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The Effects Of Long-Term Treadmill Exercise Training On Amyloid-Beta Plaque Levels and Behavioral Profile in 15-Month Tg2576 Mice

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THE EFFECTS OF LONG-TERM TREADMILL EXERCISE TRAINING ON AMYLOID-BETA PLAQUE LEVELS AND BEHAVIORAL PROFILE IN 15-MONTH TG2576 MICE

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By

Riya Thomas

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ABSTRACT

Alzheimer's disease (AD) is a progressive, neurodegenerative disease that is characterized by progressive loss of memory and cognitive function. The causative factors of this disease include modifications in neuronal cell metabolism, such as the progression of neurotic injury by amyloid beta (Aβ), and tau tangle deposition in the brain. Recent studies show that brain atrophy and other pathologic conditions are responsible for dementia in older individuals (80 years of age or older). Non-pharmacological interventions may have a role in both the prevention and slowing disease progression in AD. The role of exercise training in disease prevention has been evaluated in large epidemiological studies even though much less is known about the potential benefit of exercise training in patients already diagnosed with AD. Several studies in transgenic mouse models of AD suggest exercise training reduces soluble and insoluble Aβ levels in the brain. This study assessed the effectiveness of long-term exercise training (12-months) in an AD transgenic mouse model, Tg2576. Amyloid plaque levels and behavioral profile of mice were evaluated after 12 months of training. Mice that received daily exercise training demonstrated beneficial behavioral profile and reduced plaque counts in a dose-dependent manner.

KEYWORDS: Alzheimer's disease, exercise training, cognition, Aβ plaques, Tg2576 mice

This abstract is approved as to form and content

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Dr. Benjamin F. Timson
Chairperson, advisory Committee
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INTRODUCTION

Neurodegenerative Diseases

Neurodegenerative diseases are characterized by selective and symmetric loss of neurons in motor, sensory, and cognitive systems (Gorman, 2008). Description of the patterns of cell loss and the identification of disease-specific cellular markers have aided in nosologic classification. Senile plaques, neurofibrillary tangles, neuronal loss, and acetylcholine deficiency define Alzheimer's disease (AD), depletion of dopamine characterizes Parkinson's disease (PD), cellular inclusions and swollen motor axons are found in amyotrophic lateral sclerosis (ALS), and γ-aminobutyric acid containing neurons of the neostriatum are lost in Huntington's disease (HD) (Martin, 1999).

In some neurological diseases such as AD, genes have been found which lead to a clinical and pathological syndrome, with only variations in the age at onset and the rate of progression. In other disorders such as Downs's syndrome, errors in DNA replication resulting in an increased number of trinucleotide repeats are associated with selective patterns of neurodegeneration (Martin, 1999). In these disorders, heterozygotes are as severely affected as homozygotes, suggesting that the biochemical change caused by the mutation results in toxicity to the cell (Martin, 1999). In contrast, in autosomal recessive disorders such as Friedreich's ataxia, the manifestations of the disease are caused by a cellular loss of function due to the failure of protein production. These conditions are most likely caused by abnormalities in the transport, degradation, and aggregation of proteins that lead to cell-specific changes and ultimately to neuronal death, probably by apoptosis (Mattson, 2000; De Strooper, 2010).

The identification of specific genetic and environmental factors responsible for these diseases has strengthened evidence for a shared pathway of neuronal death, which involves oxidative stress, stressed calcium homeostasis, mitochondrial dysfunction and activation of cysteine proteases called caspases (Brady and Morfini, 2010). These cascades are counteracted by survival signals, which suppress oxygen radicals and stabilize calcium homeostasis and mitochondrial function. With the identification of mechanisms that either promote or prevent neuronal apoptosis, new approaches need to be developed for preventing and treating neurodegenerative disorders such as AD (Li et al., 2015).

Overview of AD

In 1901, Dr. Alois Alzheimer's patient, Auguste Deter had strange behavioral symptoms such as short-term memory loss. In April 1906, after her death, Alzheimer along with two Italian physicians used Nissl staining techniques to identify amyloid plaques and neurofibrillary tangles in her brain. Later in November 1906, Dr. Alzheimer's speech at the physicians' conference in Germany about the pathology and clinical symptoms of pre-senile dementia was groundbreaking.

AD usually begins in the seventh to ninth decade of life, but an early-onset familial form is now well recognized where disease symptoms are manifested in the fifth and sixth decades (Thorvaldsson et al., 2011). The disease affects over 4 million people in the United States, resulting in over 100,000 deaths per year. The risk of AD rises sharply with advancing age, and as life expectancy increases, the effect of the disease will also increase (Price et al., 2009).

Brain changes associated with AD lead to growing trouble with memory, thinking and reasoning, making judgments and decisions, planning and performing familiar tasks, vision loss, and changes in personality and behavior (Shankar et al., 2008). Symptoms of AD progressively deteriorate over time. In many instances, AD patients start developing symptoms earlier but only receive an official diagnosis years later. Post diagnosis patients enter the second stage of the disease where they develop mild cognitive impairment which will last two to ten years. Alzheimer's dementia occurs during the final stages where there is severe damage and this is the most extreme form of the disease. The similarity of pathologic findings in the different AD groups provides evidence for a common pathophysiological pathway in AD (Lippa et al., 1996). With respect to the severity of changes and location of tangle-bearing neurons, the disease progresses in six stages: transentorhinal stages I–II: clinically silent cases; limbic stages III–IV: incipient AD; neocortical stages V–VI: fully developed AD (Sperling et al., 2011).

Pathology of AD

The neuropathological features of neurofibrillary tangles and senile plaques described by Dr. Alzheimer, in 1907, result in the progressive loss of memory and cognitive function (Harman, 2006). These modifications are indications of the cause of AD, especially neuronal cell metabolism in the form of neuritic injury, and neuronal cell death affecting the brain (Serrano-Pozo et al., 2011).

Recent studies show that brain atrophy and other pathologic conditions are responsible for dementia in the oldest persons (80 years of age or older). AD may be primarily a disorder of synaptic failure where hippocampal synapses begin to decline in patients with mild cognitive impairment in whom residual synaptic outlines show

compensatory increases in size. As the disease progresses synapses are disproportionately fewer relative to neurons (Loo et al., 1993). The core symptoms of AD include cognitive impairment, decline in the ability to perform daily activities, and manifestation of behavioral and psychological symptoms of dementia (BPSD).

There is progressive brain cell death that occurs over a course of time in AD and the total brain size shrinks, where the tissue has progressively fewer nerve cells and connections. While these cannot be observed in the brains of living people affected by AD, postmortem results show tiny inclusions in the nerve tissue called plaques. Cerebral plaques that are burdened with Aβ, dystrophic neuritis in neocortex, and neurofibrillary tangles in medial temporal-lobe structures are important pathological features of AD (Christen, 2000) (Figure 1).

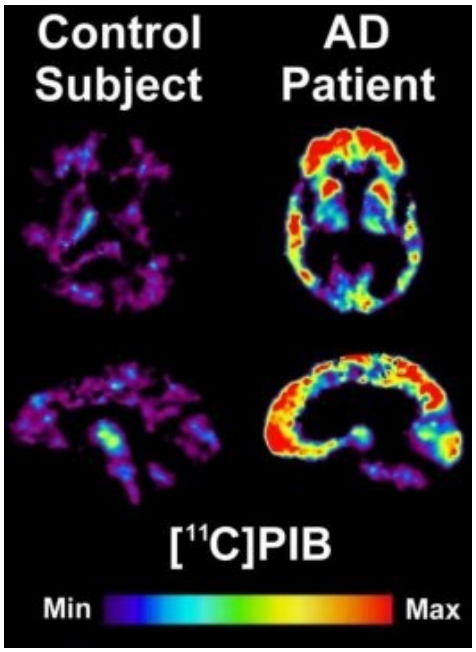

Figure 1. PET image comparison of normal versus AD brain. Positron Emission Tomography (PET) scan images of normal brain and AD brains with amyloid plaques (Credit: University of Pittsburgh, PET Amyloid Imaging Group).

Amyloid Cascade Hypothesis of AD

There currently exist several hypotheses concerning the development and progression of AD. The most studied and widely accepted is the amyloid cascade hypothesis. This hypothesis suggests that the deposition of a protein, the amyloid-beta (Aβ) peptide in the brain, is a crucial step that ultimately leads to AD (Saunders et al., 1993; Lippa et al., 1996; Sperling et al., 2011). The aggregation of Aβ into toxic oligomers and eventually plaque later in life is directly related to soluble Aβ peptide concentrations in the brain during midlife (Bero et al., 2011). Soluble Aβ concentration in the brain depend on the relative rates of which the peptide is produced and cleared. The aggregation of Aβ into oligomers and eventually plaque is shown in Figure 2.

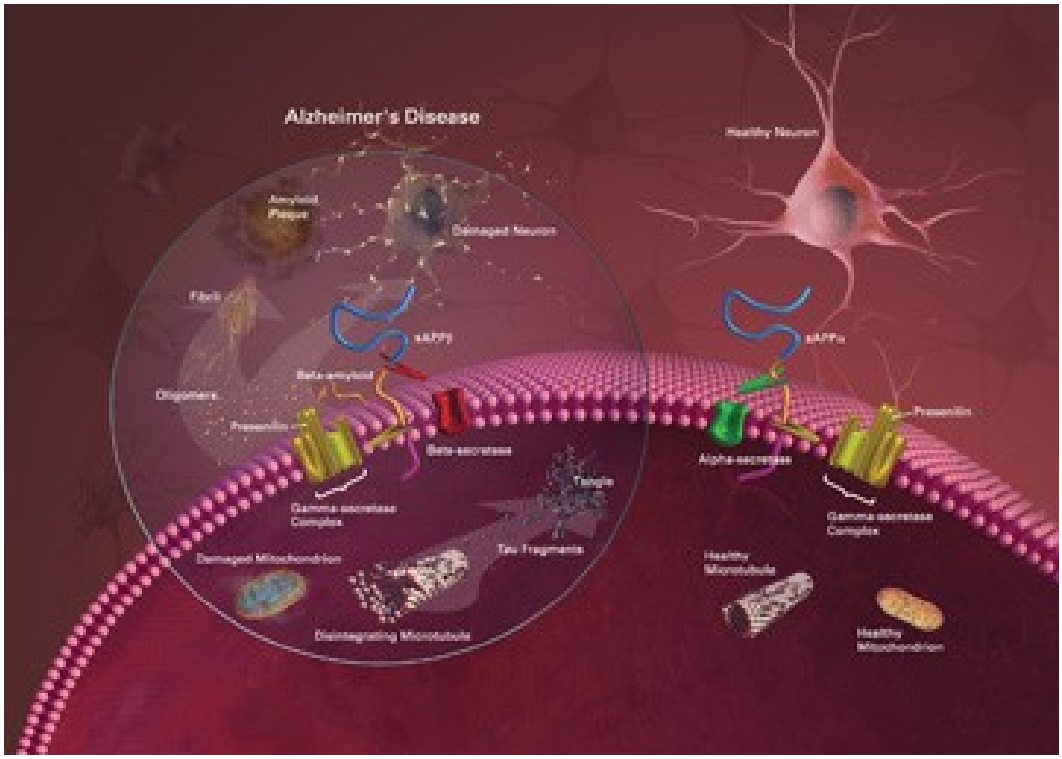

Figure 2. Plaque formation. Soluble Aβ aggregates into toxic soluble oligomers, then insoluble fibrils, and finally into plaque in the extracellular space in the brain (Image courtesy of the National Institute on Aging/National Institute of Health).

Aβ Production. Aβ is produced when two different enzymes cleave the 771 amino acid amyloid precursor protein (APP) which is crucial for neuronal development and synaptic connection (Mattson, 2000). The enzymes beta-secretase and gamma-secretase sequentially cleave APP producing Aβ, which can be 38, 40 or 43 amino acids long (Fig 1) (Querfurth and LaFerla, 2006). The Aβ is 42 amino acid form is more hydrophobic than the shorter forms and is more likely to aggregate (Yang et al., 2003). The accumulation of Aβ in the brain leads to damage of neurons which in turn triggers inflammatory responses as the brain attempts to repair itself (Harman, 2006).

The concept of Aβ-derived soluble toxic oligomers has been proposed to account for the neurotoxicity of the amyloid-β peptide. These intermediate forms lie between free, soluble Aβ monomers and insoluble amyloid fibrils (Zhang-Neus et al., 2006). Soluble Aβ oligomers are organized into different structures ranging from dimers (Walsh et al., 2002) to dodecamers (Lesne et al., 2006) and include; trimers (Walsh et al., 2002; Glabe, 2006), tetramers (Walsh et al., 2002; Glabe, 2006), pentamers and decamers (Ahmed et al., 2010), and Aβ-derived diffusible ligands (ADDLs) (Hepler et al., 2006, Lambert et al., 1998). Additionally, the role of different cleavage variants, Aβ1–40 and Aβ1–42, has been under investigation for some time. They are the two dominant Aβ peptides produced by β-secretase and γ-secretase. In vitro, Aβ1–40 tends to be more stable in isolation and remains in the monomer stage longer before it aggregates to form fibrils. Aβ1–42 tends to remain in a mix of monomer, trimer, and tetramer form until it aggregates into fibrils (Sengupta et al., 2016).

Toxic, soluble Aβ species in different forms have been isolated from transgenic mouse brains and the human brain in vitro under various experimental conditions (Karran

et al., 2011; Mattson, 2000; Oddo et al., 2003). Dodecamers appear to be diffuse throughout the tissue and exert toxic effects in cultures. Aβ oligomers contain mainly replicates of Aβ trimers and were identified in the brains of Tg2576 mice (Lesne et al., 2006). This soluble aggregate of Aβ appears to impair memory function in these animals, irrespective of neuronal loss and leads to synaptic dysfunction in humans (Lesne et al., 2006). Consistent with its toxic effects, these Aβ oligomers were also found to correlate with markers of synaptic dysfunction and levels of hyperphosphorylated tau (Sengupta et al., 2016). This suggests that toxicity is dependent on the size, aggregation state, and diffusion of Aβ oligomers.

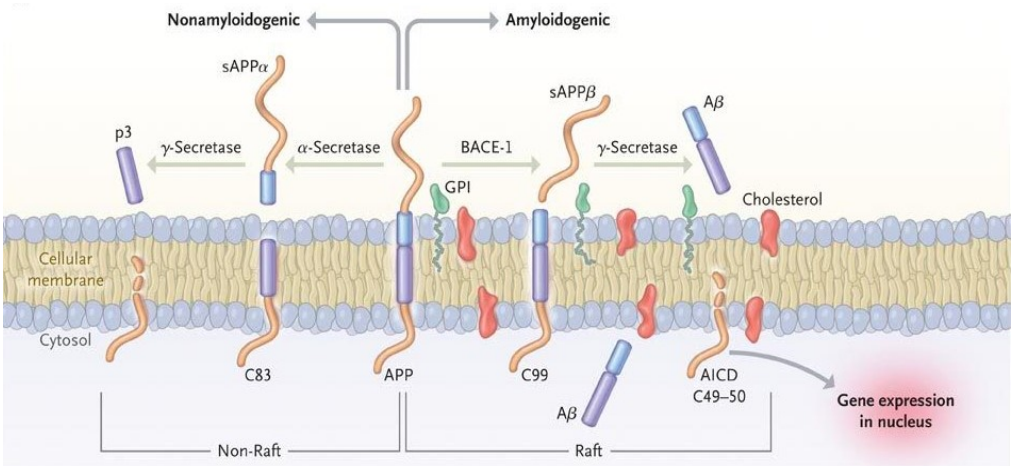

Figure 3. Processing of Amyloid Precursor Protein. In Panel A, α-secretase cleaves the amyloid precursor protein (APP) initiating the nonamyloidogenic processing. sAPPα and a carboxy-terminal fragment, C83 is then produced and C83 is further digested by γ secretase, liberating the amyloid intracellular domain (AICD). β-secretase beta-site amyloid precursor protein cleaving enzyme 1 (BACE-1), cleaves APP releasing sAPPβ and C99, initiating the amyloidogenic processing. Further C99 is digested by γ secretase releasing Aβ. Reproduced with permission from (Querfurth and LaFerla, 2006), Copyright Massachusetts Medical Society.

Aβ Clearance. Aβ clearance from the brain occurs through enzymatic and non-enzymatic pathways. Non-enzymatic pathways include 1) the bulk flow of the interstitial fluid (ISF) into the CSF by ISF drainage pathway through perivascular basement membranes, (Cserr, 1983; Hladky and Barrand, 2014; Bedussi et al., 2015) 2) the uptake by microglial or astrocytic phagocytosis (Jones et al., 2013; Fu et al., 2014), and 3) the transport across the blood brain barrier mediated by clearance receptors such as low-density lipoprotein receptor-related protein 1 (LRP1) (Jones and Shusta, 2007; Storck et al., 2016; Fiala et al., 1998), very low-density lipoprotein receptor (VLDLR) (Holtzman et al., 2012; Deane et al., 2009) and P-glycoprotein localized predominantly on the abluminal side of the cerebral endothelium (Terasaki and Ohtsuki, 2005; Cornford and Hyman, 2005).

Enzymatic clearance pathways include the Aβ-degrading enzymes neprilysin (Yoon and Jo, 2012; Baranello et al., 2015; Hafez et al., 2011), insulin-degrading enzyme (IDE) (Duckworth et al., 1998; Bates et al., 2009), and matrix metalloproteinase-9 (MMP9) (Miners et al., 2008; Yoon and Jo, 2012).

Aβ is trafficked via the interstitial fluid (ISF) into the CSF via bulk flow; however, this has been shown to be a minor route for Aβ clearance in mice, as it only accounts for approximately 10% of Aβ clearance (Fagan et al., 2009). Microglia, the brain's local macrophages, play an essential role in Aβ clearance through their ability to take up and degrade soluble and fibrillar forms of Aβ (Rogers et al., 2002; Lee and Landreth, 2010; Jiang et al., 2008). Both microglia and astrocytes secrete proteinases, including IDE, that mediate the degradation of Aβ peptides in the extracellular environment (Butovsky et al., 2007). Microglial cells have neuroprotective actions by

producing neurotrophic factors and by eliminating Aβ from the brain by phagocytosis before formation of senile plaques (Kurochkin and Groto, 1994; Simard and Rivest, 2006; Harry and Kraft, 2008).

The other route for Aβ clearance is direct trafficking out of the brain via the blood–brain barrier (BBB) into peripheral circulation (Shaw et al., 2009). Astrocytes, a component of BBB plays a role in plaque maintenance and Aβ clearance (Lee and Landreth, 2010; Kraft et al., 2013). Animal experiments demonstrated that astrocytes internalize Aβ by receptors such as LRP1, which has a function to transcytose ligands across the BBB. LRP1 also has the capability to uptake Aβ bound to apolipoprotein (Zhao et al., 2009). Aβ1-40 is cleared rapidly across the BBB via LRP1 while Aβ1-42 is removed across the BBB at a slower rate (Thal, 2012; Deane et al., 2009; Storck et al., 2016).

The brain contains vigorous essential Aβ clearance mechanisms (Tanzi et al., 2004). Aβ peptides are proteolytically degraded within the brain principally by neprilysin (Iwata et al., 2004; Baranello et al., 2015; Hafez et al., 2011) and IDE (Kurochkin and Goto, 1994; Duckworth et al., 1998; Bates et al., 2009). Administration of inhibitors of these proteinases into the brain results in substantial elevation of Aβ levels in the brain and stimulation of plaque deposition (Dolev et al., 2004). On the other hand, overexpression of neprilysin or IDE lowered brain Aβ levels and reduced plaque formation (Lehman et al., 2003). It has been argued that the principal approach of Aβ42 clearance from the brain is through its proteolytic degradation because this peptide is not efficiently transferred through the vasculature (Jiang et al., 2008).

Other Hypotheses of AD

While the amyloid cascade hypothesis is currently the most widely accepted hypothesis of AD, other hypotheses have been proposed. These hypotheses tend to incorporate the development of plaques that are found between the dying cells in the brain; from the build-up of a protein, Aβ (Harman, 2006; Fulda et al., 2010), neuritic plaques (NPs) (Serrano-Pozo et al., 2011; Li et al., 2016), tau protein dysfunction (Mondragon-Rodriguez et al., 2014), and intracellular neurofibrillary tangles (NFTs) (De Strooper, 2010).

Studies on the correlation of NFTs and NPs to cognitive deterioration in AD have yielded differing results (Nelson et al., 2007). For this reason, NFTs and NPs may contribute only slightly to dementia, or NFTs and NPs are less relevant to cognitive decline in older patients with AD than other pathologic processes (De Strooper, 2010; Nelson et al., 2007). A study quantitatively assessing the numbers of intracellular and extracellular NFTs in AD brains and of controls, found that in some cases, NFTs were not removed from the brain throughout the disease process (Mattson, 2000). This finding provides evidence that the protein constituent of NFT is resistant to proteolytic removal due to extensive cross-links. Additionally, correlation between the number of NFTs and neuronal loss indicates there are at least two distinct mechanisms responsible for neuronal death in AD that is related to the presence of neurofibrillary pathology (Yang et al., 2003).

Another proposed pathological component of AD, paired helical filaments of tau are formed first in the intracellular space of the neuron, and coalesce to form NFTs in the soma and axonal processes. At the same time or a little later, precursor proteins (either in

the dimeric or higher aggregated forms) are deposited in the extracellular space associated with the pre-synaptic axonal processes. These deposited proteins form the amyloid fibrils which may either crystallize within the center of a plaque, or the fibrils or their precursor subunits may migrate and accumulate around and within the walls of small blood vessels (De Strooper, 2010).

Aβ aggregation and deposition triggers a process that leads to neuronal loss via the formation of paired helical filaments of tau (Saido et al., 1995,1996; Fagan et al., 2009). Nonetheless, it remains to be clarified how deposited Aβ triggers or accelerates tau pathology and how tau pathology ultimately leads to neuronal loss (Fiala et al., 2007). However, a recent study demonstrated that the formation of intracellular amyloid-like fibrils resulted in the seizing of key intracellular proteins, which then led to the imbalance of essential cellular processes (Mucke and Selkoe, 2012). It is possible that intracellular tau aggregates act in a similar fashion (Glabe, 2006). Tau pathology follows a regular neurological path that correlates with the observed sequence of cognitive defects. Tau pathology also correlates with neuronal loss and cerebral atrophy, which in turn correlates with dementia. In addition, preclinical evidence suggests that in the absence of plaques, tau pathology can spread throughout the brain (Hardy, 2005).

In summary, Aβ aggregation and deposition is a very early event that can trigger tau pathology and will do so in many individuals, but the deposition of Aβ does not correlate with the presence of neurofibrillary tangles, cell loss or dementia. Given the current knowledge discussed above, and recognizing that there are considerable gaps in our understanding of AD pathology, one hypothesis that accommodates most of the known data is that Aβ aggregation and deposition triggers a process that leads to neuronal

loss. Nonetheless, it remains to be clarified how deposited Aβ ultimately leads to neuronal loss.

Genetic Aspects of AD

Early studies in familial AD (FAD) identified a locus on the long arm of chromosome 21, the region known to be duplicated in Down's syndrome, that encodes for APP. Subsequently, seven different mutations in the same gene have been found, all of which increase the production of Aβ 1–43 leading to fibrillar aggregation toxic to neurons (Shen and Kelleher, 2007).

Autosomal dominant mutations that cause early onset familial AD (eFAD) occur in three genes: presenilin 1 (PSEN1), presenilin 2 (PSEN2), and APP (Mattson, 2000). PSEN1 and PSEN2, are homologous proteins that can form the catalytic active site of gamma-secretase. Also apolipoprotein E (*APOE*) gene represents the major genetic risk factor for AD (Pastor et al., 2003). Humans contain three common *APOE* alleles: *APOE2*, *APOE3* and *APOE4*. The *APOE4* allele is responsible for the age of disease onset, *APOE3* is neutral, and *APOE2* may be protective (Dal Forno et al., 2002; Karran et al., 2011). Compared to individuals lacking *APOE4* alleles, the risk for people who possessed one *APOE4* allele were at a 2-3-fold increase while individuals with two *APOE4* alleles were at a 12-fold increase in AD onset (Kim et al., 2009). A series of experiments in some APP transgenic mice that had the human *APOE* genes indicate that these associated proteins facilitate the clearance of amyloid-β, with *APOE3* and *APOE4* being increasingly less effective at clearing Aβ (Allan and Ebmeier, 2011; Karran et al., 2011).

Other AD risk factor genes are being discovered via genome-wide association (GWA) studies. Of interest, the genes that cause the autosomal dominant form of the disease have not been detected as risk factors in the GWA studies that have been conducted so far (Van Broeckhoven and Kumar-Singh, 2006). The most likely explanation for this is that the common genetic variation around these three genes is simply not sufficient to alter their expression in a way that increases the risk of AD (Jones et al., 2010).

The amyloid cascade hypothesis appears to explain and incorporate several key points that are relevant to the disease process, including the pathology, the phenotypes mediated by the genes that cause autosomal dominant disease, and the genetic risk caused by the *APOE* gene (Morris et al., 2010). However, this hypothesis does not consider the interaction of Aβ with tau. This was clarified, to some degree, by the discovery that mutations in the tau gene could cause dementia in the frontal lobe. Thus, tau pathology itself can cause neuronal loss. This observation places tau pathology downstream of Aβ pathology (Dal Forno et al., 2002).

For the FAD-linked APP gene mutations, there are potential effects that pre-dispose individuals to early onset AD. If the amount of Aβ42 is increased sufficiently, then early onset AD can result. For the amyloid-β C-terminal APP mutations, the increase in the Aβ42/Aβ40 ratio is sufficient to cause early onset AD (Querfurth and LaFerla, 2006). For the presenilin mutations, the increase in the ratio of longer Aβ42 or Aβ43 versus shorter Aβ40 or Aβ38 species, even in the framework of an overall reduction in amyloid-β production, is sufficient to cause early onset AD (Winkler et al., 2012). A recent study confirmed that Aβ43 is at least as pathogenic as Aβ42, and studies in

patients demonstrated that the ratio of Aβ peptides in the CSF of carriers of the presenilin 1 mutation was substantially different from control patients and patients with sporadic AD (SAD) (Lippa et al., 1996).

Increases in the Aβ42/Aβ40 ratio or increases in the levels of Aβ42 or Aβ43 in the brain will cause individuals to develop AD (Bernstein et al., 2009; Lee and Han, 2013). However, the duration of the disease appears to be unchanged by genetic factors. This implies that changes in the amount of Aβ or Aβ ratios are not important modifiers of the disease process (Pike et al., 1993; Glabe, 2006). The reduction in Aβ42 levels in the CSF is an early event in the disease process (Fagan et al., 2006, 2009) but there are very few studies demonstrating that once Aβ deposition has occurred, the amount of deposition can be correlated with neuronal loss, paired helical filaments of tau or dementia.

Therapeutic Strategies

It is always preferable to treat any disease as early as possible. If the trigger event is the onset of Aβ deposition, then testing the amyloid cascade hypothesis would require a clinical trial in cohorts with normal scans of people who are at risk of developing AD (De Meyer et al., 2010). This represents a huge clinical challenge, including the selection of the clinical trial population and ethical considerations (Karran et al., 2011).

An effective treatment for SAD depends on the interpretation of the disease pathways. Prospective studies show that cognitive leisure activity and training can lower the risk of dementia (Verghese et al., 2003). Findings from these studies provide support for the concept of building a cognitive backup. Epidemiological studies suggest that simple lifestyle changes may be adequate to slow the onset and progression of AD (Pope,

2003; Beckett et al., 2015; Scarmeas et al., 2009; Buchman et al., 2012; Scarmeas et al., 2011; Larson et al., 2006; Lindsay et al., 2002; Podewils et al., 2005; Abbott et al., 2004; Yoshitake et al., 1995; Ravaglia et al., 2008).

There are several strategies that are being developed to identify risk factors for the development of AD. Plasma cortisol levels, a widely used physiological marker of the stress state, have been correlated with the progression of AD (Dong and Csernansky, 2010; Csernansky et al., 2006). There have been reports of chronic isolation stress which leads to accelerated plaque development in certain mouse models of AD (Dong et al., 2008; Rothman and Mattson, 2010; Hebda-Bauer et al., 2013; Cordner and Tamashiro, 2016). Exercise training reduces oxidative stress and improves neuron regulation which counteracts damages from stress and age related neuronal degeneration (Kiraly and Kiraly, 2005; Cho and Roh, 2016). Friedland et al. (2001) validated that patients with AD were less active, both intellectually and physically, in midlife and that sedentariness was associated with a 150% increased risk of developing AD (Friedland et al., 2001). Similarly, Laurin et al. (2001), also demonstrated that physical activity was protective against the development of cognitive impairment (Laurin et al., 2001). Exercise training may be a major protective factor but without clinical guidelines, its prescription and success with treatment adherence remain elusive (Kiraly and Kiraly, 2005). The remainder of this review will focus on physical activity and exercise training as a preventative measure against the development of chronic disease in general and AD specifically.

Physical Activity and Disease

Physical activity has been shown to elicit positive effects in the prevention and delay of chronic diseases such as cardiovascular disease, depression, diabetes, and breast cancer (Morris et al., 1973; Dunn and Dishman, 1991; Knowler et al., 2002; Friedenreich and Cust, 2008). The most universally studied of the above-mentioned diseases is cardiovascular disease, and the role that physical activity plays in it has been well-defined. Extensive study into neurological diseases has provided evidence indicating that the risk factors for cardiovascular disease overlaps with AD (Kesaniemi et al., 2001). Several epidemiological studies have shown that the switch from a sedentary to a physically active lifestyle reduces AD risk in humans (Fletcher et al., 1996; McGough et al., 2011; Vreugdenhil et al., 2012).

Physical Activity and Exercise Training. According to Casperson et al. (1985), physical activity could be divided into categories that are mutually exclusive of each other. Physical activity can be divided into light, moderate, or moderate intensity; those that are willful or compulsory; or those that are weekday or weekend activities. These are adequate ways of subdividing physical activity and the only condition is that the subdivisions be mutually exclusive and that they sum to the total caloric expenditure due to physical activity which may relate to a specific aspect of health (Casperson et al., 1985). For example, physical activity of high intensity in a structured, planned, and repetitive manner could result in an exercise training effect leading to an improved physical fitness state, and better health. Existing work in the field of exercise physiology suggests that normal daily physical activity (light physical activity) alone does not reduce

the risk of diseases but an exercise training strategy typically results in a state of increased physical fitness (Haskell, 1994).

Physical Activity in AD. It is becoming apparent that several risk factors for cardiovascular disease are also risk factors for neurodegenerative diseases including AD. Lower motivation of AD patients to exercise training regimens could be an obstacle for clinical studies, although this can be overcome, at least in part, by experimental studies (Selkoe and Schenk, 2003). Wilson and his group reported that physical activity for only several hours a week was associated with the risk of development of AD in small populations (Wilson et al., 2002). Epidemiological studies show that midlife physical activity was associated with delayed AD onset (Fratiglioni et al., 2004; Coltman and Berchtold, 2002; Andel et al., 2008). Physical activity improved cognition in adults with AD and those with cognitive impairment and dementia (Heyn, 2004; Smith et al., 2014; Etneir et al., 2006; Colcombe and Kramer, 2003; Brown et al., 2013). Also, intervention studies suggest that physical activity may have an impact in reducing AD risk although volume and intensity of exercise training have not been defined.

Physical Activity and AD Transgenic Mouse Models

Because human epidemiology investigations are limited in their ability to study mechanisms related to AD, investigators have developed transgenic mouse models of the disease to use in this area of research. These studies, summarized in Table 1, have investigated physiological mechanisms underlying the effects of physical activity and exercise training on delaying the onset and slowing the progression of the disease process.

Table 1: Physical activity paradigms used in AD transgenic mouse models

Study	Mode	Intensity	Duration	Frequency	Length
Adlard et al 2005	Wheel Running	3-7-6.0 m/min	12 hr/day	7 days/week	5 months
Wolf et al 2006	Wheel Running	Not reported	12 hr/day	7 days/week	8.5 months
Cracchiolo et al 2007	Wheel Running	Not reported	12 hr/day	7 days/week	3 months
Nichol et al 2007	Wheel Running	2.4 m/min	12 hr/day	7 days/week	2 months
Parachikova et al 2008	Wheel Running	Not reported	12 hr/day	7 days/week	3 weeks
Richter et al 2008	Wheel Running	2.0 m/min	12 hr/day	7 days/week	10 weeks
Um et al 2008	Treadmill Running	13.2 m/min	60 min/day	5 days/week	16 weeks
Cho et al 2010	Treadmill Running	13.2 m/min	60 min/day	5 days/week	16 weeks
Cho et al 2015	Treadmill Running	10 m/min	60 min/day	5 days/week	5 weeks
Um et al 2011	Treadmill Running	12.0 m/min	60 min/day	5 days/week	3 months
Kang et al 2013	Treadmill Running	10 m/min	60 min/day	5 days/week	5 weeks
Gimenez-Llort et al 2010	Treadmill Running	4.2 m/min	30 min/day	5 days/week	5 weeks
Ke et al 2011	Treadmill Running	10 m/min	60 min/day	5 days/week	5 weeks
Liu et al 2011	Treadmill Running	11 m/min	30 min/day	5 days/week	5 months
Liu et al 2013	Treadmill Running	11 m/min	30 min/day	5 days/week	5 months
Garcia-Mesa et al 2011	Wheel Running	Not reported	12 hr/day	7 days/week	6 months
Garcia-Mesa et al 2012	Wheel Running	4.0 m/min	12 hr/day	7 days/week	6 months
Garcia_Mesa et al 2016	Wheel Running	3.3 m/min	12 hr/day	7 days/week	3 months
Xiong et al 2015	Treadmill Running	15 m/min	300 m/day	6 days/week	5 months
Zhao et al 2015	Treadmill Running	11 m/min	30 min/day	5 days/week	5 months
Yuede et al 2009	Wheel Running	10.9 m/min	60 min/day	5 days/week	4 months

Yuede et al 2009	Treadmill Running	10.9 m/min	60 min/day	5 days/week	4 months
Moore et al 2016	Treadmill Running	15 m/min and 32 m/min, 10% grade	60 min/day	5 days/week	3 months

Overview of Physical Activity in AD Transgenic Mouse Models. Physical activity as a method of decreasing cardiovascular disease risk has been studied using animal models for more than 50 years. This body of work is highly sophisticated in its construction of physical activity paradigms which assess the intensity, volume, and mode of physical activity and exercise training and the relationship of those variables to reducing cardiovascular disease risk. In contrast, physical activity has been investigated as an intervention against AD pathology in transgenic mouse models for only slightly over a decade beginning with the study of Adlard et. al. published in 2005. Except for Moore et al. (2016) these studies have not quantified physical activity in a manner that would allow the assessment of the effect of physical activity level on reducing risk of developing AD pathology.

Physical activity and exercise training studies using AD transgenic mouse models have utilized running wheels or motor driven treadmills in the administration of these treatments. As with all treatment programs, there are advantages and disadvantages to each mode of physical activity. Running wheels are advantageous in that they allow for voluntary activity for the animals, however, their disadvantage is that they do not allow for precise regulation of the intensity nor the volume of the activity in which the animals engage. With running wheels, it is not difficult to quantify the distance a mouse covers during a period of time (i.e., a 12 or 24 hour period), but it is not possible to know

anything about the manner in which the mouse covered that distance. For example, some mice will run in the wheel at a constant, slow speed, whereas, others will run in bursts of high speed followed by periods of no activity.

Another problem with wheel running is that there is a wide variation in running distance among animals within a study, making data interpretation difficult because health benefits of physical activity and exercise training are related to the amount of physical activity performed (Bherer et al., 2013; Noble et al., 1999). Treadmill running, on the other hand, allows for precise regulation of exercise training, but has been criticized as being a stressful intervention (Yanagita, et al., 2007). This could be detrimental in the study of AD as it has been demonstrated that stress accelerates plaque development and cognitive decline in AD transgenic mice (Dong et al., 2004; Yuede et al., 2009). The work of Yuede et al. (2009), comparing voluntary wheel running and treadmill running to sedentary controls does not fully support this concern however. Plaque counts were found to be lower in Tg2576 mice subjected to wheel running than those subjected to treadmill running. However, mice running on treadmills had lower plaque counts than sedentary mice, indicating treadmill running was an improvement over a sedentary lifestyle. Wheel running mice did have lower plaque count than treadmill running mice. In addition, however, hippocampal volume was similar between wheel running and treadmill running mice and both were greater than sedentary mice.

It is important in studies of physical activity and exercise training that some measure of improved physical fitness is assessed to demonstrate a training effect. This generally takes the form of an increase in maximum oxygen consumption of the animal or an increase in one of the mitochondrial oxidative enzymes in a working skeletal

muscle, the classic being citrate synthase (CS). To date only Moore et al. (2016) have done this in a study using an AD transgenic mouse model. When comparing wheel running to treadmill running, it has been demonstrated in rats that treadmill running results in increased soleus muscle CS activity (Bedford et al., 1979; Hilty et al., 1989), whereas, voluntary wheel running does not demonstrate this even when animals are divided into high and low running groups (Rhodes et al., 2005; Yancey and Overton, 1993; Noble and Farrell, 1994). Therefore, future study of physical activity and exercise training effects on delaying the onset and slowing the progression of the disease process in AD transgenic mouse models should utilize treadmill running to precisely regulate exercise intensities and volume.

Effects of Physical Activity on Soluble Aβ and Plaque. The Aβ peptide is normally present in the interstitial fluid of the brain in soluble form throughout life (Cirrito et al., 2003). In AD pathology, Aβ aggregates into toxic soluble oligomers and eventually insoluble plaque in a concentration dependent manner (Bero et al., 2011). Therefore, strategies, including physical activity, aimed at reducing soluble Aβ in the brain prior to disease onset have been investigated as a method of delaying the onset and slowing the progression of AD pathology.

AD transgenic mouse studies investigating the effects of physical activity on amyloid pathology have been equivocal. Physical activity has been shown to either decrease (Adlard et al., 2005; Lararov et al., 2005; Um et al., 2008; Yuede et al., 2009; Liu et al., 2013; Tapia-Rojas et al., 2016) or not change (Cracchiolo et al., 2007) brain amyloid plaque levels. Likewise, brain soluble Aβ levels have been found to decrease (Adlard et al., 2005; Um et al., 2008) or not change (Yuede et al., 2009) with physical

activity. A likely explanation for the equivocation is the differences in the physical activity paradigms among the studies. As stated above, some used wheel running others treadmill running. It is entirely possible that the variation in intensity of physical activity in these studies accounted for the different results.

To address this likelihood, Moore et al. (2016) conducted a study to more thoroughly investigate the effect of physical activity dose on soluble Aβ levels in the hippocampus and cortex of Tg2576 mice. Three groups were utilized, a sedentary group, a moderate intensity treadmill running group (15 m/min on a level treadmill), and a high intensity group (32 m/min, 10% treadmill grade). All animals in the treatment groups ran for 60 minutes per day, five days per week, for 12 weeks. The sedentary animals were placed on a stationary treadmill for an equal amount of time each day. The results demonstrated a clear dose-response of Aβ40 and Aβ42 to exercise training intensity. Additionally, the study demonstrated that the reduction of Aβ40 and Aβ42 was due to increased clearance as measured by a dose dependent upregulation of the Aβ degrading enzymes neprilysin, IDE, and MMP9, the blood brain barrier transport protein, LRP1, and the molecular chaperone, HSP70. The moderate intensity treadmill running protocol in this study was more intense than the physical activity protocols in most of the previous studies summarized in Table 1. This indicates a likely minimum physical activity intensity threshold below which the effect of the physical activity is not realized and suggests that investigators in the future much more precisely assess physical activity levels in the study of physical activity effects on AD pathology.

It is also likely that the differences among studies investigating physical activity on amyloid plaque in AD transgenic mice can be attributed to differences in physical

activity intensity as described above for soluble Aβ. At this point no study has been made of the effect of physical activity intensity on amyloid plaque. It will be the purpose of the study undertaken here to do so.

Physical Activity on Tau. Studies of the effect of physical activity on Tau and NFPs have been limited compared with those investigating Aβ. Belarbi et al. (2011) looked at the effects of 9 months of voluntary physical activity in THY-Tau22 mice and there was prevention of memory alterations, cholinergic defects, inflammatory and metabolism related modifications. Treadmill running has been shown to ameliorate the accumulation of phosphorylated tau in AD mouse models (Stranahan et al., 2012). In a long-term exercise training study, general locomotor and exploratory activity were exhibited in P301S tau transgenic mice along with reductions in full-length and hyperphosphorylated tau (Ohia-Nwoko et al., 2014).

Physical Activity and other factors. Symptoms such as weight loss, fatigue, decreased food intake, and weakness have been demonstrated in AD patients who are inactive. Physical activity in the form of a structured exercise paradigm may be beneficial to AD patients by improving learning, plasticity, angiogenic factors, cerebrovascular function and lower production of Aβ42 (Walsh et al., 2002).

High levels of Aβ42 deposition mediates apoptosis of neuronal cells or apoptosis itself. But exercise training improves neuronal function by modulating neurogenesis, synaptic plasticity, learning, and memory (Kuperstein et al., 2010). However, the mechanisms by which apoptosis is modulated by exercise training in AD remains uninvestigated. De Strooper (2011) evaluated exercise training to determine whether it could prevent or improve cell death in the brains of transgenic (Tg) mice, as found in AD

patients. The study demonstrated that caspases, Bax protein levels, and cytochrome c were significantly reduced in Tg mice brains that were exercised trained compared to sedentary control.

Pro-apoptotic protein (cytochrome c and Bax) reduction post exercise training in APP Tg mice may be mechanistically related to lower Aβ42 levels which may be mediated through apoptotic pathway inhibition (Anderson et al., 1996). Importantly in this study, the Tg mice brains had elevated levels of Bcl-2, an apoptotic protein, compared to sedentary control. This observation is critical because a previous study demonstrated that exercise training can result in neuronal resistance against oxidative and apoptotic damages mediated through reduction in Aβ-induced apoptosis (Molteni et al., 2002). Therefore, these results suggest that apoptosis of neurons responsible for AD pathogenesis can be modulated by exercise training which increases the clearance of Aβ.

Neurotrophic factors such as NGF, BDNF and CREB, which are known to link adult hippocampal neurogenesis and hippocampal function were decreased in AD and are clinically associated with Aβ42 depositions in the brain of AD animals and patients. Therefore, establishing the rationale for using treadmill exercise training as non-pharmacological approach for elevating neurotrophic factors has received the greatest attention from AD investigators (Lesne et al., 2006).

Chen and Russo-Neustadt (2009) demonstrated that hippocampal NGF, BDNF and CREB levels were down-regulated in Tg mice compared with non-Tg mice, but they were up-regulated after treadmill exercise training, indicating that treadmill exercise training increased levels of hippocampal NGF, BDNF and its downstream effectors CREB, which are important for cognitive function in Tg-NSE/PS2m mice. The

underlying molecular mechanisms could indicate that treadmill exercise training has the potential to enhance cognitive function in Tg-NSE/PS2m mice, possibly through an increased level of NGF, BDNF and CREB, which are important for neurogenesis in the hippocampus. This result indicates that treadmill exercise training has a better ability to induce antioxidant enzymes to cope with the oxidative stress formed in the brain of Tg mice with AD.

Heat shock proteins (HSPs) are ubiquitously expressed intracellular proteins that protect proteins and DNA from stress-induced damage. HSP-70 has been reported to inhibit the self-assembly of poly-glutamine proteins into amyloid-like fibrils. However, till recently, it was not known whether HSP-70 increases within the brains of AD-transgenic mice during exercise training (Hardy, 2005). Jones et al. (2010) showed that hippocampal HSP-70 level was down-regulated in Tg mice compared with non-Tg mice, and that the level of this protein in Tg mice was up-regulated after treadmill exercise training (Jones et al., 2010). Moore et al. (2016) demonstrated that HSP-70 was elevated by treadmill exercise training in a dose-dependent manner. These findings indicate that treadmill exercise training contributes to a neuroprotective effect on the brain of Tg mice with AD, suggesting that increased level of HSP-70 protein following treadmill exercise training may benefit the brain, making it more resistant to stress induced cell damage (Mosser et al., 1997; Broer et al., 2011).

Exercise training is suggested to reduce Aβ accumulation in cortical areas of AD transgenic mouse by increasing proteolytic degradation by proteasome. The exact molecular mechanisms underlying these favorable effects of exercise training in brain are not well known, but the MAPK, PI3K, and PI/Akt signaling pathways and the

transcription factor CREB have been involved at the molecular level (De Strooper, 2010). Exercise training also can change the function of glutamatergic systems, increasing NMDA receptor in the hippocampus, which are crucial in learning and memory processes. The health and psychological benefits elicited by regular physical activity in older adults contribute to healthy aging. Therefore, physical exercise training is a potential intervention to preserve or ameliorate cognitive function and behavior in AD (Khachaturian et al., 2011).

Taken together, any or all of the above-mentioned proteins are potential triggers for the neuronal cell death and survival. Therefore, studies investigating death and survival of neuronal cells exposed to AD would be a step in the search for therapy leading to recovery from AD and explanation of molecular mechanism responsible for the effect of therapeutic interventions such as exercise training to repress neuronal cell death (Yuan et al., 2003; Kang et al., 2013).

In summary, several studies in transgenic mouse models of AD suggest exercise training reduces soluble and insoluble Aβ levels in the brain. Epidemiological studies suggest that physical activity exerts beneficial effects on humans. But as reviewed extensively here, the lack of a specific exercise paradigm raises the question of quantifying exercise training in a controlled experimental setting to address dose-dependent beneficial effects. This study is the second part of an investigation into the dose response of exercise training on AD pathology. In the first part, Moore et al. (2016) demonstrated that three months of exercise training in young Tg25756 mice reduced soluble Aβ40 and Aβ42 and upregulated multiple Aβ clearance proteins in an exercise training dose dependent manner. This study extended the treadmill running employed by

Moore et al. (2016) to 12 months in the same mouse model and investigated its effect on amyloid plaque levels in the cortex and hippocampus and on cognitive function.

MATERIALS AND METHODS

Animals

This study was approved on 17 September 2010 by the Institutional Animal Care and Use Committee (IACUC), protocol number 10024. Tg2576 mice, from our in-house colony, expressing human APP695 with double mutations K670N and M671N driven by the hamster prion protein [147] were used for this study. Hemizygous male Tg2576 mice maintained on C57B6/SJL background were crossed with wild type C57B6/SJL females. Only male Tg2576 offspring were used in this study. Animals were housed individually in Optimize ventilated cages and provided food and water *ad libitum*. Tail snips were acquired at the time of weaning, 3 weeks of age, to confirm transgene expression. Genotyping was performed using a QIAGEN QIAamp Mini Kit (cat. no. 51304, Valencia, CA) to purify DNA, followed by RT-PCR analysis.

Experimental Design

Animals in high intensity (HI) and low intensity (LOW) groups were placed on a motor driven treadmill (Columbus Instruments, Columbus, OH) with an electric grid for motivation for 1 hour each day, 5 days per week, for 12 months. LOW animals ran at a speed of 15 m/min, 0% grade. HI animals ran at a speed of 32 m/min, 10% grade, as we have previously observed that most animals are able to run at this speed for one hour daily (Moore et al., 2016). Sedentary (SED) animals were removed each day from their home cage and placed on an immobile treadmill to keep handling and environmental stimuli consistent among groups.

Table 2: Summary of experimental design.

	HI	LOW	SED
Age at beginning of exercise training	3 months	3 months	3 months
Run time	12 months	12 months	N/A
Speed	32 m/min,10% grade	15 m/min, 0% grade	N/A
Age at time of behavioral analysis	14 months	14 months	14 months
Age at end of Exercise training	15 months	15 months	15 months

Body Weights and Food Intake

Body weights of animals were monitored once at the end of every month during the 12-month exercise training strategy. Baseline weights were recorded prior to the training strategy. At the end-point, food consumption was monitored over 24 hrs prior to sacrifice. All animals were individually housed so 30 grams of food was weighed and provided to their home cages. 24 hrs later, food was weighed again and subtracted from the previous weight.

Tissue Preparation

Animals were sacrificed at the end of month 12 of treatment by isoflurane. Brains were promptly removed and immersed in 4% paraformaldehyde for 24 hrs then transferred to a 30% sucrose solution to store for later histological analysis. The soleus muscle was collected from each hind limbs and snap frozen on dry ice, and then stored at -80 °C for citrate synthase analysis.

Citrate Synthase Analysis

Following sonication in CellLytic MT buffer, soleus muscle citrate synthase (CS) activity was assessed (Sigma Aldrich Citrate Synthase Enzyme Activity Kit, Appendix A-1). Absorbance was measured at 412 nm on a BioTek Epoch plate reader and values for left and right soleus muscles of each animal were averaged. Total protein was quantified (Thermo Scientific™ Pierce™ BCA™ Protein Assay).

Tissue Sectioning and Amyloid Plaque Staining

Brain hemispheres were mounted for sectioning using Tissue-Tek® Optimal Cutting Temperature (O.C.T.) compound (Electron Microscopy Sciences, Hatfield, PA). Using a cryostat (Microm HM550, ThermoFischer Scientific, Walldorf, Germany), coronal sections 40 μm thick were taken at the level of the hippocampus (5.5 mm deep) and every 8th section was saved for staining.

DAB Staining. Sections were fixed and stained using mHJ3.4B, a biotinylated reporter antibody specific to N-terminus of Aβ. After incubation with streptavidin-HRP, Aβ plaques were detected by DAB and viewed using light microscopy. Plaque area in the cortex and hippocampus was determined using NIH Image J software. Images were captured for plaque staining at 40X magnification (Appendix A-2).

Thioflavin S Staining. Plaque staining was also analyzed with Thioflavin S staining. Slides were incubated in filtered 1% aqueous Thioflavin-S (Sigma) for 5 min, dehydrated twice in 70% ethanol for 5 min, and washed twice in PBS for 2 min. Mosaic images were captured for amyloid plaque staining at 10X magnification (Appendix A-2).

Behavioral Analysis

Mice were subjected to behavioral analyses during the last two weeks of treatment in the form of open field, Morris water maze (MWM) and novel object recognition (NOR) testing to evaluate spatial and recognition memory. Behavioral analysis was tracked in real time by an overhead camera and videos were analyzed using the ANY-Maze software (Stoelting Co).

Open Field. Mice were placed in the center of a white box (l38.5xw30xh30 cm) and allowed to move freely for 10 min. The distance traveled and average speed were measured using the Any-Maze software (Appendix A-3).

Morris Water Maze. Morris Water Maze testing was conducted in a 150 cm diameter pool divided into four equal quadrants with the "goal quadrant" containing a submerged platform that the mice had to find. Mice had a 7 day acquisition period with four 60 second trials per day. Two days of cued trials first took place in which a tennis ball denoted platform location. Four trials per cued day occurred where the submerged platform was cycled through each quadrant. Mice were started opposite to the goal quadrant in each of these trials. Five days of place trials then followed the cued trials. For each of these successive trials, black shaped cues were placed on the walls surrounding the pool and mice were started in a different quadrant using a random start pattern. In both the cued and place trials latency (time to find platform) was recorded with a maximum of 60 seconds allowed. If the animal did not find the platform within 60 seconds it was led to the platform. All animals, whether or not they found the platform, were allowed to remain on the platform for 30 seconds. A single probe trial was evaluated after a one hour delay following the last place trial. The platform was removed

for the probe trial and percentage of time spent in the goal quadrant was recorded. All MWM trials were evaluated using ANY-maze video tracking system (Stoelting Co., Wood Dale, IL) (Appendix A-4).

Novel Object Recognition. Mice were first acclimated to the test area for 20 minutes, one day before testing. On test day, each mouse had a sample trial and a test trial. During the sample trial, the mouse was exposed to two identical objects for 10 minutes before returning to the home cage. After a 50 minute delay, the mouse was put back into the test area for the test trial with one of the objects from the sample trial and a novel object and exposed to the objects for 10 minutes. Test areas were cleaned with 70% ethanol after each trial. Sample and trial sessions were recorded using ANY-maze software. The time the animal spent with each object was assessed using a manual stopwatch. Active investigation was described as facing the object within close proximity (2 cm) and/or touching the object with forepaws. The amount of time spent investigating the objects was recorded and the time spent investigating the novel object as compared to the familiar object was analyzed as a measure of recognition memory (Leger et al., 2013) (Appendix A-5).

Statistical Analysis

Experimenters were blinded during data analyses. Repeated measures ANOVA was used to determine whether there was significant effects of treatment between objects in object recognition test with exercise treatment (SED, LOW, HI) as the between-subjects factor and the two objects (top, bottom; familiar, novel) as the within-subjects factor. Repeated measures ANOVA was also used to determine whether there was significant effects of treatment status between places trial days in MWM with exercise

treatment as the between-subjects factor and the five days of place trials as the within-subjects factor. One-way ANOVAs were performed to evaluate treatment effects on total investigation times in sample and test trials for object recognition and probe trails using MWM. One-way ANOVA was also used to analyze treatment effects on anxiety levels by total distance in the arena and time spent in the center zone in the open-field test.

For body weights and food consumption, One-way ANOVA was used to evaluate treatment effects between groups. Citrate synthase levels and amyloid-beta plaque levels were analyzed using One-way ANOVA between groups as well.

All analyses were performed using GraphPad Prism version 6 with statistical significance set at $p < 0.05$. If ANOVA suggested significant effects of group, Tukey's *post-hoc* comparison was used to evaluate between group differences. All data are presented as mean +/- SEM.

RESULTS

Body Weight and Food Intake

Sedentary (SED), High Intensity Exercise (HI), and Low Intensity Exercise (LOW) groups all progressively increased in body weight throughout the course of the study (Figure 4). Body weight did not differ among groups during the course of the study (Appendix B-1).

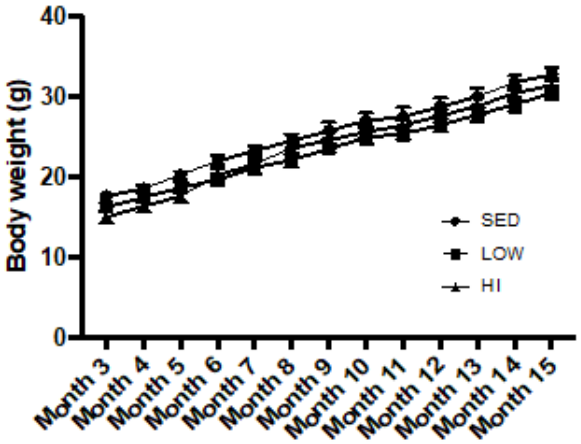

Figure 4: Body weight increased similarily in mice in all three groups during the 12 month duration of the study. Mice were three months old when training began. (SED, n=4; LOW, n=9; HI, n=5).

At the 14 month time point, food intake was assessed to check for a potential exercise training effect on appetite. There were no differences in food intake among groups over 24 hours (Figure 5, Appendix B-2).

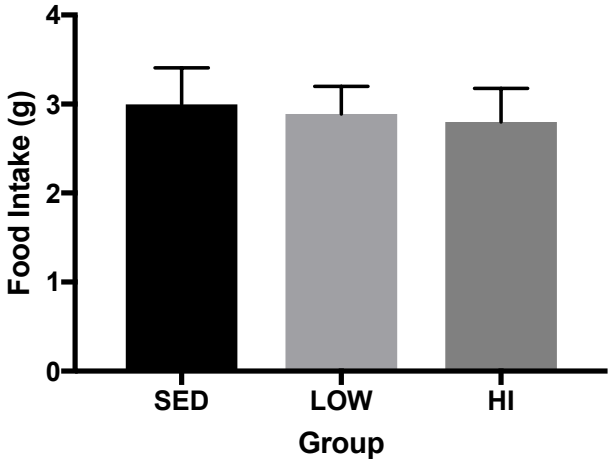

Figure 5: Food intake of mice, monitored over one 24-hour period, at the end of the treatment program. Error bars represent SEM (SED, n=4; LOW, n=9; HI, n=5). Groups are not statistically different from one another.

Citrate Synthase

Citrate synthase (CS) activity measurement is a standard test for assessing an exercise training effect in mice. CS activity was significantly different among all three groups (Figure 6). The HI group had a 110% increase in CS activity compared to the LOW group and a 425% increase compared to the SED group. The LOW group had a 149% increase in CS activity compared to the SED. Thus, indicating a clear dose response of the treadmill exercise paradigm in the groups (Appendix B-3).

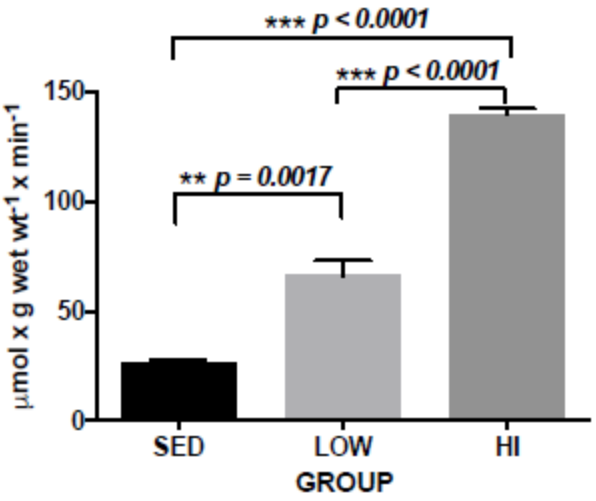

Figure 6: Citrate Synthase Activity. Values are expressed as activity of a gram of soleus muscle/min. Error bars represent SEM (SED, n=4; LOW, n=9; HI, n=5). One-way ANOVA and Tukey's post hoc analysis: **significant at $p < 0.0017$, ***significant at $p < 0.0001$.

Amyloid Plaque

As Tg2576 mice are a model of AD, amyloid plaque levels were assessed after 12 months of exercise training via immunohistochemistry methods. Amyloid plaque load was impacted by exercise training in both the cortex and hippocampus in a dose dependent manner (Figure 7). The HI group had significantly lower percent area in the cortex than the LOW (45.2%) and SED (58.5%) groups and the LOW group was significantly lower than SED (24.4%). In the hippocampus the HI group plaque area was significantly lower than the LOW (83.3%) and SED (88.6%) groups and the LOW group was significantly lower than SED (31.4%). Representative images of plaque in the cortex are shown in Figure 8 (Appendix B-4).

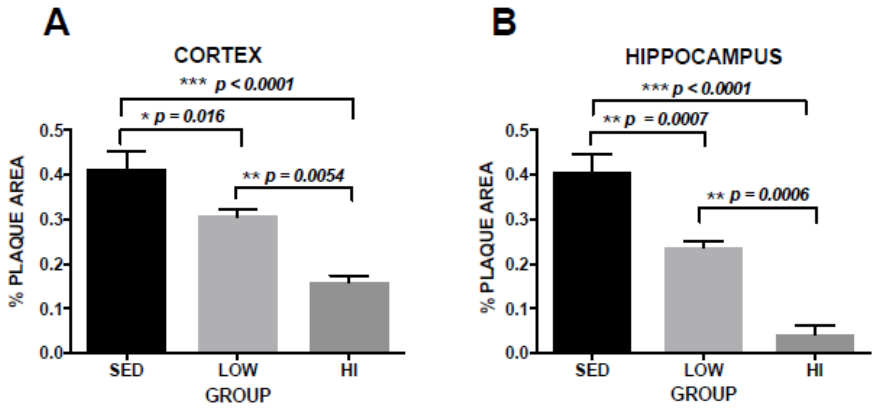

Figure 7: A. Percent area of amyloid plaque in cortex. Values are expressed as percentage of area in the cortex covered by amyloid plaques. Error bars represent SEM (SED, n=6; LOW, n=6; HI, n=5). One-way ANOVA and Tukey's post hoc analysis: * significant at p <0.016, ** significant at p < 0.0054, *** significant at p < 0.0001. **B.** Percent area of amyloid plaque in hippocampus. Values are expressed as percentage of are in the hippocampus covered by amyloid beta plaques. Error bars represent SEM (SED, n=6; LOW, n=6; HI, n=5). One-way ANOVA and Tukey's post hoc analysis: ** significant at p < 0.0007 between the SED and LOW groups and ** significant at p < 0.0006 between the LOW and HI groups, *** significant at p < 0.0001

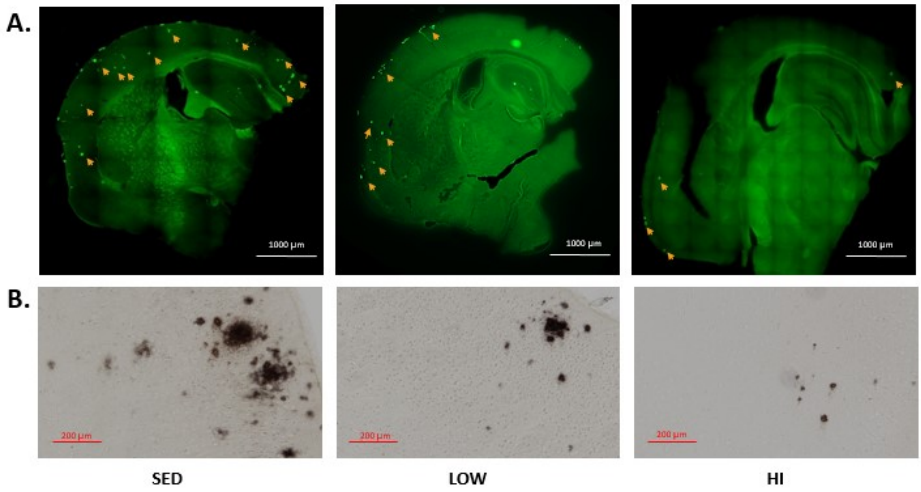

Figure 8: A. Representative images of ThioS staining of amyloid plaques at 10X magnification. Orange arrows point towards the plaques in the cortex. Amyloid plaques are lower in the HI group compared to the LOW and SED groups. Plaques are also lower in the LOW group compared to the SED group. **B.** Representative images of DAB staining of amyloid plaques in the cortex at 40X magnification. Amyloid plaques are significantly lower in the HI group compared to the LOW and SED groups. LOW group has lower plaques compared to SED group.

Behavior

Open Field. A potential confounding factor for the beneficial effects of high intensity treadmill exercise is whether the exercise routine modulates anxiety-like behavior. In the open field test, there were no differences in total distance covered and distance in middle grid between the HI, LOW, or SED groups (Figure 9, Appendix B-5).

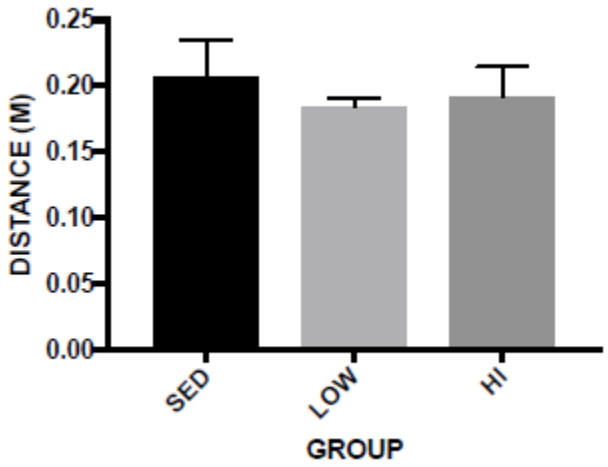

Figure 9: Open Field Test – Locomotor Activity. Values are expressed as total distance covered in the open field arena in area units. Error bars represent SEM (SED, n=4; LOW, n=9; HI, n=5). One-way ANOVA. No significant differences between groups.

Morris Water Maze. Spatial memory was investigated using a Morris water maze test. In the acquisition phase (Figure 10A), The HI group latency to the platform was significantly lower on days 3, 4, and 5 compared to day 1. The LOW and SED groups latency to the platform was significantly lower on day 4 compared to day 1 with LOW being lower on day 5, but SED day 5 latency was not significantly different from day 1. In the probe trial (Figure 10B and C), the HI group had a significantly faster time to the platform location and spent a longer time in the target quadrant compared to the

LOW and SED groups. The LOW group had a significantly faster latency to the platform location than the SED group, however, the time spent in the target quadrant was not different between the LOW and SED groups. Swimming speed was not different among groups (Figure 10D, Appendices B-6 and B-7).

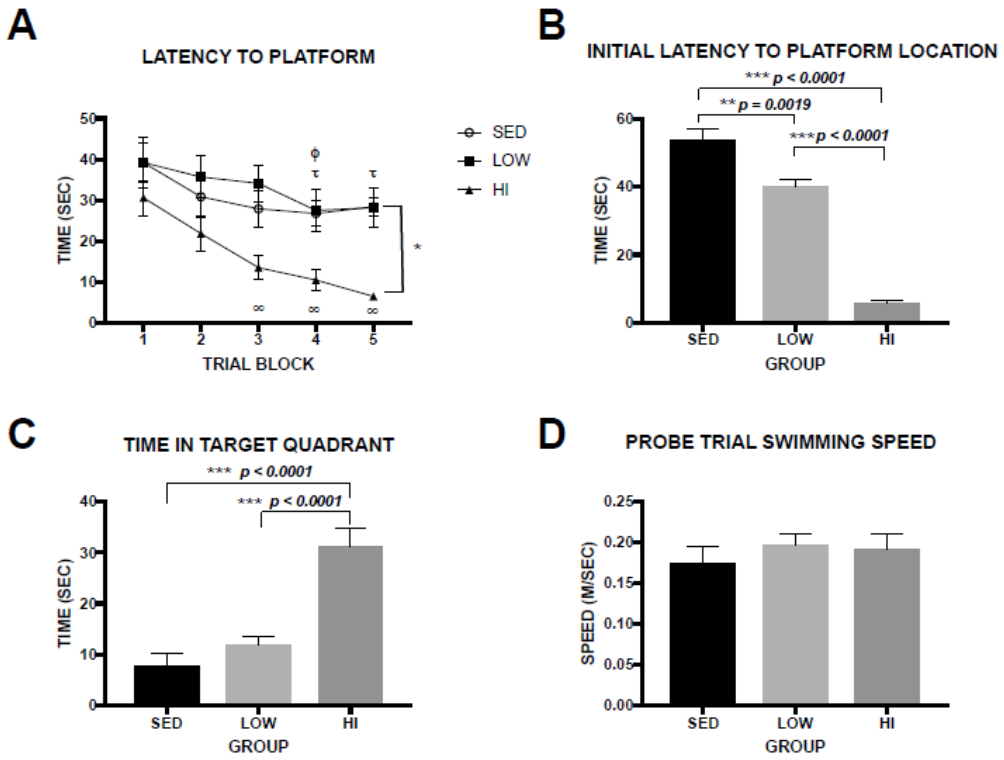

Figure 10: A. Morris water maze-acqusition phase. The HI group improved performance compared to the LOW and SED groups. Error bars represent SEM (SED, n=5; LOW, n=10; HI, n=6). **B.** Morris water maze-probe trial. The HI group had lower latency to platform compared to the LOW and SED groups. Error bars represent SEM (SED, n=5; LOW, n=10; HI, n=6). One-way ANOVA and Tukey's post hoc analysis: ** significant at $p < 0.0019$, *** significant at $p < 0.0001$. **C.** Morris water maze-probe trial. The HI group spent more time in target quadrant compared to the LOW and SED groups. Error bars represent SEM (SED, n=5; LOW, n=10; HI, n=6). One-way ANOVA and Tukey's post hoc analysis: *** significant at $p < 0.0001$). **D.** Morris water maze-probe trial. Values expressed as swim speed in meters per second. Error bars represent SEM (Sed, n=5; Mod, n=10; HI, n=6). One-way ANOVA. No significant differences between sedentary, moderate, and high intensity groups.

Novel Object Recognition. All groups investigated the similar objects equally at approximately 50% in the sample trial (Figure 11A). In the test trial, the high intensity mice investigated the novel object significantly more compared to the LOW and SED groups which investigated the novel object and familiar object equally. Total object investigation time did not differ among groups (Figure 11C, Appendices B-8 and B-9).

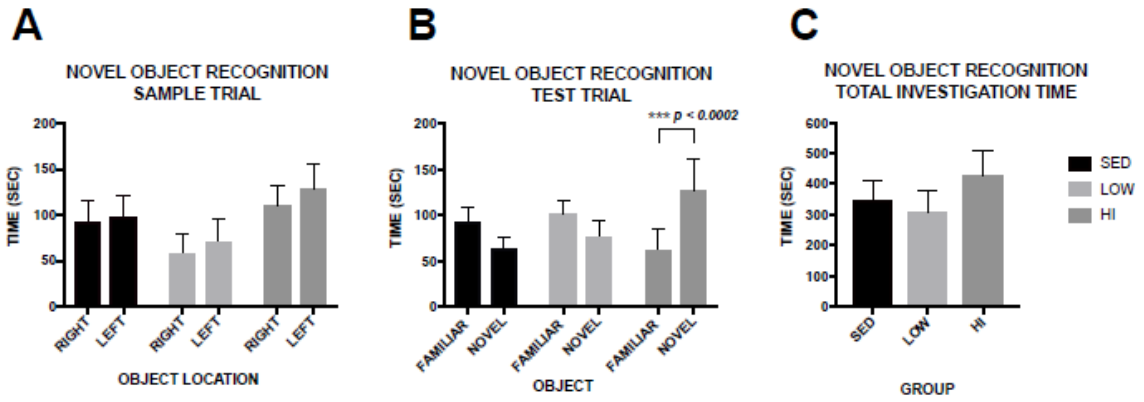

Figure 11: A. Object recognition test-sample trial. All groups spent the same amount of time investigating the two similar objects within groups. Error bars represent SEM (SED, n=9; LOW, n=10; HI, n=7). No significant differences existed among groups. **B.** HI group spent more time investigating the novel object compared to the familiar object. One-way ANOVA ad Tukey's post-hoc analysis: *** significance at $p < 0.0002$. Error bars represent SEM (SED, n=9; LOW, n=10; HI, n=7). No significance existed between novel and familiar object recognition in LOW and SED groups. Object recognition test-testing phase. **C.** All groups had similar total investigation times. No significant differences existed between groups. Error bars represent SEM (SED, n=9; LOW, n=10; HI, n=7).

DISCUSSION

Soluble Aβ is present in the interstitial fluid in the brain throughout life and aggregates into amyloid plaques later in life in a concentration dependent manner (Lomakin et al., 1997). Bero et al. (2011) published a definitive study on this topic demonstrating, using microdialysis, a tight relationship between ISF Aβ early in life and subsequent plaque deposition in aged mice in four different areas of the brains of Tg2576 mice. The findings of Bero et al. (2011) formed the conceptual basis for the two part study performed in our lab of which the work reported here is the second part. In the first part, Moore et al. (2016) found that soluble Aβ levels early in life were lower in the cortex and hippocampus of Tg2576 mice that were exercise trained by treadmill running. They further demonstrated that this effect was dose-dependent, in other words, the greater the exercise training intensity the lower the soluble Aβ levels in both brain areas assessed. This study utilized the exercise training paradigm of Moore et al. (2016), but rather than stopping the training period at six months of age it was continued until 15 months of age to determine whether or not the amyloid plaque levels in the 12 month exercise trained animals were reduced in a similar dose-dependent manner as the soluble Aβ levels in the younger trained mice. This study also determined the effects of the exercise training program on behavioral tests of anxiety, spatial memory, and recognition memory.

Body Weight and Food Intake

High intensity treadmill exercise training, as employed in this study, could potentially affect food intake and body weight of the mice. Studies throughout literature overlook these factors during or post physical activity training, possibly because of lower

running speeds or length of exercise regimen. Therefore, in the present study we addressed this issue to identify potential negative peripheral effects of 12 months of continuous treadmill exercise training.

It has been speculated that prolonged physical exercise could affect body weight (Chaput et al., 2011). Therefore, we monitored body weight once each month over the 12-month exercise regimen. All groups progressively increased in weight, without any significant differences among groups throughout the duration of the study. Various studies report that wild-type and transgenic mice increase in weight as they age, indicating that weight-increase over the lifetime of male mice is a natural effect (Inui, 2000; Ellacott et al., 2010; Gargiulo et al., 2014; List et al., 2014). The effect of exercise training on body weight in mice is equivocal with some indicating that body weight does not change in exercise trained mice and is lower than sedentary control mice (McMullan et al., 2016; Toti et al., 2011), whereas, others (Evangelista et al., 2015; Kemi et al., 2002) found no difference in body weight between sedentary and exercise trained mice. The data from this study are similar to those of Evangelista et al. (2016) and Kemi et al. (2002). The effect of exercise training on body weight in mice varies with different studies for reasons that are not entirely clear. It may have something to do with the intensity, duration and mode of the exercise training programs. A concern for us was that the high intensity training regimen for a continuous 12-month duration may be stressful to the animals, which may lead to weight loss. But the high intensity exercise trained mice gradually increased in weight just as the low and sedentary groups, further indicating that the mice adapted to the exercise intensity training regimen, which did not

affect their ability to naturally gain weight as they aged. However, mechanisms related to metabolism and weight gain/loss in exercise trained mice remains to be elucidated.

At the 14 month time point, food intake in all groups was assessed to determine whether or not exercise training affected appetite. There were no differences in food intake assessed 24 hours after the last day of training in any of the groups. These data indicate that the exercise regimen employed in this study did not affect food intake despite increased activity during exercise training between the high intensity, low intensity, and sedentary groups. Future experiments monitoring food consumption more regularly are needed to determine mechanisms relating to exercise treatment.

Assessment of Exercise Training

With the exception of Moore et al. (2016) from our laboratory no previous studies investigating the role of physical activity in delaying the onset or slowing the progression of Alzheimer's pathology in AD transgenic mouse models attempted to assess the degree of an exercise training effect. Like our pervious study we used citrate synthase activity in this study as a measure of an exercise training effect. We found a 149% increase in CS activity in the low intensity group compared to sedentary, a 425% difference between the high intensity group compared to sedentary, and a 110% increase in activity of the high intensity group compared to the low intensity group. These results indicate a clear dose-dependent exercise training effect in the animals in this study.

When using citrate synthase activity as a marker of an exercise training effect it is important that muscle samples are not harvested immediately following an exercise bout as it has been demonstrated that citrate synthase activity is elevated following an acute exercise bout (Leek et al., 2001). It is therefore necessary to allow the mice sufficient

recovery between the last exercise bout and removal of the soleus muscle so the data are not influenced by the final exercise bout. It is also important, however, to not wait too long following the final exercise bout and muscle harvest as this could result in a detraining effect. In this study we waited 72 hours following the final exercise bout to harvest the soleus muscle as it appears from previous reports that this is a sufficient period to allow citrate synthase activity to return to steady state following acute exercise (Siu et al., 2003).

The increases in CS activity in this study in both exercise trained groups compared to the sedentary group are greater than those of our previous study (Moore et al., 2016) where we found a 39% increase in CS activity in the low intensity group compared to sedentary and a 146% increase in CS activity in the high intensity group compared to the sedentary group. A possible explanation for this is that the mice in the previous study performed the exercise training program for three months whereas, the mice in the current study exercise trained for 12 months. The absolute numbers for the high and low intensity groups are not much different between the two studies, however, with high intensity group CS means being 128 and 139 μmol min^{-1} g wet $weight^{-1}$ for the previous and current studies, respectively and the low intensity group CS means being 75 and 66 μmol min^{-1} g wet $weight^{-1}$, respectively for the previous and current studies. The sedentary groups differed by a fairly large margin between the studies (52 vs. 26 μmol min^{-1} g wet $weight^{-1}$, respectively for the previous and current studies) which is the reason for the large percent difference between the studies in the low and high intensity group comparisons to the sedentary group. The reason for the large difference between sedentary group CS activity in these two studies is not readily apparent, although one

possibility could be the age difference as citrate synthase activity has been reported to be lower in gastrocnemius muscle of sedentary aged mice compared to sedentary young mice (Ryan et al., 2010).

To date, the neuroscience community has not quantified the impact of physical activity paradigms in the context of exercise training effects in studies investigating its impact in reducing risk of pathology associated with neurodegenerative diseases. It is highly likely that the intensity and/or volume of the physical activity conducted by an individual is directly related to risk reduction of chronic neurological diseases such as Alzheimer's as this is the case with other chronic diseases, primarily cardiovascular disease (Garber et al., 2011). In fact, this was demonstrated by our previous study (Moore et al., 2016). For this reason it is important that future studies investigating the effect of physical activity on reducing risk of neurological pathology assess the degree of a training effect, or lack thereof, produced by the physical activity paradigm utilized in the study.

Amyloid Plaque

Aβ deposition is a hallmark of AD pathogenesis. Aβ is normally present in the ISF of the brain in soluble form throughout life (Cirrito et al., 2003). In AD pathology, Aβ aggregates into toxic oligomers and eventually insoluble plaque in a concentration dependent manner (Bero et al., 2011). Therefore, strategies, including physical activity, aimed at reducing soluble Aβ in the brain prior to disease onset have been investigated as a method of delaying the onset and slowing the progression of AD pathology.

In the previous study from our laboratory, Moore et al. (2016) demonstrated that three months of exercise training in the form of treadmill running at two different

intensities (15 m/min on a level grade and 32 m/min on a 10% grade for one hour per day, five days per week) reduced ISF concentrations of soluble Aβ40 and Aβ42 in a dose dependent manner in the cortex and hippocampus of young Tg2576 mice. It was further demonstrated that a number of soluble Aβ clearance proteins were upregulated, the degree of which was also exercise training dose-dependent, indicating that the lower soluble Aβ levels were the result of increased Aβ clearance through multiple mechanisms. These mechanisms included; 1) increased activity of Aβ degrading enzymes NEP, IDE, and MMP-9, 2) upregulation of LRP1 increasing Aβ efflux from the brain across the blood-brain barrier, and 3) upregulation of HSP-70 increasing Aβ stabilization preventing aggregation. The work of Bero et al. (2011) suggests that if Tg2576 mice were subjected to the same exercise training protocol as used by Moore et al. (2016) for a prolonged period of time there should be a dose-dependent reduction in amyloid plaque load later in life. The results of this study support that hypothesis as the percentage of the area containing amyloid plaques in both the cortex and hippocampus of the mice used in this study was significantly decreased in an exercise intensity dose-dependent manner. Taken collectively, the results of this study and those of Moore et al. (2016) provide support for the concept that brain amyloid plaque levels late in life are closely related to soluble Aβ levels in early life and that strategies employed to keep ISF soluble Aβ levels low early in life do, in fact, lead to reduced plaque levels in later life.

The data from this study provide clarification to previous studies into the effects of physical activity interventions on amyloid plaque load in AD transgenic mice which have been equivocal. Most studies have reported reduced amyloid plaque load as a result of physical activity (Adlard et al., 2005; Lazarov et al., 2005; Um et al., 2008,2011;

Yuede et al., 2009; Liu et al., 2013), however others have not found physical activity to reduce amyloid plaque load (Cracchiolo et al., 2007). None of these previous studies have considered the issue of the dose-dependence of exercise training on amyloid plaque development. It is therefore, likely that the disparate results are due to differing intensities of the physical activity paradigms employed in these studies. For this reason it is recommended that all future studies investigating physical activity effects on reducing AD pathology assess the exercise training impact using some objective measure such as citrate synthase activity.

Another reason for differences among the studies mentioned above could be the method used to deliver the physical activity as some studies utilized running wheels (Adlard et al., 2005; Lazarov et al., 2005; Cracchiolo et al., 2007; Yuede et al., 2009) and some used motor driven treadmills (Um et al., 2008,2011; Yuede et al., 2009; Liu et al., 2013; Moore et al., 2016). It has been demonstrated that emotional stress accelerates amyloid plaque development in Tg2576 mice (Dong et al., 2004, 2008) and argued that treadmill running is not a good method of physical activity in studies using mice as it is an emotionally stressful intervention (Yanagita et al., 2007). Wheel running, on the other hand, is an activity that the mouse can perform at a time and intensity of its choosing and thus has the advantage of not being emotionally stressful. On the other hand, exercise training intensity cannot be controlled with running wheels as distance run on running wheels is highly variable among animals and is of low intensity as it has been demonstrated in rats that voluntary wheel running does not result in increased citrate synthase activity compared to sedentary control animals even when the animals are divided into high and low running groups (Nobel et al., 1999). A previous study from our

laboratory compared wheel running and treadmill running interventions in Tg 2576 mice and found that plaque counts were lower in wheel running mice that treadmill trained mice who ran at the same average speed as the wheel running mice. The running speed was extremely low, however (average of 10.9 m/min on a level treadmill), which likely did not produce an exercise training effect which unfortunately was not measured in that study. In future studies, investigating exercise training as a strategy for delaying the onset and slowing the progression of AD pathology it will be necessary to use treadmill running to control exercise intensity and volume, both of which are important contributors in AD risk reduction.

Behavioral Assessment

A prolonged forced exercise training may induce anxiety in mice which may affect treadmill running (Svensson et al., 2016). The open field test, an indicator of anxiety-like behavior, did not reveal any differences between the groups. This indicates that 12 months of forced exercise training did not cause anxiety in the mice and therefore, was unlikely to have an impact on the cognitive performance in the Morris Water Maze (MWM) or Novel Object Recognition (NOR) tests. These data are consistent with reports from Yuede et al. (2009) where forced treadmill exercise did not exhibit significant anxiety-like behavior in the open field test.

In the MWM test the HI exercise trained group learned the location of the platform faster than the LOW and SED groups as their latency to the platform in the place trials significantly improved from day 1 to day 3 and continued to improve throughout the five days. Analysis of simple main effects showed the performance of the LOW group significantly improved from day 1 to day 4 and five while the SED group

showed an indication of improved performance from day 1 to day 4 (day 1 to day 5 comparison was not significant). In the probe trial the latency to the platform location was far less for the HI exercise trained group than the LOW and SED groups and the latency to platform for the LOW group was significantly less than the SED group. Significantly improved spatial memory function is also demonstrated in the HI group by the increased time spent in the target quadrant relative to the LOW and SED groups. Swimming speed was not different among groups demonstrating that it was not a factor contributing to differences among groups in latency to platform during the place trial nor latency to platform location during the probe trial. To further evaluate behavioral changes resulting from the exercise training paradigms we subjected the mice to a NOR test to assess recognition memory. As with the MWM test, the HI group performed significantly better than LOW and SED, with the HI mice spending more time investigating the novel object than the familiar object compared to the LOW and SED groups. The LOW and SED groups did not differ in time spent investigating the novel and familiar objects.

Direct comparisons between our MWM and NOR data and those of others studying transgenic AD mice subjected to exercise training treatments using treadmill running are limited. Um et al. (2008) subjected NSE/APPsw mice who ran 13.2 m/min on a level treadmill for 60 minutes a day, five days a week for 16 weeks and a sedentary group to a MWM test. Their reported mean initial latency to the platform location times in the probe trial for the sedentary and exercise trained groups were approximately 55 and 35 seconds, respectively (times estimated from Fig 3c in their manuscript). Interestingly, these are very similar to the times for our SED (53.5 s) and LOW (39.9 s) groups. As for NOR, the only direct comparison is to the study of Yuede et al. (2009) from our

laboratory. In that study a group of Tg2576 mice subjected to treadmill running at an average speed of 10.9 m/min on a level treadmill for 60 min/day, five days/week did not differ from sedentary mice in preference for the novel object. This was also the case in this study with the LOW to SED comparison. The data from this study are in agreement with the previous studies with respect to behavior demonstrated in MWM and NOR testing in AD transgenic mice subjected to low intensity treadmill running.

Taken collectively, our MWM and NOR data point to a clear dose-response of Tg2576 mice to exercise training. In all cases, high intensity exercise training results in significant, and dramatic, improvement in performance on both spatial and recognition memory tasks compared to SED mice. Low intensity exercise training does improve spatial memory over the sedentary condition as demonstrated by the improved latency to the platform location in the probe trial of the MWM test indicating this treatment is beneficial over the sedentary condition, however this difference is subtle.

The mechanisms through which the "stair-step" dose response effect of the exercise training paradigm reduces amyloid plaque levels and improves cognitive function remains unknown. Although mechanistic and signaling targets such as glucose uptake, DNA repair, upregulation of neurogenic factors such as BDNF, increased levels of clearance proteins, and upregulation of angiogenesis are a few of the processes that may be modulated because of chronic physical activity, the mechanisms by which they are modulated remains uninvestigated. One way to measure a dose response could be to investigate cerebral blood flow. Although not consistently reported, increased cerebral blood flow to the brain post exercise may lead to increased brain metabolism (Querido and Sheel, 2007). Increased cerebral blood flow may also reduce the hypoxic condition of

AD brains, as hypoxia facilitates AD pathogenesis by up-regulating *BACE1* gene expression (Sun et al, 2006), and may also modulate blood-brain barrier permeability, regional brain glucose content, and local glucose utilization.

SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

In summary, these experiments have demonstrated that intensity and duration of exercise training are important factors in dissecting the link between physical activity and Alzheimer's disease, and thus relevant to designing exercise training paradigms. The exercise training interventions in this study helped to address the beneficial effects of long-term treadmill exercise interventions in improving cognitive function and decreasing amyloid-beta plaque levels in a dose-dependent manner in an AD transgenic mouse model. More importantly, the dose response was quantified and demonstrated by a metabolic assay which has been absent in previous studies in the field of AD and neuroscience. The low intensity treadmill running protocol in this study was more intense than the physical activity protocols in most of the previous studies (summarized in Table 1). This indicates a likely minimum physical activity intensity threshold below which the effect of the physical activity is not realized and suggests that investigators in the future need to precisely assess physical activity levels in the study of physical activity effects on AD pathology. Therefore, future study of physical activity and exercise training effects on delaying the onset and slowing the progression of the disease process in AD transgenic mouse models should utilize treadmill running to precisely regulate exercise intensities and volume.

This study demonstrated the beneficial effects of a long-term (12-month) treadmill exercise training regimen in cognitive improvement and amyloid plaque reduction in 15-month old Tg2576 mice. Reports suggest that reducing soluble Aβ concentrations subsequently reduces oligomerization and plaque levels in AD mouse models as aging progresses. Even though physical activity has been used as an

intervention to reduce plaque levels and improve cognition in AD mouse models (Parachikova et al., 2008), the prophylactic impact of exercise training at varying intensities for a prolonged period in reducing amyloid plaque is not established. Moore et al. (2016) demonstrated dose response reduction of soluble Aβ levels in the cortex and hippocampus of 6-month old Tg2576 mice after 3 months of exercise training, starting at 3 months of age. They completed the exercise training regimen prior to amyloid plaque development which is typically about nine months of age in Tg2576 mice. The current study was a follow-up to the Moore study, utilizing the same experimental design but with a long-term exercise training regimen.

For the first time, these experiments demonstrate that the intensity of exercise training is imperative to elicit a dose response on cognitive performance and amyloid plaque levels in an AD mouse model. An exercise training regimen in AD mouse models becomes relevant for potential exercise interventions in AD patients. Whether exercise training at varying intensities can be used as a preventative, treatment, or reversal strategy against the pathogenesis of AD needs to be investigated in human patients. The exercise training regimen in this study was for 12 months beginning before Aβ oligomerization began. In human patients, the age at which Aβ aggregation begins is difficult to detect. Firstly, novel techniques need to be developed to detect early levels of Aβ before aggregation. Lastly, a quantified exercise training regimen needs to be employed to elicit beneficial responses. Whether a long-term exercise training regimen like the one used in this study could be beneficial in human AD patients is still uninvestigated. In addition, the dose and duration of exercise intervention, and extent of

beneficial effects post exercise intervention are important questions that need to be addressed in the field of AD.

Furthermore, mechanistic pathways of physical activity in AD pathogenesis remain cryptic. Exercise training interventions which are quantified could shed light into APP processing, Aβ production, aggregation, and clearance in the brain. Understanding the structural and molecular characteristics of different Aβ species and its interactions with clearance/degradation molecules due to treadmill exercise may increase knowledge of potential therapeutic targets. Additionally, utilizing mouse models with mutated APP and altered clearance mechanisms could help define factors affecting the trafficking and processing of Aβ.

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APPENDICES

Appendix A: Protocols

Appendix A-1. Tissue protein extraction from soleus muscles with CellLytic™ MT and protein quantification with Pierce™ BCA protein assay kit.

Post isolation of left and right soleus muscle from the hind limbs of mice, the CellLytic kit, catalog number C 3228, from Sigma Aldrich was utilized for tissue protein extraction. Quantification of protein content in muscle was conducted through Pierce BCA protein assay kit from Thermo Fisher Scientific, catalog number 23225.

Appendix A-2. NIH ImageJ analysis of amyloid plaques

Procedure

Mosaic images of Thioflavin-S stain

1. Procure mosaic images at 10X magnification on a Zeiss Axio Imager M1 under identical exposure settings in the FITC channel
2. Equally threshold the images using the "Threshold" feature
3. For all representative mosaic images, set the boundaries of each individual brain section including the hippocampus and cortex using the mosaic function
4. Obtain sequential images that encompasses the entire area of the tissue boundary (between 25 and 50 images per section)
5. Stitch the individual images using the stitch function to produce a single image

Single images of DAB stain

1. Procure single images at 40X magnification on a Zeiss Axio Imager M1 under identical exposure settings in the brightfield channel
2. Export the single images onto NIH ImageJ software
3. Indicate brain regions of interest using the "Pencil" feature
4. Quantify Aβ plaques in the cortex and hippocampus using the "Analyze Particles" feature
5. Compare group means for the percentage of brain area measured

Appendix A-3. Open Field Test

Procedure

1. Place mice in the center of a white box (l38.5xw30xh30 cm) and allow to move freely for 10 min
2. Using Stoelting Anymaze software, measure the distance traveled and average speed

Appendix A-4. Morris Water Maze Test

Procedure

Morris Water Maze testing was conducted in a 150 cm diameter pool divided into four quadrants with the "goal quadrant" containing a submerged platform that the mice had to find. Mice had a 7 day acquisition period with four 60 second trials per day. If the animal did not find the platform within 60 seconds, lead it to the platform and allow to remain on the platform for 30 seconds. All MWM trials were evaluated using ANY-maze video tracking system (Stoelting Co., Wood Dale, IL).

I. Cued Trial
    1. Conduct two days of cued trials in which a tennis ball denotes platform location
    2. Conduct four trials per cued day where the submerged platform is cycled through each quadrant
    3. Place mice in quadrant opposite to the goal quadrant in each of the trials and record the time, distance, and speed covered

II. Acquisition Trial
    1. Conduct acquisition trial for five consecutive days. For each of the successive trails, place black shaped cues on the walls surrounding the pool
    2. Place the mice in a different quadrant using a random start pattern and record the time, distance, and speed

III. Probe Trial
    1. Conduct a single probe trial after a one hour delay following the last acquisition trial. Remove the platform.
    2. Place the mice in the quadrant opposite the goal quadrant and record the time, distance, and speed

Appendix A-5. Novel Object Recognition Test

Procedure

Mice were first acclimated to the test area for 20 minutes one day before testing

1. During the sample trial, place the mouse into the test arena for 10 minutes and expose to two identical objects
2. After ten minutes, return the mouse to its home cage
3. After a 50 minute delay, introduce the mouse back into the test area for 10 minutes during the test trial with one of the objects from the sample trial and a novel object
4. Record time spent investigating the objects for 10 minutes during the sample trial and test trial using Stoelting Anymaze software

Appendix B: Raw Data

Appendix B-1. Body weight raw data (All values in grams).

Animal #	Months												
Sedentary Group	3	4	5	6	7	8	9	10	11	12	13	14	15
1	17	18	20	22	23	25	26	28	28	29	31	31	33
2	19	19	21	22	23	25	26	28	29	31	32	34	35
3	17	18	19	20	22	22	23	24	24	26	27	30	31
4	18	20	21	24	25	26	28	28	29	29	30	32	32
Low Intensity Group													
1	19	19	22	23	25	26	26	28	28	30	32	34	35
2	16	17	18	20	21	23	24	26	27	28	30	31	31
3	16	17	19	21	22	24	24	26	27	28	29	29	30
4	16	17	19	19	22	20	22	22	22	24	24	28	30
5	18	20	19	18	18	18	21	21	21	22	25	26	29
6	14	15	16	18	19	22	23	25	25	26	27	27	28
7	15	16	18	20	22	22	24	26	26	27	28	30	31
8	15	16	16	17	17	19	22	23	25	25	26	26	28
9	18	20	21	21	25	25	26	27	28	28	29	30	32
High Intensity Group													
1	14	16	18	19	19	23	26	27	27	29	31	32	34
2	13	15	15	16	18	21	21	23	24	25	25	28	30
3	16	15	17	20	23	25	25	24	24	27	28	31	31
4	17	19	20	23	25	24	26	27	27	28	29	30	30
5	15	17	18	23	23	25	26	25	27	29	31	33	33

Appendix B-2. Food intake raw data (values are in grams).

Animal #	Food Intake
Sedentary Group	
1	2
2	4
3	3
4	3
Low Intensity Group	
1	2
2	2
3	4
4	3
5	2
6	4
7	4
8	2
9	3
High Intensity Group	
1	3
2	2
3	3
4	2
5	4

Appendix B-3. Citrate synthase activity raw data (values are in µmoles/min g wet weight).

Animal #	CS Activity
Sedentary Group	
1	24.44023
2	27.97466
3	26.06958
4	28
Low Intensity Group	
1	88.79949
2	68.84706
3	81.92579
4	70.06199
5	35.19393
6	55.61629
7	42.82353
8	96.07964
9	56.73935
High Intensity Group	
1	146.1271
2	144.478
3	138.4242
4	132.1587
5	137.4479

Appendix B-4. Amyloid plaque % area.

Animal #	Cortex	Hippocampus
Sedentary Group		
1	0.380000	0.584000
2	0.300000	0.393500
3	0.467000	0.303000
4	0.525500	0.333500
5	0.487000	0.377000
6	0.304000	0.446000
Low Intensity Group		
1	0.280500	0.249000
2	0.311000	0.205000
3	0.258500	0.291000
4	0.328000	0.213000
5	0.297500	0.247000
6	0.367000	0.221000
High Intensity Group		
1	0.1810	0.0860
2	0.1250	0.0000
3	0.1165	0.0260
4	0.1800	0.0975
5	0.1910	0.0040

Appendix B-5. Open field test raw data (distance covered in area units).

Animal #	Total distance	Middle field distance
Sedentary Group		
1	0.284	0.056
2	0.154	0.013
3	0.21	0.052
4	0.177	0.017
Low Intensity Group		
1	0.222	0.015
2	0.186	0
3	0.2	0.021
4	0.196	0.035
5	0.181	0.045
6	0.176	0.051
7	0.171	0.014
8	0.167	0.031
9	0.158	0.025
High Intensity Group		
1	0.162	0.037
2	0.157	0.013
3	0.252	0.117
4	0.147	0.101
5	0.242	0.117

Appendix B-6. Morris water maze acquisition phase time to platform raw data (values are in seconds).

Animal #	Day 1	Day 2	Day 3	Day 4	Day 5
Sedentary Group	28.75	22.5	21.75	22.25	23.75
1	58.25	49.25	45.25	37.5	36
2	35.25	34.75	25.5	30	31.5
3	25.75	25	24.5	20.75	24.25
4	48	22.75	22.5	23.25	26.5
5	54.5	60	52.25	47	39.5
Low Intensity Group					
1	48.75	45.5	30.25	17.25	19.75
2	50.25	60	60	51.25	60
3	25.25	29.5	33	32	28.75
4	24.75	16.25	19.75	7.75	18.75
5	60	59.75	60	60	44.25
6	48	34.5	30.25	16.5	21.25
7	60	46.25	40	32	27
8	17.5	15.25	9	9	13.25
9	31.5	17	25.75	9.75	6.25
10	28.5	27	31.5	28.5	44.75
High Intensity Group					
1	35.25	24	23	23.75	9.75
2	14	6.5	6.5	6.25	4.25
3	27.75	14	12.25	6	4.5
4	25.5	19.5	9	8.25	6.5
5	36.5	30.75	22.25	10.75	6
6	44.5	36.25	8	7.75	7.75

Appendix B-7. Morris water maze probe trial raw data.

Animal #	Time in target quadrant (s)	Latency to platform (s)	Distance traveled (m)	Avg. swimming speed (m/s)
Sedentary Group				
1	6	48.8	13.974	0.233
2	4.9	39.2	7.403	0.123
3	19.1	54	14.837	0.247
4	7.8	60	9.67	0.161
5	8.17	59	8.6	0.145
6	0	60	11.4	0.133
Low Intensity Group				
1	24.2	39.4	14.372	0.24
2	12	45.4	11.023	0.184
3	12.6	38.2	11.171	0.186
4	13.8	52.6	9.029	0.151
5	13.9	32.1	11.099	0.185
6	4.6	60	13.065	0.218
7	6.4	60	9.554	0.159
8	10.63	30.9	9.002	0.159
9	5.44	36	10.59	0.146
10	14.9	49	7.587	0.184
High Intensity Group				
1	41.2	5	13.138	0.219
2	28.2	8.4	14.138	0.236
3	22.5	5	13.642	0.227
4	38.2	6.2	13.225	0.22
5	20.35	7	13.645	0.236
6	36.1	3	14.118	0.287

Appendix B-8. Novel objection recognition test raw data (values are investigation times in seconds).

Animal #	Right side	Left side	Novel	Familiar
Sedentary Group				
1	130	132	103	111
2	95	123	65	157
3	10	13	67	121
4	106	105	59	55
5	17	15	17	27
6	30	17	37	37
7	184	189	83	140
8	59	112	18	53
9	200	175	120	126
Low Intensity Group				
1	3	4	72	85
2	129	217	115	135
3	59	67	48	78
4	12	11	52	91
5	14	20	32	74
6	3	7	4	6
7	11	18	152	133
8	190	170	165	191
9	61	51	70	96
10	26	34	34	46
11	8	9	79	95
12	25	56	24	69
High Intensity Group				
1	167	220	153	110
2	122	125	267	143
3	130	158	180	112
4	9	15	25	6
5	101	132	83	24
6	167	187	11	5
7	76	67	169	38

Appendix B-9. Novel objection recognition test raw data (values are percent of investigation time).

Animal #	Right side	Left side	Novel	Familiar
Sedentary Group				
1	49.6	50.4	48.1	51.9
2	43.6	56.4	29.2	70.8
3	43.5	56.5	35.6	64.4
4	50.2	49.8	51.8	48.2
5	53.1	46.9	38.6	61.4
6	63.8	36.2	50.0	50.0
7	49.3	50.7	37.2	62.8
8	34.5	65.5	25.3	74.7
9	53.3	46.7	48.9	51.1
Low Intensity Group				
1	42.9	57.1	45.9	54.1
2	37.3	62.7	46.0	54.0
3	46.8	53.2	38.1	61.9
4	52.2	47.8	36.4	63.6
5	41.2	58.8	30.2	69.8
6	30	70	40.0	60.0
7	37.9	62.1	53.3	46.7
8	51.4	48.6	46.3	53.7
9	54.5	45.5	42.3	57.7
10	43.3	56.7	42.5	57.5
11	47.1	52.9	45.4	54.6
12	30.9	69.1	25.8	74.2
High Intensity Group				
1	43.2	56.8	58.2	41.8
2	49.4	50.6	65.1	34.9
3	45.1	54.9	61.6	38.4
4	37.5	62.5	80.6	19.4
5	43.3	56.7	77.6	22.4
6	47.2	52.8	68.8	31.2
7	53.1	46.9	81.6	18.4