DO REPEATED BOUTS OF INFLAMMATION LEAD TO SUSTAINED ELEVATION OF AMYLOID BETA IN THE BRAIN?

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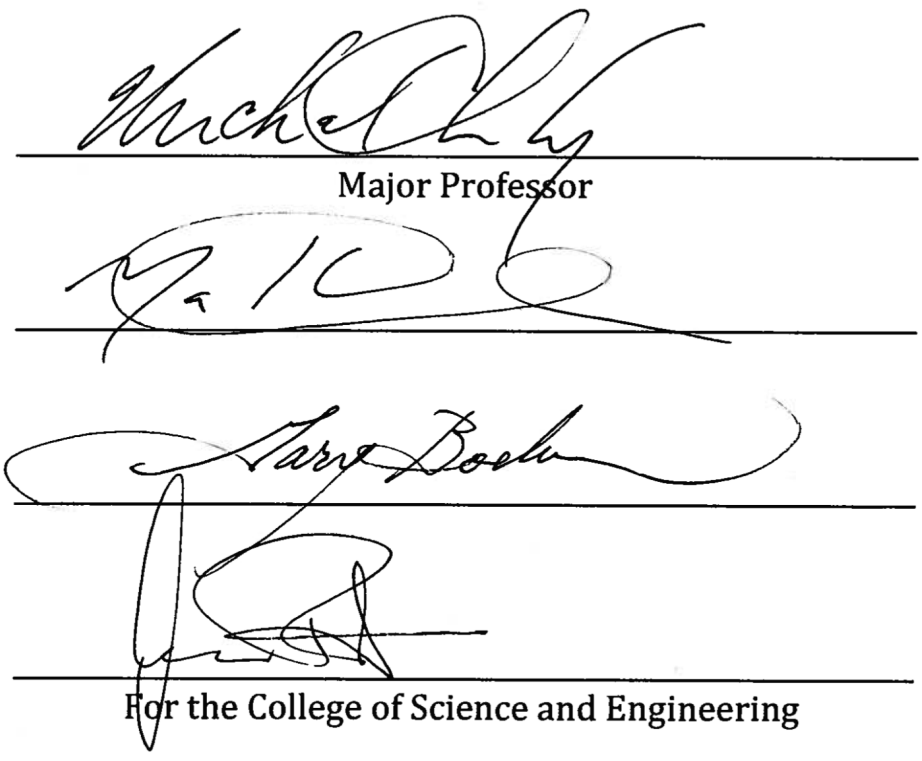

Major Professor

For the College of Science and Engineering

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TABLE OF CONTENTS

LIST OF FIGURES

DO REPEATED BOUTS OF INFLAMMATION LEAD TO SUSTAINED ELEVATION OF AMYLOID BETA IN THE BRAIN?

1. Introduction

There is a dragon that is rearing its ugly head and an absence of any dragon slayer. The dragon is Alzheimer's disease and literally wreaking havoc on the brains of millions in the absence, at present, of any means to combat the beast. Alzheimer's currently affects over 5 million Americans and is predicted to affect roughly 16 million by 2050 (Alzheimer's Fact Sheet 2014). 1 in 3 individuals over the age of 85 live with the disease and another American is stricken with Alzheimer's disease (AD) every 67 seconds, increasing to every 33 seconds by 2050 (Alzheimer's Fact Sheet 2014). Alzheimer's impact is widespread as it not only immediately affects those who have succumbed to the disease, but it also has considerable ramifications for those involved in the lives of the diseased to the tune of an additional $9.3 billion in health care costs due primarily to the physical and emotional toll of caregiving itself (Alzheimer's Fact Sheet 2014). It is currently the 6th leading cause of death for individuals over the age of 60 and the 5th leading cause of death for those over the age of 85, incurring costs to an already overburdened health care system that triple those of other diseases impacting the elderly (Alzheimer's Fact Sheet 2014).

Alzheimer's is primarily and initially characterized by dementia. There are a variety of ranges and types of dementia: Alzheimer's being the most common. This disease, in fact, first becomes of concern when individuals begin showing signs of mild cognitive dysfunction such as memory loss that eventually affects daily life activities including confusion with surroundings, challenges in completing customary tasks, challenged language skills, diminished motivation and depression,

and ultimately difficulty with simple physical issues such as walking and swallowing, thus necessitating confinement to a bed (2014 Alzheimer's Disease Facts and Figures). What spotlights Alzheimer's (and a few other dementias) to most dementia disorders is the fact that dementia associated with Alzheimer's disease is irreversible (2014 Alzheimer's Disease Facts and Figures). Thus we have an indefatigable ugly beast.

1.1 Hallmarks of Alzheimer's disease

Biomarkers for Alzheimer's disease (AD) are relatively difficult to determine in life; therefore, Alzheimer's is determined particularly in a clinical setting. While there are a variety of clinical tests used to determine AD, there is, however, apparently no real use for more diagnostic tools until there is accessibility of therapies proven to ameliorate the symptoms (Tarawneh and Holtzman, 2012). It is estimated that biological markers actually precede cognitive dysfunction by about a decade or more. It is therefore of precedence that AD is pinpointed in affected individuals before clinical symptoms clearly manifest themselves, so that the disease is caught at a time when it is more amenable to treatment (Tarawneh and Holtzman, 2012). It is paramount then to sort causes of AD and the factors that lead to the appearance and increase of the biological markers.

The two main biological hallmarks of AD are amyloid beta (Aβ) plaques consisting of aggregates of Aβ, and neurofibrillary tangles (NFTs) composed of aggregates of hyperphosphorylated tau. While both exert significant impact on AD pathology, Aβ is more oft than not considered to be the more defining culprit even to the extent that it initiates and influences the formation of NFTs. It is argued whether

such “amyloid hypothesis” is valid or if Aβ and hyperphosphorylated tau work via different pathways (Small and Duff, 2008). There appears to exist stronger evidence in favor of the “amyloid hypothesis”, though Aβ and hyperphosphorylated tau cause destruction through different mechanisms to downstream targets. Tau is a gene that under normal conditions is understood to function as a microtubule stabilizer as well as a regulator of motor-driven axonal transport; however, if hyperphosphorylated under pathological conditions, tau becomes detached from microtubules resulting in catastrophe to microtubule stabilization. The hyperphosphorylated tau then aggregates in somatodendritic compartments of neurons leading to formation of NFTs (Ittner and Gotz, 2011). The dependence of Aβ on tau for progression of disease has been evident in a variety of mouse models. For instance, tau knockout mice actually demonstrated protection from Aβ excitotoxicity in neurons suggesting Aβ dependence on tau for disease progression.

Other mouse models indicate that the opposite is not as cogent; in other words, tau does not drive AD pathology. Consider for example the APP transgenic mouse. This model exhibits the overexpression and mutation of amyloid precursor protein (APP) (Balducci and Forloni, 2011). APP is a transmembrane protein expressed in neurons as well as a few other cell types in the periphery. It is the abnormal cleavage of APP that leads to AD pathology in the production of Aβ. It was found in APP transgenic mice that tau was hyperphosphorylated in parallel to Aβ formation. Another model under consideration is that one exhibiting a mutation in the MAPT gene, which encodes tau protein resulting in hyperphosphorylation of tau and disturbance of microtubule stabilization. In this model only NFT pathology was

exhibited, with an absence of Aβ plaque pathology (Ittner and Gotz, 2011). In light of these studies, however, there continues disputation on the weight of impact of Aβ versus tau. Notwithstanding, both sides confirm that Aβ and tau lead to disease progression through differing and multiple downstream targets. Due to limits in space for covering these mechanisms in depth, Aβ and its relationship to AD pathology will be the emphasis of this study. The location of Aβ sets the stage for the connection of its biological pathology to its clinical manifestations as well as forms a foundation for determining causal factors.

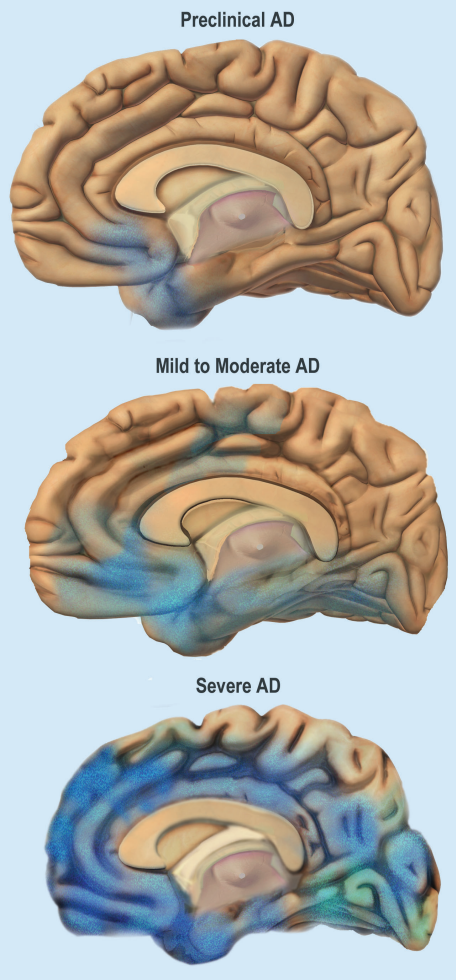


Figure 1. AD plaques and tangles spread through the brain. (National Institute on Aging)

1.2 Imaging

Brain imaging has illustrated the whereabouts of neurodegeneration in AD pathology. Atrophy is shown to occur predominantly in the medial temporal lobe (Johnson et al., 2012). It typically begins in the entorhinal cortex then progressing to the hippocampus, amygdala, and parahipppocampus (Johnson et al., 2012) (Figure 1). Longitudinal MRI studies show that this atrophy is already significant by the time of clinical diagnosis so much so that it now is included as a biomarker of AD at a predementia state (Johnson et al., 2012). Though amyloid beta plaques and

neurofibrillary tangles stand as the hallmarks of AD and its development, the degree of atrophy stands as a marker of disease progression (Johnson et al., 2012). Whereas structural magnetic resonance imaging (MRI) points out areas of atrophy, functional MRI (fMRI) measures neuronal activity. Both atrophy as well as neuronal activity are affected by the disease processes associated with AD. Using fMRI has demonstrated a decrease in hippocampal activity associated with new learning in AD patients (Johnson et al., 2012). The activity of the default network particularly the "posterior cingulate/precuneus, with the hippocampus" is negatively affected at rest in patients with MCI and AD (Johnson et al., 2012). This affected area, in fact, involves the identical areas of the brain involved in the largest concentration of Aβ aggregates (Johnson et al., 2012). Even in cognitively normal older individuals, positron emission tomography (PET) imaging shows a deficit of the default network during memory tasks and at rest when amyloid deposition takes place (Johnson et al., 2012). Fludeoxyglucose PET (FDG PET) is a brain analysis technique associated with synaptic activity. FDG PET data has also demonstrated that amyloid deposition has already made significant impact by the time the AD diagnosis has been made in an individual, indicating that the disease has been progressing long before symptoms of cognitive dysfunction (Johnson et al., 2012). Amyloid PET shows a 96% outcome of individuals with AD having Aβ deposition, indicating the existence of only very few AD patients that do not show Aβ deposition; however, this variance may be due to factors such as sensitivity variation, incorrect AD diagnosis, etc. (Johnson et al., 2012). The combined results of these imaging techniques demonstrate yet another

confirmation of the connection of amyloid beta burden, particular areas of the brain, and the development of AD.

1.3 Amyloid beta

So what is Aβ and what is it all about? If AD is a dragon, then Aβ, at least the dysregulation of Aβ, is the fiery torch that is the source of most of the beast's destructive effects. Aβ, at non-pathological levels may actually play a role in the homeostasis of neuronal activity in areas supporting mnemonic processes. Aβ, at normal levels, is in fact thought to enhance learning and memory via enhancement of long-term potentiation (LTP) and therefore synaptic plasticity (Morley et al., 2009). However, numerous mouse studies have demonstrated that Aβ in excess attenuates LTP, whereas less Aβ actually assists LTP. Additionally, mouse studies demonstrated that models with very low levels of Aβ paralleled a decrease in LTP (Morley et al., 2009). It would appear that the significance in the function of Aβ works according to a U shaped curve. Morley et al.,, in trials using experimentally naïve CD-1 strain of mice, demonstrated that these mice following injection of low level $A\beta_{1\text{-}42}$ peptide performed superior to the controls in T-maze foot shock avoidance tests as well as object recognition tests, indicating that Aβ plays a role in improvement of learning and memory (Morley et al., 2009). Conversely, Morley and colleagues discovered that if these mice were administered compounds resulting in the inhibition or blocking of Aβ, such as use of antibodies to Aβ, antisense to Aβ, or inhibitors to Aβ, learning and memory were impaired, indicated by their delay in reaching criterion as compared to control mice (Morley et al.,). These results further support the idea that $A\beta_{1\text{-}42}$ may have a role in assisting LTP and therefore learning and memory.

The culpability of Aβ in AD comes in the form of an overexpression of Aβ, particularly the peptide fragment $A\beta_{1\text{-}42}$. This fragment exists *in vivo* in the form of monomers, oligomers, and fibrils. Because Aβ has been connected with the formation of amyloid or senile plaques in the brains of AD patients and synaptic dysfunction has been associated with the cognitive deficits seen in AD patients, studies have monitored with intent the various forms of Aβ and their link to synaptic dysfunction. It has been shown in APP transgenic mice that cognitive deficits and neuroanatomical pathology may long precede the formation of plaques (Mucke and Selkoe, 2012). Though there exists neuritic pathology in the immediate vicinity of the plaques, it is becoming increasingly evident that the destruction accomplished by the AD beast begins with the initial overload of Aβ in the synapse upon its inaugural snip from amyloid precursor protein (APP). In fact, studies, such as those noted by Selkoe (2002) and Mucke (2010), verify the regulation of pre- and post-synaptic activity by Aβ oligomers. Indeed, the mere fact that transgenic mice expressing human APP (hAPP mice), therefore exhibiting a higher than normal load of Aβ from start, experience cognitive deficits as well as synaptic defects before the appearance of any plaques, strongly suggests that the causal factor is the Aβ in some soluble assembly form and its destruction of synaptic plasticity (Mucke and Selkoe, 2012; Roberson, 2011).

Though the complete function and pathway in which Aβ works its destruction is not completely sorted, it is suggested that one path of impact is through the depression of N-methyl-D aspartate receptors (NMDARs) and a-amino-3-hydroxy-5-methyl-4 receptors (AMPARs). These particular receptors are found to be in

profound correlation with induction of LTP or long term depression (LTD) depending on the activation level and resultant Ca^{2+} influx. In order for LTP to exhibit, the recruitment of AMPARs and growth of dendritic spines must occur; on the other hand, LTD will exhibit following shrinkage of dendritic spines with resultant synaptic loss (Mucke and Selkoe, 2012). Thus, overexpression of Aβ may lead to the blockage and down regulation of key receptors in the processes of learning and memory in the brain. Aβ may also work through the depression of particular neurotransmitters such as glutamate, a key neurotransmitter also involved in activation and desensitization of NMDARs and therefore synaptic plasticity (Mucke and Selkoe, 2012). It would therefore appear that inhibiting the production of Aβ or at least clearing the excess load of Aβ in AD brain would have profound effects on improved cognitive function.

1.4 Secretase enzymes

Scientists and researchers have therefore pressed forward in pursuit of causal factors in the overproduction of Aβ. $Aβ_{1-42}$, the 42 amino acid fragment found most associated with the pathology of AD, is cleaved from APP by particular secretase enzymes. Simply put, APP is cleaved by α-secretase or β-secretase initially, followed by cleavage performed by γ-secretase. If cleavage initially occurs with α-secretase, then there occurs progression of the nonamyloidogenic pathway. If, however, cleavage occurs via β-secretase, then follows progression of the amyloidogenic pathway. When cleavage occurs via β-secretase, various size soluble Aβ peptide fragments result. Roughly 10% of those produced are the length $Aβ_{1-42}$, the fragment that aggregates quite easily into extracellular fibrils and is associated with

the pathology of AD (Richardson and Burns, 2002). BACE is the β-site APP-cleaving enzyme that is to blame for the β-secretase activity initiating formation of Aβ. This would put it in position as a therapeutic target for the amelioration of AD; however, it has been demonstrated that BACE interacts with other substrates as well and therefore its elimination could have detrimental effects on normal brain development and function (Evin, Barakat and Masters, 2010). γ-secretase is closely linked to the presenilins, a family of genes whose mutations have been connected with direct involvement in early onset AD. It is the mutations in the presenilins that result in changes in the proportions of $A\beta_{1\text{-}42}/A\beta_{1\text{-}40}$. Indeed, APP is the only substrate through which γ-secretase brings about AD pathology. This might then also lead to it being a potential therapeutic target as well; however, it too, when drastically reduced has been demonstrated to result in neuronal dysfunction (Strooper et al., 2012).

1.5 Production and clearance of amyloid beta

Considering the production of Aβ and the necessity of a homeostatic balance of levels of this peptide for optimal learning and memory, it becomes apparent that the proper levels of production and clearance of this peptide must be maintained. As Aβ is produced in normal cellular respiration, the brain as well as the periphery normally functions in the clearance of Aβ from the body. Existing on the surface of neurons and macrophages in the brain are various lipoprotein receptors that act in clearance of Aβ. Low-density lipoprotein receptor-related protein (LRP) is one such receptor on the surface of neurons that functions in the lysosomal degradation of Aβ upon binding. An alteration in the blood brain barrier leads to

accumulation of Aβ in the brain and therefore reduced clearance. LRP functions in the mediation of Aβ transport across the BBB (Erickson et al., 2012).

Another receptor, located on the surface of macrophages, is RAGE (receptor for advanced glycation end products) which functions in the endocytosis and transcytosis of Aβ into the brain (Uden et al, 2002). Whereas LRP functions in the clearance of Aβ from the brain, RAGE functions in the trancytosis of Aβ from the periphery into the brain. The binding of RAGE with Aβ therefore enhances levels of Aβ in the brain leading to increased pathology of progressive AD (Deane, 2003). There exist numerous other receptors that function in much the same way as LRP and RAGE in the amelioration as well as pathology of Aβ.

Also present in the brain are Aβ degrading proteases (AβDPs). As the name implies, these also function in the degradation of Aβ. Known as the most potent of the AβDPs is neprilysin (NEP). NEP is a membrane protein located at the presynaptic and postsynaptic membranes a location that makes it convenient to accessing and degrading Aβ (Shirotani et al., 2001; Iwata et al., 2001; Iwata et al., 2004). Though there are numerous degrading proteases with Aβ as substrate, it has been found in various studies that levels of NEP as well as insulin-degrading enzyme (IDE) particularly are impactful and which levels correlate inversely with levels of Aβ, though it may be that these proteases degrade the monomeric form of Aβ more efficiently than the oligomeric form (Leissring, 2003; Carty, 2013)

In fact, numerous studies have been conducted to demonstrate the critical activity of various proteases, peptidases, and other enzymes in the degradation of the Aβ peptide. These mechanisms are shown to decrease substantially with age

thus accounting for the association of sporadic AD as an age-related disease (Seido and Leissring, 2012).

1.6 Inflammation

One path that has become increasingly apparent in the pathogenesis of AD, though the detail functions remain to be elucidated, is the role of inflammation in this disease process. What led scientists on this journey was the discovery of the presence of inflammatory mediators surrounding amyloid plaques in the brains of AD patients upon autopsy (Englehart, 2004). Additionally, epidemiologic studies indicated that individuals who had long-term experience in taking NSAIDs were at an appreciably lower risk for acquiring AD than those who had not (Tuppo and Arias, 2005; Aisen 2000, 2003).

It has indeed been shown that pro-inflammatory cytokines, such as interleukin 1 beta (IL-1β) and tumor necrosis factor alpha (TNFα) as well as IFNγ (interferon gamma), not only induce Aβ production but also exacerbate a vicious cycle of neuroinflammation. *In vitro* studies have demonstrated an increase in β-secretase processing of APP upon introduction of IL-1, while other mouse studies exhibiting a knockout of the receptor antagonist for IL-1 also showed increased Aβ pathology (Shaftel et al., 2008). IL-1 was also demonstrated to contribute to pronounced disruption of the tau protein resulting in its hyperphosphorylation, therefore another avenue by which IL-1 correlates to exacerbation of AD pathology (Shaftel et al., 2008).

The contribution of TNFα and IFNγ is seen particularly in a study conducted by Blasko and colleagues (Blasko et al., 2008). This team found that when both TNFα

and IFNγ were added to human cells in culture that Aβ generation was accomplished, though these cytokines did not produce such results individually. This evidence however still begs the question whether cytokines precede Aβ production in life or merely act as amplifiers of AD pathology (Blasko et al., 2008). Several ways in which cytokines amplify AD pathology are through mechanisms of β-secretase activity that, in turn, increases secretion of $A\beta_{1-42}$, as well as through the increased expression of APP (Lee et al., 2008).

Magaki and colleagues studied the presence of pro-inflammatory cytokines and anti-inflammatory cytokines in normal elderly versus patients with mild cognitive impairment (MCI) as well as patients with mild AD. In cell culture, it was indicated that the presence of pro-inflammatory cytokines, particularly IL-1β and IL-6 was decreased in mild AD versus MCI. Furthermore, it was noted that ratios of pro-inflammatory to anti-inflammatory cytokines after stimulation by LPS were decreased in MCI subjects versus control, probably due to the increase in anti-inflammatory cytokines stimulated by the pro-inflammatory condition. Levels of these cytokines were then near normal levels at the mild AD stage following their alteration during MCI. These findings indicate support of the hypothesis that inflammation or alteration of the immune response may indeed precede AD. This outcome correlates with other studies that have demonstrated a progressive decline in cytokine production upon LPS stimulation with developing stages of AD, thus lower production at advanced stage AD compared to moderate AD and so on (Magaki, 2007).

In addition, data also exists that indicates Aβ as a culprit guilty of actually initiating a vicious neuroinflammatory cycle by calling forth an army of microglia, astrocytes, and neurons which are inclined to launch an all out inflammatory response involving the production of a host of inflammatory mediators. Once these cytokines and other inflammatory mediators are produced, they then cause adverse effects that are capable of inducing and exacerbating AD pathology. Activated microglia, astrocytes and neurons exist in the surrounding areas of AD plaques (McGreer and McGreer 2001). Suo et al., found that Aβ worked in vitro to directly induce the secretion of IL-1β as well as IFN-γ from endothelial cells in a dose-dependent manner (Suo et al., 1998). The results of Suo's work also indicated that $A\beta_{1\text{-}42}$ had significant impact on endothelial cells in particular thus adversely impacting the integrity of the blood brain barrier, another factor linked to Aβ dysfunction in AD (Suo et al., 1998).

1.7 Inflammation in a mouse model

Such effects are noted in transgenic mouse models of AD pathology as well. Transgenic AD mice were shown to express higher levels of proinflammatory cytokines in the brain (Lim, 2000; Abbas, 2002; Tehranian, 2001) Additionally, when transgenic mice were administered anti-inflammatory treatments, they demonstrated improved condition and prolonged onset of disease (Lim, 2000; Lim 2001; Jia, 2013; Capiralla, 2012). In addition, studies using transgenic mice showed further that peripheral inflammation exacerbated and even induced an earlier onset of neurodegeneration. These models have provided a foundation for inflammatory research (Kahn et al., 2012). Having said that, since less than 10% of Alzheimer's

cases are actually genetically related ("Learning to live." 2011), research has persisted in nontransgenic mice in order to determine a link between inflammation and AD pathology that more closely resembles the typical human AD condition. Lee et al., found that mice that were administered serial injections of LPS proved to exhibit significant cognitive deficits as well as notable increase in hippocampal levels of Aβ as compared to the saline control mice (Lee et al.,). Kahn and colleagues in a follow-up study also found a link between induction of inflammation via 7 days of LPS injections and significant elevation of Aβ in the hippocampus of mice as well as hippocampal-dependent cognitive deficits (Kahn et al., 2012). Further, Kahn and colleagues established that after 15 resting days post LPS injections, these mice continued to show significant elevation of Aβ in the hippocampus, though trending toward baseline. These discoveries invoke further question and research as to the length of continuance of significant levels of Aβ as well as what might occur if a second bout of acute inflammation were administered when the levels were still significantly elevated. In an attempt to mimic chronic inflammation, the condition consistent with AD pathology in humans, several bouts of acute inflammation could be administered in a mouse model.

1.8 Hypotheses

Therefore, the purpose of this study was to determine if there is a point in time at which Aβ levels return to baseline following 7 days of LPS injection. We hypothesized that Aβ does indeed return to baseline at some point in the future. This study also set out to determine if a second bout of acute inflammation would raise the levels of Aβ significantly higher than those following the initial bout and if

they would take longer to return to baseline level. The hypothesis was that these results would indeed occur, giving stronger foundation to the link between increased exposure to inflammation and increased susceptibility to AD.

2. Materials and Methods:

2.1 Mice

The C57BL/6J mouse strain was the model used, as it was the same used by Kahn et al., and was found to exhibit no known physical confounders for the study (Kahn et al., 2012). Male C57BL/6J mice were used. These mice had been bred in the TCU vivarium under proper animal care and safety controls approved by the Institutional Animal Care and Use Committee (IACUC) of Texas Christian University. All mice used in this study had both food and water available *ad libitum,* were on the same light schedule (lights on daily from 0700 to 1900), and were housed in groups of two to four in standard cages (12.5cm x 15cm x 25cm).

2.2 Contextual Fear Conditioning

A quintessential method used to determine cognitive behavior in mice after induction of inflammation is contextual fear conditioning (CFC). CFC is a hippocampal-dependent task used to measure cognitive deficits in the mice. CFC has a variety of approaches. One common approach consists of a training day that includes acclimation time as well as consolidation time following an adverse stimulus (usually a mild shock) and a testing day characterized by an established resting time in the unit. The units (Coulbourn Instruments, Whitehall, PA, 7Wx7Dx12H) utilize black polka dot walls as well as an olfactory cue, in this case

peppermint oil diluted with water at a ratio of 1:10. Mice experienced shock via an electrified grid floor as their behavior was monitored and analyzed using the fear conditioning software. Freezing behavior was monitored utilizing the FreezeFrame™ (ActiMetrics Software, Wilmette, IL).

The protocol used for the contextual fear conditioning is in accordance with Kranjac et al.,. (2011). Visual and olfactory cues were shown to enhance freezing time (Kranjac et al.,, 2011). Notably, an olfactory cue used in this way is an indicator of the integrity of the hippocampus (Otto, 2006).

On training day, each mouse was given 120 seconds to acclimate to the unit, followed by a 2-second 0.7mA shock. Then the mouse remained in the unit another 60 seconds with no shock before removal, a period of consolidation or memory imprinting. Testing day merely involved monitoring the freezing behavior of the mice over a 120s time interval (in the absence of the shock). Freezing time was then analyzed following the completion of all mice undergoing testing. Freezing time in rodents is an innate response to fear. This freezing time was analyzed using ANOVA procedures (Statview 5.0, SAS, Cary, NC) applying Group (LPS/SAL) as independent variables. All statistical analyses used alpha level of 0.05..Freezing time is indicative of whether the mouse remembered the context in conjunction with the shock. Prolonged freezing is correlated with better learning and memory; whereas the lack of freezing correlates to cognitive deficits.

2.3 Tissue collection and amyloid beta analysis

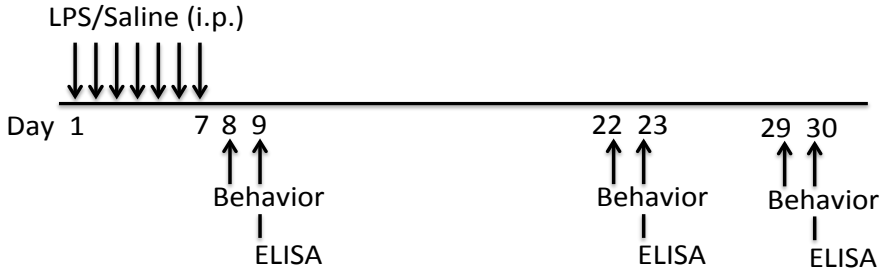
Following fear conditioning, the mice were euthanized according to IACUC-approved methods and the hippocampus removed. Hippocampal tissue was

removed and lysed with protein extraction solution (PRO-PREP, Boca Scientific, Boca Raton, FL) containing protease inhibitors. This solution was then immediately frozen on dry ice and stored overnight in -80C. The lysate protein levels were determined using the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA), and protein content normalized. Finally, Aβ ELISA (COVANCE Chemiluminescent BetaMark™ x-42 ELISA KIT) was performed in order to determine Aβ content of the samples. A series of standards and unknown samples were assayed in duplicate following manufacturers guidelines and the plate read at the optical density of 620nm to detect levels of Aβ in the samples.

2.4 Experiment 1 procedure

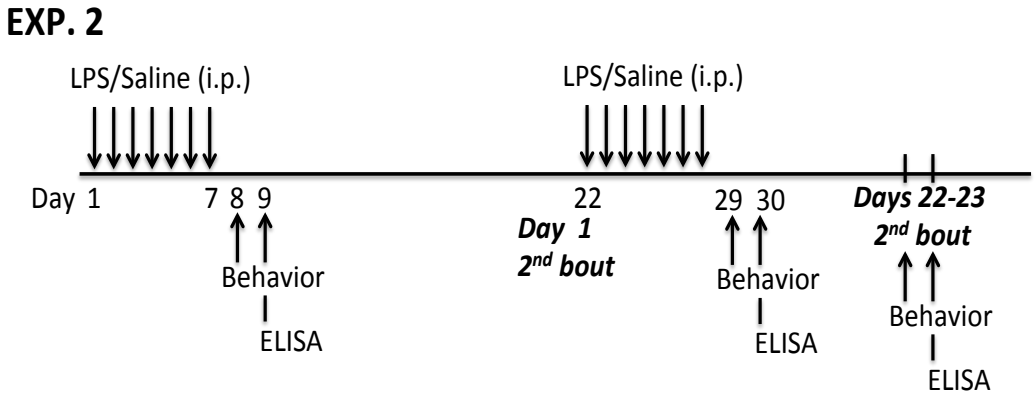
For experiment 1, a total of 42 animals were used. On Days 1-7, 21 control animals received one intraperitoneal (IP) injection of saline daily and 21 animals received one IP injection of 250μg/kg LPS. Twenty-four hours following final injection, seven control mice and 7 LPS mice were submitted to a training day of CFC followed by 24 hours later by the testing day. CFC testing was immediately succeeded by hippocampus tissue removal. The remaining mice were allowed a 15-day resting period. At 15 days following the final injection another 14 mice were submitted to the two days of CFC immediately succeeded by hippocampus removals. Finally, the remaining 14 mice were given 7 more resting days. This time point was chosen to match the end of a second bout of inflammation proposed in experiment 2. At this time, 22 days following the final injection, the remaining mice underwent 2 days of CFC immediately followed by hippocampus removals. See timeline below:

EXP. 1



2.5 Experiment 2 procedure

For experiment 2, testing for Aβ levels and cognitive behavior with a second bout of inflammation, an additional 42 mice were used. Experiment 2 began with 7 consecutive days of injections similar to that in experiment 1. Again at 1-2 days following final injection, CFC followed by hippocampus tissue removal was performed on 7 animals from each treatment group. Remaining animals were given 15 days of sedentary recovery at which time (day 22 they were administered a second bout of 7 consecutive days of LPS or saline injections identical to the first bout. At 1-2 days following the final injection of the second bout, CFC and tissue removal for Aβ detection was performed on 7 animals from each treatment group. The remaining animals were allowed sedentary recovery in their home cages for an additional 15 days, followed by 2days of CFC and subsequent hippocampus tissue removal. See timeline below:



2.6 Statistics

Statistical analyses were performed using one-way analysis of variance (ANOVA) following each Aβ ELISA and for CFC data in both experiments. Alpha level was set at 0.05.

3. Results:

3.1 Experiment 1

On training day, there was no difference in activity between groups, suggesting that the animals receiving LPS no longer exhibited sickness behavior due to induced inflammation. Sickness-related behavior in animals receiving a single injection of LPS has routinely been shown to cause lethargy and significantly elevated freezing of animals. 24 hours after training, we found a marked increase in freezing time in the control group on testing day than in the LPS group, indicating that LPS-induced inflammation did exert negative impact on cognitive function ($f_{(1,12)}$=8.475, $p<0.05$) (Figure 2. a). These findings are similar to previous findings in our lab (Kahn et al., 2012; Weintraub et al., 2013). Abeta ELISA results at this time

point demonstrated significant elevation ($f_{(1,10)}$=14.327, p<.01)) of Aβ at 2 days following the final injection in the LPS animals compared to controls (Figure 2. b).

The remaining mice remained in their home cage for an additional 15 days following the final injection. At this time point, another 14 mice (7 LPS and 7 saline controls) were submitted to CFC. We showed that behavior at this time point was returning to normal and no longer showed significance in cognitive dysfunction between groups ($f_{(1,10)}$=1.589, ns) (Figure 1. c). Abeta ELISA results at 16 days post final injection showed a continued significant elevation of Aβ ($f_{(1,10)}$=9.291, p<.05) compared with controls (Figure 2. d).

To determine if there was a time point further out at which Aβ levels would return to a level no longer significantly different than saline controls following a bout of acute inflammation, we added an additional time point beyond that of the confirmed elevation of Aβ. The remaining 14 mice were housed an additional 7 days (22 days after the final injection) and then submitted to CFC training and testing. Again, the LPS-treated mice did not demonstrate a significant cognitive dysfunction ($f_{1,12)}$=4.431, p>0.05) (Figure 1. e). Aβ ELISA results at 23 days post final injection showed no continued significance of effect of an acute bout of LPS induced inflammation on levels of Aβ ($f_{(1,12)}$=.127, ns) compared with controls (Figure 2. f).

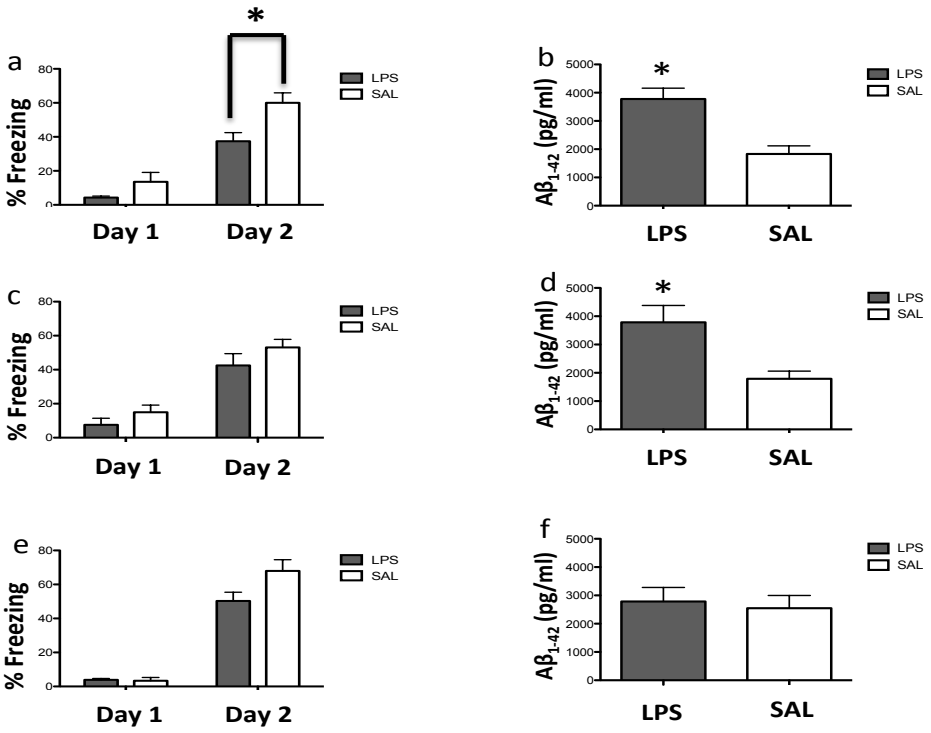


Figure 2. Levels of $A\beta_{1\text{-}42}$ in correlation with contextual fear conditioning results at 2, 16, and 23 days post injections with LPS and saline. a) CFC results indicate significance in cognitive dysfunction in the LPS mice compared with controls on Day 2 following final injection that correlated with significantly elevated $A\beta_{1\text{-}42}$ levels in the murine hippocampus on the same day (b). c) Learning deficits show improvement at 16 days post final injection in LPS mice though $A\beta_{1\text{-}42}$ levels at this time point remained significantly elevated (d). e) At 23 days post final injection, possibly due to technical difficulties, CFC results again show a near significance in cognitive dysfunction in LPS mice compared with saline controls on testing day (Day 2) although $A\beta_{1\text{-}42}$ levels at this time point were approaching baseline as demonstrated by its close similarity to that of saline controls. The similarity between LPS animals and control on Day 1 of b, d, and f illustrates absence of sickness behavior. Values are expressed as mean ±SEM. *$p<0.05$.

3.2 Experiment 2:

Since it has been demonstrated in our lab previously as well as in experiment 1 that levels of Aβ are at least trending toward baseline, yet still elevated, at 15 days post final injection, it was this time point that was chosen for the initiation of a second bout of acute inflammation. Again, CFC showed no sickness behavior at each

time point on training day. At testing day 2 days following the first round of injections, CFC did produce different results than what is commonly demonstrated in that cognitive dysfunction was not significant in the LPS-treated group compared to saline controls ($f_{(1,12)}$=.286, ns) (a, Day 2) (Figure 3). Aβ levels at this time point were in agreement with previous data in that they were significantly elevated in the LPS group compared to saline controls ($p<.05$) (Figure 3).

There was no significant difference in cognitive function between treatment groups at 2 days following the second bout of injections ($f_{(1,12)}$=.394, ns) (c, Day 2) (Figure 3). Interestingly, Aβ levels at this time point were no longer significantly elevated in the LPS-treated group, as they were after the first bout of inflammation ($p>.05$) (d) (Figure 3). The remaining mice were given a recovery period of 15 days following the final injection of the second bout of inflammation, and then submitted to CFC and Aβ measurement. Again, there were no significant differences in levels of Aβ ($p>.05$) (f) (Figure 3) or cognitive function ($f_{(1,10)}$=2.257, ns) (e, Day 2) (Figure 3) at this time point.

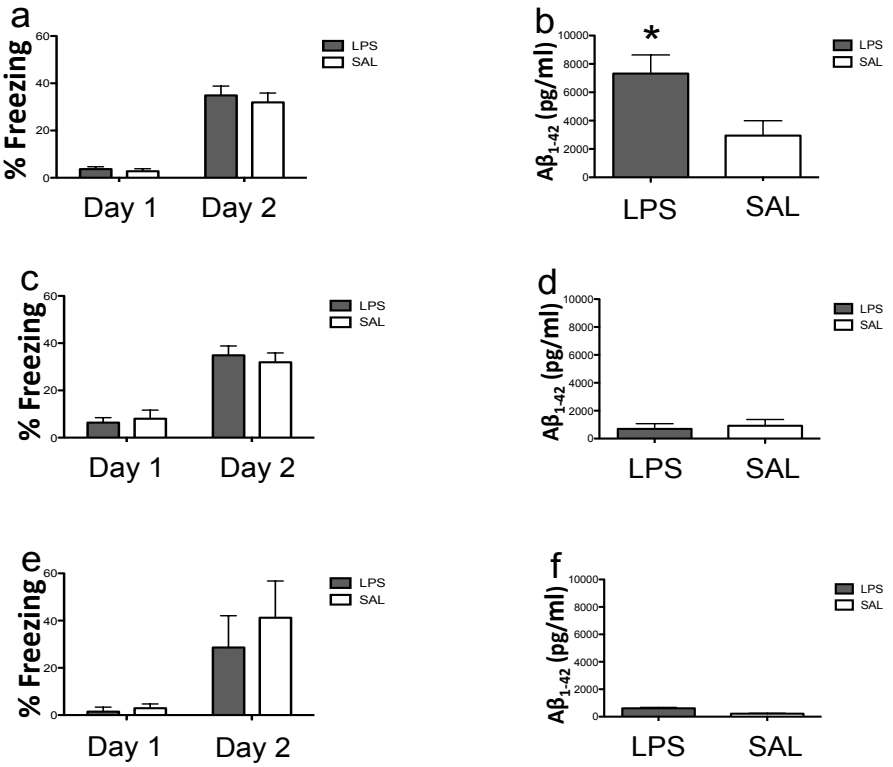


Figure 3. Levels of $A\beta_{1\text{-}42}$ in correlation with contextual fear conditioning at time points following one and two bouts of injections in LPS injected animals compared to saline controls. a) Behavior did not show significant dysfunction at 2 days following one bout of LPS (Day 2) though Aβ levels in murine hippocampus were significantly higher than saline controls at this time point (b). (c) At 2 days following a second bout of LPS injections, there was no significant difference in cognition between LPS animals and saline controls. Also, Aβ levels were similar to that of saline controls at this time point (d). These effects were also evident at 16 days following the second bout of injections (e, Day 2)(f). Values are expressed as ±SEM. *$p<0.05$.

4. Discussion:

4.1 Experiment 1

The first experiment of this study was conducted in order to determine some time point at which levels of Aβ would no longer be in significant elevation to that of saline controls. Since previous studies in our lab demonstrated that levels of Aβ had declined, however were still significantly elevated, at 16 days post final injection, a

time point beyond that was chosen (Kahn, 2012). The 23 days post final injection time point was chosen specifically in view of the time points proposed for data collection in experiment 2, a time point directly following a proposed second bout of inflammation. It was hypothesized that Aβ would no longer be significantly elevated compared to saline controls by 23 days post injections.

We first confirmed that a single acute bout of inflammation leads to significant increase of Aβ in the hippocampus of mice and that this rise in Aβ corresponded with significant cognitive dysfunction. This data confirmed studies conducted previously in our lab (Kahn, 2012; Weintraub, 2013). Secondly, at 15 days post final injection, we demonstrated that Aβ levels are still significantly elevated compared with controls, though this study found cognitive dysfunction only to be trending and not significant. Our lab has previously shown through regression analysis that hippocampal-Aβ accumulation was responsible for 30.4% of the variability of freezing behavior (Weintraub et al., 2014). Therefore, the attenuation of cognitive dysfunction at this point may be the result of restorative processes of other mechanisms in the brain while Aβ levels are declining before they reach levels of controls. The fact that there no longer remained elevated Aβ at 23 days post final injection, accompanied by lack of cognitive dysfunction in animals induced with inflammation suggests that there does exist a point in time at which Aβ levels tend to return to baseline if not exacerbated by other means.

4.2 Experiment 2

We confirmed in this experiment that levels of Aβ were still significantly elevated following one bout of LPS-induced inflammation; however, there was

discrepancy with our results for cognitive dysfunction at this time point. Since cognitive dysfunction has shown significance in each experiment conducted in our lab using this model as well as in the first experiment of this study, we attribute the discrepancy of these results to a misplaced switch discovered on the CFC equipment during this time. This may have accounted for malfunction of the shock and therefore inconsistency with mouse behavior compared to typical performance.

It has been shown that chronic inflammation in humans corresponds with a higher risk of Alzheimer's disease later in life (Tuppo and Arias, 2005). Due to difficulty in administering chronic inflammation in mice, we chose to attempt to mimic chronic inflammation via administration of a repeated bout of acute inflammation at a point at which Aβ was still significantly elevated and study the accumulated effects on levels of Aβ and cognitive function.

Since our first experiment confirmed that levels of Aβ were still significantly elevated at 15 days following one round of injections, our second experiment determined if adding another acute bout of inflammation at that time would exacerbate levels of Aβ. We hypothesized that a second bout of LPS induced inflammation would lead to a greater concentration of Aβ in hippocampal lysate taken from such mice as compared with those who had only received one bout of LPS. It was also assumed that the level of Aβ would remain significantly elevated an additional 15 days following the second bout of inflammation. Unexpectedly, we found that there were no differences in CFC performance and levels of Aβ between the treatment groups, and it appeared that Aβ levels and cognitive function remained unaffected after 15 additional days of recovery.

Due to these unexpected findings, we examined the weight changes of the mice during the first bout of inflammation compared with those during the second bout of inflammation. It has been shown that mice administered LPS injections will experience a rapid and significant loss in weight due to sickness-related loss of appetite, followed by weight gain by the end of the seven days of treatment. This weight gain is likely due to the attenuation of sickness in the mice as a result of endotoxin tolerance. It has been demonstrated that sickness behaviors occur following injection with LPS, most evident up to 4 days of administration (Kahn, 2012). Sickness behaviors include loss of appetite, decreased activity, and other depressive activities (Cunningham, 2008). In order to eliminate the confounding effect of inflammation on learning and memory, studies were run to determine the length of time that the pro-inflammatory cytokines IL-1β and IL-6 levels were elevated following administration of LPS. It was demonstrated that levels of cytokines returned to baseline by the seventh day of injections. This was accompanied by the demonstration that following 7 consecutive days of LPS injections, mice no longer displayed sickness behavior in the open field test as had been observed by these same animals at 1 and 4 days of injections (Kahn, 2012).

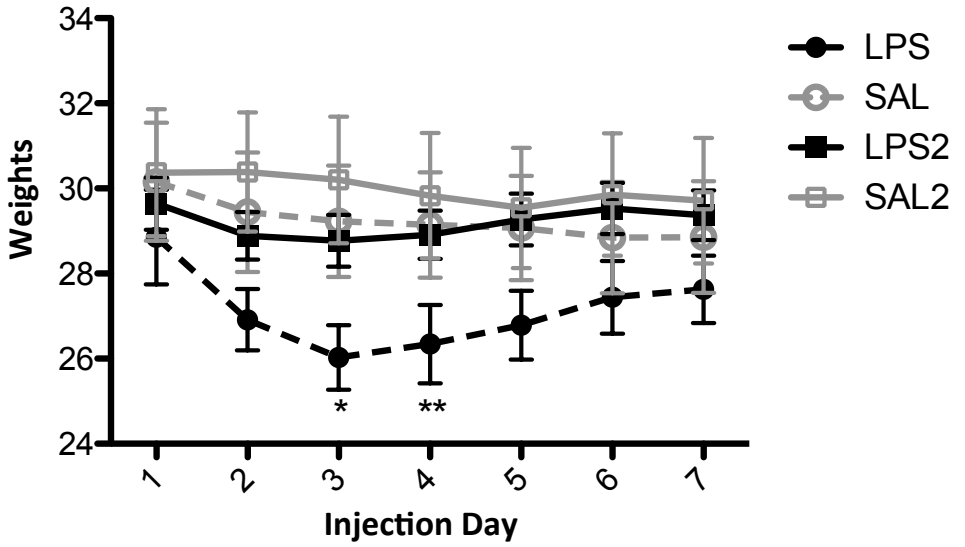


Figure 3. Weight variation between groups of mice within bouts 1 and 2 of injections. Weights of LPS mice at day 3 of injections during bout 1 were significantly lower than that of LPS mice during bout 2 and than saline mice of either bout. At day 4 weights were still close to significance. Values are expressed as ±SEM. *p<.05. **p>.05.

By day 7 of LPS administration, endotoxin tolerance has set in. Endotoxin tolerance is the reaction of the immune system to prevent such an inflammatory state that would result in septic shock. Septic shock is the progression of inflammation to a point of tissue damage, organ failure, and eventual death. Indeed, the weight changes during the first bout of LPS administration clearly demonstrated these phenomena, as the LPS-treated mice lost significant weight by day 3 of injections compared with saline controls and remained near significance on day 4, though weights were returning toward initial weight by day 7. In contrast, there was no significant difference in weight changes between LPS- and saline-treated mice for any day during the second bout of inflammation. It is possible that the cells in the peritoneal cavity that responded to the LPS during the first bout of inflammation,

remained tolerant of the endotoxin for a longer period than we had expected. If they remained tolerant of LPS, then the second round of treatment would result in little inflammation, and thus explain the non-significant loss in weight over the second treatment period.

An alternative explanation may be a protection offered by B-1 cells. These cells are known to have a memory cell function as well as to result in proliferation and secretion of immunoglobulins upon LPS administration. Genestier et al demonstrated by observing four B cell subsets in mice that murine B-1 cells were induced by TLR agonists (like LPS) to proliferate into plasma cells. They also demonstrated that consequence of LPS administration produced a more voluminous proliferative response particularly in murine B-1 and MZ (marginal zone) B cells and IgM was the main isotype produced (Genestier et al., 2007). Genestier et al., further noted that B-1a cells were responsible for the production of natural antibodies in response to treatment by TLR agonists, LPS in particular, and concluded that murine B-1 cells and MZ B cells functioned in much the same way as human memory B cells (Genstier et al., 2007). Kawahara and colleagues demonstrated that B-1 cells in the peritoneal cavity migrate to the spleen upon activation by LPS and down regulate Mac-1 (macrophage-1 antigen-complement receptor) to become IgM antibody-secreting cells (Kawahara, 2003). Further, Barbeiro et al found that B-1 cells utilize the IL-10 pathway to regulate cytokine activity, notably they diminish the production of TNF-α and IL-6 via the IL-10 pathway upon LPS administration (Barbeiro, 2011). More recently, Popi and colleagues demonstrated that B-1 cells might actually transform into “macrophages” upon LPS stimulation. Though it was afore thought

that macrophages only stem from monocytes, Popi and colleagues suggested another source. They found certain phenotypic similarity to B-1 cells on "macrophages" proliferating in mice devoid of monocyte-derived macrophages upon LPS stimulation. These B-ICDPs (B-1 cell-derived phagocytes) exhibit as a key element in the peritoneal macrophage population upon LPS stimulation (Popi et al., 2012). The results of these studies taken together, particularly the tendency of these B-1 cells to prevent septic shock as well as act similarly to memory B cells, suggests that the B-1 cell community might be culpable for the inhibition of inflammatory response seen during the second bout of LPS administration. There apparently remains a memory innate immunity at this time point.

Further studies should be conducted using mechanisms other than LPS stimulation in order to induce inflammation via pathways that evade those involved in endotoxin tolerance and trained immunity if we are to elucidate more effectively the role of inflammation in production of Aβ. For example, we are currently conducting a study using poly I:C a viral mimetic in place of LPS to induce inflammation in the mice. Previous studies in our lab have shown that poly I:C induced inflammation also leads to significantly elevated levels of Aβ in the hippocampus; however continued poly I:C administration does not result in endotoxin tolerance (Weintraub, 2014). This may be due to the fact that poly I:C induces inflammation through the TLR3 receptor while LPS works through the TLR4 receptor. Numerous studies have shown that the TLR4 receptor may be negatively affected by various signals upregulated to keep the inflammatory response from

getting out of hand upon subsequent introduction to LPS (Biswas, 2009; Foster, 2009).

Other studies possible could involve administering either LPS or poly I:C during a first bout of inflammation and switching to the other during the second bout of inflammation, thus stimulating inflammation through different receptors. Human cells undergo inflammation from a variety of sources, so this method could mimic more closely what occurs during normal exposure of humans to various pathogens. None-the-less, the importance remains in identifying the link between inflammation and Alzheimer's disease that specifically leads to sustained elevated levels of Aβ and the progression of Alzheimer's pathology. Such a link will give scientists a specific cause to target in developing both treatment and prevention strategies for this tenacious beast.

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ABSTRACT

DO REPEATED BOUTS OF INFLAMMATION LEAD TO SUSTAINED ELEVATION OF AMYLOID BETA IN THE BRAIN?

By

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One of the main biological hallmarks of Alzheimer's disease is the presence of amyloid beta plaques. Amyloid beta (Aβ) is a peptide fragment cleaved from the amyloid precursor protein on the membranes of neurons which accumulates in the synapses and blocks signaling of neurotransmitters, resulting in damage and death to neurons and ultimately atrophy in the brain. Aβ concentration is particularly apparent in the hippocampus therefore having negative impact on learning and memory, characteristics of Alzheimer's disease. Scientists have found a strong link between chronic inflammation and the dysregulation of Aβ resulting in the increasing influx of Aβ in the brain without compensatory clearance. Our study sought to determine how long Aβ remained elevated in the hippocampus of mice following 7 days of lipopolysaccharide LPS-induced inflammation. We injected C57BL/6J mice with 7 days of LPS or saline and assessed cognitive function and levels of Aβ at 2, 16, and 23 days following injections and found that by 23 days Aβ was returned to levels similar to controls.

We then sought to determine if a repeated bout of LPS-induced inflammation at some point following the first bout when Aβ was still significantly elevated would result in an even greater elevation of Aβ as well as greater cognitive dysfunction than that following the first bout. Unexpectedly, we found that Aβ levels in the LPS-injected mice were similar to saline controls following the second bout of inflammation. Either endotoxin tolerance or the activity of B-1 cells in the peritoneal cavity may explain the unexpected result.