A POTENTIAL ROLE FOR LPS-INDUCED INFLAMMATION IN THE INDUCTION OF ALZHEIMER'S DISEASE-RELATED PATHOLOGY AND COGNITIVE DEFICITS

by

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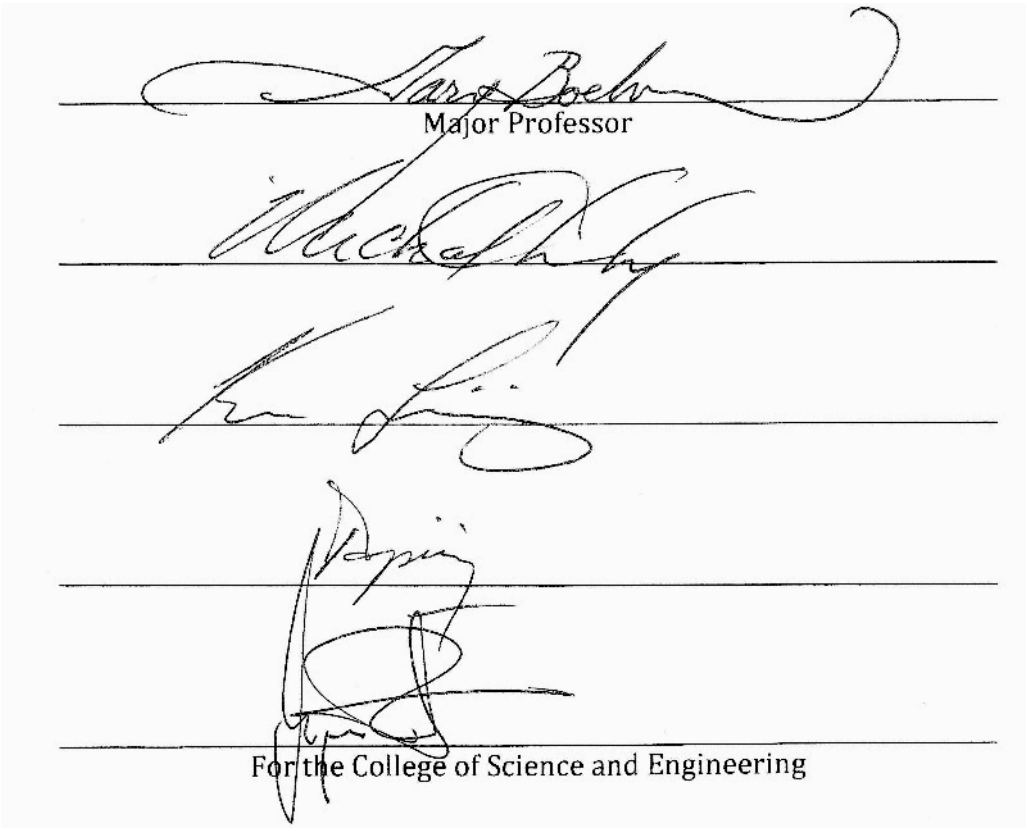

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1. Introduction

In 1906, Alois Alzheimer first described a patient who presented with symptoms of erratic behavior, language problems, and memory loss (Stelzman, Schnitzlein, & Murtagh, 1995). After the patient's death, Alzheimer performed an autopsy in which he described abnormal protein clumping and tangled fibers, now recognized as the senile plaques and neurofibrillary tangles (NFTs) that are two of the biological hallmarks of Alzheimer's disease.

According to 2011 statistics presented by the Alzheimer's association, an estimated 5.4 million American's are currently living with Alzheimer's disease (AD), at an annual cost of 183 billion dollars in medical expenses and lost wages. It is also estimated that by 2030, 7.7 million Americans will develop this disease unless medical breakthroughs are found to treat or prevent it. AD typically presents with mild, usually short-term memory deficits, which can progress into more severe forms of dementia. One of the pathological markers of AD is the presence of plaques in areas such as the cortex, hippocampus, and amygdala (Heneka & O'Banion, 2007). Many neurodegenerative disorders, including AD, affect the hippocampus, a well-studied portion of the mammalian brain known to function in cognition and behavior both in humans (Squire, 1993) and other non-human animals (Eichenbaum, 1996).

A common feature in the emerging research on neurodegenerative diseases, such as AD, is the impact of the inflammatory process on the progression of the disease (Minghetti, 2005). According to McGeer and McGeer (2001), the increased number of self-destructing

neurons, referred to as autotoxicity, is accompanied by chronic inflammation. While most commonly thought of as peripheral responses to infection, inflammatory processes have been associated with diseases of the central nervous system (CNS) as well (Schwab & McGeer, 2008). Chronic inflammation of the CNS is a common component of Parkinson's disease and AD. In fact, the presence of amyloid-Beta$_{1\text{-}42}$ peptide (A$\beta_{1\text{-}42}$), found in the insoluble plaque formations associated with AD (Heneka & O'Banion, 2007), can activate microglia that then produce pro-inflammatory cytokines and chemokines (Schwab & McGeer). Numerous animal models have been used try to determine not only the biological pathways of inflammation in AD (Akiyama et al., 2000; Hickman, Allison, & El Khoury, 2008; Jaeger et al., 2009), but the behavioral effects of the inflammation as well (Lee et al., 2008, Thirumangalakudi et al., 2008). Cell culture, brain imaging, and behavioral research are also being used to understand the pathology of AD in humans (Bowman et al., 2007; Suo et al., 1998).

Several behavioral paradigms have been designed to assess learning and memory, and used specifically to demonstrate deficits in hippocampus-dependent learning in rodent models of AD. Most of these paradigms are associated with some form of relational learning (Eichenbaum, 1996), which is often measured in rodents as learned performance in spatial learning tasks (Morris, 1982, Olton, 1987). Most scientific research focusing on the relationship between AD pathology and behavior is conducted using genetically altered mice. More than ten genetically altered mouse models have been generated in an attempt to produce specific Alzheimer's disease-like pathologies, none of which occur in the wild type mouse (Duff & Suleman, 2004). And while genetically altered mice are an effective way of studying the progression of AD, they are expensive and involve labor-intensive upkeep.

From the time of fertilization, all cells in these animals have some form of the genetic mutation initiated by the investigator. Therefore it is highly likely that any of these gene mutations that involve proteins that are important in development, could adversely affect development. Specifically, mutations in genes that have roles in neural development could adversely affect behavior. Using genetically modified animals to study the progression of AD-like pathology is logical; however, utilizing these animals for behavioral studies may lead to data that is possibly confounded by developmental differences.

One experiment involving a non-transgenic animal has recently revealed an increase in the amount of $A\beta_{1-42}$ found in the hippocampus and cortex of an out-bred (ICR) mouse (Lee et al., 2008). These mice were injected with the endotoxin Lipopolysaccharide (LPS), a pro-inflammatory bacterial mimetic made from the cell wall of gram-negative bacteria (Wittmann et al., 2008). Lee et al. determined that multiple injections of LPS, which induced inflammation, produced both an increase in $A\beta_{1-42}$ plaque build-up and, with one injection of LPS, showed significant disruptions in hippocampal learning and memory behavior. This study was one of the first to attempt to link inflammation with AD pathology and deficits in learning, and is the impetus for this thesis.

1.1. Alzheimer's disease: pathology

One of the trademark neuropathologies for a diagnosis of AD is the presence of NFTs (Murphy & Levine, 2010). NFTs are composed of both hyperphosphorylated tau, a cytoskeletal protein that plays an important role in the formation and stabilization of microtubules, and paired helical filaments (Markesbery, 2010). The formation of NFTs is thought to occur from a disruption in these microtubules (Cras et al., 1995). Cytoskeleton

microtubules direct cellular development and intracellular trafficking, and abnormal accumulation of hyperphosphorylated tau protein leads to the destabilization of these microtubules and the formation of NFTs (Rocher et al., 2010). It is now possible to use Positron Emission Tomography (PET) to digitally represent the presence of NFTs in the adult brain. This technique is an important tool in the diagnosis of AD (Marksberry, 2010).

The other important trademark neuropathology for the diagnosis of AD is the presence of amyloid plaques (Murphy & Levine, 2010). The formation of amyloid-beta plaques results from the breakdown of the cell membrane amyloid precursor protein (APP), which is cleaved by several enzymes, to create Aβ peptides of various lengths. Of the several peptides that are formed, the hydrophobic $A\beta_{1-42}$ is thought to have the most profound affect in the formation of extracellular AD plaques (Heneka & O'Banion, 2007). The production of Aβ peptides occurs from the cleavage of APP by two protease enzymes, β-secretase and γ-secretase, that cleave Aβ into various length peptides (Murphy & LeVine, 2010). The genetically-linked, early onset form of AD appears to involve mutations found in APP or in the γ-secretase-altering enzymes presenilin-1 (PS-1) or presenilin-2 (PS-2) (Murphy & LeVine), leading to increased production of the longer forms of Aβ (Seiffert et al., 2000). These longer forms, as opposed to soluble shorter forms of Aβ ($A\beta_{1-40}$), are insoluble and are more difficult to clear from the brain. As they accumulate they form the senile plaques associated with various forms of dementia, including AD (Murphy & Levine; Selkoe, 2001).

Previous studies have tried to determine if Aβ plaques and NFTs are the cause of the dementia associated with AD pathology; however, others have indicated that the presence of these pathologies correlates poorly with the severity of the disease (Wilcock & Esiri, 1982).

Alternatively, dendrite size and branching has been used as an indicator of both function and recovery of function, following lesion studies in animals (Gillani et al., 2010; Papadopoulos et al., 2006). Einstein, Buranosky, and Crain (1994) discovered that dendrite differences, such as branching and size, were not significant when comparing dendrites located in brain regions with plaques and those located in brain regions without plaques. However, Harris et al. (2010) discovered that APP transgenic mice showed cognitive deficiencies in the Y-maze and in the Morris water maze and had Aβ-induced synaptic impairments demonstrated by decreased long-term potentiation (LTP). LTP is a cellular phenomenon that models long-term memory formation. Cissé et al. (2010) hypothesized that soluble Aβ lead to cognitive deficits by reducing NMDA receptor signal strength and in turn inhibiting memory formation. In both of these studies, the increased Aβ peptides altered LTP through the inhibition of NMDA receptors and alteration in synaptic strength and stability.

As previously mentioned, animal models are often used to study the biological and behavioral pathologies associated with various forms of dementia. To study Alzheimer's disease in rodents is complicated because there is no single rodent strain that exhibits all forms of AD pathology (Duff & Suleman, 2004). Therefore, AD transgenic mice are being developed with human transgenes that force the onset of AD-like pathologies. One mouse model that exhibits the most similarities to human AD pathology is the triple transgenic Alzheimer's mouse, 3xTg-AD, which includes transgenic mutant forms of PS1, APP, and Tau. By 6-months of age, 3xTg-AD mice display a build up of both plaques and NFTs in areas such as the hippocampus, cortex and amygdala. At this same time, a cognitive decline begins to appear, based on Morris water maze performance. Because these pathologies are not visible at 2-months of age, it has been suggested that an age-dependent increase in the

plaque and tangle development correlates with cognitive dysfunction in this mouse model (Oddo et al., 2003, Rodriguez et al., 2008).

Another study of AD-like pathology utilized the Ts65Dn mouse, a model of Down's syndrome (DS), to determine the effects of estrogen on biological markers that are related to AD (Hunter et al., 2004). Estrogen replacement has been studied as a potential therapy for the cognitive decline found in AD in post-menopausal women (Craig & Murphy, 2009; Janicki & Schupf, 2010). And like patients with AD, patients with DS develop plaques and tangles, and show a gradual cognitive decline (Granholm, Sanders, & Crinc, 2000**;** Yates, Simpson, Maloney et al., 1980). The Ts65Dn mouse model, although not directly designed for the study of AD, is useful, due to the fact that it develops plaques and NFTs similar to AD. Previous findings, utilizing female Ts65Dn mice revealed that infusion of estrogen reduced cognitive impairments associated with the AD-like pathology that occurs in this mouse. (Bimonte & Denenberg, 1999). The cognitive decline was seen as an inability to work under increased cognitive load demonstrated using the water-based radial arm maze. However, Hunter et al. revealed that, unlike in female Ts65Dn mice, estrogen did not improve the cognitive decline of the DS male mouse, but did decrease the amount of hyperphosphorylated tau, one of the biological hallmarks of AD. Unfortunately, the authors also discovered that hippocampal $A\beta_{1\text{-}42}$ levels increased in the DS mouse regardless of treatment.

Although these mouse models, as well as other transgenic and knock-in animals, allow for the study of AD by inducing pathologies similar to those found in humans, they are complicated to breed and house. And as previously stated, the use of mice genetically manipulated during early development may add confounding variables to behavioral testing.

For these reasons, the ability to find methods to induce Alzheimer's-like pathologies in a non-transgenic animal may prove beneficial for biological and behavioral research.

1.2. Lipopolysaccharide-induced inflammation

Cytokines are proteins that are secreted primarily by cells in the immune system and are used to regulate the host's response to an infection (Dinarello, 2000). Cytokines can be pro-inflammatory or anti-inflammatory and can either exacerbate or reduce the inflammation, respectively. Interestingly, cytokines produced in the periphery in response to a pathogen can affect the brain. One way this occurs is through a neural route, in which vagus nerve afferents relay information from the periphery resulting in central production of cytokines by astrocytes and microglia (Ferrari et al., 2006; Lee et al., 1993). Another way this can occur is through a humoral pathway that involves peripherally produced cytokines entering the brain through the circumventricular organs and the choroid plexus, or active transport across the Blood-Brain Barrier (BBB) (Konsman, Parnet, & Dantzer, 2002). Goshen and Yirmiya (2007) note that some of the stressors that will induce cytokine production in the brain are the activation of innate immunity, disease, and psychological and physical stress. Furthermore, the production of these cytokines can lead to changes in levels in neurotransmitters and in the homeostasis of the neuroendocrine system, such as increases in the activity of the hypothalamic-pituitary adrenal axis, a key stress response pathway. In addition, pro-inflammatory cytokines can lead to a number of behavioral changes often termed "sickness behaviors" (Dantzer & Kelley, 2007). These sickness behaviors include loss of appetite, reduced activity, anhedonia, and withdrawal from social activities. Rather than use a live pathogen to induce these pro-inflammatory cytokines, and the resultant cascade of biological

responses, many studies utilize LPS to trigger an immune response (Dantzer, 2004). LPS is a bacterial mimetic derived from cell wall of gram-negative bacteria. For example, when LPS is injected intraperitoneally (i.p.) it binds to Toll-like receptor 4 (TLR4) on peritoneal macrophages, resulting in the production of IL-1 and other pro-inflammatory cytokines (Konsman, Parnet, & Dantzer, 2002).

1.3. Inflammation and Alzheimer's disease

Previous and evolving research over the last 20 years supports the idea that the brain is no longer considered an immuno-privileged site. In other words, it is now well understood that the central nervous system and the immune system interact at both the central and peripheral levels (Akiyama et al., 2000). Because of the reaction of the brain to inflammation, both peripherally and centrally, questions have arisen as to whether or not inflammation in the brain can lead to or exacerbate AD, or whether it is merely a side effect of the disease (Akiyama et al., 2000; Hickman, 2008; Jaeger et al., 2009). A number of studies implicate neuroinflammation as a contributor to the pathogenesis of AD (Hickman, Allison, & El Khoury, 2008; Lee et al., 2008). For example, one of the primary responders to neurodegeneration is the microglial cell. Many researchers hypothesize that activated microglial cells, along with pro-inflammatory cytokines and the complement system, may be the main contributors to increased neuroinflammation seen in AD (Selkoe, 2001). Extracellular signaling molecules activate microglial cells, leading the release of various pro-inflammatory cytokines such as IL-1β, thus resulting in an increased inflammatory response (Akiyama et al., 2000; Heneka & O'Banion, 2007). According to Blasko et al. (1999), the proinflammatory cytokine IL-1β can increase the processing of APP and thereby increase Aβ

formation, as indicated by data from human cell culture. IL-1β produced by activated microglia can also bind to receptors on the surface of the microglial cell that produced it; an autocrine activity that results in even more IL-1β synthesis as well as synthesis of other pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6). Additionally, evidence suggests that aggregated Aβ itself can activate microglia (Heneka & O'Banion, 2007), which enhances the inflammatory response to the Aβ by the production of inflammatory mediators, such as the previously mentioned TNF-α and IL-6 (McGeer & McGeer, 2010). In fact, TNF-α can be found at sites of brain injury, and thus has been described as an indicator of damage. TNF-α has also been detected in plaques of post-mortem AD brains, and increased levels of TNF-α correlate with progressive hallmark symptoms of AD (McCoy & Tansey, 2008). Furthermore, mild cognitive deficits have been detected with chronic elevations of IL-6 (Dugan et al., 2009). A study comparing mice that over express IL-6 to wild type mice discovered that those mice overexpressing IL-6 showed significantly lower LTP in the dentate gyrus of the hippocampus (Bellinger, Madamba, Campbell, & Siggins, 1995), leading the authors to speculate that elevated IL-6 plays a role in the cognitive impairments associated with neurodegenerative disorders.

As previously mentioned, there is evidence that some peripherally made cytokines are able to cross protective barriers such as the blood-brain barrier (BBB) and act directly on tissues in the brain using active transport systems (Banks, Kastin, & Broadwell, 1995). Entrance of cytokines such as IL-1β and IL-6 into the brain, potentiate the inflammatory response in brain tissues. Not only do these inflammatory cytokines lead to increased production of Aβ in the brain, but they also alter the movement of Aβ across the BBB. Jaeger et al. (2009) showed that LPS-induced inflammation leads to increased Aβ levels in

the brain. They proposed that these increases may be due to a) excess APP breakdown, b) a decrease in the efflux of Aβ peptides out of the brain, or c) an increase in the influx of Aβ from peripheral sources, possibly due to altered BBB transport systems. To test which of these possibilities was correct. Jaeger et al. (2009) injected radioactively tagged Aβ, either peripherally via intravenous injection, or centrally via intracerebroventricular (ICV) injection. In testing whether LPS-induced peripheral inflammation could alter the BBB, they discovered that mice that received three injections of LPS, at times = 0, 6 and 24 hours, had an increased amount of Aβ in the brain due to increased blood-to-brain transport and a decreased clearance of Aβ from the brain. These data help support the idea that peripheral inflammation leads to production of peripheral Aβ and its transport into the brain causes an increase in central Aβ. Sutcliffe et al. (2011) recently discovered that in APP mice, brain deposited Aβ originated in the periphery. The authors used the cancer drug Gleevec, which acts as a γ-secretase inhibitor, through a complex mechanism, to block APP cleavage, and demonstrated a reduction in Aβ levels in the brain and periphery, suggesting that this drug may be an effective treatment for patients showing symptoms of AD. Importantly, Gleevec does not cross the BBB at an effective level. Therefore, in this study Gleevec blocked peripheral Aβ production in the APP mouse, which lead to significant reduction in brain levels of Aβ.

Twenty-five years of evidence suggests that peripheral inflammation and the associated inflammatory mediators are linked to increased production and deposition of amyloid-beta by increasing neural inflammation (Akiyama et al., 2000). In a study by Lee et al. (2008), a single injection of LPS was able to increase the amount of $A\beta_{1-42}$ found in the hippocampus and cortex of ICR mice. An even larger increase in $A\beta_{1-42}$ was attributed to the

three or seven days of consecutive injections of LPS. Several injections of LPS also led to the accumulation of senile plaques in the hippocampus. Furthermore, Lee et al. demonstrated cognitive deficits following a single injection of LPS. This study demonstrated that LPS-induced inflammation produced an increase in $A\beta_{1-42}$, formations of senile plaques, and cognitive deficits, similar to the pathologies that can also be seen in several transgenic models of Alzheimer's disease. While an increase in $A\beta_{1-42}$ following multiple injections of LPS is a novel finding, single injections of LPS have previously been shown to decrease cognitive function (Gahtan & Overmier, 2001; Kohman et al., 2007; Sparkman et al., 2005b).

1.4. Alzheimer's disease: learning & memory

Alzheimer's Disease usually begins with mild memory deficits, typically affecting short-term memory (Heneka & O'Banion, 1997). This gradual decline of cognitive abilities involves a loss of synapses and neurons, as well as increases in NFTs and senile plaques (Murphy & LeVine, 2010) These degenerative pathologies may lead to the confusion and increased memory loss in the elderly population that are diagnosed with AD, dementia, vascular dementia (VAD), and other cognitive disorders. According to the NIH Alzheimer's Disease Fact Sheet (http://www.nia.nih.gov), AD destroys the cognitive abilities of thought, memory recall, and simple every day life skills. These symptoms, and the diagnosis of Alzheimer's disease are most commonly seen after the age of 65, with the exception of familial early-onset Alzheimer's disease. Early-onset Alzheimer's disease, which is diagnosed before the age of 65 (Wain et al., 2009), has many genetic factors associated with the pathology. According to Mattson, Maudsley, and Martin (2004), the presence of plaques and NFTs in brain regions such as the hippocampus and the amygdala, disrupts cognitive

functioning**.**

Numerous studies have attempted to link learning deficiencies with increased Aβ production. According to Filali, Lalonde, and Rivest (2009), the accumulation of Aβ results in reduction of synapse density and number of neurons in the same area. These molecular changes also correlate with memory impairment. The authors discovered, using a battery of cognitive tests, that the APPswe/PS1 mouse had impaired learning of the passive avoidance test and left-right discrimination learning. In a study of mice that overexpress human APP, deficits in learning were evident during contextual fear conditioning in mice as young as 20 weeks of age (Comery et al., 2005). In a study by Chen et al. (2000), PDAPP mice that express increased APP were used to determine if the β-amyloid plaque formation would alter learning. The results indicated that as the plaque burden in the mouse increased, cognitive function decreased.

However, there is considerable disagreement in the literature regarding the affect of brain Aβ levels on learning and memory. Morley et al. (2010) modulated brain levels of Aβ using various techniques, and demonstrated an "inverted-U" relationship. They found that animals with intermediate levels of Aβ performed better at both the T-maze and Novel Object Recognition tests than did animals with low levels of Aβ or high levels of Aβ. Extremely low and extremely high amounts of Aβ produced worsened performance.

1.5. Modeling AD in rodents: learning & memory behavioral paradigms

In order to explore possible differences in cognitive functioning in experimental rodent models, a number of behavioral paradigms have been developed. The following paradigms have been used to assess learning and memory differences in animals that have

been altered either by genetic mutation or by an introduced pharmacologic or immune challenge.

1.5.a. The passive avoidance test

Passive avoidance is demonstrated when a rodent learns to avoid a species-typical behavior (i.e., crossing to the dark portion of the chamber) in order to also avoid a linked aversive stimulus (i.e., mild foot-shock). One version of this behavioral task utilizes one day of training and one day of testing. This paradigm consists a two-chamber apparatus in which a guillotine door separates the two chambers. The normal behavior of a mouse is to move from a lighted compartment to a darkened compartment when the guillotine door is raised. When the mouse crosses into the darkened chamber, the door shuts, a mild shock is given through the grid floor, and the paradigm ends. On the second day, the mouse is placed in the lighted side of the chamber and latency to cross into the dark chamber is recorded. Other permutations of this task exist. For example, Yirmia, Winocur, and Goshen (2002) used an appetitive version of the passive avoidance task in which animals that had been previously water deprived, learned to avoid a waterspout that had been electrified, even though they needed to rehydrate.

When Lee et al. tested LPS and saline injected mice in this apparatus, they found on the second day, that animals injected with LPS moved to the dark compartment faster than those injected with vehicle, possibly demonstrating a lack of learning from training day. It is also important to mention that the passive avoidance paradigm might be difficult to analyze when sickness behavior is present. In such a case, animals that are sick may not move to the darkened compartment. If this occurs on Day 2, the behavior could be misinterpreted as

learning. Alternatively, such behavior on Day 1 could be used as an indicator of sickness behavior.

1.5.b. The Morris water maze

The Morris water maze (MWM) consists of a large round tub filled with water. A platform is placed in a specific quadrant of the pool, hidden slightly below the surface of the water that has been rendered opaque by adding non-toxic paint. For location learning purposes extra-maze cues are placed on the surrounding walls. Although the location of the platform remains the same, the mouse starting points are varied (Yirmiya, Winocur, & Goshen, 2002). According to Morris (1984), the maze was designed to provide evidence that spatial learning occurs in the rat-based on its ability to use extra-maze cues to locate an escape platform. Lee et al. (2008), used the MWM to show that animals injected with LPS had increased escape latency as well as increased total swimming distance, but no change in swim speed when compared to the vehicle treatment group. The author interpreted these differences as learning deficit in the LPS-treated group. The PSAPP mouse that over expresses APP, has also previously been shown to have a learning deficit in the MWM when compared to non-transgenic mice (Arendash et al., 2001).

1.5.c. Contextual fear conditioning

Contextual fear conditioning (CFC) is a learning mechanism in which a neutral context, such as the conditioning apparatus, is paired with an aversive stimulus, such as a shock (Anagnostaras, Josselyn, Frankland, & Silva, 2000). Multiple pairings of these stimuli render the once neutral context aversive (Anagnostaras, Maren, & Fanselow,1995; Phillips &

LeDeaux, 1992). In rodents, this learned fear leads to the innate action of freezing when there is no way to escape (Wahlsten, 2011), and damage to the hippocampus leads to decreased freezing time in the CFC apparatus (Rudy and Pugh, 1998). Results from Phillips & LeDoux (1992), and numerous other studies, indicate that the hippocampus plays an integral role in the learning of the stimuli associations in CFC (Anagnostaras, Maren, & Fanselow,1995; Fanselow, 2000; Pugh et al., 1998). In addition, administration of LPS or IL-1β can also lead to decreased freezing time in rats (Pugh et al., 1998), suggesting a deficit in learning the pairing of the unconditioned stimulus and conditioned stimulus. However, it is important to note that studies involving inflammation and the CFC paradigm must consider the timing of the inflammation and the pairing of inflammation to the training and testing portion of the task when interpreting performance. Animals that exhibit sickness behaviors tend to remain inactive, which, in the testing portion of CFC, could be interpreted as better memory.

1.6. Determining sickness behavior in mice: Open field paradigm

Utilizing open field boxes is a simple but effective way to observe the motor activity of mice (Choleris, 2001). Activity levels, such as movements and distance traveled, are observable and quantifiable because the device measures all movements in the X, Y, and Z plane. In addition, the time spent in the center of the box versus the outside walls can also be used as a measure of anxiety level. David et al. (2009) demonstrated that mice receiving chronic corticosterone treatment spent significant less time in the center of the open field box than did vehicle controls. However, chronic administration of fluoxetine or imipramine produced antidepressive/antianxiolitic effects in the corticosterone treated animals that

completely abolished the anxiety behavior measured by the open field apparatus. In a study performed by Swiergiel and Dunn (2007), both IL-1β and LPS, injected 60 minutes and 120 minutes respectively before behavior, resulted in decreased motor activity of mice in the open field apparatus. These authors also reported that animals who had received IL-1β and LPS spent less time in the center of the apparatus possibly indicating the animals might have a higher level of anxiety. However, it is important to note that the interpretation of higher anxiety was likely confounded by the sickness behavior of decreased motor activity in animals along the box's border. Such confounding behavior must be considered when interpreting data collected using the open field paradigm.

1.7. Summary and hypotheses

The studies presented below focus on the potential effects of repeated injections of LPS on $A\beta_{1-42}$ expression, cognitive deficits, and Alzheimer's-like pathology. In the previously mentioned study of LPS-induced amyloid-beta deposition, Lee et al. (2008) demonstrated, that a single dose of LPS resulted in an increase in $A\beta_{1-42}$ in the brain, and that 7 consecutive days of LPS injections resulted in AD-like plaques in ICR mice. They also showed that one injection of LPS resulted in behavioral changes. Using both the passive avoidance and the Morris water maze, the authors demonstrated that LPS produced significant memory impairments in both behavior paradigms. However, the authors failed to explain that these changes might possibly be attributed to "sickness behaviors" as the injection of LPS was given only four hours prior to testing. In addition, the MWM protocol used by Lee et al. was non-traditional in that they allowed the mice to learn the maze for three days prior to being given LPS. Furthermore, Lee et al. did not test the behavioral effects

of multiple days of LPS injections. It is therefore unlikely that the authors can attribute behavioral effects produced by a single acute inflammatory dose of LPS to AD-like pathology. It is our goal to produce peripheral inflammation and determine if this inflammatory state leads to AD-like pathology, and if that AD-like pathology results in cognitive impairments that can be distinguished from the effects of sickness behavior. Utilizing the same LPS and ELISA protocols used by Lee et al. (2008) we will determine if a more extended inflammation leads to increased $A\beta_{1-42}$ and learning deficits. However, we will use the common laboratory mouse strain C57BL/6J because the ICR mouse used by Lee et al. is also known to have forms of visual impairments that can affect performance in many rodent behavioral paradigms (Adams, Fitch, Chaney, & Gerlai, 2002). We hypothesize that seven consecutive days of peripheral LPS administration will lead to elevated levels of $A\beta_{1-42}$ and, like with only one injections of LPS, will cause cognitive impairment.

2. Methods

2.1. Subjects

Male C57BL/6J mice from the TCU vivarium will be utilized in all experiments. All animals will be housed and treated in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996), and in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Texas Christian University.

2.2. Housing

All subjects will be housed in groups of three or four in standard cages (12.5cm x

15cm x 25cm). All experimental groups and control groups will be on the same light schedule, lights on at 0600 and lights off at 1800, and both food and water will be available *ad libitum*.

2.3. Biological assays

2.3.a. LPS injections and tissue preparation

To determine if LPS could increase $A\beta_{1-42}$ and alter behavior in the C57BL/6J mouse, injections of LPS or Saline were given for seven consecutive days prior to all behavioral and biological assays. The LPS strain was derived from *Escherichia Coli* (Serotype: 055:B5; Sigma-Aldrich, St. Louis, Missouri). In all experiments, LPS was injected intraperitoneally (i.p.) at a weight-dependent dose of 250 µg/kg.

At the appropriate times after completion of LPS treatment, mice were euthanized in accordance with IACUC-approved methods, and hippocampal tissue samples were extracted and prepared for protein assay and ELISA procedures. The removed tissues were homogenized with protein extraction solution (PRO-PREP, Boca Scientific, Boca Raton, FL.) containing protease inhibitors, and were allowed to further lyse for 30 minutes on ice. The lysate was centrifuged at 15,000 rpm for 30 minutes and the clear lysate removed for *DC* Protein Assay (Bio-Rad Laboratories, Hercules, CA.).

2.3.a. 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 were used, and made in the same buffer as in the lysates. 5µl of the standards and the

samples were pipetted into the 96 well plate with 25µl of reagent A' and 200 µl of reagent B. After 15 minutes, the plate was placed in the plate reader (BMG LabTech FLUOstar Omega, Cary, NC) and the optical density of the plate was read at 750nm. The results of the protein assay were used to normalize protein content.

2.3.b. Aβ ELISA procedure

The BetaMark $A\beta_{x-42}$ ELISA (Covance Research Products, Dedham, Massachusetts) 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 diluted 2:1 with working incubation buffer, which includes the HRP-labeled detection antibody. 100µl of each dilution of the standard curve, in duplicate, and 100µl of each unknown sample in triplicate to the plate and was incubated over night at 2–8 degrees Celsius. 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 minutes at room temperature in the dark. After this incubation period, the plate was read at the optical density of 620nm.

2.3.c. Statistical analysis of $A\beta_{1-42}$ levels

In experiment 1, Aβ levels were analyzed using the non-parametric one-sample t-test. Because all Aβ levels from saline treated animals were below the detectable limit of the

standard curve from the ELISA kit, each sample was given the lowest value on the standard curve (7.8pg/ml). This step eliminates any variance between samples and forces the use of a non-parametric test for these samples. A one-sample t-test was used to compare the fold increase of Aβ levels following three or seven injections of LPS. After making adjustments in the Aβ analysis procedure, we were then able to use a conventional analysis of variance (ANOVA) for all other experiments. In these experiments Fisher's PLSD post-hocs were used to find differences after significant main effects were determined.

2.4. Behavioral paradigms

2.4.a. Passive avoidance test protocol

Passive avoidance behavior was assessed using eight fully automated Gemini II shuttlebox units manufactured by San Diego Instruments (San Diego, CA). The apparatus consists of two compartments (20.64cm W x 25.08cm L x17.14cm H) separated by a guillotine door, where one compartment is dark and the other is illuminated. The floor is made of stainless steel rods so that a light shock can be delivered as an aversive stimulus. This test was run for 2 consecutive days, training day on day 1 and testing day on day 2. On the training day, each mouse was placed in the illuminated compartment facing away from the dark compartment. At this time, the guillotine door separating the two compartments was in the "down" position. After 60, seconds the guillotine door was raised and the mouse had the ability to cross into the darkened compartment. Once the mouse fully entered the darkened compartment, the guillotine door closed, and a mild shock (0.4 mA for 2 seconds**)** was delivered. Step-through latency was determined as the amount time it took the mouse to enter the dark compartment. No shock was delivered during this trial. If the dark

compartment was not entered into within 300 seconds, the trial was terminated. The behavioral data was analyzed using analysis of variance (ANOVA) procedures (Statview 5.0, SAS, Cary, NC) in which Treatment was the independent variable. The alpha level used for all statistical analyses was 0.05.

2.4.b. Open field protocol

The animal was placed in an open field arena (27.9 cm × 27.9 cm) that was completely enclosed and illuminated, and allowed to explore the arena for twenty minutes. After completing the twenty-minute task, the mouse was returned to its home cage, and the open field boxes were cleaned thoroughly. Motion in the X, Y, and Z planes of the open field boxes was recorded, along with the amount of time spent in the open center ("in zone") of the box versus against the walls or corners ("residual zone"). The Activity Monitor (Med Associates, Inc., St. Albans, VT) computer recording program was used to record the animal's movement inside the boxes. The behavioral data was analyzed using analysis of variance (ANOVA) procedures (Statview 5.0, SAS, Cary, NC) in which Treatment was the independent variable. The alpha level used for all statistical analyses was 0.05.

2.4.c. Morris water maze protocol

A circular white pool was filled with water and the water was dyed with commercially available white tempera paint. A platform was placed in one quadrant just beneath the water's surface. On the first trial, the training trial, mice were placed in the water and were allowed to find the platform and then remain on the platform for 10 seconds, after which the mouse was returned to it's holding cage. Any mouse that did not find the platform

before the 120-second time limit was guided to the platform and allowed to remain on the platform for 10 seconds. This process was repeated four times per day for four days, followed by a probe trial, performed on the fifth day. The probe trial consisted of two trials in which animals were allowed to swim for two minutes each trial in the pool that did not have an escape platform. Twenty-four hours after the probe trial, reverse Morris water maze was started (day 6). In this paradigm, the platform is placed back in the pool; however, it is placed in a quadrant diagonally opposite from the original location in MWM. The reverse Morris water maze protocol was conducted for 3 days, followed by another probe trial, on day 9. For all probe trials, the computer recorded the total amount of time the animal swam in the quadrant that previously held the platform. During days 1–4 and 6–8, escape latency, swim distance, and swimming speed were recorded by the Accutrak system (EZ Video Automated Tracking System, AccuScan Instruments Inc. Columbus, Ohio). The behavioral data were analyzed using standard repeated-measures analysis of variance (ANOVA) procedures (Statview 5.0, SAS, Cary, NC) in which Treatment was a between-subjects variable and Testing Day was a with-in subjects (i.e., repeated measures) variable. For both probe trials, data was analyzed using analysis of variance (ANOVA) procedures (Statview 5.0, SAS, Cary, NC) in which Treatment was the independent variable. The alpha level used for all statistical analyses was 0.05.

2.4.d. Contextual fear conditioning protocol

Contextual fear conditioned mouse freezing behaviors were monitored using a Freeze Monitor System and software (San Diego Instruments, San Diego, CA). The unit (26.5cm x 26.5cm x 17.8cm) uses an electrified grid floor through which the aversive stimulus, a 0.7mA

shock, is delivered. The unit is also equipped with a small light bulb (24 VCD) attached to the ceiling, and infrared photo beams along the side walls, allowing the system to monitor movement of the animal. Our protocol of contextual fear conditioning consisted of a training session on day 1 and a testing session on day 2. Twenty-four hours after the 7th injection of LPS, mice were placed in the CFC chamber for training. The training session began with a 90 second acclimation period, followed by a 2 second 0.7mA shock. Animals then remained in the apparatus for a 90 second inter-trial interval (ITI), followed by a 2 second shock. One more 90 second ITI, was followed by a shock, and a final 90 seconds of no shock. The entire training day lasted a total of 366 seconds. On the testing day, no shocks were delivered, though the system continuously recorded the movement of the animal for 90 seconds. Animals were considered to be "freezing" when they did not break photo beams for any 2-second interval of time. Previous experiments our lab have revealed that the incorporation of an olfactory contextual cue and a salient wall design (black polka dots) increased the freezing time suggesting an increase in the learning of the context-shock pairing. The olfactory cue used in this study was peppermint oil (Now Foods, Bloomingdale, IL) and was mixed with water at ratio of 1:10, and placed in a container underneath the grid floor. The time freezing in the first 90-second bin was analyzed using analysis of variance (ANOVA) procedures (Statview 5.0, SAS, Cary, NC), in which Treatment was the independent variable. The alpha level used for all statistical analyses was 0.05.

2.5. List of experiments

Experiment	N	# of Groups	Dose of LPS (μg/kg)	# of injections	Brain Tissue Collection	Behavioral Testing
Experiment 1	24	4	0, 250	3,7	Yes	n/a
Experiment 2	72	3	0, 250	7	Yes	n/a
Experiment 3	37	4	0, 250	1,7	No	PA
Experiment 4	31	4	0, 250	1,7	No	OF
Experiment 5a	21	2	0, 250	7	No	MWM/RMWM
Experiment 5b	24	2	0,250	7	Yes	MWM/RMWM
Experiment 6	16	2	0, 250	7	No	CFC

Table 1. List of all experiments completed. PA-passive avoidance; OF-open field; MWM; Morris water maze; RMWM- reverse Morris water maze; CFC- contextual fear conditioning.

3. Results

3.1. Appearance of animals and weight loss from LPS

Animal weights were measured daily during the seven days of injections, for both LPS and saline groups. Animals that received seven injections of LPS displayed classical-sickness related symptoms such as weight loss, lethargy, piloerection, and decreased grooming, that continued until day 4 or 5. On day 1 both groups showed no significant difference in starting weight ($F_{(1,127)}$ =.338, ns). A repeated measures ANOVA revealed a significant treatment effect ($F_{(1,127)}$ =5.310, $p < .05$), and weight x treatment interaction, ($F_{(6,762)}$ =80.983, $p <.0001$); (Data pooled across all experiments).

3.2. Experiment 1: LPS-induced $A\beta_{1-42}$ production

A pilot study was performed to determine if repeated i.p. injections of LPS would lead to increased $A\beta_{1-42}$ in the hippocampus of the C57BL/6 mouse. Four groups were used to determine if there was a difference in the amount of $A\beta_{1-42}$ in the hippocampus. Groups 1

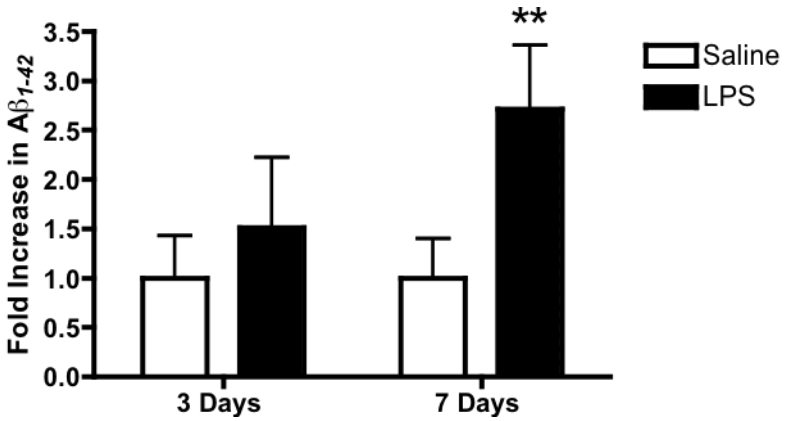

Figure 1. LPS-induced $A\beta_{1\text{-}42}$ production. 7 days, but not 3 days, of LPS injection significantly elevates $A\beta_{1\text{-}42}$ in the mouse hippocampus. ** compared to saline control, $p<0.01$.

and 2 received 3 consecutive days of LPS or saline injections respectively. Groups 3 and 4 received 7 consecutive days of LPS or saline injections respectively. Twenty-four hours after the final injection, the mice were euthanized by IACUC approved protocols and hippocampal tissue samples were extracted and prepared for protein assay and ELISA procedures. The results, as shown in Figure 1, from the pilot biological assay revealed that mice receiving 3 consecutive injections of LPS showed no difference in amount of $A\beta_{1\text{-}42}$ in the hippocampus as compared to the saline group. However, and more importantly, 7 consecutive injections of LPS produced a significant increase in the amount of $A\beta_{1\text{-}42}$ in the hippocampus as compared to the saline group ($t_{(5)}= 5.008$, $p <0.01$).

3.3. Experiment 2: Temporal clearance of LPS-induced $A\beta_{1\text{-}42}$

In a second experiment three, groups of animals were used to determine the temporal clearance of LPS-induced $A\beta_{1\text{-}42}$ in the hippocampus. Groups 1 and 2 received 7 consecutive

days of LPS or saline, respectively. Twenty-four hours later the mice were euthanized and hippocampal tissue samples were extracted. Group 3 received 7 consecutive days of LPS injections and hippocampal tissue was removed after 15 days of recovery in their home cage. After normalizing for protein levels, we again found, that 7 days of LPS injections resulted in

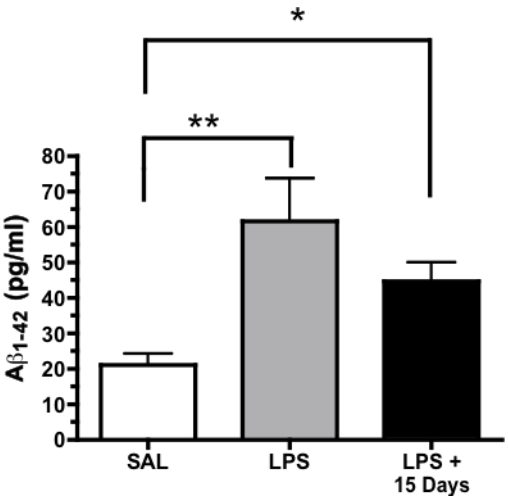

Figure 2. LPS-induced $A\beta_{1-42}$ production and clearance. 7 days of LPS injection significantly elevates $A\beta_{1-42}$ in the mouse hippocampus. 15 days following the last injection, $A\beta_{1-42}$ remains significantly elevated as compared to the saline control group. * compared to saline control, $p<0.05$. ** compared to saline control, $p<0.01$.

a significant elevation in $A\beta_{1-42}$ in the hippocampus ($F_{(2,10)}= 6.732$, $p < 0.01$) as compared to saline controls (Figure 2). After running Fisher's PLSD post-hoc analysis, Aβ elevation was still significant after 15 days of sedentary recovery in the home cage when compared to the saline group ($p <0.05$), but was not significantly different from the LPS group (Group 1).

3.4. Experiment 3: Passive avoidance behavior

To assess whether 7 consecutive days of LPS administration could result in sickness behaviors that might confound our assessment of cognitive differences, we utilized a passive

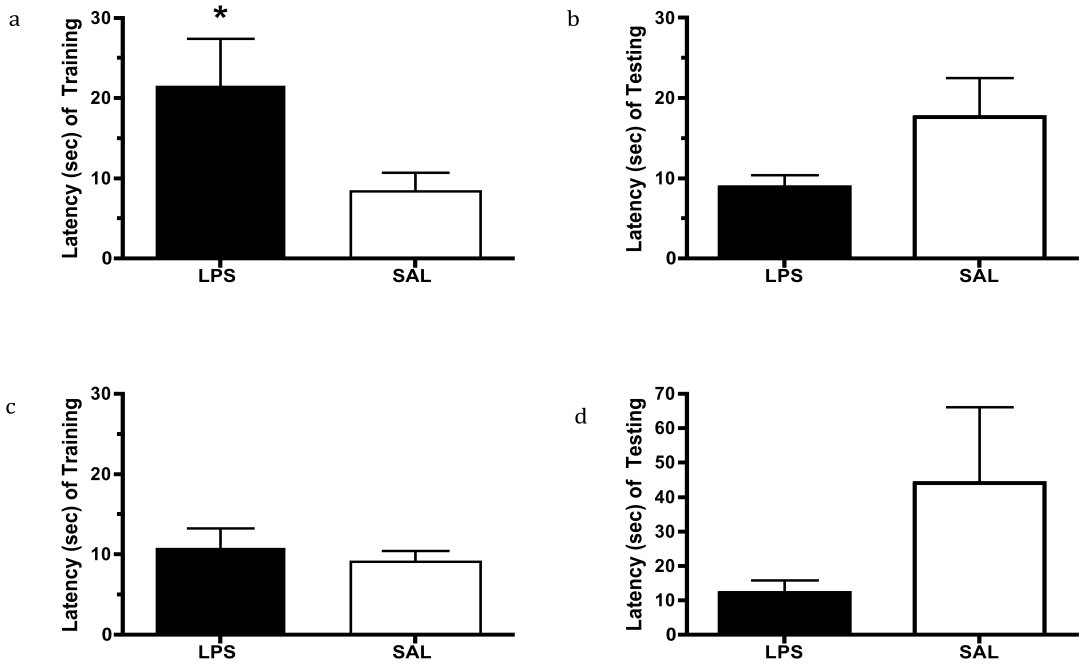

Figure 3. Behavior in the passive avoidance paradigm following LPS administration. **a)** 1 injection of LPS significantly increases latency to cross during the training day of the Passive Avoidance paradigm suggesting the possibility of the presence of sickness behavior. **b)** On testing day of the Passive Avoidance paradigm, animals who received one injection of LPS do not significantly differ from animals who received one injection of saline. **c)**. 7 injections of LPS does not increase latency to cross on training day in the passive avoidance paradigm. **d)**. On testing day of the Passive Avoidance paradigm, animals who received 7 injections LPS do not significantly differ from animals who received 7 injection of saline. * compared to saline control, $p<.05$.

avoidance paradigm specifically focusing on the training day to measure the animal's innate instinct to move to a darkened compartment. Group 1 received a single injection of LPS four hours prior to being placed in the passive avoidance apparatus. This positive control for sickness behavior was used because LPS-induced sickness behaviors have been documented to be at their peak 4 hours after injection (Sparkman, et al. 2005a). As a control, a second group received 1 injection of saline four hours prior to being placed in the passive avoidance apparatus. Groups 3 and 4 received 7 consecutive days of LPS or saline injections, respectively. Results from this experiment (Figure 3) indicated animals that received one

injection of LPS might be displaying sickness behavior on day 1, demonstrated by an increased latency to cross, when compared to animals that received 1 injection of saline ($F_{(1,17)}$= 4.632, $p < 0.05$). On day 2, there were no significant differences in latency to cross for the two treatment groups ($F_{(1,17)}$= 2.692, ns), suggesting that there was no difference in learning. Perhaps more importantly, 7 days of LPS administration did not result in any differences in latency to cross on day 1 when compared to saline controls ($F_{(1,16)}$=.403, ns). Unfortunately, there was also no difference on day 2 testing of latency to cross, suggesting once again that there was no measurable difference in learning between animals receiving LPS and those receiving saline ($F_{(1,16)}$=1.616, ns), at least using this paradigm.

3.5. Experiment 4: Open field behavior

In order to confirm our interpretations that following 7 days of LPS administration sickness behaviors were no longer still present, as detected using a passive avoidance paradigm, we utilized an open field paradigm to measure animal activity levels. Groups 1 and 2 received either a single injection of LPS or saline, respectively, four hours prior to the open field task. Groups 3 and 4 received 7 consecutive days of LPS or saline, respectively, prior to the open field task. Using this paradigm, we found that the animals that received one injection of LPS moved significantly less ($F_{(3,28)}$= 11.713, $p < 0.01$), had decreased vertical movements ($F_{(3,28)}$= 3.789, p <0 .05), and had a significant decrease in time spent "in zone", in the center of the open field box, ($F_{(3,28)}$= 3.033, p <0.05), as compared to all other groups (Figure 4). These behaviors may all be indicative of sickness behavior. More importantly, the animals that received 7 consecutive days of LPS injections were not significantly different from those animals who received 7 consecutive days of saline in terms of distance

moved ($F_{(1,14)}$=3.787, ns), vertical counts ($F_{(1,14)}$= 1.259, ns), or time spent "in zone" ($F_{(1,14)}$= .489, ns). These data suggest that animals that had received 7 consecutive days of LPS injections no longer display sickness behaviors (Figure 4).

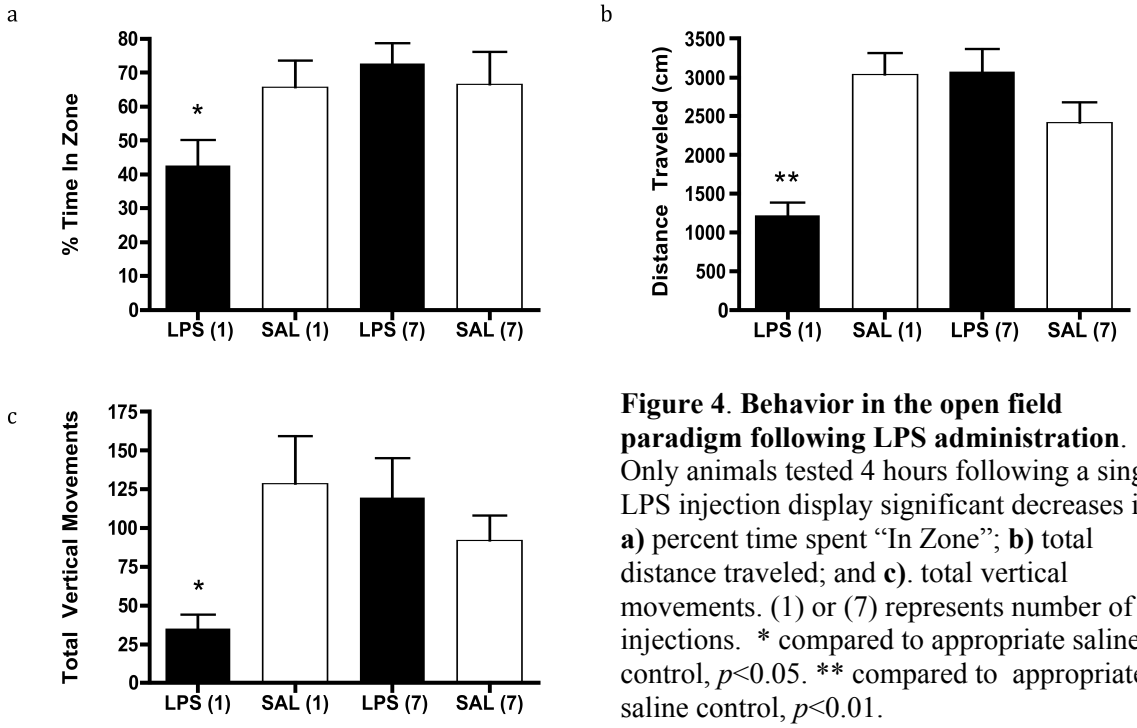

Figure 4. **Behavior in the open field paradigm following LPS administration**. Only animals tested 4 hours following a single LPS injection display significant decreases in **a)** percent time spent "In Zone"; **b)** total distance traveled; and **c)**. total vertical movements. (1) or (7) represents number of injections. * compared to appropriate saline control, $p<0.05$. ** compared to appropriate saline control, $p<0.01$.

3.6. Experiments 5a & 5b: Morris water maze and ELISA

The Morris water maze was chosen to assess the function of the mouse hippocampus in learning. In our version of this task, 4 days of training to locate an escape platform was followed by a probe trial, followed by three days of reversal learning and an additional probe trial. Animals were divided into four groups to assess both learning and Aβ levels following LPS administration. Groups 1 and 2 received 7 days of LPS and saline, respectively, and were used to determine Aβ levels in the hippocampus, prior to the start of the MWM

paradigm (ie. 24 hours after the last LPS injection). Groups 3 and 4 also received 7 days of LPS or saline, respectively, and were then subject to the 9 day MWM paradigm. On day 10, hippocampi from these animals were removed and Aβ levels determined. As shown in Figure 5, during the 4 days of training, there was a main effect of day on escape latency ($F_{(1,3)}$= 31.976, p<0.0001), and swimming distance ($F_{(1,3)}$= 34.919, p<0.0001), indicating learning for all groups of animals. However, no main effects of treatment were found in escape latency

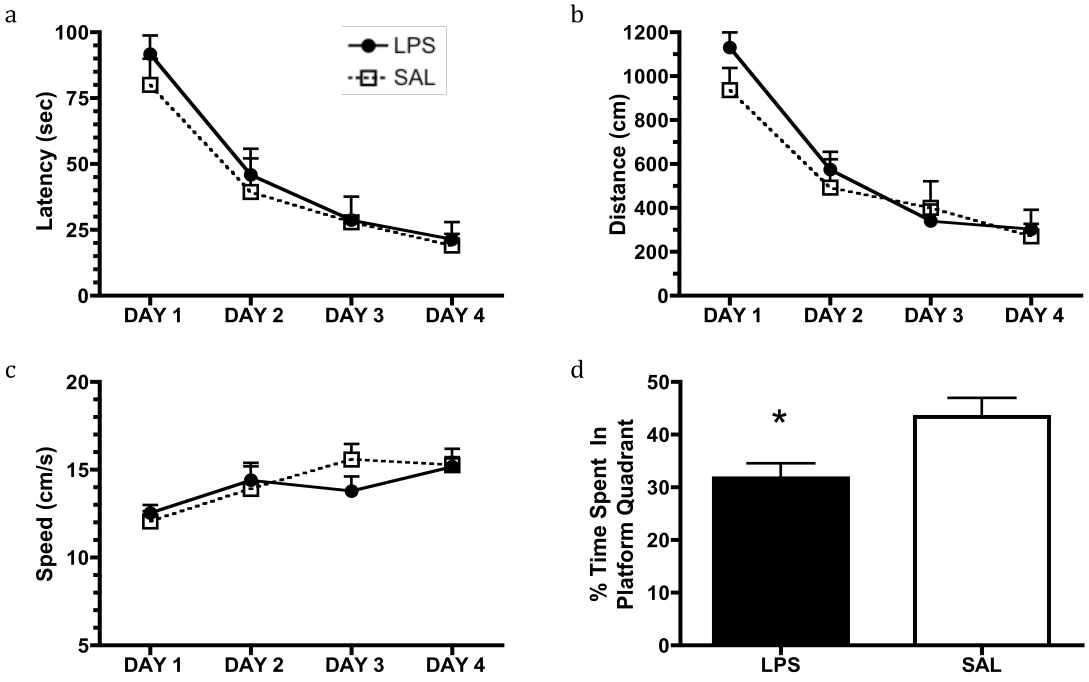

Figure 5. Performance in the Morris water maze following 7 consecutive days of LPS administration. a) Total latency to reach the platform significantly decreased over the 4 days of the test, with a significant main effect for day, p<0.0001, but no difference was found between treatment groups at any time point. **b**) Total distance traveled to reach the platform significantly decreased over the 4 days of the test, with a significant main effect for day, p<0.0001, but no difference was found between treatment groups at any time point. **c**) Total speed to reach the platform significantly decreased over the 4 days of the test, with a significant main effect for day, p<0.01, but no difference was found between treatment groups at any time point. **d**). On Day 5 after LPS injections, animals receiving LPS spend significantly less time in the quadrant that previously contained the escape platform. *compared to saline control, p<0.05.

($F_{(1,19)}$= .468, ns), swimming distance ($F_{(1,19)}$= .706, ns), or swimming speed ($F_{(1,19)}$= .085, ns), during the first 4 days of MWM. Further, we failed to find an interaction effect for any

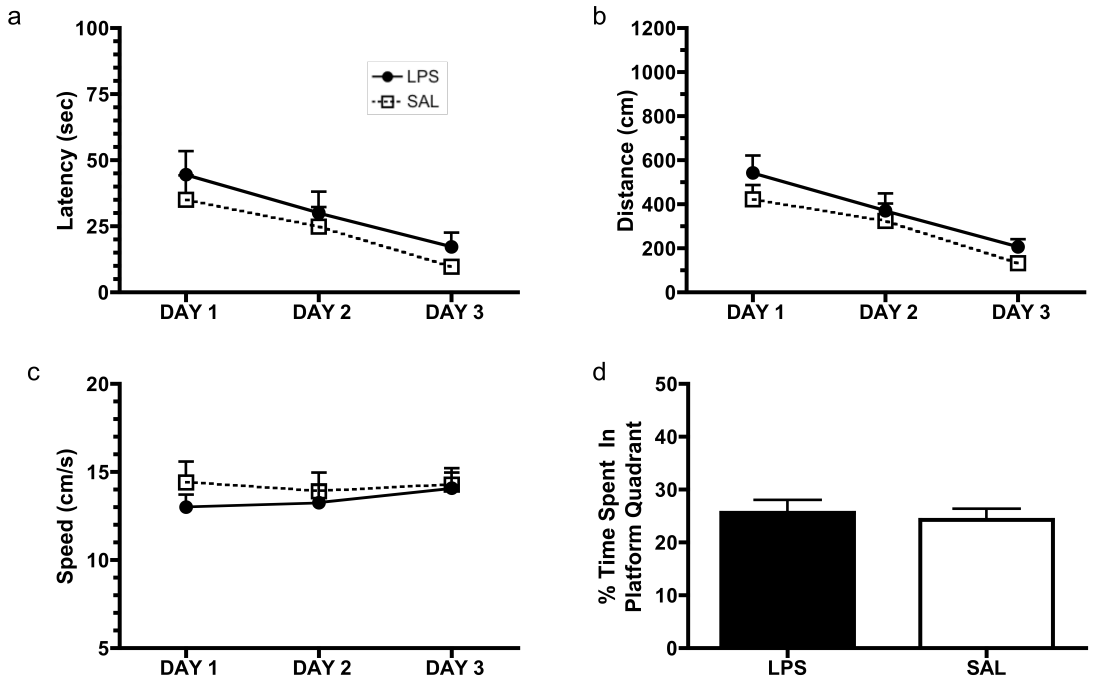

Figure 6. **Reversal learning performance in the Morris water maze**. Total latency to reach the platform significantly decreased over the 4 days of the test, with a significant main effect for day, $p<0.0001$, but no difference was found between treatment groups at any time point. **b**) Total distance traveled to reach the platform significantly decreased over the 4 days of the test, with a significant main effect for day, $p<0.0001$, but no difference was found between treatment groups at any time point. **c)**. There was no main effect, by day, found for total speed to reach the platform. There was also no difference found between treatment groups at any time point. **d**). On Day 4, the probe trial, there were no significant differences in amount of time spent in the platform quadrant.

of the measures. However, LPS treated animals displayed a significant reduction in the time spent in the platform quadrant on the day 5 probe test ($F_{(1,19)}= 6.479$, $p < 0.05$). Three days of reversal training, as shown in Figure 6, also produced a main effect of day on escape latency ($F_{(1,2)}= 18.723$, $p<0.0001$), and distance ($F_{(1,2)}= 21.770$, $p<0.0001$) but not speed ($F_{(1,2)}= .638$, ns). No treatment or interaction effects were found, nor was there a difference on the probe trial that followed reversal training ($F_{(1,19)}= .157$, ns).

Our assessment of Aβ production confirmed our previous findings that 7 days of LPS administration leads to significant elevations in hippocampal Aβ ($F_{(3,19)}= 9.208$, $p<0.01$).

Interestingly, a comparison of treatment groups using Fisher's PLSD indicates that only when measured after 7 consecutive injections of LPS, is Aβ significantly elevated over 7 days of saline ($p < 0.01$). When measured after the 9 days of swim testing in the MWM, the hippocampal Aβ levels had returned to saline control levels (Figure 7).

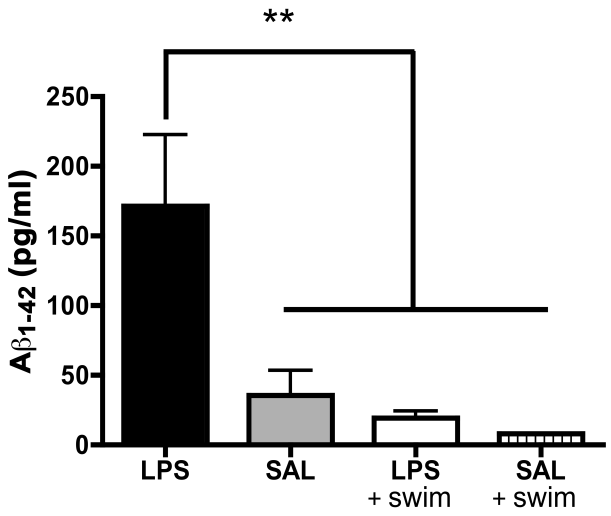

Figure 7. Levels of $A\beta_{1-42}$ after participation in Morris water maze. After 7 days of LPS administration, we see a main effect of treatment on level of $A\beta_{1-42}$ in the hippocampus ($F_{(3,19)}= 9.208, p<.01$). We determined that 7 days of LPS administration resulted in significantly higher levels of Aβ as compared to saline controls. However, following participation in 9 days of MWM, Aβ levels returned to baseline . ** compared to saline control, $p<0.01$.

3.7. Experiment 6: Contextual fear conditioning

We used contextual fear conditioning, to determine if any memory consolidation differences in learning could be identified between two experimental groups of animals; one that received 7 consecutive days of LPS injections and another received 7 consecutive days of saline as a control. Our ANOVA revealed that no significant differences in freezing time existed on the training day between our groups ($F_{(1,14)}= 0.904$, ns). However, animals receiving 7 injections of LPS exhibited significantly more freezing time ($F_{(1,14)}= 9.497$, $p<0.01$) than their saline controls on the testing day (see Figure 8), indicating a deficiency in memory consolidation.

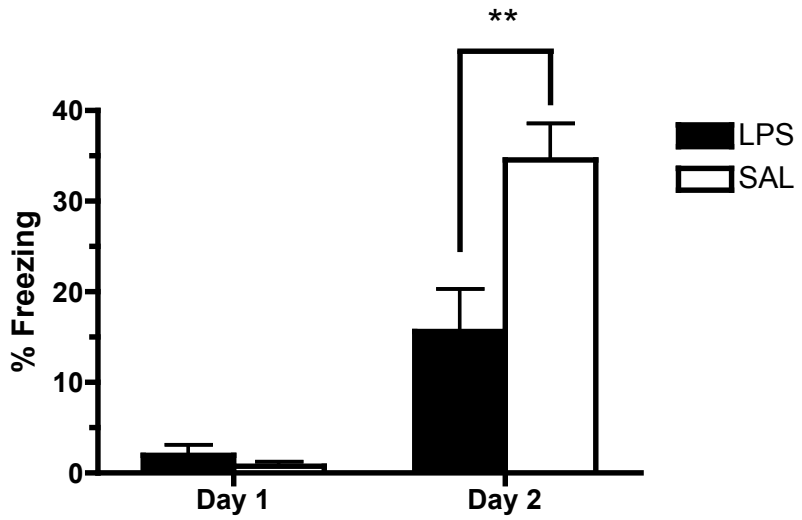

Figure 8. Percent time freezing in contextual fear conditioning. 7 consecutive days of LPS injections significantly decrease percent time freezing in the contextual fear conditioning paradigm, on testing day. ** compared to saline control, $p<0.01$.

4. Discussion

The purpose of the performed studies was to determine if LPS-induced peripheral inflammation could lead to elevations in $A\beta_{1-42}$ and if these elevations correspond to behavioral changes and/or cognitive deficits. Previous data suggests that the immune system can modify numerous physiological processes, including learning and behavior (Danzer 2004; Dantzer & Kelley, 2007; Yirmiya & Goshen, 2011). A recent study by Lee et al., (2008) demonstrated that LPS-induced peripheral inflammation produced both an increase in $A\beta_{1-42}$ in the hippocampus as well as disruptions in behavior in the ICR strain of mouse. However, using behavioral paradigms that involve vision may be confounded by the deficits in the visual system previously reported in the albino ICR mouse (Adams et al., 2002). Therefore, our project attempted to replicate the findings by Lee et al. in the C57BL/6J mouse strain, and to extend these findings with more extensive behavioral and cognitive data, specifically with behavioral data collected after 7 consecutive injections of LPS.

Our findings demonstrate that 7 consecutive days of LPS administration results in significant elevations in $A\beta_{1-42}$ in the hippocampus. The production of $A\beta_{1-42}$ following 3

consecutive days of LPS injections was not significantly elevated, suggesting that the total duration of the inflammatory response produced by LPS may influence the level of $A\beta_{1-42}$ production. While Lee et al. were able to produce significant $A\beta_{1-42}$ production following a single LPS injection in the ICR mouse; our data suggests that the C57BL/6J mouse may require a more substantial LPS load or a longer duration of inflammation to produce significant $A\beta_{1-42}$. Unfortunately, our inability to demonstrate a significant increase in $A\beta_{1-42}$ production following 3 days of LPS may have been influenced by the small number of animals in this pilot study. One interesting finding was that 15 days of recovery, following the 7 consecutive LPS injections, allowed for some $A\beta_{1-42}$ clearance, however levels of Aβ still remained significantly elevated as compared to the saline treated animals. Our findings suggest that a more thorough study of the relationship between LPS load and the temporal regulation of $A\beta_{1-42}$ production and clearance is needed in the future.

In order to determine if elevations in $A\beta_{1-42}$ following 7 days of LPS administration correspond to deficits in learning or memory, we proposed to adopt several of the behavioral paradigms previously used by Lee et al. However, it was first necessary to determine if the repeated administration of LPS could lead to motor deficits or motivational changes often associated with "sickness behaviors" that would limit our interpretation of performance in learning tasks, as this was not tested in Lee et al. Our initial assessment of passive avoidance learning suggested that we might use the training day (Day 1) to indicate whether an animal enacts an innate behavior to move to a darkened compartment. We hypothesized that an animal that was exhibiting sickness behaviors would lack the motivation to move to the darkened chamber and would exhibit an increased latency to cross. In fact, animals that received only one LPS injection four hours prior to passive avoidance training demonstrated

a significant elevation in escape latency suggesting that these animals lacked the motivation to cross. Our result conflicts with that of Lee et al., as they showed no significant differences in latency to cross 4 hours after a single injection of LPS when compared to saline controls. However, many other studies have shown that the inflammatory and behavioral effects of LPS are highest four hours after administration (Gahtan & Overmier, 2001; Kohman, 2007, Sparkman 2005a; Sparkman, 2005b). In addition, our results suggest that, as expected, a single dose of LPS 4 hours prior to performance of the open field task led to a significant decrease in total movement, including time spent in the center of the box and rearing, implicating sickness behavior.

It was our goal to determine whether or not we could perform behavior testing with animals that had received 7 injections of LPS. Once again, using the passive avoidance paradigm, four hours after the seventh consecutive day of LPS injection, animals no longer demonstrated an elevation in escape latency. Additionally, animals receiving 7 consecutive days of LPS did not display differences in total movement, time in the center zone, or rearing events when tested in the open field, suggesting that sickness behaviors were no longer evident. It is our interpretation that these animals no longer demonstrate sickness behaviors. We speculate that after 7 consecutive days of LPS administration, mice display endotoxin tolerance, and behavior correlates thereof, in which monocytes and macrophages, which respond to LPS, become refractory to successive endotoxin challenge (Biswas & Lopez-Collazo, 2009). The exact mechanism for this tolerance is still not completely understood, however it has been proposed that the reduced endotoxin response is a physiological adaptation that prevents an over-exuberant inflammatory response that could lead to endotoxin shock.

If our interpretation is correct, and animals following 7 days of LPS administration are no longer exhibiting sickness behaviors, then assessment of various phases of learning and memory using performance-based paradigms will prove more reliable. In fact, using the Morris water maze, we demonstrate that after 7 days of LPS, animals learn the escape task at the same rate as control animals. Not only does this indicate that LPS-treated animals are able to learn the task, they also did not exhibit motor deficits or lack of motivation to swim that might be considered a sickness behavior. However, while the LPS and saline treated animals perform similarly in the four training days of the MWM, LPS treated animals spent significantly less time in the target quadrant during the probe trial. As many investigators suggest that probe trial performance is a good indicator of strength of memory (Morgan, 2009), these data indicate that our LPS treated animals displayed a learning deficit. An alternative explanation, however, could be that animals treated with LPS do not display the same perseverance as control animals, or that there may be motivational differences (Cunningham & Sanderson, 2008). Interestingly, an additional 3 days of reversal training, followed by a new probe trial, revealed that the behavioral differences seen in the first probe trial were no longer evident. When we tested hippocampal Aβ, levels after the 9 days of swimming in the MWM, we found the Aβ that had been significantly elevated after the injection series had decreased to baseline saline levels, indicating a clearance of Aβ and leading us to speculate that the elimination of Aβ might have allowed the animals to perform better in the later stages of our WMW protocol. The results from MWM may also indicate that although acquisition was not negatively affected by increased Aβ, evidenced by no differences between treatment groups in the 4 days of latency data, consolidation memory may be altered as demonstrated by the first probe trial. To test this hypothesis, we used

contextual fear conditioning, a classical conditioning paradigm that pairs a neutral context with an aversive stimulus (Whalsten, 2011). The results from this experiment demonstrated that after 7 consecutive injections of LPS, the memory of the aversive context was less retrievable, as demonstrated by a significantly decrease in freezing time, as compared to animals injected with saline.

Yirmiya & Goshen (2011) reviewed a large body of evidence that implicates elevated pro-inflammatory cytokines in the impairment of learning and memory, independent of the effects of sickness behaviors. Although our study does not analyze the peripheral or central levels of various cytokines, we are confident that our 7 days of LPS administration leads to inflammation. Nonetheless, we must exercise caution when inferring the cause of the learning deficits we present. It is possible that central IL-1β levels remain elevated after 7 days of LPS administration, leading to the learning deficits we detected. Although possible, this seems unlikely, as the animals no longer display behaviors indicative of ongoing inflammation. It will be important in the future to measure the presence of central and peripheral cytokines in order to determine their possible contribution to the cognitive impairments shown in our model.

We propose that the elevated levels of $A\beta_{1-42}$ present in the hippocampus following 7 days of administration of LPS leads to cognitive impairments. Such a hypothesis is not without precedent (Cissé et al. 2010; Harris et al., 2010; Morley et al., 2010,). To test this hypothesis, it will be necessary to block APP breakdown following of our LPS-induced inflammation to determine the contribution of Aβ to learning deficits in this model. One potential mechanism will be the use of imatinib (Gleevec), which binds to a gamma-secretase activating protein and reduces its activity, thus decreasing the production of Aβ peptides (He

et al., 2010). It is interesting to note that although imatinib poorly crosses the BBB, its function in reducing peripheral Aβ production has been shown to lead to significant decreases in brain Aβ levels. These findings suggesting that transport of Aβ peptides into the brain is an important mechanism in the buildup of Aβ in the brain, at least in the APP mouse (Sutcliffe et al., 2011).

One other interesting finding of our study was the rapid elimination of Aβ from the hippocampus following 9 days of swimming in the MWM. Considering that 15 days of sedentary recovery following our LPS administration was not sufficient to reduce Aβ levels back to baseline, we propose that exercise is an effective mechanism for clearance of brain Aβ, or for reducing peripheral production. It is remarkable that only 9 days of swimming, at an average of less than 8 minutes per day, can cause such dramatic reduction in Aβ. Perhaps the increase in cardiac output associated with exercise leads to an increase in clearance of Aβ from the brain. Or, it is possible that exercise helps to reduce any lingering inflammation once the LPS injections cease. In addition, we must consider that while the animals were swimming in the MWM, they were also learning. Could the complex mechanisms associated with learning actually aid in the reduction of Aβ from the hippocampus? It will be important in the future to tease away the effects of exercise from learning in order to determine the true nature of Aβ elimination from the hippocampus in our model.

In summary, these data build upon previous findings that showed multiple injections of endotoxin increase brain Aβ levels and alter cognitive function. With this model of inflammation-induced cognitive impairment, we will conduct further research on the effects of inflammation on specific types of memory, the involvement of specific peripheral and central cytokines, and possible alterations in brain pathology. In addition, this model will

allow us to further evaluate possible preventive treatments that will target either inflammation or Aβ production in an attempt to eliminate cognitive deficits. Understanding inflammatory effects on cognition and physiology is imperative for future research of neurodegeneration and disease.

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ABSTRACT

A POTENTIAL ROLE FOR LPS-INDUCED INFLAMMATION IN THE INDUCTION OF ALZHEIMER'S DISEASE-RELATED PATHOLOGY AND COGNITIVE DEFICITS

by

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Alzheimer's disease (AD) is characterized by neuronal cell death in regions of the adult brain, including the hippocampus, due to formation of amyloid-beta (Aβ) plaques and neurofibrillary tangles. Inflammation has been implicated in the onset and progression of these pathologies. Our study was designed to create an animal model of peripheral inflammation-induced AD-like pathologies using the bacterial endotoxin Lipopolysaccharide (LPS). C57BL/6J mice were given intraperitoneal injections of LPS or saline for 7 days. Hippocampal tissue from animals receiving LPS contained significantly higher levels of $A\beta_{1-42}$ than did control animals. We also demonstrated that one injection of LPS leads to sickness behavior, but 7 days does not, implicating endotoxin tolerance. To determine if elevation in $A\beta_{1-42}$ might inhibit learning, cognitive testing in both MWM and CFC, revealed learning deficits in LPS treated mice. In summary multiple injections of LPS resulted in increased $A\beta_{1-42,}$ in the hippocampus and cognitive deficits in mice.