ALleviation of central accumulation of amyloid-beta and prevention of cognitive dysfunction following peripheral inflammation

by

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ALLEVIATION OF CENTRAL ACCUMULATION OF AMYLOID-BETA AND PREVENTION OF COGNITIVE DYSFUNCTION FOLLOWING PERIPHERAL INFLAMMATION

by

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In 1907, Alois Alzheimer published an article in which he described a female patient who had lived in an asylum and presented with behaviors that could not be classified as a common mental illness. He discussed her rapid memory loss, disorientation, and paranoid thoughts. Alzheimer described her as appearing completely helpless and unable to understand her situation. She was able to name objects that were placed in front of her, but then was unable to recall the objects after the session. After the death of the patient, Alzheimer performed an autopsy and noted the symmetrical atrophy of the brain, as well as cellular changes discovered with, then novel, silver impregnation dye and microscopy.

Today this grouping of pathologies is referred to as Alzheimer’s disease (AD), currently affects 5.4 million Americans, and represents the 6th leading cause of death in the US ("2013 Alzheimer's Disease Facts and Figures," 2013). This number is expected to continue to rise and, if no therapeutic advances are made to combat this disease, an estimated 6.7 million Americans will develop AD by 2025. There are two forms of AD, sporadic AD, the more commonly occurring form diagnosed after the age of 65, and familial or early-onset AD, diagnosed before the age of 65. Although a number of the genetic defects that lead to familial AD are now known, the cause of the more common form of AD, sporadic Alzheimer’s disease, remains unknown.
At autopsy, an AD-affected brain can appear atrophied, especially in brain regions associated with learning and memory (Heneka & O'Banion, 2007). This reduction in mass has been associated with degeneration of synaptic connection and death of neurons (Mattson, 2004). A common feature in the emerging research on neurodegenerative diseases, such as AD, is the impact of inflammatory processes on progression of the disease (Minghetti, 2005) and the clinical manifestations of AD (Eikelenboom, Rozemuller, & van Muiswinkel, 1998). Chronic inflammation of the CNS is a common component of Parkinson’s disease (PD) and AD. In fact, the presence of amyloid-beta peptide (Aβ), which can be found in the insoluble plaque formations associated with AD (Heneka & O'Banion, 2007) has the ability to activate microglia, innate immune cells that reside in the CNS, that subsequently produce pro-inflammatory cytokines and chemokines (Schwab & McGeer, 2008). Aβ, activated microglial cells, and pro-inflammatory cytokines can all lead to increased central inflammation.

Numerous animal models have been generated to examine not only the relationship between inflammation and AD (Akiyama et al., 2000; Jaeger et al., 2009; Kahn et al., 2012) but the behavioral effects of the inflammation as well (Kahn et al., 2012; Lee et al., 2008; Thirumangalakudi et al., 2008). One of our previous experiments, involving a non-transgenic animal model, revealed an increase in the amount of Aβ in the hippocampus and cognitive deficits on hippocampus-dependent tasks, following administration of the endotoxin lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria (Kahn et al., 2012). We determined that seven consecutive days of a single, daily peripheral injection of LPS, produced an increase in central Aβ, and led to significant disruptions in hippocampus-dependent learning. Our study attempted to
link inflammation with AD-like pathology, and is the impetus for this dissertation, as we will seek to test the hypothesis that the increase in hippocampal Aβ is in fact to blame for the cognitive decline. We will then investigate four ways that could potentially decrease central Aβ in hopes of rescuing inflammation-induced cognitive deficits.

**Alzheimer’s disease pathology**

Clinically, AD patients first present with mild cognitive impairments, such as short-term memory loss, but as the disease progresses patients can lose their ability to perform basic functions and intellectual abilities (Mayeux & Stern, 2012). Alzheimer’s disease is also characterized by the trademark neuropathology of both neurofibrillary tangles and beta-amyloid plaques (Hochstrasser, et al., 2013; Masters & Selkoe, 2012; Murphy & LeVine, 2010). The formation of both the amyloid plaques and neurofibrillary tangles are not only believed to be histological markers of this disease, but also possible causative mediators in the degenerative nature of AD (Hochstrasser et al., 2013). However, contradictory evidence suggests that the presence of these plaques and tangles may not be the cause of this disease nor necessary for disease progression. Previous research has shown that increased levels of Aβ plaques correlate poorly with disease severity (Heneka & O'Banion, 2007).

Tau, a cytoskeletal protein that plays an important role in the formation and stabilization of microtubules, is found predominantly in axons but has also been seen, at much lower levels, in dendrites (Ittner & Gotz, 2011). Neurofibrillary tangles (NFTs) are composed of both hyperphosphorylated tau and paired helical filaments (Markesbery, 2010). Hyperphosphorylated tau can accumulate and aggregate in the somato-dendritic
areas of neurons and can form NFTs, which have been shown to interfere with normal neuronal functions (Ittner & Gotz, 2011; Ittner, Ke, & Gotz, 2009). NFTs may have a role in the progression of AD by aggregating more tau and other proteins, thereby causing a greater loss in the normal function of tau (Ballatore, Lee, & Trojanowski, 2007).

The Aβ hypothesis of AD theorizes that Aβ, the protein fragment found in senile plaques of patients diagnosed with AD, is the catalyst of a cascade of cellular events leading to cerebral injury and neuronal loss (J. Hardy & Selkoe, 2002). The formation of Aβ plaques results from the cleavage Amyloid Precursor Protein (APP), a transmembrane protein with a single transmembrane domain. APP is sequentially cleaved by two protease enzymes, β-secretase and γ-secretase (Seiffert et al., 2000) producing various lengths of peptides that can accumulate to form the senile plaques associated with various forms of dementia (Murphy & LeVine, 2010). Importantly, there is a direct correlation between elevated levels of Aβ and AD progression (Mucke & Selkoe, 2012).

Currently, there are two diagnoses of AD, sporadic AD occurring after the age of 65 and accounting for over 90% of all AD cases, and the more genetically-linked version, familial or early onset AD, occurring in less than 10% of AD cases. The most common cause of familial AD involves mutations in the genes that encode presenilin-1 (PS-1) or presenilin-2 (PS-2), which are part of the γ-secretase complex (De Strooper, Iwatsubo, & Wolfe, 2012; Murphy & LeVine, 2010; Wolfe et al., 1999), that leads to increased production of the longer Aβ peptides through altered activity of γ-secretase (Seiffert et al., 2000; Wolfe et al., 1999). These longer peptides, as opposed to more soluble and shorter Aβ peptides, tend to aggregate and are more difficult to clear from the brain. According to Muller and Zheng (2012), understanding the cleavage processes that occur
under normal APP processing, during which Aβ is released, may be extremely helpful in developing AD therapeutic targets. β-secretase (BACE) and γ-secretase have been the more recent targets for the development of new AD therapeutic drugs, due to their mechanisms of action which, through a series of cascades, alter the amount of Aβ produced (Eisele et al., 2007; Schenk, Basi, & Pangalos, 2012; Seiffert et al., 2000; Sutcliffe, Hedlund, Thomas, Bloom, & Hilbush, 2011). In a recent review of the structure and function of BACE, elevated levels were proposed as a potential early biomarker and potential therapeutic target of sporadic AD (Evin, Barakat, & Masters, 2010) as BACE has been found to be elevated in brains of AD patients (Holsinger et al., 2002). An earlier study of APP transgenic mice, previously shown to have an increased number of plaques in the brain, were crossed onto BACE knockout mice. This resulted in a significant decrease in the number of plaques previously seen (Ohno et al., 2004). Interestingly, He et al. (2010), demonstrated that production of Aβ could be reduced by altering the activity of gamma secretase activating protein (GSAP), thus decreasing γ-secretase activity and the concomitant cleavage of APP into Aβ.

**Inflammation**

Inflammation is a component of the body’s initial innate response to intruding pathogens and harmful toxins. One non-specific response to these stimuli originates from pattern recognition receptors on immune cells designed to recognize general structural components that are present and exposed on the surface of broad classes of invading pathogens. Binding of these so called pathogen-associated molecular patterns, or PAMPs, to these receptors leads to activation of immune cells such as neutrophils and
macrophages. Immune cell activation also leads to the production of cytokines and chemokines, proteins secreted to help regulate a host’s response to infection (Dinarello, 1996). Cytokines are commonly classified by functional activity, such as pro-inflammatory or anti-inflammatory. As the name suggests, pro-inflammatory cytokines facilitate coordination of both the peripheral and central inflammatory response.

Although the brain was once believed to be an immune-privileged area, we now know that cytokines produced in the periphery in response to a pathogen can affect the brain in several ways (McGeer & McGeer, 1996). One way this occurs is through a neural route, in which inflammation in the periphery stimulates vagus nerve afferents to relay information to the brain resulting in central production of cytokines, primarily through the activation of astrocytes and microglia (Ferrari et al., 2006; Lee, Liu, Dickson, Brosnan, & Berman, 1993). Another way cytokines can affect the brain is through a humoral pathway that involves peripherally produced cytokines entering the brain through the circumventricular organs and the choroid plexus, or active transport of peripherally-made cytokines across the blood-brain barrier (BBB; Dantzer et al., 2000; Katsuura, Arimura, Koves, & Gottschall, 1990; reviewed in: Konsman, Parnet, & Dantzer, 2002).

Increased pro-inflammatory cytokine production leads to a number of behavioral changes, such as alterations in mood, decreased exploration, decreased eating, etc., often grouped together and referred to as “sickness behaviors” (Dantzer & Kelley, 2007). Increased “sleepiness” is also a characteristic of sickness behavior (Dantzer & Kelley, 2007), and interestingly, levels of both pro-inflammatory cytokines IL-1 and TNF have been shown to be elevated during infection (Krueger et al., 2007). In a review of sleep
literature, Bollinger et al. (2010) concludes that sleep improves immune response in most immune cells, indicating the importance of sleep in normal immune function and the importance of increased sleep during illness. Moreover, elevations in pro-inflammatory cytokine levels can also alter cognitive function (Cunningham et al., 2009; Cunningham & Sanderson, 2008; Kranjac et al., 2012; Pugh et al., 1998; Sparkman, Kohman, Scott, & Boehm, 2005).

A common way to initiate inflammation and the resultant cascade of biological responses, in the absence of a live pathogen, is through administration of purified lipopolysaccharide (Dantzer, 2004; Konsman et al., 2002). Lipopolysaccharide (LPS) is a component of the cell wall of gram-negative bacteria and binds to Toll-like receptor (TLR)-4, as well as other protein components of this receptor complex, leading to a cascade of signaling pathways that result in increased pro-inflammatory cytokine production (Dantzer, 2004). TLR-4 signaling leads to the production of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6, among others. IL-1β can be secreted by monocytes, macrophages, endothelial and epithelial cells and can cause local tissue destruction, increased vascular permeability, and fever through an IL-1β-dependent increase in the transcription of IL-6. Macrophages also make TNF-α, which is known to enhance vascular permeability and fever, as well as induce apoptosis. In addition, TNF-α has appetite suppressing properties, and has been implicated in cachexia, also known as wasting syndrome, producing the symptoms of weight loss, fatigue, weakness, and muscle atrophy (Tisdale, 2009). IL-6 is secreted by macrophages and endothelial cells and effects the liver and influences cellular proliferation and antibody secretion utilized in adaptive immunity. One important function of IL-6 is the alteration of the body’s
temperature set point through its effects on the hypothalamus, leading to fever.

Temporal alterations in the levels of cytokines and chemokines have been studied after a single and multiple i.p. injections of LPS. It appears that peak levels of IL-1\(\beta\), IL-6, and TNF-\(\alpha\), and the chemokines monocyte chemotactic protein (MCP)-1 and macrophage inflammatory protein (MIP)-1\(\alpha\) (discussed later), in serum occur around 4 hours after a single injection, followed by a return to basal levels around 24 hours post-injection (Cunningham et al., 2009; Erickson & Banks, 2011; Kahn et al., 2012; Kranjac et al., 2012). Multiple injections of LPS have also been used to more closely resemble longer bouts of inflammation and inflammatory diseases (Erickson & Banks, 2011; Kahn et al., 2012; Lee et al., 2008; Nguyen, D'Aigle, Gowing, Julien, & Rivest, 2004). The effect of serum cytokine levels in one study of three LPS injections, given over a 24 hour period (injections were given at 6h and 24h, following the first injection) revealed that unlike 24 hours after a single injection of LPS, levels of IL-1\(\beta\), TNF-\(\alpha\), IL-6, MCP-1, and MIP-1\(\alpha\) were all still significantly elevated following the multiple injections (Erickson & Banks, 2011). Similarly, we demonstrated that elevated levels of central cytokines were detected 4 hours after a single injection of LPS, and 4 hours after the last injection of 4 consecutive days of LPS injections. However, cytokines were no longer significantly elevated 4 hours after seven consecutive days of single injections (Kahn et al., 2012). We speculated that this reduction in the inflammatory response occurred due to endotoxin tolerance, a phenomenon believed to develop in order to mitigate consequences of chronic inflammation and possible septic shock (Dobrovolskaia & Vogel, 2002).

Bacterial septic shock is believed to develop from a system-wide overproduction of pro-inflammatory cytokines. Symptoms of septic shock include, but are not limited to,
decreased blood pressure, fever, wide spread blood clotting, and death. In one study of sepsis, a single administration of high-dose LPS (10 mg/kg) led to widespread tissue destruction, prolonged central inflammation and neuronal death. Three months after this LPS-induced sepsis, cognitive deficits were still apparent on the radial-arm maze paradigm (Semmler et al., 2007).

An extensive review by Yirmiya and Goshen (2011) describes countless studies that have investigated the relationship between inflammation and behavior. For example, intracerebroventricular (i.c.v.) administration of IL-1β 1hr prior to spatial water maze training led to memory impairment displayed during first trial of the following day (Oitzl, Josephy, & Spruijt, 1993). In another study, learning a contextual fear conditioning (CFC) paradigm was disrupted by social isolation, which was also discovered to increase IL-1β in the hippocampus (Pugh et al., 1999). Pugh et al. (1999) also discovered that administration of IL-1 receptor antagonist (IL-1ra), after conditioning, decreased the previously observed deficits in CFC. Fiore et al (1996) showed that animals over-expressing TNF-α demonstrated learning impairments on the passive avoidance paradigm. When animals were administered TNF-α (i.c.v.) daily for one week prior to MWM, they too displayed learning deficits (Bjugstad, Flitter, Garland, Su, & Arendash, 1998). In addition, i.p. administration of LPS has produced learning and memory deficits measured by several behavioral tasks, including two-way active avoidance (Sparkman, Kohman, et al., 2005), CFC (Kranjac et al., 2012; Pugh et al., 1998), passive avoidance (Lee et al., 2008), auditory cued fear condition (Pugh et al., 1998), and Morris water maze (Lee et al., 2008; Sparkman, Kohman, et al., 2005; Sparkman, Martin, Calvert, & Boehm, 2005). Multiple injections of LPS have also been used to more
closely resemble longer bouts of inflammation and inflammatory diseases (Kahn et al., 2012; Lee et al., 2008; Nguyen et al., 2004; Villaran et al., 2010).

**Endotoxin Tolerance**

As described above, endotoxin tolerance is believed to be an adaptation of the innate immune system, protecting the host from sepsis (Dobrovolskaia & Vogel, 2002; Gantner & Singh, 2007). In 1946 Paul Beeson first reported a decreased inflammatory response in humans after each successive dose of typhoid vaccine, used to slow the progression of a syphilitic infection (Beeson, 1946). LPS was later demonstrated to produce similar activation of TLRs (Gantner & Singh, 2007) as well as display effects of endotoxin tolerance (Dobrovolskaia & Vogel, 2002). Endotoxin tolerance protects an organism from lethal doses of endotoxin after previous exposure to sub-lethal concentrations (Lehner et al., 2001). Therefore, repeated injections of endotoxin can be delivered in a laboratory setting with minimal risk of septic shock.

Experiments using repeated injections of LPS are designed to model more chronic illnesses with extended durations of inflammation (Kahn et al., 2012; Lee et al., 2008; Nguyen et al., 2004; Sheng et al., 2003). In our previous study, we demonstrated that 24 hours after seven consecutive daily injections of LPS, animals no longer displayed sickness behaviors, as demonstrated by normal activity in open field boxes, and a return of peripheral and central cytokines to basal levels (Kahn et al., 2012). We theorized this display of reduced sickness behaviors and lower levels of inflammatory mediators was indicative of endotoxin tolerance. This inferred tolerance in 7 days to repeated injections...
led us to run cognitive tests on LPS-administered animals without sickness as a confounding variable in our cognitive results.

**AD and inflammation**

There appears to be an overwhelming presence of inflammatory mediators in an AD brain as compared to a normal aging brain (Akiyama et al., 2000; Delacourte, 2006; Eikelenboom et al., 1998; Heneka & O'Banion, 2007), along with higher levels of pro-inflammatory cytokines and chemokines reported in plasma of AD patients (Akiyama et al., 2000; Hochstrasser et al., 2013; Magaki, Mueller, Dickson, & Kirsch, 2007). Other studies have shown that inflammation can lead to the induction of Aβ deposition that can, in turn, induce neuronal cell death (Mucke & Selkoe, 2012; Shankar & Walsh, 2009), glial cell activation (Schubert et al., 2000), and subsequent release of additional proinflammatory cytokines (Hickman, Allison, & El Khoury, 2008).

There are several cytokines associated with AD, including IL-1β, TNF-α, and IL-6 (Heneka & O'Banion, 2007). Activated microglial cells, described below, secrete IL-1β, (Akiyama et al., 2000; Heneka & O'Banion, 2007), which has been shown to increase the processing of APP (Buxbaum et al., 1992; Dinarello, 1996) and increase activity of acetylcholinesterase, leading to further cholinergic decline and the memory dysfunction associated with AD (Li et al., 2000). In addition, IL-1β has also been shown to enhance microglial activation leading to a further increase in their IL-1β production (Griffin et al., 1998). Further indicating a link between inflammatory mediators and AD, the addition of two cytokines, TNF-α and interferon (INF-) γ, onto neuroblastoma cells led to the increased production of Aβ peptides *in vitro* (Blasko et al., 1999). TNF-α has been
previously found at sites of brain injury and detected in plaques of post-mortem AD brains (McCoy & Tansey, 2008). Additionally, increased levels of TNF-α correlate with progressive hallmark symptoms of AD (McCoy & Tansey, 2008), and TNF receptor-1 knockout mice are protected from both AD and PD (He et al., 2007; Sriram et al., 2002). In addition to the upregulation of cytokines, chemokines such as monocyte chemotactic protein (MCP)-1 and macrophage inflammatory protein (MIP)-1α levels have also been shown to be increase in AD (El Khoury et al., 1998; El Khoury & Luster, 2008; Xia & Hyman, 1999). MIP-1α and MCP-1 are important in that they signal the recruitment of macrophages and neutrophils to the site of inflammation, and in the CNS, may regulate microglial migration and recruitment of astrocytes to areas of neuroinflammation (Heneka & O'Banion, 2007). Evidence also suggests that Aβ itself can activate microglia, which further enhances the inflammatory response to the Aβ (Heneka & O'Banion, 2007).

As the innate immune cell of the central nervous system, microglia cells continuously sample the environment for foreign pathogen or injury (Luo & Chen, 2012). After environmental changes are detected, microglia cells are activated, and therefore change shape and secrete pro-inflammatory cytokines at the site of injury (Gehrmann, Matsumoto, & Kreutzberg, 1995; Luo & Chen, 2012; Perego, Fumagalli, & De Simoni, 2011). Microglia cells are antigen-presenting cells and can become phagocytosing cells after detection of neuronal or synaptic degeneration (Gehrmann et al., 1995). Microglia can perform both protective and destructive functions. For instance, in developing brains, microglia increase neurotrophin release to regulate neuronal differentiation (Markus, Patel, & Snider, 2002). However, increased levels of microglia activation in various
neurodegenerative diseases, such as AD and PD, potentiate neuronal damage as they increase pro-inflammatory cytokine levels, as well as other neurotoxic substances (Bi et al., 2005; Luo & Chen, 2012). Previous studies in AD mouse models have shown that microglia cells migrate towards Aβ plaques, increasing pro-inflammatory cytokine production and further potentiating AD pathology (Frautschy et al., 1998; Krabbe et al., 2013). In cell culture, the addition of Aβ led to increased microglia phagocytosis of Aβ (Bolmont et al., 2008). However the efficacy of this role still remains uncertain as increased recruitment of microglia to Aβ plaques does not lead to increased clearance (Lee & Landreth, 2010).

There are numerous studies attempting to determine the relationship between inflammation and the progression of AD. However, we still do not know the impact that inflammation has on AD. It is unknown whether inflammation is involved in the onset or progression of AD, or just a side-effect of pathological changes (Wyss-Coray & Rogers, 2012). One theory posits that the presence of aggregated, misfolded, or misphosphorylated proteins may be the source of ongoing inflammation in AD brains (Heneka & O'Banion, 2007). There are a number of diseases that have been associated with inflammatory mechanisms, ranging from fevers to chronic low-grade inflammation.

There are numerous diseases that are associated with chronic inflammation and highly correlated with AD. One longitudinal study demonstrated that the onset of mid-life type 2 diabetes mellitus correlates with a higher risk of cognitive impairment (Luchsinger et al., 2007). Human longitudinal studies of hypertension (Iadecola & Davisson, 2008; Iadecola, Park, & Capone, 2009; Reitz, Tang et al., 2007) and obesity (Misiak, Leszek, & Kiejna, 2012; Whitmer et al., 2008) show higher incidence rates of cognitive impairment.
and AD. And, it has also been discovered that cardiovascular disease can occur along
side AD and further aggravate cognitive and physical problems (Mayeux & Stern, 2012).

The amyloid cascade hypothesis of AD and Aβ-induced synaptic dysfunction

In their seminal paper of the amyloid cascade hypothesis of AD, Hardy and
Selkoe (2002) discuss the original studies and findings that led them to the idea that
amyloid deposition was the primary event in AD pathology. These findings included the
discovery of Aβ in the meningeal blood cells of AD patients (Glenner & Wong, 1984),
recognizing Aβ as the primary component of senile plaques (Masters et al., 1985), and
cloning of the APP gene (Kang et al., 1987). Although Aβ plaques were originally
thought to cause the detrimental effects (Hardy & Higgins, 1992; Selkoe, 1991), various
forms of Aβ, such as monomers, dimers, tetramers and oligomers, have now been shown
to be potentially neurotoxic (Mucke and Selkoe, 2012). One study, in vitro, revealed that
aggregated Aβ peptides can destroy cholinergic neurons, which are known to play a role
in learning and memory function (Whitehouse et al., 1982). In a study of young APP
transgenic animals, mice displayed poor performance on the Y-maze, both in
spontaneous alternation and working memory paradigms, even though there was no
evidence of amyloid plaque formation during the time of testing (Holcomb et al., 1999).
The profound effects of Aβ accumulation and aggregation may contribute to the
cognitive decline in Alzheimer’s patients, however, the exact mechanism in which this
occurs is still unknown (Mucke & Selkoe, 2012).

A number of potential mechanisms that lead to Aβ-induced disruption of synaptic
transmission and network dysfunction have been discovered. As previous mentioned, Aβ
leads to increased levels of pro-inflammatory cytokines such as IL-1β. Elevated levels of IL-1β increase production of IL-6 by astrocytes (Rossi & Bianchini, 1996), and such elevated levels of IL-6 have been associated with mild cognitive impairments (Dugan et al., 2009) and increased neurodegeneration (Campbell et al., 1993). Elevated levels of IL-6 have also been shown to significantly reduce long-term potentiation (LTP), a cellular phenomenon that many consider a model of long-term memory formation, in the dentate gyrus of the hippocampus (Bellinger, Madamba, Campbell, & Siggins, 1995). However, although overexpression of IL-6 in the hippocampus of APP transgenic mice led to massive neuroinflammation, reduced amyloid deposition and normal cognitive function were also discovered (Chakrabarty et al., 2010). Aβ has been shown to cause decreased transsynaptic activity in cortical-hippocampal connections (Harris et al., 2010), fewer excitatory postsynaptic potentials (Larson, Lynch, Games, & Seubert, 1999) and reduced LTP; (Selkoe, 2002; Shankar et al., 2007). These studies were performed in APP transgenic mice wherein the synaptic dysfunction occurred prior to plaque formation (Selkoe, 2002).

Another theory of Aβ-induced synaptic dysfunction involves the partial blockage of N-methyl-D-aspartate (NMDA) receptors (NMDARs; Mucke & Selkoe, 2012). NMDARs are a type of glutamate receptor involved in synaptic plasticity and memory formation (Li & Tsien, 2009). The partial blockage of NMDA receptors by Aβ, through unknown mechanisms, may lead to Aβ-facilitated long-term depression (LTD; Li et al., 2009). LTD, similar to LTP, is also a theoretical cellular and molecular component of learning involving dendritic spine elimination and/or decreased spine volume to allow for the creation of more enhanced synapse connections created during LTP (Nicholls et al.,
In studies of cultured cortical neurons, Aβ increased endocytosis of NMDARs, reduced signaling to cyclic-AMP response element binding protein (CREB), important for long-term memory formation (Chen et al., 2003), and reduced surface-expressed NMDARs (Snyder et al., 2005). According to Mucke and Selkoe (2012), understanding how Aβ effects synaptic transmission and which Aβ peptides initiate these effects, may lead to new therapeutic routes to relieve cognitive dysfunction associated with AD.

Because mice do not naturally develop pathological markers of AD, transgenic mice have been developed to express mutant human genes that force the onset of AD-like pathologies and offer a way to study the effects of Aβ on the brain (Duff & Suleman, 2004). While some of these transgenic mice have already been mentioned, one mouse model in particular exhibits the most similarities to human AD pathology, the triple transgenic Alzheimer’s mouse (3xTg-AD). 3xTg-AD mice have mutant forms of PS1, APP, and Tau that lead to a build up of both plaques and NFTs in areas such as the hippocampus, cortex and amygdala by six months of age. Because these pathologies are not visible at 2-months of age, it has been suggested that an age-dependent increase in the plaques and tangles correlates with cognitive deficits apparent during acquisition in the Morris Water Maze (Oddo et al., 2003; Rodriguez et al., 2008). Although a study of AD transgenic mice, in which an overexpression of APP lead to increases in Aβ plaque deposition around 12 months of age, revealed cognitive deficits in MWM, there were no cognitive deficits throughout the life span in the hippocampus-dependent object recognition task (Chen et al., 2000).

While studying of the effects of inflammation in a non-transgenic mouse, Lee et al. (2008) found that a single injection of LPS led to an increase in the amount of Aβ
found in the hippocampus and cortex. An even larger increase in Aβ occurred following three or seven consecutive days of LPS administration. Several injections of LPS also led to the accumulation of senile plaques in the hippocampus (Lee et al., 2008). Furthermore, Lee et al. demonstrated cognitive deficits following a single injection of LPS, although some of the deficit may be attributed to sickness behavior associated with the inflammatory response. The cognitive differences may also be attributed to cognition deficits from increased pro-inflammatory cytokines levels. In our recently conducted series of experiments, seven consecutive days of peripheral injections of LPS in non-transgenic animals led to a significant increase in hippocampal-Aβ as well as cognitive impairment on two well known hippocampus-dependent tasks, MWM and contextual CFC, that could not be confounded with sickness behaviors or cytokine-induced cognitive disruption, due to the occurrence of endotoxin tolerance (Kahn et al., 2012). We hypothesized that the cognitive disruption may have been due to the elevation in hippocampal Aβ.

**AD Therapeutic Modalities**

A majority of research conducted on AD has been directed at understanding the disease pathology, in order to find treatments, with many treatments aimed at reducing or eliminating Aβ peptide (Schenk et al., 2012). Several studies have focused on reducing central Aβ by decreasing γ-secretase activity (He et al., 2010; Sutcliffe et al., 2011), decreasing BACE activity (Ohno et al., 2004), and decreasing inflammation using non-steroidal anti-inflammatory drugs (NSAIDs; Chen et al., 2003; Tuppo & Arias, 2005) and steroidal anti-inflammatories (Aisen et al., 2000; Aisen & Pasinetti, 1998). Further, non-
pharmaceutical interventions, such as exercise (Cho et al., 2003; Cotman & Berchtold, 2002; Cotman, Berchtold, & Christie, 2007), and dietary interventions (Lee et al., 2009; Parachikova, Green, Hendrix, & LaFerla, 2010) have been studied, all with the ultimate goal of alleviating the symptoms of AD. For the purposes of this dissertation, we examined whether several potential therapeutic modalities could reduce the level of Aβ, and Aβ-induced cognitive deficit, following inflammation.

**Summary and overall hypotheses**

The studies presented below focus on potential therapeutic mechanisms to decrease APP processing or increase clearance of Aβ, in order to prevent cognitive dysfunction after repeated administration of LPS. Our prior study examining LPS-induced Aβ deposition demonstrated that seven consecutive injections of LPS resulted in an increase in hippocampal Aβ and cognitive deficits in two hippocampus-dependent tasks (Kahn et al., 2012). We hypothesized that the increased amount of Aβ was responsible for these cognitive deficits. Further, we hypothesized that treatments outlined in this document would decrease the amount of Aβ accumulation in the hippocampus after peripheral inflammation, either by blocking Aβ creation or increasing Aβ clearance. This would enable us to determine whether Aβ was in fact the cause of cognitive deficits in this model of AD-like pathology.
Chapter 2.

Experiment 1

IMATINIB METHANESULFONATE REDUCES HIPPOCAMPAL AMYLOID-
BETA AND RESTORES COGNITIVE FUNCTION FOLLOWING REPEATED
ENDOTOXIN EXPOSURE

Our lab has previously shown that repeated LPS administration in the periphery of
a mouse leads to increased central hippocampal-Aβ and cognitive deficits in
hippocampus-dependent tasks (Kahn et al., 2012), both pathologies associated with
Alzheimer’s disease. However, from that experimental design, we were only able to
theorize that the deficits were due to the accumulation of Aβ in the hippocampus. In
order to gather evidence either for or against this connection, we needed to find a way to
decrease production of Aβ without altering the inflammatory processes following LPS
administration.

There are currently three common approaches to targeting Aβ as a way to reduce
or prevent AD (Schenk et al., 2012). They include inhibiting the production of Aβ,
preventing the aggregation of Aβ into plaques, and increasing clearance of Aβ. The
secretase enzymes involved in APP processing have become major therapeutic targets for
drugs intended to combat AD pathology (Muller & Zheng, 2012). APP is cleaved by both
BACE, in the extracellular domain, and γ-secretase, in the transmembrane domain
(Seiffert et al., 2000). Most of the transgenic animal models of AD have mutated PS-1
and PS-2 genes, similar to mutations found in familial AD (Hutton & Hardy, 1997), and
it is these presenilins that make up a portion of γ-secretase (De Strooper et al., 1998). In
an earlier study of N-{(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1,1-
dimethylethyl ester (DAPT), a non-selective γ-secretase inhibitor, APP transgenic mice
displayed a significant decrease in cortical Aβ three hours after the dose was given
(Dovey et al., 2001). However, when a later version of DAPT, Semagacestat, was tested
by the pharmaceutical company Eli Lilly (Indianapolis, IN), the phase-3 trials were
discontinued because of adverse side-effects (Schenk et al., 2012), consisting of skin
rash, hair color changes, and gastrointestinal effects.

In a groundbreaking study, He et al. (2010), discovered that when they decreased
the concentration of γ-secretase activating protein (GSAP) in neuroblastoma cells that
overexpressed APP, the level of the toxic form of Aβ decreased. This reduction in Aβ
was due to decreased γ-secretase activity concomitant to the reduced levels of GSAP. He
et al. (2010) then tested the drug imatinib (IM), an abl-specific tyrosine kinase inhibitor,
that they had previously shown reduced the level of Aβ in APP mice. They found that
IM interferes with the interaction of GSAP and γ-secretase, leading to reduced γ-secretase
activity and the resulting decrease in Aβ. IM was originally developed as a treatment for
chronic myelogenous leukemia and is the active component of the FDA-approved anti-
cancer drug Gleevec™. A more recent study showed that IM decreased both plasma and
brain levels of Aβ in wild-type mice (Sutcliffe et al., 2011). However, because Gleevec™
does not cross the blood-brain-barrier (He et al., 2010), Sutcliffe et al. proposed that the
decrease in central Aβ came from a decrease of peripheral production of Aβ. In support
of this idea, they speculated that the liver was a major producer of Aβ in mice, as they
determined two-to-eight fold higher levels of presenilin-2 transcription, in the liver as
compared to other tissues, including the brain (Sutcliffe et al., 2011).
Experiment 1: Hypothesis

The present study tested the hypothesis that IM would decrease the production of Aβ in our LPS-induced inflammatory model. Further, we proposed that because IM does not cross the blood-brain barrier (BBB), that any decreases in hippocampal-Aβ would be due to decreases in peripheral Aβ production and transport across the BBB (He et al., 2010). We hypothesized that IM treatment would 1) block peripheral Aβ production, resulting in a decrease in hippocampal-Aβ and 2) rescue learning otherwise disrupted by LPS treatment. We also hypothesized that 3) decreases in hippocampal-Aβ would not be due to anti-inflammatory effects of IM, but rather to the reduction of Aβ formation.

Experiment 1 Methods

Subjects

Male C57BL/6J mice (4–6 month old), bred in the TCU vivarium from a breeding stock obtained from Jackson Laboratory (Bar Harbor, ME), were utilized in all experiments. All animals were housed and cared for in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2010), and in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of TCU.

Housing

All subjects were housed in groups of three or four in standard cages (12.5 cm x 15 cm x 25 cm). All experimental groups and control groups were on the same light
schedule, lights on at 0600 h and lights off at 1800 h. Both food and water were available \textit{ad libitum}.

\textit{Treatment conditions}

Following the experiment, intraperitoneal (i.p.) injections of 20 mg/kg IM or volume-equivalent saline were administered twice a day, for 14 days (days 1–14). During days 7–14, an additional single injection of 250 µg/kg LPS or saline was also administered once per day. There were four treatment groups: IM-saline, IM-LPS, saline-saline, and saline-LPS.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{timeline.png}
\caption{Injection Timeline for IM experiment.} Animals were administered 14 consecutive days of IM or saline twice daily. The last 7 days, animals were also administered LPS or saline once daily. 4 hours after the first LPS or saline injection, serum was collected to assess cytokine and Aβ_{1-42} levels (ELISA). 24 hours following the 14 days of injections, animals were trained in CFC. 24hrs later they were tested in CFC. Immediately following testing, hippocampus tissue was collected for Aβ_{1-42} ELISA analysis. Sal= saline, LPS= lipopolysaccharide, IM= imatinib.
\end{figure}

\textit{Serum Isolation}

To assess whether i.p. administration of LPS led to peripheral production of Aβ, and whether pre-treatment of IM could alter the production of Aβ, blood from the tail vein of each mouse was collected 4 h after the first LPS injection. After the blood was collected in microfuge tubes, it was placed on ice and left untouched for 30 min to allow for clotting. The tubes were then warmed to 37 °C. The clots were dislodged from the
side of the tubes using cotton tipped applicators and then the tubes were placed in the centrifuge at 135 g for 30 min. The serum was then removed and placed in a new tube. Aβ level in serum was detected using an Aβ ELISA, described below.

*Tissue preparation*

Following completion of pharmacological treatments and cognitive testing, mice were euthanized in accordance with IACUC-approved methods, and hippocampal tissue samples were extracted and homogenized with protein extraction solution (PRO-PREP, Boca Scientific, Boca Raton, FL) containing protease inhibitors and immediately frozen on dry ice. The samples were stored overnight in -80 °C, to allow for further lysing of the cells. The next day, the lysate was centrifuged at 15,000 rpm for 30 min and the clear lysate was removed for *DC Protein Assay* (Bio-Rad Laboratories, Hercules, CA.).

*DC Protein Assay*

The *DC Protein Assay* utilizes a working reagent that is used with detergent-based buffers. To prepare the protein standard curve, dilutions ranging from 0.2 – 1.5 mg/ml of γ-globulin were used, and made in the same buffer as the lysates. Standards (5 µl) and samples (5 µl) were pipetted into the 96 well plate with 25 µl of reagent A’ and 200 µl of reagent B. After 15 min, the plate was placed in the plate reader (BMG LabTech FLUOstar Omega, Cary, NC) and the optical density of each sample was read at 750 nm. The results of the protein assay were used to normalize protein content.
**Aβ ELISA procedure**

The BetaMark Aβ_{1-42} ELISA (Covance Research Products, Dedham, MA) is a 48-h procedure that utilizes a 96-well antibody-coated plate, into which both samples and standards of known concentrations are placed. To perform this ELISA, a preparation of working incubation buffer, 1X wash buffer, and standard intermediates were made prior to the start of the assay. Next, the standard diluent was used to reconstitute the Aβ standard and to prepare the standard curve. The samples were then be diluted 2:1 with working incubation buffer, which includes the HRP-labeled detection antibody. Each dilution of the standard curve (100 µl) was pipetted in duplicate, and 100 µl of each unknown sample in triplicate was added to the plate and was incubated over night at 2–8 °C. On the following day, the wells were washed 5 times with the 1X wash buffer. After the washes, 200 µl of TMB, a substrate for the HRP enzyme, was added to each well. The plate was then be incubated for 45 min at room temperature in the dark, and read at the optical density of 620 nm.

**Statistical Analysis Aβ Levels**

Aβ levels, from the ELISA, were analyzed using two-way analysis of variance (ANOVA). In this experiment, Fisher’s PLSD post-hoc tests were used to determine differences after a significant main effect of groups. In order to compare groups from different plates, all protein concentrations were normalized by plate protein concentration (pg of Aβ/mg of protein).
Cytometric Bead Array

Blood was collected from the tail vein of mice 4 h after the first LPS injection. Serum was isolated and used to measure peripheral pro-inflammatory cytokines IL-6 and TNF-α, as well as chemokines, MCP-1, and MIP-1α, by Cytometric Bead Array (CBA; BD Biosciences, San Jose, CA), in accordance with kit instructions. Briefly, known concentrations of the cytokines and chemokines will be incubated with fluorescent beads in filter-bottom microtiter plates to produce a standard curve of mean fluorescence intensities. Serum samples will be incubated with the same master bead mixtures. After incubation, beads will be washed using a vacuum manifold to extract wash buffer from all 96 wells equally through the filter bottom, while retaining the beads. Beads will then be resuspended in the assay buffer, and the mean fluorescence data will be collected on a FACSCalibur flow cytometer (BD Biosciences) using CellQuest Pro software (BD Biosciences), and analyzed using FCAP Array software (Soft Flow, Inc., New Brighton, MN).

Contextual fear conditioning

Freezing behavior in mice was monitored using the FreezeFrame™ software (ActiMetrics Software, Wilmette, IL). The units (Coulbourn Instruments, Whitehall, PA, 7W x7Dx 12H) use an electrified grid floor through which the aversive stimulus is delivered. Our protocol began 24 h after the 7th injection of LPS or saline, and consisted of a training session (day 1) and a testing session 24 h later (day 2). The training session had a 120-s acclimation period, followed by a single 2-s 0.7 mA shock. Animals remained in the apparatus for 60 s following the aversive stimulus. On testing day, no
shocks were delivered, though the system recorded the movement of the animal for 90 s. Freezing behavior was monitored, and total freezing time (in seconds) was collected as the dependent variable.

Previous experiments in our lab have revealed that the incorporation of an olfactory contextual cue (peppermint oil 1:10 in water) and a wall design (black polka dots) increased freezing time suggesting enhanced acquisition of the fear after context-shock pairings (Kahn et al., 2012; Kranjac et al., 2012). The time freezing was analyzed using two-way ANOVA procedures (Statview 5.0, SAS, Cary, NC), in which Group (LPS/SAL) and Treatment (EX/SED) were the independent variables. The alpha level used for all statistical analyses was 0.05. Any significant main effects or interactions were further analyzed by Fisher’s PLSD post-hoc tests.

**Experiment 1 Results**

*Two weeks of IM administration does not alleviate LPS-induced weight loss*

In order to determine whether twice-daily administration of IM for the week prior to, and during the week of repeated LPS exposure, would alter weight loss as compared to LPS alone, animal weights were recorded daily prior to injections for the four treatment groups: IM-saline (n=9), IM-LPS (n=9), saline-saline (n=10), and saline-LPS (n=10). As expected, during the week of co-administration of treatment (LPS or saline) and condition (IM or saline) injections, there was a minimal but statistically significant main effect of Treatment (LPS, saline; $F(1,34)= 36.71, p<0.0001$), on total weight loss. There was no significant main effect of Condition (IM, saline; $F(1,34)= 1.47, ns$),
however, there was a significant interaction of Treatment x Condition ($F(1,34)= 4.18$, $p<0.049$). As previously observed in earlier experiments, Fisher’s PLSD post-hoc analysis revealed that LPS-treated animals lost significantly more weight as compared to saline-treated animals ($p<0.0001$). By day 14 of injections, there were no longer significant main effects of Treatment ($F(1,34)= 3.85$, $ns$) or Condition ($F(1,34)= 2.62$, $ns$), nor any significant interaction of Treatment x Condition ($F(1,34)= 2.96$, $ns$). Our results indicate that administration of IM does not appear to alleviate LPS-induced sickness-related weight loss, and therefore appear not to interfere with the pro-inflammatory effects of LPS (Figure 2).

![Figure 2. Inflammation-induced weight loss despite IM administration.](image)

Animals that received LPS lost significantly more weight than animals that received saline, regardless of IM treatment. Means with different letters (a,b,c,d) are significantly different ($p<0.05$) from each other. Bars represent mean ±SEM. SAL= saline, LPS= lipopolysaccharide, IM= imatinib.

Two weeks of twice daily IM reduces LPS-induced plasma Aβ levels

To assess whether administration of LPS leads to peripheral production of Aβ, and whether pre-treatment of IM could alter the production of Aβ, serum was collected 4 h after the first LPS injection and measured using an Aβ ELISA, for three treatment
groups: IM-LPS (n=5), saline-LPS (n=5), and the control group (saline-saline; n=3).

There was a main effect of Treatment ($F(2,10)= 9.05, p<0.05$), and Fisher’s PLSD post-hoc analysis revealed that administration of saline for 7 days followed by a single injection of LPS, on day 8, displayed a significant elevation of plasma Aβ ($p<0.01$), when compared to animals that were first pre-treated for 7 days with IM, and when compared to the saline-injected control animals (Figure 3). These results indicate that a single LPS injection elevates Aβ in serum, and pre-treatment and co-administration of IM blocks this elevation. Interestingly, the elevation in serum-Aβ occurred only 4 h after a single injection of LPS was administered, therefore showing that LPS-administration quickly leads to elevated Aβ levels in the periphery.

Figure 3. IM reduces serum Aβ following LPS administration.
Administration of IM for one week prior to a single i.p. injection of LPS blocks the peripheral production of Aβ measured in the serum. Animals receiving saline prior to LPS demonstrated significant elevation in serum Aβ. Letters that are different (a,b) represent significant differences ($p<0.01$). Bars represent mean ± SEM. Control= saline (SAL), LPS= lipopolysaccharide, IM= imatinib.

$LPS$-induced inflammation is observed in plasma despite two weeks of twice-daily IM administration

To show that co-administration of IM for 7 days prior to endotoxin exposure would not alter the inflammatory process triggered by LPS administration, it was necessary to assess plasma levels of pro-inflammatory cytokine levels 4h after the first injection of LPS. As hypothesized, there was no significant main effect of Treatment (IM
or saline) on the levels of pro-inflammatory cytokines (IL-6 and TNF-α) or chemokines (MCP-1 and Mip-1α), indicating that cytokine and chemokine levels in animals injected with IM-saline did not differ from those injected with saline-saline (Figure 4). Also as hypothesized, analysis revealed a significant main effect of Condition (LPS or saline) for IL-6 $(F(1,28)= 35.984, p<0.0001)$, TNF-α $(F(1,28)= 16.304, p<0.01)$, MCP-1 $(F(1,28)= 51.152, p<0.0001)$, and Mip-1α $(F(1,28)= 6.524, p<0.05)$. Specifically, we found significantly increased levels of pro-inflammatory cytokines and chemokines in both the animals treated with saline for 7 days, followed by a single injection of LPS, and those treated with IM for 7 days, followed by a single injection of LPS. These results indicated that 7 days of IM treatment does not interfere with the production of these pro-inflammatory cytokines and chemokines, indicating that potential explanations, based upon the reduction in Aβ, could not be confounded by a possible reduction in peripheral inflammation. This finding negates the potential confound that the observed reduction in Aβ is a result from an overall reduction in peripheral inflammation.

**Figure 4.** LPS-induced inflammation was observed despite IM co-administration. Administration of IM for one week prior to the first i.p. injection of LPS did not block the resulting inflammation. Letters that are different (a,b) represent significant differences $(p<0.05)$. Bars represent mean ±SEM. Sal or IM administered for 14 days; Sal or LPS administered for the final 7 days. SAL = saline, LPS = lipopolysaccharide, IM = imatinib.
Two weeks of twice daily IM reduces LPS-induced hippocampal Aβ accumulation

To determine whether twice-daily administration of IM for the week prior to, and during the week of repeated LPS exposure, could decrease in the amount of Aβ in the hippocampus, tissue was analyzed using an Aβ ELISA for the four treatment groups: IM-sal (n=10), IM-LPS (n=10), sal-sal (n=10), and sal-LPS (n=10). As expected, there was a main effect of Condition (LPS or saline; \( F(1,36)=15.14, p<0.001 \)), a main effect of Treatment (IM or saline; \( F(1,36)=5.44, p<0.05 \)), and a significant interaction of Condition x Treatment (\( F(1,36)=6.58, p<0.05 \)) on Aβ production. There were no significant increases in Aβ when animals were injected with either saline-saline or IM-saline. Fisher’s PLSD post-hoc analysis revealed that i.p. administration of saline-LPS led to a significant increase in hippocampal Aβ, compared to animals that were administered 2 weeks of twice-daily IM and one week of LPS (\( p<0.05 \)). These results indicate that IM does in fact lead to a decrease in hippocampal Aβ accumulation (Figure 5).

![Figure 5. LPS-induced Aβ production was blocked by IM co-administration.](image)

Following 7 consecutive days of LPS treatment, Aβ was significantly elevated in the mouse hippocampus (\( p<0.05 \)). Prior treatment with IM blocked the elevation in Aβ in the hippocampus. Bars represent mean ±SEM. Sal= saline, LPS= lipopolysaccharide, IM= imatinib.
Two weeks of twice-daily IM administration restores cognitive function following LPS exposure

To assess whether twice-daily administration of IM for 2 weeks, with concomitant LPS administration on the second week, could restore cognitive function following LPS exposure, freezing behavior in the hippocampus-dependent CFC task was analyzed for the four treatment groups: IM-sal (n=10), IM-LPS (n=10), sal-sal (n=10), and sal-LPS (n=10). As anticipated, there were no significant main effects of Condition (LPS or saline; \( F(1,36)= 0.13, \text{NS} \)), Treatment (IM or saline; \( F(1,36)= 3.31, \text{NS} \)), or interaction of Condition x Treatment (\( F(1,36)= 0.84, \text{NS} \)) on behavior during the training day. This indicated that there were no significant behavioral differences in the four experimental groups. On the testing day, as hypothesized, there was a main effect of Condition (\( F(1,36)= 5.40, p<0.05 \)), a main effect of Treatment (\( F(1,36)= 9.17, p<0.01 \)), and a significant interaction of Condition x Treatment (\( F(1,36)= 4.25, p<0.05 \)) on freezing behavior. Fisher’s PLSD post-hoc analysis revealed that animals treated for 7 days with LPS (sal-LPS group) displayed significantly less freezing compared to all other groups (\( p<0.05 \)). Thus, we found that when animals were pre-treated with twice-daily IM in conjunction with LPS, cognitive function was rescued (Figure 6). This freezing behavior was not significantly different from animals administered saline-saline or IM-saline, suggesting that LPS-induced cognitive deficits may be due to the accumulation of significant levels of Aβ peptide in the hippocampus.
Experiment 1: Imatinib Discussion

The presented study tested the three hypotheses that twice-daily IM treatment would 1) block LPS-induced peripheral Aβ production, resulting in decreased hippocampal-Aβ accumulations as well as 2) rescue LPS-induced cognitive deficits. We also hypothesized that, 3) the decrease in hippocampal-Aβ would not be the result of anti-inflammatory effects of IM, but rather from reduced production of Aβ. This study demonstrated that IM, when pre-administered and co-administered with LPS, reduced hippocampal-Aβ production, did not alter LPS-induced inflammation, and prevented LPS-induced learning deficits during CFC.

Sutcliffe et al. (2011) suggested that a major source of rodent Aβ found in the brain, originates from the periphery, and more specifically they speculated that it was produced in the liver. Sutcliffe et al. determined that peripheral administration of imatinib reduced central accumulation of Aβ, and that central administration of imatinib was not
necessary for this decrease to occur in mice. A study utilizing Sprague-Dawley rats that had been centrally and bilaterally injected with Aβ, followed by peripheral injections of imatinib every 2 days over a 12-day period, revealed that the inclusion of imatinib rescued cognitive impairment in MWM (Cancino et al., 2008). The combination of results from Sutcliffe et al. and Cancino et al., led us to develop the injection timeline for our experiment. IM or saline was administered for 14 consecutive days, and during the final 7 days co-administration of LPS or saline was added.

Results from the Aβ ELISA from our experiment indicated that IM blocked the elevation in Aβ found in the hippocampus following LPS-induced inflammation (Figure 5). Because IM was assumed to poorly penetrate the BBB (He et al., 2010), our results suggested that LPS-induced peripheral inflammation led to peripherally produced Aβ that was then transported into the brain, leading to cognitive deficits. Alternatively, a reduction in Aβ could occur if IM reduced the LPS-induced inflammatory response. This alternative hypothesis is unlikely as our results from serum cytokine analysis indicated a significant elevation of pro-inflammatory cytokines and chemokines even when IM is co-administered with LPS (Figure 4). Most importantly, co-administration of imatinib with LPS reduced Aβ accumulation in the hippocampus. This reduction in Aβ coincided with rescued cognitive function in CFC, that had been disrupted following an inflammatory response to multiple LPS injections (Figure 6). Additionally, IM+LPS treated animals with reduced Aβ also demonstrated cognitive function superior to animals treated with SAL+LPS, indicating that a reduction in Aβ can rescue behavioral deficits induced by multiple LPS injections. These data support our hypotheses that accumulation of Aβ
results in decreased cognitive function, and therefore decreasing the production of Aβ results in rescued cognitive function.

Sutcliffe et al. (2011) suggested that a major source of rodent Aβ originated from the periphery and more specifically the liver. They were able to show that mouse liver has a higher level of the presenilin-2 mRNA, compared to other tissues including the brain (Sutcliffe et al., 2011). To further test Sutcliffe et al.’s hypothesis, we assessed whether i.p. injections of LPS were in fact increasing peripheral production of Aβ. Following the pre-treatment week with IM, serum was collected 4 hours after the first LPS injection was administered. Interestingly, results from an Aβ ELISA revealed that animals administered saline for 7 days, followed by a single injection of LPS displayed a significant elevation of plasma Aβ compared to animals that were first pre-treated for 7 days with IM, and to those that received saline. Interestingly, we found a significant reduction in peripheral Aβ as early as 4 h after a single i.p. injection of LPS.

We can only speculate that the increase in Aβ is originating in the periphery, as we did not perform a central Aβ ELISA following one injection of LPS, after a pre-treatment week with IM. However, it is highly unlikely that LPS can initiate central Aβ production in only 4 h. Further, according to He et al. (2010), IM poorly crosses the BBB. Even if LPS-induced inflammation were capable of increasing BBB permeability (Mayhan, 1998), this inflammation came after one week of IM administration. Thus it seems highly unlikely that IM blocked central Aβ production in our experiment. A future study to determine BBB permeability after 7 consecutive injections of LPS may be necessary to determine if the IM reduced Aβ peripherally, centrally, or in both.

Imatinib, sold by Novartis under the brand name Gleevec™, is an FDA approved
pharmaceutical that has been on the American market since 1990. Future research using IM as a potential therapeutic agent for AD has the potential to increase after 2015, when the active patent on the ingredients, and accessibility and use of this drug in research can increase. This affords scientists the opportunity to study a population of people treated with IM for over 20 years. Although the rodent work conducted with IM provides an interesting and novel way to decrease Aβ accumulation, expanding this research to a human population is vital. Targeted research on an aging population that has taken prescribed and regulated doses of IM may lead to better understanding of IM effects on AD.
CHAPTER 3:

Experiment 2

IBUPROFEN DOES NOT REDUCE ALZHEIMER’S-LIKE PATHOLOGY OR REVERSE COGNITIVE DEFICITS WHEN CO-ADMINISTERED WITH LPS

Neuroinflammation can occur after brain injury, infection, or ischemia. However, it is also known to occur during aging and is exacerbated during neurodegenerative disease such as AD and Parkinson’s disease (Hein & O'Banion, 2009). Prostaglandins (PGs) are released by activated vascular endothelial cells in response to pro-inflammatory cytokines (Hein & O'Banion, 2009), and have strong physiological effects such as the ability to regulate hormones and potentiate inflammation. NSAIDs exhibit anti-inflammatory effects by inhibiting cyclooxygenase (COX) activity, which reduces the production of PGs (Kaufmann, Andreasson, Isakson, & Worley, 1997). Interestingly, long-term use of NSAIDs has been shown to reduce the risk of AD, presumably through COX inhibition (Chen et al., 2003; Tuppo & Arias, 2005).

There are two main isoforms of COX proteins, COX-1 and COX-2. COX-1 is constitutively expressed (Quan, Whiteside, & Herkenham, 1998), while COX-2 expression is upregulated after inflammation, such as that produced by LPS (Elmquist et al., 1997). Quan et al. (1998) reported increased and rapid COX-2 mRNA expression in central endothelial cells after peripheral LPS injections in rats. Post-mortem, human AD brains were collected, sectioned, and stained for COX-2 activity (Hoozemans et al., 2001). Analysis of these human samples revealed an increase in COX-2 activity in the temporal and frontal cortex. AD patients also experience significant increases in COX-2
independently of total plaque accumulation. This evidence suggests, that an increase in COX-2 activity occurs regardless of degree of the disease pathology.

A review by Pasinetti (2002) examined many studies in which NSAIDS, such as ibuprofen (IB) and indomethacin, decreased the production of Aβ both in vivo and in vitro. However, in this review, there were also reports of other NSAIDs that either did not work or, if they did work, were given at doses high enough to be potentially toxic. One important limitation discussed by the author is the use of patients who have progressed too far into AD pathology to be helped by anti-inflammatory measures. Therefore, the authors suggested further testing of NSAIDs before declaring NSAIDs as therapeutic AD drugs.

In a study examining the effects of COX inhibitors on learning and inflammation, mice were trained in the hippocampus-dependent passive avoidance task (Jain, Patil, Kulkarni, & Singh, 2002). Immediately after training, mice were injected (i.p) with LPS (50 µg/mouse). Rofecoxib (1.9 mg/kg, p.o.), a COX-2 selective inhibitor was given orally for 7 days after the LPS injection. Treatment with Rofecoxib prevented the learning deficits, at testing, as compared to mice given only the LPS injection. Although not tested during these experiments, it was suggested that reducing inflammation could decrease AD-related cognitive dysfunction. In a slightly different study of LPS (250 µg/kg) and COX inhibitors, IB, a nonselective COX inhibitor, was given 1h prior to a peripheral injection of LPS, and spatial memory deficits on MWM were reduced (Shaw, Commins, & O'Mara, 2005). However, LPS was administered 4 h prior to testing and therefore cognitive deficits, found in the LPS (only)-injected mice, may be confounded by performance factors associated with sickness behaviors. The sickness behaviors may
have been attenuated by IB, as IB was given 1 h prior to LPS administration, and therefore possibly decreased the inflammatory response to LPS.

To examine possible therapeutic effects of COX inhibitors on AD pathology, research has been conducted utilizing multiple transgenic animal models of AD (Kotilinek et al., 2008) and on animals that have had Aβ injected directly into the brain (Joo et al., 2006). In the Tg2576 mouse, in which Aβ levels are elevated at around 6 months of age, low doses of IB or naproxen (nonselective COX inhibitors) and Merck Frosst tricyclic (COX-2-selective inhibitor) were given for 4 weeks prior to MWM training. All groups of NSAID-treated transgenic mice, regardless of the NSAID, had faster acquisition of the platform location, spent more time in the proper quadrant during the probe trial as compared to non-treated transgenic mice, and were not significantly different from age-matched wild-type mice. In a study of the 3xTg-AD mouse, in which Aβ accumulation develops around 4 months of age, IB was added to rodent chow and fed to the animals daily between 1-5 months of age. Mice fed with ibuprofen-supplemented chow displayed significantly better memory performance in MWM compared to untreated transgenic control mice, and displayed similar cognitive abilities in MWM when compared to wild-type control mice (McKee et al., 2008). With few exceptions, such as in Pasinetti (2002), mentioned above, and a study utilizing the 5XFAD transgenic mouse, in which IB was not effective at reducing plaque deposition (Hillmann et al., 2012), the majority of IB literature alludes to the efficacy of IB on AD pathology.
**Experiment 2: Hypothesis**

We hypothesized that IB could 1) decrease the accumulation of hippocampal-Aβ and 2) protect against LPS-induced cognitive deficits. These hypotheses are based on the expectation that IB could decrease the inflammatory response to LPS in the periphery and reduce inflammation-induced Aβ production.

**Experiment 2: Methods**

**Subjects**

Male C57BL/6J mice (4–6 month old), bred in the TCU vivarium from a breeding stock from Jackson Laboratory (Bar Harbor, ME), were utilized in all experiments. All animals were housed and cared for in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2010), and in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of TCU.

**Treatment conditions**

A single injection of 250 µg/kg LPS (*Escherichia coli* serotype: 055:B5; Sigma-Aldrich, St. Louis, MO) or saline was administered once a day for 7 consecutive days. Injections of IB, or a weight-equivalent amount of saline, were given 4 h after the daily injection of LPS, for 7 consecutive days. IB was injected intraperitoneally (i.p.) once a day at a dose of 20 mg/kg, a dose previously used in mice (Vargas, Bustos-Obregon, & Hartley, 2011). This procedure resulted in four treatment groups: sal/sal (n=9), and sal/IB (n=9), LPS/IB (n=9), LPS/sal (n=8).


**Contextual fear conditioning**

Freezing behavior in mice was monitored using the FreezeFrame™ software (ActiMetrics Software, Wilmette, IL). The units (Coulbourn Instruments, Whitehall, PA, 7Wx7Dx12H) use an electrified grid floor through which the aversive stimulus is delivered. Our protocol began 24 h after the 7th injection of LPS or saline, and consisted of a training session (day 1) and a testing session 24 h later (day 2). The training session had a 120-s acclimation period, followed by a single 2-s 0.7 mA shock. Animals remained in the apparatus for 60 s following the aversive stimulus. On the testing day, no shocks were delivered, though the system recorded the movement of the animal for 90 s. Freezing behavior was monitored, and total freezing time (in seconds) was collected as the dependent variable.

Previous experiments in our lab have revealed that the incorporation of an olfactory contextual cue (peppermint oil 1:10 in water) and a wall design (black polka dots) increased the freezing time suggesting an increase in the learning of the context-shock pairing (Kahn et al., 2012; Kranjac et al., 2012). The time freezing was analyzed using analysis of variance (ANOVA) procedures (Statview 5.0, SAS, Cary, NC), in which Group (IB/Saline) and Treatment (LPS/Saline) will be independent variables. The alpha level that was used for all statistical analyses was 0.05. Any significant main effects and interactions were further analyzed by Fisher’s PLSD post-hocs.

**Tissue preparation**

Following completion of injections and CFC, mice were euthanized in accordance with IACUC-approved methods, and hippocampal tissue samples were extracted and
homogenized with protein extraction solution (PRO-PREP, Boca Scientific, Boca Raton, FL) containing protease inhibitors and immediately frozen on dry ice. The samples were stored overnight in -80 °C for further lysing. The next day, the lysate was centrifuged at 15,000 rpm for 30 min and the clear lysate was removed for DC Protein Assay (Bio-Rad Laboratories, Hercules, CA.).

**DC protein assay**

The DC Protein Assay utilizes a working reagent that is used with detergent-based buffers. To prepare the protein standard curve, dilutions ranging from 0.2 mg/ml – 1.5 mg/ml of γ-globulin was used, and made in the same buffer as in the lysates. Standards (5 µl) and samples (5 µl) were pipetted into the 96 well plate with 25 µl of reagent A’ and 200 µl of reagent B. After 15 min, the plate was placed in the plate reader (BMG LabTech FLUOstar Omega, Cary, NC) and the optical density of each sample was read at 750 nm. The results of the protein assay were used to normalize protein content prior to ELISA procedures.

**Aβ ELISA procedure**

The BetaMark Aβx-42 ELISA (Covance Research Products, Dedham, MA) is a 48-hour procedure that utilizes a 96-well antibody coated plate, into which both samples and standards of known concentrations are placed. To perform this ELISA, a preparation of working incubation buffer, 1X wash buffer and standard intermediates were made prior to the start of the assay. Next, the Standard Diluent was used to reconstitute the Aβ standard and to prepare the standard curve. The samples were then diluted 2:1 with
working incubation buffer, which includes the HRP-labeled detection antibody. Each
dilution of the standard curve (100 µl) was pipetted in duplicate, and 100 µl of each
unknown sample in triplicate was added to the plate and was incubated over night at 2–8
°C. On the following day, the wells were washed 5 times with the 1X wash buffer. After
the washes, 200 µl of TMB, a substrate for the HRP enzyme, was added to each well. The
plate was then incubated for 45 min at room temperature in the dark and read at the
optical density of 620 nm.

**Statistical analysis Aβ levels**

Aβ levels from the ELISA were analyzed using two-way analysis of variance
(ANOVA). In these experiments Fisher’s PLSD post-hoc tests were used to determine
differences after a significant main effect of groups was found. In order to compare
groups from different plates, all protein concentrations were normalized by plate protein
concentration (pg of Aβ/ mg of protein).

**Experiment 2: Results**

*Changes in animal weight, day 1 - day 7, following IB and LPS administration*

To determine whether co-administration of IB with repeated LPS exposure could
lead to a decrease in peripheral inflammation, we first needed to show if there was an
effect of IB administration on weight changes. As expected, on day 1, weight was not
significantly different for Treatment (LPS or saline; \( F(1,31)= 3.85, \ ns \)), Condition (IB or
saline; \( F(1,31)= 0.16, \ ns \)), or their interaction (\( F(1,31)= 0.22, \ ns \); Figure 7A). However,
following co-administration of LPS or saline and IB or saline, there was a significant
main effect of Treatment (LPS or saline; $F(1,31)= 55.56, p<0.001$) on weight. Though there was no main effect of Condition (IB or saline; $F(1,31)= 0.50, ns$), there was a significant interaction of Treatment x Condition ($F(1,31)= 6.99, p<0.01$), that revealed a significant differences in weight loss between animals that had received sal-sal and sal-IB. In this case it appears as if saline-IB-injected animals lose more weight ($mean= 0.72, SEM=0.09$) as compared to saline-saline administered animals ($mean=0.19, SEM= 0.14$), where as LPS-IB ($mean= 1.48, SEM= 0.24$) and LPS-saline ($mean=1.77, SEM= 0.128$) weights do not significantly differ (Figure 7B). This was unexpected, as we hypothesized that the anti-inflammatory effects of IB would alleviate some of the sickness-related behaviors. However, on the 7th and final day of injections, there were no main effects of Treatment (LPS or sal; $F(1,31)= 1.70, ns$), Condition (IB or sal; $F(1,31)= 0.208, ns$), nor a significant interaction of Treatment x Condition ($F(1,31)= 0.01, ns$; Figure 7C).

Because there were no longer weight differences on day 7 we waited 24 h, as in previous experiments, and then began cognitive training and testing in CFC.
Analysis of freezing behavior following co-administration of LPS and IB

In order to assess whether co-administration of IB could restore cognitive function following LPS exposure, freezing behavior in the hippocampus-dependent CFC task was analyzed. Although there was a significant main effect of Treatment (LPS or sal; $F(1,31)= 5.04, p<0.03$), the difference on training day was negligible (saline mean= 0.36, LPS mean= 1.66). There was no significant difference in the main effect of Condition (IB or sal; $F(1,31)= 1.03, ns$), or interaction of Condition x Treatment ($F(1,31)= 0.14, ns$) on behavior during the training day. Because there were significant behavioral differences in our four experimental groups prior to testing, we used the training day freezing percentage as a covariate when we ran the analysis on testing day freezing.
percentage, to help account for a possible base-line difference. On the testing day, contrary to our hypothesized results, there were no main effects of Treatment ($F(1,27)= 0.21, ns$), main effect of Condition ($F(1,27)= 1.20, ns$), or a significant interaction of Condition x Treatment ($F(1,27)= 0.79, ns$) on freezing behavior (Figure 8). Analysis of CFC resulted in inconclusive results, as either LPS/SAL administration failed to inhibit learning of CFC or SAL/SAL-administered animals did not adequately learn the CFC paradigm.

![Graph showing freezing behavior in CFC following LPS and IB co-administration](image)

**Figure 8. Analysis of freezing behavior in CFC following LPS and IB co-administration.** Following 7 consecutive days of LPS treatment, there was a significant difference in CFC behavior on training day as LPS-administered mice froze significantly more ($p< 0.05$; mean=1.66%) compared to saline injected mice (mean=0.36%). Due to this difference, we used training behavior as a covariate in analysis of testing day freezing behavior and no significant differences were discovered. Bars represent mean ±SEM. SAL= saline, LPS= lipopolysaccharide, IB= ibuprofen.

**IB does not block LPS-induced hippocampal Aβ accumulation**

We were not able to arrive at any conclusions on possible effects of IB on LPS-induced CFC deficits, as either our LPS/SAL-administered animals did not display disrupted learning or our SAL/SAL control group did not adequately learn the cfc paradigm, observed numerous times in other experiments with similar protocols. However, we were still interested in determining whether co-administration of IB with repeated LPS exposure inhibited peripheral inflammation, and therefore blocked or
reduced the accumulation of Aβ in the hippocampus. After completion of the Aβ ELISA data revealed, as expected, a main effect of Treatment (LPS or sal; \(F(1,31)= 54.18, p<0.001\)), however, no main effect of Condition (IB or sal; \(F(1,31)= 0.07, ns\)), or interaction of Treatment x Condition \((F(1,31)= 3.71, ns)\), on Aβ accumulation (Figure 9). As we hypothesized, there were no significant increases in Aβ when animals were injected with either SAL/SAL (\(mean= 392.25, \text{SEM}= 40.22\)) or SAL/IB (\(mean= 328.81, \text{SEM}= 37.92\)). However there was also no significant difference in hippocampal Aβ in animals that were administered LPS/IB (\(mean= 682.11, \text{SEM}= 37.92\)) and LPS/SAL (\(mean= 598.89, \text{SEM}= 35.97\)). Our results indicate that one-week of once daily IB administered i.p. 4 h after LPS-injections does not affect the production of Aβ or the hippocampal accumulation of Aβ.

**Figure 9.** LPS-induced Aβ production was not blocked by IB co-administration. Following 7 consecutive days of LPS treatment, Aβ was significantly elevated in the mouse hippocampus \((p<0.05)\). However, the addition of ibuprofen does not lead to a significant reduction in Aβ. Means with different letters (a,b) are significantly different from each other. Bars represent mean ±SEM. SAL= saline, LPS= lipopolysaccharide, IB= ibuprofen

### Experiment 2: Ibuprofen Discussion

Ibuprofen is an over-the-counter available NSAID that is used to reduce fever and lessen other sickness-related symptoms, including sore muscles. We were interested in the effect ibuprofen may have had on decreasing the LPS-induced accumulation of Aβ seen in previous studies (Kahn et al., 2012; Lee et al., 2008; Weintraub et al., 2013).
Unfortunately, the data did not confirm our hypothesis, as co-administration of ibuprofen and LPS did not reduce levels of hippocampal-Aβ as compared to animals injected with LPS alone. Although, the level of freezing in our saline control groups was less than previously observed in other studies, some learning did occur as the freezing percentages, for all groups, increased during testing day compared to training day levels. Despite this, our behavior data was inconclusive after the control group, saline-saline, did not learn the paradigm adequately.

In past studies, utilizing AD transgenic mice, significant reductions in Aβ plaque formation following ibuprofen administration has been reported (Lim et al., 2000; Yan et al., 2003). In a similar study, 11-month-old AD transgenic mice (Tg2576) were fed ibuprofen in rodent chow for 16 weeks, which led to a 60% reduction in Aβ plaque load in the cortex (Yan et al., 2003). Unfortunately, prolonged ibuprofen usage can lead to lower gastrointestinal problems (Laine, Smith, Min, Chen, & Dubois, 2006), and increased risk of renal failure (Perneger, Brancati, Whelton, & Klag, 1994). For these reasons, this study was designed to determine the efficacy of short-term NSAID administration on LPS-induced Aβ accumulation.

For seven consecutive days, animals received an injection of either LPS or saline (1000 h). Ibuprofen or saline was then also administered 4h later (1400 h). Twenty-four hours following the final injection day, CFC was performed to reveal any cognitive differences that may have developed from the four treatments (Figure 8). Our lab has shown on numerous occasions that following 7 consecutive injections of either LPS or saline, sickness behaviors are no longer observed (Kahn et al., 2012; Weintraub et al., 2013). We have shown that both peripheral and central cytokines are no longer elevated.
during CFC training or testing (Kahn et al., 2012). Although weight data from day 7 showed that where the groups no longer differed, suggesting endotoxin tolerance, CFC training data suggested there may have been a small group difference. Because of this significant difference on training day behavior, we used training day freezing behavior data as a covariate during the analysis of CFC testing day freezing behavior. On testing day, we did not find significant main effects or an interaction effect, indicating that there were no differences in cognitive ability among animals of the different treatment groups. Unfortunately, our control group (SAL/SAL) as well as our SAL/IB group did not appear to learn the behavior task adequately, as their freezing percentages were 29.1% and 26.6% respectively.

Although our behavior did not successfully demonstrate learning differences in the way we had hypothesized, or as previously described in other studies, we decided to proceed with the Aβ ELISA. If we found that IB blocked Aβ production, then we would readdress the learning paradigm in a subsequent experiment. Unfortunately, administering ibuprofen 4h after each LPS injection, did not reduce the LPS-induced formation of hippocampal-Aβ (Figure 9), as compared to mice administered saline 4 h after LPS administration. As expected, peripheral LPS-administration increased accumulation of hippocampal-Aβ, however, animals that had been co-administered ibuprofen did not have significantly less Aβ than those co-administered LPS and saline. Perhaps there was not enough ibuprofen in the system to lead to a reduction in Aβ. Although similar to humans, taking ibuprofen when symptoms of sickness appear, our 4-h time point, we did not continue to deliver ibuprofen multiple times in the same day, as humans might do if they continued to feel ill.
Although the majority of studies utilizing ibuprofen to decrease Aβ load in transgenic animals have proven effective, not all have done so. For example, a study using 5xFAD mice, mice which display learning deficits and Alzheimer’s associated pathologies before 4 months of age (Jawhar, Trawicka, Jenneckens, Bayer, & Wirths, 2012), ibuprofen did not lead to an overall reduction of Aβ (Hillmann et al., 2012) similar to our results following inflammation. The authors theorized that these mice were not affected by ibuprofen, due to altered PS-1. The human mutation chosen to make this transgenic animal resulted in resistance of γ-secretase protein changes (Hillmann et al., 2012). In an earlier study, investigators examined an alternative hypothesis of ibuprofen and subsequent reduction on Aβ, unrelated to anti-inflammatory effects. The researchers discovered the IB has subtle modulating affects on γ-secretase enzymes (Weggen et al., 2001). The affect on γ-secretase led to a change in the Aβ isoform produced. In this study, ibuprofen, along with indomethacin and sulindac sulphide, was added to cells transfected with both human APP and mutant PS-1 (Weggen et al., 2001). These specific NSAIDs reduced the amount Aβ42 produced, but unlike in previous studies where the change was believed to occur from inhibiting COX activity, a subtle alteration in γ-secretase activation was discovered. Weggen et al. also discovered that this change in γ-secretase was not due to changes in the total amount of APP processed, but an increase in the production of the less destructive isoform of Aβ, Aβ38, by cleaving APP in a different location. These less hydrophobic peptides of Aβ can be more readily removed from the brain, and are therefore less detrimental to the brain. In further support of the theory that ibuprofen works by altering γ-secretase, an epidemiological study reviewing populations of AD patients and NSAID usage revealed that low-dose levels of
NSAIDs, at sub-anti-inflammatory levels, were effective at reducing AD pathology (Broe et al., 2000). Therefore, this reduction in AD pathology may be due to factors unrelated to the anti-inflammatory effects of NSAIDs.

Interestingly, studies treating AD transgenic mice with NSAIDS in which positive outcomes were found, such as a reduction in Aβ, involved NSAID administration that lasted for at least 4 consecutive weeks. Although not the aim of this study, it may be interesting in the future to determine if chronic administration of ibuprofen, possibly through the addition of an ibuprofen-treated chow, could lead to a reduction of hippocampal-Aβ in our inflammation model. We may also want to consider administering IB twice a day, like imatinib, as human dosage allows for IB administration every four hours. We could then test for decreased levels of Aβ1-42, in the ELISA we use now, or find an ELISA specific to Aβ in shorter isoforms. Although IB may not be effective at reducing LPS-induced Aβ with its anti-inflammatory processes, using IB to change γ-secretase activity could result in forms of Aβ that are more easily removed from the brain.
CHAPTER 4:

Experiment 3

TWO WEEKS OF VOLUNTARY EXERCISE REDUCES ALZHEIMER’S-LIKE PATHOLOGY, AFTER INFLAMMATION, IN MICE

Data from human subjects, 65 years of age or older, indicated an inverse correlation between physical activity and AD; subjects that were self-reported physically active in the initial data collection phase (1991–1992) later showed the lowest incidence of AD when the follow-up phase was conducted 5 years later (Laurin, Verreault, Lindsay, MacPherson, & Rockwood, 2001). To determine the effects of physical activity on the development of pathology in AD transgenic animals, numerous studies have been conducted using both forced exercise and voluntary exercise. In two of these studies, utilizing the Tg2576 AD transgenic mice, exercise was used in an attempt to alleviate AD pathology (Nichol, Parachikova, & Cotman, 2007; Yuede et al., 2009). Yuede et al. (2009) examined whether 16 weeks of forced treadmill running or voluntary running differentially affected plaque formation, hippocampal volume, and behavior in transgenic animals. Both voluntary and forced exercise resulted in larger hippocampal volumes, when compared to sedentary controls indicating that physical exercise leads to increased neurogenesis.

Similar increased hippocampal volumes have been seen in human AD patients with higher levels of physical fitness levels (Burns et al., 2008). However, Yeude et al. also showed that only animals in the voluntary running group displayed a decrease in number of plaques and improved memory in the novel object paradigm. The authors suggest that forced exercise may limit some of the positive outcomes from physical
activity, possibly due to stress. Forced exercise has been previously shown to elevate corticosterone levels (Hoffman-Goetz, Spagnuolo, & Guan, 2008), which can lead to destruction of neurons in the hippocampus (Mcewen, Weiss, & Schwartz, 1968).

Finally, in a study of voluntary running, three weeks of running rescues water radial arm maze deficits in aged Tg2576 mice (Nichol et al., 2007). Interestingly, physical exercise brought the cognitive abilities of the Tg2576 mice to the same level as the wild-type animals in a water radial arm maze.

Previous studies in wild type mice have demonstrated increased behavioral performance on tasks such as MWM (Vaynman, Ying, & Gomez-Pinilla, 2004) and radial arm maze (Schweitzer, Alessio, Berry, Roeseke, & Hagerman, 2006) following exercise. These changes in performance have been theorized to occur from exercise enhanced neurogenesis in the dentate gyrus of the hippocampus (Farmer et al., 2004). Other theories posit that exercise can enhance the availability of growth factors, such as brain derived neurotrophic factor (BDNF; Berchtold, Chinn, Chou, Kesslak, & Cotman, 2005) and insulin-like growth factor-1 (Trejo, Carro, & Torres-Aleman, 2001), which can enhance plasticity. In addition, previously conducted rodent studies have shown that synapse density and hippocampal neurogenesis increases after physical exercise (Kohman et al., 2011; Wu et al., 2008; Wu et al., 2007).

One way to decrease inflammation without the use of pharmaceuticals is through regular physical exercise (Cho et al., 2003; Cotman & Berchtold, 2002; Cotman et al., 2007). In a murine study of the effects of peripheral injections of LPS on hippocampal neurogenesis and learning, Wu et al. (2007), discovered that after 5 weeks of treadmill running, mice displayed an increased performance in the MWM. Interestingly, there was
also increased proliferation of neural progenitors and increased differentiation of neuronal progenitor cells into neurons. Wu et al., (2007) also showed increases in BDNF and its receptor, tyrosine receptor kinase B (TrkB), after physical exercise, which are both normally reduced after LPS administration. BDNF is known to be involved in numerous neurophysiological processes such as neurogenesis, synaptogenesis, and cell survival (Mattson, Maudsley, & Martin, 2004). Induction of BDNF expression may be a possible mechanism by which one may rescue cognitive deficits related to AD-like pathology. In an experimental study of infection and exercise, six weeks of voluntary running decreased the memory impairments from infection with \textit{E. coli} in rats. Interestingly, BDNF levels were significantly increased in the hippocampi of these animals, as compared to sedentary (locked wheel) animals (Barrientos et al., 2011).

In 2011, we tested animals using MWM and reverse MWM following 7 consecutive injections of LPS or saline, which led us to confusing yet novel results. After the nine days of swimming, animals that had been administered a single injection of LPS for seven consecutive days, no longer displayed elevated levels of A\textsubscript{\textbeta} (Figure 10). Interestingly, this small amount of exercise appeared to somehow facilitate the removal of A\textsubscript{\textbeta}. We believe the removal of A\textsubscript{\textbeta} was facilitated, and not just naturally
removed, as our lab had previously published results depicting significant elevations in Aβ following 15 sedentary days after the cessation of LPS injections (Kahn et al., 2012).

Unlike most exercise studies during which exercise training is followed by testing to determine the possible protective effects of exercise, we had a different idea. We hypothesized that exercise after LPS administration would lead to increased levels of BDNF, and prevent LPS-induced cognitive deficits. Based on preliminary data (Vinson, 2013) our results indicated that mice receiving LPS but not given access to exercise had significantly more Aβ in the hippocampus as compared to all other groups, which mirrored our hypothesis. We also hypothesized that exercise would increase the level of BDNF in the hippocampus, which might account for recovery of cognitive function later in the experiment. However, administration of LPS or saline followed by either the exercise or sedentary conditions revealed no significant differences in BDNF mRNA levels. In addition, we did not find differences in percent time freezing between any groups, in the CFC paradigm, indicating that exercise did not prevent previously seen cognitive deficits after LPS-administration.

As previously mentioned, APP transgenic mice exhibit decreased amyloid production and rescued cognitive function when forced overexpression of IL-6 occurred (Chakrabarty et al., 2010). Interestingly, exercise results in a substantial increase in IL-6 levels due to its production by the working muscles (Pedersen, 2011; Woods, Vieira, & Keylock, 2009). One human study investigating the effect of exercise on inflammatory mediators determined that participants in an exercise group, as well as a group that was infused with IL-6, displayed significantly less TNF-α than the sedentary control group, following an injection of LPS (Starkie, Ostrowski, Jauffred, Febbraio, & Pedersen, 2003).
These results suggest that a possible benefit of exercise is a transient increase in IL-6 that may act to suppress the release of proinflammatory mediators such as TNF-α.

In a compelling study, Hickman et al. (2008) demonstrated that microglia cells can be neuroprotective and neurodegenerative in function. Numerous studies have demonstrated that at the site of plaque deposits, microglia phagocytose Aβ (Frautschy et al., 1998; Lee & Landreth, 2010; Rogers, Strohmeyer, Kovelowski, & Li, 2002). However, it has also been theorized that, as AD progresses and AD pathology increases, microglia become less efficient at removing Aβ (Hickman et al., 2008). Microglia can be neuroprotective by eliminating Aβ, which can delay disease progression. Microglia can also be neurodegenerative, increasing pro-inflammatory cytokine activation and promoting Aβ accumulation (Hickman et al., 2008). Interestingly, as mice age, microglia lose their ability to degrade Aβ but not their ability to increase inflammation through pro-inflammatory cytokine release.

**Experiment 3: Hypothesis**

As numerous papers have shown that exercise can lead to the reduction of Aβ, we hypothesized that voluntary physical exercise would decrease the accumulation of Aβ following 7 consecutive injections of LPS. As we were previously unable to show that exercise increased BDNF, we needed to find another mechanism that could explain the increased degradation of Aβ following exercise. Based on previous work that both LPS and Aβ increase microglia activation, we also hypothesized that voluntary physical exercise would increase the activation of microglial cells resulting in increased central Aβ clearance.
Experiment 3: Methods

Subjects

Male C57BL/6J mice (4–6 month old), bred in the TCU vivarium from a breeding stock from Jackson Laboratory (Bar Harbor, ME), were utilized in all experiments. All animals were housed and cared for in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2010), and in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of TCU.

Treatment conditions

Experiment 3a:

Animals were housed in their home cages from 0700 h to 1800 h (light cycle), and were then moved into private cages with either access to a running wheel, for those in the exercise condition, or no running wheel, for those in the sedentary condition. They remained in these private cages from 1800 h to 0700 h (dark cycle). Singly housing these mice during the dark cycle allowed us to track the individual running revolutions for each mouse, while avoiding possible depressive-like states by group-housing during the light cycle (Dong et al., 2004). Injections of 250 µg/kg LPS (Escherichia coli serotype: 055:B5; Sigma-Aldrich, St. Louis, MO) or saline were administered once a day for 7 consecutive days. For experiment 3a, animals were placed in one of four groups (Figure 13), exercise-LPS (EX-LPS), exercise-saline (EX-SAL), sedentary-LPS (SED-LPS), and sedentary-saline (SED-SAL).
Animals in EX-LPS and EX-SAL had running wheels placed into their cages for 7 days following the 7th injection of LPS or SAL, during the dark cycle. Animals in the SED-LPS and SED-SAL groups were placed in their private cages as well during the dark cycle, but no running wheel was accessible.

Figure 11. Organizational chart for exercise experiments. Methodology and treatment in both experiment 1* and experiment 2**. CON = control mice, EX= exercise mice, SED= sedentary recovery mice.
Experiment 3b:

Animals were housed in their home cages from 0700 h to 1800 h (light cycle), and were then moved into private cages with either access to a running wheel, for those in the exercise condition, or no running wheel for those in the sedentary condition. They remained in these private cages from 1800 h to 0700 h (dark cycle). Singly housing these mice during the dark cycle allowed us to track the individual running revolutions for each mouse, while avoiding possible depressive-like states by group housing during the light cycle. Injections of 250µg/kg LPS (Escherichia coli serotype: 055:B5; Sigma-Aldrich, St. Louis, MO) or saline were administered once a day for seven consecutive days. For experiment 3b, animals were placed in one of six groups (Figure 13), Control-LPS (CON-LPS), control-saline (CON-SAL), exercise-LPS (EX-LPS), exercise-saline (EX-SAL), sedentary-LPS (SED-LPS), and sedentary-saline (SED-SAL). Twenty-four hours after the 7th injection of LPS or SAL, CON-LPS and CON-SAL animals were tested using the CFC paradigm, followed by hippocampus removal, in order to provide a true injection-only control group. Animals in EX-LPS and EX-SAL had running wheels placed into their cages for 14 days following the 7th injection of LPS or SAL, during the dark cycle. Animals in the SED-LPS and SED-SAL groups were placed in their private cages as well during the dark cycle, but no running wheel was accessible. A second batch of animals followed the same treatment procedure, however, after the completion of the exercise portion, animals were transcardially perfused, and their brains were collected for immunohistochemistry, as described below.
Tissue preparation

Following completion of exercise treatments, mice were euthanized in accordance with IACUC-approved methods, and hippocampal tissue samples were extracted and homogenized with protein extraction solution (PRO-PREP, Boca Scientific, Boca Raton, FL) containing protease inhibitors and immediately frozen on dry ice. The samples were stored overnight in -80 °C, to allow for further lysing of the cells. The next day, the lysate was centrifuged at 15,000 rpm for 30 min and the clear lysate was removed for DC Protein Assay (Bio-Rad Laboratories, Hercules, CA.).

Aβ ELISA procedure

The BetaMark Aβ_{x-42} ELISA (Covance Research Products, Dedham, MA) is a 48-hour procedure that utilizes a 96-well antibody coated plate, into which both samples and standards of known concentrations are placed. To perform this ELISA, a preparation of working incubation buffer, 1X wash buffer and standard intermediates were made prior to the start of the assay. Next, the Standard Diluent was used to reconstitute the Aβ standard and to prepare the standard curve. The samples were then diluted 2:1 with working incubation buffer, which includes the HRP-labeled detection antibody. Each dilution of the standard curve (100 µl) was pipetted in duplicate, and 100 µl of each unknown sample in triplicate was added to the plate and incubated over night at 2–8 °C. On the following day, the wells were washed 5 times with the 1X wash buffer. After the washes, 200 µl of TMB, a substrate for the HRP enzyme, was added to each well. The plate was then be incubated for 45 min at room temperature in the dark and read at the optical density of 620 nm.
**Immunohistochemistry**

Following completion of the 2-week running protocol, mice were anesthetized with a mixture of ketamine (120 mg/kg) and xylazine (16 mg/kg). They were then transcardially perfused, first by exsanguination with cold phosphate buffered saline (PBS; pH 7.4) for 7 min, followed by fixation with cold 4% paraformaldehyde (PFA; pH 7.4) for 5 min. Brains were then extracted and placed in a tube containing cold 4% PFA. These brains were later sectioned using a vibratome (Leica Biosystems), slicing at 40 µm, and sections were placed in a 48-well plate containing .03% sodium azide in PBS.

Before staining, free-floating sections were rinsed with DI water (for 10 min, including three water changes) to remove any trace of sodium azide. 1mL of sodium hydrobromide (0.5%PBS) was added to each section and was incubated at room temperature for 30 min. The sections were then washed with PBS for 30 min. 500 µl of .3% H_2O_2 in 10% MeOH in PBS was added to each section followed by a 30 min room temperature incubation period. The sections were then washed in PBST for 30 min. The rabbit IgG ABC Kit (Vectastain, Vector Laboratories, Inc. Burlingame CA) was used to block the sections overnight at 4 °C. The following day, 500 µl of primary antibody, rabbit anti-Iba1 (ionizing calcium-binding adaptor molecule 1; 1:4000, Wako Chemicals USA, Inc., Richmond, VA) was added and incubated overnight at 4 °C. 24 h later, the sections were washed with PBST for a total of 35 min, with several changes of PBST. Then 500 µl of secondary antibody (anti-rabbit) was added using the same Vectastain kit mentioned above, following kit instructions, and incubated overnight in 4 °C. On the final day, the sections were washed for 35 min in PBST. Then, once again, using the Vectastain kit,
500 µl of signal amplification substrate, using a mixture of Reagent A and B from the kit, as per instructions, was added to the sections for 30 min. Following this signal amplification step, the sections were washed for 35 min with PBS. Using liquid diaminobenzidine (DAB)-plus substrate kit (Invitrogen, Camarillo, CA) as per manufacturer instructions, a chromogenic reaction was detected under a microscope. Sections were then mounted onto slides with VectaMount (Vector Laboratories, Inc., Burlingame, CA), and a coverslip was added. For quantification of Iba-1-positive cells in the hippocampus, every second rostral coronal section, including the dentate gyrus of the hippocampus, was used. Iba-1 positive cells were visualized using a light microscope (Zeiss AxioImager, Carl Zeiss, Jena, Germany) with a camera (AxioCam MRm, Carl Zeiss, Jena, Germany) attached, and cells were counted using Image J software (National Institute of Health, Bethesda, MD).

**Experiment 3a: Results (running 1 week)**

*No difference between groups in running after 1 week of voluntary running*

A repeated measures ANOVA, on total revolutions from individual running wheels, collected during a single week of running, following LPS or saline injections, revealed that there was no significant main effect of Treatment (LPS or sal; $F(1,84)=0.06$, *ns*). As expected there was a significant main effect of Exercise by day ($F(6,84)=25.22, p<0.0001$), as mice ran significantly more each day. There was also no significant interaction of Treatment x Exercise ($F(6,84)=0.25, ns$). Post-hoc analysis using Bonferroni multiple comparisons discovered no significant differences in running between treatment groups, for all 14 days. Our results indicate that, as expected and
previously seen in our experiments after seven consecutive injections of LPS, animals no longer display sickness behaviors by the completion of the injections. Further, previous exposure to either LPS or saline injections prior to an exercise protocol did not alter the behavior of the animals (Figure 12A).

1 week of voluntary running does not reduce LPS-induced hippocampal Aβ accumulation

As expected, there was a significant main effect of Treatment (LPS or sal; $F(1,26)=10.60, p < 0.01$) on LPS-induced Aβ accumulation. However there was no effect of Condition (Sed or Ex; $F(1,26)= 0.046, \text{ns}$) nor an interaction effect of Treatment x Condition ($F(1,26)= 0.887, \text{ns}$) on clearance of hippocampal-Aβ. Our result indicate that although, once again, we were able to increase the level of hippocampal Aβ accumulation after LPS injections, and this level of Aβ accumulation remained elevated in the LPS-SED group, one week of voluntary wheel running was not able to decrease the level of hippocampal Aβ, as hypothesized (Figure 12B). We next hypothesized that perhaps one week of running was not a sufficient amount of exercise to reduce Aβ accumulation, and therefore decided to increase the voluntary running period to two weeks.
Experiment 3b: Results (running 2 weeks)

No difference between groups in running after 2 weeks of voluntary running

A repeated measures ANOVA on total revolutions from individual running wheels collected during two consecutive weeks of running, following LPS or saline injections, revealed that there was no effect of Treatment (LPS or sal; $F(1,169)=1.19$, ns). As expected there was a significant main effect of Exercise by day ($F(13,169)=36.18, p<0.0001$), as mice ran significantly more each day. There was also no interaction of Treatment x Exercise ($F(13,169)=1.32$, ns). Post-hoc analysis using Bonferroni multiple comparisons discovered no significant differences between treatment groups on any day of the exercise period. Our results indicate that, as expected and previously seen after one week of voluntary running, animals injected with LPS no longer appear to display sickness behaviors by the completion of the injections, as their day 1 revolution...
data does not differ from saline-injected mice. These results also revealed that exposure to either LPS or saline injections prior to an exercise protocol did not alter the behavior of the animals (Figure 13A).

2 weeks of voluntary running reduces LPS-induced hippocampal Aβ accumulation

In order to determine whether 2 weeks of voluntary running would lead to a decrease in hippocampal Aβ accumulation after 7 consecutive days of LPS injections, hippocampal tissue was analyzed using an Aβ ELISA. As expected, there was a significant main effect of Treatment (LPS or sal; $F(1,34)=37.28$, $p < 0.0001$) on total accumulation of $A\beta$. In addition, 2 weeks of wheel running produced a significant main effect of Condition (Cont, Sed, Ex; $F(2,34)= 9.21$, $p < 0.001$) as well as a significant interaction of Treatment x Condition ($F(2,34)= 5.67$, $p <0.01$). Interestingly, after two weeks of running, animals injected with LPS had levels of hippocampal-Aβ that did not significantly differ from those as saline-injected animals, regardless of whether they exercised or not. Therefore, although 1 week of voluntary running was not able to reduce the hippocampal-Aβ accumulation seen after one week of LPS injections, two weeks of voluntary wheel running was able to significantly decreased the amount of Aβ in the hippocampus (Figure 13B).
**LPS increased the number of activated astrocytes following 2 weeks of voluntary running**

Immunohistochemistry was used to assess whether administration of LPS or voluntary exercise could alter the number of activated microglia, designated with Iba-1 staining. There was a main effect of Treatment (LPS or sal; $F(1,16)=27.47$, $p<0.0001$) as LPS-administration resulted in increased numbers of activated microglia. However, the main effect of Condition (EX or SED) only approached significance; mice that spent two weeks in sedentary recovery had less activated microglia than those in the exercise condition ($F(1,16)=3.695$, $p=0.07$). There was also no interaction effect of Treatment (LPS or sal) and Condition (EX or SED) on number of activated microglia. Interestingly,
following 2 weeks of either exercise or sedentary recovery, mice that had received 7 consecutive injections of LPS had significantly more activated microglia as compared to mice that had been administered saline (Figure 14).

Figure 14. LPS-administration, followed by 2 weeks of exercise or sedentary recovery, leads to an increase in activated microglia. Two weeks of voluntary exercise, following LPS-administration, significantly increased the number of Iba-1 positive microglia ($p<0.0001$). However, there was no significant effect of exercise on Iba-1 positive microglia, though it approached significance ($p=0.07$) in saline-injected animals. Bars represent mean ±SEM. Letters that are different (a,b,c) represents significant differences ($p<0.05$). CON= control mice EX= exercise, SED= sedentary.
Exercise remains an effective tool to reduce inflammation (Wu et al., 2011), increase neurotrophic factors (Cotman et al., 2007) and increase cognitive function (Barrientos et al., 2011; Kohman et al., 2011). We hypothesized that voluntary exercise would lead to increased Aβ clearance and a recovery of cognitive function. Interestingly, one week of voluntary wheel running was insufficient to enhance Aβ clearance, but two weeks of exercise was sufficient to eliminate the LPS-induced accumulation of Aβ in the hippocampus. Unfortunately, we were unable to perform cognitive testing in order to show cognitive improvement following exercise. Preliminary studies proved that exercise produces hyperactive animals, which could not show learning in the CFC.
paradigm, as this task requires the animal to freeze as an indicator of learning (data not shown). In addition, the use of tasks such as MWM or water radial arm maze further increase exercise in animals, and therefore may lead to increased clearance, as seen in our previous work. Finding a testing paradigm that does not involve freezing or exercise as part of the protocol is important in order to determine cognitive differences in these animals. One option is the novel object paradigm, although we have not found the appropriate parameters to use this task.

Most exercise studies involve training, in which animals are first exercised, then the protective effects of exercise are evaluated (Barrientos et al., 2011; Ferreira, Real, Rodrigues, Alves, & Britto, 2011; Wu et al., 2011). We chose an alternative approach in which exercise, following the production of Aβ, could enhance clearance of Aβ and prevent the cognitive deficits associated with hippocampal-Aβ accumulation. This idea stemmed from a previous experiment in which we realized we could no longer detect hippocampal Aβ following a nine day MWM procedure. One hypothesis, for reduced levels of Aβ in all treatment groups, was exercise from the MWM had helped increase clearance of Aβ. This decrease in Aβ accumulation is similar to findings in multiple peer reviewed articles in which AD transgenic mice displayed decreased levels of Aβ after running in a voluntary exercise paradigm, ranging from 3 weeks to 5 months, all (Adlard, Perreau, Pop, & Cotman, 2005; Maesako et al., 2012; Nichol et al., 2008).

During our experiments we determined that two weeks, but not one week, of voluntary wheel running reduced the accumulation of Aβ in LPS-treated mice to levels of saline-treated controls. One possibility for this reduction may be attributed to the large difference in average distance run, in both the LPS- and Saline- administered mice.
between 1 and 2 weeks of exercise (Table 1). Our findings were similar to those stated above in which levels of Aβ were reduced in AD transgenic mice models that had been exercised. In contrast to our findings, one study, utilizing an AD transgenic mouse, found that after three weeks of voluntary running there were no differences in total Aβ or APP (Parachikova et al., 2008). However, there was a significant increase in CXCL12, a chemokine shown to enhance cognitive function and that is down regulated in AD transgenic mice. Parachikova et al. suggested a possible inflammatory modulation could alter cognitive deficits in AD.

**Voluntary Running Distance**

<table>
<thead>
<tr>
<th>Weeks Exercising</th>
<th>Treatment</th>
<th>Total Revolutions (SEM)</th>
<th>Total Miles (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SAL</td>
<td>66104.25 (18645.49)</td>
<td>19.93 (5.62)</td>
</tr>
<tr>
<td>1</td>
<td>LPS</td>
<td>713650.38 (9198.53)</td>
<td>21.51 (2.77)</td>
</tr>
<tr>
<td>2</td>
<td>SAL</td>
<td>174047.30 (31997.84)</td>
<td>52.47 (9.65)</td>
</tr>
<tr>
<td>2</td>
<td>LPS</td>
<td>192688.90 (35645.10)</td>
<td>58.09 (10.75)</td>
</tr>
</tbody>
</table>

**Table 1. Wheel running descriptive statistics.** Weeks exercised, group, total revolutions, and total miles for both the 1 and 2 week exercise groups. SAL= saline, LPS= lipopolysaccharide.

This is contrary to our data as we had a significant decrease in overall Aβ. However, we also hypothesized that changes in inflammation, from exercising, facilitated the removal of Aβ. An example of inflammation-induced Aβ clearance was found in the hippocampus of APP transgenic mice that also overexpressed IL-6. This overexpression of IL-6 resulted in massive neuroinflammation. However, reductions in amyloid
deposition and normal cognitive function were also discovered (Chakrabarty et al., 2010). Interestingly, the increase in IL-6 was accompanied by an increase in microglia-mediated phagocytosis of Aβ, indicating that IL-6 may actually have a beneficial role early in the disease, by enhancing clearance of Aβ, and may possibly be an explanation for our decreased levels of Aβ in our exercise groups.

The design of our experiment also differs from that of previous studies, as we do not continuously individually house the animals. In order for us to capture the total amount of exercise for each mouse, animals are individually housed during their dark (active) cycle. During the light (resting) cycle we place them back into their home cages with their cage mates. A study exploring the effects of isolation on AD pathology discovered that isolation stress accelerated Aβ plaque accumulation in Tg2576 mice (Dong et al., 2004). Our method may be a better way to capture exercise data without confounding the results with isolation stress.

There were some limitations of this study, that if redesigned, could lead to further insights on exercise-facilitated Aβ removal, changes in cognitive function, and microglia activation. Most of the research conducted on AD utilizes genetically altered animals. These transgenic animals represent less than 10% of cases in human AD patients as they are altered with genes found the more familial related form, early onset AD. The literature is limited in non-transgenic effects of AD, as rodents do not naturally develop AD pathology. However, there is a lot of research available on the effects of inflammation in non-transgenic mice, as well as the effects of exercise in these mice. We are trying to blend the transgenic AD research together with known changes in non-transgenic animals, following inflammation and exercise. Due to the both the uncommon
exercise protocol and the using non-transgenic animals for AD research, it is difficult to compare our inflammation-induced levels of Aβ accumulation, as well as the effects exercise had on Aβ clearance levels.

Lastly, we also attempted to determine whether there was a direct correlation of exercise and microglia activation. There are published data showing that in aged mice, but not in young mice like the ones used in our experiment, inhibiting microglia activation led to enhanced cognitive function in MWM (Kohman, Bhattacharya, Kilby, Bucko, & Rhodes, 2013). The authors used minocycline, an antibiotic with a side effect that inhibits microglia activation (Yoon, Patel, & Dougherty, 2012), to reduce the total number of Iba-1 (a protein expressed by activated macrophage and microglia)-positive cells. In our study, 7 consecutive injections of LPS led to increased microglia activation in both the exercised and sedentary mice. We were not able to test the cognitive differences between these groups of animals, so we do not know if the exercise was able to reverse previously seen cognitive deficits following our inflammation model. Further, although it did not appear the microglia activity was altered by exercise, we cannot determine whether microglia phenotypes were changed, and if these different phenotypes had neuroprotective or neurodegenerative attributes. As normal aging occurs, microglia have the tendency to shift phenotypes from non-primed to an inflammatory phenotype (Dilger & Johnson, 2008). Thus, studying microglia activity in younger animals, both exercised and sedentary, may lead to further understanding of the possible neuroprotective abilities of microglia.

Although we had two independent cell counters, microglia cell counting can be subjective. Currently we do not have the ability to perform design-based stereologic
studies. In order to be assured that equal areas of hippocampus were being counted in all animals, we used only a 5x magnification, which could have led to counting mistakes. However, two independent investigators produced Iba-1 positive cell counts that were not statistically different from each other, suggesting that imaging at 5x was probably sufficient. We used Iba-1 antibody as a marker of activated microglia, so in addition to activation, we may want to detect differences in microglia phenotypes in future studies. In order to do this research we would need to use antibodies to detect cell surface proteins such as CD 68, which is a marker for microglia cells in phagocytic and highly active states.

There are several other possible mechanisms to account for the reduction in Aβ we find after exercise. For instance, astrocytes have been shown to phagocytose and degrade Aβ in cell culture (Koistinaho et al., 2004). In this study the astrocytes were unable to degrade Aβ when cultured from young mice, as opposed to adult mice, and when there was a lack of apolipoprotein E (APOE). The APOE gene is part of the system needed to make lipoproteins, and more specifically a major component of very low-density lipoproteins that remove cholesterol from the blood stream. Interestingly, numerous studies have found that the e4 version of the APOE gene increases the risk for developing AD (Genin et al., 2011; Reinvang, Espeseth, & Westlye, 2013), although reasons for the increased risk are still unknown. Interestingly, after 3 or 6 weeks of treadmill running, there were increases in both total number and density of astrocytes, in comparison to non-exercised animals (Li et al., 2005). The authors concluded that increasing number and density of astrocytes leads to strengthening of the BBB, and exercise may be beneficial following stroke. It was also discovered, following 4 nights of
voluntary wheel running, fibroblast growth factor (FGF)-2 was significantly elevated (Gomez-Pinilla, Dao, & So, 1997). FGF-2 has been shown to promote astrocyte proliferation (Gomez-Pinilla, Vu, & Cotman, 1995). From these studies it appears that exercise may lead to an increase in number of astrocytes. It was also previously mentioned that astrocytes phagocytose and degrade Aβ in vitro (Koistinaho et al., 2004). It is possible that exercise may have increased astrocyte numbers and therefore increased the phagocytosis of Aβ.

Another plausible mechanism for removing Aβ involves the brain “glymphatic system”. In the human body, the lymphatic system is tubular and runs parallel to the circulatory system. It is a mechanism that links interstitial spaces, between cells, and the vasculature. It allows for movement of filtrate (liquid and debris) back into the vasculature. However, unlike the glymphatic system, described below, as filtrate moves through the lymphatics it will encounter lymph nodes, made of leukocytes, in order to fight off pathogens and increase immunity. The glymphatic system is a central pathway to move waste material from the interstitial fluid (ISF) into the cerebral spinal fluid (CSF) (Iliff et al., 2013). As the CSF flows, it can come into contact with circumventricular organs, the less protected areas of the BBB. At these points there is the possibility for debris to leak out of the CSF and into the periphery. There may be a way to test the CSF fluid of our exercised mice for Aβ levels, in order to determine if the reduction in Aβ accumulation is due to glymphatic system clearance.

There is also the possibility that Aβ is being eliminated by being cleared by the numerous phagocytic cells or liver found in the periphery. Any cell with a receptor for
Aβ can phagocytose the peptide. Aβ peptide would not, under normal conditions, be able to enter the kidney filtrate as they are too large. However, we caused inflammation for 7 days, which can lead to an altered glomerulus. Although finding the mechanism behind the clearance is necessary for fully understanding how to decrease Aβ from the brain, what is important in this study was that we did increase the rate Aβ clearance following exercise.
Chapter 5.

General Conclusion

Worldwide estimates place 35 million people affected by Alzheimer’s disease (Prince, 2013), of those 35 million, 5.4 million are Americans. Further AD is the 6th leading cause of death, and the only disease in the “top 10” for which mortality rates continue to rise (Alzheimer’s Disease facts & figures, 2012). Currently, a positive diagnosis of AD can only be made upon autopsy. At such a time, neurofibrillary tangles, made of hypophosphorlated tau, and Aβ plaques, aggregated beta sheets of the Aβ_{1-42} peptide will be present. We still do not know what causes AD, however, neurodegenerative diseases, such as AD and PD, have been shown to have central inflammation as a hallmark characteristic (Minghetti, 2005).

Aβ peptide has been shown to activate microglia, leading to increased pro-inflammatory cytokine and chemokine release (Schwab & McGeer, 2008). Aβ peptide has also been shown to lead to cognitive deficits in APP mice, and induce synaptic impairments demonstrated by decreased LTP (Harris et al., 2010). In a separate study, researchers theorized that soluble Aβ reduced NMDA receptor signal strength, inhibiting memory formation, leading to cognitive deficits in AD mice (Cissé et al., 2010). In both of these studies, accumulation of Aβ altered LTP through the inhibition of NMDA receptors and reduced synaptic strength and stability.

The accumulation of Aβ following an acute bout of inflammation was the focus of our previous experiments, and led to the creation of an acute inflammation model of AD-like pathology. Following seven consecutive days of a single LPS injection per day, there was an increase in the amount of Aβ in the hippocampus as well as cognitive
deficits on two hippocampus-dependent tasks (Kahn et al., 2012). As our previous studies attempted to connect inflammation-induced AD-like pathology, we wanted to confirm our original hypothesis, that increased Aβ accumulation was in fact the source of cognitive dysfunction. In addition, it seems reasonable that the longer Aβ remains in the brain, the higher the likelihood that it will aggregate into plaques. For this reason, we began investigating ways to either slow the production of Aβ or increase clearance of Aβ, following an acute inflammatory bout.

As we had previously theorized that some Aβ found centrally may have entered the brain from the periphery, after peripheral inflammation, we were interested in determining whether we could reduce Aβ production in the periphery, without altering the inflammatory response to LPS. An FDA approved anti-cancer drug, Gleevec™, has a documented secondary effect on AD pathology (He et al., 2010; Sutcliffe et al., 2011). Imatinib, the active ingredient in Gleevec™, has been shown to reduce GSAP activity and, and subsequently reduce activation of γ-secretase, resulting in less APP cleavage into Aβ42. We discovered that two weeks of twice-daily IM treatment would block LPS-induced peripheral Aβ production, resulting in decreased hippocampal-Aβ accumulation, as well as rescued LPS-induced cognitive deficits (Weintraub et al. 2013).

We also discovered that this decrease in hippocampal-Aβ was not the result of anti-inflammatory effects of IM, as cytokine production was unaltered when LPS was administered after the week of IM or saline injections. We still do not know whether IM is able to cross the BBB, but we were able to reduce central Aβ accumulation. Therefore, regardless of where this occurs, reducing central Aβ led to diminished cognitive deficits.
As we were able to reduce Aβ without affecting the inflammatory response to LPS, we were also interested to see if we could stop Aβ accumulation by reducing the inflammatory response. Human research has shown that long-term intake of NSAIDs, which are commonly used, inexpensive, and over-the-counter, reduces the risk of AD (Chen et al., 2003; Tuppo & Arias, 2005). Unfortunately, our data did not confirm our hypothesis, as co-administration of ibuprofen and LPS failed to reduce levels of hippocampal-Aβ, as compared to animals injected with LPS alone. Further, our behavior data were inconclusive after the control failed to adequately learn the paradigm. We originally chose ibuprofen because it is inexpensive and easily accessible by the general public. Although most previous research compiled on AD transgenic mice did not coincide with the results from our experiments, interestingly, a study utilizing 5XFAD mice, likewise revealed that ibuprofen was not effective in reducing Aβ pathology (Hillmann et al., 2012). Although ibuprofen is an anti-inflammatory, the reason it was so effective in other AD mice was theorized to be for its secondary effect. This NSAID, like IM, changes the location of γ-secretase cleavage on APP, leading to the production of less Aβ42, and increased Aβ38 (Weggen et al., 2001). Although we did not see any reduction in total Aβ42 accumulation after one week of ibuprofen administration, it is possible that we had not attained a pharmacologically effective dose. Perhaps pretreatment of animals in a similar fashion to how we conducted the IM study, might lead to different results.

In addition to slowing the production of Aβ, there is also interest in finding an alternative way to rid the brain of Aβ, such as increasing its clearance from the brain. In our original AD and inflammation studies we used MWM and reversal MWM to detect
any cognitive deficits from Aβ accumulation, following 7 days of LPS injections. After the 9 days of swimming were completed, there were no elevated levels of Aβ detected in any group. Although that amount of exercise appeared lower than most that utilized in most exercise protocols, a study by Barrientos et al. (2011) revealed that a reduced exercise amount of .7 km per week protected infection-induced memory deficits. We hypothesized that voluntary exercise would lead to decreased hippocampal-Aβ and diminished cognitive deficit following LPS administration. Interestingly, we were only able to show significantly less Aβ after two-weeks of voluntary wheel running.

There was significant clearance of Aβ after the two weeks of sedentary recovery, an effect we expected, due to transport systems and phagocytic cells designed to degrade Aβ. However, there was also significant reduction in Aβ after exercising, emphasizing an increased rate of clearance following 2 weeks of voluntary exercise that surpasses natural clearance rates. After 2 weeks of voluntary exercise, LPS-administered mice ran an average of 58.09 miles (93.49 km), while saline-injected mice ran an average of 52.47 miles (84.44 km). It should be noted that mice in our exercise study ran much further than those in the previously mentioned study by Barrientos et al. This difference was probably due to design, as we had both younger mice and did not regulate the amount of running (Table 1).

There are other possible mechanisms, aside from microglia-assisted clearance, that could account for the decreased amount of Aβ, including elimination of Aβ by other phagocytic cells, removal of Aβ through the brain glymphatic system, alterations in Aβ uptake by via changes in microglia phenotypes, and increased Aβ removal from the brain parenchyma through efflux into the blood. We found a significant increase in the number
of activated microglia after LPS-administration, but this increase was seen regardless of exercise or sedentary grouping. Therefore, we speculated that it was possible other phagocytic cells such as astrocytes, cells that have been shown to aid in the removal of Aβ through phagocytosis of Aβ peptide (Koistinaho et al., 2004). Another plausible mechanism for removing Aβ involves the glymphatic system. In a previous study, Iliff et al. reported that the glymphatic system was a contributor to the removal of Aβ from the brain, and failure in this system could lead to plaque deposition (Iliff et al., 2012). Testing CSF fluid of our exercised mice may provide a clue to the level of Aβ in the fluid of the glymphatic system clearance.

Another alternative hypothesis, currently untested, is that microglia phenotypes change following exercise. Although LPS administration leads to increased pro-inflammatory and destructive (M1) microglia, following exercise microglia can alternatively be activated in an anti-inflammatory and repairing (M2) phenotype (Kohman et al., 2012). There is also the possibility that the Aβ may be transported out of the brain. The transporter protein low-density lipoprotein receptor related protein-1 (LRP-1) has been shown to transport Aβ out of the brain (Banks, Robinson, Verma, & Morley, 2003; Deane et al., 2008), and AD leads to altered levels of LRP-1, where there is a significant increase in receptor expression co-localized with Aβ plaques (Donahue et al., 2006). It may be also interesting to examine whether exercise increases the level of LRP-1 expression. If that occurs, finding a way to block exercise-induced LRP-1 upregulation, may prove to be a fruitful next step. If blocking LRP-1 upregulation leads to elevated levels of Aβ, then we can propose another therapeutic way to increase Aβ clearance from the brain, by upregulating LRP-1 and increasing transport of Aβ out
of the brain.

In summary, we have demonstrated possible mechanisms for reducing central Aβ accumulation following acute peripheral inflammation. Although there are still no known direct causes of sporadic AD, this work reinforces the need for further understanding of the links between inflammation and Aβ, and the importance of identifying mechanisms to increase Aβ clearance or decrease APP processing. Ultimately, this work demonstrates the positive outcomes that can occur following reduction or removal of excess Aβ from the brain. However, more studies need to be designed and completed in order to provide further explanations of the mechanisms behind these reductions in Aβ accumulation. Finding true preventative measures or new treatments for Alzheimer’s disease is imperative, as without interventions, there are a projected 16 million people that will suffer from AD by 2050.
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**VITA**

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ABSTRACT

ALLEVIATION OF CENTRAL ACCUMULATION OF AMYLOID-BETA AND PREVENTION OF COGNITIVE DYSFUNCTION FOLLOWING PERIPHERAL INFLAMMATION

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Department of Psychology
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Dissertation Advisor: Dr. Gary W. Boehm, Professor of Psychology

Alzheimer’s disease (AD) is characterized by neurodegeneration in regions of the adult brain, due to formation of amyloid-beta (Aβ) plaques and neurofibrillary tangles. Inflammation has been implicated in the onset and progression of these pathologies. Our previous studies resulted in an animal model of peripheral inflammation-induced AD-like pathology using the bacterial endotoxin lipopolysaccharide (LPS). Following 7 consecutive once-daily injections of LPS, C57BL/6J mice displayed significantly higher levels of Aβ as well as cognitive deficits in hippocampus-dependent tasks. Here we explored multiple ways to alleviate the inflammation-induced central accumulation of Aβ. Numerous studies have focused on reducing central Aβ with pharmaceuticals designed to interfere with the mechanisms that lead to production of Aβ, or by interfering with inflammation using non-steroidal anti-inflammatory drugs. Non-pharmaceutical interventions, such as exercise, have also been studied and were found to be beneficial. The experiments described in this dissertation focused on three treatments that previously demonstrated possible therapeutic value for reducing central Aβ accumulation. Treatment with Imatinib, an FDA approved drug for chronic myelogenous leukemia, resulted in
decreased Aβ accumulation and rescued cognitive deficits when the animals were first pre-treated for a week prior to LPS administration. In addition, our data suggests that the Aβ production occurs in the periphery. Next, animals treated with Ibuprofen, a popular NSAID, did not appear to have altered Aβ or reduced cognitive deficits. However, further testing is needed, as ibuprofen has proven more effective when given for extended periods of time. Finally, and most interestingly, two weeks of voluntary wheel running resulted in almost a complete elimination of central Aβ, following LPS administration, while sedentary animals still showed significant elevation of central Aβ. It is reasonable to assume that if the production of Aβ could be reduced or eliminated from the brain there may be a potential for less susceptibility to the onset and detrimental effects of AD. Two of the three studies described above showed potential to be effective at reducing AD pathology. However, further studies need to be conducted in order to further understand the mechanism behind Aβ removal and reduction.