Impact of Physical Exercise on the Hippocampal Pathology and Cognition in a Mouse Model of Alzheimer’s Disease

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Laboratory Medicine and Pathobiology University of Toronto

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Abstract

It is recognized that neuronal and vascular functions are affected during the progression of Alzheimer’s disease (AD). The accumulation of amyloid-beta peptides (Aβ) in the parenchyma and around blood vessels can contribute to neuronal and vascular compromise, and exacerbate cognitive impairment. Exercise has been described as one of the most influential modulators at preventing cognitive deficits observed in AD.

The present work examined the extent to which physical activity is capable of reducing Aβ-related pathologies. I hypothesized that physical exercise stimulates memory and neurogenesis, remodels vasculature and lessens Aβ burden in the hippocampus of TgCRND8 mouse model of amyloidosis.

All animals entered the study at 3 months of age and separate cohorts ran for 1, 2, or 3 months. My results indicate that voluntary running promotes spatial memory, neurogenesis, and cerebrovascular morphology in TgCRND8 mice. Specifically, the number of proliferating cells was significantly greater in running compared to non-running (sedentary) TgCRND8 mice as disease progressed. The number of doublecortin-positive new immature
neurons was higher in TgCRND8 mice running for 1 month, compared to sedentary transgenics. Neuronal maturation was increased after 2 months (and not 1 month) of running in TgCRND8 mice. Running reduced plaque load in the dentate gyrus and Cornu Ammonis subregions of the hippocampus only after 2 months (5 month-old mice), and not after 1 month (4 month-old mice) or 3 months (6 month-old mice) of exercise. In 6 month-old mice, cerebral amyloid angiopathy levels were significantly reduced in running compared to sedentary mice.

At 6 months of age, sedentary TgCRND8 mice had higher capillary density, capillary length, branch density, and non-capillary tortuosity compared to age-matched non-transgenics. These changes in vascular morphology suggest an adaptative response to Aβ pathology. In contrast, the vascular parameters of TgCRND8 mice running for 3 months were indistinguishable from non-transgenics animals.

Memory improvements were observed at 4, 5 and 6 months of age in TgCRND8 mice running for 1, 2, and 3 months, respectively. Running TgCRND8 mice spent more time in the novel arm of the Y-maze compared to the familiar arms.

Overall, these data indicate that as amyloidosis progresses in the hippocampus of TgCRND8 mice, physical exercise has a pronounced impact on cognition, with spatial memory rescued even when the number of new mature neurons (at 4 months) and plaque burden (at 4 and 6 months) are not significantly altered compared to sedentary transgenic animals. These data suggest that increasing neurogenesis and lowering plaque buildup may not be individually necessary to promote cognitive function.

In conclusion, this work demonstrates that exercise as a lifestyle modification can improve cognition and reduce the negative impact of AD-related hippocampal pathologies.
Acknowledgments

When I began my PhD thesis at the Aubert Lab I had no doubts that I will be a part of great team of supportive people who will apply equal measure of challenge, enthusiasm, and encouragement. What I did not expect was the extent to which you would all help me grow as a researcher and a person. I joined the lab but I gained the family. And I would like to take this opportunity to thank every one of you.

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<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Aβ</td>
<td>Amyloid-beta peptides</td>
</tr>
<tr>
<td>AβPP</td>
<td>Amyloid-beta precursor protein</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ADAS-Cog</td>
<td>Alzheimer’s Disease Assessment Scale Cognitive Subscale</td>
</tr>
<tr>
<td>ADL</td>
<td>Activities of Daily Living Questionnaire</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ApoE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>ApoJ</td>
<td>Apolipoprotein J</td>
</tr>
<tr>
<td>BABB</td>
<td>Benzyl alcohol benzyl benzoate</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2-deoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CA</td>
<td>Cornu Ammonis</td>
</tr>
<tr>
<td>CAA</td>
<td>Cerebral amyloid angiopathy</td>
</tr>
<tr>
<td>CBF</td>
<td>Cerebral blood flow</td>
</tr>
<tr>
<td>CBV</td>
<td>Cerebral blood volume</td>
</tr>
<tr>
<td>CICI</td>
<td>Chemotherapy-induced cognitive impairment</td>
</tr>
<tr>
<td>ChAT</td>
<td>Choline acetyltransferase</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CTF</td>
<td>Carboxy-terminal fragment</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>Cy</td>
<td>Cyanine</td>
</tr>
<tr>
<td>DCX</td>
<td>Doublecortin</td>
</tr>
<tr>
<td>DG</td>
<td>Dentate gyrus</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DRN</td>
<td>5-HT 5-hydroxytryptamine</td>
</tr>
<tr>
<td>fMRI</td>
<td>Functional magnetic resonance imaging</td>
</tr>
<tr>
<td>FWHM</td>
<td>Full width half maximum</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma amino butyric acid</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin type G</td>
</tr>
<tr>
<td>IVIg</td>
<td>Intravenous immunoglobulin</td>
</tr>
<tr>
<td>LHV</td>
<td>Longitudinal Hippocampal Vessel</td>
</tr>
<tr>
<td>LRP</td>
<td>Low-density lipoprotein receptor-related protein</td>
</tr>
<tr>
<td>MCI</td>
<td>Mild cognitive impairment</td>
</tr>
<tr>
<td>MMSE</td>
<td>Mini-Mental State Examination</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NA</td>
<td>Numerical aperture</td>
</tr>
<tr>
<td>NeuN</td>
<td>Neuronal nuclei</td>
</tr>
<tr>
<td>NeuroD</td>
<td>Neurogenic differentiation marker</td>
</tr>
<tr>
<td>NMDAR</td>
<td>N-methyl-D-aspartate receptor</td>
</tr>
<tr>
<td>Non-Tg</td>
<td>Non-transgenic</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NPCs</td>
<td>Neuronal progenitor cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCA</td>
<td>Posterior Cerebral Artery</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PNCAM</td>
<td>Polysialyated-neural cell adhesion molecule</td>
</tr>
<tr>
<td>QOL</td>
<td>Quality of Life in Alzheimer's disease scale</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for advanced glycation end products</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>sAβPP</td>
<td>soluble fragment of amyloid-β precursor protein</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SGL</td>
<td>Subgranular layer</td>
</tr>
<tr>
<td>SVZ</td>
<td>Subventricular zone</td>
</tr>
<tr>
<td>Tg</td>
<td>Transgenic</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>THA</td>
<td>Transverse Hippocampal Arteries</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>Trk</td>
<td>Tyrosine receptor kinase</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WD</td>
<td>Working distance</td>
</tr>
</tbody>
</table>
Dissemination of Work Arising from this Thesis

Sections of Chapter 1 have been published in Current Alzheimer’s Research. It has been modified for this thesis with permission from Bentham Science Publishers.


Role of each author:
EMC conducted literature review of the exercise section while LM wrote the metabolic intervention section. JJO contributed to writing the vasculature section. PMN and IA revised the manuscript and contributed to the final draft.

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Role of each author:
EMC and IA developed main research question. EMC led running experiments, performed immunohistochemistry, stereology, behavioural tests, and statistical analysis, and manuscript writing. KX quantified neuroblasts. IA provided materials and contributed to experimental design, analysis and manuscript writing.

Chapter 4 (in preparation for publication):

Role of each author:
EMC, BS and IA developed main research question. EMC led running experiments, performed vessel analysis, stereology, behavioural tests, statistical analysis, and manuscript writing. JJO assisted with vessel tracking. LAMT provided assistance with two-photon microscopy and image analysis. JS provided expertise with Mercox-BABB perfusions. JGS provided and consulted on vessel track software. MJ contributed to data interpretation and manuscript editing. BS and IA provided materials and analysis tools for vessel analysis, and they contributed to experimental and data analysis design, and manuscript writing.

Scientific Illustrations
Illustrations were created by Hangyu Lin, with scientific input from EMC and IA.
Chapter 1

Introduction

1.1. Thesis organization

The overarching goal of this thesis is to investigate the response of AD-related pathologies to physical activity. Specifically, we investigated neurogenic, vascular, and cognitive changes in the TgCRND8 mouse model of AD following 1, 2, and 3 months of voluntary running. This paradigm reflects a strategy aimed at modulating multiple processes at a stage with neurogenic and cognitive deficits and established amyloidosis.

Chapter 1 provides a literature review of our current knowledge of AD pathologies and their rodent models followed by a characterization of physical exercise as a preventative strategy in healthy population, and as a therapeutic strategy in AD patients. Greater attention is given to running as an intervention in mouse models of AD, particularly the TgCRND8 mouse model, and the extent to which exercise can modulate multiple AD pathologies. Rationale, general hypothesis and specific aims are established in Chapter 2. Thereafter, Chapters 3 and 4 constitute the results section of this thesis. Specifically, Chapter 3 contains data on spatial memory, neurogenesis, and amyloid-beta (Aβ) plaque accumulation while Chapter 4 describes data related to hippocampal vasculature, cerebral amyloid angiopathy (CAA), and spatial memory. Chapter 5 presents a general discussion and conclusion based on the findings in the preceding two chapters. Chapter 5 also contains a summary of research presented in this thesis (Table 5.1). Thesis considerations discussing limitations and alternative solutions are outlined in Chapter 6. Future directions, clinical relevance and final remarks in Chapter 7 conclude this thesis.
1.2. Alzheimer’s disease (AD)

1.2.1. History of AD

It has been more than 100 years since Dr. Alois Alzheimer’s first publication of a “particular malady of the cerebral cortex”. His patient Auguste Deter died at age 51 after displaying cognitive deficits including disorientation, loss of memory, and trouble reading and writing (Alzheimer, 1907). The publication included an analysis of thin brain slices stained with silver salts (Bielschowsky, 1902) that identified two distinct types of abnormal deposits inside and around the brain cells. These deposits were what modern medicine defines as Aβ plaques and neurofibrillary tangles. Analysis of brain tissue from Johann F. revealed the presence of external plaques but not tangles (Alzheimer, 1911). Remarkably, the brain sections from Johann F. were re-discovered in the late 1990s at the University of Munich’s Institute of Neuropathology and were re-examined with modern technology. Graeber et al., (1997) performed mutational screening of exon 17 of the amyloid precursor protein gene (AβPP; nomenclature in accordance with Sipe et al., 2014) and genotyping for apolipoprotein E alleles (ApoE). The patient was shown to be homozygous for ApoEε3 and lacked AβPP mutations at codons 692, 693, 713 and 717. As a result, Graeber and colleagues (1997) confirmed Alzheimer’s diagnosis as the plaque-only type of AD.

Dr. Alzheimer is now well recognized for his contribution to modern neuroscience. His pioneering work of identification of plaques and tangles along with his clinical description continue to be accepted as the pathological and clinical hallmarks of the disease that now bears his name. Perhaps his greatest success was the merging of the neurology and psychiatry fields (Devi and Quitschke, 1999).
1.2.2. Prevalence and treatment

Alzheimer’s disease belongs to a large class of dementias characterized by acquired and persistent deterioration of language, cognitive ability, visuospatial skills, and personality changes as the brain degenerates (Cummings and Jeffrey, 1984). There are many diseases classified as dementia and associated with progressive neurodegeneration: Alzheimer’s disease, vascular dementia, frontotemporal dementia, Lewy body dementia and Creutzfeldt-Jakob disease, among others (Holmes et al., 1999; Gauthier, 2006).

AD is the most common form of dementia: currently over 750,000 people in Canada live with AD and the incidence is expected to accelerate due to aging population (Alzheimer Society of Canada, 2012). Unlike other highly prevalent diseases such as diabetes or cancer, AD is unique in that, despite tremendous research efforts, there is no agreement regarding disease etiology, very few proven disease-modifying therapies and only minimally effective symptomatic therapies. Finding effective therapeutics for AD has emerged as one of the major unmet medical needs of our time (Citron, 2002).

Vascular dementia is the second most common form of dementia (after Alzheimer’s disease) and is responsible for at least 20% of dementia cases (Gorelick et al., 2011). The two main types of vascular dementia are stroke-related dementia and small vessel disease-related dementia. Additionally, vascular dementia represents a major comorbidity among many AD patients (Gorelick et al., 2011, Schneider et al., 2007, Toledo et al., 2013).

1.2.2.1. Pharmacological therapeutics

Currently there is no cure for AD and only a handful of therapeutics are approved by Health Canada for use in the clinical setting (Kerchner and Boxer, 2010). The aim of these therapeutics is to stimulate neurotransmission and by doing so, stabilize cognitive function (Kerchner and Boxer, 2010). Cholinesterase inhibitor therapy with rivastigmine, donepezil,
or galantamine is endorsed as standard first-line therapy in patients with mild-to-moderate AD. The N-methyl-D-aspartate receptor (NMDAR) antagonist, memantine, is used in the clinic in combination with a cholinesterase inhibitor for patients with moderate Alzheimer's disease, and as a monotherapy for patients with severe AD.

1.2.2.2. Immunotherapy

Several active and passive immunotherapies aimed at reducing amyloid pathology are currently being investigated. The premise of immunotherapy is to prevent aggregation and/or enhance clearance of Aβ. It has been shown by Boche et al. (2008) that amyloid was cleared within 3-4 years after immunization in AD patients, first in the parenchyma and later in the vasculature. However, the impact of active immunization on cognition remains controversial (Hock et al., 2003; Gilman et al., 2005; Holmes et al., 2008). Two other monoclonal humanized anti-Aβ antibodies (bapineuzumab and solanezumab) and intravenous antibodies such as gammagard are undergoing clinical trials (Spencer and Masliah, 2014; Wisniewski and Goñi, 2015).

1.2.2.3. Lifestyle modifications and physical exercise

As previously mentioned, there is currently no medication available to reduce the risk of developing AD or to delay its onset. Drug development is complicated by the fact that Aβ accumulation begins several decades before any clinical symptoms are detected (Baker-Nigh et al., 2015). As a result, investigations of lifestyle adaptations are gaining much needed attention. There is a multitude of lifestyle modifications currently being explored in both the clinic and in basic research, including cognitive, environmental and physical stimulation, diet and metabolism management, sleep patterns and meditation (Kempermann et al., 1997, 1998; Mattson, 2004; Milgram et al., 2006; Ju et al., 2014, Maruszak et al., 2014; Newberg et al., 2014). In order to combat a disease with such a complex pathology profile, the intervention
must be equally broad and target multiple systems simultaneously. One such multimodal intervention strategy, namely physical exercise, is the focus of this thesis. Here, I evaluated the ability of physical exercise to modulate memory, neurogenesis and vascular morphology in the presence of amyloidosis and results are presented in Chapters 3 and 4. Benefits of physical exercise on brain health in adult and aging population as well as in AD patients and rodent models are discussed in Section 1.5.

1.2.3. Causes and risk factors

Presently, the cause of sporadic AD remains unknown. Genetic mutations known to cause early onset familial AD account for less than 0.1% of all cases (Harvey et al., 2003). Autosomal dominant mutations of the AβPP and presenilin 1 and 2 genes are associated with familial forms of AD (Goate et al., 1991; Sherrington et al., 1995; Levy-Lahad et al., 1995). ApoE and more recently ApoJ have been identified as the predominant genetic risk factors for the sporadic development of AD (Corder et al., 1993; Poirier et al., 1993; Harold et al., 2009; Lambert et al., 2009). Most commonly, however, AD occurs sporadically after the age of 65, making age the greatest risk factor for AD up to the age of 95 (Kawas et al., 2000; Fjell et al., 2014; Nelson et al., 2011). Other risk factors include vascular disease, high cholesterol, head injury, depression, stress, low cognitive activity, obesity and high fat diet (Mortimer et al., 1991; Speck et al., 1995; Grootendorst et al., 2011; Kivipelto et al., 2001, 2005).

Though rare, cases of familial AD further our understanding of the importance of Aβ oligomerization in the AD pathogenesis. Further genetic evidence implicating the AβPP gene in AD etiology comes from patients who have an additional copy of the gene (Masters et al., 1985). AβPP gene is located on chromosome 21 and thus patients with trisomy 21 – better
known as Down syndrome - almost universally exhibit AD-like disorders by 40 years of age (Masters et al., 1985).

1.2.4. **Mouse models**

Mutations in the AβPP, presenilin and tau genes have been utilized in generating various mouse models recapitulating a subset of pathologies of human AD (Webster et al., 2014). Although no one animal model fully represents the progression of AD pathology observed in human patients, murine models of AD have been an invaluable tool in studying this disease. Each transgenic mouse model of AD provides different insights into the varying array of AD pathologies and cognitive deficits associated with the disease. The temporal course of pathogenesis can be different and in some instances cognitive deficits can appear prior to the appearance of significant neuropathology. Careful forethought is therefore required in the selection of an optimal model displaying AD-related deficits based on specific research interest. One such mouse model, TgCRND8, contains a mutated human AβPP gene (Chisthi et al., 2001) and has been utilized for the work presented in this thesis.

1.2.5. **Etiology**

Although the histological features of AD are well characterized, many hypotheses have been postulated regarding its primary cause. The oldest hypothesis suggests that deficits in cholinergic neurotransmission in the neocortex and hippocampus lead to cognitive impairments observed in AD patients (Lippa et al., 1980; Bartus et al., 1982). Subsequently, two alternative protein misfolding hypotheses have been put forth and suggest that either tau protein (Lee et al., 1991) or amyloid-β peptides (Glenner and Wong, 1984; Masters et al., 1985) initiate the cascade (reviewed in Wolfe, 2008). Finally, the vascular hypothesis states that disruption of cerebral vessels is detrimental not only to the delivery of oxygen and nutrients but also to the maintenance of the neurovascular unit and leads to compromised
clearance of amyloid from the brain (Hardy et al., 1986; reviewed in Iadecola, 2013). Although it is debatable which pathology occurs first, it is clear that cholinergic dysfunction, amyloid pathology, tau aggregation and vascular remodelling are all hallmarks of AD and as such constitute important therapeutic targets (Hardy and Higgins, 1992; reviewed by Carreiras et al., 2013). One of the models stipulates that the deposition of Aβ and disruption in vascular integrity are the initial pathological events (Haas and Selkoe, 2007; Armstrong et al., 2009) leading to the formation of neurofibrillary tangles, cell death, and ultimately dementia (Figure 1.1; Jellinger and Bancher, 1998). This project focuses mainly on the amyloidogenic and vascular aspects of AD in its early stages of pathology, and thus amyloid and vascular hypotheses are discussed next.

1.2.5.1. **Amyloid hypothesis**

The amyloid hypothesis postulates that the gradual changes in the steady-state levels of Aβ peptide due to enhanced production, aggregation and/or reduced clearance initiate a cascade of events leading to amyloidosis (Figure 1.1; Hardy and Higgins, 1982; Haass and Selkoe, 2007). Specifically, the augmented levels of Aβ peptides enhance oligomer formation leading to parenchymal deposits known as non-fibrillar plaques (Haass and Selkoe, 2007). As the diffuse plaques begin to acquire Aβ fibrils, local activation of astrocytes and microglia lead to initial inflammatory response (Akiyama et al., 2000). This event occurs in concert with synaptic spine loss and neurotic dystrophy (Akiyama et al., 2000). The cascade culminates in a widespread synaptic and neuronal dysfunction and cell death associated with multiple neurotransmitter deficiencies leading to progressive dementia and cognitive failure (Haass and Selkoe, 2007).
Figure 1.1 Amyloidosis. The presumed pathological pathway leading to the development of cognitive impairment observed in Alzheimer’s disease.
1.2.5.1.1. **Amyloid-β precursor protein processing**

AβPP is constitutively expressed in healthy non-diseased conditions and cleaved into three main isoforms, APP695, APP751 and APP770, of which the APP695 isoform is predominantly found in the brain (Sisoda and St. George-Hyslop, 2002). AβPP is a transmembrane glycoprotein, typically found in neuronal cells, with an intracellular carboxy terminus and extracellular amino terminus (reviewed by Thinakaran and Koo, 2008). It is processed by the Golgi apparatus and carried along the axon via fast anterograde transport (Koo et al., 1990). Inhibition of axoplastic flow during axonal damage results in accumulation of AβPP at the site of axonal injury and thus AβPP serves as a marker of axonal injury (Sherriff et al., 1994; Gentleman et al., 1995). Due to the presence of metal binding, neurotrophic, cell adhesion, and protease inhibitor domains, AβPP has been implicated as a regulator of synapse formation (Priller et al., 2006), neural plasticity (Turner et al., 2003) and iron export (Duce et al., 2000).

AβPP undergoes proteolytic cleavage by α, β and γ-secretases (Figure 1.2; Esch et al., 1990; Vassar et al., 1999; reviewed in Wolfe, 2008). Cleavage by α followed by γ-secretases prevents the generation of Aβ peptides and is thus classified as the non-amyloidogenic pathway (Sisoda and St. George-Hyslop, 2002). Alternatively, AβPP can be processed through the sequential cleavage by β and γ-secretases, classified as amyloidogenic pathway (Sisoda and St. George-Hyslop, 2002). β-secretase cleaves AβPP in the extracellular domain between Met671 and Asp672 (known as the +1 site) or between residue 682 and 683 (known as the +11 site; Sisoda and St. George-Hyslop, 2002). This cleavage generates an extracellular soluble fragment (sAβPP) and a membrane-bound carboxy-terminal fragment (β-CTF; Vassar et al., 1999). β-CTF can then be cleaved by γ-secretase generating peptides.
Figure 1.2 Schematic of AβPP processing. AβPP is a transmembrane protein that can be processed along the non-amyloidogenic pathway with α and δ secretases or along the amyloidogenic pathway with β and δ secretases. Amyloidogenic pathway generates Aβ40 and Aβ42 fragments.
of varying length between 38-43 residues (Sisoda and St. George-Hyslop, 2002).

Importantly, cleavage at residue 40 and 42 generates forms of Aβ that are implicated in AD, namely the Aβ
40 and Aβ
42 peptides. The presence of two additional amino acids, isoleucine and alanine, in the Aβ
42 peptide increases its hydrophobicity and propensity to aggregate.

Cleavage of AβPP through the amyloidogenic pathway is not a pathogenic process in itself. Rather, altering AβPP processing or clearance such that Aβ is present in increased quantities leads to pathogenesis. In particular, mutations in close proximity to the β-secretase cleavage site cause overproduction of Aβ
40 and Aβ
42 peptides and are often seen in the familial AD (Vassar et al., 1999; Wolfe, 2008). One such mutation is the Swedish (KM670/671NL) mutation which makes AβPP more favourable for cleavage by β-secretase thus increasing the production of both Aβ
40 and Aβ
42 (Mullan et al., 1992). The Indiana mutation (V717I, V717G and V717F substitutions) is another example of mutations in the AβPP gene causing increased Aβ
42 production through enhanced γ-secretase activity (Murrell et al., 1991). This mutation occurs closer to the carboxy terminal of the AβPP sequence. Ultimately, familial AD resulting from AβPP mutations, results in earlier onset of symptoms and faster disease progression than what is seen in sporadic AD (Selkoe, 2001). The Swedish and Indiana mutations are both present in the TgCRND8 mouse model used in this thesis.

1.2.5.1.2. Amyloid-β aggregation

Aβ accumulates as either parenchymal plaques or around the walls of blood vessels forming cerebral amyloid angiopathy (CAA; Figure 1.3; Weller et al., 2009). Aβ
40 is the major peptide in CAA deposits whereas Aβ
42 is predominantly present in the core of dense amyloid plaques (Miller et al., 1993; Biffi and Greenberg, 2011). Both forms of amyloid aggregates are evaluated in this thesis.
Figure 1.3 Schematic of $\text{A}\beta$ aggregation into plaques and CAA. $\text{A}\beta$ is known to aggregate as parenchymal plaques (blue arrows) or around large blood vessels as cerebral amyloid angiopathy, or CAA (red arrow).
Much work has been done to characterize the steps of the aggregation process of Aβ in order to elucidate which species of Aβ constitute the toxic forms (Figure 1.3). The process begins with Aβ monomers followed by fibrillogenesis into higher-order soluble aggregates, termed oligomers. During fibrillogenesis, Aβ undergoes a conformational change from random-coil to a β-sheet rich structure which is unstable and promotes the self-assembly into more stable oligomers. The aggregation into oligomers is the rate-limiting step and can be classified as the primary nucleation event (Jarrett, 1993; Cohen et al., 2013). Further addition of monomers gives rise to protofibrils and subsequently to fibrils. Fibrils are insoluble species and they precipitate out of solution and deposit in the brain as Aβ plaques. Before Aβ fibrils are formed, the primary nucleation event involves the formation of Aβ oligomers from Aβ monomers. However, once a critical concentration of fibrils is reached, a secondary nucleation event occurs involving the surfaces of fibrils catalysing the nucleation of new aggregates from Aβ monomers in a positive feedback mechanism (Walsh et al., 1999; Walsh and Selkoe, 2007; Cohen et al., 2013).

Soluble oligomers are thought to be the most toxic forms of Aβ, compared to fibrils and plaques (Pike et al., 1991; Buseiglio et al., 1992; Walsh and Selkoe, 2007). Neurons and dendrites in immediate proximity to plaques are known to be dystrophic, exhibit reduced activity and responsiveness to environmental stimulation compared to neurons further away from the plaques. (Koffie et al., 2009). However, this was most likely due to the presence of Aβ oligomers surrounding the plaques rather than plaques themselves.

CAA, another form of Aβ aggregate in addition to parenchymal plaques, deposits in the media and adventitia of the cerebral arteries and arterioles (and in capillaries, although to a much smaller degree). CAA deposits in the anatomically-hierarchical pattern that closely
follows the deposition of parenchymal plaques (Thal et al., 2003; Biffi and Greenberg, 2011). Specifically, leptomeningeal arteries and arterioles of the neocortex are affected first, followed by the allocortex and midbrain (Thal et al., 2003; reviewed in Hazrati et al., 2009). Basal ganglia and thalamus are affected by CAA to a much smaller degree and CAA is rarely present in the lower brainstem (Thal et al., 2003). The deposition of CAA leads to degeneration of smooth muscle cells and pericytes resulting in fibrinoid necrosis (Thal et al., 2002). Additionally, CAA can occlude vessels leading to ischemia (Biffi and Greenberg, 2011). Further, Burgess et al. (2014) showed that the ability of a vessel to change its diameter in response to hypercapnia is diminished in amyloid-coated vessels compared to non-coated vessels in TgCRND8 mice. Such thickening and stiffening of non-capillary walls disrupts cerebral blood flow (CBF), thus diminishing the drainage of interstitial fluid and solutes from the brain and is the main cause of microbleeds as well as large haemorrhage (reviewed in Zlokovic, 2011). In aged rats, chronic vascular insufficiency induced by a two vessel occlusion resulted in progressive accumulation of Aβ peptides detected by Western analysis (Bennett et al., 2000). This correlated with a shift in the localization of AβPP from neurons to extracellular deposits in brain parenchyma. General failure of drainage leads to further accumulation of Aβ resulting in loss of homeostasis of the neuronal environment (Biffi and Greenberg, 2011). Overall, the process of Aβ aggregation that lies at the foundation of the amyloid hypothesis triggers a cascade of events including cerebrovascular remodelling and neuronal damage leading to compromised cognitive abilities.

1.2.5.1.3. Amyloid-β deposition and neurotransmission in TgCRND8 mice

In comparison to other AD mouse models, the TgCRND8 mouse model exhibits early and accelerated Aβ plaque deposition (Webster et al., 2014). Aβ appears between 2 and 3 months of age with the first plaques present in the frontal cortex and the subiculum - the
most inferior part of the hippocampus (Chishti et al., 2001). By comparison, the APP23, PDAPP and Tg2576 mice develop plaques by 6, 6-9 and 9 months of age, respectively (Games et al., 1995; Hsiao et al., 1996; Sturchler-Pierrat et al., 1997; Webster et al., 2013, 2014). The progression of Aß deposition in the TgCRND8 mice and in human patients occurs in similar brain regions, with the cortex and hippocampus affected earlier in the disease process and the cerebellum unaffected until the more advanced stages of disease (Chishti et al., 2001; Thal et al., 2002). In this thesis, the plaque pathology was assessed at age of 4, 5 months (Chapter 3) and 6 months (Chapter 4).

TgCRND8 mice also develop CAA deposits in the leptomeningeal and intracerebral vessels (Ambrée et al., 2006b). Dorr et al. (2012) evaluated temporal development of CAA in TgCRND8 mice with Thioflavin-S immunostaining. Very little amyloid was detected around blood vessels in 2-3-month-old mice. By 4-6 months of age, Thioflavin-S signal was detectable around multiple cortical blood vessels. Hawkes et al. (2014) confirmed the presence of CAA at 4 months of age in the cortex and hippocampus of TgCRND8 mice. Multiple studies have since confirmed the presence of CAA in the cortex of 5-month-old (Hawkes and McLaurin, 2009; Herring et al., 2011; McLean et al., 2013; Lai et al., 2015) and 6-8-month-old TgCRND8 mice (Burgess et al., 2014). In older mice (7-12-months-old) CAA is increased in comparison to mid-age and young mice, suggesting temporal accumulation of CAA along cortical blood vessels (Dorr et al., 2012). In this body of work, hippocampal CAA was examined at 6 months of age and data are described in Chapter 4.

In addition to Aß-related pathologies, hyperphosphorylated tau proteins have been observed in the brains of 7- and 12-month old TgCRND8 mice, however tau does not aggregate into neurofibrillary tangles in these mice (Bellucci et al., 2007). TgCRND8 mice
also recapitulate some aspects of the neurotransmitter dysfunction observed in AD. Specifically, the cholinergic, GABAergic and noradrenergic systems were found to be deregulated in TgCRND8 mice at various ages. Bellucci et al. (2006) reported that 7-month-old TgCRND8 mice have decreased choline acetyltransferase immunoreactivity in the nucleus basalis and reduced basal and potassium-stimulated extracellular acetylcholine levels in the cortex. Additionally, TgCRND8 mice exhibit GABAergic neuronal loss at 6 months of age (Krantic et al., 2012; Ma and McLaurin, 2014; Albuquerque et al., 2015). Specifically, neuropeptide Y-expressing GABAergic neurons are decreased in CA1-CA2 (in pyramidal, stratum oriens, stratum radiatum and molecular layers), CA3 (in stratum oriens layer) and in the dentate gyrus (in polymorphic layer) in TgCRND8 mice as compared to non-transgenic controls (Albuquerque et al., 2015). Furthermore, somatostatin-expressing GABAergic neurons were decreased in CA1-CA2 (in stratum oriens layer) and in CA3 (in stratum radiatum layer), whereas paravalbumin neurons were significantly altered in CA3 (in stratum oriens layer) (Albuquerque et al., 2015). Lastly, Francis et al. (2012) observed a deficit in noradrenergic function in the frontal and temporoparietal cortices, hippocampus, and cerebellum in 1-month-old TgCRND8 mice when compared to non-transgenic controls. Levels of noradrenaline in the ventral mesencephalon, striatum, and brainstem were unaffected, suggesting that rostral, cortically projecting noradrenergic cells of the locus coeruleus are the most severely disrupted in this mouse model (Francis et al., 2012). Cumulatively, these findings provide further support for the TgCRND8 mice as the most suitable model of multiple pathologies present in the AD brain.

### 1.2.5.2. Vascular hypothesis

Cerebrovascular dysfunction plays an important role in the progression of AD and many demented patients have both the amyloid and vascular pathology, particularly those...
above the age of 60 (Figure 1.1; Gorelick et al., 2011, Schneider et al., 2007, Toledo et al., 2013; Jellinger and Attems, 2010). Findings from both clinical and rodent studies suggest that cerebrovascular changes that occur in the AD brain take place early on (Fischer et al., 1990; Mazza et al., 2011) and often precede (Montagne et al., 2015) clinical symptoms of the disease. Post-mortem human tissue analysis indicates morphological changes to the structure of cerebral vessels (Fischer et al., 1990) as well as damage to the blood-brain barrier (BBB) (Fiala et al., 2002; Salloway et al., 2002; Zipser et al., 2007). Functional in vivo studies indicate disruption of CBF in AD patients, reduced hemodynamic responses in activated brain areas, and impaired vascular tone (Cullen et al., 2005; Goos et al., 2009; reviewed in Mazza et al., 2011), particularly in the hippocampus (Raven et al., 2012). Finally, CAA develops in cerebral vessels altering the wall architecture and disrupting hemodynamic responsiveness (Biffi and Greenberg, 2011).

As will be discussed in Chapter 4, the aim of this thesis was to characterize the morphology of hippocampal vessels in a mouse model of AD. Literature overview of changes to the vessel structure in the AD brain are subsequently discussed.

1.2.5.2.1. Vascular morphology

Cerebral vascular morphology has profound pathological implications as “almost every disease from cancer to the common cold affects blood vessel attributes (vessel number, radius, tortuosity and branching pattern)” (Bullitt et al., 2005). Abnormalities in vessel architecture increase geometric resistance to blood flow, decreasing overall perfusion and leading to reduced blood supply (Lorthois et al., 2014). Vessel tortuosity is defined as the accumulation of curvature along blood vessel length and is an important morphometric parameter in identifying pathological state (Fischer et al., 1990). It has been shown that factors favouring vessels to become tortuous include high internal pressure, small diameter,
increased rigidity and length (Yasargil, 1987). Although the precise mechanism by which vessels become tortuous remains elusive, the clinical recognition of abnormal vascular tortuosity constitutes a part of diagnosis of many tumours, retinopathies, hypertension and diabetes (Johnson and Dougherty, 2007; Sutter and Helbig, 2003). Brown et al. (2009) performed a morphometric analysis of arteriolar tortuosity in human cerebral white matter and associated changes to vessel tortuosity with clinical vascular dementia and saw increased number of tortuous arterioles beginning at age 50. Dorr et al. (2012) showed increased tortuosity in cortical arterioles in TgCRND8 mice which occurred together with accumulating CAA. Tortuosity was reversed to non-transgenic levels with the use scyllo-inositol (Dorr et al., 2012), a compound that is known to prevent the accumulation of Aβ (McLaurin et al., 2000, 2006).

Other morphometric features of cerebral vasculature, such as density, length and diameter, have also been characterized in patients and mouse models of AD. Specifically, capillary density is affected in the AD brain, as the initial report by Meier-Ruge et al. (1980) showed increased capillary density in the hippocampus. This increase could be attributed to any of the three factors known to occur in the presence of Aβ: the shrinkage of hippocampus (Meier-Ruge et al., 1980); the angiogenic function of Aβ (Boscolo et al., 2007); the increased levels of vascular endothelial growth factor (VEGF) and transforming growth factor β (TGF-β) (Tarkowski et al., 2002; Kalaria et al., 1998; reviewed in Vagnucci and Li, 2003). Further, Gama Sosa et al. (2010) found that capillaries in the PS1 PAC M146V mouse model of amyloidosis were often thinner and irregular in shape. The findings of increased capillary density were later challenged by human and animal studies (reviewed by Bailey et al., 2004), thus it remains unknown whether capillary density is increased or decreased in the AD brain.
It is possible that the capillary density undergoes fluctuations in response to accelerating Aβ pathology. This hypothesis is further explored in Chapters 4 and 5.

Several morphological characteristics, such as tortuosity, length, diameter, volume and branching, were evaluated and are discussed in Chapter 4.

1.2.5.2.2. Vascular function: blood flow and BBB integrity

The above-mentioned structural changes to the cerebral vasculature in the AD brain have profound effects on the function of the vascular bed. Already at the pre-clinical stage, the AD brain is characterized by reduced blood supply at rest and chronic brain hypoperfusion to activated areas, a process known as functional hyperaemia (Hirao et al., 2005; Bateman et al., 2006). The reduction in CBF in AD patients ranges from 10 to 30% in the temporoparietal, frontal and posterior cingulate cortex as well as in the hippocampus (Bateman et al., 2006). This decrease was associated with severity of dementia, while the reduction in blood supply at rest is a powerful correlate of AD progression (Hirao et al., 2005; Yoshida et al., 2007).

In addition, vascular reactivity to an insult such as hypercapnia is compromised in cortical vessels of TgCRND8 mice (Dorr et al., 2012) in that the transit time was increased and more variable in 12-month-old transgenic animals when compared to non-transgenic littermates. Similarly, CBF is diminished in aged wild-type rats due to reduced vascular density (Lynch et al., 1999).

The hemodynamic changes are being established very early in the pathological process as they manifest in the population at risk of developing AD (Nicolakakis and Hamel, 2011). It is debated whether the diminished blood supply is the result of decreased demand by the diseased tissue, or a result of compromised morphology of cerebral vessels that are unable to optimally change CBF in activated areas (reviewed in Zlokovic, 2011).
Lack of BBB stability is another characteristic of compromised vascular integrity in the AD brain. BBB breakdown occurs early on in disease progression and is region-specific. Montagne et al. (2015) showed that BBB disruption begins in the hippocampus and the disruption is more pronounced in patients with mild cognitive impairment (MCI) than in age-matched control patients. In addition, both human and murine studies showed that the breakdown of BBB leads to the accumulation of potentially neurotoxic blood-derived products, such as fibrinogen, that normally do not enter the brain (Fiala et al., 2002; Ryu and McLarnon, 2009). This can potentially lead to an increased inflammatory response, causing neuronal damage, as macrophages are able to migrate through disrupted BBB (Fiala et al., 2002). BBB breakdown can also disrupt clearing of toxic products, including Aβ peptides, from the brain (Ryu and McLarnon, 2009).

1.2.5.2.3. **CAA and compromised Aβ clearance**

The vasculature constitutes a major pathway through which Aβ is removed from the brain (Shibata et al., 2000). The process occurs via the transvascular transport system involving lipoprotein receptor-related protein 1 (LRP-1) that regulates brain Aβ homeostasis (Deane et al., 2004; Bell et al., 2009). The LRP-1-mediated clearance is disrupted in the presence of vascular dysfunction, contributing to parenchymal and vascular Aβ deposition (Deane et al., 2004, Park et al., 2013). In particular, Bell et al. (2009) showed in AD patients and two mouse models that the suppression of LRP-1 in vascular smooth muscle cells is a key factor in Aβ clearance impairment. In addition, morphological changes such as network fragmentation and increased tortuosity disrupt blood flow causing failure of perivascular drainage of interstitial fluid and solutes, including Aβ, from the brain leading to increased Aβ accumulation (Mawuenyega et al., 2010; reviewed in Zlokovic, 2011). As a result of
disrupted architecture and function of cerebral vasculature, the neuronal and cognitive function is compromised in the AD brain (Farkas et al., 2006).

These lines of evidence suggest that neurodegeneration frequently occurs concurrently with pathological vessel morphology, blood flow, BBB permeability and Aβ accumulation that together propagate AD symptoms. Mitigating vascular pathologies reduces vascular lesions in AD and may delay disease progression (reviewed in Viswanathan et al., 2009). This suggests that improving vascular health is an attractive therapeutic target and warrants greater attention.

1.2.5.2.4. Vasculature in TgCRND8 mice

Knowledge of vascular morphology in TgCRND8 mice is limited. The study by Dorr et al. (2012) was the first to characterize arteriolar tortuosity in 2-3, 4-6 and 7-12 –month-old mice representing early, middle and late stage of amyloid deposition, respectively. Their findings indicated a progressive increase in tortuosity of cortical penetrating arterioles, with the largest change occurring in the middle stage of the disease, followed by saturation of this effect in the late stage. In a later study, this same group confirmed increased cortical arteriolar tortuosity in 7-month-old TgCRND8 mice (Lai et al., 2015). Further, Lai and colleagues (2015) also reported frequent vessel coiling, meandering, and short-range looping. Considering the above findings, we evaluated vascular morphology in TgCRND8 mice at 6 months of age.

Morphological changes to the cerebral vasculature are accompanied by progressive compromise of vessel function in TgCRND8 mice. Longer and more variable vascular transit time and impaired vascular response to the global hypercapnic challenge was reported in 7-12-month-old mice (Dorr et al., 2012). Further, it is primarily the cortical arterioles that exhibit a decrease in blood flow with hypercapnia, while the flow of venules and capillaries
remains largely unchanged from non-transgenic controls (Lai et al., 2015). This is also consistent with preferential CAA deposition on the arterial walls.

As mentioned earlier, BBB disruption is an important characteristic of disrupted vascular integrity in the AD brain. Although there is no difference in BBB leakage between TgCRND8 mice and their non-transgenic controls at 6-8 months of age (Burgess et al., 2014b), there are amyloid-dependent differences in the leakage kinetics present in these mice. Burgess et al. (2014b) found that amyloid-burdened vessels exhibited higher occurrence of slow leakage than non-burdened vessels when BBB permeability was induced in those mice. This suggests that the accumulation of CAA on the vessel walls modifies BBB structure and leakage parameters. Additionally, CAA-positive vessels of 6-month-old TgCRND8 mice appear to have significantly more fibrinogen than non-burdened vessels (Cortes-Canteli et al., 2010). Conversely, depletion of fibrinogen lessened CAA pathology and improved the performance on Morris water maze and Y-maze memory tasks (Cortes-Canteli et al., 2010).

In this thesis we hypothesized that physical exercise is capable of improving vascular morphology in TgCRND8 mice thus mitigating some of the pathologies present in this model. The results pertaining to the vascular hypothesis are presented in Chapter 4.

1.3. Adult neurogenesis

In this section, the importance of neurogenesis on learning and memory and its impairment in AD are discussed. Results assessing the neurogenesis status and its modulation with physical activity in 4 and 5-month-old TgCRND8 mice are evaluated in Chapter 3.
1.3.1. **History**

In the past, the scientific community firmly believed that our brain could not repair itself during adulthood. Many early neuroanatomists considered the nervous system capable of axonal regeneration yet incapable of *de novo* production of neurons. The belief that new neurons could not be generated in an adult mammalian brain was challenged in 1963 when Dr. Joseph Altman first described a process known today as adult neurogenesis. His work labelling neurons in the brain of adult rats and cats with radiolabelled thymidine proved the presence of actively dividing cells in the dentate gyrus of hippocampus. Further work by Altman and Das (1965, 1969) identified and named the rostral migratory stream as the source of adult-generated granule cell neurons in the olfactory bulb. However, this work remained largely ignored until Kaplan and Hinds (1977) and later Bayer et al. (1982) showed that the formation of new neurons occurs in the adult mammalian brain in two distinct areas – in the dentate gyrus (DG) and in the subventricular zone (SVZ). Paton and Nottebohm (1984) confirmed these findings in songbirds. These studies finally put research on adult neurogenesis into a mainstream pursuit. Many studies emerged using 5-bromo-2’-deoxyuridine, or BrdU, a thymidine analogue that incorporates into DNA of mitotic cells, supporting the presence of adult neurogenesis in the adult brain (Kuhn et al., 1996; Seki and Arai, 1993). Eriksson et al. (1998) was first to demonstrate the occurrence of neurogenesis in the adult human brain by co-labelling cells with BrdU and neuronal nuclei protein (NeuN), calbindin, and neuron specific enolase (NSE). In addition, Gould et al. (1999a) confirmed the presence of neurogenesis in adult brain of primates. Adult human neurogenesis has recently been confirmed to occur in the dentate gyrus, olfactory bulb and striatum in post-mortem human brains through the measurement of $^{14}$C - the levels of which changed during nuclear bomb testing throughout the 20th century (Spalding et al., 2013).
1.3.2. Importance for learning and memory

Today we know that adult neurogenesis is a process involving proliferation of neuronal progenitor cells (NPCs), as well as their migration, differentiation and functional integration into pre-existing neural networks (Figure 1.4). The functional relevance of adult neurogenesis remains uncertain (Kempermann et al., 2004). However, there is some evidence that hippocampal adult neurogenesis is important for learning and memory (reviewed in Neves et al., 2008). The evidence for functional significance of adult neurogenesis in learning and memory comes from a series of studies using neurogenic ablation (Wojtowicz, 2006). Winocur et al. (2006) used low dose Gamma irradiation to reduce the proliferation of NPCs in dentate gyrus of adult rats and found 4 weeks after treatment that rats performed poorly on fear conditioning and nonmatching-to-sample (NMTS) memory tests. In addition, irradiated rats showed a significant reduction in BrdU-positive proliferating cells and doublecortin (DCX)-positive immature neurons in the dentate gyrus eight weeks after irradiation. In another study with irradiation-ablated neurogenesis, Snyder et al. (2005) reported that new neurons aged 4-28 days old at the time of training are required for long-term memory in a spatial version of the water maze suggesting a role for adult neurogenesis in the long-term spatial memory formation.

Garthe et al. (2009) used temoxolomide (a DNA alkylating agent) to suppress proliferation of cells in the dentate gyrus and found an impairment in reference memory tested with the Morris water maze, indicating a lack of cognitive flexibility to cope with altered challenge. Shors et al. (2001) used methylazoxymethanol acetate (a toxin targeting proliferating cells by DNA methylation) and reduced the number of new immature and
**Figure 1.4 Schematic of neurogenesis stages.** Adult hippocampal neurogenesis occurs in two distinct niches of mammalian brain: in the subventricular zone (SVZ) and in the subgranular layer (SGL) of the dentate gyrus (DG). In the SGL, proliferating radial cells give rise to neuronal progenitor cells that differentiate into immature neuroblasts before maturing into neurons.
mature granule neurons in the dentate gyrus, which resulted in impaired performance on the fear conditioning test. All these studies point to the importance of adult hippocampal neurogenesis in the maintenance of learning and memory processes.

1.3.3. Neurogenesis and AD

Adult neurogenesis is known to be affected by various brain insults, including stroke, neurodegeneration, and trauma (reviewed in Kaneko and Sawamoto, 2009). Therefore, the restoration of adult neurogenesis and stimulation of maturation of newly formed neurons in the diseased brain is an attractive therapeutic strategy. Impaired neurogenesis and neuronal damage results from an accumulation of Aβ, imbalanced levels of several neurotransmitters and neurotrophic factors, as well as by inflammation in the brain (Kaneko and Sawamoto, 2009). Importantly, many molecules central to AD pathology, such as AβPP metabolites and presenilins, play an important physiological regulatory role in many aspects of adult neurogenesis. As such, the use of mouse models engineered with different mutations under the control of various promoters yields variable results on the fate of neurogenesis. Further, high sensitivity and responsiveness of neurogenesis requires careful examination of data with respect to extent and onset of amyloidosis, age, neurodegeneration, and various experimental conditions such as BrdU regimen and markers used to evaluate neurogenesis (Lazarov and Marr, 2010; Mu and Gage, 2011).

Mounting evidence suggests a decrease in hippocampal neurogenesis in AD patients and mouse models alike (Haughey et al., 2002a, b; Wang et al., 2004; Chevallier et al., 2005; Verret et al., 2007; Fiorentini et al., 2010). Chen et al. (2008) reported that PS1/PS2 knock-out mice exhibit neurodegeneration in the cortex and hippocampus accompanied by induced cell proliferation in the subgranular zone. However, most of these newly developed cells failed to mature, thus negating the apparent increased neurogenesis. Contrastingly, other
studies suggest increased adult neurogenesis in AD. Specifically Jin et al. (2004) found up-regulation of DCX-positive immature neurons, polysialyated-neural cell adhesion molecule PNCAM, and neurogenic differentiation marker NeuroD in 3-month-old PDGF-APP(Sw,Ind) mice, an age when neuronal loss and amyloidosis are not yet present in those mice. However, mature neuronal markers, such as the neuronal marker NeuN and the calcium-binding calbindin, were not up-regulated (Jin et al., 2004). The authors suggested that the increase in cell markers characteristic of earlier stages of neurogenesis might be a compensatory mechanism to replace lost neurons. However, the process does not amount to a functional response as cells do not reach the maturation stage. Nevertheless, the authors suggested that stimulating neurogenesis might provide an attractive treatment strategy (Jin et al., 2004). More recently, Meneghini et al. (2013) confirmed increased numbers of BrdU-positive proliferating cells and DCX-positive immature neurons in TgCRND8 mice. However, the number of NeuN-positive mature neurons was unchanged when compared to non-transgenic control animals. Increased number of neuroblasts may in part be explained by increased levels of VEGF, known to be present in brains of AD patients (Kalaria et al., 1998; Tarkowski et al., 2002). VEGF is a growth factor implicated in the stimulation of neurogenesis and administration of exogenous VEGF has been shown to increase the proliferation of cortical neuronal precursors in vitro as well as the proliferation and DCX-positive cell counts in the hippocampus of rodents (Jin et al., 2004; Zhu et al., 2003; reviewed in Welberg, 2011). Thus, the increased neurogenesis observed in some mouse models may be partially mediated by VEGF.

Overall, a vast body of work supports the notion that neurogenesis is an integral part of AD pathology and constitutes an important therapeutic target. Drawing conclusion on
whether neurogenesis is increased or decreased in the context of amyloidosis is dependent on many variables, such as cell markers, BrdU injection schedule, mouse model and the stage at which the neurogenesis process examined.

1.3.3.1. Neurogenesis in TgCRND8 mice

Several studies have suggested decreased neurogenesis in TgCRND8 mice. Herring et al. (2009) used 4-month-old female mice to measure neurogenesis, 12 days after BrdU injection. Data showed a reduction in BrdU-positive and BrdU/NeuN-positive cell number in comparison to non-transgenic animals while the number of BrdU/GFAP-positive cell numbers were increased from non-transgenic control mice (Herring et al., 2009). The decrease in BrdU-positive cell numbers could be due to impaired cell proliferation and/or survival since cells were counted 12 days after the last BrdU injection. Fiorentini et al. (2010) distinguished between cell proliferation and survival by assessing BrdU cell population in two distinct injection paradigms. Firstly, 3 and 7-month-old male and female TgCRND8 mice were sacrificed 24hrs after BrdU injection. BrdU-positive cell number was significantly diminished from non-transgenic mice, suggesting impaired cell proliferation in TgCRND8 mice. Secondly, 3 and 7-month-old TgCRND8 mice were sacrificed 5 weeks after BrdU injection and the population of BrdU-positive and BrdU/NeuN-positive cells was significantly smaller in TgCRND8 mice than in age-matched control mice suggesting impaired cell survival in transgenic mice.

Contrastingly, other studies conclude that adult neurogenesis is increased in TgCRND8 mice. For example, Meneghini et al. (2013) found increased cell proliferation in 7-month-old TgCRND8 mice as measured by quantifying BrdU-positive cells. In addition, the population of BrdU/DCX-positive immature neuroblasts was also increased in 7-month-old TgCRND8 mice when compared to non-transgenic controls. Additionally, Kanemoto and
colleagues (2014) injected 5-week-old TgCRND8 mice with BrdU and examined BrdU cell survival 1, 2, 4, 6 and 8 weeks later. At all time points examined, the population of BrdU-positive cells was higher in TgCRND8 than in non-transgenic mice. Although there were more BrdU-positive cells in TgCRND8 mice, there was a tendency towards reduction in BrdU/NeuN-positive cell numbers at 1 and 8 weeks post injection, suggesting impaired differentiation or survival of newly born neurons. Furthermore, Thomason et al. (2013) assessed cell proliferation and differentiation in young (100/121 days old) and old (200/221 days old) TgCRND8 mice. In the young mice increased cells proliferation (number of BrdU+ cells), decreased neuronal differentiation (number of BrdU+/ DCX+ cells), and unchanged cell maturation (number of BrdU+/ NeuN+ cells) was detected compared to non-transgenics. In the old mice cell proliferation, differentiation and maturation are decreased compared to non-transgenic littermates.

Both increased and decreased neurogenesis have been observed in TgCRND8 mice with varying injection and survival protocols making it difficult to assess the true relationship between AD and neurogenesis. Nevertheless, these findings indicate that when Aβ pathology is present, early stages of adult hippocampal neurogenesis appear to be increased as a compensatory mechanism aimed at repopulating lost neurons. This response is not sufficient to replace lost cells and as such new-born cells do not complete the maturation phase of neurogenesis. As a result, compensatory mechanism aimed at stimulating neurogenesis is a transient process that is overpowered with accumulating Aβ.

The stimulation of neurogenesis with physical exercise in 4- and 5-month-old TgCRND8 mice is evaluated in Chapter 3 while the compensatory mechanism is further discussed in section 5.3.
1.4. Cognition in the context of AD

1.4.1. Clinical studies

Clinical findings demonstrate that impairment in cognitive ability involves multiple brain structures (Elias et al., 2000) and typically begins several years before AD diagnosis (Bäckman et al., 2004; Whalley et al., 2000; Snowdon et al., 1996). Early cognitive impairments related to episodic memory (Bäckman et al., 2001), working memory (Baddeley et al., 1986, 1991), executive functioning (Albert et al., 2001), verbal ability (Jacobs et al., 1995), visuospatial skill (Small et al., 1997), attention (Linn et al., 1995), and perceptual speed (Fabrigoule et al., 1998) are noted during disease progression. Tasks assessing episodic memory, such as word recall and face recognition, are particularly useful at identifying at-risk individuals in pre-clinical stages of AD (Small et al., 1997; Elias et al., 2000). It has become increasingly clear that identifying and targeting early cognitive deficits is critical to achieving the optimal impact of treatment (Salmon et al., 2002).

Anatomical research implicates the hippocampus as one of areas of particular interest in the development and progression of cognitive ability in AD. It has been known since the seminal work with Henry Gustav Molaison, known as H.M. patient, that the hippocampus is crucial to learning and memory processes (Scoville and Milner, 1957). Subsequent lesion and imaging research confirmed the hippocampus as a crucial region for encoding, storage and retrieval of episodic information (Braak and Braak, 1995; Fox et al., 1996). It is also one of the first sites where histopathological changes take place during AD progression, along with the entorhinal cortex and followed by the medial temporal lobe (Braak and Braak, 1995).

Patients in the early stages of AD often develop non-cognitive neuropsychiatric disorders, such as depression, apathy and agitation while delusions, hallucinations, aggression and sleep disturbances are present in advanced AD (Lyketsos et al., 2011;
McKeith and Cummings 2005). These disorders contribute to cognitive decline and are a source of great distress to patients and caregivers drastically affecting the quality of life leading to institutionalization.

1.4.2. Modelling cognition in rodents

Many rodent behavioural tasks have been designed to assess a cognitive domain representative of those examined through neuropsychological testing conducted in humans. Some cognitive deficits have been studied extensively, such as reference, spatial and working memory, and executive function. By contrast, episodic memory has received much less attention. Still, other cognitive aspects affected in AD patients and involving language capabilities simply cannot be modelled in mice.

Working memory is perhaps one of the best modelled aspects of AD deficits (reviewed in Webster et al., 2014). Clinically, assessments of working memory rely on verbal-based visuospatial tests (Kaplan et al., 1978; Benedict et al., 1998; Spreen and Strauss, 1998; Delis et al., 2000). In rodents, spatial-based working memory tasks are frequently utilized and are considered depictive of the visuospatial working memory tests used clinically (Benton, 1992; Benedict and Groninger, 1994). The most widely used paradigms are maze-type tasks, such as Y-maze and T-maze which test spatial working memory (Blodgett and Mccutchan, 1947; Glickman and Jensen, 1961). These tasks rely on the foraging behaviours of mice and their tendency to explore novel environments. Another common maze-type test evaluating spatial working memory is the Morris water maze (Morris et al., 1982). Several AD mouse models have been characterized with the Morris water maze and many show hippocampal-dependent learning deficits, including acquisition of spatial memory and long-term spatial memory impairment (Webster et al., 2014). Other examples of spatial working memory tests for mice include Radial Arm Maze, Radial Arm
Water Maze and Barnes Maze (Olton and Samuelson, 1976; Olton et al., 1979; Diamond et al., 1999; Barnes et al., 1979).

The hippocampus and cortex are the primary brain regions responsible for spatial memory performance in both mice and humans (reviewed in Webster et al., 2014). The hippocampus provides animals with a spatial map of their environment - the dorsal hippocampus being responsible for retrieval, processing short-term spatial memory and transferring memory from short term to longer delay periods (O'Keefe and Dostrovsky, 1971). The parietal cortex is involved in the transformation of sensory information coordinates into action or effector coordinates by updating the spatial representation of the body within the environment (Colby and Goldberg, 1999). The entorhinal cortex contains a topographically organized map of the spatial environment made up of grid cells and stores spatial information obtained from the environment as a durable representation in the brain (O'Keefe and Dostrovsky, 1971; Hafting et al., 2005). Lastly, the prefrontal cortex processes the short-term spatial memory used to guide planned search behaviour and is believed to join spatial information with its motivational significance (Pratt and Mizumori, 2001).

As will be presented in Chapters 3 and 4, the focus of this thesis was the evaluation of working memory with the spatial component assessed via Y-maze task performance. A brief literature review of cognitive ability of TgCRND8 mice is presented in the next section.

Other types of memory known to be impaired in AD patients, such as executive function, cognitive flexibility, attention and episodic memory, are also being modelled and tested in mice. Executive function encompasses a wide range of higher cognitive processes, such as reasoning, planning, cognitive flexibility, sequencing, response inhibition and abstract concept formation (Albert et al., 2001). Cognitive flexibility is a form of executive
function most commonly modelled in mice and tested with reversal learning and response inhibition tasks. In both patients and mice, the dorsolateral and orbital prefrontal cortices are important for the executive function (Weinberger et al., 1986; Brigman et al., 2005). Attention has also been modelled in mice and tested with an operant box chamber equipped with a stimulus and reward system (Muir et al., 1996). Episodic memory refers to the ability to encode and recall personal past and experiences and has been the least explored in mice, as up until recently it was believed that this type of memory is unique to humans (Tulving and Markowitsch, 1998; Webster et al., 2014).

The most comprehensive approach is to evaluate the multicognitive phenotype with overlapping behavioural tasks. In this body work, we assessed spatial working memory and present data from this analysis in Chapters 3 and 4. Alternative behavioural tasks of memory function are discussed in section 6.1.

1.4.3. Memory impairments in TgCRND8 mice

TgCRND8 mice exhibit early cognitive deficits. Specifically, these mice have impaired spatial memory, as tested on the Morris water maze, starting at 3 months of age (Janus et al., 2000; Chishti et al., 2001; Görtz et al., 2008; Richter et al., 2008; Ambrée et al., 2009), yet they are not impaired on this task when tested before development of amyloid plaques (6-8 weeks of age; Chishti et al., 2001; Hyde et al., 2005). In addition, deficits in the alternation and novel arm recognition in the Y-maze task were detected at 6 months of age (Hyde et al., 2005; Ma and McLaurin, 2014; Burgess et al., 2014a). Together, these data suggest that the onset of spatial memory impairment occurs concomitantly with the deposition of Aβ in the brains of these animals and worsens as Aβ pathology increases (Chishti et al., 2001; Hyde et al., 2005). As such, in our study TgCRND8 mice were evaluated every month for memory performance starting at age of 3 months.
Reference memory deficits, as measured by the Barnes maze, are also evident at 3 months of age (Görtz et al., 2008; Richter et al., 2008; Ambrée et al., 2009). A similar time course of deficit development is observed in the recognition memory and fear conditioning (Görtz et al., 2008; Richter et al., 2008; Ambrée et al., 2009; Greco et al., 2010; Hanna et al., 2012; Steele et al., 2014). TgCRND8 mice display impaired object recognition at 2 months of age (pre-plaque) and the deficit is maintained at 6-8 months of age when plaque pathology is in an advanced stage (Francis et al., 2012; Greco et al., 2010).

TgCRND8 mice also exhibit non-cognitive deficits that can be attributed to amyloid pathology. Among them is an array of stereotypic behaviours displayed in the home cage (Ambrée et al., 2006a, 2009). Additionally, open field and elevated-plus maze tests suggest healthy levels of anxiety until approximately 5 months of age (Ma and McLaurin, 2014; Görtz et al., 2008). After this stage, TgCRND8 mice exhibit lower anxiety than non-transgenic littermates. Lastly, disturbances in circadian rhythm and sleep patterns have been recorded by Ambrée et al. (2006a).

1.5. Physical exercise

Amyloid accumulation, vascular compromise, and neuronal damage and cell death are just a few of many pathologies that act in concert to elicit cognitive impairments observed in AD. As such, it is imperative that the treatment strategy is as broad as the disease it aims to combat. As will be discussed in subsequent sections of this chapter, physical exercise is one such approach that is gaining scientific recognition.

1.5.1. Historical perspective

The concept that physical exercise improves health and well-being of an individual is not new. In fact, the Greek word *physis* means *nature or natural* and became the root of *physic* – the term used for medicine in the past and it is where the modern word *physician* is
derived from (after Berryman, 2010). Hippocrates, who wrote two books on the exercise regimen stated that "eating alone will not keep a man well; he must also take exercise. For food and exercise (...) work together to produce health" (Hippocrates; after Jones, 1953). Furthermore, many physicians of the Renaissance age - Christobal Mendez, William Buchan, and Clement Tissot among them - were of belief that "exercise deserves lofty praise as a blessed medicine that must be kept in high esteem" (Mendez, 1553; after Berryman, 2010).

In addition to benefiting patients, physical exercise was also prescribed to the general public. Physical education in the 1800s was led by physicians who focused predominantly on anthropometric measurements teaching health lectures while supervising the creation of new gymnasiums built on university campuses and regarded as "Palaces of Health" (Hackensmith, 1966; Van Dalen and Bennett, 1971). With the invention of organized sports at the end of 19th century, physical exercise began to shift away from medical and into sport faculties. As a result, places once filled with rehabilitation equipment gave way for games and sports played by skilled and healthy people. Consequently, physicians were replaced by coaches, and exercise lost its standing amongst the medical and scientific community.

Physical exercise regained its popularity as a medical treatment in the 1970s. Basic research followed this trend and in 1980s and 90s began to regard exercise as intervention on par with pharmaceutical strategies giving a foundation to public health awareness and a return of the physician as an administrator of exercise. Just like Hippocrates and many Renaissance physicians believed, the view that exercise is in fact medicine was accepted by physicians of the modern generation. In fact, in 2007, Ronald Davies, the President of The American Medical Association asked his colleagues if they “learned that a single prescription
could prevent and treat dozens of diseases, such as diabetes, hypertension, and obesity, would you prescribe it to your patients? Certainly.” (after Berryman, 2010).

As exercise is regaining its place among medical faculties, it is important to gather expertise and knowledge on the basic roles that exercise plays in sustaining health, delaying disease onset, and treating existing conditions. We will then know the mechanisms behind the concepts our forefathers embraced centuries ago.

1.5.2. Prevention and treatment

While discussing physical exercise as a modulator of brain health in the context of AD, an important distinction between prevention and intervention should be made.

Epidemiological and prospective studies focusing on early-life activity interpret physical exercise as a preventative measure. These studies support the cognitive reserve hypothesis, which stipulates that an active brain has a larger reserve to compensate for pathology experienced later in life than an inactive one (Fratiglioni et al., 2004; Staff et al., 2004). Building this reserve relies on activity, including physical, before the disease onset (Lytle et al., 2004). Indeed, people who are physically active in mid- and late life have a lower risk of developing cognitive impairments and dementia. Specifically, Middleton et al. (2011) reported that women who were physically active at any point over their life had a lower risk of cognitive impairment in late life. In addition, studies in mice which began to exercise long before plaque development show that active mice fare much better on cognitive tasks than sedentary controls when tested after amyloid pathology develops (Adlard et al., 2005; Yuede et al., 2009; Tapia-Rojas et al., 2015). Lastly, Merkley et al. (2014) evaluated the effects of early exposure (1-month-old) to one month of voluntary physical activity on the hippocampal neurogenesis of 11-month-old wild-type rats. It was found that the rate of neuronal maturation and survival during a 4 week period after cell division was enhanced up
to 11 months of age (the end of the study period) suggesting long-term effects of early-life exposure to exercise on hippocampal neurogenesis (Merkley et al. 2014).

Intervention studies, by contrast, focus on AD patients who were inactive in earlier years and who began physical exercise at a time when cognitive impairments are evident. Encouragingly, recent research on AD and physical activity has demonstrated the feasibility of physical training even in severely demented patients in nursing homes (Rolland et al., 2008). Specifically, aerobic exercise, twice a week, has been reported to slow disease progression in nursing home residents (Rolland et al., 2007) suggesting that physical activity interventions for older adults with dementia might be of significant benefit for cognition and behaviour (Heyn et al., 2004). Meta-analysis by Heyn et al. (2004) showed that in people with cognitive impairment or dementia, exercise training improved behaviour disturbances, as well as physical and cognitive function. The mean time required to achieve these results was short, with most training programs lasting less than 4 months. Additionally, the intensity, frequency and duration of exercise regimen in AD patients were generally much shorter and less challenging than in preventative studies. Yet, even with much less exercise, these patients were able to benefit from physical exercise and improve their memory despite low levels of cognitive reserve. Expanding on the cognitive reserve concept to explain this paradox, one can hypothesize that the more cognitively impaired or at risk an individual is, the easier it may be to elicit a noticeable improvement. Recent findings by Etnier et al. (2007) indicated a greater cognitive effect of exercise in women who carry a mutation on the ApoE gene (a genetic risk factor for AD), while little effect was observed in non-carriers. Further evidence supporting this hypothesis comes from basic research studies. Nichol and colleagues (2007) found that aged Tg2576 mice exhibited positive cognitive effects of an
exercise regime sooner than wild-type mice, perhaps due to their greater impairment. These findings lend hope to those patients looking to improve their cognitive function who have not exercised prior to AD diagnosis, while suggesting that the threshold for exercise-induced improvement may be lower in cases of Alzheimer pathology than in age-related decline in healthy population.

This thesis sought to evaluate physical exercise at a time that addressed treatment of cognitive decline rather than its prevention. As such, physical exercise was commenced at 3 months of age - a time point when amyloidosis and cognitive deficits have already developed in TgCRND8 mice.

1.5.3. Environmental enrichment vs. physical exercise in rodent models

In many rodent investigations, researchers frequently utilize environmental enrichment in which a running wheel is only one of many components. Several studies have shown that housing animals in cages equipped with objects providing physical as well as tactile, visual, dietary, auditory, and vestibular stimuli augments numbers of dendritic spines and branches, synaptic size, stimulates hippocampal neurogenesis, and increases their performance on memory tests (Bennett et al., 1964; Rosenzweig and Bennett, 1996). In fact, housing animals in an enriched environment provided the first evidence that adult neurogenesis could be enhanced (Kempermann et al., 1997, 1998). However, the combination of environmental enrichment and physical exercise makes it difficult to delineate which components specifically augment neurogenesis and memory (Kempermann et al., 1997, 1998; van Praag et al., 1999; Rossi et al., 2006; Schloesser et al., 2010; Sun et et al., 2010). Additionally, complex environments and housing multiple animals in one cage makes tracking of physical activity impossible. Answers regarding the differential effects of environment and running come from studies that directly compare groups of mice housed in
standard, running-only, enriched-only and running plus enriched cages. For example, Kobilo et al. (2011) found that cell proliferation, neuronal survival and neurotrophin levels were enhanced only when running wheels were accessible. It was also found that exercise is the critical factor mediating increased brain-derived neurotrophic factor (BDNF) levels and hippocampal neurogenesis (Kobilo et al., 2011). Subsequently, Mustroph and colleagues (2012) found that the combination of enrichment and running did not significantly increase hippocampal neurogenesis any more than running alone did. Further, animals housed in the running-only condition were the only group to show enhanced acquisition on Morris water maze (Mustroph et al., 2012).

These findings suggest that environmental enrichment and physical activity share common features pertaining to synaptic plasticity, but that the increment in hippocampal neurogenesis and augmented memory performance are unique to physical activity. For this reason, it is important to distinguish between the two housing manipulations in experimental design and, more importantly, in interpretation of results. Since our outcome measures focused on cognitive improvements and increases in neuronal maturation, we selected running as the intervention group and sedentary conditions as the control group.

**1.5.4. Benefits of physical exercise in aging**

As part of the healthy aging process, the brain undergoes many structural and functional changes (reviewed in Pacheco et al., 2015). For example, the ability to learn new tasks decreases in aged rats (Barnes, 1979; Gage et al., 1984; Smith et al., 2000). On the cellular level, synaptic strength and plasticity are reduced in aged rats (Geinisman et al., 1992; Barnes, 1994) and hippocampal neurogenesis is diminished in age-dependent manner in rats (Kuhn et al., 1996; Heine et al., 2004). In elderly healthy humans, imaging studies have shown hippocampal atrophy (West, 1993; Small et al., 2002). These consequences of
aging can be prevented or their onset may be delayed with the introduction of physical exercise (Kronenberg et al., 2006). For example, exercise in healthy adults (40 to 59 years of age) was associated with fewer cases of dementia when subjects were re-examined at 70 to 85 years of age (DeFina et al., 2013). Furthermore, it was found that in 71 – 93-year-old physically capable and cognitively healthy men who engaged in moderate physical activity in the form of walking yielded a lower risk of developing dementia when evaluated 3 and 6 years later; risk of developing dementia was assessed at a follow-up examination 3 and 6 years after the start of the experiment (Abbott et al., 2004). Men who walked 0.25 miles per day or less experienced a 1.8-fold greater risk of dementia compared with those who walked more than 2 miles per day (Abbott et al., 2004). In another study, Muscari et al. (2010) examined 120 healthy adults aged 65 - 74 with the Mini-Mental State Examination test (MMSE) after 12 months of a supervised exercise regimen. The control group showed a significantly greater decline on the MMSE compared to the exercising group, which experienced a very small decline. In a similar study involving 259 cognitively healthy women aged 70 and over, Klusmann and colleagues (2010) found that the group that exercised for 6 months performed significantly better on a cognitive training challenge than the sedentary control group. Lastly, Sofi et al. (2010) performed a systematic review of 15 prospective studies on non-demented individuals aged 65 and over and found a significant protective effect of physical activity against cognitive decline with a 38% risk reduction in exercising group compared to the sedentary group.

Despite the abundance of prospective epidemiological studies, many of them come with caveats and limiting factors. For example, the definition of physical activity and its assessment vary considerably between studies, with some studies rigorously measuring
energy expenditure, frequency and duration of each exercise bout and relative intensity type of activity while others include self-reported activities such as gardening and yard work (reviewed in Lautenschlager et al., 2012).

Animal research investigating the impact of exercise on the aging brain provides a more controlled environment and advances our knowledge of the complexity of underlying mechanisms. In their seminal work, van Praag et al. (2005) showed that exercise enhances learning and hippocampal neurogenesis in 20-month old wild-type mice. In this experiment, mice remained sedentary until the age of 19 months. After one month of running, aged runners showed faster acquisition and better retention on the Morris water maze task than age-matched sedentary mice. In addition, cell proliferation and the number of mature neurons (as measured by BrdU- and BrdU/NeuN-positive cells, respectively) was augmented in old runners compared to age-matched sedentary mice. Subsequent studies examined mice running throughout middle age. For example, Marlatt et al. (2012) examined a group of mice that begun running at age of 9 months. After 6 months of running, mice performed significantly better on the Morris water maze task and displayed increased neurogenesis and BDNF levels. Similarly, Wu et al. (2008) evaluated mice that begun a 6-week exercise paradigm at ages of 8 and 12 months. Running mice at both ages had significantly elevated levels of BrdU-positive mitotic cells and BrdU/DCX-positive new-born immature neurons when compared to sedentary controls. In addition, running stimulated BDNF and its tyrosine receptor kinase B (TrkB) expression in both age groups in comparison to sedentary controls (Wu et al., 2008). These results suggest that physical exercise introduced in middle age is capable of stimulating neurogenesis, neurotrophic factors and cognitive performance.
1.5.5. Benefits of physical exercise in AD

In pathological conditions such as AD the brain exhibits accumulation of Aβ, compromised cerebrovascular morphology and function as well as progressive neuronal damage that act together to exacerbate cognitive deficits (Bruel-Jungerman et al., 2009). Physical exercise is considered a strong modulator counteracting multiple pathological events occurring in the AD brain thus delaying the onset and/or slowing down its progression. As will be discussed in Chapters 3 and 4, this thesis evaluates physical exercise as a form of intervention strategy aimed at amyloid pathology and neuronal, vascular and cognitive deficits. The literature pertaining to the effects that physical exercise can exert on Aβ, neurogenesis, vascular health and cognition is discussed below.

1.5.5.1. Physical exercise and Aβ

There is growing evidence that physical exercise mediates Aβ accumulation and thus modulates the onset of AD pathology. Additionally, the changes in Aβ levels due to running occur in the hippocampus – a neurogenic niche of the brain implicated in learning and memory processes. Thus, running emerges as an attractive intervention and prevention strategy aimed at modulating Aβ burden in the brain.

There have been several studies conducted in mouse models of AD investigating whether physical exercise can prevent Aβ deposition. Adlard et al. (2005) was first to report that 5 months of voluntary exercise, beginning at 1 month of age (pre-plaque stage) in TgCRND8 mice, reduced Aβ plaque load in the frontal cortex and the hippocampus. Also, in TgCRND8 mice at pre-plaque stage, Ambrée and colleagues (2006) showed that CAA is reduced with preventative exercise. It must be noted that in this study a running wheel was part of the enriched environment cage. Recently, Tapia-Rojas et al. (2015) reported that running prevented the accumulation of amyloid burden in the hippocampus of
APPswe/PS1ΔE9 mice. Lastly, exercise introduced at 5 months of age (pre-plaque stage) was associated with significantly lower brain plaque number in 9-month-old Tg2576 mice when compared to sedentary mice (Yuede et al., 2009). These data suggest that running may decrease Aβ burden.

The studies mentioned above investigate exercise as a prevention of Aβ accumulation. By contrast, intervention studies are much less successful at lowering amyloid burden. For example, Richter et al. (2008) attempted, unsuccessfully, to modulate Aβ in TgCRND8 mice after its onset. Our interventional investigation of effects of physical exercise on Aβ included several modifications to exercise paradigm and outcome measures than those reported by Richter and colleagues (2008); these modifications are reported and contrasted with Richter et al. (2008) study in Chapter 3.

1.5.5.2. **Physical exercise and cerebral vasculature**

Physical exercise is known to change the biochemistry, morphology and function of the brain, including its vasculature (reviewed in Schmidt et al., 2013). Most of our knowledge on how cerebral vasculature benefits from exercise comes from functional and imaging studies examining CBF. By contrast, morphological assessments, particularly of the hippocampal vasculature, are much more scarce (Schmidt et al., 2013). However, in agreement with the vascular hypothesis discussed earlier, cerebrovascular impairment is central to AD pathology and vascular compromise is a powerful predictor of progression to AD (Hirao et al., 2005; Yoshida et al., 2007). As such, studies exploring the effects of physical exercise on vessel morphology are essential.

Evidence of modulating cerebrovasculature with exercise in humans comes from functional magnetic resonance (fMRI) imaging of CBF (Mazza et al., 2011). These studies have demonstrated increased CBF and blood volume in the cortical regions of rodent and
primate models following exercise. For example, Swain and colleagues (2003) found an increase in cortical capillary density after 30 days of running and increased cerebral blood volume (CBV) in the II/III layer of the motor cortex in 6- to 12-month-old rats. In primates, Rhyu et al. (2010) revealed an increased vascular volume fraction of the motor cortex in adult monkeys exercising for 5 months. Reductions in CBF have been coupled to hypometabolism, and considered an indicator for cerebral functional impairments, including cognitive dysfunction that occurs in AD (Mori et al., 1994).

Modifications to cerebrovasculature mediated by physical exercise include reduced tortuosity in healthy adults (Bullitt 2009), lowered arterial stiffening and improved endothelial function in mice (Fleenor, 2010; Durrant 2009). These modifications are thought to be partially mediated by VEGF (Van Der Borght et al., 2009). As mentioned earlier, VEGF is a hypoxia-inducible growth factor that is known to influence angiogenesis – a process promoted following 3 days of voluntary running (Van Der Borght et al., 2009).

The above studies suggest that physical exercise can promote cerebrovascular health and function, which are compromised in AD. By preserving cerebral vasculature, physical exercise maintains neurogenesis, neurotransmission and synaptic plasticity in the AD brain. The effects of physical exercise on hippocampal vascular morphology in TgCRND8 mice are reported in Chapter 4 of this thesis. Examining the function of cerebral vasculature and angiogenesis following a period of physical exercise is discussed in Chapter 6.

1.5.5.3. Physical exercise and hippocampal neurogenesis

Most of our knowledge about the modulating effects of exercise on neurogenesis comes from rodent studies, as evaluating neurogenesis in human subjects in vivo is not feasible. As discussed in section 1.5.4, studies in wild-type mice have shown that exercise can prevent a decline in hippocampal neurogenesis at all ages, regardless of when exercise
regimen was introduced (reviewed in Vivar et al., 2013). Given the widespread loss of neurons observed in the AD brain, interventions aimed at increasing hippocampal neurogenesis to repopulate lost cells is an attractive strategy. However, similarly to the disparate results under sedentary conditions, there is lack of concordance with regard to the effects of physical exercise on neurogenesis in mouse models of AD. As discussed earlier, the genetic model, age of the mice, duration of exercise regimen, and the type of the intervention all influence the outcome. In general, mice showing early neurogenesis deficits benefit the most from physical exercise, particularly if it was introduced before amyloid pathology had developed. For example, in the 3xTg-AD model, reduced neurogenesis (Rodríguez et al., 2008) was improved by running (Rodríguez et al., 2011). Additionally, physical activity improved synaptic plasticity in this model (García-Mesa et al., 2011). More recently, Tapia-Rojas and colleagues (2015) observed that running APPswe/PS1ΔE9 mice showed increased number of immature and mature neurons in the hippocampus and exhibited increased cell proliferation when compared to sedentary transgenic mice, indicating that running increased neurogenesis. In the 18-month-old APP23 mice, running increased the number of DCX-positive cells when compared to APP23 sedentary mice (Mirochnic et al., 2009). However, these mice do not exhibit genotype-driven changes in neurogenesis at this age (Wolf et al., 2006). In the same study it was observed that APP23 mice running from the age of 2 months for a period of 9 months exhibited no change in adult hippocampal neurogenesis, with a down-regulation of hippocampal and cortical growth factors.

Taken together, these results indicate that, just like modulation of neurogenesis under baseline conditions, the effects of exercise on neurogenesis vary between different mouse
models of AD. Nevertheless, transgenic mice that exhibit neurogenesis deficits benefit greatly from exercise-induced stimulation of neurogenesis.

Augmenting neurogenesis via physical exercise occurs partially through the manipulation of a supportive environmental milieu, including many growth factors known to stimulate neurogenesis (reviewed by Vivar et al., 2013). BDNF is a neurotrophic factor widely involved in synaptic plasticity (Kuipers et al., 2006; Leal et al., 2014), neurogenesis (Bekinschtein et al., 2011), survival of newly-developed neurons (Lipsky and Marini, 2007), and in learning and memory (Yamada et al., 2003). BDNF levels are known to significantly increase in the dentate gyrus in response to exercise (Nepeer et al., 1995; Arsenijevic et al., 1998). Nepeer and colleagues (1995) showed that short-term exercise (2-7 days) increased BDNF mRNA levels in the rat hippocampus. In TgCRND8 mice, BDNF levels are known to be reduced, even at young age (Francis et al., 2012), suggesting that these mice stand to benefit from physical activity. In humans, aerobic endurance has been shown to increase BDNF, after a single bout, five-week, and three-month aerobic program (Zoladz et al., 2008; Rasmussen et al., 2009; Seifert et al., 2010). In this context, physical exercise provides a strong regenerative intervention in promoting the birth of new neurons by modulating a favourable milieu rich in BDNF for neurogenesis to occur.

1.5.5.4. Physical exercise and cognitive deficits

Several studies have shown that exercise restores spatial memory in mouse models of AD. Nichol et al. (2007) showed that 3 weeks of voluntary running in 16-18-month old Tg2576 mice resulted in significant improvement in their performance on the water radial arm maze task when compared to sedentary transgenics. This group later reported a significant improvement in the same task in symptomatic ApoE4 mice after 6 weeks of wheel running (Nichol et al., 2009). Improved spatial memory in ApoE4 mice was attributed
to an increased expression of BDNF and its TrkB receptor detected in running mice. Similarly, prevention studies conducted on TgCRND8 (Adlard et al., 2005) and APPswe/PS1ΔE9 mice (Tapia-Rojas et al., 2015) showed that physical exercise can prevent the decline in spatial memory tested with the Morris water maze task. While Adlard et al. (2005) attributed the improvement in memory to reduced hippocampal plaque burden, Tapia-Rojas and colleagues (2015) reported increased neurogenesis as well as reduced plaque deposition in running transgenics. In contrast, studies where running begun after plaque pathology and cognitive deficits are present resulted in lack of significant improvements to spatial memory, as measured by the Barnes maze in 5-month-old TgCRND8 mice following 2 months of running (Richter et al., 2008). In this study, plaque burden (quantified in neocortex and hippocampus combined) was also unaffected by running.

The varying results stem from multitude of factors in experimental design, equipment used, mouse models, and outcome measures. It appears that running has the greatest potential on improving cognition if it is introduced before or at the onset of amyloid pathology. It is also apparent that physical exercise exerts its beneficial effects on cognition via multiple pathways. Thus, it is difficult to determine a single mechanism of action by which running improves memory. Instead, it is becoming apparent that it is a combination of neurogenic, vascular and amyloid pathologies that are modulated by running, that together result in improved memory. Nevertheless, the difficulty in characterizing a mechanism of action should be viewed as a great therapeutic opportunity rather than a challenge to the research field. For the above reasons, examining multiple pathologies simultaneously gives the best insight into the extent and magnitude of action that exercise impairs on the amyloid-burdened brain.
Chapter 2

Research Objectives

2.1. Rationale

Currently, there is no medication available to treat AD. Drug development is complicated by the fact that many therapeutic targets, such as Aβ accumulation and neuronal dysfunction, begin several decades before clinical symptoms are detected. In addition, multiple pathologies arise simultaneously propagating cognitive deficits and the interactions between them remain largely unknown. The overarching goal of this thesis is, therefore, to investigate multiple pathologies in TgCRND8 mouse model of AD and their malleability with an intervention strategy in the form of physical activity.

2.2. Hypothesis

I hypothesize that physical exercise stimulates memory and neurogenesis, remodels vasculature and lessens Aβ burden in the hippocampus of TgCRND8 mouse model of amyloidosis.

2.3. Specific Aims

This hypothesis was tested by completing the following specific aims using the TgCRND8 mice entering the studies at 3 months of age.

1. Test the benefits of physical exercise on spatial memory, neurogenesis and hippocampal plaque load after 1 and 2 months of running (Chapter 3).

2. Assess the effects of physical activity on the hippocampal vascular morphology, cerebral amyloid angiopathy, plaque load and spatial memory following 3 months of running (Chapter 4).
Chapter 3

A comparative study evaluating the impact of physical exercise on disease progression in a mouse model of Alzheimer’s disease.

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Journal of Alzheimer’s Disease, Maliszewska-Cyna E, Xhima K, Aubert I. A comparative study evaluating the impact of physical exercise on disease progression in a mouse model of Alzheimer’s disease, Copyright (2016), with permission from IOS Press.
3.1. Abstract

Evidence suggests that physical exercise can serve as a preventive strategy against Alzheimer’s disease (AD). In contrast, much less is known about the impact of exercise when it is introduced after cognitive deficits are established. Using the TgCRND8 mouse model of amyloidosis, we compared the effects of exercise as an intervention strategy aimed at altering disease progression. Voluntary running for 1 month or 2 months was introduced in 3-month old TgCRND8 mice, which exhibit amyloid-beta (Aβ) plaque pathology and cognitive deficits at this age. Specifically, we examined Aβ plaque load, spatial memory, and neurogenesis in the dentate gyrus in the hippocampus. After 1 month of running, TgCRND8 mice spent more time in the novel arm of the Y-maze compared to the familiar arms, indicating improved memory. The levels of doublecortin (a marker of immature neurons) were increased in TgCRND8 mice running for 1 month, but with no significant difference in the number of new mature neurons or plaque burden. As the disease progressed, running prevented further deficits in the Y-maze performance and hippocampal neurogenesis and it reduced plaque load pathology in TgCRND8 mice running for 2 months, compared to non-running transgenics. Therefore, the impact of running on memory, neurogenesis and amyloid pathology was of greater significance when sustained through later stages of the disease.

**Keywords:** Alzheimer’s disease; physical exercise; neurogenesis; spatial memory; amyloid pathology
3.2. Introduction

Alzheimer’s disease (AD) is characterized by the progressive accumulation of amyloid-beta peptides (Aβ), neuronal loss and cognitive impairments (Masters et al., 1985; Kaneko et al., 2009). The hippocampus, a neurogenic brain area involved in learning and memory (Scoville and Milner, 1957), is severely affected in AD (Kaplan and Hinds, 1977; Neves et al., 2008). The accumulation of Aβ in the hippocampus can contribute to impaired neurogenesis, neuronal loss and cognitive deficits (Mattson, 2000; Haughey et al., 2002a, b).

Several AD pathologies are lessened by physical exercise when introduced prior to disease onset in mouse models (Adlard et al., 2005; Yuede et al., 2009; Tapia-Rojas et al., 2015) and in humans (Buchman et al., 2012; Lautenschlager et al., 2012). Recently, Tapia-Rojas et al. (2015) evaluated the benefits of exercise introduced prior to the accumulation of amyloid plaques and detection of cognitive deficits in APPswe/PS1ΔE9 mice. Exercise, used as a preventative measure, reduced amyloid load and astrogliosis, increased neurogenesis and improved spatial memory (Tapia-Rojas et al., 2015).

The benefit of physical activity has also gained recognition as a therapeutic intervention for those who are suffering from mild cognitive impairments (MCI) and are at risk of developing AD (Intzandt et al., 2015). Evidence supports the link between physical exercise and improved cognitive ability in pre-clinical dementia and MCI (Scherder et al., 2005; Lautenschlager et al., 2010; Smith et al., 2010; Baker et al., 2010; Hahn et al., 2011). However, for many clinical applications, exercise can be considered as an intervention i.e. starting after the onset of AD pathology (reviewed in Lautenschlager et al., 2010; Smith et al., 2010). Nichol et al. (2007) showed that three weeks of wheel running improved cognitive performance in aged Tg2576 mice. Other rodent studies where running was introduced in the
presence of plaque pathology and cognitive deficits show a lack of significant improvements to amyloidosis, neurogenesis and spatial memory (Richter et al., 2008; Mirochnic et al., 2009).

To further evaluate the potential of physical exercise as a treatment option, we used the TgCRND8 mouse model of amyloidosis and introduced voluntary running as an intervention strategy in 3-month-old mice, an age where Aβ pathology and spatial memory deficits are present (Chishti et al., 2001; Hyde et al., 2005). Memory, neurogenesis and Aβ deposition were evaluated in two independent cohorts of mice, exposed to two different exercise durations. After 1 month of running, TgCRND8 mice showed signs of memory improvement and increased expression of the immature neuronal marker doublecortin (DCX) in the hippocampus relative to non-running transgenic animals. The benefits of exercise after 2 months of running included a significant augmentation in memory performance, increased hippocampal neurogenesis, and decreased amyloid pathology in running TgCRND8 mice compared to non-running transgenics. Overall, our data support the use of physical exercise as relevant disease-modifying intervention, even at stages of established AD pathology.

3.3. Methods

Animals

We used the TgCRND8 mouse model of amyloidosis maintained on the C57BL/6 and C3H hybrid background and expressing the Swedish (KM670/671NL) and Indiana (V717F) mutations of the amyloid-β precursor protein (AβPP) (Chishti et al., 2001). All procedures were conducted in accordance with guidelines established by the Canadian Council on Animal Care and protocols approved by the Sunnybrook Research Institute Animal Care Committee.
**Exercise paradigm and monitoring**

Three-month-old TgCRND8 mice and non-transgenic littermates (Non-Tg) were randomly assigned to either standard housing (non-running group) or to a cage equipped with a spinning disk (running group; BioServ Fast Trac and Mouse Igloo) and bicycle counter (CatEye, Strada Cadence CC-RD200). Figure 3.1A illustrates the age at which animals enter the study (3-month-old), time at which they were pulsed with 5-bromo-2’-deoxyuridine (BrdU, 50 mg/kg) for the first 10 days of the experiment to study neurogenesis, their running paradigm, behavioural testing schedule and termination ages (4- and 5-month-old). All animals were singly housed and their weight and usage of spinning disks were monitored every other day. Mice were housed in a 12 hrs (07:00-19:00) light-dark cycle in a room maintained at 22°C with ad libidum access to food and water. A total of 17 animals per cohort were used for behavioural tests, daily activity, and weight monitoring. Brains isolated from 7 animals per cohort were used for immunohistochemistry. Brains from the remaining animals were allocated to other studies. Details on the number and sex of animals used in the current study are provided in Figure 3.1B.
Figure 3.1 Experimental paradigm. (A) Timeline of the study. At 3 months of age, mice were randomly assigned to either running or non-running groups. Mice were injected with 50 mg/kg BrdU for the first 10 days of the study. Behavioural tests were performed at 3, 4 and 5 months of age in independent cohorts of animals. 4- and 5-month-old mice ran for 1 and 2 months, respectively. (B) Table illustrating the number of animals, females and males, in each cohort. (C) Average distance ran by TgCRND8 mice (dark blue bar) and non-Tg littermates (pale blue bar). (D) Weight of animals at 3, 4, and 5 months of age. Statistics: (C) Student t-test, (D) 2-way ANOVA. Significance: *p<0.05. Data represent the mean ± SEM. N=17 per group.
Behavioural tests

Two separate cohorts of animals were used for the 1 month and 2 months running paradigms in order to conduct all behaviour tests on naïve mice.

Exploration of a novel environment

The open field test consists of exploration of a novel environment (cage dimensions: 20 x 40 cm) for a period of 10 minutes. Each experiment was video-recorded (Logitech Webcam Pro 9000) and analysed with the Videotrack Go system (Viewpoint Life Sciences, Montreal, Canada). During video analysis, the area of the open field was divided into a centre field (7 x 26 cm) and a peripheral ring (6.5 cm from the border of the open field); distance travelled and time spent in each area were quantified. Four-month old animals from all cohorts were evaluated for exploration of the novel environment.

Spatial working memory

Early cognitive deficits in AD patients relate to episodic memory. However, in mice with AD pathology, impairments in spatial working memory are generally the first to be observed and they are also the best studied and modeled (Webster et al., 2014). The Y-maze, T-maze (Glickman and Jensen, 1961; Dellu et al., 1992) and Morris water maze (Morris et al., 1982), are commonly used for testing reference and spatial working memory in mice. Here, we used the Y-maze task, which has been validated by our group and others in TgCRND8 mice (Hyde et al., 2005; Ma and McLaurin, 2014; Burgess et al., 2014). In comparison to the Morris water maze, the Y-maze is less stressful and requires minimal training, thereby minimizing potential confounds of stress hormones and the learning process on outcome measures (Gould et al., 1999; Harrison et al, 2009; Kennard et al., 2011; Schoenfeld et al., 2012).
Mice are inquisitive in nature (Granon et al., 1996), and those with intact working memory typically spend more time in the novel arm compared to the two familiar arms of the Y-maze (Dellu et al., 2000). Here, the Y-maze paradigm consisted of a 10-minute acquisition session in which naïve animals explored two arms (familiar arms 1 and 2) of the Y-maze with the third arm blocked, followed by a 90 minutes of rest in a home cage. Animals were then returned to the Y-maze for a 5-minute retention session with access to all three arms of the maze, including arm 3, which is referred to as the novel arm. The test was video-recorded (Logitech Webcam Pro 9000) and analysed with the Videotrack Go system (Viewpoint Life Sciences) to measure the time spent in each arm. Time spent in the centre between the three arms was not included in the analysis. Separate cohorts of mice were evaluated with the Y-maze task at 3, 4, and 5 months of age.

**Immunohistochemistry for cell survival and differentiation**

Mice were deeply anesthetized with a cocktail of 150 mg/kg ketamine and 10 mg/kg xylazine administered with an intraperitoneal injection. Animals were perfused transcardially with 0.9% saline followed by 4% paraformaldehyde (PFA). Brains were removed, post-fixed in 4% PFA for 24 hrs and equilibrated in 30% sucrose. They were sectioned coronally at 40 μm using a sliding microtome and stored in cryoprotectant at -20°C.

To quantify the extent of neurogenesis, the number of cells immunopositive for BrdU and mature neuronal nuclei (NeuN) antigen were measured. Astrogenesis was quantified as the number of cells immunopositive for BrdU and glial fibrillary acidic protein (GFAP). Immature neurons were characterized as cells immunopositive for DCX.

For BrdU/NeuN/GFAP immunolabelling, sections were incubated in 2N HCl for 30 min at 37°C for antigen retrieval, and then neutralized in borate buffer (pH 8.5) for 10 min.
After rinsing with PBS and blocking with 1.5% BSA, 2% donkey serum and 0.15% TritonX-100 for 1 hr, sections were incubated with antibodies against GFAP (1:500, AbDSerotec, AHP1468), NeuN biotinylated (1:200, Chemicon, MAB377B) and BrdU (1:400, Serotec, OBT0030) overnight at 4°C. Sections were rinsed and incubated with secondary antibodies appropriate for the primary antibodies used, i.e. donkey-anti-goat IgG Cy5 (1:200, Jackson Immunolabs, 705-175-147) for GFAP, streptavidin Alexa Fluor 488 (1:200, Jackson Immunolabs, 016-540-084) for NeuN and donkey-anti-rat IgG Cy3 (1:200, Jackson Immunolabs, 712-165-153) for BrdU.

For DCX immunolabelling, sections were blocked with 10% donkey serum and 0.25% TritonX-100 for 1 hr and incubated with anti-DCX antibody for 48 hrs at 4°C (1:200, Santa Cruz, sc-8066). Sections were washed and incubated with donkey anti-goat Cy3 secondary antibody (1:200, Jackson Immunolabs, 705-165-147).

**Imaging and quantification**

For each animal, three sections (1 in 12 series) containing the dentate gyrus (DG) were used to quantify BrdU cell population and colocalization of BrdU with markers for neurogenesis and astrogenesis. An adjacent set of 3 sections per animal (1 in 12 series) was used for quantitative analysis of DCX immunoreactivity. For each immunostain, all sections were processed at the same time and imaged using the same background and threshold settings.

Immunofluorescence was detected by confocal microscopy at 63X magnification and visualized using the LSM Image Browser (Zeiss Axiovert 100M, LSM510). Fluorochromes DyLight488, Cy3 and Cy5 were excited at 488, 561 and 633 nm wavelengths, respectively. Z-stack images with 1.6 µm optical section thickness were obtained.
BrdU cells in the DG were assessed by counting all BrdU-positive cells in 6 fields of view captured at 63X magnification per section in 3 sections per animal. For the quantification of colocalization, five BrdU-positive cells were imaged for each DG (right and left) on the 3 sections per animal, for a total of 30 cells per animal. BrdU-positive cell was first identified by first detecting Cy3 (Figure 3.4, column A) and then NeuN or GFAP (Figure 3.4, column B and C) colocalization was determined by combining the sequential visualization of Cy2 and Cy5 channels (Figure 3.4).

DCX signal was imaged at 20X magnification with a Zeiss spinning disk microscope (CSU-W1; Yokogawa Electric, Zeiss Axio Observer.Z1 - Carl Zeiss, Don Mills, Ontario, Canada) coupled to Axiocam camera and operated with Zen 1.1.2 software. The Cy3 fluorochrome was excited at a wavelength of 561 nm. Tiled Z-stack images of the entire dentate gyrus were acquired with 0.5 \( \mu \text{m} \) optical section thickness, and then projected to obtain a maximum intensity image (Figure 3.6). The number of DCX-positive pixels in the dentate gyrus was quantified using ImageJ image analysis software, as previously described (Lee et al., 2006). Briefly, the DCX signal was processed using a 10-pixel-wide rolling-ball subtraction, a two-pixel-wide median filter, and an automatic threshold to determine the number of DCX-positive pixels.

**Aβ immunohistochemistry and stereology**

Immunostaining against Aβ plaques and quantification using design-based stereology were carried out as described previously (Jordão et al., 2010). Briefly, sections were incubated in anti-Aβ 6F/3D primary antibody (1:400, DakoCytomation, M0872) followed by biotinylated donkey-anti-mouse IgG secondary antibody (1:100, Jackson Immunolabs, 715-001-003) and subsequently incubated with streptavidin horseradish peroxidise and 3, 3’-
diamidobenzidine (Vectastain Elite ABC Kit and DAB Peroxidase Substrate Kit, respectively, both from Vector Laboratories). Immunolabelled sections were analysed with StereoInvestigator software (MBF Bioscience, Williston, VT, USA) operating a Zeiss Imager M1 microscope coupled to a digital camera and motorized stage.

For each hemisphere, the hippocampal region was divided into the dentate gyrus (DG) and Cornu Ammonis (CA) (Figure 3.7A and B, respectively). Total plaque numbers and mean cross-sectional plaque areas were estimated using the Optical Fractionator and the Nucleator probes, respectively. The mean surface area of Aβ was calculated by multiplying estimates from the Optical Fractionator and the Nucleator probes. Plaque quantification in the CA was based on 6-7 sections per animal sampled at an interval of 1 in 8 with 300 µm x 300 µm sampling grid and 150 µm x 150 µm counting frame. As a result, 25% of CA surface area was sampled with an average of 147 hits per animal and 0.09 coefficient of error (CE Gundersen). Plaque quantification in the DG was based on 12-13 sections per animal at an interval of 1 in 4 with 300 µm x 300 µm sampling grid and 300 µm x 300 µm counting frame. These parameters covered the entire DG for each section, with an average of 207 hits per animal and 0.07 coefficient of error (CE Gundersen).

Statistics

Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA) was used for statistical analysis and generation of graphs. All graphs are presented as mean ± SEM. Genotype difference in daily use of spinning disks by the mice was analysed with the Student t-test. Gaussian distribution of data sets was confirmed by Shapiro-Wilk normality test. Differences in Aβ plaque load, DCX, and neurogenesis were assessed with a 2-way ANOVA. Differences in time spent in novel and familiar arms were analysed with a 1-way
ANOVA whereas differences in time spent in novel arm were analysed with a 2-way ANOVA. Differences in weight were analysed with a 2-way ANOVA. The Bonferroni correction was used as a post-hoc test. Statistical significance (alpha) was set at 0.05, and defined as *p<0.05, **p<0.01, and ***p<0.001.

3.4. Results

Running and weight monitoring

On average, the daily distance run (Figure 3.1C) by TgCRND8 mice (9.9 ± 1.7 km/day) was not statistically different from their Non-Tg littermates (8.0 ± 1.1 km/day; t(32)=1.4, p=0.2). We recorded a 98% compliance to running with 47/48 mice utilizing the spinning disk daily and running was sustained throughout the experiment. The weight of TgCRND8 non-running mice increased between 3 and 5, and 4 and 5 months of age (Figure 3.1D, F(3,192)=11.0, *p<0.05). No other significant changes were found in weight comparison between groups.

Open field test

No significant differences in the total distance of exploration were found between TgCRND8 and Non-Tg littermates, running and non-running, at 4 months of age (Figure 3.2A). The distances travelled by 4-month-old mice in the centre and peripheral fields were not statistically different between groups (Figure 3.2B and C). This data suggests that mice across all groups do not differ in the levels of locomotion, exploration and anxiety-related behaviour, supporting the use of the Y-maze as a cognitive task based on exploration.
Figure 3.2 Baseline behavioural assessment. (A-C) Open-Field test in 4-month-old TgCRND8 mice and non-Tg littermates, running and non-running. (A) Total distance covered in a novel environment by TgCRND8 and non-Tg mice. (B) Distance covered in the centre and (C) periphery of the novel environment. (D) In the Y-maze, 3-month-old non-Tg mice spent significantly more time in the novel arm (solid bar) compared to familiar arms (pattern bar). In contrast, in age-matched TgCRND8 mice, the time spent in the novel arm was not significantly different than the time spent in the familiar arms. Statistics: 1-way ANOVA. Significance: *p<0.05. Data represent the mean ± SEM. N=17 per group.
Y-maze task

Comparing the time spent in novel and familiar arms indicates whether spatial working memory using the Y-maze test is impaired (failure to recognize the novel arm) or preserved (capacity to recognize the novel arm) within each group of mice. At 3 months of age, TgCRND8 mice have no significant preference for the novel arm compared to the familiar arms (Figure 3.2D, \( t_{(32)} = 1.6, p = 0.13 \)), which is indicative of cognitive deficits. In contrast, Non-Tg littermates differentiate the novel arm from the familiar arms (Figure 3.2D, \( t_{(32)} = 2.3, *p = 0.04 \)).

The impact of running is clear in Figure 3, with running TgCRND8 mice being able to recognize the novel arm, as indicated by greater time spent in the novel arm compared to familiar arms of the maze (Figure 3.3A, 4 months, \( F_{(3,126)} = 4.4, *p = 0.01 \); 5 months, \( F_{(3,126)} = 2.9, *p = 0.03 \)). By contrast, TgCRND8 non-running mice failed to recognize the novel arms of the maze at 4 and 5 months of age (Figure 3.3A, \( F_{(3,126)} = 1.2, p = 0.5 \); \( F_{(3,126)} = 0.3, p = 0.9 \), respectively). As expected, Non-Tg mice spent more time in the novel arm compared to the familiar arms (Figure 3.3A, 4 months running, \( F_{(3,126)} = 2.2, *p = 0.02 \) and non-running, \( F_{(3,126)} = 2.1, *p = 0.01 \); 5 months running, \( F_{(3,126)} = 3.2, *p = 0.04 \) and non-running, \( F_{(3,126)} = 3.6, **p = 0.005 \)).
Figure 3.3. Y-maze performance is improved with running in TgCRND8 mice. Mice were assessed with the Y-maze task for spatial working memory by quantifying time spent in the novel and familiar arms of the maze. (A) Running TgCRND8 mice, at both 4 and 5 months of age, showed a significant preference for the novel arm compared to the familiar arms. In non-running TgCRND8 mice, no statistical difference was observed between the time spent in the novel compared to the familiar arms. Running and non-running 4- and 5-month-old non-Tg animals spent significantly more time in the novel arm compared to the familiar arms. (B) Time spent in the novel arm in 4 and 5-month-old non-running and running mice was evaluated against time spent in novel arm by 3-month-old TgCRND8 mice (dotted line). Non-running TgCRND8 mice showed progressive decline in time spent in novel arm whereas running TgCRND8 mice maintained their performance on the Y-maze task. Statistics: (A) 1-way ANOVA, (B) 2-way ANOVA. Significance: *·p<0.05, **p<0.01. Data represent the mean ± SEM. N=17 per group.
Data from the Y-maze can be used to evaluate whether spatial recognition memory (time spend in novel arm only) is different between groups. As the disease progresses, the performance of non-running TgCNRD8 declines (Figure 3.3B, 5-month compared to 3 month-old TgCRND8 mice, indicated by the dotted line, \( F(1,126)=1.8, ^*p=0.04 \)). Non-Tg littermates at 4 and 5 months of age show no statistical difference in the Y-maze performance compared to 3 month-old non-transgenics (comparing Figure 3.2D and 3B, 4 months, \( F(1,126)=1.1, p=0.2 \), 5 months, \( F(1,126)=0.2, p=0.8 \)). Compared to their non-Tg littermates, non-running TgCRND8 mice exhibit deficits in the Y-maze test, spending less time in the novel arm (Figure 3.3B, 4 months, \( F(1,126)=2.6, ^*p=0.01 \), 5 months, \( F(1,126)=2.9, ^{++}p=0.008 \)). This impairment was abolished by running, as the time spent in the novel arm by running TgCRND8 mice and Non-Tg running littermates was not statistically different (Figure 3.3B, 4 months, \( F(1,126)=1.0, p=0.3 \); 5 months, \( F(1,126)=1.5, p=0.2 \)). Furthermore, 5-month-old running TgCRND8 mice spent more time in the novel arm compared to non-running TgCRND8 mice (Figure 3.3B, \( F(1,126)=1.8, ^*p=0.03 \)).

**Neurogenesis: Cell survival, differentiation and maturation**

Cells dividing in the dentate gyrus at 3 months of age incorporated BrdU and they were quantified at 4 and 5 months of age, as surviving proliferating cells (BrdU alone), and differentiating into neurons (BrdU/NeuN) or astrocytes (BrdU/GFAP) (Figures 3.4 and 3.5).

**BrdU**

The levels of cell proliferation and survival in the dentate gyrus were first evaluated in all groups (Figure 3.5A). The effects of running were significant at 5 months of age in TgCRND8 mice, with the number of BrdU-positive cells being greater compared to age-matched non-running TgCRND8 mice (Figure 3.5A, \( F(3,48)=7.7, ^*p=0.01 \)). This significant
difference between the running and non-running groups was not observed in 4-month-old running TgCRND8 mice, most likely because of the increased number of BrdU-positive cells in 4 month-old TgCRND8 mice compared to age-matched Non-Tg mice (Figure 3.5A, $F_{(3,48)}=2.8, ^{+}p=0.02$). In Non-Tg mice, running for 1 and 2 months increased the number of BrdU-positive cells (Figure 3.5A, 4-month-old, $F_{(3,48)}=4.0, ^{*}p=0.04$; 5-month-old, $F_{(3,48)}=3.3, ^{***}p=0.0003$).
BrdU/NeuN

In TgCRND8 mice, the impact of running on neurogenesis was observed at 5 months of age, after 2 months of running, with greater number BrdU/NeuN-positive cells compared
to age-matched non-running TgCRND8 mice (Figure 3.5B, F(3,48)=13.5, **p=0.003). The deficit in BrdU/NeuN-positive cells found in 5-month-old non-running TgCRND8 mice compared to age-matched Non-Tg mice (Figure 3.5B, F(3,48)=4.8, ++p=0.003) was abolished by running (Figure 3.5B, F(3,48)=0.6, p=0.6, running 5 month-old TgCRND8 mice compared to Non-Tg littermates). Similarly, running also prevented the decline in NeuN/BrdU-positive cells observed with disease progression in TgCRND8 mice (Figure 3.5B, F(3,48)=2.0, ^p=0.02; in contrast to running TgCRND8 mice at 4 and 5 months of age, F(3,48)=1.2, p=0.3).

In 4 and 5-month-old Non-Tg mice, the number of BrdU/NeuN-positive cells was greater in running cohorts compared to non-running age-matched mice (Figure 3.5B, 4-month-old, F(3,48)=5.1, ***p=0.0002; 5-month-old, F(3,48)=5.7, ***p=0.0002).

**BrdU/GFAP**

In contrast to the BrdU/NeuN population in the dentate gyrus (Figure 3.5B), a smaller number of newborn cells express BrdU and GFAP (Figure 3.5C). The population of BrdU/GFAP-positive cells increased from 4 to 5 months of age in Non-Tg mice (Figure 3.5C, F(3,48)=3.2, ^p=0.02), with no statistical increase in TgCRND8 mice (F(3,48)=1.2, p=0.3). Running from 3 to 5 months of age in TgCRND8 mice and their Non-Tg littermates, maintained relatively low numbers of BrdU/GFAP-positive cells compared to non-running mice (Figure 3.5C, TgCRND8, F(3,48)=4.0, **p=0.008; Non-Tg, F(3,48)=4.2, *p=0.04).

**DCX**

Quantitative analysis of DCX immunostaining, labelling immature neurons, revealed an increased number of DCX-positive pixels in running compared to non-running mice at 4 months of age in TgCRND8 mice (Figure 3.6A, C, and E, F(1,24)=2.7, *p=0.01) and Non-Tg mice (Figure 3.6B, D, and E, F(1,24)=3.4, **p=0.009). There was no difference in the number
of DCX-positive pixels between running TgCRND8 and running Non-Tg mice (Figure 3.6A, B and E, $F_{(1,24)}=1.7$, $p=0.2$) and between non-running TgCRND8 and non-running Non-Tg mice (Figure 3.6C, D and E, $F_{(1,24)}=0.4$, $p=0.7$).

Figure 3.6. Immature neurons in the dentate gyrus. (A-D) Representative images of brain sections immunostained for DCX in the dentate gyrus of (A,C) TgCRND8 and (B,D) non-Tg mice. Scale bar: 100 μm. (E) At 4 months of age, the number of DCX-positive pixels was significantly greater in running TgCRND8 and non-Tg mice compared to their respective non-running groups. Statistics: 2-way ANOVA. Significance: *$p<0.05$, and **$p<0.01$. Data represent the mean ± SEM. N=7 per group.

Exercise and Aβ plaque pathology

Aβ plaque pathology in TgCRND8 mice was quantified as Aβ plaque surface area (Figure 3.7C), number (Figure 3.7D), and mean size (Figure 3.7E). Aβ plaque load was examined in two major regions of the hippocampal formation, namely the dentate gyrus (DG) (Figure 3.7A, yellow line) and Cornu Ammonis (CA) (Figure 3.7B, red line).

Running had a significant impact on plaque pathology in TgCRND8 mice exercising from 3 to 5 months of age. Specifically, running reduced the progression of Aβ pathology as measured by the surface area occupied by Aβ plaques (Figure 3.7C; DG, $F_{(1,24)}=3.4$, *$p=0.03$; CA, $F_{(1,24)}=3.6$, **$p=0.004$), number of plaques (Figure 3.7D; DG, $F_{(1,24)}=4.8$, **$p=0.005$; $F_{(1,24)}=3.5$, CA *$p=0.02$) and plaque size (Figure 3.7E; $F_{(1,24)}=2.8$, CA *$p=0.02$) compared to
non-running in 5-month-old mice. Running for 1 month, from the age of 3 to 4 months, had no significant effect on the surface area, number, or size of Aβ plaques in the hippocampus (Figure 3.7C-E).

As the disease progresses, Aβ plaque pathology increased significantly in the hippocampal formation (DG and CA) in both running and non-running mice (Figure 3.7C-E). Specifically, comparing 4- and 5-month-old mice, significant increases were found regarding the surface of Aβ (Figure 3.7C, $F_{(1,24)}=8.5, ^{+++}p<0.001$), number of plaques (Figure 3.7D, $F_{(1,24)}=11.8, ^{+++}p<0.001$), and mean plaque size (Figure 3.7E, $F_{(1,24)}=14.1, ^{p}<0.05$).

Taken together, these data suggest that amyloid pathology accumulates in both running and non-running TgCRND8 mice. However, running considerably slows down the development of plaque pathology in TgCRND8 mice.

3.5. Discussion

To further evaluate the potential of physical exercise as a treatment for AD, we introduced voluntary running as an intervention strategy in 3-month-old TgCRND8 mice, an age where cortical Aβ pathology and spatial memory deficits are present (Chishti et al., 2001; Hyde et al., 2005). In summary, we found that in TgCRND8 mice, running for 1 month (between 3 and 4 months of age) rescued spatial working memory and increased expression of immature neurons in the dentate gyrus. In comparison, 2 months of running (between 3 and 5 months of age) had a greater impact by maintaining improvements in memory, reducing hippocampal Aβ accumulation, and increasing neurogenesis as defined by the population of new mature neurons.

Neurogenesis in mouse models of AD has been reported as being increased or decreased depending on the model, disease stage and methodology (Perry et al., 2012). Several murine studies report increased cell proliferation and number of immature new
Figure 3.7. The effects of running on Aβ plaque load. (A,B) Representative images of Aβ immunohistochemistry on brain sections taken from a 5-month-old non-running TgCRND8. For Aβ quantification, the hippocampal formation was divided into two regions: (A) the dentate gyrus (DG, yellow line) and (B) the Cornu Ammonis (CA, red line). (C-E) During disease progression, Aβ pathology increases from 4 to 5 months of age within the running and non-running groups, respectively. (C) Aβ surface area and (D) the number of plaques in the DG and CA, as well as (E) mean plaque size in the CA, were significantly lower in 5-month-old running compared to non-running TgCRND8 mice. Statistics: 2-way ANOVA. Significance: *p<0.05, **p<0.01 and ***p<0.001. Data represent the mean ± SEM. N=7 per group.
neurons at early stages of Aβ pathology (Jin et al., 2004; Meneghini et al., 2013). Other studies report diminished number of newborn neurons reaching maturation (Mattson, 2000; Haughey et al., 2002a; Wang et al., 2004; Chevallier et al., 2005; Verret et al., 2007; Fiorentini et al., 2010), partly due to impaired survival of newly generated neurons (Verret et al., 2007). Chen and colleagues (2008) reported increased neurogenesis at early stages of neurodegeneration but not at late stages, suggesting that dynamic changes in neurogenesis were correlated with the severity of neuronal loss in DG, and perhaps serve as compensatory mechanism following neurodegeneration.

We compared the number of BrdU-positive cells, the status of immature neurons, and the number of new mature neurons at one and two months after BrdU pulse. Firstly, in non-running mice, a greater population of BrdU-positive cells was found in 4-month-old TgCRND8 compared to Non-Tg mice. This increase in BrdU-positive cells may represent an initial compensatory mechanism to enhance cell proliferation in response to APP/Aβ exposure, as proposed by Chen and colleagues (2008) and previously observed in TgCNRD8 mice at early stages of the disease (Krantic et al., 2012). Our results indicate that this initial increase in proliferation does not promote neurogenesis, as no corresponding increase in DCX or BrdU/NeuN-positive cells is observed, possibly because of the toxic environment composed of Aβ (Haughey et al., 2002a, b; Perry et al., 2012). Running significantly increased the number of BrdU-positive cells in 4-month-old Non-Tg mice, thereby abolishing the difference previously observed with non-running TgCRND8 mice. Secondly, in running mice, the number of DCX-positive pixels significantly increased at 4 months of age (Figure 3.6). Furthermore, the number of BrdU/NeuN positive cells in both TgCRND8
and Non-Tg mice (with exception noted in 4-month-old TgCRND8) significantly increased with running (Figure 3.4B).

Both NeuN (Figure 3.4B) and DCX (Figure 3.6) signals are prominent in 4 and 5-month-old mice, with the population of BrdU/NeuN cells being significantly smaller than the population of NeuN and DCX cells. This is expected as BrdU only incorporates into cells that are proliferating at the time of pulse. The colocalization of BrdU with NeuN identified cells that incorporated BrdU following its injection at 3 months of age (Fig 1A), differentiated into neurons, and survived until 4 and 5 months of age (Figure 3.4A-D). Taken together, this data indicates that physical exercise has a significant impact on the development of immature and mature neurons. Our findings of increased neurogenesis with physical exercise are in line with previous reports using adult and aged wild-type mice that found physical exercise augments the rate of hippocampal neuronal differentiation, maturation and survival (van Praag et al., 1999, 2005; Kronenberg et al., 2003; Van der Borght et al., 2007; Vivar et al., 2013). Similarly, in the APP23 transgenic mice, physical exercise was able to increase the number of newborn granule cells in the dentate gyrus (Mirochnic et al., 2009). Our data demonstrate that the levels of neurogenesis in TgCRND8 running mice were not statistically different than in age-matched running Non-Tg mice. In contrast, we observed a significantly lower number of newly-differentiated mature neurons in non-running TgCRND8 mice at 5 months of age, compared to 4 months of age (Figure 3.5B), suggesting that the neuronal maturation and/or survival process is compromised as the pathology progresses. These data suggests that exercise can support the differentiation of newborn cells into mature neurons despite the presence of Aβ.
Recent reports propose a causative relationship between augmented adult neurogenesis and improved memory (Rodriguez et al., 2008; Chuang et al., 2010). For example, Rodriguez et al. (2008) showed that the new hippocampal neurons are important in generating memory episodes, while cognitive stimuli are known to promote the survival of newborn cells. The relevance of DCX-positive neuroblasts in forming new memories is gaining support, as suggested by Vukovic and colleagues (2013). Indeed, the selective reduction of DCX positive cells in a knock-in mouse model impaired spatial memory acquisition in an active place avoidance test.

In our study, the relationship between memory function, as measured in the Y-maze, and levels of neurogenesis appears to be stronger in TgCRND8 mice than in non-transgenic mice. Indeed, in 4-month-old TgCRND8 mice, 1 month of running improved Y-maze performance and significantly increased DCX immunostaining. An increase in the number of BrdU/NeuN-positive cells in TgCRND8 mice was seen after 2 months of running. Our data in TgCRND8 mice suggests that running first increases DCX levels and then the number of BrdU/NeuN-positive cells, at 4 and 5 months of age, respectively. Both immature (DCX-positive) and mature (BrdU/NeuN-positive) neurons have the potential to contribute to the memory improvements observed in TgCRND8 mice. In contrast, running in non-transgenic mice increased neurogenesis (DCX levels and the number of BrdU/NeuN-positive cells) without having a significant impact on Y-maze-related memory functions, compared to non-running mice. This finding is consistent with previous work in non-transgenic mice investigating the effect of physical exercise on Y-maze performance (Llorens-Martin et al., 2010). It remains to be established whether our exercise paradigm could improve memory on a more challenging spatial test such as the Morris water maze or Barnes maze in non-
transgenic mice. Indeed, previous studies suggest that exercise-induced improvements in learning and memory can be correlated with enhanced hippocampal neurogenesis in non-transgenic mice (reviewed in Vivar et al., 2013). Specifically, Marlatt et al. (2012) showed that 15-month-old mice running for 6 months had significantly increased BrdU, DCX and BrdU/NeuN cell populations compared to sedentary control animals. In addition, running mice performed significantly better on the Morris water maze task than non-running counterparts. In future studies, it would be of interest to establish which measurements of memory best correlate with exercise-induced neurogenesis in transgenic and non-transgenic mice. Furthermore, mechanisms other than neurogenesis are stimulated by physical exercise and can also contribute to improving cognition. Modulating vasculature, reducing neuroinflammation and preventing the loss of cholinergic neurotransmission may all play a role in circumventing AD-like pathologies, and may directly or indirectly support increased neurogenesis and cognition (Matsuoka et al., 2001; Vaucher et al., 2002; Bellucci et al., 2006; Wu et al., 2006; Cotman et al., 2007; Parachikova et al., 2008; Dorr et al., 2012).

As the disease progressed in TgCRND8 mice, running prevented the decrease in performance observed in the Y-maze (Figure 3.5B, ^ age effect, comparing the time spent in the novel arm at 3 and 5 months of age). Our findings are in contrast with results reported by Richter et al. (2008) and may be due to several differences in experimental design. Firstly, we used the Y-maze while Richter and colleagues used the Barnes maze to evaluate spatial memory performance. Secondly, the running equipment used for the mice was different. We used low-resistance spinning disks, which resulted in increased compliance with only 1 mouse not using the spinning disk out of 48 mice in our study compared to 13 non-exercisers out of 54 mice in Richter and colleagues’ study (2008). The low-resistance spinning disks
that we used also allowed mice to run longer distances compared to standard metal wheels (9 km/day versus 1.4 km/day) (Creer et al., 2010). These experimental differences may be of significance in the search of sufficient levels of physical activity translating into improved cognitive performance.

The effects of exercise on Aβ burden have been previously evaluated in AD mouse models. In Tg2576 and TgCRND8 mice, exercise lowered Aβ plaque pathology when it was introduced in a pre-plaque stage of disease progression (Adlard et al., 2005; Yuede et al., 2009; Tapia-Rojas et al., 2015) but not when animals began to exercise following Aβ plaque deposition (80-day old TgCRND8 for a period of 10 weeks) (Richter et al., 2008). By contrast, our 2 month-long exercise paradigm reduced Aβ load when initiated in 3-month-old TgCRND8 mice as intervention measure in the post-plaque stage of disease progression. Contrastingly, no changes were detected after 1 month of running. In both running and non-running mice TgCRND8 mice, the plaque number, size and surface area increased as animals aged from 4 to 5 months, with a reduced degree of increase observed with running. This suggests that physical exercise is capable of significantly attenuating hippocampal Aβ plaque burden and it can potentially be of significance in promoting neurogenesis through different mechanism in TgCRND8 mice, including decreased amyloidosis.

Physical exercise appears to be an effective multimodal intervention strategy against AD, as we were able to show in a mouse model of amyloidosis that running is able to promote an environment with reduced Aβ, enriched neuronal maturation, and improved cognitive performance. Physical voluntary exercise could serve as a lifestyle-modifying intervention to counteract AD pathologies and potentially improve the quality of life of AD patients.
Chapter 4

The impact of physical exercise on hippocampal vasculature and memory in a mouse model of Alzheimer’s disease.

In the previous chapter I demonstrated that running, introduced at 3 months of age in TgCRND8 mice, improved spatial memory in 4- and 5-month-old mice (running for 1 and 2 months, respectively). Other Aβ-related pathologies where differently affected by running, depending on the age at which the exercised paradigm ended. More precisely, in 4-month-old mice, the number of new mature neurons and plaque burden were not affected by running. It was only in 5-month-old mice that the hippocampal plaque burden was reduced and neuronal maturation increased, compared to sedentary TgCRND8 mice. In light of the finding that neither plaque reduction nor increased neuronal maturation were necessary to improve spatial memory following 1 month of running, I set out to examine what other pathology best correlates with spatial memory. The next section of this thesis evaluates the extent to which physical exercise can modulate hippocampal vascular morphology and CAA burden, which becomes reliably detectable at 6 months of age. I also asked the question whether a 3-month exercise regimen (from 3 to 6 months of age) can maintain the beneficial effects observed at 5 months of age on plaque pathology and cognition.

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4.1. Abstract

The cerebral vasculature is an important therapeutic target for Alzheimer’s disease (AD). Physical exercise has the potential to elicit beneficial effects on the cerebrovasculature and lessen cognitive decline associated with AD. Here, we examined the effects of voluntary running on hippocampal vascular morphology, cerebral amyloid angiopathy (CAA), plaque load, and spatial memory in TgCRND8 mice and their non-transgenic littermates. Firstly, we assessed the impact of amyloidosis on the hippocampal vasculature of non-running (sedentary) mice and found that TgCRND8 mice have higher capillary density (71%), capillary length (69%), branch density (29%), and tortuosity of larger vessels (6%) compared to non-transgenic littermates. Then, we established that three months of running rendered the vasculature morphology in TgCRND8 mice comparable to that of non-transgenic mice. Running also lowered hippocampal CAA burden (50%) without reducing parenchymal plaque load. Finally, Y-maze memory performance was significantly better in running compared to sedentary TgCRND8 mice. No statistical distinction was observed between running TgCRND8 mice and non-transgenic littermates. These data suggest that running could be an effective strategy aimed at maintaining the cerebrovasculature and promoting cognitive ability in cases of AD.

Keywords: Alzheimer’s disease, physical exercise, hippocampal vasculature, capillaries, cerebral amyloid angiopathy, spatial memory.
4.2. Introduction

Cerebrovascular integrity is fundamental to neuronal activity (Iadecola, 2010) and the disruption of vessel structure and function plays an important role in mild cognitive impairment (MCI) and Alzheimer’s disease (AD) (Snowdon et al., 1997; Lim et al., 1999; Vermeer et al., 2003). Changes to human cerebral vascular morphology include increased tortuosity, thickening of arteriolar walls, microaneurysms, and modified vessel wall composition (Nagasawa et al., 1979; Fischer et al., 1990; Thore et al., 2007). In mice, amyloidosis affects capillary density and geometry resulting in thinner and irregularly shaped capillaries (Paris et al., 2004a; Lee et al., 2005; Gama Sosa et al., 2010). These morphological alterations are associated with decreases in cerebral blood flow (CBF), blood-brain barrier dysfunction, and cerebral amyloid angiopathy (CAA) in AD patients (Prohovnik et al., 1988; Hock et al., 1997; Jagust et al., 1998; Mentis, et al., 1998; Bailey et al., 2004; Hirao et al., 2005; Bateman et al., 2006; Montagne et al., 2015). To date, cerebrovascular dysfunction in AD has been investigated mainly in cortical areas (Prohovnik et al., 1988; Hock et al., 1997; Jagust et al., 1998; Mentis, et al., 1998; Bailey et al., 2004; Hirao et al., 2005), and in the hippocampus of MCI patients (Fischer et al., 1990; Hirao et al., 2005; Montagne et al., 2015). The relevance of the hippocampus in AD (Fjell et al., 2014) and the limited knowledge of morphological modifications to its vasculature during disease progression make it an important yet understudied research area.

Physical exercise is known to change the biochemistry, morphology and function of the brain, including its vasculature (Schmidt et al., 2013). Modifications to the cerebrovascular morphology mediated by physical exercise include reduced tortuosity in healthy adults (Bullitt et al., 2009). In animal studies, exercise lowered arterial stiffening and improved endothelial function (Durrant et al., 2009; Fleenor et al., 2010), and it increased
CBF and blood volume in cortical regions (Swain et al., 2003; Rhyu et al., 2010). In mouse models of AD, physical exercise reduces cortical and hippocampal amyloid-beta peptides (Aβ) plaque pathology and promotes cognition (Adlard et al., 2005; Nichol et al., 2007; Yuede et al., 2009; Tapia-Rojas et al., 2015; Maliszewska-Cyna et al., 2016). However, the effects of physical activity on the vasculature in the context of AD pathology is less known.

The present work investigated the effects of physical exercise on hippocampal vascular morphology, Aβ pathology, and spatial working memory in the TgCRND8 mouse model of amyloidosis. We found that, sedentary TgCRND8 mice have higher capillary density, length, branching, tortuosity, CAA burden, and impaired spatial working memory compared to non-transgenic littermates. Three months of voluntary running in TgCRND8 mice resulted in vessel morphology comparable to that of non-transgenic animals. In addition, TgCRND8 runners had lower CAA burden than sedentary littermates. Lastly, running TgCRND8 mice exhibited no spatial memory deficits compared to non-transgenic littermates.

Cumulatively, this work suggests that physical exercise may be an effective therapeutic strategy for maintaining a healthy hippocampal vasculature leading to preserved cognitive function.

4.3. Methods

Animals

We used the TgCRND8 mouse model of amyloidosis maintained on a C57BL/6 and C3H hybrid background and expressing the Swedish (KM670/671NL) and Indiana (V717F) mutations of the amyloid precursor protein gene (Chishti et al., 2001). All procedures were conducted in accordance with the guidelines established by the Canadian Council on Animal Care and protocols approved by the Sunnybrook Research Institute Animal Care Committee.
**Exercise paradigm and monitoring**

A total of 36 mice, sex- and age-balanced, were used in this experiment. Three-month old TgCRND8 mice and their non-transgenic (Non-Tg) littermates were randomly assigned to either standard housing (non-running, sedentary, group) or to a cage equipped with a spinning disk (running group; BioServ Fast Trac and Mouse Igloo) and bicycle counter (CatEye, Strada Cadence CC-RD200). All animals were singly housed and their weight, food and water intake were monitored every other day. Running animals had free access to a spinning disk for a period of three months (Figure 4.1A) and wheel activity was monitored every other day. TgCRND8 and Non-Tg mice were compliant to the running regimen, with no significant difference in the average daily distance run between groups (Figure 4.1B, p=0.6). Wheel activity was sustained throughout the experiment.

**Y-maze task**

Y-maze, a type of memory test frequently employed to assess spatial working memory in rodents (Glickman and Jensen, 1961; Dellu, et al., 1992), relies on rodents’ natural preference for novelty exploration (Granon et al., 1996). Spatial memory is evaluated by the time mice spend in the novel arm relative to the familiar arms (Dellu et al., 2000). Longer exploration of the novel arm compared to that of the two familiar arms is thus an indication of functioning spatial memory.

At 6 months of age, animals were assessed for spatial memory performance using the Y-maze task. Male and female mice were tested on the Y-maze task and there was no sexual dimorphism detected in the non-Tg and TgCRND8, sedentary and running mice (Appendix A). The paradigm consisted of a 10-minute acquisition session in which animals explored two arms of the Y-maze, followed by a 90-minute inter-trial-interval (Figure 4.1C).
Figure 4.1. Experimental paradigm and methodology. (A) Experimental timeline illustrating age at which animals entered the study (3-month-old), running duration (3 months), Y-maze testing and methoxy-XO4 injection (24 hrs before termination) and Mercox-BABB perfusion (6-month-old). (B) Daily distance ran was not significantly different between Non-Tg mice (light blue) and TgCRND8 mice (dark blue). Data represent the mean ± SEM. N=9 per group. (C) Y-maze task consisted of a 10-minute habituation phase in two arms, 90-minute inter-trial-interval, and 5-minute testing phase in 3 arms of the maze. (D) Post-fixed brains were cleared to allow for whole-brain imaging. (E) 5-6 non-overlapping images were obtained from the hippocampal region. (F) Hippocampal vascular network illustrated in a composite of 11 scans. PCA - Posterior Cerebral Artery; LHV - Longitudinal Hippocampal Vessel; THA - Transverse Hippocampal Arteries; Arrow – capillary. Scale bar: 100 μm.
Animals were then returned to the Y-maze for a 5-minute retention session with access to all arms of the maze, including the novel arm. The test was video-recorded (Logitech Webcam Pro 9000) and analysed with the Videotrack Go system (Viewpoint Life Sciences). The time spent in each arm was measured to assess whether animals exhibited a preference towards the novel arm.

**Methoxy-XO4 injection**

1,4-bis(49-hydroxystyryl)-2-methoxybenzene (or methoxy-X04), a Congo red derivative, was used as an Aβ-binding probe (Klunk et al., 2002). TgCRND8 mice were intraperitoneal injected 24 hrs before sacrifice with 2 mg/kg of methoxy-XO4 in 0.1 ml of phosphate-buffered saline (PBS) solution containing 5% Cremophor.

**Merox-BABB perfusion and brain clearing**

Merox perfusion and benzyl alcohol benzyl benzoate (BABB, 1:2 ratio) optical clearing methodologies were adapted from earlier work (Meyer et al., 2008; Parra et al., 2010). Briefly, animals were deeply anesthetized with a cocktail of 150 mg/kg ketamine, 10 mg/kg xylazine and 25 mg/kg acepromazine administered with an intraperitoneal injection. Following anesthesia, animals were trancardially perfused with PBS mixed with 5 μl/ml heparin, followed by 4% paraformaldehyde (PFA), at a rate of 1 ml/min. This was followed by perfusion with Merox-BABB-Nile Red solution (10 ml Merox 2 fixative, 3 g Merox catalyst, 10 ml BABB, 1g Nile Red fluorescent dye), prepared fresh, and filtered twice with a 0.8 μm syringe filter; perfusion rate was maintained at 2 ml/min. The brain was then dissected and post-fixed in 4% PFA overnight at 4°C. Brains were dehydrated with methanol at increasing concentrations and cleared with BABB for 72 hrs at room temperature. This
method resulted in optical clearing of the brain (Figure 4.1D). The brains were stored at 4°C in BABB solution and imaged within 5 days post clearing.

**Two-photon fluorescence microscopy**

Two-photon fluorescence microscopy at 780 nm excitation was used to image 5 to 6 non-overlapping regions in the hippocampus (Figure 4.1E, F). Fluorescence emission from methoxy-XO4 and Nile Red was collected in the ranges of 435–485 nm and 515–560 nm, respectively (Greenspan et al., 1985; Koenigsknecht-Talboo et al., 2008). Imaging was performed using FV1000MPE microscope (Olympus) equipped with a water-immersion 25X objective (Olympus; WD=1.98 mm, NA=1.05) with the 509 by 509 μm² field of view, the matrix size was set to 512 x 512, and dwell time to 4 μs/pixel. Images of varying depth (900 - 1600 μm) were acquired in the coronal plane, starting from -0.94mm from Bregma, every 2.5 μm. The point spread function (PSF) was estimated at 0.34 by 0.34 microns in plane and 1.25 microns through plane.

**Image processing**

Microscopy data were deconvolved in the AutoQuantX2 software. Blind deconvolution algorithm with total iterations of 20 was used to deconvolve all images. A hippocampal region of interest (ROI) was then manually identified using Imaris 7.7.1 software and areas outside of ROI were excluded from further analysis (Figure 4.1E, grey mask).

**Automated vessel tracking**

Identification of the 3D hippocampal vascular network allows a variety of morphological parameters to be quantified. Each two-photon fluorescence microscopy dataset underwent a seeded multi-scale segmentation based on the procedure adapted from
(Rennie et al., 2011) to automatically identify vessel-like structures in the image. Briefly, the 3D datasets were blurred with a Gaussian smoothing filter of full width half maximum (FWHM) of 3 μm and isotropically resampled. Multiple seeds were then automatically placed in the centre of tubular structures. The segmentation process then involved searching the centre lines of branching segments in 3D grayscale dataset. Produced vascular tree is composed of all segments as well as their connectivity, which can then be used to assess morphological features of each segment. Vessels were defined as segments connecting to branch points.

The vessels were categorized as capillaries (Figure 4.1F, arrow) and non-capillaries, with abluminal diameter between 5-8 μm and 8-30 μm, respectively (Mishra et al., 2014). Capillary density and length, together with non-capillary measurements of branch density and tortuosity were used to characterize the morphology of the hippocampal vascular network. Vessel density was defined as the number of branch-to-branch vessels divided by the volume of ROI. Tortuosity was defined as a ratio of the vessel length and the 3D Euclidean distance between the vessel’s endpoints (Figure 4.2A, B) (Bullitt et al., 2003). A perfectly straight vessel has tortuosity of 1, whereas larger values indicate more tortuous vessels.

**Amyloid load estimation**

Methoxy-XO4-positive Aβ signal was visualized and manually segmented using Imaris 7.7.1 software (Bitplane). To ensure the separation of vascular and parenchymal amyloid, two surfaces were created for plaques and CAA (Figure 4.4H; CAA in blue, plaque in green) and CAA surfaces were deleted from plaque quantification. The filament tool was used to manually segment CAA-covered vessels identified by colocalizing Mercox-positive
vessels with methoxy-XO4-positive CAA signal. Identified CAA-burdened blood vessels were manually traced with the aid of the automatic depth recognition function. The surface tool was used to enumerate the volume of manually-segmented Aβ. CAA coverage was quantified as mean volume of CAA normalized to the tissue volume. Plaque burden was estimated based on plaque total surface area, mean plaque size and total plaque number. Plaque surface area was quantified as sum of surface area of all plaques and normalized to tissue volume.

**Data analysis and statistics**

SPSS software (Version 16.0. Chicago, SPSS Inc.) was used for statistical analysis and Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA) was used for generating bar plots. All data are presented as mean ± SEM. Means were taken across approximately 2,300 vessels per animal, with 9 animals per group. Student’s *t*-test and ANOVA were used to determine statistical significance between two and more groups, respectively. Statistical differences were set at *p*<0.05, **p**<0.01, and ***p***<0.001 after correction for multiple comparisons with Bonferroni post-hoc analysis.

**4.4. Results**

**Vascular morphology**

Overall, capillary density and length, branching density, and tortuosity were higher in sedentary TgCRND8 mice compared to Non-Tg littermates. Running led to vascular morphology in TgCRND8 mice which was comparable to that observed in Non-Tg mice (Figure 4.3).
Figure 4.2. Hippocampal vasculature. (A, B) Representative 2-photon microscopy images of hippocampal vasculature in 6-month-old mice. (A) Vessels of relatively low tortuosity in Non-Tg mice. (B) Vessels of relatively high tortuosity in TgCRND8 mice. Tortuosity was defined as a ratio of the vessel length (red line) and the 3D Euclidean distance between the vessel’s endpoints (blue line). (C-F) Representative 2-photon fluorescence microscopy images of hippocampal vasculature filled with Nile Red in Mercox-BABB suspension in the four groups of mice used in this study. Scale bar: A = 30 μm; F = 70 μm.
Figure 4.3. Quantification of hippocampal vascular morphology. (A-D) Non-Running TgCRND8 mice (dark orange) compared to Non-Tg littermates (light orange) had increased (A) capillary density, (B) capillary length, (C) branch density, and (D) non-capillary tortuosity. Significance of genotype effects are noted as *p<0.05, **p<0.01, and ***p<0.001. In response to running, vascular measurements in TgCRND8 mice (dark blue) became indistinguishable from sedentary Non-Tg littermates (light orange). Within the TgCRND8 population, running mice (dark blue) had significantly lower (A) capillary density and (C) branch density compared to non-running transgenics (dark orange). Significance of running effects: **p<0.05. Data represent the mean ± SEM. N=9 per group.
In sedentary animals, our data suggest elevated angiogenesis in TgCRND8 compared to Non-Tg mice (Figure 4.2C, E; Figure 4.3A-C). This is supported by a $71 \pm 10\%$ higher capillary density ($^{**}p=0.002$), $69 \pm 8\%$ higher capillary length ($^{***}p=0.001$) and $29 \pm 6\%$ higher branch density ($^*p=0.02$) in TgCRND8 compared to Non-Tg mice (Figure 4.3A, C). In addition, the tortuosity of non-capillary vessels (8-30 µm diameter) was more pronounced in sedentary TgCRND8 mice compared to Non-Tg littermates (Figure 4.3D; $^{**}p=0.002$).

In TgCRND8 mice, running maintained vessel morphology at comparable levels to that of Non-Tg littermates (Figure 4.2C, D, F). Firstly, capillary density in running TgCRND8 mice was indistinguishable from sedentary ($p=0.7$) and running Non-Tg mice ($p=0.7$). Furthermore, capillary density in running TgCRND8 mice was $57 \pm 15\%$ lower than in sedentary transgenics (Figure 4.3A; $^\#p=0.03$). Capillary length in running transgenics was not significantly different from sedentary ($p=0.4$) and running Non-Tg (Figure 4.3B; $p=0.2$). Additionally, branch density in running TgCRND8 mice was indistinguishable from sedentary ($p=0.5$) and running Non-Tg mice ($p=0.5$), and $35 \pm 9\%$ lower than in non-running TgCRND8 mice (Figure 4.3C; $^\#p=0.03$). Lastly, running did not affect tortuosity in TgCRND8 mice compared to sedentary ($p=0.1$) and running non-transgenics controls (Figure 4.3D; $p=0.3$). Power analysis indicated that 13 more animals per group are required for tortuosity to reach statistically significant difference between running TgCRND8 and sedentary Non-Tg mice (alpha 0.05, beta 0.8).

Running had no detectable effect in Non-Tg mice, on any of the measured morphological features of hippocampal vasculature. Specifically, there was no significant difference between sedentary and running Non-Tg mice in capillary density ($p=0.3$), capillary length ($p=0.6$), branch density ($p=0.2$) and tortuosity ($p=0.5$) (Figure 4.3A-D).
Hippocampal cerebral amyloid angiopathy

We quantified the levels of CAA (Figure 4.4A, D, arrow) and Aβ plaque load (Figure 4.4H, I, green). Running TgCRND8 mice had 50 ± 18% lower CAA coverage compared to sedentary TgCRND8 mice (Figure 4.4J; **p=0.009). The plaque load was not significantly different between running and non-running transgenics (Figure 4.4 K; total surface area: p=0.3; not illustrated: mean plaque size: 7128 ± 1004 for sedentary and 6603 ± 691 for running, p=0.7; total plaque number: 3162 ± 889 for sedentary and 3200 ± 849 for running, p=0.9).

Spatial memory

Three months of voluntary running maintained memory performance in TgCRND8 mice compared to sedentary transgenics. Specifically, running TgCRND8 mice spent 36 ± 11% more time in the novel arm compared to the time spent in the familiar arms (Figure 4.5; **p=0.009). Time spent in the novel arm by running transgenics mice was indistinguishable from sedentary (p=0.8) and running non-transgenics mice (p=0.3) suggesting preservation of spatial memory. By contrast, non-running TgCRND8 mice failed to recognize the novel arm from two familiar arms (Figure 4.5; p=0.6) indicating compromised spatial memory. Additionally, sedentary TgCRND8 mice spent significantly less time in the novel arm compared to the other three groups of mice (Figure 4.5; ##p<0.01).

In the non-transgenic mice, both running and non-running mice spent significantly more time in the novel arm than in familiar arms (Figure 4.5; non-running Non-Tg: *p=0.02; running Non-Tg: **p=0.008).
Figure 4.4. CAA is decreased in running TgCRND8 mice. (A-F) Representative 2-photon fluorescence microscopy images of (A, D) Mercox-BABB-filled hippocampal blood vessels, and (B, E) Methoxy-XO4-labelled Aβ burden in 6-month-old TgCRND8 mice. (C, F) Merged images illustrate CAA-burdened (arrow) and unburdened vessels (arrowhead). (G) CAA-burdened vessels (red) were manually reconstructed with Imaris 7.7.1 software. (H) CAA (blue) and Aβ parenchymal plaques (green) were segmented separately. (I) Merged image illustrating CAA-burdened vessels (red), CAA (blue), and Aβ plaques (green). Scale bar: C = 70 μm; F, I = 30 μm. (J) CAA coverage was significantly lower in running compared to non-running TgCRND8 mice. (K) Hippocampal plaque surface area was not affected by running. Significance: "p<0.01. Data represent the mean ± SEM. N=9 per group.
4.5. Discussion

The present study investigated the effects of physical exercise on hippocampal vascular morphology, Aβ burden, and spatial memory in TgCRND8 mice. Vasculature in sedentary TgCRND8 mice was characterized by higher capillary density and length, vessel branching and non-capillary tortuosity compared to non-transgenic littermates. Three months of voluntary running resulted in preserved vessel morphology (i.e. keeping vascular parameters measured in TgCRND8 mice not significantly different from Non-Tg littermates).

Furthermore, CAA burden, but not plaque load, was significantly lower in running TgCRND8 mice compared to sedentary transgenics. Finally, TgCRND8 running mice performed significantly better than non-running transgenics in the Y-maze spatial task.
Increasing evidence suggests that cerebrovascular dysfunction plays an important role in the progression of AD (reviewed by Gorelick et al., 2011). Indeed, many AD patients have vascular pathology (Schneider et al., 2007; Toledo et al., 2013), particularly those above the age of 60 (Jellinger and Attems, 2010). Abnormalities of the cerebrovasculature may lead to increased vascular resistance and decreased tissue perfusion (Bullitt et al., 2003). Meier-Ruge et al. (1980) showed increased capillary density in the hippocampus of AD patients, which could be attributed to the shrinkage of hippocampus (Meier-Ruge et al., 1980) or to the angiogenic function of Aβ (Boscolo et al., 2007). The findings of higher capillary density were later challenged by human and animal studies (Bailey et al., 2004), thus it remains unknown whether capillary density is increased or decreased in the AD brain. In rodents, studies have shown a 20-30% decrease in hippocampal and cortical vascular density in Tg2576 and APPswe/PS1ΔE9 models (Paris et al., 2004a; Lee et al., 2005), as well as thinning and irregular geometry of hippocampal capillaries associated with hippocampal atrophy (Gama Sosa et al., 2010). In our study, 6-month-old sedentary TgCRND8 mice, at a relatively early stage of CAA deposition (Dorr et al., 2012), exhibited higher capillary density, length, branching and tortuosity compared to Non-Tg littermates. Without a detectable shrinkage of hippocampal volume in TgCRND8 mice at the age that we studied (Chishti et al., 2001; Kobayashi and Chen; 2005), the above results suggest the stimulation of angiogenesis in 6-month-old non-running TgCRND8 mice (Burri and Tarek, 1990; Risau, 1997; Gambino et al., 2002). Future immunohistochemical studies of angiogenic markers could confirm the status of angiogenesis in the hippocampus of 6-month-old TgCRND8 mice.
The effects of Aβ on cerebral vasculature have been described as pro- or anti-angiogenic, most likely depending on the severity of Aβ pathology and brain region examined. Our experiments were conducted in animals with relatively early vascular and CAA pathology, particularly in the hippocampus (Dorr et al., 2012; Hawkes et al., 2014). Indeed, a decreased vascular density in the somatosensory cortex, a brain region with more advanced parenchymal and vascular Aβ pathology than hippocampus, was found in these mice (Thomason et al., 2015). Hippocampal and cortical data suggest that early pro-angiogenic effect is transient in nature and resolves with accumulating CAA (Paris et al., 2004a, b). Decreased metabolic needs of progressively impaired neuronal networks could also contribute to reduced angiogenesis in later stages of pathology (Zlokovic, 2011). In our study, three months of running (from 3 to 6 months of age) resulted in comparable capillary density and length, as well as vessel branch density, between running TgCRND8 mice and non-transgenic controls. These results suggest that physical exercise maintains vascular characteristics in TgCRND8 mice at levels comparable to the ones observed in non-transgenic animals. We hypothesized that exercise-induced maintenance of cerebrovasculature is at least partially due to lower CAA burden (50%) observed in running TgCRND8 mice, as healthy larger vessels contribute to the fully functioning vascular network capable of efficiently removing Aβ from the brain. Thus, physical exercise appears to counteract the accumulation of Aβ on the vessel walls. It was previously reported that TgCRND8 mice housed in enriched cages containing running wheels had reduced whole brain CAA (Ambrée et al., 2006; Herring et al., 2011). Additionally, Lazarov et al. (2005) showed that APPswe/PS1ΔE9 mice exposed to an enriched environment that also included running wheels exhibited significant reduction in Aβ load in the cortex and hippocampus.
compared to mice housed in standard cages. Furthermore, Lazarov et al. (2005) recorded the most significant reduction in Aβ burden in mice housed enriched-environment with high activity levels suggesting that exercise plays a significant role in modulating Aβ deposition. Moreover, Dorr and colleagues (2012) have previously shown that Aβ clearance using scyllo-inositol in TgCRND8 mice lowers vessel tortuosity and improves brain vascular function in TgCRND8 mice. The current study suggests that physical exercise leads to lower CAA burden, which may contribute to reducing the angiogenic response in TgCRND8 mice (Kalaria et al., 1998; Biron et al., 2011). Higher CAA in sedentary TgCRND8 mice may also contribute to increased hypoxic conditions and to the activation of pro-angiogenic response (Kalaria et al., 1998). Supporting this possibility, whole-brain hypervascularisation was reported in the Tg2576 mouse model of amyloidosis (Biron et al., 2011).

Maintaining proper vascular network and minimizing CAA coverage may contribute to preserved cognitive function in AD and MCI (Iadecola, 2010). Indeed, it has been shown that the presence of vascular deficits is a powerful predictor of progression to AD and results in a significant deterioration of cognitive function in patients (Snowdon et al., 1997; Lim et al., 1999; Vermeer et al., 2003; Weller et al., 2009). It is well recognized that physical activity stimulates cognitive function in healthy adult and aging population and in AD patients (Kramer and Erickson, 2007). In mouse models of AD, physical activity is known to stimulate memory (Adlard et al., 2005; Nichol et al., 2007; Yuede et al., 2009; Tapia-Rojas et al., 2015; Maliszewska-Cyna et al., 2016). Running, initiated at 3 months of age in TgCRND8 mice, significantly improved Y-maze performance at 5 months of age (Maliszewska-Cyna et al., 2016) and at 6 months (current study). Of note, running reduced hippocampal plaque pathology in 5 month-old (Maliszewska-Cyna et al., 2016) but not 6-
month-old TgCRND8 mice, whereas CAA burden was diminished at 6 months of age (current study). These results indicate that physical exercise is efficient at lessening hippocampal plaque pathology at relatively early stages of disease progression (Adlard et al., 2005; Yuede et al., 2009; Tapia-Rojas et al., 2015; Maliszewska-Cyna et al., 2016). It is possible that as pathology progresses, maintaining a healthy vascular network and lower CAA burden with physical exercise has functional consequences on spatial working memory. Strengthening the link between vascular morphology and spatial memory is detected correlation between lower capillary density and length and time spent in the novel arms (density: R^2=-0.51, *p=0.03; length: R^2=-0.55, *p=0.02). It remains to be determined whether the impact of running on cognition and CAA persists at later stages of disease progression. No correlation was found between wheel activity and vascular parameters or Y-maze performance.

Running in non-transgenic mice produced no detectable change to hippocampal vascular measurements made here. One possible explanation is that, instead of recruitment of new capillaries, running in healthy mice induces temporary changes CBF which would not have been detected with an *ex vivo* morphology study conducted here (Kuschinsky and Paulson, 1992; Hudetz et al., 1996; Bouras et al., 2006). Changes in the flow distribution are usually regulated in the capillary network through transient vessel caliber adjustments (Kuschinsky and Paulson, 1992; Hudetz et al., 1996), thus it is possible that running non-transgenic animals exhibit temporary fluctuations in CBF without modulating the structure of vascular network.

We examined the 3D vascular network morphology and CAA deposition in the intact hippocampus. This approach allowed for the assessment of a large number of vessels.
Indeed, employing semi-automated vessel tracking resulted in the evaluation of the morphology of approximately 2,300 vessels (Figure 4.2C-F). In addition, network analysis allows for the measurements of parameters, such as capillary length, density, and vessels branching and tortuosity. These parameters can be difficult, and at time impossible, to accurately measure using brain sectioning, immunostaining and imaging, often based on 2D approaches (Lazarov et al., 2005; Ambrée et al., 2006; Herring et al., 2011).

In summary, this study provides a detailed assessment of the hippocampal vascular network and its response to exercise and progressive amyloidosis. We showed that hippocampal capillary density and length, vascular density and non-capillary tortuosity are higher in TgCRND8 mice compared to non-transgenic littermates. Three months of physical exercise resulted in maintained vessel morphology, lower CAA, and preserved spatial memory. Normalized vascular network morphology and lower CAA in response to physical exercise may be associated with the restoration of physiological levels of CBF, which in turn, could contribute to improved spatial memory in running TgCRND8 mice. It remains to be established whether exercise induces similar vascular changes in other brain regions.

Our data suggests that physical exercise is an attractive lifestyle strategy capable of lessening CAA burden, maintaining vessel morphology and spatial memory. In light of limited therapeutic options to combat AD, promoting vascular health through exercise seems an excellent way of preventing or delaying the onset of vascular and cognitive impairments.
Chapter 5

General discussion

5.1. Summary of research findings

In this thesis I tested the hypothesis that physical exercise stimulates memory and neurogenesis, remolds vasculature and lessens Aβ burden in the hippocampus of TgCRND8 mouse model of amyloidosis. The experimental paradigm and main findings are summarized in Figure 5.1 and Table 5.1, respectively.

In Chapter 3, I demonstrated that spatial memory and the number of immature neurons were higher after one month of running in TgCRND8 mice, while hippocampal plaque burden was reduced, and neuronal maturation as well as spatial memory were increased after two months of running. These data suggest that the greatest benefit of physical exercise is observed on spatial memory, and that memory can be promoted independently of plaque reduction or increased neurogenesis. In Chapter 4, I first characterized the vasculature in sedentary TgCRND8 mice and found higher capillary density, length and branching than in Non-Tg mice. This could suggest increased angiogenesis in sedentary TgCRND8 mice, possibly as an adaptive response to Aβ. I then showed that 3 months of running maintained capillary density and vessel branching at levels comparable to Non-Tg animals and it lessened CAA coverage on the vessel walls. Further, exercise-mediated stabilization of vessel morphology and lower CAA could contribute to improved spatial memory in running TgCRND8 mice, as capillary density and length correlated with improved spatial memory. This effect is most likely mediated by the restoration of physiological levels of CBF and/or BBB permeability.
Figure 5.1 Experimental timeline. In all experiments the animals entered the study at age of 3 months and after a period of 1, 2, or 3 months of running behavioural testing was conducted. Following behaviour, animals were perfused and brain tissue was collected for analysis.
### Table 5.1 Summary of findings.

<table>
<thead>
<tr>
<th>Running duration</th>
<th>Mouse age</th>
<th>Pathology</th>
<th>Marker / Test</th>
<th>Non-Tg R</th>
<th>TgCRND8 NR</th>
<th>TgCRND8 R</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 month</td>
<td>4-months-old</td>
<td>Cell proliferation</td>
<td>BrdU</td>
<td>improved from nonTg NR</td>
<td>increased from Non-Tg NR at Non-Tg R levels</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Neuronal differentiation</td>
<td>DCX</td>
<td>improved from nonTg NR</td>
<td>normal improved from Tg NR</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Neuronal maturation</td>
<td>BrdU/NeuN</td>
<td>improved from nonTg NR</td>
<td>normal at Non-Tg R levels</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Plaque burden</td>
<td>6F3D</td>
<td>N/A</td>
<td>present present</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Spatial working memory</td>
<td>Y-maze</td>
<td>normal</td>
<td>impaired from Non-Tg NR normal</td>
<td></td>
</tr>
<tr>
<td>2 months</td>
<td>5-months-old</td>
<td>Cell proliferation</td>
<td>BrdU</td>
<td>improved from nonTg NR</td>
<td>normal improved from Tg NR</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Neuronal differentiation</td>
<td>DCX</td>
<td>low</td>
<td>low</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Neuronal maturation</td>
<td>BrdU/NeuN</td>
<td>improved from nonTg NR</td>
<td>impaired from Non-Tg NR improved from Tg NR</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Plaque burden</td>
<td>6F3D</td>
<td>N/A</td>
<td>higher lower</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Spatial working memory</td>
<td>Y-maze</td>
<td>normal</td>
<td>impaired from Non-Tg NR normal</td>
<td></td>
</tr>
<tr>
<td>3 months</td>
<td>6-months-old</td>
<td>Vessel morphology</td>
<td>Mercox-BABB</td>
<td>normal</td>
<td>higher higher than Non-Tg NR at Non-Tg levels</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CAA</td>
<td>Methoxy-XO4</td>
<td>N/A</td>
<td>higher lower</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Spatial working memory</td>
<td>Y-maze</td>
<td>normal</td>
<td>impaired from Non-Tg NR normal</td>
<td></td>
</tr>
</tbody>
</table>

Summary of research findings categorized by the duration of exercise paradigm. Difference due to genotype or treatment are indicated for each pathology.
5.2. Modulators of cognition

Progressive loss of cognitive function is one of the most important symptoms of AD (Braak and Braak, 1995). Results described in Chapters 3 and 4 indicate that stimulated neurogenesis, conserved vasculature, and lowered Aβ burden play a role in restoring memory function (Table 5.1). Nevertheless, improvements in cognition may be due to other mechanisms associated with AD and affected by physical exercise yet not evaluated in this thesis. Managing stress and synaptic plasticity are two such processes in which exercise can have great impact.

5.2.1. Reduced stress

Stress is considered a risk factor in the development of AD (Machado et al., 2014) and reducing its impact on the brain by physical exercise is thus a compelling alternative strategy for elevating cognitive deficits.

In animal models stress is known to accelerate and intensify AD-like pathologies compared to non-stressed transgenics (Machado et al., 2014). For example, Tg2576 mice exhibited decreased hippocampal cell proliferation and more advanced plaque deposition after 6 months of isolation stress compared to age-matched non-stressed transgenics (Dong et al., 2004, 2008). Further, restraint for 2 hours during a 16-day stress period resulted in increased levels of Aβ plaque deposition, tau hyperphosphorylation, and neuritic atrophy of cortical neurons in 10-month-old Tg2576 mice (Lee et al., 2009). Finally, using 8 months of isolation stress in APPV717I-CT100 mouse model of amyloidosis, Jeong et al. (2006) found that stress accelerated cognitive decline, as measured by the passive avoidance and the social transfer of food preference tests. Additionally, these mice exhibited increased number and density of vascular and parenchymal Aβ, and elevated neurodegeneration and tau hyperphosphorylation (Joeng et al., 2006). However, in TgCRND8 mice, Yuan et al. (2013)
failed to observe any adverse effects on hippocampal or cortical plaque pathology following 2 months of restraint stress in 6-month-old mice. Although the effects of stress on Aβ in TgCRND8 mice requires further examination, there is an overwhelming indication in other rodent models that stress is an important component in the acceleration of AD pathology and thus constitutes an important therapeutic target.

Physically active rodents are more resistant and resilient to the negative impact of stress on physical and mental health (Taylor et al., 1985; Brown and Siegel 1988; Dishman et al., 1998). In my examination of associative memory, running mice (both transgenic and non-transgenic) had unusually low freezing scores during fear conditioning testing (data not shown). Generally speaking this would be associated with impaired associative learning. However, I have considered the possibility that it could also be due to a higher resilience to a mild electric shock in the running groups. Literature appears to support this concept that physical activity lowers stress response in rodents. Specifically, it was shown that rats housed with running wheels for 6 weeks prior to the exposure to an acute, intense stressor were protected against stress-induced immunosuppression (Moraska and Fleshner, 2001), and inflammatory cytokines elevations (Speaker et al., 2011). Further, running rats were protected against affective deregulation including anxiety, social avoidance, and learned helplessness observed with the shuttle box escape test (Greenwood et al., 2003, 2005). Together, mediating stress responses leads to neural adaptations that functions to constrain, but not prevent (which would be maladaptive) the stress response.

Two possible mechanisms of running-induced neuronal adaptation to stress are the modulation of GABAergic tone and increased expression of growth factors. Running enhances local inhibitory mechanisms in the hippocampus, including increase in stress-
induced activation of hippocampal interneurons, expression of vesicular GABA transporter (vGAT), and extracellular GABA release during cold water swim stress (Schoenfeld et al., 2013). Conversely, blocking GABA receptors in the ventral hippocampus with the antagonist bicuculline, reversed the anxiolytic effect of running (Schoenfeld et al., 2013), suggesting that running improves anxiety regulation by engaging local inhibitory mechanisms in the ventral hippocampus. This has important implications for AD, where GABAergic dysfunction and successive loss of GABAergic neurons are known to occur (Krantic et al., 2012). We can thus propose that stabilizing GABAergic tone via physical exercise could prevent neuronal dysfunction and potentially preserve spatial memory.

Reduced blood BDNF is observed in depressive patients and is increased after antidepressant treatment (Martinowich et al., 2007; Molendijk et al., 2011). In a rat model of stress using repeated corticosterone injections, voluntary wheel running for 14 days restored hippocampal cell proliferation, attenuated learning impairment, and enhanced expression of BDNF protein (Yau et al., 2011). Furthermore, intraventricular BDNF infusion protected the hippocampus and cortex from ischemic damage and insulated septal cholinergic neurons from axotomy-induced death. Recently, Hutton and colleagues (2015) showed that a combination of diet and exercise (but not exercise alone) had beneficial effects on hippocampal function following four weeks of unexpected stressors. Specifically, physically active mice receiving dietary supplements exhibited higher number of DCX-positive immature neurons, as well as increased hippocampal BDNF mRNA and VEGF levels. Because TgCRND8 mice also exhibit deficits in BDNF mRNA (beginning at 6 weeks of age; Francis et al., 2012), counteracting BDNF deficits with running is a compelling mechanistic candidate by which memory can be improved.
5.2.2. Improved synaptic plasticity

In addition to stimulating the generation of new neurons, physical exercise also maintains of existing neurons. Improving synaptic plasticity in the form of activity-dependent modulation of synaptic strength – a process known to be compromised in AD - provides a neurophysiological mechanism for hippocampal-dependent learning and memory.

Synaptic loss and dysfunction is an early pathological feature of AD that correlates with memory impairment (Terry, 2000; Scheff et al., 2007; reviewed in Saura et al., 2015). Functional MRI studies have shown a decrease in activity and connectivity in the hippocampus and cortex during episodic memory tasks in AD patients (Press et al., 1989; Small et al., 1999; Pariente et al., 2005). APP transgenic mice exhibiting spatial and contextual memory impairments also show changes in long-term potentiation (LTP), a form of synaptic plasticity thought to be the cellular basis of learning and memory (Bliss and Lømo, 1973). APP overexpressing mice exhibit disrupted hippocampal synaptic plasticity prior to amyloid plaque pathology, suggesting that disruption of neural circuits is plaque-independent (Dodart et al., 1999; Hsia et al., 1999; Jacobsen et al., 2006).

Synaptic loss affecting several neuronal populations and neurotransmitter systems is another hallmark of AD pathology (recently reviewed by Saura et al., 2015). In addition to the cholinergic dysfunction, glutamatergic and GABAergic systems are impaired in an activity-dependent manner (Lacor et al., 2004; Deshpande et al., 2009). These deficits are paralleled by the loss of synapses through postsynaptic mechanisms involving deregulation, removal and mistargeting of extrasynaptic glutamate receptors (Shankar et al., 2007; D’Amelio et al., 2011) and contribute to cognitive impairment in AD (Yao et al., 2003; Blalock et al., 2004; Berchtold et al., 2014).
Running influences synaptic plasticity on many levels, including inducing LTP and strengthening postsynaptic mechanisms. Recordings from DG brain slices showed greater LTP in the runners compared to sedentary mice (van Praag et al., 1999; O’Callagahan et al., 2007; Vasuta et al., 2007). *In vivo* recordings showed enhanced LTP in the DG of running rats compared to non-runners (Farmer et al., 2004). Running also reversed the age-related LTP decline in rodents, which correlated with improved memory, enhanced hippocampal neurogenesis and increased BDNF levels (O’Callaghan et al. 2009; Marlatt et al. 2012). Further, physical exercise increased the concentration of the NR2A subunit of the NMDAR complex at the postsynaptic terminals, suggesting that exercise can increase the contribution of NMDA subunits to LTP (Vasuta et al. 2007). Taken together, these studies suggest that physical exercise is capable of restoring compromised synaptic plasticity in the AD brain by modulating LTP and maintaining postsynaptic function.

**5.3. Compensatory mechanisms in AD**

Several compensatory mechanisms have been suggested to occur in AD as a way for the brain to adapt to Aβ-induced toxic insults (Smith et al., 2007). The model can partially explain the plateau stage of memory decline observed in the preclinical stage of AD. Many investigations found mild impairment of episodic memory 4 years before AD diagnosis followed by a period of 3 years where memory performance did not change (Bäckman et al., 2001, 2004; Small et al., 1997, 2003; Cerhan et al., 2007). In a recent study, Granger et al. (2016) showed that 5-month-old TgCRND8 male mice compensated for accumulating Aβ by switching to increasingly effective navigational search strategy in the Morris water maze task. By employing such coping strategy, mice performed significantly better on the task indicating higher cognitive reserve, even in the presence of Aβ (Granger et al., 2016).
There are many processes thought to support the compensatory mechanism. One model suggests neurogenesis or strengthening of existing synaptic connections maintains memory performance. Another theory proposes angiogenesis as a response to hypoxic conditions present in early AD to supply activated brain areas with proper blood supply. Up-regulation of neurotransmitters observed in brain regions affected early in AD progression has been proposed as an alternative compensatory mechanism.

The relevance of my results to the compensatory mechanism are discussed below.

5.3.1. Transient up-regulation of BrdU-positive cells

In an Aβ-burdened brain neurogenesis has been reported as either increased or decreased, suggesting a transient compensatory response. Jin et al. (2004) was the first to show in J20 mice increased incorporation of BrdU and expression of immature neuronal markers in the dentate gyrus. These changes occurred before Aβ deposition were detected. Further study revealed an increase in cell proliferation and differentiation in J20 mice induced by oligomeric Aβ (López-Toledano and Shelanski, 2007). Chen et al. (2008) found stage-dependent dynamic changes in neurogenesis in PS1/PS2 mice in which cell proliferation was significantly enhanced at early stages of neurodegeneration, whereas the survival of newly generated neurons was diminished in later stages. These studies suggest that the full maturation of neurons may be hampered by Aβ (Schaeffer et al., 2009; Thomason et al., 2013). My results showed higher numbers of BrdU-positive cells in sedentary TgCRND8 compared to Non-Tg mice at earlier (4 months of age) though not later (5 months of age) stages of disease progression (Figure 3.5). This data is supported by previous findings by Kanemoto et al. (2014) who examined cell survival in 5-week-old TgCRND8 mice 1, 2, 4, 6 and 8 weeks after BrdU injection. At all time points examined, the population of BrdU-positive cells was higher in TgCRND8 than in non-transgenic mice.
Although there were more BrdU-positive cells in TgCRND8 mice, there was a tendency towards reduction in BrdU/NeuN-positive cell numbers at 1 and 8 weeks post injection, suggesting impaired differentiation or survival of newly born neurons. Together, our data as well as that of others point to a reactive mechanism occurring in early stages of AD-like pathology (at 4 months of age in TgCRND8 mice). Newly-generated cells are small in number and are therefore not capable of mediating the global repair necessary to maintain cognitive function.

Although the endogenous compensatory mechanism re-establishing the proper number of hippocampal neurons is ineffective and ultimately fails, it provides an insight into possible therapeutic targets. Physical exercise can stabilize Aβ burden thereby supporting and perhaps prolonging the neurogenic compensatory mechanism.

5.3.2. Early strengthening of synaptic plasticity

Alternative to the recruitment of new neurons is the strengthening of existing ones through synaptic activity modulation. Functional MRI imaging studies revealed decreased activity of the medial temporal lobe and hippocampus in patients with advanced AD (Press et al., 1989; Small et al., 1999; Pariente et al., 2005). By contrast, MCI and early AD patients exhibit hyper activation of the hippocampus and entorhinal cortex during face-name, visual object and verbal associative memory tasks (Kircher et al., 2007; Hämäläinen et al., 2007; Saura et al., 2015). These enhancements may constitute a compensatory mechanism as an attempt to maintain memory at the beginning of the disease process. Furthermore, asymptomatic patients who are at risk of developing AD due to presenilin-1 mutations, exhibit higher activation within the hippocampus, frontal and temporal cortices during memory tests (Bassett et al., 2006; Mondadori et al., 2006; Yassa et al., 2008). These deficits are present several years before clinical symptoms appear. In rodent studies, TgCRND8 mice
exhibit normal LTP measured in the CA1 region at 9 weeks of age (before plaques appear) and increased LTP and synaptic excitability in the CA1 region at 20 weeks of age when hippocampal plaques are robust (Jolas et al., 2002). Cumulatively, these findings indicate that the increase in synaptic activity compensates for neuronal dysfunction in preclinical stages of AD – a process that ultimately fails as neuronal damage progresses.

5.3.3. **Transient increase in angiogenesis**

Vascular changes manifest in individuals with MCI or cognitively healthy people carrying the ApoEε4 allele, the most consistent genetic risk factor for AD (Corder et al. 1993; Poirier et al., 1993; Strittmatter et al., 1993; Nikolakasis and Hamel, 2011). My study expands the knowledge of early increases in vascular density in response to Aβ. Higher capillary density, length and branching (Figure 4.3) might be occurring together with the increased expression of angiogenic factors such as VEGF, TGF β, and tumor necrosis factor α (TNF α) (Tarkowski et al., 2002; Kalaria et al., 1998; Vagnucci and Li, 2003). Furthermore, Vagnucci and Li (2003) proposed that angiogenesis and neovascularization occur in response to impaired cerebral perfusion in early AD.

Experiments assessing vessel morphology were conducted on animals with relatively early vascular pathology (Dorr et al., 2012). Although further work examining earlier and later time points is required, the data presented here partially suggest a transient compensatory mechanism in which angiogenesis is augmented in early stages of amyloidosis. It remains to be established whether hippocampal capillary density decreases with progressing Aβ pathology, and/or whether Aβ impacts capillary density in similar fashion in other brain regions. Quantification of cortical vascular architecture of 6-month-old TgCRND8 mice - a brain region with more advanced amyloid pathology - showed lower capillary density and length (Thomason et al., 2015). The presence of an increased
hippocampal vessel density with lower Aβ burden and a decreased cortical vessel density and higher Aβ burden in the same animal may suggest a transient pro-angiogenic role of amyloid. Augmented angiogenesis most likely disappears as Aβ accumulates and is replaced by an anti-angiogenic response in later stages of pathology (Paris et al., 2004a, b). It remains to be established whether hippocampal capillary density decreases in older TgCRND8 mice, when Aβ is more pronounced.

In conclusion, there is emerging evidence of an angiogenic compensation occurring in the preclinical and early stages of AD. My data showing higher capillary density, volume and length support the angiogenic compensatory mechanism. Further work is required to establish whether the observed increase in the capillary density is mediated by VEGF; this proposal is explored in Chapter 6. Additionally, examining capillary density at later time points would shed light on the transiency of the vascular compensatory mechanism.

5.3.4. Fluctuations in growth factors and neurotransmitters

Several studies suggest that levels of cortical choline acetyltransferase (ChAT) activity are maintained or elevated in preclinical AD patients, whereas patients with moderate to severe AD show reduced levels of ChAT activity (Davis et al., 1999; DeKosky et al., 2002; Ikonomovic et al., 2005). Moreover, recent studies show preservation or elevation of neurotrophic factors, including the nerve growth factor (NGF) and BDNF in pre- or early AD (Durany et al., 2000; Egan et al., 2003; Mufson et al., 2003; Peng et al., 2004). Specifically in TgCRND8 mice, Francis et al. (2012) found fluctuations in hippocampal BDNF mRNA levels: decreased at 6 weeks and 6-8 months and increased at 9 weeks of age. Such phasic fluctuations in growth factors may occur in response to progressive Aβ pathology. Early deficits in BDNF reported by Francis and colleagues (2012) is consistent with the onset of Aβ-induced dysfunction in brain regions crucial for memory function (Thal
et al., 2002). However, this acute reduction in BDNF mRNA may be short lived as tissue levels of BDNF mRNA rebound in response to a moderate load of intracellular Aβ (Arvanitis et al., 2007). In support of this concept, BDNF mRNA was found to be up-regulated in glial cells surrounding plaques in APP23 transgenic mice (Burbach et al., 2004). The transient increase in BDNF may reflect a compensatory neuroprotective response (Lindvall et al., 1992). However, it is a temporary response as the continuous exposure to increasing Aβ results in decreased neuronal BDNF expression (Burbach et al., 2004; Arvanitis et al., 2007). The fluctuations in ChAT activity and BDNF levels suggest that the brain instigates a regional adaptive response, particularly early in the disease course. This pattern of initial stimulation might correspond with a period of stabilization of memory performance in preclinical AD (Small et al., 1997, 2003; Cerhan et al., 2007).

In summary, compensatory mechanisms observed in pre-clinical and early AD include neurogenic, angiogenic, and synaptic responses aimed at mitigating initial insult from Aβ. This endogenous response provides hope for a window of opportunity for neuronal rescue (Akwa et al., 2005) and behavioral intervention (Greenaway et al., 2006) during an identifiable preclinical period and has important therapeutic implications (Smith et al., 2007). Furthermore, physical exercise can play a supportive role and prolong the duration of the compensation thereby extending therapeutic window.

5.4. Exercise in combinational therapy for neurological disorders

Physical exercise is a simple, highly accessible and well-tolerated intervention that is being explored as a treatment strategy in AD and other diseases1. It is often stated that physical exercise can elicit effects on par with pharmacological agents (Berryman, 2010). Such statement, however, suggests a competitive approach where only one of two treatments

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1 www.clinicaltrials.gov
can be used. Exercise can act as a powerful adjuvant therapy and can be applied in conjunction with pharmacological agents, resulting in additive effect from both therapies. Combinational therapies are being explored in AD, depression and chemotherapy and are discussed in subsequent sections.

5.4.1. Combinational therapy in AD

In the field of AD, the combinational therapy is being explored in over 20 clinical trials in which rivastigmine and donepezil acetylcholinesterase inhibitors or memantine NMDAR antagonist drugs are combined with physical exercise. Following a period of a combined and monotherapy, patients are evaluated with a multitude of cognitive tests, such as Quality of Life in Alzheimer's disease scale (QOL), Activities of Daily Living Questionnaire (ADL), Mini-Mental State Examination test (MMSE), Alzheimer’s Disease Assessment Scale Cognitive Subscale (ADAS-Cog) and Clock Drawing Test. In a clinical trial with 40 patients with mild to moderate AD, a rivastigmine transdermal patch was used in combination with an exercise program consisting of aerobic, flexibility, strength and balance movements performed twice a week for 6 months (Aguiar et al., 2014). Patients receiving the combinational therapy performed better on QOL and did not worsen on the ADL compared to patients randomized to a monotherapy. In another clinical trial, Matsuda (2007) showed that patients receiving donepezil plus cognitive stimulation therapy scored significantly better on MMSE test than patients on donepezil alone. Recently, Andersen et al. (2012) employed a combination of physical and cognitive stimulation coupled with donepezil and one year later saw improvement on ADAS-Cog and Clock Drawing Test. By contrast, Chapman et al. (2004) was not able to demonstrate cognitive benefits of donepezil plus cognitive-communication stimulation therapy compared to monotherapy. Although the results from the above studies are mixed, the field of pharmacology and exercise
combination therapy targeting AD pathology is relatively new and is currently under intense investigation. For example, clinical trials with exercise and galantamine and memantine are undergoing clinical trials and results are unavailable as of writing of this document\(^1\).

Data from other diseases, such as depression and cancer, with a more established research on adjuvant therapy and greater success in mediating cognitive improvements enforces the need for further examination of combination therapy in AD.

### 5.4.2. Combinational therapy in depression

The effect of physical activity on depression has been the subject of research for several decades, and the literature on the subject continues to grow as “exercise on prescription” has become popular in the primary care of patients with depression (Lawlor and Hopker, 2001). Several plausible mechanisms for how exercise affects depression have been proposed. One such mechanism involves BDNF, the most abundant of the neurotrophins in the brain (Lindsay et al., 1994). BDNF expression is a target of several antidepressant treatments and is an important agent for recovery and protection against stress-induced neuronal damage. Direct infusion of BDNF has been shown to cause functional recovery from depression in mice (Siuciak et al., 1997). Furthermore, chronic antidepressant treatment has been shown to elevate cortical and hippocampal BDNF mRNA levels (Nibuya et al., 1995) and physical exercise has been evidenced to augment BDNF levels and counteract BDNF deficits in depression (Russo-Neustadt et al., 2000).

Subsequently, Russo-Neustadt and colleagues (2001) showed that one week of treatment with tranylcypromine combined with voluntary running augmented hippocampal BDNF levels above baseline levels in rats and improved their performance on the forced swim test. Furthermore, the results of combination therapy were significantly higher than any of the two treatments alone. It appears then that physical exercise and antidepressants have an additive
effect and can act in synergy to up-regulate growth factors expression. Such combination therapy is being explored and applied in the clinic in the development of better psychiatric treatments.

5.4.3. Combinational therapy in chemotherapy

In the field of cancer treatment, there is strong support for an association between administration of commonly used chemotherapeutic agents and an increased risk for cognitive impairment (Argyriou et al., 2011). Chemotherapy-induced cognitive impairment (CICI), commonly known as "chemobrain" or "chemofog" is a relatively common event affecting 70% of long-term cancer survivors that, in most of the cases, remains underdiagnosed, thereby adversely affecting the quality of life of cancer patients (van Dam et al., 1998; Castellon et al., 2004; Vardy et al., 2008). The new paradigm for defining successful cancer therapy is the multidisciplinary approach balancing oncologic efficacy of chemotherapy with late and long-lasting side effects (Alvarez et al., 2007). As CICI is currently treated pharmacologically, novel non-pharmacological interventions such as physical exercise are being explored. It has been shown in rodents that cognitive impairment brought about by 5-fluorouracil and oxaliplained chemotherapy agents commonly used to treat colorectal cancer had detrimental effects on hippocampal-dependant tasks such as novel object recognition, contextual fear conditioning and Morris water maze measured (Fardell et al., 2013a, b). These impairments were prevented by physical exercise administered immediately after chemotherapy, suggesting that physical exercise is beneficial in ameliorating chemotherapy-induced cognitive impairments. Ferguson et al. (2012) suggested that cognitive-behavioral interventions, including physical exercise, may provide the most benefit for patients with CICI. Lastly, Winocur et al. (2014) evaluated hippocampal neurogenesis and cognitive deficits in running and sedentary rats which received three
injections of methotrexate and 5-fluorouracil chemotherapeutic drugs. In rats receiving chemotherapy and housed in exercise cages, neurogenesis was not suppressed and Morris water maze performance was indistinguishable from non-chemotherapy treated mice. Without the exercise component, chemotherapy-treated mice exhibited reduced neurogenesis and impaired cognition (Winocur et al., 2012, 2014).

The etiology of chemotherapy-induced cognitive impairments is multifactorial, and more than one treatment modality may be required. Physical exercise has broad neurobiological effects and has been shown to be beneficial in other populations with cognitive impairments similar to those reported after chemotherapy, and thus may offer the best hope for cancer survivors.

The use of combination therapies has demonstrated success in improving the treatment of depression and cancer. In AD, comprehensive therapies benefiting from an additive effect of pharmacological and non-pharmacological interventions are beginning to be explored. Exercise may also provide invaluable support to monotherapies that would otherwise fail in clinical trials. Research from the past few decades revealed an extensive network of interactions involved in AD that results in multiple pathologies encompassing neuronal, vascular, amyloid and cognitive components. This suggests that a multidirectional therapeutic approach rather than a single target-based approach may be more feasible and more effective for the treatment of AD.

5.5. Conclusion

The work presented here demonstrates the great extent to which physical exercise can modulate brain morphology and function, even in the presence of Aβ. As outlined in Specific Aim 1, we evaluated spatial memory, neurogenesis and parenchymal Aβ following 1 and 2 months of running in TgCRND8 mice (at 4 and 5 months of age). In Specific Aim 2, we
assessed hippocampal vascular pathology, defined as vessel morphology and CAA burden, following 3 months of running in transgenic mice (at 6 months of age).

Overall, these data indicate that as amyloidosis progresses in the hippocampus of TgCRND8 mice, physical activity can mitigate cognitive, neuronal, and vascular pathologies. Exercise was found to have the most consistent impact on cognition, with spatial memory being rescued at all ages, even when neurogenesis (at 4 months) and plaque burden (at 4 and 6 months) remained unaffected in running TgCRND8 mice. These data suggest that stimulated neurogenesis and lowered plaque buildup may not be individually necessary to promote cognitive function.

Increased cell proliferation and angiogenesis in sedentary TgCRND8 mice compared to non-transgenic mice may indicate an adaptive response to Aβ. More specifically, the levels of proliferating cells at 4 months and capillary density at 6 months of age were increased in TgCRND8 mice compared to non-transgenic littermates. These changes may constitute an endogenous mechanism aimed at compensating for neuronal and vascular dysfunction, though more studies are required to assess whether such adaptive response indeed takes place in a pre-symptomatic and early AD.

Running-induced modifications of several AD-related pathologies, and memory function in particular, suggest that physical activity constitutes a valuable treatment strategy. Exercise may prove to be a powerful adjuvant aimed at ameliorating the broad spectrum of pathologies, ultimately maintaining cognitive function in AD patients.
Chapter 6

Thesis considerations

6.1. Beyond Y-maze and spatial memory

Chapters 3 and 4 contain the analysis of the spatial memory performance in TgCRND8 and non-transgenic mice. Animals were assessed at the start of the experiment (3 months of age) as well as after 1, 2, and 3 months of voluntary running (Figure 5.1). TgCRND8 mice showed spatial memory deficits while running restored spatial memory to non-transgenic levels at all time points.

We chose the Y-maze test for several reasons. Similar to the Morris water maze, the Y-maze evaluates reference and working memory with a spatial component. TgCRND8 mice are known to have impaired performance in both tasks (Y-maze: Hyde et al., 2005; Burgess et al., 2014a; Ma and McLaurin, 2014; Morris water maze: Chishti et al., 2001; Janus, 2004; Hanna et al., 2009). However, the Morris water maze is relatively stressful and it requires a significant amount of training. Stress hormones and the learning process of the Morris water maze can complicate interpretation of neurogenesis outcome measures (Gould et al, 1999b; Harrison et al., 2009; Kennard and Woodruff-Pak, 2011; Schoenfeld and Gould, 2012). For the above reasons, we selected the Y-maze task as the optimal test to assess spatial memory in TgCRND8 mice. A more comprehensive and thorough approach would be to assess different types of memory, each confirmed with a battery of behavioural tasks. I originally employed a novel object recognition task to assess short-term spatial recognition memory (Ennaceur and Delacour, 1988; Vaucher et al., 2002; Hammond et al., 2004), however, the test proved unsuccessful due to inadequate saliency of selected objects (data not shown). I
also assessed cued and contextual memory with the fear conditioning task (Kim and Fanselow, 1992; Phillips and LeDoux, 1992; Maren and Fanselow, 1996) and I was able to detect the effect of genotype but not of the running (data not shown). One possible explanation is that exercise causes mice to be more resilient to shock-induced stress (section 5.3.1; Moraska and Fleshner, 2001; Speaker et al., 2011). Overall, Barnes maze would be most suitable task for expanding my assessment of spatial memory. This test, which is considerably more complex and cognitively more challenging for mice than the Y-maze, could detect a difference in memory performance in running and non-running non-transgenics. This difference was not detected with a relatively less-challenging Y-maze test (Figures 3.7 and 4.5).

Assessing different types of memory and cognitive function would create a cognitive phenotype of running TgCRND8 mice. Examination of executive function would be a suitable complement to spatial memory studies as it encompasses a wide range of higher cognitive processes, such as reasoning, planning, cognitive flexibility, sequencing, response inhibition and abstract concept formation (Webster et al., 2014). Executive function is known to depend on the dorsolateral and orbital prefrontal cortices (Weinberger et al., 1986; Brigman et al., 2005) whereas spatial memory is predominantly hippocampal-dependent (Glickman and Jansen, 1961), thus providing a comprehensive view of cognitive deficits in two anatomical regions highly relevant to AD pathology. Employing a touch screen-automated cognitive test would reveal whether exercise can mitigate impaired attention, cognitive flexibility, and increased response inhibition known to occur in TgCRND8 mice (Romberg et al., 2013).
6.2. Cortical plaques and Aβ oligomers

The primary focus of my research has been on the hippocampal region. However, it is highly possible that the cortex – another region with high relevance to AD (Braak and Braak, 1995) – is also affected by physical exercise (Kramer and Erikson, 2007). My results showed that running lessened hippocampal plaque burden at 5 months, yet plaques were unaffected by exercise at 4 and 6 months of age (Figures 3.7 and 4.4). It can be suggested that at 4 months of age the level of hippocampal plaque pathology is too low for running to have a significant difference, or perhaps 1 month of running is insufficient to make a biologically-relevant impact. Quantification of cortical plaques - a region with more advanced amyloid pathology (Chishti et al., 2001) – would provide support for this claim. It can be further suggested that at 6 months of age, the severity of hippocampal plaque pathology could overpower the effects of running. Extrapolating from the hippocampal data, one could expect a statistically significant impact of running on lowering cortical plaque load at 4 and perhaps 5 months of age, and no effect at 6 months of age.

Amyloid plaque buildup is only one step in the process of amyloidosis resulting from abnormal APP cleavage, oligomerization, fibrilization, aggregation and clearance (Sisoda and St. George-Hyslop, 2002; Figure 1.3). Physical exercise could potentially modulate amyloidosis at any one of the aforementioned stages. Thus, assessing the effects of running on Aβ oligomers would further our knowledge about the effects of physical exercise on amyloid pathology. I attempted to characterize the effects of running on hippocampal and cortical oligomers by quantifying their levels in 5-month-old TgCRND8 mice, though the assessment was unsuccessful due to technical challenges (data not shown).
Evaluation of cortical plaques and quantification of oligomers in both brain regions could shed light on the observed cognitive improvement in 4-month-old running TgCRND8 mice. In this group, 1 month of running resulted in improved spatial memory and higher number of immature DCX-positive neurons yet the number of NeuN mature neurons and plaque burden remained unaffected. I postulated that the augmented memory function was likely due to higher levels of neuroblasts. However, it is also possible that running affects all species of Aβ in both brain regions. In summary, it is possible that running-induced improved memory following 1 month of running is the combined result of increased neuroblasts, and reduced cortical/hippocampal oligomers and plaques.

6.3. In vivo vasculature analysis and angiogenesis

Ex vivo assessment of vasculature was chosen as a first step in the examination of its morphology due to relatively limited knowledge of vessel structure in the context of amyloidosis and physical exercise. In vivo imaging of cortical vessels undergoing a hypercapnic challenge would extend our knowledge of the function of vasculature in this mouse model and the regulation of CBF after a period of running. These in vivo experiments could be then correlated with the ex vivo assessment of cortical vessel morphology (currently at the analysis stage) to link the vessel structure and function in the context of amyloidosis and physical activity.

It has been proposed that, in amyloidosis, angiogenesis occurs in response to impaired cerebral perfusion (Vagnucci and Li, 2003) and increased VEGF, TGFβ, and TNFα (Tarkowski et al., 2002; Kalaria et al., 1998; Vagnucci and Li, 2003). Results described in Chapter 4 show increased hippocampal capillary density, length, and branching, possibly mitigated by stimulated angiogenesis. To confirm the presence of new vessels,
immunohistochemical staining of hippocampal tissue with an antibody against angiogenic markers, such as CD105 (Nassiri et al., 2011), could be performed. Higher levels of CD105 immunohistochemistry in sedentary TgCRND8 compared to Non-Tg mice would provide evidence for stimulated angiogenesis. Further studies would be required to elucidate the mechanisms promoting angiogenesis in TgCRND8 mice. These studies are discussed in the next Chapter.
Chapter 7

Future directions

The following sections focus on new research directions that can be considered in the context of this thesis.

7.1. Beyond vessel morphology and function

Robust brain functioning and information processing require a highly-coordinated signal transduction involving not only healthy neurons and blood vessels, but also intact BBB (reviewed in Zhao et al., 2015). In the pathological context associated with amyloidosis, BBB breakdown contributes to the onset and progression of cognitive decline due to the accumulation of blood-derived neurotoxins and compromised Aβ homeostasis (Bennett et al., 2000; Sagare et al., 2013). The disruption of vascular and BBB integrity can drive the initial pathogenic events that lead to neuronal injury. Conversely, the neuronal deficits and resulting diminished metabolic need may cause changes to CBF and disintegration of BBB. It would be valuable to investigate whether BBB disruption is the cause or consequence of neuronal damage and Aβ accumulation. It must be noted that TgCRND8 mouse model does not exhibit detectable BBB leakage, even at 8 months of age (Burgess et al., 2014b). As it was mentioned in section 1.2.4, great care must be taken when selecting a mouse model to answer a particular research question as each transgenic model represents a different array of temporal and spatial subsets of AD pathologies. Therefore, I propose to use another mouse model, namely the Tg2576 mice (Hsiao et al., 1996). These mice have multiple vascular deficits including reduced CBF at 18 but not 8 months of age (Sihn et al., 2007) and reduced levels of P-glycoprotein at 3 months of age suggesting early
signs of BBB compromise (Hartz et al., 2010; reviewed in Palmer and Love, 2011). In addition, Tg2576 mice exhibit age-associated spatial learning deficits beginning at 12 months of age (Hsiao et al., 1996; Kawarabayashi et al., 2001). Simultaneously, hemizygous mice develop numerous parenchymal Aβ plaques starting at 9 months of age along with vascular amyloid at 18 months of age (Hsiao et al., 1996; Kawarabayashi et al., 2001). Cumulatively, the Tg2576 model appears to be suitable to gain insight into the potential pathogenic links between BBB dysfunction, neuronal injury and neurodegeneration, which still await to be directly explored. Analyzing BBB breakdown at various stages of disease progression might shed light on the discourse whether BBB deficits are a cause or a consequence of neuronal damage and/or other pathologies observed in AD. Lastly, lifestyle factors such as physical exercise, hold promise at modulating these relations and possibly maintaining and restoring the highly-sensitive and vulnerable balance between neurons, vessels and BBB.

7.2. Growth factors

Findings of higher neurogenesis and angiogenesis mediated by exercise and amyloidosis, respectively, could be explained mechanistically by examining expression levels of growth factors, namely BDNF and VEGF.

It has been well established that BDNF promotes the differentiation and survival of neurons both in vitro and in vivo (Cotman and Berchtold, 2002) and it has neuroprotective effects against ischemic damage (Yau et al., 2011) and stress (Siuciak et al., 1997). The expression of BDNF is known to be compromised in TgCRND8 mice (Francis et al., 2012) and physical exercise is capable of significantly stimulating the expression of BDNF in the healthy, aged, and AD brain (Vivar et al., 2013). Future studies should aim to evaluate the levels of BDNF in TgCRND8 mice following a period of voluntary running. These results could be evaluated in comparison with BDNF-overexpressing mice, such as the Tg(Camk2a-
Bdnf)A9Stl (Huang et al., 1999) or BDNF-Tg mice (Tolwani et al., 2002). Further, crossing TgCRND8 mice with BDNF-overexpressing mice would create a double transgenic model of amyloidosis with constitutively overexpressing BDNF. These mice would be compared to TgCRND8 mice with BDNF augmented via physical exercise. These studies would provide evidence that the exercise-induced increased neurogenesis is, at least partially, governed by augmented BDNF expression, even in the presence of Aβ.

VEGF is another growth factor that could provide a mechanistic insight into higher neurogenesis (Figure 3.5) and capillary density (Figure 4.3). VEGF is a growth factor implicated in the stimulation of neurogenesis (reviewed in Welberg, 2011) and angiogenesis (reviewed in Ferrara and Kerbel, 2005) and its expression is increased in early AD (Kalaria et al., 1998; Tarkowski et al., 2002). Furthermore, administration of exogenous VEGF induces the proliferation of cortical precursors in vitro and the proliferation and DCX-positive neuroblasts in hippocampus of rodents (Jin et al., 2004; Zhu et al., 2003). Examining VEGF expression following a period of exercise could provide a mechanistic explanation for observed increased neurogenesis and capillary density in 5- and 6-month-old TgCRND8 mice, respectively.

7.3. Clinical relevance

The purpose of the research presented here was to examine the extent to which physical activity can modify hippocampal neurogenesis, vasculature and spatial memory in the presence of amyloidosis. The results demonstrate the potency by which running can improve hippocampal neuronal and vascular plasticity. Further, this research shows that upon initial insult the brain makes an attempt at counteracting the hostile environment and tries to heal itself. Physical exercise is able to support these actions and delay disease onset thus widening therapeutic window.
Currently, poor lifestyle is responsible for almost two-thirds of disease globally (Lee et al., 2012; Hallal et al., 2012). The World Health Organization has identified a lack of exercise as one of the most important lifestyle factors relating to disease (Lee et al., 2012; Hallal et al., 2012). Physical activity is a safe and easily available intervention that can combat existing conditions on par with pharmaceuticals (Lee, 2007). Rather than juxtaposing exercise against pharmacologics, we can harness the benefits of both for a combined treatment of many diseases, including AD. The safety and relative ease of incorporating exercise into daily routine provides further support for implementing such lifestyle modifications in AD patients. Importantly, exercise paradigms do not have to be physically challenging as even modest programs have demonstrated to be beneficial to brain health in elderly patients.

### 7.4. Closing remarks

The research presented here supports the notion that physical exercise provides resiliency against Aβ-induced pathologies and promotes cognitive function. Physical activity has the potential to target multiple pathologies arising during the development and progression of AD. To battle such complex disease of which the causes, mechanisms and cures remain elusive, robust and multimodal treatments are necessary. This thesis is part of the larger effort aimed at providing a comprehensive treatment paradigm for AD patients.
Appendix A

Figure A. Y-maze performance in male and female 6-month-old mice.
(A) Sedentary TgCRND8 mice are impaired on the Y-maze task as they do not distinguish between familiar and novel arms of the maze (n=17, p=0.4). Evaluation of potential sex differences revealed that sedentary males and females do not differentiate between familiar and novel arms of the maze indicating spatial memory deficits (Females n=8, p=0.5, Males n=9, p=0.4).
(B) Running TgCRND8 mice spend significantly more time in the novel arm than in familiar arms of the maze indicating spatial memory preservation (n=17, p=0.01). There was no sexual dimorphism observed in the Y-maze performance of running transgenics. Though not statistically significant due to low n numbers, both females and male running transgenics showed trend towards preference for the novel arm (Females n=8, p=0.08, Males n=9, p=0.09). Power analysis indicated that 4 more females and 3 more males are required to reach statistical significance with 0.05 alpha and 0.8 beta errors.
Chapter 8

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