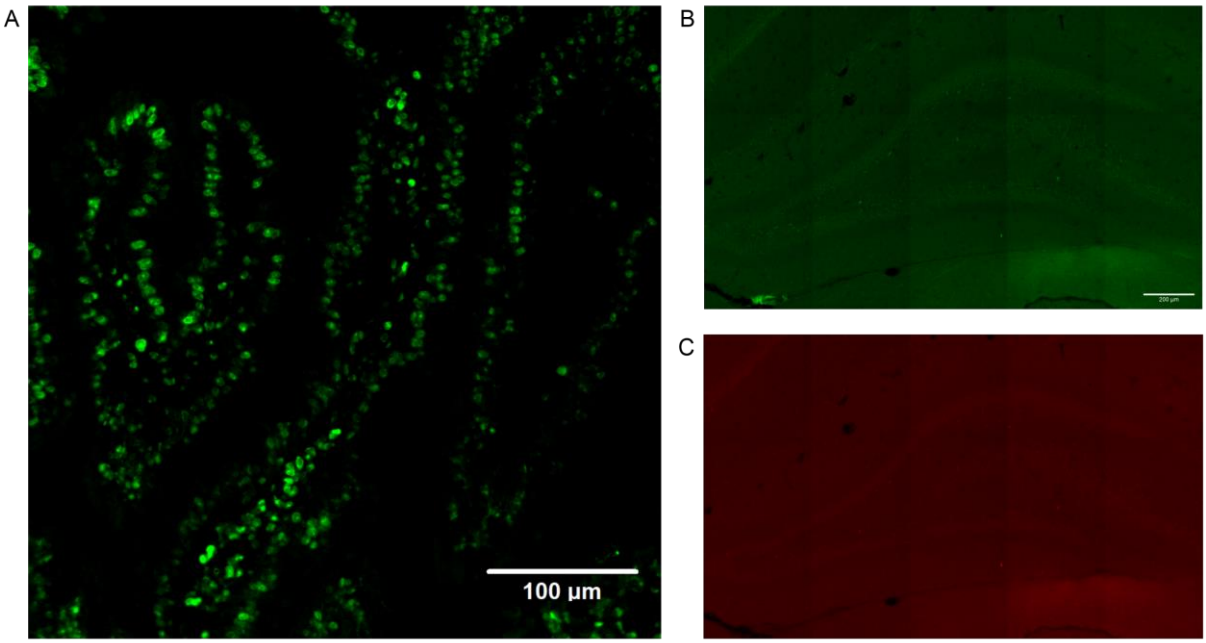


*Figure 13: **A**: Positive anti-BrdU control (intestines). **B**: Negative anti-BrdU control. **C**: Negative anti-NeuN control.*

3.1.1 Treatment effect of EYP on adult neurogenesis

Quantification was completed in each hemisphere in each section included in each rat's dorsal DG, giving a total sum of detected BrdU+/NeuN+ cells in the individual hemispheres (figure 14A: left hemisphere, figure 14B: right hemisphere) and a total number of neurons identified in the individual hemispheres (figure 14C: left hemisphere, figure 14D: right hemisphere).

The BrdU+/NeuN+ quantification confirmed that treatment with EYP and SYP both positively affected adult neurogenesis in the McGill-R-Thy1-APP rats compared to saline only treatment (figure 14A and B). One-way ANOVA analysis with Holm-Bonferroni post-hoc test revealed a significant rise in newborn neurons compared to the control group by a mean of 264.7% for SYP treated rats and 363.2% for EYP treated rats in the left hemisphere, and by a mean of 325.0% for the EYP treated rats in the right hemisphere. SYP treatment tended to increase neurogenesis in the left hemisphere, though this was not statistically significant ($p = 0.08$). Additionally, there was a tendency that the effect of EYP treatment gave rise to a more considerable increase of newborn neurons compared to SYP treatment, however, this was only significantly demonstrated in the left hemisphere ($p = 0.05$), and as a trend in the right hemisphere ($p = 0.2$). There was no significant difference between the left or right hemisphere ($p > 0.05$). The quantification of the total

number of neurons revealed no significant difference between the experimental groups or the hemispheres ($p > 0.05$, figure 14C and D).

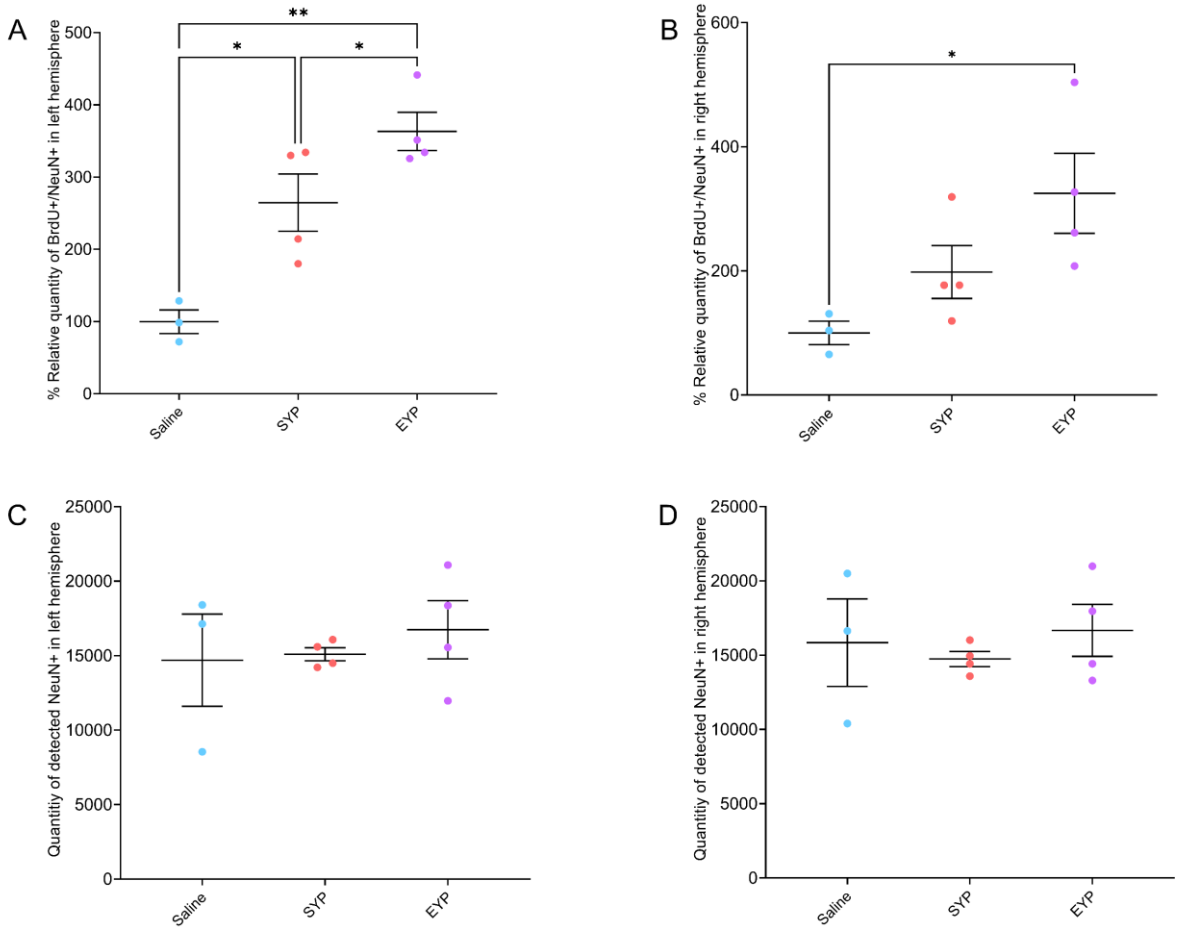


*Figure 14: **A** and **B:** The treatment effect of EYP and SYP on newborn neurons in a rat model of AD. % Relative quantity of BrdU+/NeuN+ in the two experimental groups (SYP and EYP, n=4) compared to the control group (saline, n=3), in left and right hemisphere respectively. **C** and **D:** The treatment effect of EYP and SYP on the total amount of neurons in a rat model of AD, in left and right hemisphere respectively. There were no significant changes in the total amount of NeuN+ cells in the two experimental groups (SYP and EYP, n=4) than in the control group (saline, n=3). All data are presented as mean ± SEM (standard error of the mean), variance was tested with a one-way ANOVA with Holm-Bonferroni post-hoc test (for **A** and **B**) * $p \leq 0.05$, ** $p \leq 0.01$*

3.2 Treatment effect on mRNA expression

Given that the injected plasma treatments seemingly increased neurogenesis in the dorsal DG of the hippocampus, RT-qPCR was performed using the right intermediate DG to identify potential genes contributing to the achieved effects and markers indicating other effects as angiogenesis and inflammation. The fold change in expression of the gene of interest relative to the housekeeping gene HPRT was calculated and normalized against the control group (saline). Tissue from exercised rats had already been collected, therefore "exercised rats" was included as an experimental group in the RT-qPCR analysis to address if exercised AD rats were comparable to EYP treated AD rats.

A one-way ANOVA test with Holm-Bonferroni post-hoc test was used to assess if the treatments led to any variance between the groups due to gene expression changes in the

AD rats. The calculated mRNA fold changes revealed no significant gene expression variation between experimental groups (figure 15).

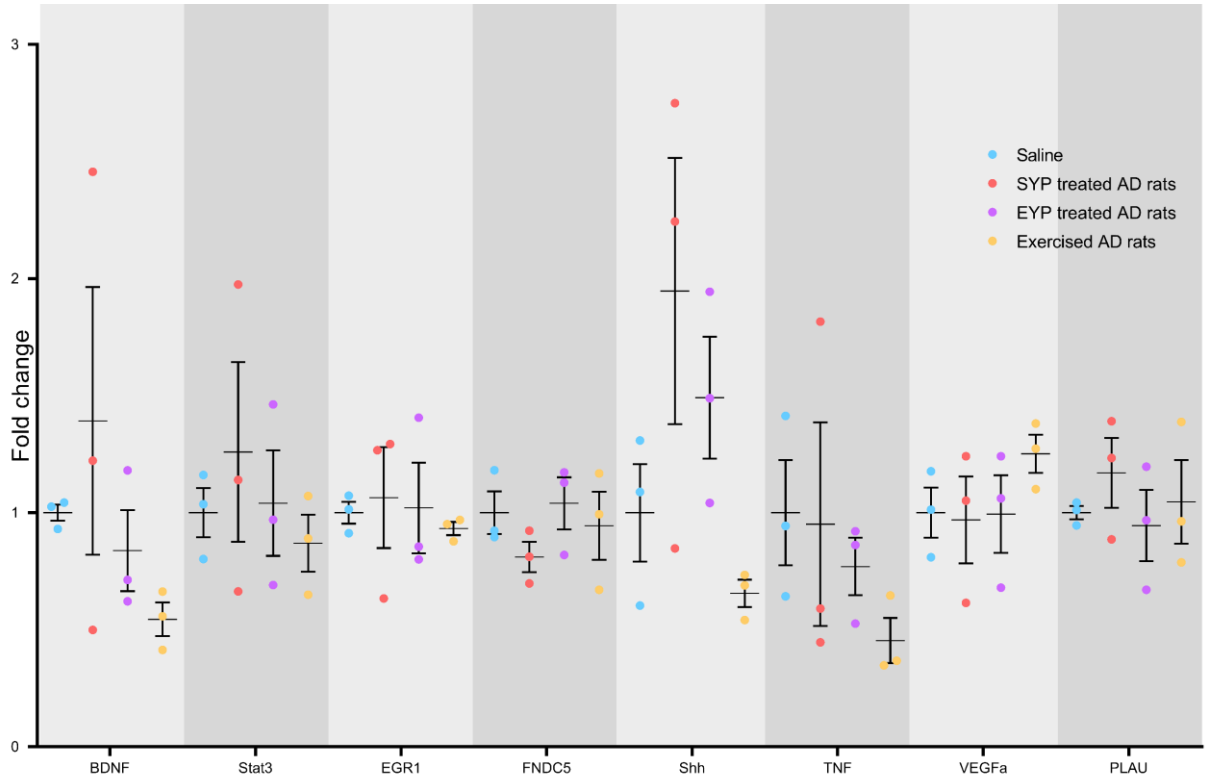


Figure 15: RT-qPCR results displayed as scatterplot of all genes together for comparison. The effect of treatment of all genes of interest presented as fold change relative to the housekeeping gene (HPRT) and normalized against the control group (saline). N= 3 for all groups. All data are presented as mean ± SEM (standard error of the mean), variance was tested with one-way ANOVA analysis.

The mRNA changes of neurogenic markers revealed a trend of upregulation of the gene expression of *Shh* in SYP compared to exercised ($p = 0.1$). All other variances between the experimental groups for neurogenic markers were not significant ($p > 0.05$). Both SYP and EYP treatment displayed an increased gene expression of *Shh* compared to saline by approximately 100% and 50%, respectively. On the contrary, the exercised group displayed a 35% decrease (figure 16E). The results of expression of *Bdnf* suggests a tendency of downregulation in EYP treated and exercised rats, however not significantly ($p > 0.05$). The variation within SYP treated rats made the results challenging to conclude, however, there might be a tendency of up-regulation of *Bdnf* (figure 16A). SYP treatment seemed to increase the expression of *Stat3* (figure 16B) and decrease the expression of *Fndc5* (figure 16C). There were no trends in gene expression changes of *Fndc5* and *Stat3* in neither the remaining experimental groups nor in the change of gene expression of *Egr1* (figure 16D).

The gene expression analysis and statistical analysis results suggested no significant difference in the inflammatory marker gene expression in response to treatments or exercise, *Tnf* ($p > 0.05$, figure 16F). The most substantial change in the expression level was between the two groups: saline and exercised rats, approximately 45% decrease for the exercised group. *Tnf* tended to be downregulated in both EYP treated rats and exercised rats compared to saline. The mRNA level of *Tnf* in SYP treated rats was seemingly

comparable to the control group, indicating that SYP did not regulate the gene expression of *Tnf*.

The results from the RT-qPCR analyses targeting genes for angiogenesis did not differ significantly between groups ($p > 0.05$). Relative to the control group, these results suggested no significant fold change in neither the expression of *Plau* (figure 16G) nor *Vegfa* (figure 16H) for the experimental groups. However, the results indicated a tendency of upregulation of *Plau* in the SYP treated AD rats and a tendency of upregulation of *Vegfa* in the exercised AD rats.

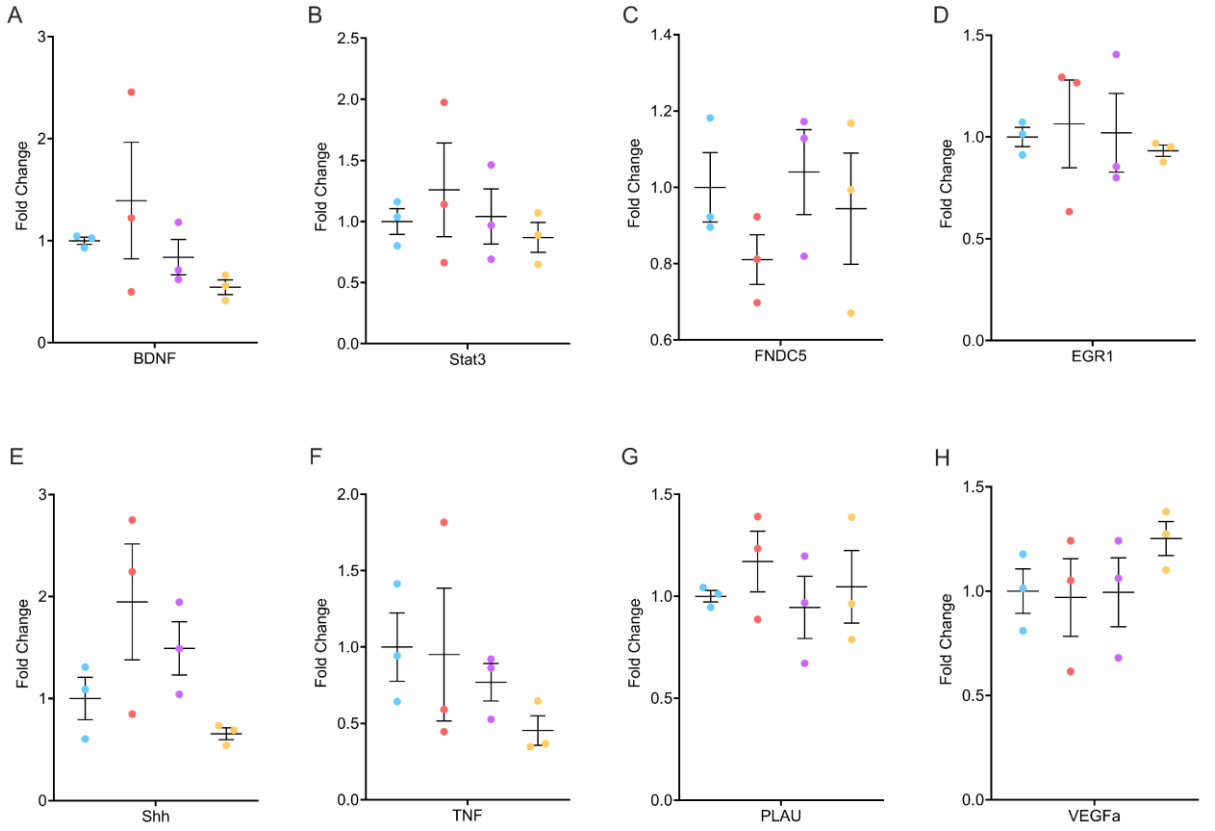


*Figure 16: RT-qPCR results displayed as **individual scatterplots** for each target gene - expressing the fold change relative to the housekeeping gene (HPRT) and normalized against the control group (saline). All data are presented as mean ± SEM (standard error of mean).*

4 Discussion

The results in this thesis are among the first to demonstrate upregulated adult neurogenesis in the DG of the hippocampus in an AD rat model injected with exercised plasma, due to the inherent potential of exercised-plasma factors. There was a clear trend of upregulated expression of *Shh* in response to plasma treatment. Thus, this neurogenic molecular marker is suggested as a trigger involved in mechanistic pathways mediating beneficial effects by promoting adult neurogenesis in the AD brain.

4.1 "Exercised plasma" infusions increase neurogenesis in the AD brain

4.1.1 Neurogenesis (BrdU)

Previous plasma exchange studies have suggested the circulatory system as an essential provider of systemic factors that seems to rejuvenate an aged brain (86, 122, 126, 128), mitigate pathological conditions one can relate to AD (126), or ameliorate AD cognitive dysfunction (129). Here, the obtained results were in line with previous findings of beneficial effects from young plasma on aged mice. The findings presented in figure 14A and B implied that systemic factors within EYP resemble the efficacy of physical exercise on adult neurogenesis (58, 128). Both plasma treatments increased neurogenesis in the AD rats, indicating an overall effect of young plasma injections, and the difference between SYP and EYP indicated that exercised plasma is more effective than sedentary plasma. The difference of effect between the treatments was statistically significant in the left hemisphere ($p = 0.05$), and in the right hemisphere there was a trend ($p = 0.2$) of increased newborn neurons due to the systemic factors from EYP compared to SYP. The amplified effect on adult neurogenesis in the EYP group suggests an added benefit of exercise. The increased neurogenesis could be due to higher concentrations of active molecules in EYP than SYP, or an additive effect of another molecule working through different pathways. The underlying mechanisms remain to be investigated. By these findings, the potency of EYP treatment was supported and could be utilized as an approach to enhance brain health.

4.1.2 Total neuron number (NeuN)

Quantification of neurons in the dorsal DG of the hippocampus (figure 14C and D) indicated no difference between control and experimental groups ($p > 0.05$.) The number of newborn neurons was relatively small compared to the total number of neurons, hence the increase of adult neurogenesis might not reflect the overall NeuN+ cells. Interestingly, in a similar study on aging rats, they found exercised plasma to increase astrocytes by 2-fold compared to a control group (126). This finding might suggest that EYP increased the proliferation of astrocytes and neurons and that the total amount of astrocytes could be a potential target of interest in future studies. Other possibilities causing an unchanged total neuron count could be the low sensitivity quantification method, that the short period of treatment did not induce detectable differences in the overall neuron number.

To further break down the immunostaining results, the two hemispheres were assessed independently to consider hemispheric asymmetry. It is known that there is functional differentiation in the hippocampus, both on the septotemporal axis as well as lateral hemispheric asymmetry (69, 70). There was an indication of a greater effect of both EYP and SYP in the left than the right hemisphere, though this was not statistically significant. These results could be evaluated in conjunction with previously conducted cognitive testing

or MRI results for these rats to assess similar trends of lateralized hemispheres, such as cognitive ameliorations or increased hippocampi volume.

4.1.3 Immunohistochemical methodology

Based on previous findings of adult neurogenesis (59, 60, 85, 166), the dorsal DG of the hippocampus was assessed for neuron and neurogenesis quantification in the sections. Both hemispheres were imaged, analysed and quantified to exclude hemispheric differences. The quantitative estimation of neuronal cells represents 1/6 of the total number of neurons in the dorsal DG for each rat.

Positive and negative control sections were stained to ensure the validity of the fluorescence staining in the newly established immunohistochemistry protocol. All brain sections were internal positive tissue controls for anti-NeuN, as brain tissue is expected to express the targeted antigen. The external positive (tissue/antigen) control for anti-BrdU ensured that the immunohistochemistry method was working and was optimized and verified that the negative results were valid. The positive control was intestines from a rat in this project, it also functioned as an internal control of the BrdU injection procedure and a negative tissue control for anti-NeuN as the intestines do not express the targeted antigen.

Some isolated examples of small regions of non-specific BrdU staining in a few rats were noticed while processing the images. In these cases, the ROI for cell counting was altered to exclude these regions. Suboptimal perfusion with paraformaldehyde was observed in two of these rats particularly, suggesting that this might be a possible cause.

A standardized quantification approach of adult neurogenesis using designed-based stereology has been suggested as the optimal method of choice to obtain rigorous and reproducible results (166). Within the timeframe for this master project, a stereology-based method was not feasible. On the one hand, the applied method was less precise and sensitive than stereology, hence there was a possibility of undetected small differences. On the other hand, an advantage was that this analysis method can be semiautomated to increase the high throughput further. Additionally, the script and its predetermined thresholds minimised user bias. In the end, the effect size was large, and lower precision compared to stereology was not an issue. The relative quantification results obtained with the conducted method in this project were decided representative of actual values.

4.2 Molecular mechanisms of neurogenesis

Shh has previously been linked with promoting the proliferation of adult neural stem cells (137). The findings in this project suggested a notably increase in the gene expression of *Shh* due to SYP treatment. There is a possibility that the Shh pathway was involved in increasing the adult neurogenesis detected by immunohistochemistry (figure 14A). However, as SYP induced the biggest fold-change of *Shh* expression, the treatment effects did not correspond in the two methods. The most significant increase in BrdU+/NeuN+ cells was due to EYP, which could indicate several pathways and signals causing the effect on adult neurogenesis. However, further validation is required to see if the expression of *Shh* increases as a result of EYP injections. Previous studies have found the administration of Shh to stimulate neural stem cell proliferation (138), which supports the notion that Shh signalling was somehow involved in the increased neurogenesis detected in this study. Nevertheless, this gene is worth extra investigation in future studies.

Particularly within the experimental group treated with SYP, there was high interindividual variability, this could have impeded the results. BDNF is a neurogenesis promoter and was expected to be upregulated comparable to similar studies (129), contrary, suppression of *Stat3* has led to beneficial effect in previous studies (142), hence a decrease could indicate a beneficial response. There were no trends in neither the analysis of *Bdnf* (figure 16A) nor *Stat3* (figure 16B). Despite this, the gene expression of *Bdnf* and *Stat3* in one particular rat increased as a response to SYP compared to the other rats. These results could imply that this specific rat had a greater effect of the treatment. Overall, there seemed to be a correlation of the change of expression of *Bdnf* and *Stat3*, most of the rats expressed similar changes in both of the genes. Similarly, downstream effects of Stat3 on neurotrophin signalling have previously been found (143), this could support the findings of correlated change of gene expressions and plausibly indicate that a common pathway was affected by the treatments and regulated these genes.

Interestingly, except for the *Tnf* mRNA expression in one particular rat in the SYP group, which displayed a considerable increase, the results suggested a tendency of down-regulation of *Tnf* (figure 16F). The fold change values from SYP varied, and the mean was skewed; 2 out of 3 rats were found to have decreased mRNA expression of *Tnf,* while the mean suggested no effect of treatment. Overall, the results pointed to a down-regulation of *Tnf* in the hippocampus from exercised and plasma-treated AD rats, with the most substantial change from the exercised group. A decrease in this group was expected as exercise has been found to have anti-inflammatory attributes in AD (103, 104). The results indicated a tendency of counteracted metabolic syndrome, nevertheless, a more prominent effect for the remaining experimental groups was projected, similar to a study where exercised plasma dampened inflammation in aged mice (126).

There were little to no change in the gene expression of *Fndc5* (figure 16C) and *Egr1* (figure 16D), this could mean that the mechanisms of protective effects of neither SYP nor EYP do involve these two factors, although this is contradictory to other studies (122). Angiogenic stimulation was anticipated in the treated groups based on previous findings on genes as a response to exercise (110-113). Additionally, upregulating the Shh signalling pathway has previously been shown to stimulate cerebral angiogenesis (140), one demonstrated mechanism is interaction with the plasminogen system and, subsequently, induction of angiogenesis (139). These previous studies support the finding of increased *Shh*, despite this, minimal effects were found on *Plau* (figure 16G) and *Vegfa* (figure 16H). However, to provide additional knowledge on how vascular plasticity is affected by treatment, fMRI is a planned analysis in the clinical study.

Based on the results from the RT-qPCR, there were no indications of correlation between the gene response in exercised AD rats and exercised-plasma-treated AD rats. The present study did not reveal a clear marker indicating a pathway from blood to increased neurogenesis. However, the increased expression of *Shh* could demonstrate such a connection between increased adult neurogenesis and systemic factors. Comprising the animal study this project was based on, proteomics analysis has been performed to characterize the proteome of the blood, and interestingly, some of the findings could be associated with increased gene expression of *Shh*. The proteome revealed increased plasminogen, as well as other complement cascade related proteins. While these discoveries were contradictory to decreased expression of complement proteins in the study by Miguel et al. (126) on aging mice, it could present a noteworthy missing link

between factors in the blood, genetic modifications and cellular alterations. Complement activation is a double-edged sword, with exacerbating effects on disease and protective attributes in maintaining brain homeostasis by the clearance of Aβ plaques. Activation of the complement cascade is a sign of inflammation and could contribute to neurotoxic glial cell activation and the driver of pathology (102). Nevertheless, in this framework and conjunction with increased adult neurogenesis, a complement activation could be a protective response of the injected plasma, furthermore, contribute to the alleviation and counteraction of AD pathology.

4.2.1 RT-qPCR methodology

Large variations within one group might be due to individual differences. This variation was a challenge, especially with few rats within the experimental groups. Optimally the gene expression analyses should be performed on tissue from both hemispheres as hemispheric lateralization might skew the results somehow. However, the majority of targeted genes did not provide statistically significant variation of gene regulations and other genes might be targeted in the left intermediate hemisphere for future studies to reveal the cause of increased adult neurogenesis. This strategy minimized the number of animals needed in accordance with animal research ethics, which could outweigh the potential limit of hemispheric lateralization. More rats could be included to make results easier to interpret and to increase the statistical power, however, this was not feasible in this study. The results obtained conceivably represent the truth with little change in expression of the targeted genes from treatment.

4.3 Limitations and advantages

The capacity to perform these experiments was limited by the rate at which we were able to breed the recipient rats and wait for them to reach the required age (2-7 months). As a result, the experiments were very time consuming, and this was a contributing factor to the low sample size. A general challenge with animal models is the translational aspect and the difficulty of recreating human AD pathology. The AD rat model used in this study (McGill-R-Thy1-APP) does not exhibit substantial neuronal loss or tau pathology, which was a weakness. On the other hand, it has been determined comparable to human pathology as cognitive impairment becomes prominent. Behavioural experiments are a focus of the overall animal study, which is partly the reason why this specific model was chosen. Additionally, this AD model develops neuroinflammation, Aβ accumulation and amyloid plaque is present throughout the hippocampus. Overall, it was a good model for AD concerning Aβ pathology but less regarding neuronal loss (2, 154). The translational value of the results might be limited due to the inadequate exhibition of a fully recreated human AD pathology, which is the case for all animal models. However, it is possible that health science innovations such as the 7 Tesla MRI (7T MR) can decrease this gap between basic research and translational research.

A limitation of the study could be that the time from the last BrdU injection until sacrifice varied from 48 hours till 96 hours. Previous studies have found decreased expression of BrdU positive cells corresponding with increased time interval between last injection of BrdU and sacrifice (167, 168). In this study, one rat was sacrificed after 96 hours post last injection, the same rat displayed the lowest BrdU signal within the EYP treated group. Conversely, the rat with the greatest BrdU signal was sacrificed after 48 hours. These might be coincidences, and this was not a trend for the remaining rats. However, future

studies could include a standardized time interval between last injection of BrdU and sacrifice to exclude this variable.

Another limitation of the study was the low statistical power caused by the low sample size and large interindividual variance, that could lead to an undetected effect, due to a minor difference and large variance (i.e., a type two-error). This could be that a marker that was decided not significantly affected in response to treatments in this study might reveal to be significantly affected if more rats were included in the study. Lastly, this was a multiple comparison study, and the more hypotheses that were checked, the more the probability of detecting false positives increased. To counteract this, Holm-Bonferroni post-hoc tests were implemented to reduce the risk of incorrectly identifying a statistically significant variance (i.e., a type one-error). Another approach to lower the risk of type one-error would have been to set a lower significance level, e.g. $\alpha = 0.01$, which would decrease the chance of a false positive from 5% to 1%. However, this would also decrease the power of the analysis to detect a difference, the analysis would be less sensitive. For the purpose of finding markers of potential interest, the set significance level was decided sufficient, however, a more thorough assessment of these markers is needed to show an effect of change in gene expression.

4.4 Future directions

This present study is a part of the animal study allied to the clinical project "ExPlas - Exercised Plasma to Treat Alzheimer's Disease". The results obtained will be supplemented by additional data from the animal study and provide valuable information to support the clinical study ExPlas.

During the work with this project, an essential immunohistochemistry protocol for detecting and quantifying neurogenesis and neuronal number has been established. This will be used for further work on tissue collected in the animal study. Identical analyses on neurogenesis and gene expression will be executed on experimental groups treated starting at the age of 2 months to evaluate if treatment prior to the development of amyloid pathology has a similar effect. It is hypothesized that there might be amplified effects in the youngest experimental group, which would be of greater translational value to primary intervention studies. Additionally, the immunostaining will be performed on exercised rats, as previous findings in the animal study showed high-intensity interval training to be associated with accumulation of Aβ plaques. The remaining series of brain sections can be used for additional immunostaining, first priority will be to detect amyloid plaque to assess if there has been a reduction in amyloid pathology as a result of EYP treatment. Another potential target could be glial cells to determine how different cells in the CNS are affected by plasma treatment.

Unquestionably there are unresolved molecular processes in blood that cause the proliferation of neural cells, which these mRNA analyses did not uncover. The presence of the common denominator in EYP and SYP needs to be further assessed in future studies. Other genes could be targeted and analyzed in conjunction with the obtained results from the proteomics analysis. Correlations between blood proteome and mRNA expression in the hippocampus would be an indication of systemic factors crossing the BBB and possibly accelerating adult neurogenesis. For future studies, the mechanism that mediated the beneficial effects on adult neurogenesis needs to be elucidated to display the translational value and clinical utility of the study.

5 Conclusion

To conclude, quantification obtained from the analysis of BrdU/NeuN immunofluorescence staining confirmed that treatment with EYP resemble the efficacy of physical exercise on adult neurogenesis seen in other AD models. Moreover, as the response was amplified in the experimental group receiving EYP compared to SYP treatment, we can conclude that exercised plasma provides an additional benefit on the generation of new neurons.

The analyses of mRNA expression of neurogenesis, inflammation and angiogenesis markers suggest a difference in gene expression of *Shh* in AD-rats treated with SYP. The finding of an increased neurogenic marker indicates an association between changes in the systemic environment after exercise and beneficial effects in the hippocampus. Despite this, the molecular genetic mechanisms behind the increase of adult neurogenesis generated by exercised-plasma-factors require further investigation.

The finding of increased neurogenesis in response to EYP reveals a possible novel therapeutic strategy for AD. This is one of the first studies to detect preventative and conceivably therapeutic attributes of EYP treatment. EYP treatment seemed to stimulate counteraction of neurodegeneration by increasing adult neurogenesis, demonstrated in a transgenic rat model of AD. Additionally, the results indicate that exercise-induced factors give rise to additional advantageous effects compared to sedentary young plasma. Targeting the systemic environment with exercise-induced blood-borne factors could be a step towards slowing disease progression, mitigating AD proteinopathies and improving cognitive functions. If future studies support our findings of exercised plasma to benefit the AD brain, then further research is needed to decipher the underlying molecular mechanisms. Eventually, this knowledge might be applied in the development of next-generation AD drugs.

References

1. Zhao C, Deng W, Gage FH. Mechanisms and functional implications of adult neurogenesis. Cell. 2008;132(4):645-60.
2. Leon WC, Canneva F, Partridge V, Allard S, Ferretti MT, DeWilde A, et al. A novel transgenic rat model with a full Alzheimer's-like amyloid pathology displays pre-plaque intracellular amyloid-beta-associated cognitive impairment. J Alzheimers Dis. 2010;20(1):113-26.
3. DeTure MA, Dickson DW. The neuropathological diagnosis of Alzheimer's disease. Mol Neurodegener. 2019;14(1):32.
4. Livingston G, Huntley J, Sommerlad A, Ames D, Ballard C, Banerjee S, et al. Dementia prevention, intervention, and care: 2020 report of the Lancet Commission. Lancet. 2020;396(10248):413-46.
5. Selbaek G. Dementia risk: time matters. Lancet Public Health. 2021;6(2):e85-e6.
6. Dementia: a public health priority. who.int; 2012.
7. Blennow K, de Leon MJ, Zetterberg H. Alzheimer's disease. Lancet. 2006;368(9533):387-403.
8. Palop JJ, Chin J, Roberson ED, Wang J, Thwin MT, Bien-Ly N, et al. Aberrant excitatory neuronal activity and compensatory remodeling of inhibitory hippocampal circuits in mouse models of Alzheimer's disease. Neuron. 2007;55(5):697-711.
9. Association As. 2020 Alzheimer's Disease Facts and Figures. Alzheimer´s Association; 2020.
10. Riedel BC, Thompson PM, Brinton RD. Age, APOE and sex: Triad of risk of Alzheimer's disease. J Steroid Biochem Mol Biol. 2016;160:134-47.
11. Aisen PS, Cummings J, Jack CR, Jr., Morris JC, Sperling R, Frolich L, et al. On the path to 2025: understanding the Alzheimer's disease continuum. Alzheimers Res Ther. 2017;9(1):60.
12. Vermunt L, Sikkes SAM, van den Hout A, Handels R, Bos I, van der Flier WM, et al. Duration of preclinical, prodromal, and dementia stages of Alzheimer's disease in relation to age, sex, and APOE genotype. Alzheimers Dement. 2019;15(7):888-98.
13. Skogli E, Karttinen E, Stokke OM, Vikøren S. Samfunnskostnader knyttet til Alzheimers og annen demenssykdom. 2020. Report No.: Menon-publikasjon nr 64/2020.
14. Zissimopoulos J, Crimmins E, St Clair P. The Value of Delaying Alzheimer's Disease Onset. Forum Health Econ Policy. 2014;18(1):25-39.
15. Cummings JL, Morstorf T, Zhong K. Alzheimer's disease drug-development pipeline: few candidates, frequent failures. Alzheimers Res Ther. 2014;6(4):37.
16. Kocahan S, Dogan Z. Mechanisms of Alzheimer's Disease Pathogenesis and Prevention: The Brain, Neural Pathology, N-methyl-D-aspartate Receptors, Tau Protein and Other Risk Factors. Clin Psychopharmacol Neurosci. 2017;15(1):1-8.
17. Nabers A, Perna L, Lange J, Mons U, Schartner J, Guldenhaupt J, et al. Amyloid blood biomarker detects Alzheimer's disease. EMBO Mol Med. 2018;10(5).
18. Palmqvist S, Janelidze S, Quiroz YT, Zetterberg H, Lopera F, Stomrud E, et al. Discriminative Accuracy of Plasma Phospho-tau217 for Alzheimer Disease vs Other Neurodegenerative Disorders. JAMA. 2020;324(8):772-81.
19. Cummings J, Feldman HH, Scheltens P. The "rights" of precision drug development for Alzheimer's disease. Alzheimers Res Ther. 2019;11(1):76.
20. Livingston G, Sommerlad A, Orgeta V, Costafreda SG, Huntley J, Ames D, et al. Dementia prevention, intervention, and care. Lancet. 2017;390(10113):2673-734.
21. Guerreiro R, Bras J. The age factor in Alzheimer's disease. Genome Med. 2015;7:106.
22. Kukull WA, Higdon R, Bowen JD, McCormick WC, Teri L, Schellenberg GD, et al. Dementia and Alzheimer disease incidence: a prospective cohort study. Arch Neurol. 2002;59(11):1737-46.
23. Fratiglioni L, Launer LJ, Andersen K, Breteler MM, Copeland JR, Dartigues JF, et al. Incidence of dementia and major subtypes in Europe: A collaborative study of population-based cohorts. Neurologic Diseases in the Elderly Research Group. Neurology. 2000;54(11 Suppl 5):S10-5.
24. The incidence of dementia in Canada. The Canadian Study of Health and Aging Working Group. Neurology. 2000;55(1):66-73.
25. Hickman RA, Faustin A, Wisniewski T. Alzheimer Disease and Its Growing Epidemic: Risk Factors, Biomarkers, and the Urgent Need for Therapeutics. Neurol Clin. 2016;34(4):941-53.
26. Bekris LM, Yu CE, Bird TD, Tsuang DW. Genetics of Alzheimer disease. J Geriatr Psychiatry Neurol. 2010;23(4):213-27.
27. Campion D, Dumanchin C, Hannequin D, Dubois B, Belliard S, Puel M, et al. Early-onset autosomal dominant Alzheimer disease: prevalence, genetic heterogeneity, and mutation spectrum. Am J Hum Genet. 1999;65(3):664-70.

28. Tanzi RE, Bertram L. Twenty years of the Alzheimer's disease amyloid hypothesis: a genetic perspective. Cell. 2005;120(4):545-55.
29. Tiwari S, Atluri V, Kaushik A, Yndart A, Nair M. Alzheimer's disease: pathogenesis, diagnostics, and therapeutics. Int J Nanomedicine. 2019;14:5541-54.
30. Van Cauwenberghe C, Van Broeckhoven C, Sleegers K. The genetic landscape of Alzheimer disease: clinical implications and perspectives. Genet Med. 2016;18(5):421-30.
31. Kim J, Basak JM, Holtzman DM. The role of apolipoprotein E in Alzheimer's disease. Neuron. 2009;63(3):287-303.
32. Bettens K, Sleegers K, Van Broeckhoven C. Genetic insights in Alzheimer's disease. Lancet Neurol. 2013;12(1):92-104.
33. Farrer LA, Cupples LA, Haines JL, Hyman B, Kukull WA, Mayeux R, et al. Effects of age, sex, and ethnicity on the association between apolipoprotein E genotype and Alzheimer disease. A meta-analysis. APOE and Alzheimer Disease Meta Analysis Consortium. JAMA. 1997;278(16):1349-56.
34. Povova J, Ambroz P, Bar M, Pavukova V, Sery O, Tomaskova H, et al. Epidemiological of and risk factors for Alzheimer's disease: a review. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2012;156(2):108-14.
35. Baumgart M, Snyder HM, Carrillo MC, Fazio S, Kim H, Johns H. Summary of the evidence on modifiable risk factors for cognitive decline and dementia: A population-based perspective. Alzheimers Dement. 2015;11(6):718-26.
36. Norton S, Matthews FE, Barnes DE, Yaffe K, Brayne C. Potential for primary prevention of Alzheimer's disease: an analysis of population-based data. Lancet Neurol. 2014;13(8):788-94.
37. Walker KA, Power MC, Gottesman RF. Defining the Relationship Between Hypertension, Cognitive Decline, and Dementia: a Review. Curr Hypertens Rep. 2017;19(3):24.
38. Kivipelto M, Helkala EL, Laakso MP, Hanninen T, Hallikainen M, Alhainen K, et al. Midlife vascular risk factors and Alzheimer's disease in later life: longitudinal, population based study. BMJ. 2001;322(7300):1447-51.
39. Gabin JM, Tambs K, Saltvedt I, Sund E, Holmen J. Association between blood pressure and Alzheimer disease measured up to 27 years prior to diagnosis: the HUNT Study. Alzheimers Res Ther. 2017;9(1):37.
40. Zlokovic BV. The blood-brain barrier in health and chronic neurodegenerative disorders. Neuron. 2008;57(2):178-201.
41. Chatterjee S, Peters SA, Woodward M, Mejia Arango S, Batty GD, Beckett N, et al. Type 2 Diabetes as a Risk Factor for Dementia in Women Compared With Men: A Pooled Analysis of 2.3 Million People Comprising More Than 100,000 Cases of Dementia. Diabetes Care. 2016;39(2):300-7.
42. Gudala K, Bansal D, Schifano F, Bhansali A. Diabetes mellitus and risk of dementia: A meta-analysis of prospective observational studies. J Diabetes Investig. 2013;4(6):640-50.
43. Macauley SL, Stanley M, Caesar EE, Yamada SA, Raichle ME, Perez R, et al. Hyperglycemia modulates extracellular amyloid-beta concentrations and neuronal activity in vivo. J Clin Invest. 2015;125(6):2463-7.
44. Kim DJ, Yu JH, Shin MS, Shin YW, Kim MS. Hyperglycemia Reduces Efficiency of Brain Networks in Subjects with Type 2 Diabetes. PLoS One. 2016;11(6):e0157268.
45. Rom S, Zuluaga-Ramirez V, Gajghate S, Seliga A, Winfield M, Heldt NA, et al. Hyperglycemia-Driven Neuroinflammation Compromises BBB Leading to Memory Loss in Both Diabetes Mellitus (DM) Type 1 and Type 2 Mouse Models. Mol Neurobiol. 2019;56(3):1883-96.
46. Obesity and overweight. who.int; 2020.
47. Whitmer RA, Gunderson EP, Quesenberry CP, Jr., Zhou J, Yaffe K. Body mass index in midlife and risk of Alzheimer disease and vascular dementia. Curr Alzheimer Res. 2007;4(2):103-9.
48. Gonzalez K, Fuentes J, Marquez JL. Physical Inactivity, Sedentary Behavior and Chronic Diseases. Korean J Fam Med. 2017;38(3):111-5.
49. Buttar HS, Li T, Ravi N. Prevention of cardiovascular diseases: Role of exercise, dietary interventions, obesity and smoking cessation. Exp Clin Cardiol. 2005;10(4):229-49.
50. Rashid MH, Zahid MF, Zain S, Kabir A, Hassan SU. The Neuroprotective Effects of Exercise on Cognitive Decline: A Preventive Approach to Alzheimer Disease. Cureus. 2020;12(2):e6958.
51. Rosenberg A, Mangialasche F, Ngandu T, Solomon A, Kivipelto M. Multidomain Interventions to Prevent Cognitive Impairment, Alzheimer's Disease, and Dementia: From FINGER to World-Wide FINGERS. J Prev Alzheimers Dis. 2020;7(1):29-36.
52. Winblad B, Amouyel P, Andrieu S, Ballard C, Brayne C, Brodaty H, et al. Defeating Alzheimer's disease and other dementias: a priority for European science and society. Lancet Neurol. 2016;15(5):455-532.
53. Shah H, Albanese E, Duggan C, Rudan I, Langa KM, Carrillo MC, et al. Research priorities to reduce the global burden of dementia by 2025. Lancet Neurol. 2016;15(12):1285-94.

54. Pedersen BK. The diseasome of physical inactivity--and the role of myokines in muscle--fat cross talk. J Physiol. 2009;587(Pt 23):5559-68.
55. Cotman CW, Berchtold NC, Christie LA. Exercise builds brain health: key roles of growth factor cascades and inflammation. Trends Neurosci. 2007;30(9):464-72.
56. Larson EB, Wang L, Bowen JD, McCormick WC, Teri L, Crane P, et al. Exercise is associated with reduced risk for incident dementia among persons 65 years of age and older. Ann Intern Med. 2006;144(2):73-81.
57. Tari AR, Norevik CS, Scrimgeour NR, Kobro-Flatmoen A, Storm-Mathisen J, Bergersen LH, et al. Are the neuroprotective effects of exercise training systemically mediated? Prog Cardiovasc Dis. 2019;62(2):94-101.
58. van Praag H, Christie BR, Sejnowski TJ, Gage FH. Running enhances neurogenesis, learning, and long-term potentiation in mice. Proc Natl Acad Sci U S A. 1999;96(23):13427-31.
59. Vivar C, Peterson BD, van Praag H. Running rewires the neuronal network of adult-born dentate granule cells. Neuroimage. 2016;131:29-41.
60. Bolz L, Heigele S, Bischofberger J. Running Improves Pattern Separation during Novel Object Recognition. Brain Plast. 2015;1(1):129-41.
61. van Praag H, Shubert T, Zhao C, Gage FH. Exercise enhances learning and hippocampal neurogenesis in aged mice. J Neurosci. 2005;25(38):8680-5.
62. van Praag H, Kempermann G, Gage FH. Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. Nat Neurosci. 1999;2(3):266-70.
63. Bherer L, Erickson KI, Liu-Ambrose T. A review of the effects of physical activity and exercise on cognitive and brain functions in older adults. J Aging Res. 2013;2013:657508.
64. Bouchard J, Villeda SA. Aging and brain rejuvenation as systemic events. J Neurochem. 2015;132(1):5-19.
65. Swain RA, Harris AB, Wiener EC, Dutka MV, Morris HD, Theien BE, et al. Prolonged exercise induces angiogenesis and increases cerebral blood volume in primary motor cortex of the rat. Neuroscience. 2003;117(4):1037-46.
66. Maass A, Duzel S, Brigadski T, Goerke M, Becke A, Sobieray U, et al. Relationships of peripheral IGF-1, VEGF and BDNF levels to exercise-related changes in memory, hippocampal perfusion and volumes in older adults. Neuroimage. 2016;131:142-54.
67. Morland C, Andersson KA, Haugen OP, Hadzic A, Kleppa L, Gille A, et al. Exercise induces cerebral VEGF and angiogenesis via the lactate receptor HCAR1. Nat Commun. 2017;8:15557.
68. Anand KS, Dhikav V. Hippocampus in health and disease: An overview. Ann Indian Acad Neurol. 2012;15(4):239-46.
69. Moser MB, Moser EI. Functional differentiation in the hippocampus. Hippocampus. 1998;8(6):608-19.
70. Jordan JT. The rodent hippocampus as a bilateral structure: A review of hemispheric lateralization. Hippocampus. 2020;30(3):278-92.
71. Toni N, Teng EM, Bushong EA, Aimone JB, Zhao C, Consiglio A, et al. Synapse formation on neurons born in the adult hippocampus. Nat Neurosci. 2007;10(6):727-34.
72. Buzsaki G, Moser EI. Memory, navigation and theta rhythm in the hippocampal-entorhinal system. Nat Neurosci. 2013;16(2):130-8.
73. Leonard BW, Amaral DG, Squire LR, Zola-Morgan S. Transient memory impairment in monkeys with bilateral lesions of the entorhinal cortex. J Neurosci. 1995;15(8):5637-59.
74. Gomez-Isla T, Price JL, McKeel DW, Jr., Morris JC, Growdon JH, Hyman BT. Profound loss of layer II entorhinal cortex neurons occurs in very mild Alzheimer's disease. J Neurosci. 1996;16(14):4491-500.
75. Altman J, Das GD. Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J Comp Neurol. 1965;124(3):319-35.
76. Eriksson PS, Perfilieva E, Bjork-Eriksson T, Alborn AM, Nordborg C, Peterson DA, et al. Neurogenesis in the adult human hippocampus. Nat Med. 1998;4(11):1313-7.
77. Rudnitskaya EA, Kozlova TA, Burnyasheva AO, Kolosova NG, Stefanova NA. Alterations of hippocampal neurogenesis during development of Alzheimer's disease-like pathology in OXYS rats. Exp Gerontol. 2019;115:32-45.
78. Hollands C, Bartolotti N, Lazarov O. Alzheimer's Disease and Hippocampal Adult Neurogenesis; Exploring Shared Mechanisms. Front Neurosci. 2016;10:178.
79. Palmer TD, Willhoite AR, Gage FH. Vascular niche for adult hippocampal neurogenesis. J Comp Neurol. 2000;425(4):479-94.
80. Cameron HA, Woolley CS, McEwen BS, Gould E. Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat. Neuroscience. 1993;56(2):337-44.
81. Deng W, Aimone JB, Gage FH. New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory? Nat Rev Neurosci. 2010;11(5):339-50.
82. Cameron HA, McKay RD. Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. J Comp Neurol. 2001;435(4):406-17.

83. Dayer AG, Ford AA, Cleaver KM, Yassaee M, Cameron HA. Short-term and long-term survival of new neurons in the rat dentate gyrus. J Comp Neurol. 2003;460(4):563-72.
84. van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH. Functional neurogenesis in the adult hippocampus. Nature. 2002;415(6875):1030-4.
85. Jinno S. Topographic differences in adult neurogenesis in the mouse hippocampus: a stereology-based study using endogenous markers. Hippocampus. 2011;21(5):467-80.
86. Katsimpardi L, Litterman NK, Schein PA, Miller CM, Loffredo FS, Wojtkiewicz GR, et al. Vascular and neurogenic rejuvenation of the aging mouse brain by young systemic factors. Science. 2014;344(6184):630-4.
87. Pensalfini A, Albay R, 3rd, Rasool S, Wu JW, Hatami A, Arai H, et al. Intracellular amyloid and the neuronal origin of Alzheimer neuritic plaques. Neurobiol Dis. 2014;71:53-61.
88. D'Andrea MR, Nagele RG, Gumula NA, Reiser PA, Polkovitch DA, Hertzog BM, et al. Lipofuscin and Abeta42 exhibit distinct distribution patterns in normal and Alzheimer's disease brains. Neurosci Lett. 2002;323(1):45-9.
89. Sanabria-Castro A, Alvarado-Echeverria I, Monge-Bonilla C. Molecular Pathogenesis of Alzheimer's Disease: An Update. Ann Neurosci. 2017;24(1):46-54.
90. Evin G, Weidemann A. Biogenesis and metabolism of Alzheimer's disease Abeta amyloid peptides. Peptides. 2002;23(7):1285-97.
91. Shibata M, Yamada S, Kumar SR, Calero M, Bading J, Frangione B, et al. Clearance of Alzheimer's amyloid-ss(1-40) peptide from brain by LDL receptor-related protein-1 at the blood-brain barrier. J Clin Invest. 2000;106(12):1489-99.
92. Deane R, Wu Z, Sagare A, Davis J, Du Yan S, Hamm K, et al. LRP/amyloid beta-peptide interaction mediates differential brain efflux of Abeta isoforms. Neuron. 2004;43(3):333-44.
93. Hardy JA, Higgins GA. Alzheimer's disease: the amyloid cascade hypothesis. Science. 1992;256(5054):184-5.
94. Doig AJ. Positive Feedback Loops in Alzheimer's Disease: The Alzheimer's Feedback Hypothesis. J Alzheimers Dis. 2018;66(1):25-36.
95. Naseri NN, Wang H, Guo J, Sharma M, Luo W. The complexity of tau in Alzheimer's disease. Neurosci Lett. 2019;705:183-94.
96. Bierer LM, Hof PR, Purohit DP, Carlin L, Schmeidler J, Davis KL, et al. Neocortical neurofibrillary tangles correlate with dementia severity in Alzheimer's disease. Arch Neurol. 1995;52(1):81-8.
97. Brown BM, Peiffer J, Rainey-Smith SR. Exploring the relationship between physical activity, beta-amyloid and tau: A narrative review. Ageing Res Rev. 2019;50:9-18.
98. Fischer R, Maier O. Interrelation of oxidative stress and inflammation in neurodegenerative disease: role of TNF. Oxid Med Cell Longev. 2015;2015:610813.
99. Augusto-Oliveira M, Arrifano GP, Lopes-Araujo A, Santos-Sacramento L, Takeda PY, Anthony DC, et al. What Do Microglia Really Do in Healthy Adult Brain? Cells. 2019;8(10).
100. Kinney JW, Bemiller SM, Murtishaw AS, Leisgang AM, Salazar AM, Lamb BT. Inflammation as a central mechanism in Alzheimer's disease. Alzheimers Dement (N Y). 2018;4:575-90.
101. Amor S, Puentes F, Baker D, van der Valk P. Inflammation in neurodegenerative diseases. Immunology. 2010;129(2):154-69.
102. Morgan BP. Complement in the pathogenesis of Alzheimer's disease. Semin Immunopathol. 2018;40(1):113-24.
103. Lu Y, Dong Y, Tucker D, Wang R, Ahmed ME, Brann D, et al. Treadmill Exercise Exerts Neuroprotection and Regulates Microglial Polarization and Oxidative Stress in a Streptozotocin-Induced Rat Model of Sporadic Alzheimer's Disease. J Alzheimers Dis. 2017;56(4):1469-84.
104. Do K, Laing BT, Landry T, Bunner W, Mersaud N, Matsubara T, et al. The effects of exercise on hypothalamic neurodegeneration of Alzheimer's disease mouse model. PLoS One. 2018;13(1):e0190205.
105. Shibuya M. Brain angiogenesis in developmental and pathological processes: therapeutic aspects of vascular endothelial growth factor. FEBS J. 2009;276(17):4636-43.
106. Profaci CP, Munji RN, Pulido RS, Daneman R. The blood-brain barrier in health and disease: Important unanswered questions. J Exp Med. 2020;217(4).
107. Fischer VW, Siddiqi A, Yusufaly Y. Altered angioarchitecture in selected areas of brains with Alzheimer's disease. Acta Neuropathol. 1990;79(6):672-9.
108. Thomas KA. Vascular endothelial growth factor, a potent and selective angiogenic agent. J Biol Chem. 1996;271(2):603-6.
109. Ambrose CT. Neuroangiogenesis: a vascular basis for Alzheimer's disease and cognitive decline during aging. J Alzheimers Dis. 2012;32(3):773-88.
110. Olfert IM, Breen EC, Mathieu-Costello O, Wagner PD. Skeletal muscle capillarity and angiogenic mRNA levels after exercise training in normoxia and chronic hypoxia. J Appl Physiol (1985). 2001;91(3):1176-84.

111. Steinman J, Sun HS, Feng ZP. Microvascular Alterations in Alzheimer's Disease. Front Cell Neurosci. 2020;14:618986.
112. Breen EC, Johnson EC, Wagner H, Tseng HM, Sung LA, Wagner PD. Angiogenic growth factor mRNA responses in muscle to a single bout of exercise. J Appl Physiol (1985). 1996;81(1):355-61.
113. Olfert IM, Breen EC, Mathieu-Costello O, Wagner PD. Chronic hypoxia attenuates resting and exercise-induced VEGF, flt-1, and flk-1 mRNA levels in skeletal muscle. J Appl Physiol (1985). 2001;90(4):1532-8.
114. Moreno-Jimenez EP, Flor-Garcia M, Terreros-Roncal J, Rabano A, Cafini F, Pallas-Bazarra N, et al. Adult hippocampal neurogenesis is abundant in neurologically healthy subjects and drops sharply in patients with Alzheimer's disease. Nat Med. 2019;25(4):554-60.
115. Shohayeb B, Diab M, Ahmed M, Ng DCH. Factors that influence adult neurogenesis as potential therapy. Transl Neurodegener. 2018;7:4.
116. Pedersen BK. Physical activity and muscle-brain crosstalk. Nat Rev Endocrinol. 2019;15(7):383-92.
117. Wrann CD, White JP, Salogiannnis J, Laznik-Bogoslavski D, Wu J, Ma D, et al. Exercise induces hippocampal BDNF through a PGC-1alpha/FNDC5 pathway. Cell Metab. 2013;18(5):649-59.
118. Moon HY, Becke A, Berron D, Becker B, Sah N, Benoni G, et al. Running-Induced Systemic Cathepsin B Secretion Is Associated with Memory Function. Cell Metab. 2016;24(2):332-40.
119. Pedersen BK. Exercise-induced myokines and their role in chronic diseases. Brain Behav Immun. 2011;25(5):811-6.
120. Pluvinage JV, Wyss-Coray T. Systemic factors as mediators of brain homeostasis, ageing and neurodegeneration. Nat Rev Neurosci. 2020;21(2):93-102.
121. Villeda SA, Luo J, Mosher KI, Zou B, Britschgi M, Bieri G, et al. The ageing systemic milieu negatively regulates neurogenesis and cognitive function. Nature. 2011;477(7362):90-4.
122. Villeda SA, Plambeck KE, Middeldorp J, Castellano JM, Mosher KI, Luo J, et al. Young blood reverses age-related impairments in cognitive function and synaptic plasticity in mice. Nat Med. 2014;20(6):659-63.
123. Castellano JM, Mosher KI, Abbey RJ, McBride AA, James ML, Berdnik D, et al. Human umbilical cord plasma proteins revitalize hippocampal function in aged mice. Nature. 2017;544(7651):488-92.
124. Sha SJ, Deutsch GK, Tian L, Richardson K, Coburn M, Gaudioso JL, et al. Safety, Tolerability, and Feasibility of Young Plasma Infusion in the Plasma for Alzheimer Symptom Amelioration Study: A Randomized Clinical Trial. JAMA Neurol. 2019;76(1):35-40.
125. Pedersen BK, Saltin B. Exercise as medicine - evidence for prescribing exercise as therapy in 26 different chronic diseases. Scand J Med Sci Sports. 2015;25 Suppl 3:1-72.
126. De Miguel Z, Betley MJ, D. W, Lehallier B, Olsson N, Bonanno L, et al. Exercise conditioned plasma dampens inflammation via clusterin and boosts memory2019 2021/05/06]. Available from: https://www.biorxiv.org/content/10.1101/775288v1.full.
127. Tari AR, Nauman J, Zisko N, Skjellegrind HK, Bosnes I, Bergh S, et al. Temporal changes in cardiorespiratory fitness and risk of dementia incidence and mortality: a population-based prospective cohort study. Lancet Public Health. 2019;4(11):e565-e74.
128. Horowitz AM, Fan X, Bieri G, Smith LK, Sanchez-Diaz CI, Schroer AB, et al. Blood factors transfer beneficial effects of exercise on neurogenesis and cognition to the aged brain. Science. 2020;369(6500):167-73.
129. Kim TW, Park SS, Park JY, Park HS. Infusion of Plasma from Exercised Mice Ameliorates Cognitive Dysfunction by Increasing Hippocampal Neuroplasticity and Mitochondrial Functions in 3xTg-AD Mice. Int J Mol Sci. 2020;21(9).
130. Sukhatme VP, Cao XM, Chang LC, Tsai-Morris CH, Stamenkovich D, Ferreira PC, et al. A zinc finger-encoding gene coregulated with c-fos during growth and differentiation, and after cellular depolarization. Cell. 1988;53(1):37-43.
131. Qin X, Wang Y, Paudel HK. Inhibition of Early Growth Response 1 in the Hippocampus Alleviates Neuropathology and Improves Cognition in an Alzheimer Model with Plaques and Tangles. Am J Pathol. 2017;187(8):1828-47.
132. Rabiee F, Lachinani L, Ghaedi S, Nasr-Esfahani MH, Megraw TL, Ghaedi K. New insights into the cellular activities of Fndc5/Irisin and its signaling pathways. Cell Biosci. 2020;10:51.
133. Muller P, Duderstadt Y, Lessmann V, Muller NG. Lactate and BDNF: Key Mediators of Exercise Induced Neuroplasticity? J Clin Med. 2020;9(4).
134. Jodeiri Farshbaf M, Ghaedi K, Megraw TL, Curtiss J, Shirani Faradonbeh M, Vaziri P, et al. Does PGC1alpha/FNDC5/BDNF Elicit the Beneficial Effects of Exercise on Neurodegenerative Disorders? Neuromolecular Med. 2016;18(1):1-15.

135. Bond CW, Angeloni N, Harrington D, Stupp S, Podlasek CA. Sonic Hedgehog regulates brain-derived neurotrophic factor in normal and regenerating cavernous nerves. J Sex Med. 2013;10(3):730-7.
136. Echelard Y, Epstein DJ, St-Jacques B, Shen L, Mohler J, McMahon JA, et al. Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. Cell. 1993;75(7):1417-30.
137. Ahn S, Joyner AL. In vivo analysis of quiescent adult neural stem cells responding to Sonic hedgehog. Nature. 2005;437(7060):894-7.
138. Jiao J, Chen DF. Induction of neurogenesis in nonconventional neurogenic regions of the adult central nervous system by niche astrocyte-produced signals. Stem Cells. 2008;26(5):1221-30.
139. Teng H, Chopp M, Hozeska-Solgot A, Shen L, Lu M, Tang C, et al. Tissue plasminogen activator and plasminogen activator inhibitor 1 contribute to sonic hedgehog-induced in vitro cerebral angiogenesis. PLoS One. 2012;7(3):e33444.
140. Chen SD, Yang JL, Hwang WC, Yang DI. Emerging Roles of Sonic Hedgehog in Adult Neurological Diseases: Neurogenesis and Beyond. Int J Mol Sci. 2018;19(8).
141. Reichenbach N, Delekate A, Plescher M, Schmitt F, Krauss S, Blank N, et al. Inhibition of Stat3-mediated astrogliosis ameliorates pathology in an Alzheimer's disease model. EMBO Mol Med. 2019;11(2).
142. Chen E, Xu D, Lan X, Jia B, Sun L, Zheng JC, et al. A novel role of the STAT3 pathway in brain inflammation-induced human neural progenitor cell differentiation. Curr Mol Med. 2013;13(9):1474-84.
143. Ng YP, Cheung ZH, Ip NY. STAT3 as a downstream mediator of Trk signaling and functions. J Biol Chem. 2006;281(23):15636-44.
144. Yaffe K, Haan M, Blackwell T, Cherkasova E, Whitmer RA, West N. Metabolic syndrome and cognitive decline in elderly Latinos: findings from the Sacramento Area Latino Study of Aging study. J Am Geriatr Soc. 2007;55(5):758-62.
145. Carroll S, Dudfield M. What is the relationship between exercise and metabolic abnormalities? A review of the metabolic syndrome. Sports Med. 2004;34(6):371-418.
146. Heneka MT, Carson MJ, El Khoury J, Landreth GE, Brosseron F, Feinstein DL, et al. Neuroinflammation in Alzheimer's disease. Lancet Neurol. 2015;14(4):388-405.
147. Fillit H, Ding WH, Buee L, Kalman J, Altstiel L, Lawlor B, et al. Elevated circulating tumor necrosis factor levels in Alzheimer's disease. Neurosci Lett. 1991;129(2):318-20.
148. Daneman R, Prat A. The blood-brain barrier. Cold Spring Harb Perspect Biol. 2015;7(1):a020412.
149. Provias J, Jeynes B. Reduction in vascular endothelial growth factor expression in the superior temporal, hippocampal, and brainstem regions in Alzheimer's disease. Curr Neurovasc Res. 2014;11(3):202-9.
150. Giri M, Shah A, Upreti B, Rai JC. Unraveling the genes implicated in Alzheimer's disease. Biomed Rep. 2017;7(2):105-14.
151. Poon CH, Wang Y, Fung ML, Zhang C, Lim LW. Rodent Models of Amyloid-Beta Feature of Alzheimer's Disease: Development and Potential Treatment Implications. Aging Dis. 2020;11(5):1235-59.
152. Duyckaerts C, Potier MC, Delatour B. Alzheimer disease models and human neuropathology: similarities and differences. Acta Neuropathol. 2008;115(1):5-38.
153. Kitazawa M, Medeiros R, Laferla FM. Transgenic mouse models of Alzheimer disease: developing a better model as a tool for therapeutic interventions. Curr Pharm Des. 2012;18(8):1131-47.
154. Heggland I, Storkaas IS, Soligard HT, Kobro-Flatmoen A, Witter MP. Stereological estimation of neuron number and plaque load in the hippocampal region of a transgenic rat model of Alzheimer's disease. Eur J Neurosci. 2015;41(9):1245-62.
155. Mutations - APP KM670/671NL (Swedish) Alzforum [2020/11/15]. Available from: https://www.alzforum.org/mutations/app-km670671nl-swedish.
156. Mullan M, Crawford F, Axelman K, Houlden H, Lilius L, Winblad B, et al. A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of beta-amyloid. Nat Genet. 1992;1(5):345-7.
157. Citron M, Oltersdorf T, Haass C, McConlogue L, Hung AY, Seubert P, et al. Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production. Nature. 1992;360(6405):672-4.
158. Mutations - APP V717F (Indiana) Alzforum [2020/11/15]. Available from: https://www.alzforum.org/mutations/app-v717f-indiana.
159. Tamaoka A, Odaka A, Ishibashi Y, Usami M, Sahara N, Suzuki N, et al. APP717 missense mutation affects the ratio of amyloid beta protein species (A beta 1-42/43 and a beta 1-40) in familial Alzheimer's disease brain. J Biol Chem. 1994;269(52):32721-4.

160. Murrell J, Farlow M, Ghetti B, Benson MD. A mutation in the amyloid precursor protein associated with hereditary Alzheimer's disease. Science. 1991;254(5028):97-9.
161. Wojtowicz JM, Kee N. BrdU assay for neurogenesis in rodents. Nat Protoc. 2006;1(3):1399-405.
162. Kuhn HG, Eisch AJ, Spalding K, Peterson DA. Detection and Phenotypic Characterization of Adult Neurogenesis. Cold Spring Harb Perspect Biol. 2016;8(3):a025981.
163. Eminaga S, Teekakirikul P, Seidman CE, Seidman JG. Detection of Cell Proliferation Markers by Immunofluorescence Staining and Microscopy Imaging in Paraffin-Embedded Tissue Sections. Curr Protoc Mol Biol. 2016;115:14 25 1-14 25 14.
164. Gusel'nikova VV, Korzhevskiy DE. NeuN As a Neuronal Nuclear Antigen and Neuron Differentiation Marker. Acta Naturae. 2015;7(2):42-7.
165. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402-8.
166. Zhao X, van Praag H. Steps towards standardized quantification of adult neurogenesis. Nat Commun. 2020;11(1):4275.
167. Sauerzweig S, Baldauf K, Braun H, Reymann KG. Time-dependent segmentation of BrdU-signal leads to late detection problems in studies using BrdU as cell label or proliferation marker. J Neurosci Methods. 2009;177(1):149-59.
168. Taupin P. BrdU immunohistochemistry for studying adult neurogenesis: paradigms, pitfalls, limitations, and validation. Brain Res Rev. 2007;53(1):198-214.

Appendix IA

Immunostaining protocol detecting neurogenesis (anti-BrdU and anti-NeuN)

Day 1

Deparaffinisation of control section

1. 2 x 5 min Xylen
   2 x 1 min 100%
   1 x 1 min 96%
   1 x 1 min 80%
   H_2O

Note: Carry out the procedure with a negative control section in a 6-well plate with strainers, the remaining free-floating tissues are in "braincups", while the positive BrdU control is fixed on a slide. All rinsing and incubations should be performed on a plate shaker at room temperature unless otherwise stated. Step 12 and beyond should be performed in the dark.

2. Transfer the free-floating brain sections to a petridish filled with DMSO when selecting accurate sections for staining
3. Preheat sodium citrate buffer (10 mM sodium citrate, 0.05% Tween, pH 6.0) to 60 °C in a water bath
4. Transfer sections into Eppendorf tubes containing preheated sodium citrate buffer, and immerse into the water bath for 30 min
5. After the incubation is done, remove the tubes and slides from the water bath and retrain sections in the solution until room temperature is reached
6. Preheat water bath and 1M HCl to 45 °C
7. Rinse section 3 x 5 min in TBS
8. Denature DNA by transferring the sections into Eppendorf tubes containing preheated HCl, and immerse into the water bath for 30 min
9. Rinse sections in Borate buffer (0.1 M) for 10 min
10. Rinse sections 3 x 10 min in TBS
11. Incubate with 10% goat-serum in TBS-Tx for 60 min.
12. Incubate sections with primary antibodies: BrdU mouse monoclonal antibody (1:100 in TBS-Tx) and NeuN rabbit monoclonal antibody (1:3000 in TBS-Tx) for 24 hours at 4 °C.

Day 2

13. Rinse sections 3 x 10 min in TBS-Tx.
14. Incubate sections with fluorescent conjugated secondary antibodies, Goat Anti-Mouse IgG H&L Alexa Flour 488 and Goat anti-rabbit IgG H&L Alexa Flour 594, (1:100 in TBS) for 1 hour
15. Rinse section 3x5 min in TBS. For later mounting, keep in TBS at 4 °C covered with aluminium foil.
16. Mount on Superfrost glass slides while sections are floated in TBS - let dry for at least 30 min.
17. Add Vectashield and place the coverslip. Seal the coverslip using nail polish.
18. Mounted sections can be stored (in the dark) 4°C for up to 6 months

Appendix IB

Solutions for the immunostaining protocol detecting adult neurogenesis

DMSO
100 ml: 312.5 mL 400 mM phosphate buffer + 467.5 mL H_2O + 200 mL glycerine + 20 mL DMSO. Store at 2 – 8 °C.

Sodium citrate buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0)
1 L: 2.94 g trisodium citrate (dihydrate) + 1 L distilled H_2O. Mix to dissolve.
Adjust to pH 6.0 with 1 M HCl. Add 0.5 mL Tween-20 and mix well. Store at 2 – 8 °C.

Tris-buffered saline (TBS) pH 8.0
1500 mL: 9.09 g Tris + 13.44 g NaCl in 1500 mL H_2O
Adjust to pH 8.0 with HCl (2.0 M). Store at 2 – 8 °C.

TBS-Tx (0,5%) pH 8.0
500 mL TBS: In a ventilated hood, add 2.5 mL Triton X-100 and mix well. Store at 2 – 8 °C.

1 M HCl
50 mL: 4.2 mL 37 % HCl in 45.8 mL H_2O
Store at room temperature in a well-ventilated place for an extended period of time.

2 M HCl
50 mL: 8.4 mL 37 % HCl in 41.6 mL H_2O
Store at room temperature in a well-ventilated place for an extended period of time.

0.1 M Borate buffer pH 8.5
0.62g boric acid + 75 mL H_2O
Adjust to pH 8.5 with 10 M NaOH, and then add H_2O to a total volume of 100 mL. Store at 2 – 8 °C.

Primary antibody (BrdU)
BrdU mouse monoclonal antibody, Invitrogen. 0.1 mg/mL. LOT # 2125239. Store at 2 - 8°C.

Secondary antibody (BrdU)
Goat Anti-Mouse IgG H&L Alexa Flour 488, abcam. 2 mg/mL. LOT # GR3225138-1. Store at 2 - 8°C.

Primary antibody (NeuN)
NeuN rabbit monoclonal antibody, Abcam. 0.82 mg/mL. LOT # GR3275122-3. Store at 2 - 8°C.

Secondary antibody (NeuN)
Goat pAb to rabbit IgG Alexa Fluor 594, Abcam. 2 mg/mL. LOT # GR3323881-1. Store at 2 - 8°C.

Goat serum
Normal goat serum, Invitrogen. LOT # 1115869A. Store at -20°C.

VECTASHIELD
Antifade mounting medium. Vector laboratories. LOT # ZF0612. Store at 2 - 8 °C.

Appendix IIA

Validated primers for mRNA analyses

Table 1: Primer design and sequence overview

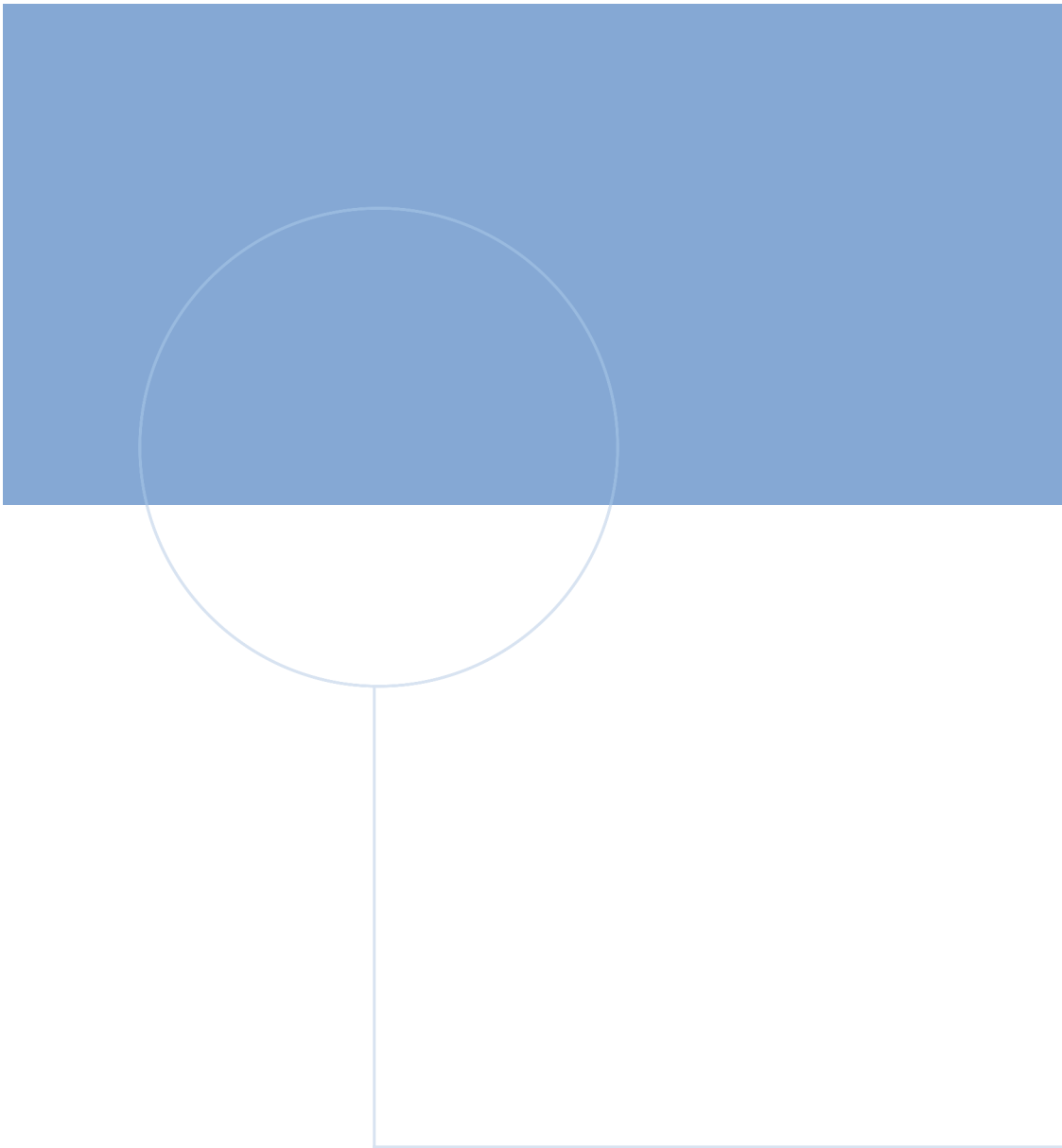
Primer name	**Sequence (5'→3')**
rno - *Stat3*	F: AGGAGGGCAGTTTGAGTCGC
	R: ACAGGCGGACAGAACATAGG
rno - *Shh*	F: GCTCTGTGAAAGCAGAGAACTCC
	R: GCGAAGAGTGGGCTCGGTC
rno – *Fndc5*	F: GTCTCCTCCGAAGCAAGATATGA
	R: TTTCACACACAGCCTTCGGT
rno - *Vegfa*	F: GAACGTACTTGCAGATGTGAC
	R: GGTTAATCGGTCTTTCCGGTG
rno - *Plau*	F: ACAGATTCCTGCTCGGGAGA
	R: GGTGGACAGCACAAGCAAAG
rno – *Bdnf*	F: GTCGCACGGTCCCCATTG
	R: ACCTGGTGGAACTCAGGGT
rno – *Egr1*	F: CAGCTGACCACAGAGTCCTTTT
	R: GTTGGTCATGCTCACAAGGC
rno – *Tnf*	F: ATGGGCTCCCTCTCATCAGT
	R: GCTTGGTGGTTTGCTACGAC
rno - *Hprt*	F: ACAGGCCAGACTTTGTTGGAT
	R: GGCCACAGGACTAGAACGTC

Appendix IIB

cDNA synthesis cycling conditions

Table 2: Thermal cycling conditions for RT-qPCR, Thermo Scientific™ NanoDrop 2000 (Thermo Fisher)

Cycle Step	**Temperature (°C)**	**Time (min)**	**Number of Cycles (n)**
Initial Denaturation	95.0	15.0	1
Denaturation	94.0	0.250	42
Annealing Extension	55.0	0.500	42
Elongation	71.0	0.500	42
Melt curve generation	65.0	0.083	1
	95.0	0.833	1



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