Summer 2021

Exercise Training Rescues Increased Brain ISF aβ Caused by Chronic Isolation Stress in APP/PS1 Transgenic Mice

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EXERCISE TRAINING RESCUES INCREASED BRAIN ISF Aβ CAUSED BY CHRONIC ISOLATION STRESS IN APP/PS1 TRANSGENIC MICE

A Master's Thesis

Presented to

The Graduate College of

Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Cell & Molecular Biology

By

Lydia M. Holtmann

May 2021

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Biomedical Sciences

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ABSTRACT

Alzheimer's Disease (AD) is a progressive brain disorder that destroys memory and cognition thought to be initiated through the build-up of the amyloid-beta (Aβ) peptide. The Aβ peptide aggregates, slowly forming into insoluble plaque substances that destroy the brain and worsen patient's symptoms over time. Studies have shown that chronic isolation stress (CIS) increases the Aβ peptide soluble and insoluble levels in the brain and that exercise training decreases these levels in transgenic mouse models of AD. We sought to determine if an exercise training regimen would rescue the increase in Aβ levels caused by CIS in the APP/PS1 transgenic mouse model of AD. Ninety-six APP/PS1 mice, 48 male and 48 female, were initially assigned to eight groups of twelve. Four groups of male and four female were utilized to assess sex differences in Aβ response. Each sex group was divided into four treatment groups; socially housed sedentary, socially housed exercise trained, CIS sedentary, and CIS exercise trained. Socially housed animals were housed in groups of 4-5 mice in normal size cages. CIS mice were housed alone in cages 1/3 the size of normal cages. Exercise trained mice ran for 60 minutes per day, five days per week, for 10 weeks on a level motor driven treadmill at a speed of 20 m/min. The exercise training regimen began at three months of age. Two-way analysis of variance revealed no sex differences in Aβ levels or exercise training responses so all animals were collapsed into the four treatment groups for further analysis. No differences were found among groups in soleus muscle citrate synthase levels indicating no exercise training effect took place in this study. Insoluble Aβ levels were greater in the CIS sedentary mice than all other groups. The data support the findings of others that CIS results in increased insoluble Aβ levels in AD transgenic mice. The novel finding of the study is that treadmill running rescues the increase in insoluble Aβ resulting from CIS. Our goal of assessing Aβ production and clearance markers to investigate possible mechanisms for this response was not accomplished due to the lab shutdown by the COVID-19 pandemic.

KEYWORDS: Alzheimer's Disease (AD), amyloid-beta (Aβ), chronic isolation stress, APP/PS1 transgenic mice, exercise training, citrate synthase, COVID-19

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ACKNOWLEDGEMENTS

Over two and a half years ago I met a crazy old man in my favorite coffee shop in Omaha, NE, where he (somehow) talked me into beginning a master's program to "better prepare myself" for the clinical world. Finally, here I am with a thesis that has survived not only the constant scrutiny of this crazy man, what seems like EVERY speed bump you could hit along the way in the lab, but to top it all off, a global pandemic. For this reason, there are many thanks to be given.

To begin, I want to thank my parents who allowed me to move in to their "empty nester" home at 22. I'm not sure at this point that I will be able to move out, the food is just too good. A special thanks goes out to all of the undergraduates that participated in this project, specifically Mikala Sisco, Garrett Kliethermes, Weston Phipps, Hadyn Tish, and Reid Morrissey. I know that running mice on treadmills can be a tedious task and I can never thank them enough for how much they helped me with this project. I am extremely thankful to Dr. Lyon Hough for being a part of my thesis committee and advising me on the specifics of Aβ and CSF flow. I cannot emphasize enough how grateful I am to have had Dr. Scott Zimmerman as my mentor and friend for the past two years. I already miss sitting in his office, drinking coffee and chatting about soccer and Aβ.

Most importantly, I want to thank Bailey Curry and Dr. Benjamin Timson. Bailey, thank you for being my partner through this entire process. I truly do not know how I would have finished without you by my side. I have learned so much from you and cannot wait to see where life takes you. I am so lucky for this journey to have brought me one of my best friends. Lastly, Timson...Thank you for being the crazy man who talked me into this. It has changed me forever into a better student and person. Although I cannot even count the number of times that I was in your office regretting it, I am confident that this was one of the best decisions I have made. Thank you for helping me through it all and being the greatest advisor I could ask for. I am forever in debt to you. I am so lucky to have you in my life! Next stop, PA school.

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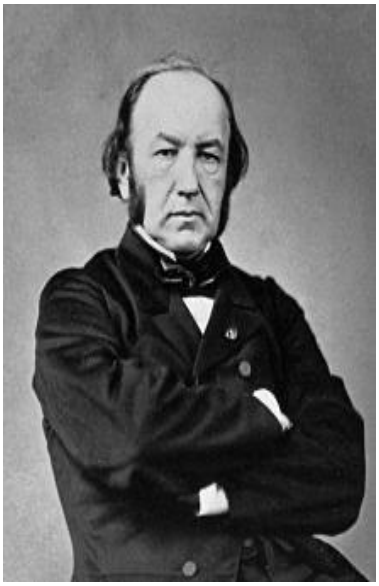
INTRODUCTION- STRESS AND CHRONIC DISEASE

The relationship between stress and chronic disease has captured the attention of scientists and physicians for over 100 years (Figure 1). Hans Selye is typically given credit for first introducing the idea that all chronic disease results from the body's inability to adequately adjust to stresses incurred over time. He termed this response the General Adaptation Syndrome (GAS), a concept that forms the basis for our understanding of chronic disease processes to this day (Selye, 1950). Selye built his GAS formulation on the concepts of fight-or-flight and homeostasis developed by Walter Cannon in his classic works, *The Emergency Function of the Adrenal Medulla in Pain and the Major Emotions* (Cannon, 1914) and *Organization for Physiological Homeostasis* (Cannon, 1929). Cannon, in turn, formulated his homeostasis concept from Claude Bernard's classic concept of "le milieu interieur" described in his timeless publication, *An Introduction to the Study of Experimental Medicine* (Bernard, 1957).



Claude Bernard
1813-1878

Walter Cannon
1871-1945

Hans Selye
1907-1982

Figure 1. Claude Bernard, Walter Cannon, and Hans Selye. Three giants in the fields of physiology and medicine whose work led to the understanding of the relationship between stress and chronic disease.

Claude Bernard – Le milieu interieur

Claude Bernard is widely considered the father of modern physiology and experimental medicine. Prior to Bernard's work at the College of France during the middle part of the 19th century, the life sciences, especially physiology, were considered science only marginally while physics, astronomy, and chemistry were considered the "hard sciences" in that the scientific method could be applied rigidly only to these areas of study. Medicine, at the time, was considered an art rather than a science. Bernard argued in his classic writings, *Introduction to the Study of Experimental Medicine*, published in French in 1865 (translated into English by H.C. Greene with Introduction by L.J. Henderson in 1927, and reprinted in 1957 with a new Foreword by I.B. Cohen), that physiological research was subjected to the scientific method and it was the science that formed the basis of medicine (Bernard, 1957). In his 1927 Introduction (p. viii-ix), Henderson, states, "*This science, of which he (Bernard) is the founder, was destined to remain undeveloped until long after his death. Today, with the aid of a physical chemistry unknown to the contemporaries of Claude Bernard, it is fulfilling the promise which he alone could clearly see*."

Prior to Bernard it was commonly believed that the internal environment of plants and animals was controlled by the external environment. In *Introduction*, Bernard first introduced his concept of the milieu interieur.

> "*Considered in the general cosmic environment, the functions of man and of the higher animals seem to us, indeed, free and independent of the physio-chemical conditions of the (external) environment, because its actual stimuli are found in an inner, organic, liquid environment. What we see from the outside is merely the result of physico-chemical stimuli from the inner environment; that is where physiologists must build up the real determinism of vital functions*." (p. 79)

The concept of le milieu interieur was Claude Bernard's most significant contribution to the study of physiology and ultimately to the scientific basis of medicine. It formulated the basis of Cannon's concept of homeostasis and Selye's General Adaptation Syndrome.

Walter Cannon – Fight-or-Flight and Homeostasis

Walter Cannon performed many experiments in the Laboratories of Physiology in the Harvard Medical School during the early 1900s that expanded Bernard's *milieu interieur*. From these experiments he developed the physiological basis for the idea of fight-or-flight and introduced the concept of homeostasis.

The British psychologist, William McDougall, suggested in his book, *An Introduction to Social Psychology* (1908) that the emotion of fear is associated with the instinct for flight and the emotion of anger or rage is associated with the instinct to fight. Cannon conducted a number of experiments that elucidated the physiological contributions of the sympathetic nervous system and the adrenal medulla to the fight-or-flight response described in broad general terms by McDougall. He described these experiments in his paper, *The Emergency Function of the Adrenal Medulla in Pain and the Major Emotions* (Cannon, 1914). In the introduction Cannon states,

> "*During the past three years, in a series of investigations conducted in this laboratory, we have attempted to gain insight into the meaning of the changes wrought by adrenalin or increased adrenal secretion, and in this paper I propose to discuss the bearings of our results*."

Cannon's experiments demonstrated that adrenalin is liberated normally during fear and rage in animals. He further demonstrated that increases in splanchnic nerve activity increased secretion of adrenalin from the adrenal medulla and the effects of this increased activity resulted in multiple important changes in function to include: 1) a shifting of blood flow from abdominal

vessels to vessels of the lungs, heart, skeletal muscle, and the central nervous system, 2) an increase in cardiac vigor, and 3) an increase in blood sugar content.

In "*discussing the bearings of our results"*, Cannon's main goal of the paper was to integrate the physiological outcomes of increased adrenal medullary and sympathetic nerve activity with a purposeful function for the organism. In this regard, he brought McMillian's concept of the human reactions to fear and rage from the realm of social psychology into the realm of physiology and gave birth to the fight-or-flight sympathetic response that we know today. Cannon rightly connected the increased liberation of glucose from the liver with the increased demand for skeletal and cardiac muscle contraction necessary for the fight-or-flight response of the organism to the emotions of fear and rage. He proposed that an event eliciting fear or rage in an animal or human would trigger a rapid response of the sympathetic nervous system and the adrenal medulla that would provide the animal with the necessary resources for quick and strong muscle contractions necessary for fight-or-flight. Interestingly, Cannon speculated in a footnote (p. 367) that, "*If these results of emotion are not "worked off" by action, it is conceivable that the excessive adrenalin and sugar in the blood may have pathological effects*." This speculation proved prophetic and had a significant impact on the work of Selye.

Cannon (1929) proposed a definition and description for his homeostasis concept in the introduction of his paper, *Organization for Physiological Homeostasis*, stating, "*To Claude Bernard belongs the credit of first giving to these general ideas a more precise analysis. He pointed out that in these animals with complex organization the living parts exist in the fluids which bathe them which constitutes the 'milieu interieur'*…." (pp. 399). In putting forth his description of the concept Cannon stated the following (p. 400):

> *"The highly developed living being is an open system having many relations to its surroundings - in the respiratory and alimentary tracts and*

through surface receptors, neuromuscular organs and bony levers. Changes in the surroundings excite reactions in this system, or affect it directly, so that internal disturbances of the system are produced. Such disturbances are normally kept within narrow limits, because automatic adjustments within the system are brought into action and thereby wide oscillations are prevented and the internal conditions are held fairly constant. The term "equilibrium" might be used to designate these constant conditions. That term, however, has come to have exact meaning as applied to relatively simple physico-chemical states in closed systems where known forces are balanced. In an exhaustive monograph L.J. Henderson (1928) has recently treated the blood from this point of view, i.e., he has defined, in relation to circumstances which affect the blood, the nice arrangements within the blood itself, which operate to keep its respiratory functions stable. Besides these arrangements, however, is the integrated cooperation of a wide range of organs - brain and nerves, heart, lungs, kidneys, spleen – which are promptly brought into action when conditions arise which might alter the blood in its respiratory services. The present discussion is concerned with the physiological rather than the physical arrangements for attaining constancy. The coordinated physiological reactions which maintain most of the steady states in the body are so complex, and are so peculiar to the living organism, that (it is) suggested that a specific designation for these states be employed – ***homeostasis****.*

Homeostasis, the concept solidified by Cannon based on the insights of Bernard, is a central tenant of physiology. The body has numerous regulatory mechanisms that maintain a relatively constant internal environment, within narrow limits, in the face of a changing conditions in the external environment. Selye proposed a cumulative challenge unresolved over time leads to disease.

Hans Selye – General Adaptation Syndrome

Through his observations and investigation of disease processes, Selye (1936) connected the ideas of Bernard and Cannon stating, "..*since the syndrome as a whole seems to represent a generalized effort of the organism to adapt itself to new conditions, it might be termed the*

'general adaptation syndrome."' It was not until 1950 that he explained his theory in depth in an article entitled, *Stress and the General Adaptation Syndrome* (Selye, 1950). He stated,

> "...k*eynote of* this *unification was the tenant that all living organisms can respond to stress as such, and that in this respect the basic reaction patterned is always the same, irrespective of the agent used to produce stress."*

The General Adaptation Syndrome (GAS) is a three-stage physiological process that the body undergoes when exposed to stressful conditions (Figure 2). These stages include the alarm reaction stage, the stage of resistance and the stage of exhaustion. The alarm reaction stage is the body's immediate response to a stressor resulting in the activation of the sympathetic system and the hypothalamic/pituitary/adrenal (HPA) axis. Sympathetic activation produces the fight-or-flight response, a concept introduced by Cannon (1914). The sympathetic system is activated by the secretion of norepinephrine (NE) and epinephrine (E) from the adrenal medulla and the release of NE from the sympathetic division of the autonomic nervous system (ANS). Upregulation of the sympathetic system increases cardiac output and blood pressure and heightens awareness to external threats. The HPA axis is a system of feedback interactions that leads to an increase in plasma cortisol levels. The cascade begins when the stressor triggers the release of corticotrophin releasing factor (CRF) into the hypothalamic-hypophyseal portal system. CRF stimulates the secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary which, in turn, initiates the release of glucocorticoids from the adrenal cortex, the most important of which is cortisol. The primary action of cortisol is to promote glycogenolysis in the liver to increase plasma glucose concentrations. Together the sympathetic system and the HPA axis function to meet the increased energy need of the body in response to a stressor during the

alarm reaction stage of GAS by increasing the delivery of oxygen and glucose to the affected organs and tissues.

Once the stressor is removed the person enters the stage of resistance. The burst of sympathetic activity subsides resulting in cardiac output and blood pressure returning to normal. Elevated plasma cortisol levels will feedback on the hypothalamus, reducing the secretion of CRF and return the HPA axis activity to normal. If the stressor persists the body will enter the stage of exhaustion. If this stressor or stressors persist, the body will continue to secrete stress hormones, i.e, epinephrine and cortisol, leading to problems that manifest if there is not enough time for recovery. During the stage of exhaustion, the body's energy reserves become depleted. Selye (1950) concluded that individuals who reach the stage of exhaustion have an increased risk of developing chronic diseases.

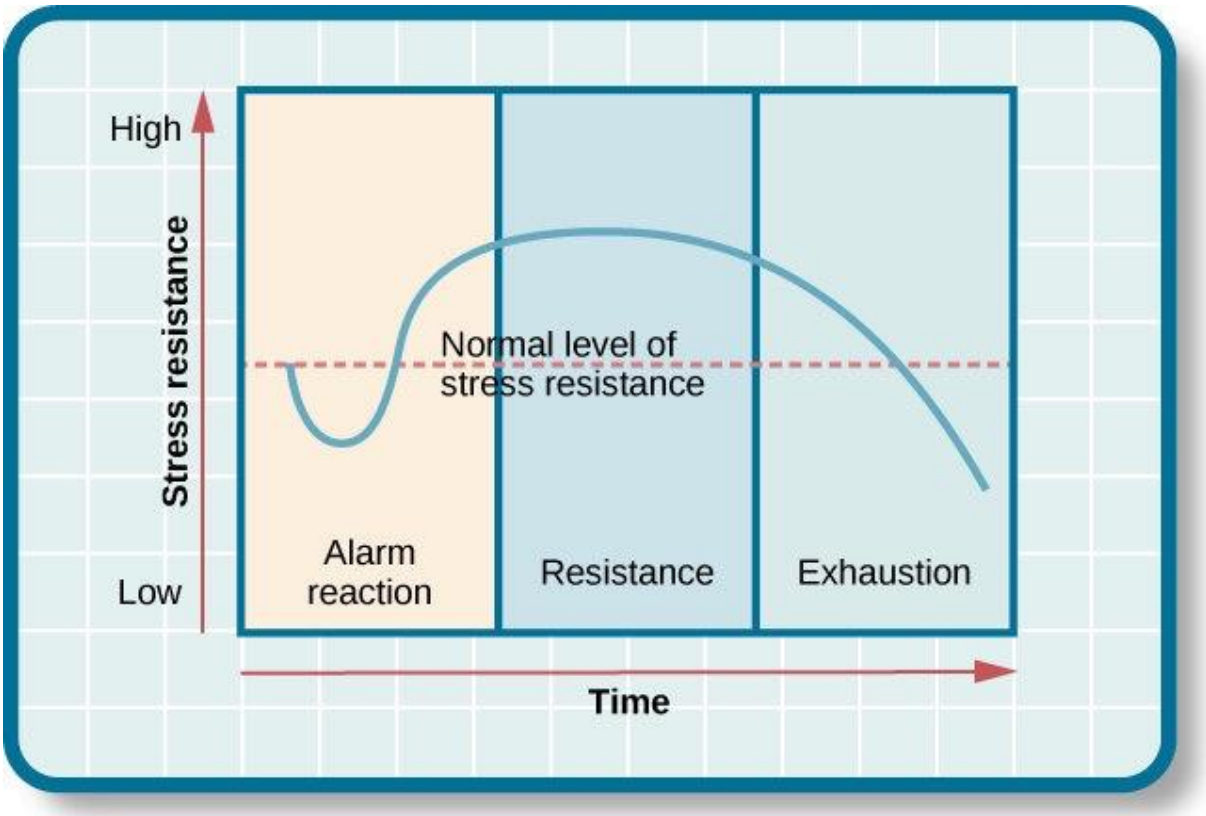


Figure 2. General Adaptation Syndrome. This figure depicts Selye's three stages of the General Adaptation Syndrome, alarm reaction, resistance, and exhaustion. Adapted from Weiser et al., 2014

ALZHEIMER'S DISEASE

Selye's global concept of the relationship between long term, unresolved stress and chronic disease has been supported by stress links to cardiovascular disease, anxiety, depression, some cancers, and HIV/AIDS. More recently stress has been identified as a factor increasing the risk of Alzheimer's disease (AD).

Alzheimer's Disease (AD) is an irreversible, progressive brain disorder that slowly destroys memory and thinking and eventually the ability to carry out the simplest tasks (NIH, 2018). AD is the most common cause of dementia and accounts for about 60-80% of dementia cases (Alzheimer's Association, 2018). Dementia is not a disease, but the decline in memory or thinking skills that can eventually affect one's daily activities. AD is a progressive disease where dementia symptoms worsen over time. AD has no current cure, although treatments are available to help slow the decline of cognitive function.

Symptoms and Diagnosis

Two subtypes of AD exist based on the age of onset: early-onset AD (EOAD) and late-onset AD (LOAD). EOAD accounts for approximately 1% to 6% of all cases with an onset from 30 to 60 years, while LOAD is the most common form of AD, with an onset of 60 years or later (Bekris et al., 2010). Both EOAD and LOAD present clinically as dementia that begins with a gradual decline of memory and slowly increases until the symptoms become incapacitating. Currently, the diagnosis of AD is based on clinical history, neurological examination, and neuropsychological tests (Bekris et al., 2010). The criteria for dementia as specified in the *Diagnostic and Statistical Manual of Mental Disorders,* third edition (revised) and fourth edition,

require that a patient have cognitive loss in two or more domains, such as memory, language, calculations, orientation, and judgement. In addition, the loss must be of sufficient severity to cause social or occupational disability (Kawas et al., 2003).

Petersen et al., (1999) describe probable AD as occurring when a patient demonstrates an insidious onset and gradual progression of dementia symptoms. AD is diagnosed when the cognitive loss is documented by neuropsychological tests and no physical signs or neuroimaging or laboratory evidence of other diseases that could cause dementia to exist, such as strokes, Parkinson's disease, subdural hematomas, or tumors. They further describe mild cognitive impairment (MCI), that frequently precedes AD, as general cognitive decline that impairs normal activities of daily living. MCI is initially reported by the patient or an informant closely associated with the patient and confirmed by abnormal performance for age on memory tests.

Risk Factors

Although there is not a single cause for AD, several factors place a person at greater risk of development for the disease. Age, genetics, family history, and other risk factors play large roles in an individual's chances of developing AD. The greatest known risk factor for AD is age, however this is not a normal part of aging (Alzheimer's Association, 2019). Individuals who suffer from AD are normally over 65 years of age, after which the risk doubles every five years until the age of 85. Family history is the second greatest risk factor for AD. Mutations in APP, PSEN1, and PSEN2 can virtually guarantee early onset familial AD, while common gene polymorphisms such as variants in the APOE gene can influence late-onset AD. Individuals with the APOE ε2 have a lower-than-normal risk of developing AD whereas individuals with APOE ε4 have a greater than normal risk of developing AD (Tanzi et al., 2012).

In addition to age and family history, a number of modifiable risk factors for AD have been identified in human epidemiological studies and meta-analyses studies. Strength of evidence of these risk factors identified in these studies varies and have been recently reviewed (Baumgart et al., 2015). Modifiable factors demonstrated to increase risk for AD include chronic stress, traumatic brain injury, mid-life obesity, mid-life hypertension, current smoking, diabetes, history of depression, sleep disturbances, and hyperlipidemia (Figure 3). Factors demonstrated to decrease risk include years of formal education, physical activity, Mediterranean diet, cognitive training, moderate alcohol consumption and social engagement.

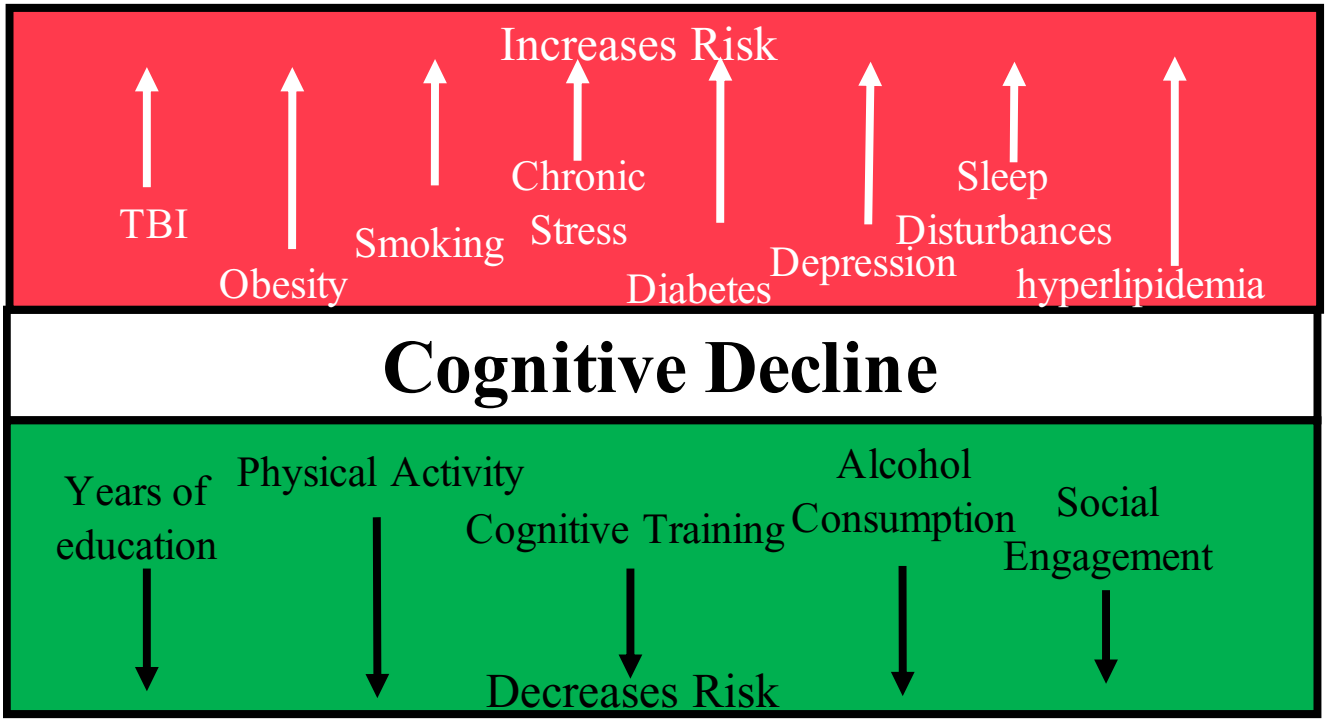


Figure 3. Modifiable Risk Factors for Cognitive Decline. Several factors including traumatic brain injury, mid-life obesity, hypertension, and others can increase risk of cognitive decline while things like formal education and physical activity decrease the risk. Adapted from Baumgart et al., 2015.

Sex Differences

An estimated 5.8 million Americans of all ages are living with AD. Of those, 5.6 million people are 65 years or older, and almost two-thirds of them are women (Alzheimer's Association, 2019). It is known that age is a risk factor of AD, however, more evidence is accumulating that sex differences exist in neurodegenerative diseases, especially AD. There are several hypotheses attempting to explain this phenomenon, both epidemiological and biological hypotheses (See Yan et al., 2018 for Review). Factors that account for these sex differences include longevity of females vs. males (Brookmeyer et al., 1998; Hebert et al., 2001), sex specific genetic interactions (Altmann et al., 2014; Janicki et al., 2014), hormones and endocrinological changes that occur with age (Morrison et al., 2006; Rocca et al., 2011), sex dimorphism in brain structures (Elbejjani et al., 2015; Sampedro et al., 2015), and an intriguing hypothesis that women respond differently to chronic stress differently than men on a cellular level.

Females are twice as likely as males to develop diseases where stress is the central etiology (Verma et al., 2011). In rodents, females show increased baseline concentrations of circulating glucocorticoids, with corticosterone being the most significant (Bangasser and Wicks, 2017). However, in humans, differences in baseline plasma cortisol levels have not been consistently found (Seeman et al., 2001; Kudielka and Kirschbaum, 2005). Both male and female rodents show similar peak levels of corticosterone during stress however, females show consistently higher corticosterone levels after the stressor occurs (Bangasser and Valentino, 2014). In patients with AD, females show higher levels of cortisol compared to males as well (Rasmuson et al., 2011).

During stress, the G-coupled protein receptor-cyclic AMP-protein kinase A(Gs-cAMP-PKA) pathway is activated by CRF. Recently, CRF signaling has shown to differ based on sex. Females show a higher sensitivity to CRF in the HPA axis than their male counterparts (Kunugi et al., 2006). Recently, Howerton et al. (2014) showed that CRF administration in male mice activated serotonergic neurons in the dorsal raphe, causing an indirect reduction in corticosterone. Bangasser et al. (2010) investigated the sex dimorphisms involved in CRF1 activation, showing that in females, prolonged CRF administration leads to activation of Gs-cAMP-PKA signaling, while in males, CRF1-β-arrestin-2 signaling predominates. Males then show downregulation and desensitization of CRF signaling while females show higher levels and sensitivity of CRF.

The mechanisms by which females are more likely to develop AD is unknown, however, there are specific situations in which women have higher levels of glucocorticoids that could predispose them to being more likely to developing the disease.

AMYLOID CASCADE HYPOTHESIS

A number of hypotheses have been put forth describing AD. The most widely accepted being the amyloid cascade hypothesis that postulates the accumulation of the Aβ peptide, resulting from an imbalance between its production and clearance, is the initiating factor in AD. An overproduction in Aβ, and/or a decreased clearance rate, leads to increased soluble Aβ in the brain interstitial fluid (ISF), that ultimately aggregates into toxic oligomers and senile plaques in the brain. These aggregates, primarily Aβ oligomers, are believed to initiate a cascade of events leading to neuron death and the cognitive dysfunction classic of the disease. For this reason, Aβ is the target of many therapies aimed at altering either the production or the clearance of this peptide.

Aβ Production

The Aβ peptide is produced by proteolytic cleavage of the amyloid precursor protein (APP). APP is a transmembrane protein that plays a role in the regulation of several important cellular functions, including cell and synaptic adhesion and promotes neuron branching (See Muller & Zheng, 2012 for review). The human APP gene has been identified in over 25 mutations that are causative of the hereditary form of familial AD (Thinakaran & Koo, 2008). APP is a member of a family of conserved type one membrane proteins, with the Aβ peptide being unique (Zheng & Koo, 2006). APP expressed in neurons is subject to fast anterograde transport to axon terminal buttons (Koo et al., 1990; Sisodia et al., 1993) where it is inserted into the plasma membrane. APP synthesized in the entorhinal cortex is transported to the hippocampus and dentate gyrus via the perforant pathway (Buxbaum et al., 1998).

APP processing. APP processing occurs via two pathways, one results in the production of Aβ, the other does not (Figure 4). A number of reviews have been published addressing the two APP processing pathways (Chow et al., 2010; De Stropper and Annaert, 2000; De Strooper et al., 2010; Querfurth and LeFerla, 2010; Thinakaran and Koo, 2008). In the nonamyloidogenic pathway APP is initially cleaved by an α-secretase, typically ADAM 9, 10, or 17 (Asai et al., 2003) releasing a large APP ectodomain (sAPPα) leaving an 83-residue carboxyl terminal fragment (C83 or CTFα) in the membrane. The CTFα fragment is hydrolyzed by γ-secretase producing p3, which is released into the ISF, and the amyloid intracellular domain (AICD) which remains membrane bound. In the amyloidogenic pathway APP is initially cleaved by beta-site amyloid precursor protein cleaving enzyme 1 (BACE-1), a β-secretase, releasing sAPPβ and a longer 99 residue carboxyl terminal fragment (C99 or CTFβ). The CTFβ fragment is cleaved by γ-secretase, releasing Aβ. Aβ can result in several different species, however those ending at position 40 (Aβ40) are the most abundant (~80-90%) followed by 42 (Aβ42, ~5-10%) (Murphy and LeVine, 2010).

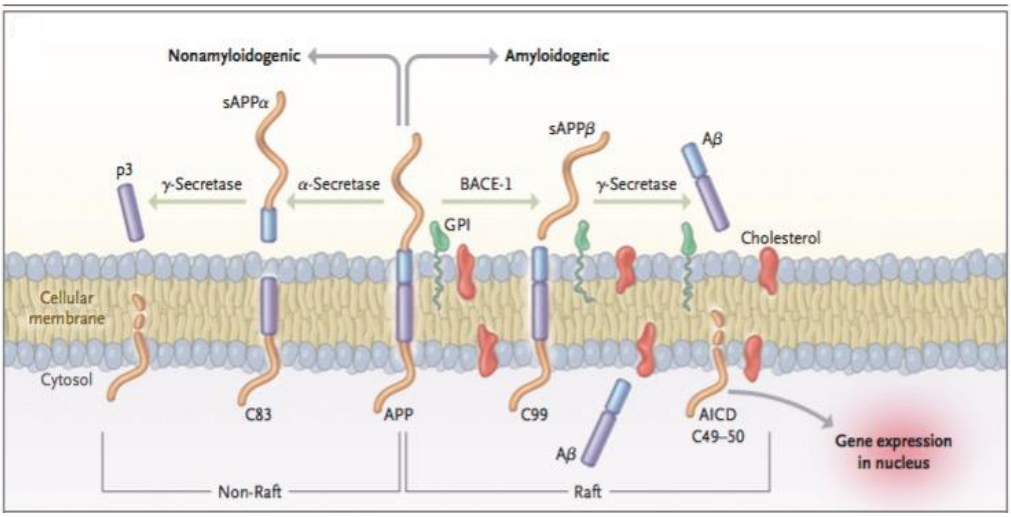


Figure 4. Amyloid Cascade Hypothesis. The amyloid precursor protein (APP) can be processed via two different pathways. In the nonamyloidogenic pathway, APP is initially cleaved by α-secretase followed by γ-secretase producing nontoxic products. In the amyloidogenic pathway APP is first cleaved by BACE-1, a β-secretase, followed by γ-secretase producing the potentially toxic Aβ peptide. From Querfurth & La

Synaptic activity is a key regulator of Aβ production. A relationship between synaptic activity and Aβ production has been studied in hippocampal slice neurons (Kamenetz et al., 2003) and *in vivo* (Cirrito et al., 2005, 2008; Kang et al., 2007). In hippocampal slices increased synaptic activity resulting from application of picrotoxin increases Aβ release and decreased synaptic activity from tetrodotoxin (TTX) administration decreases Aβ release (Kamenetz et al. 2003). *In vivo*, direct electrical stimulation of the perforant pathway (the major afferent projection from the entorhinal cortex to the hippocampus) increases hippocampal Aβ production (Cirrito et al., 2005), whereas lesion of the performant pathway decreases hippocampal Aβ production (Lazarov et al., 2002; Sheng et al., 2002). Decreasing synaptic activity by administering tetrodotoxin to the hippocampus via reverse microdialysis decreases hippocampal Aβ production (Cirrito et al., 2005; Kang et al., 2007). In addition, it has been demonstrated that areas of the brain that have a high intrinsic synaptic activity have high soluble Aβ levels and areas of the brain with low intrinsic synaptic activity have low soluble Aβ levels (Bero et al., 2011). Taken collectively, these data demonstrate a strong direct relationship between synaptic activity and Aβ production.

Aβ is not found in synaptic vesicles (Ikin et al., 1996; Marquez-Sterling et al., 1997), therefore it is not released from synaptic vesicles as a result of neuronal depolarization. APP is recycled from the terminal bouton membrane via clathrin-mediated endocytosis (Nordstedt et al., 1993) where it is spliced by BACE1 and γ-secretase in endosomes to generate Aβ (Lah and Levey, 2000; Vassar et al., 1999). Aβ is then released into the brain interstitial fluid as the endosomes are recycled to the cell surface (Koo et al., 1996). Through an elaborate set of experiments using reverse microdialysis in living Tg2576 mice Cirrito et al. (2008) were able to demonstrate that the critical component of the synaptic activity/Aβ production relationship was

endocytosis. Blocking synaptic activity with TTX reduced Aβ production as did blocking endocytosis with dynamin-DN. However, when dynamin-DN was administered first there was no further decrease in Aβ when followed by TTX administration. Conversely, when TTX was administered first Aβ levels decreased further when dynamin-DN administration followed. From these experiments the model presented in Figure 5 was constructed. Action potentials propagated down the axon open voltage gated calcium channels in the axon terminal button resulting in synaptic vesicles fusing with the membrane and releasing neurotransmitter. Clatherin-mediated endocytosis recycles full length APP into the cell in endosomes where it undergoes BACE1 and γ-secretase cleavage producing Aβ which is then released into the ISF.

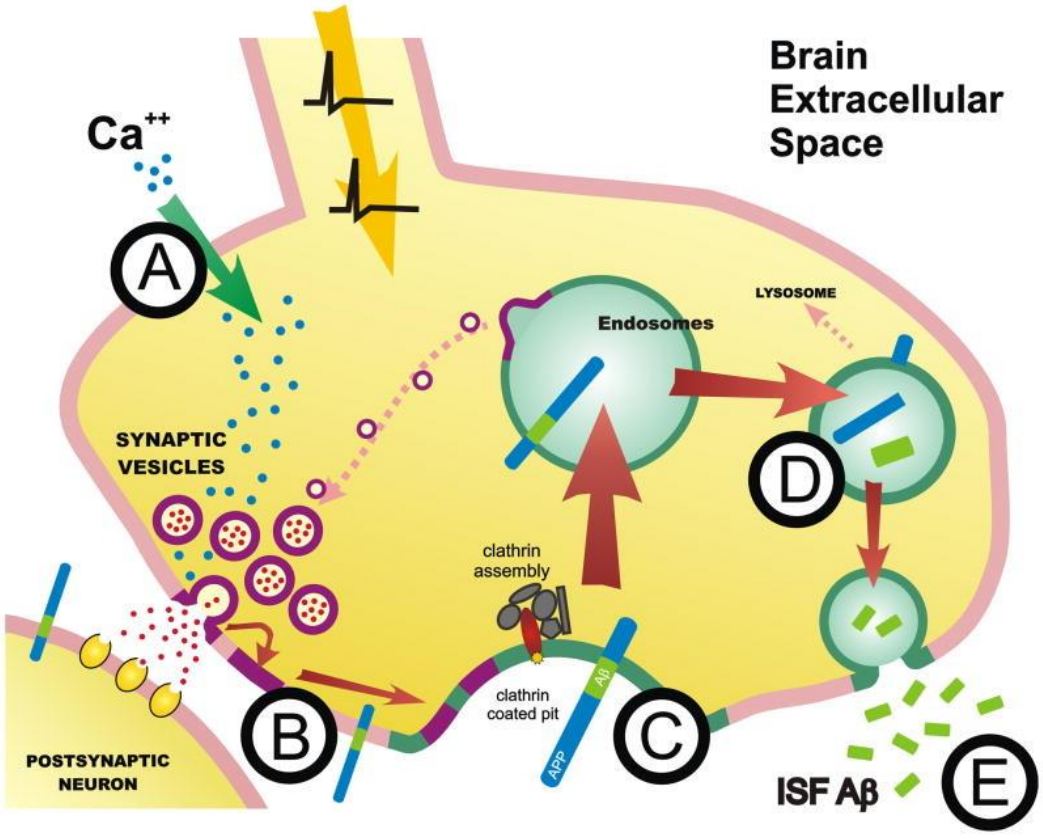


Figure 5. Synaptic Vesicle Uptake of Amyloid-Beta. Depolarization of the synaptic terminal by the action potential opens voltage gated calcium channels, resulting in calcium influx, leading to fusion of synaptic vesicles with the membrane. These vesicles are then recycled through clathrin-mediated endocytosis, internalizing APP into endosomes where BACE-1 and γ-secretase cleave APP producing Aβ that is released into the ISF.

Relative activities of α and β-secretase determine Aβ production. Production of Aβ is influenced by the relative activities of the amyloidogenic and nonamyloidogenic processing pathways of APP. For example, activation of serotonin 5-$HT_{4,\ 5,\ and\ 7}$ receptors (Fisher et al., 2016) and M1 receptors (Caccamo et al., 2006) increase α-secretase activity and reduces Aβ production, whereas NMDA receptor activation inhibits α-secretase activity and increases Aβ production (Lesne et al., 2005). Alpha secretase processing of APP consists of constitutive (unregulated) and regulated components (De Strooper and Annaert, 2000). The constitutive component takes place at the cell surface, whereas, the regulated component resides in the secretory pathway and can be activated by several mechanisms including, protein kinase C (PKC) (Buxbaum et al., 1993; Skorvronsky et al., 2000) and protein kinase A (PKA) (Pimenova et al., 2014; Fisher et al., 2016). The constitutive pathway of α-secretase cleavage of APP is thought to be minimal relative to the regulated pathway because APP is rapidly removed from the cell surface (Koo et al., 1996). BACE1 is located within the secretory pathway and the endosomal compartment (Huse et al., 2002) with very little to no activity at the plasma membrane (Chow et al., 2010). Aβ peptides generated in the secretory pathway and endosomes are secreted into the extracellular space (Greenfield et al., 1999) where they aggregate into toxic oligomers and senile plaque in a concentration dependent manner (Bero et al., 2011).

There are two thoughts about the regulation of the relative activities of the nonamyloidogenic and amyloidogenic pathways of APP processing. One is that BACE1 and α-secretase compete for APP in the secretory pathway and in endosomes (Skovronsky et al., 2000). If either enzyme is upregulated in these locations, that pathway will be favored. The other is that the activities of the two enzymes are compartmentalized within the secretory and endosomal pathways. Compartmentalization can be the result of localization, i.e., α-secretase is located in

the secretory pathway and β-secretase is localized in endosomes, or as is more likely, their activities are localized by pH activation and inactivation. BACE1 has a very tight pH optimum of 4.5 and is virtually inactive below 4.0 and above 5.0 (Ellis and Shen, 2015), whereas α-secretase is optimally active in the pH range of 6.0 – 8.0 (Reiss et al., 2011). Given these pH optima, α-secretase would be active and BACE1 would not in the secretory pathway where pH in the ER, trans-Golgi network, and secretory vesicles is typically 7.4, 6.2, and 5.5, respectively (Paroutis et al., 2004). Conversely, BACE1 would be active and α-secretase would not in endosomes where pH is typically 4.0 – 5.0 (Hu et al., 2015). pH would effectively compartmentalize α-secretase activity to the secretory pathway and BACE1 activity to endosomes. Anything resulting in the upregulation of α-secretase activity would reduce APP trafficking to the cell surface and reduce its endosomal concentration and thus Aβ production. Conversely, reduction of α-secretase activity would increase APP trafficking to the cell surface and increase its endosomal concentration and thus Aβ production. Taken collectively, the data cited above suggest that the quantity of Aβ ultimately produced is determined by the quantity of APP that is recycled from the cell surface into endosomes which, in turn, is determined by both α-secretase and synaptic activities. Thus, Aβ production is substrate (APP) limited.

Aβ Clearance

As noted previously, soluble Aβ aggregates in the brain ISF into toxic oligomers and plaque in a concentration dependent manner (Bero et al., 2011). Soluble Aβ concentration in the brain ISF depends on the balance between the rates of production and clearance of the peptide. The importance of Aβ clearance from the brain has received attention as evidence has accumulated indicating that individuals with AD have an imbalance between production and

clearance of soluble Aβ primarily due to a decrease in its clearance from the brain (Mawuenyega et al., 2010). Multiple reviews of Aβ clearance mechanisms have been published (Bates et al., 2009; Deane et al., 2009; Saido and Leisssring, 2012; Baranello et al., 2015; Tarasoff-Conway et al., 2015; Ries and Sastre, 2016). Soluble Aβ can be cleared from the brain though several parallel functioning mechanisms including, 1) enzymatic degradation, 2) transport across the blood-brain barrier (BBB), 3) ISF bulk flow via the glymphatic system. Aβ that reaches the peripheral circulation through any of the routes mentioned above is degraded in the liver or kidney.

Enzymatic degradation of Aβ within the brain. Aβ degradation is a significant clearance mechanism of the peptide and thus, plays a major role in the determination of soluble Aβ levels in the brain. Aβ degradation in the brain can theoretically take place anywhere it is potentially located, the secretory pathway, the ubiquitin proteosome system, endosomes, lysosomes, or the ISF. Both *in vitro* and *in vivo* studies have identified a number of proteolytic enzymes that degrade Aβ in its monomeric, oligomeric, and/or fibrillary form.

Enzymatic degradation involves several proteases, including neprilysin (NEP), insulin-degrading enzyme (IDE), Endothelian-Converting Enzymes (ECEs), Angiotensin-Converting Enzyme (ACE), Matrix Metalloproteinases (MMPs), cathepsin B (Cat B), and plasmin. Aβ is also degraded by α_2-macroglobulin and the proteasome, both of which are large protease complexes.

Neprilysin (NEP). NEP is the major Aβ degrading peptidase and accounts for as much as 50% of the total Aβ clearance activity *in vivo* (Saido and Leissring, 2012). It is an M13 zinc-metalloprotease (Howell et al., 1995) as well as a type II membrane-associated peptidase, which allows it to degrade large extracytoplasmic peptides like Aβ (Turner et al., 2001). It functions to

degrade monomeric Aβ in presynaptic terminals and in intracellular locations (Iwata et al., 2004; Saido and Leissring, 2012). NEP degrades Aβ in both its monomeric and oligomeric forms *in vitro* (Hama et al., 2001; Howell, et al., 1995; Kanemitsu et al., 2003). *In vivo* studies have also provided strong evidence for the Aβ degrading role of NEP. Iwata et al. (2001) demonstrated a 70-80% reduction in human-type $A\beta_{42}$ within 30 minutes that was virtually blocked by administration of the NEP inhibitor, thiophan. Furthermore, knockout of NEP in mice results in increased levels of soluble and oligomeric Aβ (Eckman et al., 2006; Huang et al., 2006; Madani et al., 2006; Farris et a;., 2007) and overexpression of NEP results in decreased levels of soluble Aβ and plaque (Leissring et al. 2003; Marr et al., 2003; Iwata et al., 2004; Poirier et al., 2006).

Insulin degrading enzyme (IDE). IDE, a zinc-endopeptidase, cleaves a number of peptides located in the cytosol, peroxisomes and at the cell surface. It only degrades monomeric forms of Aβ due to its unusual crystal structure that contains a small internal chamber (Becker and Roth, 1992). IDE is also unique in its participation in both Aβ and insulin catabolism. Investigations have found that AD patients may show reduced insulin clearance, explaining the link between diabetes mellitus and AD risk (Arvanitakis et al., 2004; Ott et al., 1999). IDE has been shown to degrade Aβ *in vitro* (Kurochkin and Goto, 1994; Vekerllis et al., 2000; Qiu et al., 1997; Qiu et al., 1998). *In vivo* studies have further characterized the role of IDE in the degradation of Aβ. IDE knock-out mice show a 64% increase in brain Aβ levels compared to wild type littermates (Farris et al., 2003; Miller et al., 2003), whereas, overexpression of IDE in transgenic mice results in reduced levels of cerebral Aβ (Leissring et al., 2003).

Matrix metalloproteinases (MMPs). Matrix metalloproteinases (MMPs), specifically, MMP-2 and MMP-9, are important Aβ degrading proteases because of their ability to degrade monomeric and fibrillar forms of Aβ in the extracellular compartment (Lorenzl et al., 2003; Yan

et al., 2006). *In vitro* studies have demonstrated that MMP-2 and MMP-9 degrade both soluble and insoluble forms of Aβ (Deb and Gottschall, 1996; Yan et al., 2006; Vetrivel et al., 2008; Liao and Van Nostrand, 2010). The role of MMPs in the degradation of Aβ *in vivo* does not appear to be as significant as NEP and IDE, however. Yin et al., (2006) found soluble $A\beta_{42}$ levels to be increased by 28% and 19% in the hippocampus of MMP-2 and MMP-9 knockout mice, respectively. Soluble $A\beta_{40}$ levels in the hippocampus of MMP-2 knockout mice were increased by 35%, whereas they were unchanged in MMP-9 knockout mice. *In vitro* experiments found both MMP-2 and MMP-9 degrade $A\beta_{42}$, but do so rather slowly (Hernandez-Guillamon et al., 2015) providing further evidence that, while MMPs degrade Aβ, their contribution to Aβ clearance *in vivo* is not as significant as other degrading proteases.

Endothelins (ECEs). Endothelins are potent vasoactive peptides produced from inactive intermediates, called big endothelins, by proteolytic processing via endothelin-converting enzymes (ECEs) (Inoue et al., 1989). Multiple ECEs have been cloned. Two of which, ECE-1 (Xu et al., 1994) and ECE-2 (Emoto and Yanagisawa, 1995), were later implicated in the degradation of Aβ. ECE-1 was first demonstrated to degrade Aβ *in vitro* when Eckman et al. (2001) showed that treating ECE expressing cell lines the phosphoramidon, a metalloprotease inhibitor, caused a 2-3-fold increase in extracellular Aβ due to an inhibition of intracellular Aβ degradation. They further demonstrated that overexpression of ECE-1 in Chinese hamster ovary cells reduces extracellular Aβ concentration by up to 90%. This group later showed that knockout, as well as inhibition with phosphoramidon, of both ECE-1 and ECE-2 results in increased levels of both $A\beta_{40}$ and $A\beta_{42}$ (Eckman et al., 2003; Eckman et al., 2006), demonstrating the ability of these two enzymes to degrade Aβ *in vivo*.

Cathepsin B (Cat B). Cathepsin B (CatB) is a cysteine protease that degrades peptides and proteins that enter the endolysosomal system by endocytosis or phagocytosis (Chapman et al., 1997). CatB becomes enzymatically active and is released via exocytosis. In patients with AD, CatB is located in most early endosomes (Cataldo et al., 1997). CatB has been shown to degrade synthetic soluble $A\beta_{42}$ monomers and insoluble $A\beta_{42}$ assemblies *in vitro* (Mueller-Setiner et al., 2006). *In vivo*, CatB overexpression in transgenic hAPP mice results in a decrease in amyloid plaque and CatB knockout results in an increase in plaque load (Mueller-Steiner et al., 2006; Wang et al., 2012).

Plasmin. Plasmin is a serine protease best known for its involvement in dissolving blood clots. Kingston et al., (1995) demonstrated *in vitro* that tissue plasminogen activator (tPa) was increased by the presence of Aβ peptide analogues suggesting the possibility that plasmin was involved in Aβ degradation. Plasmin was subsequently shown to degrade Aβ in vitro (Van Nostrand and Porter, 1999; Tucker et al., 2000). The role of plasmin in Aβ degradation *in vivo*, however, is equivocal. Aβ injected into the brains of mice genetically deficient in tPa or plasminogen persisted, while Aβ injected into the brains of wild-type mice was rapidly cleared (Melchor, etl al., 2003). It has also been demonstrated that inhibition of PAI-1, a tPa inhibitor, significantly lowers brain Aβ levels (Jacobsen et al., 2008). On the other hand, Tucker et al. (2004) reported that mice genetically deficient in plasminogen did not show increased levels of endogenous Aβ.

Angiotensin-converting enzyme (ACE). Angiotensin-converting enzyme (ACE) is a zinc metalloprotease that has been well characterized for its role in the regulation of blood pressure by converting angiotensin I to angiotensin II and inactivating bradykinin (Coates et al., 2003). It has been considered to participate in the Aβ clearance process as *in vitro* analysis has shown it

capable of degrading Aβ (Hu et al., 2001; Hemming and Selkoe, 2005; Oba et al., 2005). However, *in vivo* analysis has not demonstrated this effect. Complete inhibition of ACE in the brains of mice with enalaprilat failed to alter Aβ levels (Eckman et al., 2006). Therefore, while ACE is capable of degrading Aβ *in vitro*, does not appear to have a physiological role in this process. This is fortunate as ACE inhibitors are commonly used as blood pressure medication and elevated Aβ in brain ISF would be an undesirable side effect.

α2-macroglobulin (α2M). α2-macroglobulin (α2M), located throughout the body including the brain ISF and cerebrospinal fluid, is a highly abundant glycoprotein. α2M traps a broad range of proteases (Barrett and Starkey, 1973) in a cage-like structure via covalent-linkage of the protease to thioester bonds on α2M (Sottrup-Jensen, 1989). α2M is frequently considered to be a proteinase inhibitor as it sequesters proteinases from large substrates (Barrett and Starkey, 1973). However, smaller substrates, such as Aβ can bind to α2M (Yansheng, et al., 1997) and be degraded (Qui et al., 1996; Lauer et al., 2001; Wyatt et al., 2013). Lauer et al. (2001) speculated that once Aβ42 binds to the larger α2M complex it is directed to the active site of the caged proteinase and degraded. At present α2M has not been demonstrated to degrade Aβ *in vivo* in an animal model so the degree to which it participates in this process in living mammals has yet to be determined. α2M also plays a significant role in clearance of Aβ from the brain ISF into the circulation through the blood-brain-barrier via its interaction with LRP1 (Wyatt et al., 2013). This clearance mechanism will be discussed more thoroughly in the next section.

Proteasome. The proteasome is a large multi-subunit proteinase complex found in the cytoplasm of eukaryotic cells (Heinemeyer et al., 1997). It is comprised of a 20S functional proteolytic subunit and two 19S regulatory domains (Hilt and Wolf, 1996). The proteasome provides the cell with a mechanism of selectively degrading damaged or mis-folded proteins

(Rock et al., 1994). Early work indicated that the proteasome was inhibited by, but did not degrade Aβ (Checler et al., 2000). However, work by Zhao and Yang (2010) later demonstrated that Aβ monomers and oligomers bound to the proteasome and were degraded. It was further determined that the binding of Aβ was competitive and while it did inhibit the binding of other proteins reducing their degradation rates, it enhanced the degradation of Aβ.

In summary, enzymatic degradation is a critical function in the clearance of Aβ from the brain. Multiple proteases have been identified that degrade Aβ in its monomeric, oligomeric, and/or fibrillar forms in various locations in the brain (Table 1).

Table 1. Proteases reported to degrade Aβ in the brain.

Protease	Monomers	Oligomers	Fibrils	Location
NEP	*	*		ISF, SP
IDE	*			ISF, EN, LY
ECE 1, 2	*			ISF, SP, EN
MMP 2, 9	*		*	ISF, SP
Plasmin	*	*	*	ISF, SP
CatB	*		*	ISF, EN, LY
α_2-Macrogobulin	*			ISF, CSF
Proteasome	*	*		Cyto

Adapted from Saido and Leissring, 2012.
ISF- interstitial fluid, SP- secretory pathway, EN- endosomes, LY- lysosomes, CSF- cerebrospinal fluid, Cyto- cytoplasm

Aβ transport out of the brain. In addition to removing Aβ from the brain through enzymatic degradation, a significant amount of the peptide is cleared by transporting it from the brain ISF into the peripheral circulation. There are several mechanisms by which this is accomplished, the most prevalent of which is transport across the blood/brain barrier (BBB). Aβ is also transported into the cerebral spinal fluid (CSF) and the lymphatic system and returned to

the circulation through these sites via bulk flow. Once in the peripheral circulation Aβ can be degraded in the liver and kidneys.

Blood-brain-barrier (BBB). The BBB endothelial cells are connected by tight junctions as well as being protected by the glial barrier that consists of astroglial endfeet process that form gap junctions (Hermann et al., 2012; Thrane et al., 2014). Because of these protective measures, passage of Aβ across the BBB requires specialized transporters, including the low-density lipoprotein receptor related protein 1 (LRP1) and the receptor for advanced end glycation products (RAGE).

LRP1 is a member of the LDL receptor family that plays a major role in binding a diverse array of ligands such as apoE, α2M, tissue plasminogen activator, proteinase inhibitors, blood coagulation factors, and Aβ. This receptor is primarily involved in mediating the transcytosis of these proteins across the BBB (Deane et al., 2008., Shibata et al., 2000). LRP1 is abundantly expressed in diverse tissues including the liver, brain and other vasculatures (Shinohara et al., 2017). LRP1 is a large multi-functional receptor that regulates the endocytosis of diverse ligands and transduces several cell signal pathways by coupling with other cell surface receptors. LRP1 is the main receptor for Aβ transport across the BBB in the direction of brain to blood, however, there is a down regulation of the receptor seen in AD, increasing the amount of soluble Aβ in the brains of AD patients and transgenic mice (Deane et al., 2004; Donahue et al., 2006; Shibata et al., 2000). Several investigations support the role of LRP1 as a mediator of Aβ. Shibata et al. (2000) demonstrated that injected radioisotope-labeled Aβ is eliminated from transgenic mouse brains in an LRP1-dependent manner *in vivo*. Deletion of LRP1 in vascular smooth muscle cells increases Aβ deposition in APP/PS1 transgenic mice (Kanekiyo et al., 2013). The importance of LRP1 was supported through *in vitro* studies as well. Transgenic APP/PS1 mice that are

genetically modified to have the LRP1 gene knocked out demonstrated receptor-mediated clearance of Aβ through LRP1 in neurons and astrocytes (Kanekiyo et al., 2013; Liu et al., 2017.)

RAGE is a multiligand receptor and member of the immunoglobulin superfamily. The receptor works to bind and influx Aβ into the brain. RAGE expression is determined by the levels of its ligands; as Aβ species increase, RAGE expression also increases in the affected vessels, working as a positive feedback loop of Aβ pathology (Yan et al., 1996). RAGE mediates Aβ influx across the BBB through inflammatory and pro-coagulant activity but also by the induction of apoptosis via tumor necrosis factor (Du Yan et al., 1997).

ISF bulk flow. The CSF functions as a pseudo-lymphatic system, acting as a sink for brain interstitial solute (Hladky & Barrand, 2014). The CSF acts as a passive clearance mechanism to rid the brain of waste products, especially those of higher molecular weight like proteins. CSF enters the brain through periarterial spaces, passes into the interstitium using perivascular astrocytic aquaporin-4 (AQP4) channels, and drives perivenous drainage of ISF and solutes, it then flows to the fourth ventricle through the cerebral aqueduct, ultimately entering the subarachnoid space and cisterns (Damkier et al., 2013). In the brain, the primary goal is to move waste into the bloodstream where it can be eliminated by the kidneys and the liver.

CSF is formed by the choroid plexuses and flows from the lateral ventricles to the third ventricle via the foramina of Morno (Keep et al., 1990; Damkier et al., 2013). It then flows to the fourth ventricle through the cerebral aqueduct, ultimately entering the subarachnoid space and cisterns (Damkier et al., 2013).

Rennels et al. (1985) infused horseradish peroxidase into lateral ventricles and was able to trace it into the subarachnoid space, Virchow-Robin space, and periarterial spaces, following

the direction of blood flow. This perivascular circulation hypothesis was expanded by Iliff et al. (2012), who described the presence of the "glymphatic pathway". The glymphatic pathway functions by balancing the influx of CSF and perivenous efflux of ISF, clearing proteins, most importantly, toxic proteins like Aβ. Recently, it was also noted that lymphatics are active in this pathway (Aspelund et al., 2015).

The glymphatic pathway (Figure 6) is an organized transport system that flows through many brain regions. Beginning in the subarachnoid space, CSF flows into the brain through the perivascular space surrounding the leptomeningeal arteries (Iliff et al., 2012.; Iliff et al., 2013). The CSF is driven by arterial pulsations into brain parenchyma through perivascular spaces of the Virchow-Robin spaces, where the CSF will flow across the astroglial endfeet, specifically through AQP4 channels (Hannocks et al., 2018; Iliff et al., 2012). The AQP4 channel is expressed densely in brain parenchyma and is significant in that it mediates the transport into the interstitium, allowing the CSF to mix with ISF. Once in the interstitium, the fluid is cleared via a net movement toward the venous perivascular and perineuronal spaces (Iliff et al., 2012).

During AD, factors that affect bulk flow include molecular size, AQP4 expression and localization and sleep. Small, soluble Aβ is able to cross the membrane into the glymphatic system. Arterial pulsation allows for transport of the CSF into the interstitium in normal conditions. During neurodegenerative diseases, like AD, mechanical remodeling of the brain microvasculature contributes to arterial stiffening paired with the normal processes of aging leads to mechanical strain from increased arterial pulse wave velocity and pulse pressure (Tsao et al., 2013). Iliff et al. (2012) found a 55-65% in Aβ clearance in AQP4 knockout mice, indicating the importance of this aquaporin. It was suggested that localization of the AQP4 channel functions to decrease the resistance of the CSF-ISF exchange. Sleep has been identified as a

factor that enhances clearance of waste solutes in the brain. Xie et al. (2013) recorded a glymphatic influx increase of 95% and Aβ cleared 2-fold faster in the cortex of a sleeping/anesthetized rodent compared to an awake rodent. From this, it was inferred that drug-induced low arousal/sleep states promote Aβ clearance from the brain.

Molecular Chaperones. Chaperones are proteins that have a role in the intracellular handling of misfolded proteins, induce protein conformational changes, and act as protein transporters. Chaperones can be divided into professional or amateur chaperones depending on their function (See Wihelmus et al., 2007, for review). Professional chaperones, like the heat shock protein (Hsp) family, have specific functions in facilitating normal folding of proteins and intracellular handling of misfolded proteins. Hsp family members recognize misfolded proteins and transport them to the proteasome for degradation. Amateur chaperones are those that bind to other proteins to induce conformational changes or serve as transport proteins. Chaperones specific for the transport of Aβ in AD are professional chaperone Hsp70 and amateur chaperones ApoE, ApoJ, and α_2-Macroglobulin.

Heat shock protein 70 (Hsp70). Hsp70 functions to bind substrates to assist with folding, degradation, transport, regulation and aggregation prevention (Hartl et al., 2011). Hsp70 is able to assist with protein folding processes because of its unique structure. The substrate binding and release cycle is driven by switching Hsp70 between low-affinity ATP bound state and high-affinity ADP bound state. ATP hydrolysis, J-domain proteins, and nucleotide exchange factors are essential for chaperone activity of Hsp70 (Mayer & Bukau, 2005). Evidence suggests Hsp70 as a regulator of neurodegenerative processes, such as protein misfolding in brains of patients with AD (Meriin et al., 2005; Magrané et al., 2004). Recently, Hsp70 was identified to protect against intracellular Aβ accumulation as well as suppress the formation of neurofibrillary tangles

during AD (Dou et al., 2003; Fonte et al., 2002). Also, Hsp70 overexpression promotes clearance of Aβ (Fonte et al., 2002; Hoshino et al., 2011; Magrane et al., 2004).

Apolipoprotein E (ApoE). The ApoE genotype has long been recognized as a genetic risk factors for early and late onset AD, with the possession of the ε4 allele being one of the strongest genetic AD risk factors (Bertram et al., 2007). ApoE is produced by astrocytes and microglia in the brain but is mainly expressed in other organs like the liver and is the main ligand in receptor mediated endocytosis of HDL-like particles through LDL receptor family (Pitas et al., 1987; Grehan et al., 2001). ApoE is the major determinant of lipid transport and metabolism in the brain produced by these cells. After receptor-mediated endocytosis of ApoE containing lipoprotein particles by LDL receptors, ApoE is either degraded or recycled back to the cell surface (Rensen et al., 2000). In humans, there are three common isoforms of ApoE, ApoE2, ApoE3, and ApoE4. Investigations have found that binding of Aβ to ApoE is isoform dependent (2 > 3 >> 4) (Strittmatter et al., 1993). *In vivo*, ApoE4 induced a tenfold higher deposition of fibrillar amyloid than ApoE3, and human ApoE4 accelerated Aβ deposition in APPSwe transgenic mice relative to human ApoE3 (Holtzman et al., 2000; Carter et al., 2001). Other investigations have shown that clearance is inhibited due to Aβ forming complexes with ApoE, especially ApoE4 (Castellano et al., 2011; Deane et al., 2008, Strittmatter et al., 1993). ApoE4 dosage is associated with increased neuritic plaques in AD (Tiraboschi et al., 2004).

Apolipoprotein J (ApoJ). ApoJ, also known as clusterin (CLU), is a protein found in a wide range of tissues and body fluids. CLU functions include lipid transport, chaperone functions, inhibition of complement and regulation of the cell survival and death pathways (Humphreys et al., 1999; Wang & Eckel, 2014). Investigations on the clearance and neuroprotective role of CLU have been equivocal. Several investigations have shown CLU

binding to and altering the aggregation of Aβ, suggesting neuroprotective roles (Bell et al., 2007; Cascella et al., 2013; DeMattos et al., 2004; Merino-Zamorano et al., 2016; Narayan et al., 2014). CLU has also been found to colocalize with fibrillar Aβ deposits and prevent misfolding and aggregation with soluble Aβ (Calero et al., 2000). However, other studies show that CLU can reduce the clearance of Aβ, showing a neurotoxic role in the brain (DeMattos et al., 2002; Lambert et al., 1998; Nielson et al., 2010).

α2-macroglobulin (α2M). α2M functions as both a proteinase inhibitor and a molecular chaperone. It appears to form stable, soluble complexes between itself and the misfolded protein (French et al., 2008). α2M inhibits aggregation by binding to Aβ, preventing fibril formation and neurotoxicity, and promoting protease-mediated degradation (Hughes et al., 1998; Lauer et al., 2001). α2M in its tetrameric protease-bound form, reveals a binding site for LRP1, facilitating endocytosis and clearance mechanisms for itself and its protease (Ashcom et al., 1990). α2M has been widely documented to facilitate clearance of the Aβ peptide through this mechanism (Yerbury & Wilson, 2010; Fabrizi et al., 2001; Cascella et al., 2013). α2M is found to be colocalized with Aβ peptides in the brain during AD, for this reason, if the clearance mechanism is faulty, Aβ aggregation is seen in the brain (Thal et al., 1997).

Amyloid Cascade Hypothesis: Summary

The amyloid cascade hypothesis of Alzheimer's disease posits that the aggregation of the Aβ peptide within the brain ISF initiates a cascade leading to neuronal loss resulting in diminishing cognitive and motor function and ultimately death. Aggregation of Aβ is

concentration dependent and its soluble level within the brain ISF depends on the relative rates of production and clearance of the peptide.

Aβ is produced by the sequential cleavage of the amyloid precursor protein by β-secretase, specifically BACE1, and γ-secretase in the amyloidogenic pathway. The primary location of the BACE1 cleavage is in endosomes where the pH is within the narrow pH optimum of 4-5 for this enzyme. Given that BACE1 is primarily active in endosomes a major factor determining Aβ production is the quantity of APP that reaches the endosomes. This is determined by three factors; 1) the APP synthesis rate in the endoplasmic reticulum, 2) the activity of the nonamyloidogenic pathway of APP cleavage by α-secretase and γ-secretase in the secretory pathway, and 3) neuronal activity which results in cell surface APP being recycled in endosomes. Therefore if, for example, APP synthesis is high in the ER the quantity of APP entering the secretory pathway will be high. If α-secretase activity is low in the secretory pathway the quantity of APP in the membrane at the cell surface will be high. If neuronal activity is high there will be a large number of recycled endosomes containing significant levels of membrane bound APP upon which BACE1 will act resulting in high levels of Aβ production. Conversely, decreasing APP synthesis, increasing α-secretase activity in the secretory pathway, or decreasing neuronal activity will decrease Aβ production.

Aβ clearance from the brain ISF is critical to keeping soluble levels low and aggregation to a minimum. Clearance is accomplished by four mechanisms: 1) ISF enzymatic degradation, 2) cellular uptake and degradation, 3) transport out of the ISF into the blood through the blood-brain-barrier, and 4) ISF bulk flow through the glymphatic system. Ultimately, all soluble Aβ that does not aggregate (and some that does) is removed from the body by enzymatic degradation. While flushing Aβ out of the brain through the glymphatic system, or into the

blood across the BBB reduces its concentration in the brain however, it is still in the body and must be taken up and degraded at some other location in order to prevent its aggregation in the brain or other organs. Amyloid aggregation diseases (of which AD is one type) is not just an issue in the brain, it occurs in other organs including the liver, kidney, and heart (Zheng et al., 2019). Most organs produce amyloid and clear it into the blood. LRP1 is common to many cells in the body (see Au et al., 2017 for review) and takes up Aβ by receptor mediated endocytosis into cells that expresses it. Once taken up, it will be enzymatically degraded within that cell by Aβ degrading enzymes. The liver and kidney express LRP1 (Au et al., 2017) and thus take up and degrade significant quantities of Aβ produced and cleared into the blood by other organs including the brain. The brain contributes to the systemic regulation of amyloid by taking up Aβ from the blood across the BBB via RAGE for subsequent degradation in the ISF or after uptake into neurons and glial cells through LRP1.

A model of Aβ production and clearance is shown in Figure 6.

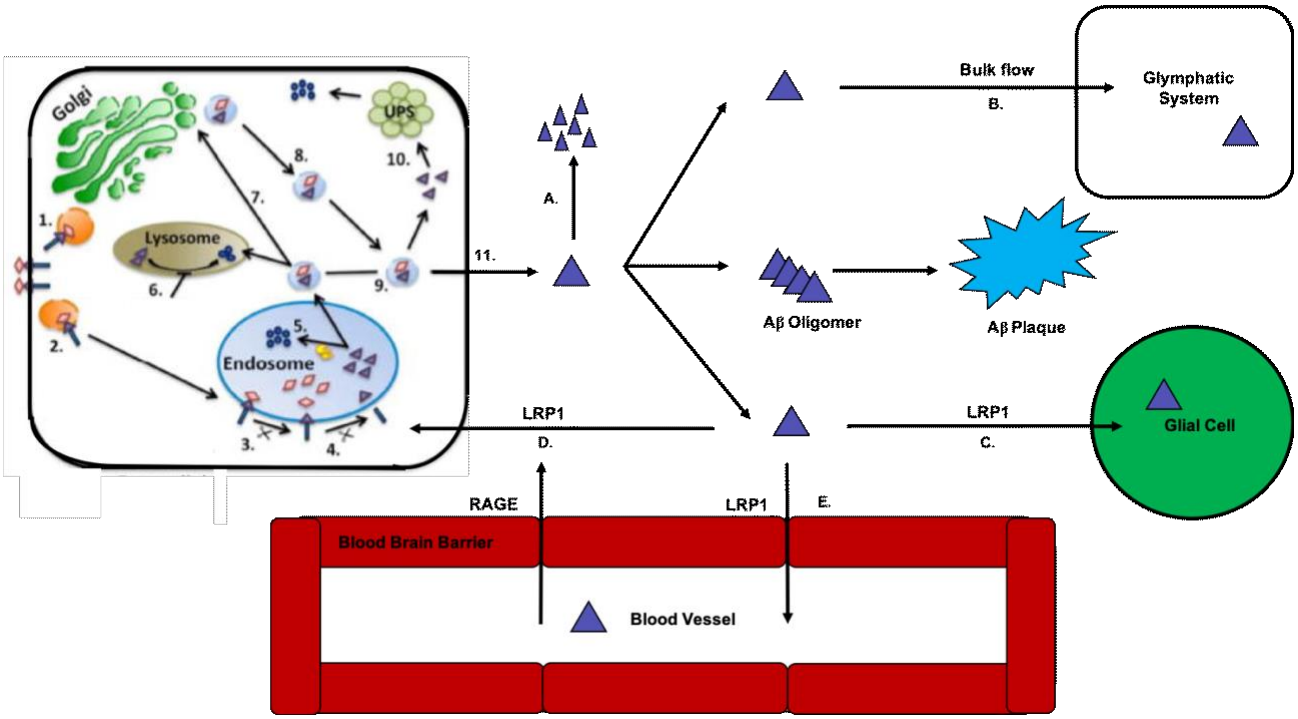


Figure 6. Model of Aβ production and clearance. APP synthesized in the ER is processed through the secretory pathway and delivered to the cell surface (Step 1). The quantity of APP that reaches the cell surface is determined, in part, by α-secretase activity in the secretory pathway in an inverse manner, i.e., greater α-secretase activity results in less APP reaching cell surface. APP reaching the cell surface is internalized into endosomes during synaptic activity (Steps 2 and 3). The acidic endosomal compartments favor BACE1 cleavage of APP, followed by γ-secretase cleavage producing Aβ (Step 4). In neurons Aβ generated in endosomes is subject to degradation by endothelin-converting enzyme (ECE) (Step 5). Aβ not degraded by ECE can be transported to the lysosome (Step 6) or the proteasome (Step 10) for degradation. Aβ not transported to the lysosome or proteasome can be released from the cell via recycling vesicles from either the Golgi (Step 7) or endosome (Steps 8, 9, and 11). Once in the ISF, Aβ can be degraded by enzymes such as NEP, IDE, MMP-9, ECE-1 and 2 (Step A). Aβ that escapes degradation can be cleared from the ISF into the blood across the BBB via LRP1 (Step E) or into the glymphatic system (Step B) or it can be taken up into glial cells (Step C) or neurons (Step D) via LRP1 and enzymatically degraded. Aβ not cleared by any of the aforementioned mechanisms will aggregate into oligomers and eventually amyloid plaque. (From Baranello et al., 2015).

Effects of Stress on Aβ: Transgenic Mouse Studies

One factor that is evident in causing an increase in brain soluble Aβ levels is stress. The physiological processes that stress drives have a serious detrimental effect on the ability to heal, cope and maintain positive quality of life and this becomes increasingly clear in cases of

neurodegenerative diseases like AD (Justice, 2018). Stress increases AD pathogenesis in many paradigms by increasing soluble Aβ levels, as well as plaque in transgenic mouse models of AD. These paradigms include chronic stress, acute stress, and post-traumatic stress.

Acute Stress. Acute stress is attained by placing mice in small restraint tubes that do not allow them to turn around. Limiting their movement through acute restraint stress has be utilized to investigate the effects of stress on Aβ levels in transgenic mice. Multiple paradigms have been utilized to show increases in Aβ levels through this process. Five hours of restraint showed increased formic acid extracted Aβ levels in 5 to 6-month-old male 3xTg-AD mice (Baglietto-Vargas et al., 2015). Aβ levels were also shown to increase in the hippocampus of living Tg2576 4-month-old mixed sex mice one hour into a three-hour restraint stress, using a microdialysis technique (Kang et al., 2007). This procedure peaked 13 hours following the procedure of the stressor, showing a 32% increase in Aβ levels. APP/PS1 transgenic female. mice also showed increase in soluble Aβ levels after one hour of restraint stress and remained elevated 15 hours following the removal of the stressor (Yuede et al., 2018).

Acute restraint stress increased BACE-1 activity and CRF concentration in the brains of AD transgenic mice (Baglietto-Vargas et al., 2015) which increased Aβ production. Kang et al. (2007) investigated the mechanism underlying the increased soluble Aβ response to acute restraint stress. Using reverse microdialysis, they observed that administering CRF increased soluble Aβ in the hippocampus, while administering corticosterone did not. This supported earlier work demonstrating exogenous administration of CRF increases firing of CA1 pyramidal neurons in the hippocampus (Aldenhoff et al., 1983) and enhances synaptic plasticity in response to excitatory input (Blank et al., 2002). Reverse microdialysis administration of TTX decreased neuronal activity and eliminated the increased soluble Aβ response to acute restraint stress (Kang

et al., 2007). These data were consistent with earlier work directly relating soluble Aβ levels to neuronal activity (Kamenetz et al., 2003; Cirrito et al., 2005). Taken collectively, the data support the concept that the increased soluble Aβ levels in response to acute stress are due to CFR induced increased neuronal activity.

Chronic Stress. Chronic isolation stress (CIS) paradigms are performed by continuously housing mice individually in cages with floor space one third that of the normal size for 3-6 months. This stressor is more severe than acute restraint stressors because of the continuous and long-term application of the stressor. Chronic restraint stress is a paradigm where the mouse is placed in a small tube that does not allow movement. When applied for six hours a day, four days a week from 3 to 11 months of age, this chronic restraint stress resulted in an increase in Aβ plaque deposition in the hippocampus and cortex of mix sex $APP_{V7171}CT100$ AD transgenic mice (Jeong et al., 2006). Chronic restraint stress was also applied for 6 hours a day, five days in a row, resulting in increased $A\beta_{42}$ and amyloid plaques in the hippocampus of female, but not male 5xFAD AD transgenic mice (Devi et al., 2010).

Other paradigms for chronic stress include exposing mice to a variety of stressors like damp bedding, sawdust from rat bedding, bell ringing, white noise, strobe lights, overnight illumination and soiled cages. Mice exposed to 2-3 different stressors for varying time periods over 24 hours have shown increased soluble Aβ and amyloid plaque deposition in the hippocampus and cortex. Examples of this include four-month-old female Tg2576 mice subjected to stressors daily for six weeks (Cuadrado-Tejedor et al, 2012), six-month-old male APP/PS1 mice subjected to stressors 2-3 days/week for four weeks (Han et al., 2016) and 12-month-old male 3xTgAD mice subjected to 6 hours of stressor a day, 2-3 days/week for 6 weeks (Rothman et al., 2012).

Another chronic stressor that is imposed is early life stress. Early life stressors were induced on the mice as early as postnatal days 2, whereas some investigations looked at the effects on pregnant mothers and their offspring. These early life stressors result in increased soluble Aβ levels and amyloid plaque deposition later in life. Increased soluble Aβ levels in the hippocampus were seen at 86 days after the stressor was removed from APP_{V7171}-CT100 AD transgenic mice following reduced bedding and wire mesh being placed one cm above their floor from postnatal days 2-9 (Lesuis et al., 2016). APP/PS1 AD transgenic mice subjected to this same paradigm saw an increased amyloid plaque deposition in the dentate gyrus at 10 months of age (Hoeijmakers et al., 2017). Stressors were also imposed prenatally by placing pregnant mothers in a 5 cm tube filled with one cm of water positioned under bright light for seven days. This stressor caused an increase in soluble Aβ levels in the offspring female, but not male, APP/PS1 mice at eight months of age.

Mechanisms for increased Aβ during chronic stress have been investigated through various studies which tend to agree that stress plays a large role in the increased production of Aβ. The increased Aβ levels are primarily due to an increased BACE1 activity and no significant change in any clearance proteins. Briones et al. (2012) demonstrated this in Wistar rats subjected to 2-3 unpredictable daily stressors. After 4 weeks, stressed rats showed an increase in $A\beta_{40}$ levels in the hippocampus, an increase in plasma corticosterone levels, and an increase in BACE1 activity compared to controls. Green et al. (2006) used an in vivo approach by administering both corticosterone and dexamethasone into 3xTg-AD mice daily for 7 days. This administration increased both $A\beta_{40}$ and $A\beta_{42}$ levels in the hippocampus, as well as increasing both BACE1 and its substrate, APP.

In summary, taken collectively, the data presented indicate that both acute and chronic stress result in increased brain ISF levels of both soluble Aβ and plaque. The primary mechanism by which this occurs is an increase in Aβ production due to increased neuronal activity, resulting from the stress induced upregulation from CRF in the brain. The preponderance of evidence indicates there is no change in brain ISF clearance rates. Thus, stress alters the Aβ production/clearance relationship in favor of production.

Effects of Physical Activity on Aβ: Transgenic Mouse Studies

Physical activity refers to any skeletal muscle contraction resulting in movement of the body that increases energy expenditure above basal levels (Casperson et al., 1985). Yuede et al. (2018) suggests that physical activity can be categorized into two classes, acute and chronic (Figure 7). Acute physical activity refers to one bout or session of physical activity and can be further divided into normal daily physical activity or exercise. Normal daily physical activity would include walking up the stairs to work or walking around the building, where exercise refers to one bout of an exercise regime, such as going on a run or lifting weights. Chronic physical activity refers to a repetitive, structured regime carried out over weeks, months or years that is intended to improve physical or psychological health (Yuede et al. 2018). Chronic physical activity can be subdivided into exercise training and sub-exercise training. Exercise training regimes are those that are of sufficient intensity, duration and frequency to improve physiological function above that of a sedentary individual or maintain a high level of function. Sub-exercise training chronic physical activity is not of sufficient intensity, duration, or frequency to improve physiological function above a sedentary individual. However, sub-exercise training may improve psychological health by reducing stress.

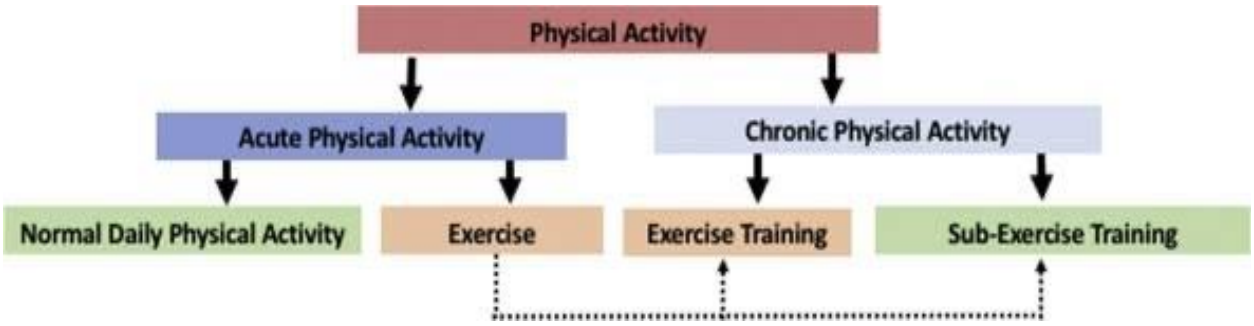


Figure 7. Physical Activity Categories. Physical activity is divided into acute and chronic physical activity, which are further divided into normal daily physical activity, exercise, exercise training, and sub-exercise training, depending on the frequency done and the level of physiological change experienced by the individual. **F**rom Yuede et al., 2018.

Results of studies investigating the effects of chronic physical activity on soluble Aβ levels and amyloid plaque load are equivocal. Most studies report that chronic physical activity reduces soluble Aβ levels (Bo et al., 2014; Cho et al., 2010; Cho et al., 2015; Ke et al., 2011; Kang et al., 2013; Liu et al., 2013; Lin et al., 2015; Um et al., 2008; Um et al., 2011; Zhao et al., 2015). However, others have not observed a change in soluble Aβ levels as a result of a chronic physical activity regime (Cho et al., 2015; Garcia-Mesa et al., 2011; Gimenez-lort et al., 2010). Similarly, the majority of studies investigating the effects of chronic physical activity on amyloid plaque load have found plaque load to be decreased as a result of the physical activity regime (Adlard et al., 2005; Bo et al., 2014; Cho et al., 2015; Garcia-Mesa et al., 2012; Garcia-Mesa et al., 2016; Ke et al., 2011; Liu et al., 2011; Maliszewska-Cyna et al., 2016; Richter et al., 2008; Tapia-Rosa et al., 2016; Yuede et al., 2009; Zhao et al., 2015). However, as with soluble Aβ levels, others have found chronic physical activity programs do not decrease plaque load (Cho et al., 2015; Cracchiolo et al., 2007; Garcia-Mesa et al., 2011; Maliszewska-Cyna et al., 2016; Mirochnic et al., 2009; Parachikova et al., 2008; Richter et al., 2008; Wolf et al., 2006; Xiong et

al., 2015; Zhao et al., 2015). One study (Jankowsky et al., 2003) found plaque load increased as a result of chronic physical activity.

The equivocal nature of the reports on the effects of chronic physical activity on soluble Aβ levels and amyloid plaque load is likely due to differences in intensity, duration and frequency of the exercise training paradigms among studies. The studies that focus on chronic physical activity utilize two types of physical activity, voluntary and forced. Voluntary chronic physical activity tracks the distance run by the mice on a running wheel, where the distance, frequency and intensity are self-selected. Forced chronic physical activity utilizes motor driven treadmills, where the speed and duration are set by the investigator. When rodents are given access to running wheels and self-select running speed, they frequently run long distances, but at low speed (intensity). It has been reported that rats allowed to run in wheels do not demonstrate an increase in citrate synthase activity even when separated into high and low distance running groups (Noble et al., 1999). Therefore, many of the studies employing wheel running in AD transgenic mice are likely sub-exercise training chronic physical activity and below an intensity threshold necessary to decrease soluble Aβ levels and plaque load.

Wheel running effects on soluble Aβ levels are sparse with only Tapia-Rojas et al. (2016) reporting a decrease in soluble Aβ and Garcia-Mesa et al. (2011) reporting no change. Alternatively, treadmill running subjects mice to greater running speeds than they typically self-select in running wheels. Studies employing this paradigm demonstrate decreased soluble Aβ levels (Bo et al., 2014; Cho et al., 2010; Cho et al., 2015; Ke et al., 2011; Lin et al., 2015; Liu et al., 2013; Kang et al., 2013; Um et al., 2008; Um et al., 2011; Zhao et al., 2015) and plaque load (Bo et al., 2014; Cho et al., 2015; Ke et al., 2011; Yuede et al., 2009; Zhao et al., 2015) with

much greater frequency than not (Cho et al., 2015; Gimenez-Llort et al., 2010; Xiong et al., 2015).

A similar pattern exists with voluntary and forced chronic physical activity effects on amyloid plaque load. Studies employing running wheels are nearly evenly distributed as to whether the physical activity paradigm decreases (Aldard et al., 2015; Garcia-Mesa et al., 2012; Garcia-Mesa et al., 2016; Maliszewska-Cyna et al., 2016; Richter et al., 2008; Tapia-Rojas et al., 2016) or does not change amyloid plaque load (Cracchiolo et al., 2007; Garcia-Mesa et al., 2011; Maliszewska-Cyna et al., 2016; Mirochnic et al., 2009; Nichol et al., 2008; Wolf et al., 2006). However, when treadmill running is utilized the vast majority of studies report a decrease in plaque loads (Bo et al., 2014; Cho et al., 2015; Ke et al., 2011; Liu et al., 2011; Zhao et al., 2015), whereas only Xiong et al., (2015) reported no change in plaque load using a treadmill running regimen.

Moore et al. (2016) investigated the effect of high and low intensity treadmill running on soluble $A\beta_{40}$ and $A\beta_{42}$ levels in young Tg2576 mice. The LOW training group ran at 15 m/min on a level treadmill while the HI intensity group ran at 32 m/min at a 10% grade, both for 60 minutes for 12 weeks, beginning at 3 months of age. Thomas et al. (2020) followed this study with an investigation of the effects of the same regimen administered for 12 months on amyloid plaque load. Soluble Aβ levels decreased significantly more in the HI intensity group compared to the LOW intensity group and the LOW intensity group relative to sedentary mice (Moore et al., 2016). Therefore, exercise training reduces soluble Aβ in a dose dependent manner.

When the running was continued for 12 months, the reduced soluble Aβ levels resulted in a dose dependent decrease in amyloid plaque load in older mice (Thomas et al., 2020). The results of these two studies confirm the speculation that soluble Aβ levels and plaque load

decrease with exercise training in a dose dependent manner. It is likely that when distance, frequency, and intensity are below a certain level the chronic physical activity becomes sub exercise training and the soluble Aβ levels and plaque load benefits are not recognized. The fact that the results of wheel running studies are evenly distributed with respect to decrease or no change in soluble Aβ levels or plaque load while treadmill studies almost all result in a decrease in these two variables support this concept.

Reduced soluble Aβ levels observed following exercise training regimen could result from either reduced production or increased clearance of Aβ. However, many studies indicate that increased clearance is the primary reason for reduced Aβ levels in humans and mice. Mawuenyega et al. (2010) investigated the balance of Aβ production to clearance rates in humans with AD versus controls. In participants with AD, there was an imbalance in the production to clearance ratios of both $A\beta_{40}$ and $A\beta_{42}$ with clearance exceeding production. The investigators concluded that individuals with AD had similar Aβ production rate, but reduced clearance rates relative to individuals without AD. Bero et al. (2011) assessed soluble Aβ and plaque levels in three different regions of the cortex in Tg2576 mice. There were large differences in soluble Aβ and plaque levels among the barrel, piriform and singular cortices, whereas total APP, CTF-β and CTF-α did not differ among regions. The data from these studies demonstrating that Aβ production rate is the same in areas where soluble Aβ and plaque levels differ significantly implies that differing clearance rates are responsible for the differing amyloid loads among regions.

As discussed earlier, clearance of Aβ occurs by several mechanisms. Enzymatic degradation is carried out by NEP, IDE, and MMP among others. Clearance also occurs through blood brain barrier receptors like lipoprotein receptor-related protein 1 (LRP1), and by

chaperones such as heat shock protein 70 (HSP70). Moore et al. (2016) assessed the effect of high and low intensity treadmill running on the expression of 18 Aβ clearance genes. From the initial blast, it was determined that mRNA expression of the genes responded differently to exercise training with the largest dose-dependent response occurring in genes coding for NEP, LRP1 and HSP70. The data support the idea that exercise training upregulates proteolytic degradation, blood brain barrier (BBB) efflux, molecular chaperones, but not uptake by microglia or astrocytes. For this reason, NEP, IDE, MMP9, LRP1 and HSP70 protein levels were measured in response to exercise training in both the hippocampus and the cortex of Tg2576 mice. It was determined that all of these proteins were upregulated in an exercise training dose dependent manner in both the cortex and hippocampus although some more robust than others.

Other studies investigating the effect of exercise training on clearance proteins have also demonstrated an increase in HSP70 (Cho et al., 2010; Koo et al., 2013; Um et al., 2008; Um et al., 2011) and LRP1 (Lin et al., 2015). On the other hand, Cho et al. (2015) did not find a difference in the Aβ degradation enzymes NEP and IDE in young 3xTg-AD mice. This is likely due to the low intensity (10 m/min) nature of the exercise training paradigm.

There has been a significant volume of work investigating the relationship between physical activity and soluble Aβ and plaque load in transgenic AD mouse models during the past 15 years. Generally, physical activity has been shown to decrease soluble Aβ and plaque load if it is of sufficient intensity, frequency and duration. The primary reason for the positive outcomes is related to upregulation of Aβ clearance proteins, while Aβ production is not significantly influenced.

Taken collectively, the data presented indicate that exercise training results in decreased brain ISF levels of both soluble Aβ and plaque. The primary mechanism by which this occurs in

an increase the clearance of Aβ from the ISF via an upregulation of multiple degrading enzymes in the ISF, an increase uptake of Aβ from the ISF through LRP1 into neurons and glial cells for degradation, and an increase in Aβ transport across the blood-brain-barrier via LRP1. In addition, exercise training enhances Aβ clearance through the upregulation of the HSP70 chaperone which prevents aggregation and delivers the peptide to intercellular locations for degradation. While there is some evidence indicating Aβ production is reduced as a result of exercise training, the preponderance of evidence indicates that it is not. Thus, with exercise training the Aβ production/clearance relationship shifts in favor of clearance.

SUMMARY AND RATIONALE FOR PROPOSED STUDY

Chronic stress increases Aβ levels in the brain ISF through a CRF-mediated increase in synaptic activity, increasing Aβ production. This stress induced impact occurs to a greater degree in females than males. Exercise training of sufficient intensity, duration, and frequency decrease Aβ levels in the brain in a dose-dependent manner through an upregulation of clearance mechanisms. To date, the interaction of stress and exercise training on ISF Aβ levels in the brain has not been studied. The goal of this study will be to determine whether or not exercise training ameliorates the stress related effects on brain ISF Aβ levels in the APP/PS1 transgenic mouse model of AD and whether or not this interaction differs with sex.

The study will include eight groups of APP/PS1 transgenic mice. Groups will be established based on a chronic stress paradigm, an exercise training paradigm, and sex. Chronically stressed animals will be housed individually in cages 1/3 the normal size, while social housed animals will live in normal sized cages with up to four littermates. Exercise trained animals will run on a motor driven treadmill at a speed of 20 m/min, 60 min/day, five days/week, for 10 weeks. Sedentary animals will be placed on an unmoving treadmill for the same time period to control for the effect of moving and handling the animals. It is hypothesized that exercise training will ameliorate the stress related effect on brain ISF Aβ levels and that the effect will be greater in females than males (Table 2).

Following the treatment paradigms, the mice were sacrificed, the brains harvested, and a number of production and clearance proteins were assessed to gain insight into the possible mechanisms resulting in any Aβ differences that may occur.

Table 2. Hypothesized Aβ Levels Relative to Social Housed Sedentary Control Following Treatment.

	Socially Housed/Sedentary	Chronic Isolation Stress/Sedentary	Chronic Isolation Stress/Exercise Trained	Socially Housed/Exercise Trained
Male	No change	Increase	No change	Significant decrease
Female	No change	Significant increase	No change	Significant decrease

MATERIALS AND METHODS

Animals

Sixty-three (63) male and female APP/PS1 transgenic mice were used in the study. All experiments were approved by the Missouri State University Animal Care and Use Committee protocol 19.01 (Appendix A). Ten male transgenic mice were graciously donated by Dr. John Cirrito, Professor of Neurology, Washington University School of Medicine in St. Louis. These mice were used to seed a breeding colony to generate mice for this study. They were initially bred to wild type females of the C57BL/C6 background. Offspring were genotyped at 21 days of age and the APP positive mice were retained for the study. APP positive females were utilized until each group was full, while males were either retained for the study or placed into the breeding protocol with wildtype females. Transgenic mice utilized in the study were tattooed on their tails with 1 to 4 marks, depending on the amount of cage mates for the purpose of identification during the study. All animals were housed in the Missouri State University vivarium and given food and water ad libitum.

Genotyping

Mice were genotyped using a QIAamp DNA mini kit from QIAGEN. Tail snips from each mouse were obtained and placed in tubes and incubated at 56 ^{o}C with a proteinase for a minimum of four hours, but not more than 24 hours. To extract DNA, RNAses and buffers specific to the kit were added and spun down to isolate the DNA for each specific mouse. Once the DNA was extracted, a master mix was produced to run the DNA on an agarose gel. The

master mix included four buffers; wild type forward, wild type reverse, trans 1 and trans 2, that all surround the APP gene to highlight it on the gel if it is present.

Once the master mix was produced and the DNA loaded, an agarose gel was run and visualized. Lanes were loaded with a ladder, a positive control, a negative control, water, and mouse DNA to identify. Lanes that had two bands represented a transgenic mouse while lanes that had one band represented a wild type mouse. The full protocol can be found in Appendix B.

Treatment Groups

Animals were separated into eight groups for the study, four groups each of male and female. Treatment conditions included chronic isolation stress, exercise training, a combination of chronic isolation stress and exercise training, and a sedentary control group. All treatments began at three months of age and continued for ten weeks. These groups are described below and shown in figure 8.

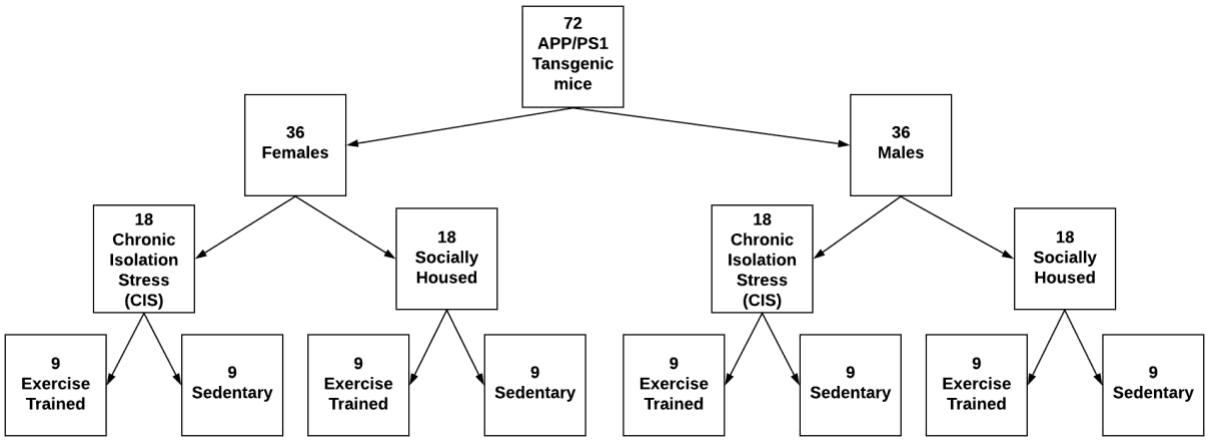


Figure 8. **Study Layout.** This study utilized six treatment and two sedentary control groups

Chronic Isolation Stress. To evaluate the effect of chronic isolation stress of Aβ levels in the cortex and hippocampus, mice were housed individually beginning at three months of age

in cages 1/3 the size of their normal housing. These mice were placed on housing racks that had them properly distanced from each other to ensure they had no interaction, neither sight nor smell of other mice. Chronically isolated mice were transported to and from the exercise training room in a manner that would avoid social interaction with other mice. Due to the isolation necessary for the study, investigators for the exercise paradigm were kept to a minimum to reduce the amount of interaction mice had with new stimuli. Mice were given food and water ad libitum.

Exercise Training. To evaluate the effect of exercise training on Aβ levels in the cortex and hippocampus, mice were run on a motor driven treadmill. Beginning at three months of age the mice ran at a pace of 20 m/min for 60 minutes a day, 5 days a week, for ten weeks. Mice were motivated to run by an electric grid at the back of the treadmill that delivered a mild shock it they touched the grid. To maintain consistency and eliminate a potential source of running was conducted at the same time each day throughout the duration of the treatment period. The exercise training protocol can be found in Appendix C.

Chronic Isolation Stress plus Exercise Training. To determine whether or not exercise training could rescue the effects of chronic isolation stress on Aβ levels in the cortex and hippocampus, a combination of these two treatments was employed. These mice were subjected to both the chronic isolation stress and exercise training paradigms described above. Mice began chronic isolation stress after their first bout of exercise training. This continued through the entirety of the exercise paradigm.

Sedentary. A sedentary control group for each sex was employed to establish a baseline for Aβ levels for comparison with treatment groups. These mice were placed on a wooden

treadmill that does not move during the 60-minute exercise training protocol. This was to ensure any stress that is induced by handling was the same for all eight groups.

Harvesting Tissues

Mice were anesthetized using isoflurane and the chest was opened by cutting the ribs along the sternum starting at the xyphoid process. Once the chest was opened the mouse was perfused by cutting the right atrium and injecting isotonic saline into the left ventricle. When perfusion was complete the skull was carefully incised from the base of the occipital lobe through the frontal lobe. The brain was then removed and placed in a petri dish on ice. With the brain on ice the cerebellum and brain stem were removed and discarded. An incision was then made along the longitudinal fissure separating the brain into hemispheres. The cortex and hippocampus were isolated from each hemisphere, weighed, and stored at -80 ^{o}C

Following removal of the skin, the soleus muscle was dissected from each leg. A blunt probe was used to penetrate the fascia on the underneath side of the Achille's tendon and moved along the posterior surface of the tibia isolating the plantar flexor muscle group (gastrocnemius, plantaris, and soleus muscles). The Achille's tendon was transected and the muscle group was pulled back exposing the dark red soleus muscle which was removed, weighed, and stored at -80 ^{o}C. A thorough description of the profusion and dissection of the brain and lower leg procedures is contained in Appendix D.

Sequential Extraction of Brain Aβ

Both the cortex and hippocampus were obtained for sequential extraction. Brains were weighed and homogenized by adding 1 μL per 1 mg of tissue weight of specified buffer and

spinning down. Extracellular, soluble intracellular, and insoluble intracellular fractions were gathered. The initial step isolated the extracellular soluble fraction. The homogenate was suspended in a PBS buffer, then centrifuged at maximum speed (20,000 g) for 25 minutes. The supernatant was then transferred to a chilled tube and stored at 4°C. Next, to isolate the intracellular soluble fraction buffer, the leftover homogenate was suspended in a Triton-100 buffer, then centrifuged at maximum speed for 25 minutes again. The same protocol was taken for storing supernatant. Finally, the insoluble fraction was suspended in a guanidine buffer, then sonicated before being centrifuged at max speed for 25 minutes again. This final fraction was then stored in a chilled tube. Specific protocols for each can be found in Appendix E.

Protein Assay: BCA

Samples were analyzed for total protein concentration before running any further testing by using a Pierce Micro BSA Assay. A curve was produced using specific buffer depending on which fraction of sample was being used (PBS, Triton, or Guanidine). Samples are diluted 1:50 into water for the samples and sample buffer for the curve. 100 µl are loaded per well in duplicates. A working reagent is created and loaded into each well and incubated at 37°C until color develops. This plate is then read at 560 nm. The full protocol for protein assay can be found in Appendix F.

ELISA

Samples were analyzed for $A\beta_{40}$ and $A\beta_{42}$ using ELISA. First, 96 well plates were coated with capture antibodies HJ2 and HJ7.4, for $A\beta_{40}$ or $A\beta_{42}$ respectively. The following day, wells were blocked and washed to avoid high background, and loaded with Aβ. Secondary antibodies,

mHJ5.1 and HRP40, were added on the third day and the samples were viewed in the plate reader at an absorbance of 650 nm. Full ELISA protocol can be found in Appendix G.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 8. Prior to analyses, all data were screened for missing values and outliers using the ROUT method (Q = 0.5%). Two-way factorial analysis of variance (ANOVA) were used analyze the citrate synthase and ELISA data. With a statistically significant interaction between main factors, simple main effects were calculated to provide clarification of statistically significant between-factor and within-factor differences. Probability value for all analyses was $p < .05$, unless otherwise stated.

Intended Methods

Western Blot. Western blots were to be used to analyze protein concentration in each fraction. Production analytes CTF-α, CTF-β, α-secretase, and BACE-1 as well as clearance markers NEP, LRP1, IDE, HSP70 and the stress indicator CRF1 were going to be measured using western blotting techniques. To do this, samples are diluted with PBS, water and 5X reducing buffer to load into individual lanes. A NuPAGE 12% Bis-Tris gel was used to run the samples until approaching the bottom. Once finished, the gel is transferred onto a nitrocellulose membrane and stained using PonS, milk-PBS T20 and the primary and secondary antibodies specific for the analyte being measured. Blots are developed using ECL solutions and stored in 1X PBS. The full protocol for western blotting can be found in Appendix H.

RESULTS

Citrate Synthase

Physical activity is defined as any movement of the body that increases total body energy expenditure above that of rest. If the increase in energy expenditure is of sufficient frequency, intensity, and duration to produce an improvement in physiological function it is defined as exercise training. Generally, to demonstrate an exercise training effect assessment of some Krebs cycle or electron transport enzyme is made. In this study, citrate synthase (CS) was assayed to assess whether or not an exercise training effect occurred as a result of the treadmill running program. The two-way ANOVA revealed no difference in CS activity among groups when comparing male vs. female mice or socially housed vs. chronically isolated mice so mice were pooled for the comparison between sedentary (SED) and mice that underwent the treadmill running program (ET). CS activity was 30.35 ± 6.93 in the SED mice and 29.92 ± 9.13 for ET mice. A t-test revealed no significant difference between the SED and ET mice (Figure 9).

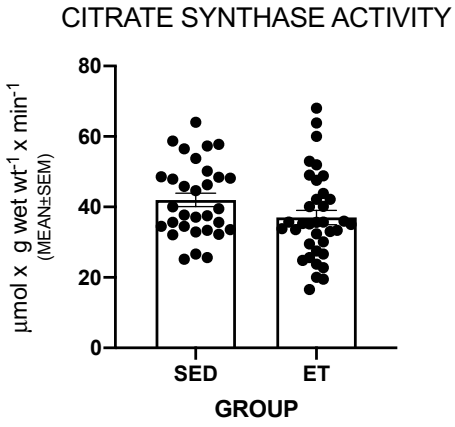


Figure 9. CS Activity was not different between sedentary and exercise trained animals (p=0.84).

Amyloid-Beta

To assess the effects of isolation stress and exercise training on amyloid beta levels in the hippocampus, $A\beta_{40}$ and $A\beta_{42}$ were assessed using ELISA after the ten-week isolation stress and exercise training regimens. A sequential extraction methodology was employed to assess soluble (triton fraction) and insoluble (guanidine fraction) Aβ levels. Two-way ANOVA revealed no difference between male and female mice in either the triton or guanidine fractions so the sexes were combined for further analysis. Neither isolation stress nor exercise training had a significant impact on the soluble $A\beta_{40}$ or $A\beta_{42}$ levels as demonstrated in the triton fraction of the hippocampus (Figure 10).

Figure 10. Soluble $A\beta_{40}$ and $A\beta_{42}$. Levels were assessed from the triton fraction of the hippocampus of APP/PS1 transgenic mice. No significant differences in either $A\beta_{40}$ or $A\beta_{42}$ were observed between mice that were socially housed and mice subjected to chronic isolation stress in both the sedentary (SED) and exercise trained (ET) groups. Nor were there differences in either soluble Aβ species between SED and ET mice.

However, in the guanidine fraction, isolation stress and exercise training played a role in the amount of insoluble Aβ40 and Aβ42 that was deposited. Insoluble $A\beta_{42}$ was significantly greater in SED mice subjected to chronic isolation stress than socially housed SED mice, p=0.0009. Insoluble Aβ42 in chronically isolated mice that underwent exercise training was

significantly lower than chronically isolated mice that were sedentary, p=0.0024. Insoluble $A\beta_{40}$ in chronically isolated mice that underwent exercise training was significantly lower than chronically isolated mice that were sedentary p=0.0047 (Figure 11). Therefore, exercise training was shown to rescue insoluble, but not soluble $A\beta_{40}$ and $A\beta_{42}$ deposition in a stressed model.

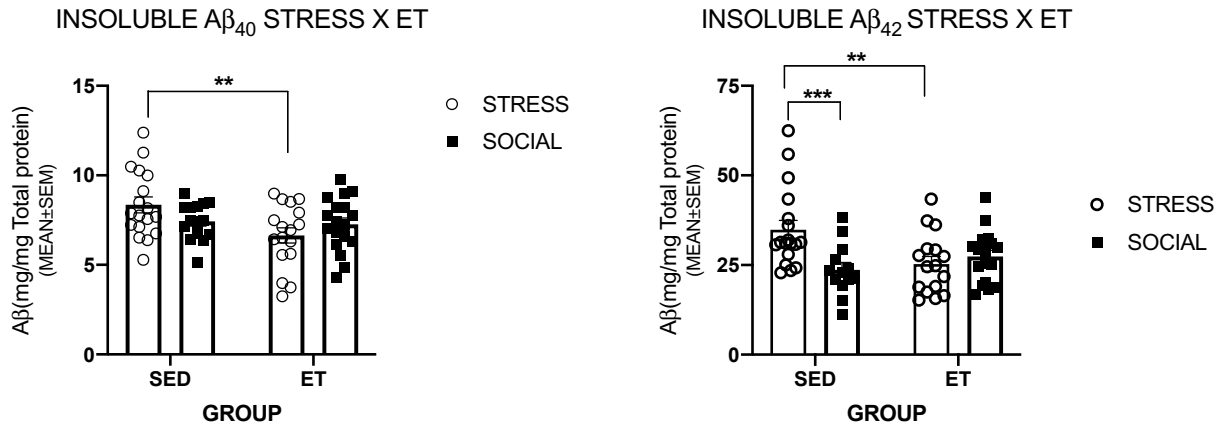


Figure 11. Insoluble $A\beta_{40}$ and $A\beta_{42}$. Levels were assessed from the guanidine fraction of the hippocampus of APP/PS1 transgenic mice. Exercise training (ET) significantly reduced insoluble $A\beta_{40}$ levels in mice subjected to chronic isolation stress, but not mice that were socially housed. Insoluble $A\beta_{40}$ levels were not significantly different between socially housed and chronically isolated mice in either the sedentary (SED) or ET groups. Insoluble AB42 levels were significantly greater in chronically stressed mice than socially housed mice. Exercise training significantly reduced insoluble $A\beta_{42}$ in chronically stressed mice compared to chronically stressed SED mice.

I planned to gain a better understanding of both the production and clearance mechanisms during this process through Western Blotting different proteins. These would have included the production markers α-secretase, BACE-1, CTF-α, and CTF-β as well as clearance markers NEP, IDE, LRP1, and HSP70 and the stress marker CRF. Unfortunately, the COVID pandemic shut down the laboratory at Washington University and Missouri State, leading to a shortening of the project's assays.

DISCUSSION

The concept of long-term stress effects on the body was introduced by Hans Selye (REF), who noted that chronic diseases form due to the body's lack of adjustment to stressors over time. Selye described the stages that the body goes through in order to deal with stress, addressing it as the "General Adaptation Syndrome." Recently, stress has been identified as a factor that increases the risk of AD due to a CRF-mediated increase in synaptic activity, increasing Aβ production (Devi et al., 2010; Green et al., 2006; Han et al., 2016; Jeong et al., 2006). Exercise training has been shown to decrease the risk of AD through an upregulation of Aβ clearance mechanisms (Bo et al., 2014; Cho et al., 2010; Cho et al., 2015; Ke et al., 2011; Kang et al., 2013; Um et al., 2008; Zhao et al., 2015). Because of those two major findings, this study was proposed to combine a stress paradigm with an exercise training regimen to determine whether or not exercise training ameliorates the stress related effects on brain Aβ levels. Hans Selye's concept of stress and its relationship with chronic diseases is supported by the main finding of this study. Mice subjected to chronic isolation stress (CIS) had significantly more insoluble hippocampal $A\beta_{42}$ than mice that were socially housed as demonstrated by the data from the guanidine fraction. Thus, this study supports the findings of others who reported increased hippocampal Aβ plaque deposition in mice subjected to chronic stress employing a variety of stress inducers (Jeong et al., 2006; Devi et al., 2010; Cuadrado-Tejedor et al., 2012; Rothman et al., 2012; Han et al., 2016). CIS did not result in an increase in soluble hippocampal Aβ in this study as no difference existed in the triton fraction between CIS and socially housed mice. APP/PS1 transgenic mice begin to develop plaque between five and six months of age and it is possible that once soluble Aβ reaches a certain level plaque begins to form and soluble levels no

longer increase. If this were the case, it would be expected that soluble Aβ levels would not be different among treatment groups once plaque began to develop as was found in this study. In support of this notion, Yuede et al. (2009) found plaque differences, but no soluble Aβ differences between sedentary and exercise trained Tg2576 transgenic mice at nine months of age, the age at which plaque begins to form in that model. Further study into this possibility to clarify this speculation would be helpful.

Physical activity has been demonstrated to positively impact fitness and health. In addition to directly impacting health, it has also been demonstrated to do so indirectly by relieving daily stress levels and thus reducing the stress effect on health described by Selye (1950). Our lab has previously demonstrated that exercise training has a significant positive impact on soluble and insoluble Aβ levels in a dose-dependent manner in the Tg2576 transgenic model of AD (Moore et al., 2016; Thomas et al., 2020).

Casperson et al. (1985) defined physical activity as any skeletal muscle contraction resulting in movement of the body that increases energy expenditure above basal levels. Yuede et al. (2018) described Physical activity as being acute or chronic. Chronic physical activity refers to a repeated, structured regimen carried out over weeks, months or years that is intended to improve physical or psychological health. Chronic physical activity that is of sufficient intensity, duration and frequency to improve physiological function above that of a sedentary individual is referred to as exercise training. Chronic physical activity that does not improve physiological function above that of a sedentary individual is referred to as sub-exercise training physical activity. Sub-exercise training physical can improve psychological health and relieve stress and thus improve health independent of an improvement of some physiological function.

As with previous studies from our laboratory, I used citrate synthase (CS) activity to assess the degree of exercise training achieved during this physical activity paradigm. Our lab previously demonstrated that exercise training results in increased CS activity in a dose dependent manner in the Tg2576 transgenic mouse model of AD (Moore et al., 2016; Thomas et al., 2020). In these studies, running speeds of 15 m/min on a level treadmill and 32 m/min on a 10% grade both resulted in increased CS activity with greater speed resulting in greater CS activity.

Based on the findings of Moore et al. (2016) and Thomas et al. (2020) I speculated that running a speed of 20 m/min on a level treadmill would result in a training effect in APP/PS1 mice. However, results from this study failed show a significant difference between the exercise trained animals and sedentary animals. This finding was unexpected and difficult to explain. It not only conflicts with our previous data with Tg2576 mice running at 15 m/min (Moore et al., 2006; Thomas et al., 2020), it also conflicts with data from our research group with APP/PS1 mice running at 15 m/min (Yuede et al., 2018).

From previous studies in our lab, I believed that the exercise training regimen would have a positive effect on the Aβ load. I expected to see a decrease in the amount of Aβ in the brain due to the intensity of the training regimen. However, no correlations were found between exercise training and Aβ concentration in our study. Because no training effect was seen in the CS assay, it makes sense that there would not be an exercise training effect on the Aβ concentrations.

The main objective of this study was to determine whether or not there was an interaction of chronic isolation stress and exercise training on Aβ levels in the hippocampus of APP/PS1 transgenic mice. The finding in this regard was that insoluble Aβ was reduced in CIS mice to the

levels of socially housed mice by the physical activity regimen for both Aβ species. Thus, although this exercise training regimen was unable to decrease Aβ concentrations in socially housed mice, it did rescue the increase in Aβ concentrations due to CIS. Because the data show no differences in Aβ concentrations due to exercise training directly, I speculated that daily treadmill running at a sub-exercise training level reduced the stress that resulted from the isolated housing condition. Several studies have shown that environmental enrichment is capable of mitigating cognitive deficits in transgenic mouse models (Balthazar et al., 2018; Jankowsky et al., 2005). Animals that are housed in "enriched environments" are shown to result in reduced Aβ levels and amyloid deposits compared to those that are raised in "standard housing" conditions (Lazarov et al., 2005). It is possible that the treadmill running served as environmental enrichment in the mice that were chronically isolated.

Our intention was to try to determine mechanisms for decreasing Aβ levels in the APP/PS1 mouse model. Initially, the goal was to exercise train 96 mice and run ELISA and western blot assays on over 15 analytes that included production, clearance and stress markers. These markers would have given us a better understanding on the mechanisms behind the stress induced increase and the exercise training decrease of Aβ. Unfortunately, these plans were cancelled due to the COVID-19 pandemic.

In summary, these experiments have demonstrated that an exercise training regimen is capable of rescuing Aβ formation in a chronically stressed mouse model. However, there are several questions left unanswered from this experiment. Future studies are needed to determine running speeds as well as specific timing for this transgenic mouse model. Two years ago, our lab began using the APP/PS1 transgenic mouse model after several years of using the Tg2576 line. A more in-depth study is needed on running speeds and citrate synthase activity in the

APP/PS1 mouse model to determine where the threshold lies for exercise training. Studies also need to be carried out to determine specific timing for the APP/PS1 transgenic mouse line plaque formation. This is crucial for experiments that want to stop testing before plaque formation or test well into the plaque formation.

Finally, to fully grasp the effects of this study, clearance, production and stress markers would need to be assessed. Several of each were intended to be assayed, however, due to the Coronavirus pandemic, the testing was shut down.

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APPENDICES

Appendix A: Missouri State University IAUCUC Approval Notice

From: Institutional Animal Care and Use Committee (IACUC)

<iacuc_no_reply@missouristate.cayuse424.com>

Date: Tuesday, March 5, 2019 at 2:30 PM

To: Zimmerman, Scott D <ScottZimmerman@MissouriState.edu>, Zimmerman, Scott D

<ScottZimmerman@MissouriState.edu>

Subject: Application to Use Live Vertebrate Animals

Project Title: **APP/PS1 Mouse Breeding Protocol**

IACUC ID: **19-010.0**

Species: **Mouse**

The above-referenced Application has been approved and the approval can be viewed at:

https://missouristate.cayuse424.com/acap/

If you have any questions, please do not hesitate to contact the Missouri State Office of Research Compliance at iacuc@missouristate.edu or 417-836-8405.

Appendix B: Genotyping

Materials

- ATL Buffer (Qiagen)
- Ethanol (Decon Labs)
- Proteinase K (Thermo Fisher Scientific)
- Mouse Tails (freshly snipped)
- 1.7 ml Eppendorf tubes
- Parafilm
- Small Animal Surgical Scissors
- Cauterizer (Bovie)
- Sharpies (for tail marks)
- Water Bath (56°C)
- Incubated Tubes containing tail fragments (from Day One)
- RNAse A (New England Biolabs)
- Buffer AL (Qiagen)
- AW1 (Qiagen)
- AW2 (Qiagen)
- Buffer AE (Qiagen)
- Spin Columns (Qiagen)
- 1.7 ml Eppendorf tubes
- 200 Proof Ethanol (Decon Labs)
- Mouse DNA
- Autoclaved water
- Gotaq (Promega)
- Primers (Integrated DNA Technologies)
- Trans1
- Trans2
- WTF
- WTR
- Agarose (Sigma)
- 125 ml Gel Rig
- Gel Combs (2; normally the 12 lane ones)
- 1x TAE (Make from 50x Stock)
- Ethidium Bromide

Protocol

1st Day (Tailsnipping)

1. Turn on water bath to 56° C.
2. Add **200 µl ofATL Buffer** into 1.7 ml Eppendorf tubes (one tube for each mouse). The buffer can be hard to pipet. Does not have to be exact.
3. Cut **0.75-0.80 cm** of mouse tail using clean scissors.
4. **Cauterize** mouse where cut was made to avoid infection.
5. Mark mouse tail with sharpie so can be identified later. Be sure to mark around entire tail. In lab notebook, be sure to put tail identification next to sample number. (e.g. sample +45, tail 2P [for 2 purple stripes])
6. **Place** tail into tube and **cut** into small pieces. **Label** tube with designated sample number on top and side of tube.
    a. Repeat steps 3 -6 for each mouse, being sure to clean scissors and area with ethanol between mice to avoid DNA contamination.
7. After all tails have been cut, add **30 µl of proteinase K**, vortex for 15 seconds.
8. Wrap **parafilm** around top of each tube
9. Incubate test tubes at 56°C in water-bath for at least **4 hours** for tissue lysis.
    a. **DO NOT LEAVE SAMPLES IN WATER BATH FOR LONGER THAN 24 HOURS!!!**

2nd Day (DNA Extraction)

10. Remove samples from water bath between 4 and 24 hours after placement.
11. Step up temperature of water bath to **70°C**.
12. Add **7 µl RNase A**(100 mg/ml), mix by vortex for 15 seconds.
13. Add **200 µl Buffer AL**, mix by vortex for 15 seconds.
14. Incubate at**70°C** water bath for **10 minutes**.
15. While samples are incubating, label QIAamp spin columns with sample numbers. Only need to label spin columns on top.
16. Add **200 µl of 100% ethanol** (AKA 200 proof ethanol), mix by vortexing for 15 seconds.
17. Set volume of pipette to **700 µl**and carefully apply all mixture in test tube (including the precipitate) to a numbered QIAamp spin column in a 2 ml collection tube without wetting the rim. Close the cap.
18. **BE SURE TO CHANGE PIPETTE TIPS BETWEEN EACH SAMPLE TO AVOID DNA CONTAMINATION!!!**
19. Centrifuge at **13k for 1 min.**Place QIAamp spin column in a new, clean 2 ml collection tube.
20. Add **500 µl buffer AW1**without wetting the rim. Close the cap.
21. Centrifuge at **8k for 1 min.**Place QIAamp spin column in a new, clean 2 ml collection tube.
22. Add **500 µl buffer AW2**without wetting the rim. Close the cap.
23. Centrifuge at **12k for 5 min.**
24. During this time, label new Eppendorf tubes with samples numbers on top and side.

25. Place the QIAamp spin column in a **numbered, clean 1.7 ml Eppendorf tube**.
26. Add **150 µl buffer AE**and incubate at **room temperature for 5 min.**
27. Centrifuge at **8k for 1 min.**
28. Discard QIAamp spin column and close cap of labeled Eppendorf tubes. The solution in the Eppendorf tubes contains the extracted DNA.
29. Double check and make sure tubes are labeled on both the top and side
30. Store samples in the -20°C freezer (tall one in PROF 367) until later use.

Genotyping Day Three: PCR & Gel

Make master mix for PCR reaction.

Reaction:

Stock	Per Sample (a)	Mastermix total
2X Gotaq Green mastermix	12.5 µL	12.5x (n)
20 pmol/µL (WTF)	0.625 uL	0.625 x (n)
20 pmol/µL (WTR)	0.625 uL	0.625 x (n)
20 pmol/ul (Trans 1)	0.625 uL	0.625 x (n)
20 pmol/ul (Trans 2)	0.625 uL	0.625 x (n)

(n = # of samples + 3 (positive cntrl, negative cntrl, and water) + transfer loss) For sample above 10 account for 1 extra (transfer loss), above 20 (3), above 30 (5) etc.

31. Vortex this mixture. KEEP ON ICE, IF YOU DO NOT YOU WILL GET HIGH CONCENTRATION OF PRIMER-DIMERS
32. Obtain 48 well PCR plate and put on ice.
33. Pipette 15.0 µl of each master mix into individual wells.
34. Place 7 ul of autoclaved water in each well.
35. Pipette 3 ul of DNA into the mouse's specified lane.
36. Take samples to general lab (room with -80), put in themocycler (black).
37. Power on, run, Z-lab, BL. Yes to heat lid.
38. This will run samples. Reaction takes 1.5 hours but protocol is set with (-4°Cfor infinity), samples may be stored like this for about 24hours. Remove samples from thermocycler.
39. Place samples in -20 °Cfreezer.

Visualization

40. Run products on 1.5% agarose gel in 1X TAE. Directions for a 125 ml prep.
    a. To make 1x TAE: 20 ml 50x TAE stock + 980 ml ddH_20
41. Put 1.87 g of agarose into 125 ml of 1X TAE in an Erlenmeyer flask.
42. Microwave until agarose is COMPLETELY dissolved
43. Heat until just boiling, do not allow to completely boil
44. Continue to heat for a few seconds at a time until floating "sparkles" in the solution go away
45. Allow flask to cool until you can comfortably hold it in your hand
46. Add 5 ul EtBr.
47. Pour into assembled electrophoresis rig, allow to solidify
48. Two combs per gel may be used if there are a lot of samples.
49. We recommend using 12 lane combs for better visualization.
50. Once gel has solidified, remove combs and fill chamber with THE SAME 1X TAE you used to make the gel.
51. Load 5 ul of each sample and run it

52. Run with 100 bp ladder, positive, and negative and water
53. 125mV for 45
54. NOTES: Gotaq master mix contains loading dye in two fronts. One runs in front of primers at 500 bp, the seconds runs around 3500. No loading dye is needed to push sample into wells.
55. Place gel into DNA transilluminator.
56. Make sure EtBr lens is in place.
57. Visualize, save screen shot in normal colors and inverted colors. Record in genotyping notebook positive mouse numbers.

Appendix C: Exercise Training

Materials

- Treadmills
- Mice
- Straws
- Foil
- 70% Ethanol
- Pipe cleaners

Protocol

1. Obtain list of mice running/shitting from running sheets provided in yellow folder on desk in Prof 370.
2. Collect necessary mice from vivarium (Prof 368).
3. Load mice on to treadmill into their specific lane.
    a. This is indicated by tape on the treadmills. Match tattoos on tails to cage cards to running sheet and to the tape on the treadmill to ensure accuracy.
    b. Sedentary mice are placed on mock treadmills that do not move. Ensure all appropriate lanes are filled and treadmill is covered.
4. Place tin foil over the top half (furthest from you) of the treadmill.
5. Insert a straw into the hole immediately following the tin foil for running mice.
    a. Check that straws do not come in contact with the running belt, and that they are not super short.
6. Set running treadmills to 20 m/min.
7. Simultaneously start the 3 running treadmills and a stopwatch.
8. Encourage mice using extra straws, and pipe cleaners. Do not let mice sit on shockers for prolonged periods of time (more than 10 seconds) without encouragement.
9. On running sheet document if mice need heavy (persistent) or light encouragement. If mice refuse to run, not RR with the time they began refusing.
10. After one hour, shut off treadmills and remove mice. As with placing mice, compare tattoos to cage cards and the tape on the lane it was removed from before putting it into cage to ensure accuracy.
11. Clean treadmills and shockers with 70% ethanol (spray bottle).
12. Dump poop trays from underneath each running treadmill.
13. Place mice back in Prof 368.

Appendix D: Harvesting Tissues

Materials

- Live mouse
- Isoflurane
- Dissection trays
- Pins
- Dissecting scissors
- Forceps
- Ethanol
- Saline
- Ice
- Petri dishes
- Microcentrifuge tubes
- Scalpel
- Spatula
- Liquid nitrogen

Protocol

1. Weigh mouse and record.
2. Set up isoflurane vaporizer.
    - Turn on oxygen.
    - Fill reservoir with isoflurane.
    - Turn on to level 4-5.
3. Bring mouse and cage card to station one and place into chamber for anesthesia.

Anesthesia/Profusion:

4. Load mouse into isoflurane vaporizer chamber, close the lid
    - Wait while mouse slows breathing
    1. Watch for heart rate to decrease to a slow/steady rhythm
5. Once anesthetized, move the mouse to the dissection tray and pin the animal's feet down
    1. Make sure mouse is void of all reflexes prior to pinning.
    2. Cover the face with makeshift isoflurane mask to ensure anesthetic is still being applied throughout procedure
6. Begin with an incision at the xyphoid process
    1. Cut towards the mouse's head, through the ribs, avoiding organs
    2. Blunt dissect through the stomach to expose the heart
    3. Once the heart is exposed, prepare 3 syringes with saline
7. Simultaneously, cut the right atrium and inject the left ventricle with saline to begin profusion
    1. Profuse the heart with all 3 syringes of saline, keeping a relatively quick pace so the mouse remains alive during this process
8. Once complete, remove the heart and dispose
9. Clean the mouse of any blood and pass the carcass and cage card to the next station

Brain Extraction:

10. Use dissecting forceps to grasp the mouse's skull and make an incision at the base
11. With scissors pointing up, cut towards the mouse's eyes, through the skull

1. Be careful not to puncture the brain

12. Blunt dissect the same area, removing the skin, exposing the skull
    1. To expose the brain, use the sides of the forceps and carefully ply open/peel back the skull
13. Once the brain is exposed, use the forceps to extract the brain from the base of the skull
    1. NOTE: The brain might stick to the skull. If so, use the forceps to pick up and move brain around until loose.
14. Place brain onto petri dish inside of an ice bucket.
15. Pass to hippocampus and cortex extraction station.
16. Pass the mouse carcass to the solei extraction station, along with cage card.

Hippocampus/Cortex Extraction:

17. Place brain onto petri dish inside of an ice bucket.
18. Remove the cerebellum and brain stem.
19. Using a scalpel separate the brain into the left and right hemispheres by slicing down the longitudinal fissure.
20. Beginning on the left hemisphere, position the medial brain surface facing away and slightly upward. Hold the cortex with a metal spatula, while using another metal spatula to gently scrap away the midbrain.
21. Look for variation in color under the cusp of the cortex. A slightly darker banana shaped region will be evident. This is the hippocampus.
22. Use the pointed end of a metal spatula to lift the hippocampus out.
    1. If successfully done, there will be a white, almost opaque tissue revealed.
23. Stretch out the cortex, and slice between the white, opaque tissue and the cortex.
24. Repeat for the right hemisphere.
25. Weigh left hippocampus and cortex, and right hippocampus and cortex.
26. Flash freeze using liquid nitrogen.
27. Store at -80°C.

Solei Extraction:

28. Place mouse in prone position.
29. Start with the left hind leg.
30. Grab the skin just below the bend, with modest force, pull down.
    1. This should remove the skin down to the ankles, revealing the gastrocnemius.
31. Slide one side of the forceps underneath the lower hind leg muscles and rub it against the tibia to separate the muscles from the bone.
32. Cut the Achilles tendon and flip back the muscles.
33. Look for variation in color. A darker red, thin muscle will be evident. This is the soleus.
34. Slide one side of the forceps under the soleus.
35. Cut the superior tendon holding the soleus to the body.
36. Using forceps, pull the soleus out.
37. Weigh the tissue and flash freeze using liquid nitrogen.
38. Store at -80°C.
39. Repeat for right hind leg.

Appendix E: Sequential Extraction

Materials

- Microcentrifuge tubes
- Ice bucket & ice
- Microcentrifuge
- 1X PBS
- Triton X-100
- 5M Guanidine-Tris
- Protease Inhibitor

Make the following solutions for extraction:

1. 1X PBS + 1X protease inhibitors
2. 1% Triton-X in PBS + 1X protease inhibitors 100 uL of Triton X-100
3. 5M Guanidine-Tris + 1X protease inhibitors pH 8.0

Protocol

1. Chill all buffers before use. Label tubes and chill on dry ice.
2. Note: important to keep all tissue frozen solid before and during weighing so it can be moved between tubes.
3. Tare scale with new tube. Add sample to tube and weigh to calculate mass of tissue. Store weighed tubes/sample on dry ice while weighing all samples
4. Add 10ul of buffer per 1mg of tissue weight for each extraction.
5. Homogenize in PBS+PI: 12 turns right, 12 left, 12 right
6. Spin at max for 25min at 4°C. Save and transfer supernatant to chilled tube. Store supernatant at 4°C.
7. Add 10ul of TX100+PI buffer per 1mg of tissue wet weight for each extraction.
8. Homogenize: 12 turns right, 12 left, 12 right.
9. Spin at max (20,000g) for 25min at 4°C. Store supernatant at 4°C in a chilled tube.
10. Add same volume of guanidine+PI as in step 4. Sonicate 12 pulses, 50% amplitude. Sonicate tip in MilliQ water to clean and dry tip between each sample. Avoid allowing the tip to touch the sides of the tube to avoid foaming.
11. Spin at max (20,000g) for 25min at 4°C. Store supernatant at 4°C in a chilled tube.

Appendix F: Protein Assay: BCA

Materials

- Pierce BCA Protein Assay Kit (Thermo Scientific)
- Samples from Sequential Extraction
- 1X PBS
- Triton X-100
- 5M Guanidine-Tris
- Protease Inhibitor
- H20

Protocol

BSA Protein Assay (Pierce Micro BSA assay)

1. Dilute TX100 samples 1:50 into water. Dilute sample buffer 1:50 for standard curve.
    a. Samples: 6 ul Sample + 294 ul H20
    b. Curve: H20/Buffer mix = 2143.75 ul H20 + 43.75 ul buffer
2. Dilute as shown in table below

Tube	Concentration µg/ml	Ratio	Mix
1	Blank		250µl H2O/Buffer
2	250	1:8	62.5µl BSA + 437.5µl H2O
3	125	1:2	250µl prev + 250µl H2O/Buffer
4	62.5	1:2	250µl prev + 250µl H2O/Buffer
5	31.25	1:2	250µl prev + 250µl H2O/Buffer
6	15.625	1:2	250µl prev + 250µl H2O/Buffer
7	7.81	1:2	250µl prev + 250µl H2O/Buffer
8	3.9	1:2	250µl prev + 250µl H2O/Buffer

3. Pipette 100µl/well, load in duplicates
4. Make working reagent (WR)
    a. 50 parts Reagent A + 48 parts Reagent B + 2 parts Reagent C = WR
    b. Note: WR for whole plate = 6 ml Reagent A + 5.76 ml Reagent B + 240 ul Reagent C
5. Load 100µl in each well
6. Cover and incubate 37°C until color develops

    a. Note: Normally around 45 minutes to 1 hour
7. Read plate at 560nm
    a. Note: Use Protein Assay 2016 protocol in the Gen5.1.11 program to read plate

Appendix G: ELISA

ELISA Methods:

Day 1

1.

Steps/calculations for coating plate:

1. Pipette 6mL coating buffer
2. Pipette 64 ul HJ2 (0.94mg/mL given stock)
3. Invert tube 4-5x
4. Set multichannel pipetter to 50ul
5. Pour solution into large well, pipette into individual wells in plate
6. Seal plate
7. Store overnight in 4◦C fridge on rotator

HJ2

0.94 mg/mL given --> 10 µg/mL

0.94x1000 = 940

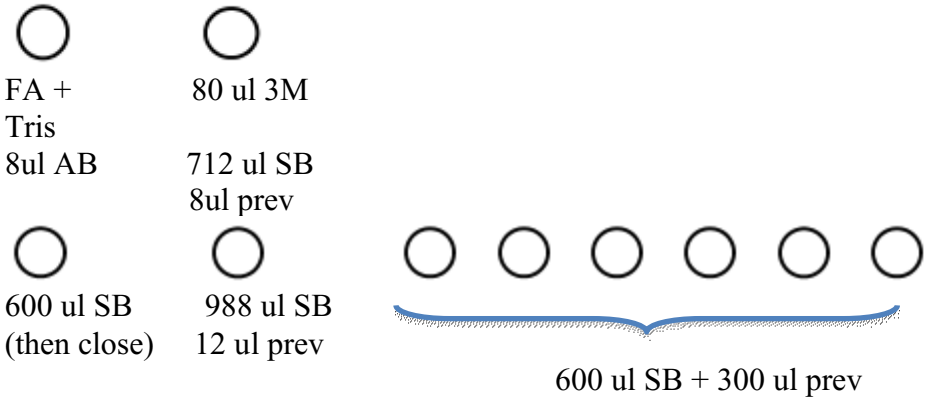
$\frac{940}{10}$ = 94 dilution factor

Coating buffer 6mL

$\frac{6000}{94}$ = 64 uL/6 mL coating buffer

Day 2

1. Make 2% BSA
   a. 100 mL of 1xPBS + 2g BSA (solid form in 4◦C fridge), stir
   b. Filter into 150 mL
   c. Place in 4◦C fridge
2. Make Sample Buffer (in 50 mL tube)
   a. 5ml 3M Tris + 250 ul 10% Azide
   b. Add 12.5 mL of 2% BSA-PBS
   c. Add 500 ul of 100xPI (in freezer)
   d. Fill to 50 mL w/ 1xPBS-0.05%Tween 20, label & chill for use
      Note: BSA & Sample Buffer expire EVERY 3 DAYS
3. Wash/Block Plate
   a. Set plate washer to 1xPBS-0.05% Tween 20
   b. Prime plate washer
   c. Wash plate, remove excess fluid, bring to bench ASAP
   d. Block with 190ul/well of 2% BSA-PBS
   e. Incubate 60 min, uncovered @ 37◦C (in heater at bench)
   f. Wait 30-40 min to make up standard samples
   g. Standard sample calculations/instructions:



(AB40 Formic Acid calculation:
(same calc for AB42)
Example: Given stock 0.98 mg/mL
(x1000) = 980 ul/mL

980)(8) = (10)(x)
7840 = 7840 x
10

C1 = given stock of AB
V1 = 8 ul
C2 = 10 ug/mL
V2 = ?

x= 784 = V2
784 - 8 = 776 ul Formic Acid/8 ul AB

1. Load Plate w/ Samples
   a. Set up vacuum w/ pipette tip
   b. When 60 min is up, wash 4x w/ 190 ul 1xPBS-Tween 20 (leave wells full)
   c. Vacuum out each well & place 50 ul from each sample in duplicate or triplicate columns
   d. Place on rotator in 4◦C fridge overnight

Day 3

1. Make 0.05% BSA-PBS-T20:

   2 / .05 = 40 dilution factor. 6 ml = 6000 ul

   6000 / 40 = **150 ul 2% BSA-PBS**

   then add **5.85 ml 1x PBS 0.1T20** (5mL then 850uL)

2. Add mHJ5.1 Biotin: 1:1000 of stock solution:

   1:1000 Concentraion
   6000 ul per plate
   6000 / 1000 = **6ul HJ5.1 Biotin**

3. Wash 4x w/ 190 ul 1xPBS T20
4. Load mHJ5.1 Biotin 1:1000 in BSA-PBS T20 (50ul/well)
5. Incubate at **37◦C** for **90 min**

6. Make 1% BSA-PBS-T20: **3mL 2% BSA-PBS + 3 mL 1xPBS 0.1 T20**

7. Add 6ul Strep Poly HRP40 1:1000 to 1%BSA-PBS-T20:

   1:1000 concentration
   6000 / 1000 = **6 ul HRP**

`

8. Wash 4x w/ 190 ul 1xPBS T20

9. Load plate with strep-Poly HRP40 mixture (50ul/well)
10. Cover
11. Put on shaker at **room temp** for **90 min**
12. Prepare sigma superslow TMB
13. Wash 4x w/ 190 ul 1xPBS T20
14. Add 50 ul/ well sigma superslow TMB
15. Read absorbance at 650nm at 2, 6, 10 min

Appendix H: Western Blot

Materials

- Gel: Invitrogen NuPAGE 4-12% Bis-Tris gel, 10 wells, 1.5mm thick
    - TC Support Center

- 1X MES SDS Running Buffer
    - 50ml 20X MES SDS Running Buffer (Invitrogen, NP0001)
    - 950ml mqH20

- 5X SDS-PAGE Sample Buffer, Reducing
    - 5ml 1M Tris, pH 6.7
    - 5ml Glycerol
    - 2g SDS
    - 0.05g Bromophenolblue
    - Fill to 20ml w/ mqH20
        - Take 1ml of above buffer + 50ul β-Merc to make reducing buffer

· 1X Transfer Buffer
    - 24.7 Tris
    - 120mM Glycine
    - 20% MeOH
    - mqH_2O
        - Parafilm cap and Store @ 4°C

- Ponceau S
    - 0.2g PonS
    - 10ml Acetic acid
    - 190ml mq H_2O

- 1X PBS-T20 (0.125%)
    - 1L Gibco 1X PBS
    - 1.25ml T20

- 3% Milk- PBST20 (0.125%)
    - 3g dry milk (crushed)
    - 100ml 1X PBS-T20 (0.125%)
    -
- Lumigen TMA-6
    - Lumigen Cat #TMA-100

Protocol

1. Turn on tube warmer to 100°C
2. Dilute samples with PBS/mqH2O and 5X reducing loading buffer, target 15ug sample/lane

a. 5X reducing loading buffer should be diluted with b-merk (50mL/1 mL of 5XRB)
b. prepare ladder: 5 mL Benchmark + 5 mL Magic Mark + 5 mL 5X LBNR (do not boil)
c. Boil samples in tube warmer for 10 minutes @ 100°C
i. Close caps of tubes and place a weight on top so they do not open during boil

3. Briefly centrifuge tubes to pellet all solution to bottom of tubes and gently vortex to mix
4. Prepare NuPAGE 4-12% Bis-Tris gel
    a. Peal tape from bottom of gel
    b. Remove comb from wells by pulling straight up
    c. Insert gel into frame with wells facing inward and clamp into place
    d. Fill inside chamber first with 1X MES running buffer then outside chamber to approx. half way up the gel
    e. Rinse wells with running buffer using a transfer pipette
5. Load ladder/samples (15-20 mL/well) and run at 100V until samples go into gel then turn up to 150V
    a. check for how samples are running (if smiley, turn down voltage)
6. Stop gel when blue buffer front reaches approx. 0.5 in from the bottom of the gel
7. Remove gel from rig and trim off curved bottom of gel and well hairs at top
8. Briefly rinse in 1X transfer buffer
9. Prepare sandwich submerged in transfer buffer:
    a. (Packet comes with paper, nitrocellulose, paper*)
    b. Hydrate all individual pieces of sandwich

Order: Black side down

Sponge
Whatman paper
Gel
Nitrocelluose
Whatman paper
Sponge
Lock clear lid

8. Insert sandwich into transfer rig: black to black
16. Add small stir bar and ice pack to chamber
17. Fill rig with 1X transfer buffer
    a. Transfer buffer should be *COLD*
    b. Make day before transfer and store in fridge
18. Transfer to nitrocellulose at 150V for 90 minutes in cold room
    a. If any error on transfer rig – run LONGER. Low chance of over transfer
19. After transfer, rinse blot with mqH2O
20. PonS blot for 5-10 min
    a. At this point, should see bands on blot
21. Distain with 3-4 mqH2O rinses
22. Block 60 minutes with 3% milk-PBS T20 (0.125%)
    a. STIR MILK!!!!!
23. Incubate 1° Ab in 1% milk-PBST20 (0.125%) overnight on shaker at 4°C

a. Primary antibody dilution begins around 1:1,000
b. This step can take place in cold room on tilter

24. Wash 10 minutes x 3 with PBS-T20 (0.125%)
25. Incubate 2° Ab in 1% milk PBS -T20 (0.125%) 90 minutes on shaker at RT
    a. Secondary Ab must be HRP conjugated
    b. Start with 1:20,000 dilution
26. Wash 10 minutes x 3 with PBST20 (0.125%)
27. Develop blot(s) with TMA6- Lumigen or West Pico (ECL solutions)
    a. When developing, take mqH2O, ECL and PBS to imager
        i. Blot goes from PBS à H2O à ECL à imager
        ii. ECL cannot be exposed to LIGHT. Keep in dark box and shut off lights!
    b. West Pico is used for low signal (gapdh, tubulin)
    c. Lumigen is used for other proteins (Ab, etc)
28. Store blots in 1X PBS at 4°C