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The Effect of Acute LPS-Induced Immune Activation and Brain Insulin Signaling Disruption in a Diabetic Model of Alzheimer's Disease

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THE EFFECT OF ACUTE LPS-INDUCED IMMUNE
ACTIVATION AND BRAIN INSULIN SIGNALING
DISRUPTION IN A DIABETIC MODEL OF
ALZHEIMER'S DISEASE

by

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A thesis submitted in partial fulfillment
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Andrew Scott Murtishaw

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ABSTRACT

The Effect of Acute LPS-Induced Immune Activation and Brain Insulin Signaling

Disruption in a Diabetic Model of Alzheimer's Disease

by

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Alzheimer's disease (AD) is a neurodegenerative disorder marked by progressive cognitive impairments and pathological hallmarks that include amyloid plaques, neurofibrillary tangles, and neuronal loss. Several well-known mutations exist that lead to early-onset familial AD (fAD). However, these cases only account for a small percentage of total AD cases. The vast majority of AD cases are sporadic in origin (sAD) and are less clearly influenced by a single mutation but rather some combination of genetic and environmental risk.

The etiology of sAD remains unclear but numerous risk factors have been identified that increase the chance of developing AD. Among these risk factors, Type II Diabetes Mellitus (DM) and chronic inflammation of the brain have been implicated as two leading risk factors. Longitudinal studies have identified that patients with T2DM have nearly twice the risk of developing AD. DM is a common metabolic disorder that affects a quarter of the elderly population with symptoms that include insulin dysregulation and altered glucose metabolism. Numerous studies link insulin resistance in the brain with an increased risk of AD. Intracerebroventricular (ICV) administration of the diabetogenic drug streptozotocin (STZ) leads to brain insulin resistance and several

AD-like pathologies including progressive deterioration of memory, increased A β load and hyperphosphorylated tau. STZ has been proposed to be a relevant animal model of sAD.

Additionally, neuroinflammation has been implicated in playing a fundamental role in the progression of the neuropathological changes observed in AD brains. Neuroinflammation is typically thought to be a result of one or more of the other AD pathologies and serves to rapidly progress the disease. Lipopolysaccharide (LPS) is capable of mounting an immune response through the activation of Toll-like receptor 4 (TLR4). Studies involving transgenic models routinely activate the immune system by administering LPS to exacerbate AD-like deficits to better understand the role of neuroinflammation in AD.

The majority of AD models rely on genetic mutations and provide valuable information regarding the role of A β and tau pathologies but do not represent the prevailing sAD. Considerable research has been conducted to help elucidate the risk factors associated with sAD, including DM and neuroinflammation. However, there is a lack of research regarding the role of neuroinflammation in this particular model of sAD. The purpose of this study was to investigate the effects of a one-time immune activation in the STZ model on learning and memory and proteins associated both with AD hallmarks and with various neurotransmitter systems. Results indicated that an acute inflammatory response played a beneficial role in spatial learning and in several of the investigated proteins. These data may help shed light on the role of brain inflammation in AD.

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CHAPTER 1

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder that currently affects nearly 5.2 million people in the United States and is expected to triple to 13.8 million by 2050 as the life expectancy continues to increase ¹. Worldwide estimates are even more staggering with a current incident rate of 35.6 million and a 2050 projection of nearly 115 million individuals ². Symptoms of AD include progressive memory loss, cognitive decline as well as behavioral perturbations ^{3,4}. Behavioral alterations include anxiety, psychoses, confusion and apathy ⁴. Pathological hallmarks of this disorder include amyloid plaques, neurofibrillary tangles (NFTs), and neuronal loss ⁵⁻⁹.

The average age of AD onset is 65 years and is typically referred to as either late-onset AD (LOAD) or sporadic AD (sAD). A small percentage of AD, 1-5%, occurs earlier and is referred to as early-onset AD (EOAD), also known as familial AD (fAD). Several well-studied genetic mutations exist that lead to fAD, including mutations in the *amyloid precursor protein*, *presenilin 1* and *presenilin 2* genes. Mutations in these genes all lead to the same outcome, increased A β peptides that result in amyloid plaque deposition. sAD accounts for the vast majority of AD cases (99%) and is thought to develop due to a number of risk factors. Two of the leading age-related risk factors for developing sAD include Type II Diabetes Mellitus (DM) ¹⁰⁻¹³ and chronic inflammation of the brain ^{14,15}.

Numerous studies have established high comorbidity between AD and DM ¹⁶⁻²⁰ and have demonstrated links between insulin resistance in the brain and AD ²¹⁻²⁴. Patients with DM have nearly double the risk of developing AD, making it one of the top risk

factors^{16,17,25}. Several mechanisms indicate overlap between AD and DM. In particular, insulin is capable of regulating the activity of GSK3- β , which is responsible for phosphorylating tau at multiple sites that are characteristic of the NFTs seen in AD²⁶⁻²⁹.

Neuroinflammation has been implicated in exacerbating AD pathologies and rapidly accelerating the disease progression^{14,30}. A growing body of research indicates that chronic inflammation facilitates both tau phosphorylation and A β deposition³¹⁻³³. Neuroinflammation has also been implicated in the worsening of learning and memory deficits seen in both normal aging and AD³⁴⁻³⁶, and is likely related to the above pathologies^{37,38}. There are benefits of short-term inflammation in the brain, including microglia that degrade A β and remove the debris of dead and dying cells, reducing the likelihood of further cell loss³⁹⁻⁴¹. Neuroinflammation can become neurotoxic, however, when the response becomes chronic and uncontrolled, leading to increased A β and senile plaque production, neuronal injury, and cell death^{30,42}.

Considerable progress has been made investigating each of the above risk factors, DM and neuroinflammation, as they each relate to AD. However, there have been no investigations capitalizing on the combination of the above risk factors to investigate their interplay with each other, leading to behavioral and pathological characteristics of AD. The purpose of this study was to evaluate the effects of neuroinflammation in a DM animal model of AD. A widely accepted diabetic model of AD, utilizing streptozotocin (STZ) to disrupt insulin production and signaling in the brain, is often used to investigate the role that DM plays in the development of sAD. The below experiment investigated the effect of acute brain inflammation in this diabetic model of sAD by inducing an inflammatory response with lipopolysaccharide (LPS), a potent immune activator. We

examined learning and memory in multiple hippocampally-dependent tasks, including the Morris water maze (MWM) and novel object recognition (NOR), to assess whether one-time immune activation was sufficient to exacerbate deficits exhibited in this DM model of AD. We also examined hippocampal protein levels of A β , phosphorylated tau (pTau), and numerous other neurotransmitter specific proteins to determine if alterations in these proteins occurred.

CHAPTER 2

REVIEW OF RELATED LITERATURE

Amyloid β Hypothesis

Amyloid plaques, also known as senile plaques, are one of the defining pathological hallmarks of AD. Senile plaques were first described in 1907 by Alois Alzheimer and have been central to the postmortem diagnosis of AD to date⁴³. Amyloid plaques accumulate extracellularly, resulting in cell damage and neuronal loss.

The A β peptide was first purified from the brains of AD and Down's syndrome individuals in 1984⁴⁴, leading to the isolation of A β from senile plaques the following year, providing the groundwork that A β forms the core of amyloid plaques⁸. The current leading hypothesis, the amyloid cascade hypothesis, posits that early A β deposition not only leads to the formation of amyloid plaques but the formation of NFTs, neurodegeneration and cognitive decline^{45,46}.

Early observations that individuals with Down's syndrome, a disorder due to three copies of chromosome 21, often develop dementia similar in nature to Alzheimer's disease and develop virtually identical amyloid plaques, initially drew the focus towards chromosome 21 as a potential locus for AD development⁴⁷. This focus on chromosome 21 led to the discovery of the *amyloid precursor protein (APP)* gene, which is responsible for the protein, amyloid precursor protein (APP), that is eventually broken down into A β peptides⁴⁸⁻⁵¹.

APP is a transmembrane protein containing a large extracellular N-terminal domain, a hydrophobic transmembrane domain and a relatively short intracellular C-terminal domain^{52,53}. Due to alternative splicing during posttranslational modification,

there are three major APP isoforms: 695, 751 and 770 (referring to the number of amino acid residues). The isoforms containing 751 and 770 amino acids are widely expressed in non-neuronal cells but appear to be present in neurons as well, whereas the 695 amino acid isoform is more commonly found in neurons than in other cell types⁵⁴. The physiological function of APP remains largely unknown despite extensive research, although it appears to be involved in cellular processes such as long-term potentiation (LTP). LTP is a mechanism widely believed to be integral to learning and memory processes by enhancing the magnitude of excitatory postsynaptic potentials thus resulting in increased synaptic efficiency⁵⁵⁻⁵⁷. Additionally, APP has been proposed to play a role in cellular adhesion due to its ability to form homodimers and colocalization with proteins known to be involved with cellular coupling, such as β 1 integrins^{58,59}.

APP undergoes a series of enzymatic events during degradation. Membrane bound APP is degraded at the plasma membrane but APP has been localized to other areas of the neuron such as the trans-Golgi network, endoplasmic reticulum and mitochondria⁶⁰. APP degradation is primarily carried out by enzymes called secretases^{61,62}. Each individual secretase (α -, β -, γ -) cleaves APP at different cleavage sites and thus, depending on which secretase exerts its activity and in which order, can result in different proteolytic fragments of various sizes.

Each secretase is an assembly of unique enzymes, allowing them to exert differential effects on APP proteolysis. Enzymes associated with α -secretase are associated with members of the ADAM (a disintegrin and metalloproteinase) family, which are known to target and cleave extracellular protein domains^{63,64}. β -secretase activity has been linked to β -site APP-cleaving enzyme 1 (BACE1), which also targets

extracellular protein regions^{65,66} while γ -secretase is composed of numerous enzymes, including presenilin 1 or 2^{67,68}.

APP is subject to two possible degradation pathways, a non-amyloidgenic (non-pathogenic) pathway which involves α -secretase and γ -secretase cleavage or a amyloidgenic (pathogenic) pathway involving β -secretase and γ -secretase activity⁶⁹. For APP to be degraded via the more prevalent non-amyloidgenic pathway, α -secretase first cleaves APP at a site approximately 83 amino acids away from the C-terminus directly within the sequence of A β . This cleavage by α -secretase results in a short, membrane retained C-terminus fragment (CTF) of 83 amino acids (C83) and a larger N-terminal soluble ectodomain fragment (sAPP α). CTF is further cleaved by γ -secretase forming yet smaller fragments, p3 and APP intracellular domain (AICD). In this non-amyloidgenic pathway, all of the cleavage byproducts are easily removed and managed by microglia and other phagocytic cells⁷⁰. The initial cleavage of APP within the A β sequence prevents the formation of A β peptides⁷¹. Alternatively, in the amyloidgenic pathway of APP processing, APP is cleaved by β -secretase at a site 99 amino acids away from the C-terminus resulting in an alternative CTF (C99) that is retained in the membrane and an alternative N-terminal soluble ectodomain fragment (sAPP β). The site of β -secretase activity begins just prior to the first amino acid residue of A β , leaving the entire A β sequence intact within the C99 fragment. The A β peptide is formed when γ -secretase further cleaves the C99 fragment releasing the A β fragment^{66,71}.

Furthermore, depending on the site of cleavage, γ -secretase can produce A β peptides of various sizes. The majority of A β produced are 40 amino acids long (A β ₄₀)

though a slightly longer variant of 42 amino acids ($A\beta_{42}$) can be formed⁷². Research supports the idea that amyloid plaques are predominately comprised of the longer variant, $A\beta_{42}$ ^{72,73}.

The majority of APP proteolysis occurs at the plasma membrane but, because APP is also found in other cellular structures, degradation can occur intracellularly as well. $A\beta$ is also produced in both endosomes and the trans-Golgi network where all three secretases can be found but where β - and γ -secretase seem to be more abundant, leading to an increased intracellular amyloidogenic processing^{74,75}.

$A\beta$ peptides appear to be part of normal brain physiology. For instance, $A\beta$ is implicated in regulating synaptic function⁷⁶. Increased neuronal activity, particularly NMDA receptor activation, appears to promote an increased production of $A\beta$ by inhibiting α -secretase while simultaneously stimulating β -secretase cleavage of APP, which in turn depresses excessive activity in a negative feedback manner⁷⁷. It has been proposed that as neurons lose sensitivity to this $A\beta$ negative feedback, the elevated activity remains unchecked, leading to excitotoxicity and a further elevation of $A\beta$ levels which can form neurotoxic fibrils⁷⁸⁻⁸¹. Supporting the theory that elevated neuronal activity increases $A\beta$ production, patients with temporal lobe epilepsy can develop large amounts of plaques in seizure focal areas as early as 30 years of age^{82,83}. Furthermore, areas of the brain that tend to show the greatest amount of $A\beta$ plaques exhibit the highest amount of resting baseline metabolic activity in both healthy and epileptic brains^{84,85}.

Not only is APP important for LTP, as mentioned earlier, it appears that $A\beta$ peptides may play a vital role as well. In vitro studies have shown that endogenous, low level $A\beta$ is necessary for the induction, but not maintenance, of LTP within the

hippocampus⁸⁶. Furthermore, Puzzo et al. demonstrated that A β ₄₂ is vital for both reference and associative memory.

While there appears to be a physiological role for A β , the balance of A β production to degradation, as well as the ratio of A β ₄₀ to A β ₄₂, appears to be crucial for the development of both amyloid plaques and of AD onset. While both A β ₄₀ and A β ₄₂ are the primary constituents in amyloid plaques, a common finding in AD patients is a shift towards an increased A β ₄₂/A β ₄₀ ratio^{73,87,88}. This shift towards greater A β ₄₂ is particularly important because A β ₄₂ appears to be the more toxic form of A β monomers due to its early deposition in amyloid plaques and the ability to aggregate into fibrils more readily than A β ₄₀^{73,87}. Differences in the initial aggregation of the two monomeric A β species, referred to as A β oligomers (A β o), have been observed. For example, when A β ₄₀ starts to aggregate, the resulting oligomers are more compact than those formed by A β ₄₂ oligomers. Interactions of the A β ₄₂ N-termini appear to allow the conformation of the oligomers to remain relatively loose due to their increased amino acid length thus freeing up the C-termini, a site necessary for protofibril and fibril formations⁸⁹.

It is these A β fibrils, characterized by a distinctive cross- β structure, that form the dense amyloid plaques within the brain and accumulate along the walls of cerebral blood vessels^{90,91}. Fibrillation is a complex pathway leading up to A β fibrillogenesis; A β fibrils are preceded by a number of intermediate structures that include A β dimers and trimers, A β o and protofibrils^{92,93}. It has been proposed that because monomeric A β is produced under normal physiological condition and may serve a functional role that the aggregation of A β is required for the peptide to become neurotoxic⁹⁴.

When referring to A β , the term “soluble” refers to any A β that is soluble in an aqueous buffer and remains soluble after high-speed centrifugation, indicating that it is not part of the fibrillar A β ⁹⁵. Thus, insoluble A β simply refers to A β fibrils and A β plaques that are not soluble in an aqueous solution. Soluble A β o are assemblies of A β monomers ranging anywhere from dimers to 24-mers, though larger A β o have been reported ^{96,97}.

Despite amyloid plaques being considered a pathological hallmark of AD, insoluble A β plaques do not correlate well to neuronal death, synaptic loss, or cognitive impairments ^{97,98}. Soluble A β o, on the other hand, do appear to correlate well with disease progression and severity of dementia ^{75,98-100}. Soluble A β o are becoming increasingly accepted as an additional pathological hallmark separate from amyloid plaques ⁶⁰. Brains and CSF of patients with AD show increased levels of soluble A β o as high as 70-fold compared to controls ^{101,102}. Elevated levels of soluble oligomers appear to be specific to brain regions associated with cognitive impairments in AD brain such as the prefrontal cortex and hippocampus but are not detectable in the cerebellum ^{103,104}.

The neurotoxicity of A β o is becoming increasingly well researched. *In vitro* studies have shown that soluble A β o, in the form of dimers and trimers, has the ability to reduce spine density of neurons at relatively low concentrations and induce long-term depression ¹⁰⁵. Low-weight A β o bind predominately to post-synaptic structures in the hippocampus resulting in changes in spine shape and reductions in spine density ¹⁰⁴. Complimenting these studies, Ono et al. ¹⁰⁶ demonstrated that A β dimers are three times more damaging than monomers, while trimers and tetramers are almost 13-fold more neurotoxic *in vitro*. Mice expressing a variant of human APP linked to AD (Tg2576)

were found to have significantly elevated levels of 12-mers (~56 kD), which correlated highly with spatial learning impairments¹⁰⁷. A β of various sizes can disrupt membrane stability and allow aberrant ion flow, such as Ca²⁺ influx which can lead to excitotoxicity^{108,109}.

As mentioned, the amyloid cascade hypothesis (ACH) is currently the leading hypothesis for the development of AD. The ACH, established in 1992, posits that A β fibril formation leads to the neuronal death, memory loss, and dementia seen in the disease⁴⁵. Due to the overwhelming research highlighting that A β fibrils and plaques do not correlate well to severity of dementia, the ACH has been modified to propose that the small, soluble A β is driving the memory loss and dementia severity^{60,110}. Specifically, the updated ACH implicates that early memory loss is due to synaptic failure rather than neuronal death and that this synaptic failure is driven by the A β rather than the fibrils^{103,110}.

As the role of A β in AD moves away from the damaging effects of extracellular A β deposition (A β plaques) and more towards intracellular accumulation of A β initiating pathological processes, the possibility that intracellular A β could be found within a neuron was initially met with skepticism. However, numerous studies have provided evidence that intracellular A β does in fact exist within the human brain¹¹¹⁻¹¹³. Furthermore, A β_{42} has been demonstrated to be the primary A β monomer located within neurons¹¹². Accumulations of intraneuronal A β_{42} have been shown to precede both NFTs and amyloid plaque formation in AD brains as well as the plaque formation in young Down's syndrome patients^{112,114}. Neurons in brain regions susceptible to AD

pathologies, such as the hippocampus and prefrontal cortex, appear to be particularly prone to accumulating A β ₄₂ peptides⁹².

Numerous animal models of AD lend support to the theory of early intraneuronal A β ₄₂ leading to other AD-like pathologies. Double transgenic mice harboring both human APP and PS1 mutation display typical plaque formation seen in AD brains, but these plaques are preceded by intraneuronal accumulation of A β ₄₂¹¹⁵. Similarly, transgenic mice with Arctic and Swedish mutations (explained below) show strong correlations between intracellular A β ₄₂ and cognitive impairments which both precede amyloid plaque formation¹¹⁶. Additionally, triple transgenic (3xTg-AD) mice (expressing mutations in PS1, APP, and tau), which are known to progressively develop both amyloid plaques and NFTs, exhibit synaptic dysfunction before plaques and NFTs and is correlated strongly with the accumulation of intraneuronal A β levels¹¹⁷.

Genetic causes of fAD have been extensively studied in the last few decades. Specific mutations in three genes (*APP*, *PS1* and *PS2*) are all known to cause fAD. The common denominator in each of these genes is that they all affect either the metabolism or stability of A β , or both. All APP mutations that are observed in fAD alter the processing of APP and thus lead to the overproduction of A β ₄₂¹¹⁸. One of the more common APP mutations, the Swedish mutation (APP^{swe}), is a double mutation that resides on APP, prior to the A β region, which leads to increased cleavage by β -secretase resulting in increased A β production¹¹⁹. Another APP mutation, the Arctic mutation (APP^{arc}) leads to a particularly aggressive form of fAD with accelerated A β production and increased A β ₀ and protofibril formation¹²⁰⁻¹²². APP^{arc} is located near the α -secretase cleavage site and affects APP processing by slightly altering the localization of

APP to areas of the cell, like endosomes and the trans-Golgi network, that favor proteolytic processing by β -secretase¹²³.

Numerous other APP mutations have been identified including the London mutation which lies just outside the $A\beta$ domain and shifts production towards $A\beta_{42}$ ¹²⁴, the Flemish and Dutch mutations which lie within the $A\beta$ sequence close to α -secretase cleavage site impairing enzymatic cleavage and thus shifting APP processing towards the amyloidgenic pathway^{125,126}. In all, ~25 specific mutations occurring on *APP* are known to cause fAD¹²⁷. Interestingly, there is one mutation located near the β -secretase site impairing amyloidgenic processing of APP in an Icelandic study that has shown to be protective against age related cognitive decline and AD¹²⁸.

Presenilin mutations account for the greatest number of fAD cases. There have been to date, over 180 pathogenic mutations in *PSN1* (housed on chromosome 14), accounting for nearly 50% of fAD cases while 13 mutations in *PSN2* (located on chromosome 1) accounts for just 1% of all fAD cases¹²⁹. The first mutations in both the *PSN1* and *PSN2* genes were discovered in 1995¹³⁰⁻¹³². As previously covered, PSN1 and PSN2 are proteins that comprise the components of the catalytic core of γ -secretase. PSN1 mutations lead to particularly aggressive early-onset AD with severe cognitive impairments that can begin as early as age 30 and often give rise to atypical symptoms such as ataxia and spastic paraparesis¹²⁷. One particularly aggressive *PSN1* mutation, L166P, generally manifests during adolescence and results in exceptionally high $A\beta_{42}$ production¹³³. As with APP mutations, PSN mutations generally lead to an increased $A\beta_{42}/A\beta_{40}$ ratio. PSN mutations are most commonly the result of missense mutations of

single amino acid substitutions throughout the PSN proteins¹³⁴⁻¹³⁶. *PSN2* mutations tend to be relatively rare and have a later age of fAD onset compared to *PSN1* mutations¹³⁷.

The genetics of sAD appears to be much more complex than fAD. The vast majority of sAD occurs with no family history of AD and is likely to be an interaction of genetic susceptibility of multiple gene with the environment. To date, there is only one proven genetic risk factor associated with sAD, the $\epsilon 4$ allele of the apolipoprotein (APOE) gene^{138,139}.

APOE is a lipoprotein that is expressed in many organs, with the highest expression occurring in the liver followed by the brain¹⁴⁰. In the brain, APOE is produced primarily by astrocytes and microglial cells and is a key lipoprotein in the regulation of lipid metabolism by directing transportation, distribution and delivery from one cell or tissue type to another through APOE receptors^{141,142}. The *APOE* gene is located on chromosome 19 and contains four exons that make up the APOE protein. APOE has three possible alleles ($\epsilon 2$, $\epsilon 3$, $\epsilon 4$) that differ only by two single nucleotide polymorphisms on exon 4¹²⁷.

APOE $\epsilon 3$ is the most common form of the allele and is present in a range of nearly 50-90% of the population, while $\epsilon 4$ and $\epsilon 2$ are expressed far less in the population, 3.35% and 1-5% respectively¹⁴⁰. APOE $\epsilon 3$ appears to confer no risk to developing AD, whereas APOE $\epsilon 4$ has been repeatedly implicated as a genetic risk factor for developing sAD^{143-144,145}. APOE $\epsilon 4$ has been found to be present in as high as 50% of patients with sAD and the presence of one copy of the allele increases chances of developing AD by three times, while carrying two copies increases that risk to nearly 12 times¹⁴³.

The mechanism by which APOE ϵ 4 increases the risk of developing AD is not entirely understood despite the recent intensity of research directed towards it. *In vitro* and in-vivo animal studies indicate that APOE plays a role in the conversion of monomeric A β into oligomers as well as a role in A β fibrillogenesis¹⁴⁶⁻¹⁴⁸.

Animal models that express human APOE have demonstrated that the various isoforms can have differing effects on A β accumulation with ϵ 4> ϵ 3> ϵ 2^{149,150}. APOE ϵ 4 is neither necessary nor sufficient for the development of AD and is thus only considered a risk factor and not causal as the genetic mutations linked to fAD. Polymorphisms in APOE alleles cannot be used alone for diagnosis or for presymptomatic diagnosis such as genetic testing of the known mutations that cause fAD¹⁵¹.

The vast majority of AD research has been directed at understanding the role that A β plays in the development of the disease. While amyloid plaques have long been considered the pathological hallmark of AD, there is still debate on their causative nature due to the lack of strong correlation with dementia severity. Given that the leading hypothesis, the ACH, has been revised to accommodate earlier A β products such as the oligomers and protofibrils, which do correlate well with dementia severity, there is little doubt that A β peptides play a role in AD pathogenesis.

Tau Hypothesis

Another major neuropathological hallmark of AD is the presence of neurofibrillary tangles (NFTs). Tau was first discovered in 1975 as a microtubule-associated binding protein that directly affected the way that tubulin was able to polymerize into microtubules¹⁵². Not until the mid 1980's were several laboratories able to identify that NFTs, seen so prominently in AD brains, were primarily composed of tau

^{7,153}. A considerable amount of research has been conducted in unraveling the role that tau plays in both a healthy brain and in AD pathology.

Microtubules, composed of tubulin subunits, are the primary support structures in eukaryotic cells ¹⁵⁴. Microtubules help maintain cell morphology, establish cellular polarity and neurite outgrowth in differentiating neurons ^{155,156}. In neurons, microtubules not only give structural stability to the cell but also serve as transportation tracts for nutrients, neurotransmitters and various organelles such as mitochondria ^{157,158}. Tau has been shown to bind to microtubules to facilitate the assembly and stability of the tubulin subunits ¹⁵⁹.

The tau gene is located on chromosome 17 and contains a total of 15 exons that can be alternatively spliced to form six possible tau isoforms, ranging from 352-441 amino acids ¹⁶⁰. Tau mRNA is readily expressed in neurons, particularly within the axon ¹⁶¹ but has also been found in oligodendrocytes, which support neurons by wrapping axons to form the myelin sheath ¹⁶². During development, only the shortest isoform of tau is expressed while the adult brain expresses all six isoforms, suggesting that tau is developmentally regulated ¹⁶³. Tau is composed of either three or four microtubule-binding domains, depending on the isoform, which is a result of alternative splicing of exon 10, located on the carboxy-terminal one-third of the protein ¹⁶⁴. The other region, the amino-terminal, comprising two-thirds of the molecule, is referred to as the flanking domain (or projection domain) and varies by isoform depending on how exons 2 and 3 are spliced, resulting in a differing number of amino acids in the terminal end of tau ^{161,165}.

Tau is a phosphoprotein, meaning that function is regulated by the addition and removal of phosphate groups, which primarily occurs on the projection domain.¹⁶⁶ The addition of phosphate groups, performed by proteins called kinases, lessens tau's affinity for tubulin and thus reduces the binding to microtubules¹⁶⁷. Phosphatases remove the phosphate groups which restore tau's affinity for microtubule binding¹⁶⁸. While tau binding has been found to provide stability to the assembled microtubules, this binding interferes with the ability of the motor protein kinesin to also bind to the microtubules¹⁶⁹. Kinesin, like other motor proteins, bind to microtubules and "walk" along the tracts with vesicles to deliver cargo vesicles to various parts of the cell^{170,171}. The interplay of constantly phosphorylating tau to allow kinesin to pass and dephosphorylating tau to provide microtubule stability is a key aspect to a healthy functioning neuron. This balance between kinase and phosphatase appears to be disrupted in AD^{172,173}.

Tau is found to be abnormally hyperphosphorylated in the brains of AD patients, about three- to four-fold higher than control brains^{7,167,174}. Tau hyperphosphorylation leads to tau disassociation from microtubules and disassembly of microtubules, which leads to impaired axonal transport and cell death^{153,174}. *In vitro* studies have shown that tau phosphorylation can occur on up to 85 residues, typically a serine or threonine amino acid; however, only about 10 sites have been identified in post-mortem control brains due to the fact that tau extracted from post-mortem tissue becomes rapidly dephosphorylated^{175,176}. There are currently ~45 known sites of tau hyperphosphorylation in AD brains, a number that far exceeds the number of identifiable sites in healthy brains^{102,166}. Theoretically, this abnormal phosphorylation could be due to decreased phosphatase activity, increased kinase activity, or a combination of the two.

In vitro studies have shown that more than a dozen different kinases and almost all known phosphatases are able to regulate tau phosphorylation^{165,177,178}. *In vivo* studies indicate that the most important kinases involved in tau phosphorylation include mitogen-activated protein kinase (MAPK), GSK3- β , cyclin-dependent kinase 5 (cdk5), cAMP-dependent protein kinase (PKA) and calcium/calmodulin-dependent kinase II (CaMK-II) which collectively are known to phosphorylate all sites implicated in AD¹⁷⁹.

GSK3- β is a kinase that has been emerging as a key player in AD research, as it serves a role in regulating tau phosphorylation in both normal physiology and pathological condition. GSK3- β has been proposed to occur via both primed and unprimed phosphorylation. Primed phosphorylation occurs when GSK3- β activity follows phosphorylation by another kinase, allowing GSK3- β to recognize the newly phosphorylated residue¹⁸⁰. Primed GSK3- β phosphorylation typically occurs at threonine-231 and affects the ability for tau to bind to microtubules. Cdk has been implicated as a kinase that can prime tau for further phosphorylation by GSK3- β ¹⁸¹. Unprimed phosphorylation occurs independent of other kinase activity at sites serine-386 and serine-404^{182,183}. All of these sites are commonly hyperphosphorylated in AD¹⁷⁹.

Protein phosphatase 2A (PP2A), one of the major tau phosphatases, appears to work against the kinase activities of MAPK, GSK3- β and CaMK-II^{184,185}. Calcineurin, also known as PP2B, is capable of dephosphorylating several sites of hyperphosphorylation and has been shown to have impaired function in AD brains¹⁸⁶. Calcineurin is the most abundant phosphatase found in the brain, particularly in the cortex and hippocampus, both areas heavily disrupted in AD progression^{187,188}.

Hyperphosphorylation affects the processing of tau in several ways. Firstly, the conformational changes that follow the phosphorylation of tau hinder the degradation by proteases thus making hyperphosphorylated tau more resistant to proteolysis than unphosphorylated tau^{178,189}. Secondly, the hyperphosphorylation is essential for the self-aggregation of tau protein into paired-helical filaments (PHFs), which in turn makes up the characteristic NFTs seen in AD^{7,190}. Hyperphosphorylation of tau results in a conformational change of increased α -helices in the secondary structure, which is found to be considerably increased in the tau isolated from PHFs¹⁹¹⁻¹⁹³. PHFs also demonstrate a characteristic β -sheet structure that is thought to be necessary in its formation¹⁹⁴. Electron microscopy studies indicate that PHF appear as two hyperphosphorylated tau proteins wound together in a left-hand helical sense and the excessive phosphorylation is the only detectable posttranslational modification between normal tau and PHF-tau¹⁹⁵.

Numerous investigations have established that PHFs are cytotoxic and likely contribute to the neurodegeneration in AD^{196,197}. The cytotoxicity of PHF could be due to the hyperphosphorylation itself or the formation of the aberrant aggregates, though it is likely a combination of both. *In vitro* studies have shown that hyperphosphorylated tau is capable of inducing apoptotic pathways resulting in cellular death¹⁹⁸. The self-aggregation of PHFs and formation of NFTs typically occur intracellularly. There is also an inverse correlation between extracellular tangles and the number of surviving neurons in areas of the AD brain with typically high intracellular NFTs^{199,200}. This suggests that the intracellular NFTs precede the cell death and the extracellular NFTs are a result of cell lysis. Abnormal aggregations of hyperphosphorylated tau also attract normal tau and other microtubule-associated proteins (MAPs), specifically MAP1B and MAP2, to the

tangles which lead to the further destabilization of microtubules as well as morphological changes in the cell that lead to disruptions in transport delivery and synaptic contacts^{201,202}.

Another form of tau, truncated tau, is known to be a major component of PHF²⁰³. Truncated tau, occurring when the carboxy terminus is cleaved typically at residue 391, has been shown to aggregate much faster and to a greater extent than wild-type tau with an intact carboxy tail^{204,205}. Tau truncation at E³⁹¹ occurs throughout the AD progression but is particularly prevalent during the later stages^{206,207}. Additionally, at least two other forms of truncated tau, cleaved at D²⁵ and D⁴²¹, have been identified to be more abundant in the brains of AD patients^{208,209}.

Various members of the caspase family, including caspase-3, have been found to cleave tau at all three truncation sites²¹⁰⁻²¹². Caspases are proteases that are induced during apoptosis and are a key participant in the proteolytic cascade leading to cell death²¹³. In neurons, caspase-3 is considered the major “killer caspase”²¹³. Caspase-3 is elevated in AD brains and has been found to be critical for A β -induced apoptosis^{211,214,215}. Though some studies indicate that tau truncation becomes more prevalent as the disease progresses, at least two studies indicate that truncation is an early event of NFTs formation as a result of accumulating A β peptides^{216,217}. Because evidence suggests that A β accumulation typically precedes NFTs formation in AD brains^{112,218} and that A β -induced neurodegeneration rarely occurs without the presence of NFTs in 3xTg mice^{117,219,220}, truncated tau may be an early step in NFTs formation as intracellular A β activate caspases.

Abnormal tau phosphorylation, amounts of tau, splicing of tau and even mutations in the tau gene can lead to a number of disorders, collectively called tauopathies¹⁶⁵. Tauopathies includes diseases such as AD, Down's syndrome and Pick's disease. Several mutations in the tau gene were discovered in 1998 in frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17), a related but distinct tauopathy, providing the first direct evidence that neurodegeneration could be linked, in part, to abnormal tau²²¹⁻²²³. There have now been 25 identified mutations associated with FTDP-17 and tend to be missense mutations that occur in the microtubule-binding domain, which leads to decreased tau affinity for microtubules, alters binding to other proteins that bind to the region, and leads to increased self-aggregation^{221,224-226}.

Several transgenic lines of mice have been established attempting to model the abnormal tau seen in AD. One of the more promising lines appears to contain the mutation P301L, a mutation based on one identified in FTDP-17 patients that is located in the microtubule-binding domain and reduces the affinity of tau for microtubules^{227,228}. Mice expressing the P301L mutation experience an age-dependent progression of tau pathology similar to that seen in various tauopathies including excessive extractable PHF-like tau aggregates, deficits in spatial reference memory, forebrain atrophy, and hippocampal neuronal loss²²⁹⁻²³¹.

Unlike A β plaques, which do not correlate well with severity of dementia, tau aggregates and NFTs do correlate well with dementia severity, cognitive decline, and regional progression of AD^{5,232,233}. The earliest noticeable neuronal loss in AD brains occurs within the entorhinal cortex of the hippocampus, which is also where NFTs are first observed. A β plaques, on the other hand, are not found within the hippocampus until

late stages of the disease ²³⁴. Tau pathology in the AD brain appears to follow a very specific pattern: starting in the entorhinal cortex, then other areas of the hippocampus, followed by the anterior temporal cortex and finally spreading into other cortical regions with the motor and sensory cortex typically being the last areas affected ^{235,236}. Severity of dementia strongly correlates with the progression of NFTs through the various brain regions ²³⁶.

Unfortunately, there are limitations to the tau hypothesis. NFTs can be seen in non-demented individuals, similar to amyloid plaques in healthy individuals ²³⁷. Additionally, animal models of tauopathies do not develop typical A β pathology, global neuronal loss, or the typical progression of NFTs through the discrete brain regions ²²⁹. Despite considerable progress investigating both of the above pathologies neither is sufficient to account for all the symptoms observed in AD

Unraveling the role that A β pathology and tau pathology have in AD development and progression will undoubtedly be key to understanding the disease. The interplay of how these two pathologies interact is already beginning to unfold. The previously discussed ACH is essentially a serial model, which posits that A β plaques leads to NFTs, leading to cellular death and neuronal loss, and has already been amended to account for A β ₀ and other intracellular A β species playing a causal role. More recently a dual pathway model has been proposed suggesting that increased A β load and increased hyperphosphorylated tau and NFTs can be linked by common upstream mechanisms, resulting in synaptic and cellular loss ²³⁸.

APOE has been proposed as one of those common upstream mechanisms. As discussed above with regards to its role in A β pathology, carrying the APOE ϵ 4 allele is

also strongly associated with an NFTs load ²³⁹. There is a strong correlation between increased tau levels found in the CSF of AD patients with APOE ε4 and the degree of cognitive decline ^{240,241}. Interestingly, one study indicated that young individuals with at least one APOE ε4 allele, but showing no signs of dementia or cognitive impairments, had significantly higher NFTs in the entorhinal cortex than age matched controls indicating the importance of the ε4 allele in the early stages of pathological development, particularly in early NFTs formation preceding cognitive impairments ²⁴².

Cholinergic Hypothesis

Cholinergic cell loss is another characteristic feature found in the brains of patients with AD. The neuronal loss seen in AD typically begins in the basal forebrain before spreading to the hippocampus and other regions of the cortex ^{243,244}. Reductions in enzymes responsible for the production and degradation of ACh, primarily choline acetyltransferase (ChAT) and acetylcholinesterase (AChE), are reduced in postmortem analyses of AD brains, which indicate that there is an overall reduction in cholinergic signaling in the cortex and hippocampus ^{245,246}. Investigations of post-mortem tissue from patients recently diagnosed with AD indicate that there is a selective disruption to the cholinergic neurotransmitter system, whereas brains from later stage AD patients demonstrate disruptions to multiple other neurotransmitter systems ^{247,248}. Additionally, the degree of cholinergic loss correlates well with the severity of dementia seen in AD patients ^{246,249}.

The reason for the selective cholinergic perturbations is currently unknown but has lead to considerable research investigating the reason that cholinergic systems appear to be disrupted first as well as towards treatments that potentiate the cholinergic system.

Early attempts focused primarily on replacing ACh precursors, such as choline and lecithin but ultimately failed to increase cholinergic activity nor did they improve AD deficits²⁵⁰. Alternative approaches investigated elevating cholinergic tone by blocking enzymatic breakdown of ACh using AChE inhibitors (AChEI). AChEI were introduced in 1997 and have demonstrated promise in slowing the progression of AD deficits compared to other investigational therapies. AChEI work by delaying the degradation of ACh released within the synaptic cleft by AChE, thereby enhancing cholinergic transmission. AChE inhibitors do not prevent the progression of the disease, but can slow the progression of symptoms for mild to moderate AD patients²⁵¹⁻²⁵⁴.

The cholinergic hypothesis is not able to account for all the deficits and pathological markers seen in AD but has provided valuable insight into some of the mechanisms that underlie the disease. Investigations into other contributing mechanisms, such as risk factors like Diabetes Mellitus and neuroinflammation, particularly as they relate to sAD, will no doubt continue to provide valuable insight into understanding the disease development and progression.

Diabetes Mellitus Risk Factor

Diabetes Mellitus (DM) is a common metabolic disorder affecting nearly 40-50% of the elderly population. DM exhibits characteristic symptoms of hyperglycemia, impaired insulin secretion and insulin resistance. Mild to severe cognitive impairments have been reported in patients with both Type I- and Type II-DM²⁵⁵⁻²⁵⁷. Additionally, the effects of DM on the brain and cognition are most pronounced in aging brains and could be due to the interaction between DM and a normally aging brain^{256,258}

Numerous studies have shown high levels of AD and DM comorbidity ^{16,17,20,25}. The lifetime risk for patients with TII-DM is thought to be around 38% in developing AD ²⁵⁹ though certain populations have been reported to have nearly double the risk ^{17,25}. Interestingly, there is an increased risk for developing DM by racial ethnicity with African-Americans and Hispanic-Americans developing DM at nearly double, 1.9 and 1.6 respectively, compared to Caucasian-Americans ^{260,261}. This increased risk for DM by race is particularly telling as this pattern is almost perfectly mirrored by the racial incident rates of AD ^{262,263}.

Because DM is a complex metabolic disorder with a varied symptomology that includes hyperglycemia, abnormal insulin signaling, cardiovascular disease and weight abnormalities, it is difficult to tease apart exactly which factors associated with DM could lead to an increased risk of AD. One factor that is increasingly implicated is insulin dysregulation and resistance. Numerous studies now link insulin resistance in the brain with increased risk of AD ^{11,18,21,22,24}.

The role of insulin in the periphery as it relates to glucose metabolism and disorders such as DM is well-studied but within the brain the role of insulin signaling is less well characterized ^{264,265}. Increasing evidence indicates that insulin is involved in several brain mechanisms independent of its glucoregulatory role including neuromodulatory roles, neurotrophic and metabolic functions, synaptic plasticity, and memory consolidation ^{22,266-269}.

The main source of insulin in the brain is produced by the pancreatic beta cells and is known to cross the blood brain barrier through transendothelial passage into the brain capillaries ²⁷⁰. The insulin transporters are unevenly distributed throughout the

brain, with areas such as the olfactory bulb showing the highest transport rate ²⁷¹. The brain is also capable of producing a small amount of insulin as evidenced from insulin mRNA found in areas such as the hippocampus, medial prefrontal cortex, thalamus and olfactory bulb ^{270,272-274}. Insulin receptors (IR) are also widely distributed throughout the brain and are found particularly densely in the hippocampus, prefrontal cortex, hypothalamus and olfactory bulbs ²⁷⁵.

Two types of IR have been identified in the mammalian brain: a neuron specific type and a peripheral type, which is also found on glial cells. Investigations of these receptors have elucidated that differences exist between IR found in brain and those in the periphery. For example, the neuron specific brain type IR is not down-regulated by insulin, whereas the peripheral type IR is down-regulated upon insulin binding ²⁷⁶. Both IR types contain an α -subunit and a β -subunit. Insulin binds to the extracellular IR α -subunit, which induces autophosphorylation of the intracellular β -subunit leading to receptor activation²⁷⁷. IR located in non-brain tissue are almost structurally identical to IR located within the brain, with the main difference being the downstream targets activated once the receptor has bound insulin ²⁷⁸.

Insulin binding is capable of regulating the activity of multiple kinases responsible for tau phosphorylation ²⁷⁹. Insulin receptor activity involves a cascade that includes Akt and GSK-3, each of which has the capacity to impact the phosphorylation state of tau. Akt is a protein that is activated via insulin signaling and inhibits GSK-3 activity by phosphorylating GSK-3 ²⁸⁰. Though originally identified for its role in glucose metabolism, GSK-3 is found throughout the body with the highest levels being in the brain ^{281,282}. Two forms of GSK-3 have been identified, GSK-3 α and GSK-3 β ²⁸³. GSK-

3β is capable of phosphorylating tau at multiple sites that are characteristic of the PHF seen in AD ^{28,29}. Interestingly, $A\beta$ peptides have been shown to activate GSK3- β and promote the hyperphosphorylation of tau, indicating a point of overlap between the two major pathologies in AD ^{284,285}. Other evidence suggests that PS1 binds both GSK3- β and tau, serving as the means to bring the two proteins into close proximity, linking both the amyloid and tau hypotheses ²⁸⁶. Increasing PS1 via transfection experiments not only increases the PS1/ GSK3- β binding interaction but also levels of phosphorylated tau ²⁸⁶. All of the above highlight that alterations in insulin signaling has the potential to impact both $A\beta$ processing and tau phosphorylation.

As previously discussed, APOE $\epsilon 4$ is implicated in playing an important role in the development of AD and also has ties to DM. One study by Luchsinger et al ¹³ found that patients with DM and APOE $\epsilon 4$ were 3.8 times more likely to develop AD, while DM patients without the APOE $\epsilon 4$ allele were 1.8 times more likely to develop AD. Another study demonstrated that DM patients with one APOE $\epsilon 4$ allele have an increased impairment of insulin sensitivity compared to controls while DM patients with two APOE $\epsilon 4$ have an even greater impairment ²⁸⁷. Post-mortem studies have shown that brains of DM+AD patients who also had at least one APOE $\epsilon 4$ allele had greater amyloid plaque deposition and NFTs than those without the APOE $\epsilon 4$ allele ²⁸⁸.

In addition to the links between insulin receptor function and AD, insulin degrading enzyme (IDE) is known to be a major degrading enzyme for extracellular amyloid peptides ^{289,290} and is upregulated by the presence of insulin ²⁹¹. IDE mRNA levels are reduced in patients with sAD within the hippocampus while patients with sAD+APOE $\epsilon 4$ have a reduction in IDE mRNA levels of nearly 50% ^{292,293}.

An animal model has been developed to mimic DM as a risk factor for developing sAD, utilizing a compound called streptozotocin (2-Deoxy-2-methylnitrosaminocarbonylamino-D-glucopyranose; STZ). When given systemically, STZ generates a cytotoxic product that is selective for pancreatic β -cells²⁷⁸. STZ enters the cell via glucose transporter (GLUT2), where it alkylizes DNA, which triggers activation of ADP-ribosylation leading to NAD^+ and ATP depletion, and ultimately leading to cell death resulting in a model DM^{294,295}. STZ has been found to exert its effects via several mechanisms including inhibition of insulin synthesis, suppression of insulin responsive cells with GLUT2 and a decrease in the ability of IR to autophosphorylate itself²⁹⁴⁻²⁹⁶.

In order to study the effects of STZ on the brain without causing systemic DM, STZ can be administered directly into the brain intracerebroventricularly (ICV). ICV administration of STZ induces a state of brain specific insulin dysregulation by inhibiting IR tyrosine kinase activity which is thought to be responsible for IR autophosphorylation^{278,297-300}. This model produces profound cognitive impairments including deficits in spatial learning, active avoidance, inhibitory avoidance, reference memory, and working memory^{298,301-303}. STZ has also been shown to increase various epitopes of hyperphosphorylated tau and increase the amounts of truncated tau^{278,304,305}. Rodent pups administered STZ exhibited striking neuronal loss, cerebral atrophy, and structures that resemble senile plaques in the brain, which is a particularly interesting finding since rats do not typically develop senile plaques even in other models of AD³⁰⁶. APP, $\text{A}\beta_{42}$ and various $\text{A}\beta_0$ have all been demonstrated to be increased following STZ administration and appear to be mediated through increased GSK-3 β activity³⁰⁷⁻³⁰⁹. While not a perfect

model of sAD, STZ-induced insulin dysregulation does show deficits associated with sAD and is helping to elucidate the mechanisms of DM and insulin dysregulation as a risk factor.

Neuroinflammation Risk Factor

Recent studies point to the involvement of neuroinflammatory response playing a fundamental role in the progression of the neuropathological changes observed in AD. There have been reports of immune-related proteins and cells located within and near amyloid plaques in AD brains since the 1980's³¹⁰⁻³¹². In the 1990's, several large observational and epidemiological studies were published suggesting that anti-inflammatory approaches, such as long-term anti-inflammatory treatment for rheumatoid arthritis, may be protective against developing AD, which has demonstrated as much as a six-fold sparing of AD³¹³⁻³¹⁶. A seminal study published in 2001³¹⁷ demonstrated that NSAIDs had the ability to prevent, or at least retard, the age of onset of AD by reducing the risk to 0.2%. This led to human trials and transgenic animal studies with non-steroidal anti-inflammatory drugs (NSAIDs) that demonstrated that NSAIDs can reduce AD pathology³¹⁸.

These epidemiological studies, retrospective observational studies and clinical trials all support the role that neuroinflammation plays, shedding light on another risk factor for developing sAD. Unlike other risk factors and genetic causes, neuroinflammation is not typically thought to be causal in nature but rather a result of one or more of the other AD pathologies and serves to rapidly progress the disease^{319,320}. Meaning that chronic brain inflammation itself may not be sufficient to develop AD, but rather, when neuroinflammation is present, the course of the disorder, namely cognitive

decline and pathological hallmarks, are exacerbated both in the magnitude and the pace at which AD progresses.

The brain was originally considered an “immune privileged” organ in early neuroscience approaches. Due to the lack of lymph nodes and the blood brain barrier, it was thought that the brain was not susceptible to inflammation or immune-activation. It is now well accepted that brain is fully capable of mounting an innate immune response. Inflammation that occurs within the brain differs from inflammation occurring within the periphery. The classical signs of inflammation such as swelling, redness, heat and pain are typically not seen in the brain but rather characterized by an increase in size and shape of microglia ^{14,321}.

Microglia are considered the first line of defense for the CNS against foreign objects and pathogens and are characterized by their ability to mount a rapid response to even the slightest change ³²¹. Microglia typically exist in a resting state, characterized by a distinct ramified morphology, where they constantly monitor the brain environment. Upon activation they demonstrate a rapid change in morphology to an amoeboid-like cell with engorged processes ^{322,323}. Activated microglia are able to express a wide range of proteins involved with a mounted immune response including major histocompatibility complex II (MHC II), pro-inflammatory cytokines, chemokines, compliment factors, reactive oxygen factors, proteases and C-reactive proteins ^{14,70,321}.

Cytokines in particular are small, nonstructural proteins secreted from a variety of both immune and non-immune cells, including neurons and glial cells ³²⁴. Cytokines have diverse roles which can include either the stimulation or inhibition of cell proliferation, apoptosis, and inflammatory responses ³²⁴. Cytokines consist of several classes including

interleukins (ILs), interferons (IFNs) and tumor necrosis factors (TNFs)^{325,326}. Cytokines can generally be classified into proinflammatory (IL-1 α , IL-1 β , TNF- α) or anti-inflammatory (IL-1ra, IL-4, IL-10); IL-6 can be either proinflammatory or anti-inflammatory depending on the amount and condition in which it was released³²⁴. There are numerous studies reporting increased levels of numerous proinflammatory cytokines including IL-1 α , IL-1 β , IL-6, TNF- α in AD brains, blood, and CSF^{15,321}.

Brain inflammation appears to be a double-edged sword, with a neuroprotective role when a limited acute-phase response occurs but with a detrimental role when a chronic response is mounted^{30,327}. The benefits of short term inflammation in the brain are significant and include microglia that degrade A β , remove the debris of dead and dying cells which reduces the likelihood of further cell loss, regulate brain development, and enhance neuronal survival^{39-41,328-330}. However, neuroinflammation can become neurotoxic when the response becomes chronic and uncontrolled leading to increased A β and senile plaque production, neuronal injury, and even cell death⁴². Chronically activated glial cells can kill adjacent neurons by secreting toxic products such as reactive oxygen intermediates, nitric oxide (NO) and proteolytic enzymes which can contribute towards the neuronal loss seen in AD^{42,70,331,332}. In disease states such as AD, Parkinson's disease and Prion disease, brain inflammation is a chronic rather than acute response characterized by an increase in not only the number and size of microglia but a sustained activation that does not readily terminate^{327,333}.

Early stage AD appears to exhibit a significant increase of activated microglia, before severe cognitive decline appears or neuronal loss is evident^{334,335}. Prior to development of symptoms, microglia are found clustered at the site of aggregated A β ³³⁶.

Furthermore, amyloid plaques contain several proteins associated with the inflammatory response including activated complement proteins and pro-inflammatory cytokines³³⁷.

Because AD and other neurodegenerative disorders increase in prevalence with age, it is interesting to note that normal aging in the brain is accompanied with an increased number of activated microglia, particularly those that overexpress IL-1 β ^{338,339}. Studies following microglial activity throughout the rodent lifespan reveal a progressive increase of microglial activation with age³⁴⁰⁻³⁴².

Microglia-mediated neurotoxicity, both in animal models and *in vitro* studies, tends to be progressive in nature, which parallels the progression in AD and other neurodegenerative diseases³⁴³⁻³⁴⁵. To demonstrate the neurotoxic role of overactivated microglia, lipopolysaccharide (LPS) is often used in animal studies. LPS, derived from the outer membrane of gram-negative bacteria, is capable of mounting an immune response through the activation of Toll-like receptor 4 (TLR4)³⁴⁶. Toll-like receptors are among the most well studied pattern recognition receptors (PRR), which are proteins that are expressed by cells of the innate immune system to recognize infectious agents and markers of cellular stress^{347,348}. There are currently 12 known TLRs and microglia have been identified expressing TLRs 1-9^{348,349}. LPS studies have demonstrated that only in the presence of microglia is there a neurotoxic effect indicating that microglia are necessary for the initiation of neuronal damage to occur^{350,351}. LPS activation of TLR4 has also demonstrated a progressive loss of neurons, particularly dopaminergic neurons^{352,353}. TLR4 activation has also been shown to have a neuroprotective role by promoting myelin repair and removing myelin debris in an animal model of brain injury following LPS administration³⁵⁴.

Additional support for the neuroinflammatory hypothesis is that head trauma and traumatic brain injury are environmental risk factors that have long been associated with AD³⁵⁵. In deceased patients, there is an increase of A β deposits 1-3 weeks post-injury in the cerebral cortex and it has been demonstrated that elevated IL-1 levels are directly responsible for this increased APP production and A β load³⁵⁵⁻³⁵⁷. IL-1 induces an increase of A β deposits, which in turn increase cytokine levels through microglial activation, thus creating a vicious, self-propagating cycle known as reactive microgliosis³⁵⁸⁻³⁶⁰. Overexpression of IL-1 has also been shown to increase IL-6 production, stimulate iNOS activity, and additional IL-1 production, further adding to the self-propagating cycle³⁶¹. Additionally, both IL-1 and IL-6 overproduction have been shown to stimulate the activation of cdk5 and kinase p38, both known to hyperphosphorylate tau^{362,363}. Reactive microgliosis is proposed to be one of the ways that neuroinflammation is implicated in accelerating the disease progression in AD brains^{41,336,364}.

Neuroinflammation appears to be initiated by A β pathology but serves a role in exacerbating both further A β production as well as tau hyperphosphorylation and NFTs formation. This dual role suggests that neuroinflammation could be a key link to the two leading AD pathologies, amyloid plaques and NFTs, as a risk factor for developing and progressing both types of AD.

Experimental Hypotheses and Implications

The vast majority of animal models of AD rely on genetic mutations that occur in fAD and provide valuable information regarding the role of A β and tau pathologies in the disease. These genetic models, however, do not represent the prevailing sporadic, late-onset AD. Considerable research has been conducted to help elucidate some of the risk

factors involved with sAD development, including DM (STZ) and neuroinflammation (LPS). Studies involving transgenic models of AD routinely activate the immune system to exacerbate AD-like deficits to better understand the role that neuroinflammation plays in AD. While considerable research has been invested in the STZ model of AD, an investigation of neuroinflammation in this particular model has yet to be conducted. Therefore, the purpose of this study was to begin a series of investigations on the effects of neuroinflammation in the DM animal model of AD. The necessary first step in these investigations was to determine whether an acute inflammatory response may be capable of altering AD like deficits in the DM animal model.

In order to disrupt insulin signaling in the brain, STZ-ICV was administered to adult male rats at a dose of 25 mg/mL (8 μ L per ventricle) consistent with the literature to induce a diabetic-like state within the brain. One week following surgery, animals received an immune challenge with a single administration of lipopolysaccharide (1 mg/mL, i.p.). Two weeks following the LPS challenge, animals began behavioral testing. Learning and memory were evaluated in the novel object recognition and Morris water maze tasks. We hypothesized that not only would the DM (STZ-treated animals) show learning and memory deficits but that the DM animals that received an immune challenge (STZ/LPS) would show more pronounced deficits in these tasks.

Following behavioral testing, we investigated protein changes consistent with those seen in AD. Specifically we evaluated A β , phosphorylated tau, receptor subunits of various neurotransmitter systems, and related proteins. We predicted that the DM animals (STZ) would show an increase in these particular protein markers and that the combination of STZ+LPS would further exacerbate these changes.

Hypothesis 1: We hypothesized that STZ, administered ICV, would replicate previous findings that lead to impairments in learning and memory, increased pTau, and increased A β peptides, consistent with AD. Furthermore, we anticipated alterations to various neurotransmitter systems that have been implicated in AD.

Implications for Hypothesis 1: If central infusion of STZ produces behavioral and AD-like pathological features, we add to the literature that suggests that 1) insulin signaling in the brain plays a role in learning and memory and 2) the disruption of insulin signaling in the brain leads to outcomes that are seen in AD, namely A β and tau pathologies, which may underlie DM as a risk factor for AD. Furthermore, replication of STZ-induced deficits allows us to investigate the role of inflammation (see Hypothesis 2) in this DM model.

Hypothesis 2: We hypothesize that by acutely activating the immune system via LPS in the STZ-model of AD, we will see an exacerbation of learning and memory deficits, as well as further elevations of both A β and tau pathologies.

Implications for Hypothesis 2: If we see an exacerbation of AD-like deficits, both behavioral and histopathological, in STZ-treated animals that also received LPS (STZ/LPS), then this experiment will add support that immune activation, even one-time, may be detrimental in a brain that is already at risk due to altered insulin signaling. If no further deficits are detected, then an acute immune activation is insufficient to have a profound negative affect on patients with DM. The possibility exists that acute immune activation will have no long term affects on this model of sAD but is a necessary first step in understanding the role of the immune system when combined with DM as risk factors.

CHAPTER 3

MATERIALS AND METHODS

Subjects

44 male Sprague-Dawley rats (n=11 per group) approximately 8 weeks in age and weighing 200-250 g were purchased from Taconic (Oxnard, CA). Rats were pair-housed in a temperature ($22 \pm 1^\circ\text{C}$) and humidity controlled facility until surgeries, after which they were individually housed with food and water available *ad libitum*. Lights were maintained on a 12:12 light/dark cycle, lights on at 7:00 am. All procedures were approved by the University of Nevada, Las Vegas Institutional Animal Care and Use Committee and carried out in accordance with NIH guidelines for the appropriate care and use of animals.

Surgery

Surgeries were performed under aseptic conditions. Anesthesia was a cocktail of ketamine (71 mg/kg) and dexmedetomidine (0.3 mg/kg) administered intraperitoneally (i.p.), as previously described^{365,366}. Bilateral intracerebroventricular infusions were performed by lowering a guide cannula into each lateral ventricle using the coordinates 0.7 mm posterior and 1.5 mm lateral and 3.5 mm ventral to the surface of the skull³⁶⁷. Using a 25 μL Hamilton syringe attached to a cannula and line, drugs were slowly infused at a rate of 1 μL every 10 seconds followed by 1 minute before removing the cannula. Burr holes were covered with dental acrylic, and the wound sutured closed. Atipamezole (0.5 mg/kg) was administered to reverse the effects of the anesthetic following the completion of the surgery. Upon ambulation, rats were administered an analgesic of Buprenorphine (0.05 mg/kg; i.p.) and then returned to the colony room.

Buprenorphine (0.05 mg/kg; i.p.) was administered for an additional two days to alleviate post-operative pain. Animals were individually housed following surgery and monitored for any post-operative complications.

Immune Activation

One week following the last surgery, animals received either saline or lipopolysaccharide (LPS) and were monitored for 4 hours for symptoms of general malaise and immune activation. Weights and temperatures were monitored every twenty-four hours following LPS administration, until there was no longer a difference in temperature, followed by a final temperature reading one week after return of temperature to baseline.

Treatment Groups

Streptozotocin (Sigma-Aldrich, St. Louis, MO) was dissolved in artificial cerebral spinal fluid (ACSF) to a concentration of 25 mg/mL. Animals received a bilateral ICV infusion of 8 μ L (per ventricle) of either ACSF or STZ. One week following surgery, animals received either saline (1 mg/mL; i.p.) or LPS (1 mg/kg; i.p.).

Behavioral Testing

One week following immune activation, animals were handled three times prior to the start of behavioral testing to reduce any possible anxiety in order to produce consistent data. Three weeks following STZ infusion, behavioral testing began consisting of open field (OF), novel object recognition (NOR), and Morris water maze (MWM). A simple timeline is provided below to show the basic outline of this experiment.

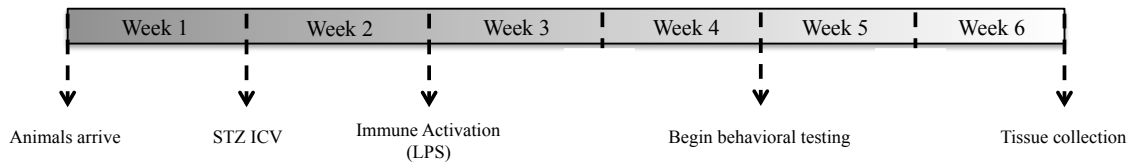


Figure 1. Experimental Timeline.

Open Field

Animals were placed in a large open chamber for five minutes to measure overall ambulatory activity and anxiety-like behavior. A tracking system (Smart, San Diego Instruments, San Diego, CA) was mounted above the chamber in order to track movement of the animal. Analyzed data included time spent in perimeter and overall distance traveled.

Novel Object Recognition

After introduction to the chamber during the open field task, NOR testing began 24 hours later. NOR consisted of a training day (Day 1) and a testing day (Day 2). During training, a pair of two identical objects (Object A) was placed in opposite corners of the chamber and animals were allowed to freely explore for five minutes, while the time spent exploring objects was recorded. Objects (Lego or PCV cylinder) were counterbalanced across groups as well as corner placements. 24 hours later, a long-term memory test was conducted consisting of a novel object (Object B) paired with an original object (Object A). Both NOR days were recorded by the tracking system and data analyzed included total time spent investigating object(s) and novel object preference. Manual scoring of object investigation time was conducted by multiple observers to ensure accurate data. Object investigation was defined as direct contact with the object or sniffing of the object within 2 cm of the animal's head.

Morris Water Maze

The Morris water maze task was conducted in a circular tank, 1.8 m in diameter and 76 cm in height, made of white polyethylene 4.7 mm in thickness (San Diego Instruments). Tap water, 48 cm deep, was maintained at a temperature of 25°C and made opaque by the addition of white non-toxic paint (Fresco Tempera Paint, Rich Art Color Company, Northvale, NJ), and changed every other day. The escape platform, a square platform 10 cm in diameter made of clear plastic, was placed in the center of one of the four quadrants (target quadrant), 30 cm from the inside wall of the maze and 1.5 cm below the surface of the water. For visible platform training, a large black and white cover was attached to the top of the platform and protruded 2 cm above the water.

Trials were recorded and captured using the video tracking system (Smart) recorded from a Sony Handycam camera connected to a Cobalt Instruments computer. Data collected for each trial consisted of a track of the animal, which includes the latency and distance traveled to locate the platform, speed of swimming, and thigmotaxis. On the probe trial the tracking system also recorded the amount of time subjects spent in each of the four quadrants of the maze, as well as the number of times the animal's path crossed over the previous platform location and its analogous location in each quadrant.

For the MWM procedure, subjects were taken individually from the colony room to a dedicated testing room containing the water maze, a computer desk, a table with the heating cage, and large geometric shapes positioned on each of the four walls, all serving as distal spatial cues. The rat was placed into the maze at one of three randomized locations, in the center of a quadrant that did not contain the escape platform (non-target quadrant). The rat was allowed to swim in the maze until it reached the hidden platform

and placed its forepaws on the platform. If after 60 seconds the animal did not locate the hidden platform, it was guided to the platform by the experimenter. The rat was given 20 seconds on the platform to orient to distal spatial cues and then placed under a heat lamp for a total of 30 seconds between trials.

Three additional trials were conducted in an identical fashion, for a total of four training trials per day. Following the fourth trial, the animal was dried and returned to its home cage. The training trials for the hidden platform were conducted until control subjects reached a latency criterion of less than 15 seconds (4-trial group mean). A probe trial was conducted twenty-four hours following achievement of this criterion. For the probe trial, the rat was placed in the maze in the same fashion as during training, but the escape platform was absent. The single probe trial was 60 seconds in duration, after which the rat was dried and returned to its home cage.

The day after completion of the probe trial, a two-day visible platform training protocol was employed. A visible platform that extends above the surface of the water (intra-maze cue) was placed into the maze instead of the hidden platform. Four trials per day were conducted for each animal in the same fashion as during the hidden platform training, with the exception that the platform location was changed on each trial. Visible platform training was conducted in order to detect any deficits in visual ability and motor function.

Tissue Collection

Animals were humanely euthanized via carbon dioxide asphyxiation the day following completion of MWM. Brains were quickly removed and the cortex,

hippocampus, and cerebellum were dissected out and flash frozen in dry ice. The dissected tissue was stored at -80° C until SDS/PAGE.

SDS-PAGE (Western Blots)

Tissue was homogenized in a non-denaturing lysis buffer consisting of 1X RIPA buffer (Cell Signaling; 20 mM Tris-HCL pH 7.5, 150 mM NaCL, 1 mM Na₂ EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, and 1 μg/ml leupeptin), 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 μg/ml aprotinin and 0.1% sodium dodecyl sulfate (SDS). Lysates were centrifuged at 15,000 x g for 15 minutes at 4°C, the supernatant were collected, and a protein assay to determine concentration was performed using the biciconinic acid method (BCA; Pierce, Rockford, IL). Samples (20 μg) were separated on 12-16% SDS-PAGE gels, depending on the protein of interest. Proteins were then electro-transferred to nitropure 45 micron nitrocellulose membranes, which were blocked in 5% milk in PBS with 0.05% and 0.1% sodium azide for two hours.

Individual membranes were then probed overnight at 4° C with one of the following primary antibodies diluted in 5% milk in PBS with 0.2% Tween (rabbit anti-amyloid oligomer 1:500, Millipore; goat anti-ChAT, 1:1,000, Millipore; rabbit anti-GABAB1, 1:2000, Cell Signaling; rabbit anti-GABAB2, 1:1000, Cell Signaling; mouse anti-GAD65, 1:2000, BD Pharmingen; rabbit anti-GIRK2, 1:1,000, Abcam; rabbit anti-GluR4, 1:1000, Cell Signaling; rabbit anti-IL-6, 1:250, Abcam; rabbit anti-NMDAR1 (NR1), 1:1000, Cell Signaling; rabbit anti-NMDA2A (NR2A), Cell Signaling; rabbit anti-NMDA2RB (NR2B), 1:1000, Cell Signaling; rabbit anti-phospho-GSK-3β, 1:500,

Cell Signaling; rabbit anti-phospho-Tau (pSer⁴⁰⁴), 1:1000, Sigma; rabbit anti-PP2A, 1:1,000, Cell Signaling; mouse anti-tau-5, 1:8,000, Millipore; mouse anti-TNFR1, 1:1000, ProteinTech; mouse or rabbit anti- β -actin, 1:20,000, ProteinTech). Detection of specific binding was performed by incubation with IRDye near-infrared secondary antibodies (1:5000 or 1:10,000, LiCor Biosciences, Lincoln, NE) for two hours at room temperature. Following washes in PBS+tween, membranes were imaged on an Odyssey CLx Infrared Imaging System (LiCor) and an average intensity was obtained for each sample. Each sample was run in duplicate with β -actin or tau to normalize protein levels.

Statistical Analyses

Differences in rectal temperature or weight were analyzed by one-way between subjects analysis of variance (ANOVA) with group as the factor. Open field data were analyzed by one-way between subjects ANOVA with group as the factor. The performance index (PI) of each group for NOR testing was analyzed by paired sampled t-tests compared to chance levels. MWM hidden and visible platform training data were analyzed by a repeated measures analysis of variance (RM-ANOVA) with days as the within subjects factor and group as the between subjects factor. Probe trial data were analyzed by one-way ANOVA with percent time in quadrant or annulus crossings as the factor. Western blot data were analyzed by one-way between subjects ANOVA with group as the factor. Tukey post-hoc comparisons of treatment groups were performed following any significant ANOVA to determine points of significance.

CHAPTER 4

RESULTS

Temperatures

Immediately prior to LPS administration, no differences in baseline temperature were found ($F_{3,38}=0.552$, $p>0.05$; see figure 2). Twenty-four hours following LPS, both LPS treated groups experienced significantly elevated temperatures ($F_{3,38}=25.185$, $p<0.001$; Tukey post-hoc: controls vs LPS $p<0.001$, controls vs STZ/LPS $p<0.001$; see figure 2). Temperatures remained elevated forty-eight hours later for both groups administered LPS ($F_{3,38}=17.691$, $p<0.001$; Tukey post-hoc: controls vs LPS $p<0.001$, controls vs STZ/LPS $p<0.001$, see figure 2). By the third day after LPS, there was still a significant difference ($F_{3,38}=4.165$, $p<0.05$, see figure 2) but Tukey post-hoc comparisons did not reveal any significant differences between controls and the other treatment groups. By ten days later, all signs of a mounted fever response had completely disappeared ($F_{3,38}=0.848$, $p>0.05$, see figure 2).

Weights

Weights were tracked along with temperatures to determine if LPS had any effect on body weight. Baseline weights prior to the administration of LPS revealed no differences ($F_{3,38}=1.296$, $p>0.05$; see figure 3). This baseline was established one week following surgery, indicating that STZ infusion had no immediate effect on weights. Immediately following immune activation, a significant difference in weights emerged with STZ/LPS having a lower body weight than controls ($F_{3,38}=3.044$, $p<0.01$; Tukey post-hoc: controls vs STZ/LPS $p<0.01$, see figure 3). Additionally, Tukey post-hoc analysis revealed that STZ-treated animals had significantly higher weights than STZ/LPS

($p < 0.05$, see figure 3).

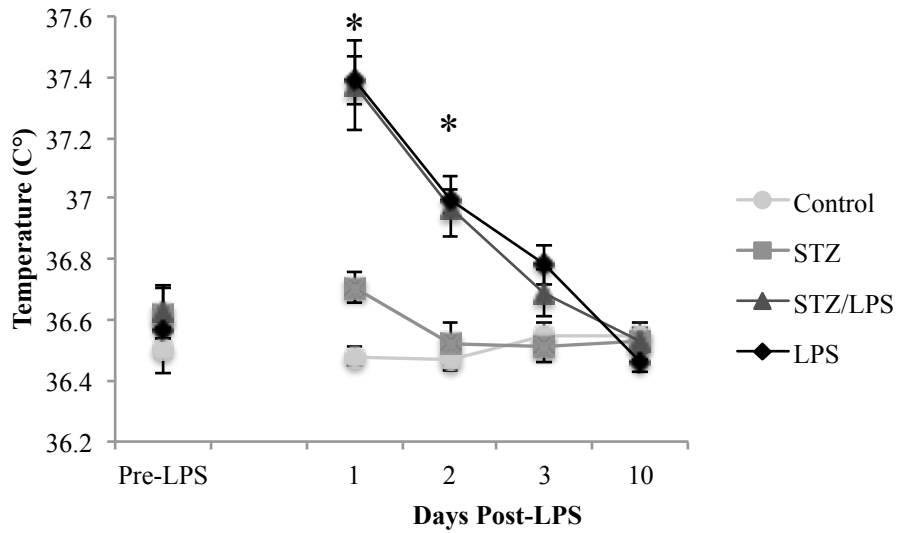


Figure 2. Temperature Data. Prior to LPS administration (Pre-LPS), no significant differences (\pm SEM) existed between treatment groups. Following LPS injection, LPS- and STZ/LPS-treated animals had significantly elevated temperatures for 48 hours, after which temperatures began to return to baseline. * = significantly different than controls ($p < 0.05$).

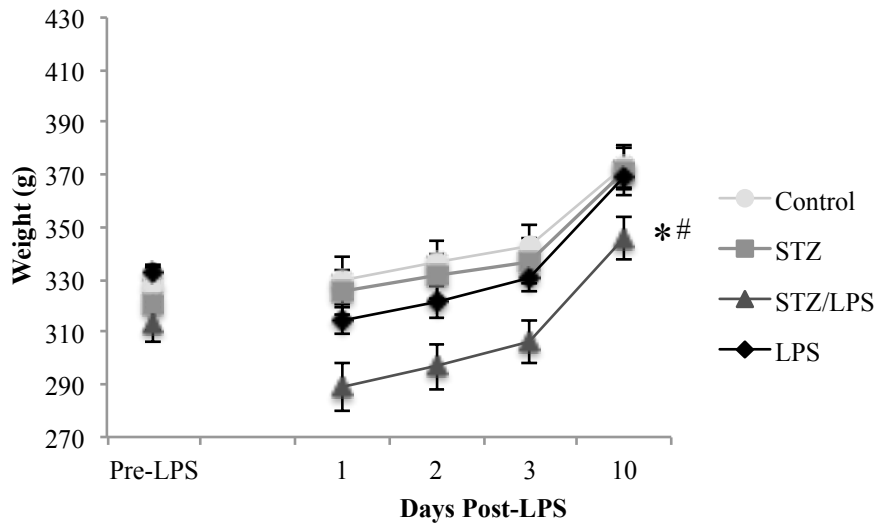


Figure 3. Weight Data. Prior to LPS administration, no significant differences in average weight (\pm SEM) were noted. Following LPS injection, STZ/LPS experienced a significantly lowered body weight. * = significantly different than controls ($p < 0.05$). # = STZ significantly different than STZ/LPS

Other than the transient increase in temperature, one-time LPS administration alone did not have any additional effects in measures of open field, spatial learning in MWM, or Western blots. We analyzed controls versus LPS-treated animals and found no additional significant differences. No differences were observed during open field in distance traveled ($F_{1,19}=3.716$, $p>0.05$) or time spent in perimeter ($F_{1,19}=2.072$, $p>0.05$). No differences were observed in Day 1 NOR exploration time ($F_{1,19}=0.046$, $p>0.05$), Day 2 Performance Index compared to controls ($F_{1,19}=0.022$, $p>0.05$), and performance in Day 2 Performance Index was above chance levels, similar to controls ($t_{10}=4.545$, $p<0.01$). No differences were observed in the MWM in hidden training latency ($F_{1,57}=1.531$, $p>0.05$), hidden training speed ($F_{1,57}=0.256$, $p>0.05$), hidden training thigmotaxis ($F_{1,57}=1.409$, $p>0.05$), visible training latency ($F_{1,82}=0.058$, $p>0.05$), visible training speed ($F_{1,82}=0.351$, $p>0.05$), and visible training thigmotaxis ($F_{1,82}=3.169$, $p>0.05$). The LPS-treated animals demonstrated a selective search during the MWM probe by spending significantly more time in the target quadrant ($F_{3,40}=26.125$, $p<0.05$; Tukey post-hocs of target quadrant versus each quadrant $p<0.01$) and in annulus crossings ($F_{3,40}=18.052$, $p<0.001$), indicating that acute immune activation did not impair any measure of learning and memory observed in this study. No significant differences between controls and LPS in levels of total oligomeric species ($F_{1,18}=3.765$, $p>0.05$) nor in the individual oligomeric species analyzed (7mer ($F_{1,18}=0.234$, $p>0.05$), 8mer ($F_{1,18}=0.197$, $p>0.05$), 9mer ($F_{1,18}=0.573$, $p>0.05$), 10mer ($F_{1,18}=2.056$, $p>0.05$), 11mer ($F_{1,18}=3.957$, $p>0.05$), 12mer ($F_{1,18}=0.049$, $p>0.05$), 16mer ($F_{1,18}=0.045$, $p>0.05$), 18mer ($F_{1,18}=0.974$, $p>0.05$), 20mer ($F_{1,18}=0.505$, $p>0.05$), 22mer ($F_{1,18}=0.082$, $p>0.05$), 24mer ($F_{1,18}=0.052$, $p>0.05$), 26mer ($F_{1,18}=1.473$, $p>0.05$)). Additionally, there were no

differences in levels of tau ($F_{1,18}=0.000$, $p>0.05$), pTau Ser404 ($F_{1,18}=0.081$, $p>0.05$), pGSK-3 β ($F_{1,18}=0.34$, $p>0.05$), NR1 ($F_{1,18}=0.528$, $p>0.05$), NR2A ($F_{1,18}=1.228$, $p>0.05$), NR2B ($F_{1,18}=0.127$, $p>0.05$), GluR4 ($F_{1,18}=0.34$, $p>0.05$), GABA_{B1a} ($F_{1,18}=0.193$, $p>0.05$), GABA_{B1b} ($F_{1,18}=0.05$, $p>0.05$), GABA_{B2} ($F_{1,18}=0.594$, $p>0.05$), GAD65 ($F_{1,18}=0.67$, $p>0.05$), ChAT ($F_{1,18}=0.308$, $p>0.05$), PP2A ($F_{1,18}=0.004$, $p>0.05$), GIRK2 ($F_{1,18}=0.955$, $p>0.05$), IL-6 ($F_{1,18}=0.002$, $p>0.05$), and TNFR1 ($F_{1,18}=0.758$, $p>0.5$).

Because the control group and LPS-treated animals were statistically indistinguishable from one another in all behavioral tasks and protein level analyses, LPS was removed from all further analyses to remove additional variance. Analysis of controls vs LPS alone was necessary to cut down costs and excessive use of animals in future experiments by ensuring that a one-time LPS injection did not result in any long-lasting measurable changes. Studies that administer LPS to transgenic AD models typically do not include an LPS-alone group³⁶⁸⁻³⁷², however, we wanted to verify that we would be able to eliminate this particular group from this study and any future acute inflammatory studies by confirming that an acute LPS administration was indeed statistically indistinguishable from controls. All analyses conducted below include only controls, STZ, and STZ/LPS.

Open Field

Open field testing was conducted to both measure overall exploratory behavior and to determine if any differences in anxiety-like behavior exists. No differences were observed in the time that the animals spent in the perimeter of the arena ($F_{2,27}=0.41$, $p>0.05$; see figure 4a). Additionally, no differences were observed in the overall distance that the animals traveled throughout the arena ($F_{2,27}= 2.165$, $p>0.05$; see figure 4b).

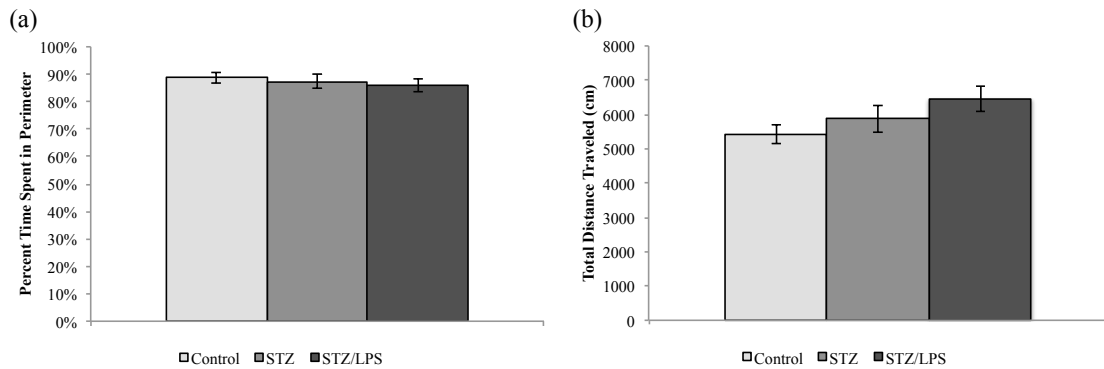


Figure 4. Open Field Data. No significant differences were observed in (a) the percent time spent (\pm SEM) in the perimeter of the open field arena or (b) the total distance travelled (\pm SEM).

Novel Object Recognition

On the first day of novel object recognition (NOR), animals were placed into the same arena used for open field with two identical objects (either identical Lego blocks or identical PVC cylinders). No significant differences were observed in the total amount of time that animals spent investigating the identical objects ($F_{2,27}=0.014$, $p>0.05$; see figure 5a). Twenty-four hours later, animals were exposed to one of the objects from the previous day and a novel object (one of the objects that wasn't previously presented). The controls spent significantly more time with the novel object than chance levels would predict ($t_9=2.268$, $p<0.05$, see figure 5b). STZ and STZ/LPS failed to investigate the novel object more than chance levels ($t_9=-.985$; $p>0.05$ and $t_9=-1.742$; $p>0.05$, respectively, see figure 5b). Additional analysis regarding the novel object performance index showed that STZ and STZ/LPS spent significantly less time with the novel object compared to controls ($F_{3,37}=6.592$, $p<0.01$; Tukey post-hoc: controls vs STZ $p<0.05$, controls vs STZ/LPS $p<0.01$, see figure 5b).

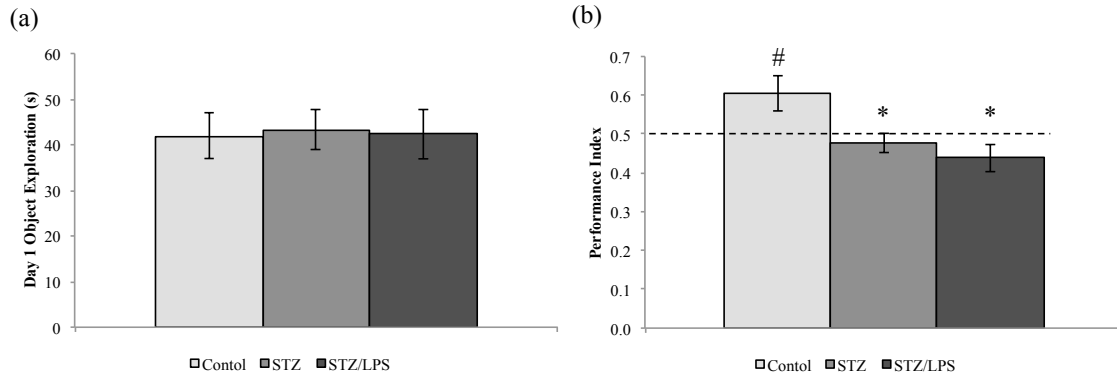


Figure 5 Novel Object Recognition Data. (a) Day one NOR, no significant differences were observed in the Day 1 total exploration (\pm SEM) of two Object A. (b) Day two NOR, controls spent significantly more time with the novel object (Object A) than the old object (Object B) than chance levels. STZ and STZ/LPS spent significantly less time exploring the novel object compared to controls. # = significantly greater than chance levels ($p < 0.05$). * = significantly different than controls ($p < 0.05$).

Morris Water Maze

Latency to locate the hidden platform across successive days was measured for six consecutive days. STZ and STZ/LPS exhibited significantly longer latencies to find the hidden platform ($F_{2,121}=10.613$, $p < 0.001$; Tukey post-hoc: controls vs STZ $p = 0.01$, controls vs STZ/LPS $p < 0.01$, see figure 6a). The overall distance traveled to locate the platform was significantly longer for the STZ group alone ($F_{2,89}=4.559$, $p < 0.05$; Tukey post-hoc: controls vs STZ $p < 0.05$, see figure 6b). However, it should be noted that technical difficulties arose with the tracking system on Day 5 of hidden training and distance data of the path length of 8 animals was lost, potentially influencing these data. No differences were identified in swim speed during hidden platform training ($F_{2,89}=1.064$; $p > 0.05$, see figure 6c). Thigmotaxis, the time spent swimming in the outer perimeter of the maze, was significantly elevated for STZ during hidden training ($F_{2,89}=10.227$; $p = 0.001$; Tukey post-hoc: controls vs STZ $p < 0.001$, see figure 6d).

Twenty-four hours after the completion of the last day of hidden training, a probe trial was conducted. The STZ group failed to demonstrate a selective search as measured by spending significantly more time in the target quadrant (where the platform was previously located) compared to all the other non-target quadrants ($F_{3,36}=6.442$, $p<0.05$; Tukey post-hoc: target vs adjacent left $p<0.05$, target vs opposite $p>0.05$, target vs adjacent right $p<0.05$, see figure 7a). Controls and STZ/LPS displayed a selective search during the probe trial (Controls: $F_{3,36}=15.427$, $p<0.001$, Tukey post-hoc: target vs adjacent left $p<0.001$, target vs opposite $p<0.001$, target vs adjacent right $p<0.001$; STZ/LPS: $F_{3,40}=11.348$, $p<0.001$; Tukey post-hoc: target vs adjacent left $p<0.001$, target vs opposite $p<0.001$, target vs adjacent right $p<0.001$, see figure 7a).

Annulus crossings, the number of times that the animal crosses the location where the hidden platform was previously located as well as the analogous location in each of the three non-target quadrants, is another measure of probe trial performance. Similarly to time spent in target quadrant, STZ failed to demonstrate a selective search when annulus crossings were analyzed ($F_{3,36}=2.468$, $p>0.05$, see figure 7b). Controls and STZ/LPS displayed a selective search during the probe trial when annulus crossings were measured (Controls: $F_{3,36}=7.382$, $p<0.001$, Tukey post-hoc: target vs adjacent left $p<0.01$, target vs opposite $p<0.01$, target vs adjacent right $p<0.001$; STZ/LPS: $F_{3,40}=8.789$, $p=0.000$; Tukey post-hoc: target vs adjacent left $p<0.001$, target vs opposite $p<0.001$, target vs adjacent right $p<0.000$, see figure 7b).

Following the probe trial, visible platform training was performed to assess visuomotor abilities. Significant differences were found in the latency to find the visible platform in the STZ/LPS group ($F_{2,121}=6.058$, $p<0.05$; Tukey post-hoc: controls vs

STZ/LPS $p < 0.01$, see figure 6a). Due to the difference in visible latency, a forepaw reach was performed but no differences in basic visual function were observed. Path length to locate the visible platform was also elevated in STZ/LPS ($F_{2,121}=6.313$, $p < 0.01$; Tukey post-hoc: controls vs STZ/LPS $p < 0.01$, see figure 6b). No differences were observed in swim speed during visible training ($F_{2,89}=1.064$, $p > 0.05$; see figure 6c). Thigmotaxis was significantly elevated for both STZ and STZ/LPS during visible training ($F_{2,121}=11.647$, $p < 0.001$; Tukey post-hoc: controls vs STZ $p < 0.001$, controls vs STZ/LPS $p < 0.001$, see figure 6d).

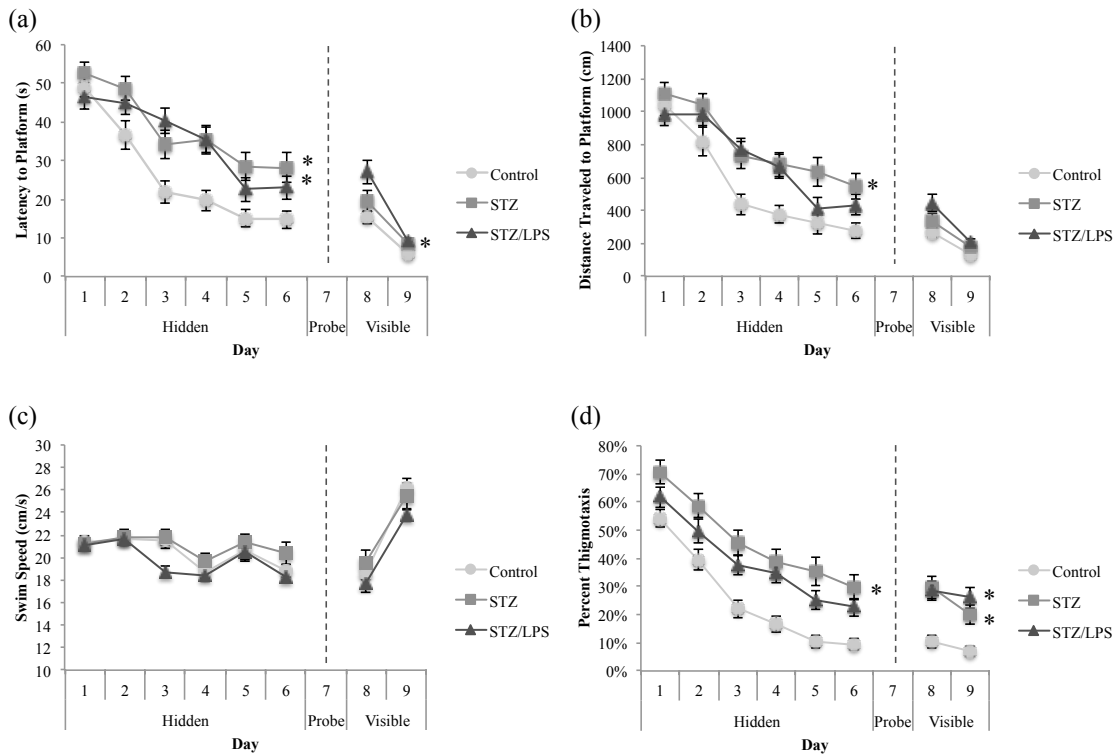


Figure 6 Morris Water Maze Data: Hidden and Visible Training. (a) Mean latency (\pm SEM) to find the platform. STZ and STZ/LPS had significantly longer latencies in hidden training, while STZ/LPS had a significantly longer visible latency. (b) Mean path length (\pm SEM) to find the platform. STZ showed a longer path length during hidden training; STZ/LPS demonstrated a longer path length during visible training. (c) Mean swim speed (\pm SEM) yielded no significant differences. (d) Percent thigmotaxis (\pm SEM). STZ displayed a significant increase in thigmotaxis during hidden training. Both STZ and STZ/LPS displayed a significant increase in perimeter swimming during visible training. * = Significantly different than controls ($p < 0.05$)

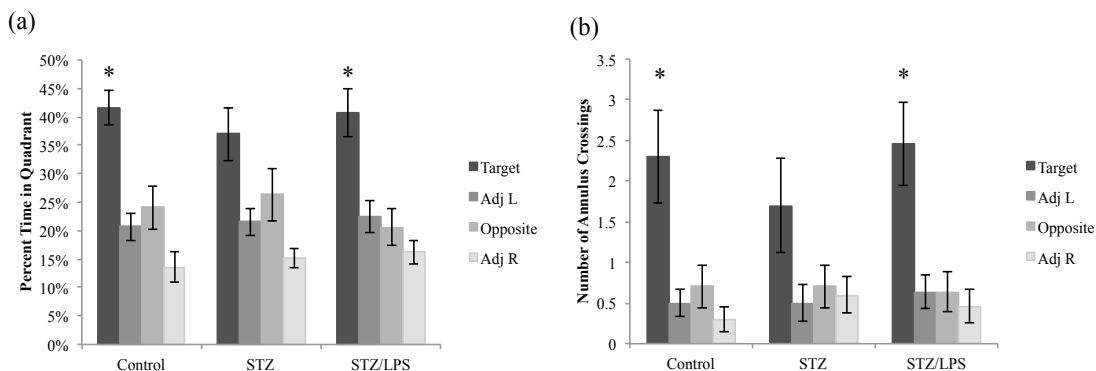


Figure 7 Morris Water Maze Data: Probe Trial. (a) Mean proportion time spent in target quadrant compared to the other non-target quadrants. Controls and STZ/LPS displayed selective searches while STZ failed to demonstrate a selective search. (b) Mean number of annulus crossings in target quadrant compared to analogous areas in non-target quadrants. Controls and STZ/LPS displayed a selective search while STZ did not demonstrate a selective search. * Target quadrant is significantly different than all three non-target quadrants ($p < 0.05$).

SDS-PAGE (Western Blots)

Protein levels of the three major pathological hallmarks: A β deposition, hyperphosphorylated tau and cholinergic loss were examined in the hippocampus. To explore the effects on A β , oligomeric A β was probed. A β oligomers can be identified in these western blots starting at 7mer and increasing up to 24mer. STZ and STZ/LPS groups showed significant elevations when all oligomeric species were combined ($F_{2,387}=26.209$, $p < 0.001$; Tukey post-hoc: controls vs STZ $p < 0.001$, controls vs STZ/LPS $p < 0.001$, see figure 8a). Post-hoc analysis also revealed that while both STZ and STZ/LPS were significantly elevated, there was a significant difference between the STZ group and the STZ/LPS group in total oligomeric A β species (Tukey post-hoc: STZ vs STZ/LPS $p < 0.05$, see figure 8a). Analysis of individual oligomeric species indicated a significant increase for both STZ and STZ/LPS, including the 11mer ($F_{2,27}=5.816$,

p<0.01; Tukey post-hoc Controls vs STZ p<0.05, Controls vs. STZ/LPS p<0.05), and 16mer ($F_{2,27}=8.943$, p<0.01; Tukey post-hoc Controls vs STZ p<0.05, Controls vs. STZ/LPS p<0.05). Interestingly, only STZ alone showed significantly elevated levels of 10mer ($F_{2,27}=3.828$, p<0.05; Tukey post-hoc Controls vs STZ p<0.05) and 22mer ($F_{2,27}=4.985$, p=0.014; Tukey post-hoc Controls vs STZ p<0.05), indicating that the acute immune response may have prevented the increase in certain A β oligomeric species observed in the STZ alone group. No differences were observed in 7mer ($F_{2,27}=2.399$, p>0.05), 8mer ($F_{2,27}=1.252$, p<0.05), 9mer ($F_{2,27}=2.872$, p>0.05), 12mer ($F_{2,27}=2.476$, p>0.05), 18mer ($F_{2,27}=1.793$; p<0.05), 20mer ($F_{2,27}=2.518$, p<0.05), 24mer ($F_{2,27}=0.504$, p>0.05) and 26mer ($F_{2,27}=1.046$, p=0.365). Please refer to Table 1 below, for a complete summary of oligomeric species.

Oligomer	STZ	STZ/LPS	F value	p value
8mer	1.76 (\pm .14)	1.37 (\pm .47)	1.252	>0.05
9mer	2.35 (\pm .13)	1.55 (\pm .36)	2.872	>0.05
10mer	2.90 (\pm.26)**	1.73 (\pm .40)	3.828	<0.05*
11mer	1.64 (\pm.15)**	1.75 (\pm.23)**	5.816	<0.01*
12mer	1.46 (\pm .10)**	1.26 (\pm .22)	2.476	>0.05
16mer	1.76 (\pm.13)**	1.50 (\pm.15)**	8.943	<0.001*
18mer	1.45 (\pm .23)	1.78 (\pm .42)	1.793	>0.05
20mer	1.7 (\pm .25)	1.47 (\pm .29)	2.518	>0.05
22mer	2.04 (\pm.27)**	1.61 (\pm .27)	4.985	<0.05*
24mer	1.07 (\pm .14)	1.17 (\pm .14)	0.504	>0.05

Table 1 A β Oligomers. Western blot data from the hippocampus showing mean A β oligomers (\pm SEM). Values shown are proportion to controls, which is set at 1. STZ and STZ/LPS showed elevated levels for species 11mer and 16mer. STZ showed further increases for 10mer and 22mer.

Levels of choline acetyltransferase (ChAT) were also evaluated to determine if there were alterations to cholinergic signaling. No differences were found in levels of ChAT between any of the groups ($F_{2,27}=1.23$, p>0.05, see figure 9a).

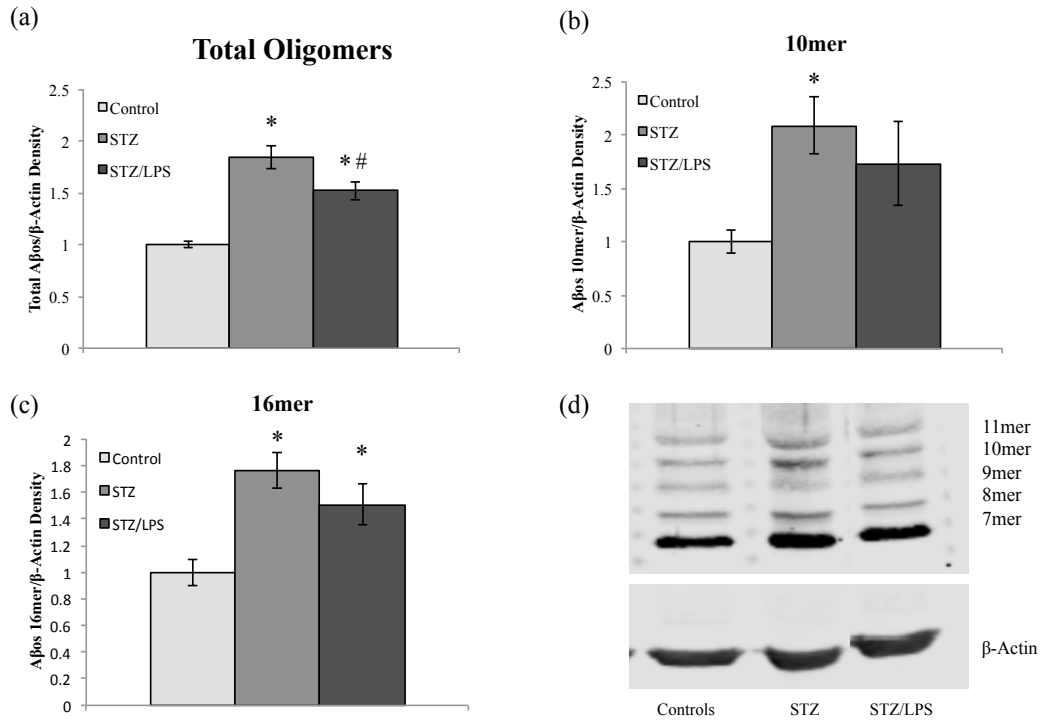


Figure 8 Western Blot Data: Aβ Oligomers (a) Total oligomers were elevated for both STZ and STZ/LPS. STZ/LPS experienced significantly less of an increase compared to STZ. (b) 10mer, a representative species of those significantly elevated in STZ but not in STZ/LPS. (c) Representative western blot of Aβ oligomers. (d) 16mer, a representative species of those significantly elevated in both STZ and STZ/LPS. * = significantly different than controls ($p < 0.05$). # = STZ significantly different than STZ/LPS ($p < 0.05$).

Evaluation of phosphorylated tau, specifically at the Serine 404 phosphorylation site, revealed significantly elevated levels in the hippocampus of both STZ and STZ/LPS ($F_{2,27}=8.83$, $p < 0.01$; Tukey post-hoc: controls vs STZ $p < 0.01$, controls vs STZ/LPS $p < 0.01$, see figure 9b). The amount of phosphorylated tau was investigated as a ratio to overall tau levels (pTau/Tau). Because overall tau levels served as the housekeeping protein, we also analyzed overall tau levels separately to ensure there was not a baseline bias when calculating pTau/Tau. Analysis of overall tau levels did not reveal any significant differences ($F_{2,27}=0.063$, $p > 0.05$, data not shown).

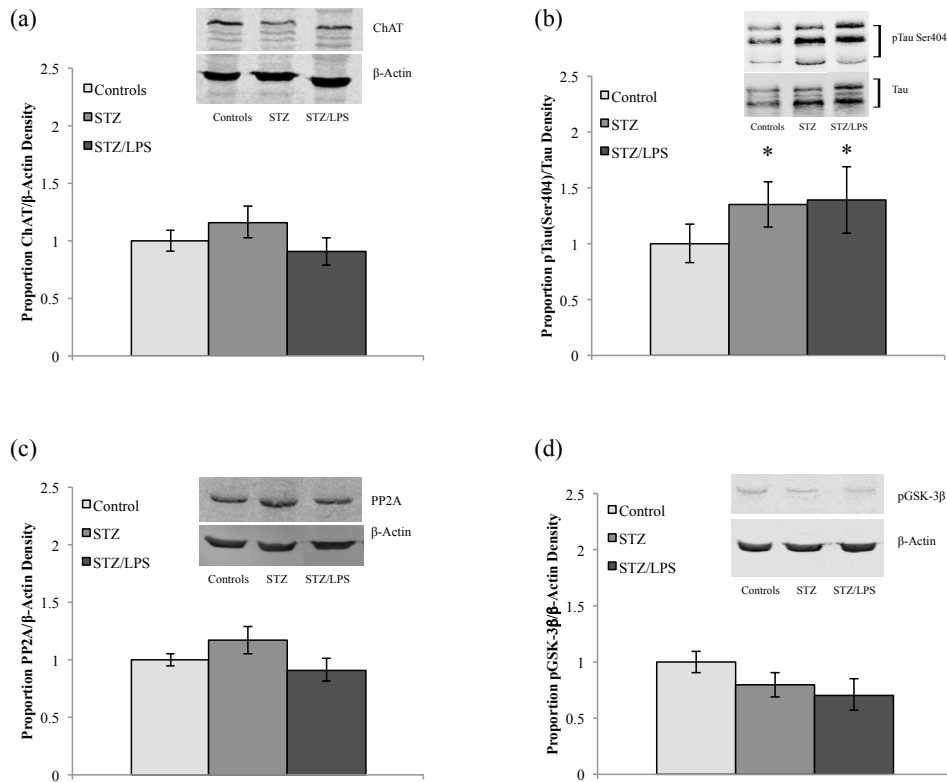


Figure 9. Western Blot Data: ChAT, pTau, PP2A, pGSK-3β. (a) Proportion (\pm SEM) and representative blot of ChAT. No differences were observed. (b) Proportion (\pm SEM) and representative blot of pTau/Tau. Levels of pTau were significantly elevated in both STZ/ and STZ/LPS. (c) Proportion (\pm SEM) and representative blot of PP2A. No differences were observed. (d) Proportion (\pm SEM) and representative blot of pGSK-3β. There was a non-significant trend towards a decrease in both STZ and STZ/LPS. * = significantly different than controls ($P < 0.05$).

Evaluations of both a phosphatase, responsible for dephosphorylation of tau, and kinase, involved in tau phosphorylation, were conducted to investigate potential mechanisms behind the elevated pTau levels. GSK-3β, a kinase that phosphorylates tau at almost every known phosphorylation site, is itself inactivated when phosphorylated. We examined phosphorylated GSK-3β and found non-significant decrease ($F_{2,27}=1.805$, $p > 0.05$, see figure 9d) indicating that a reduction in the amount of inactive phosphorylated GSK-3β would signify higher amounts of non-phosphorylated GSK-3β. This could account for the increased phosphorylated tau levels seen previously. Protein

phosphatase 2 (PP2A) is known to remove dephosphorylate tau. No significant differences were found in PP2A levels ($F_{2,27}=1.096$, $p>0.05$, 9c).

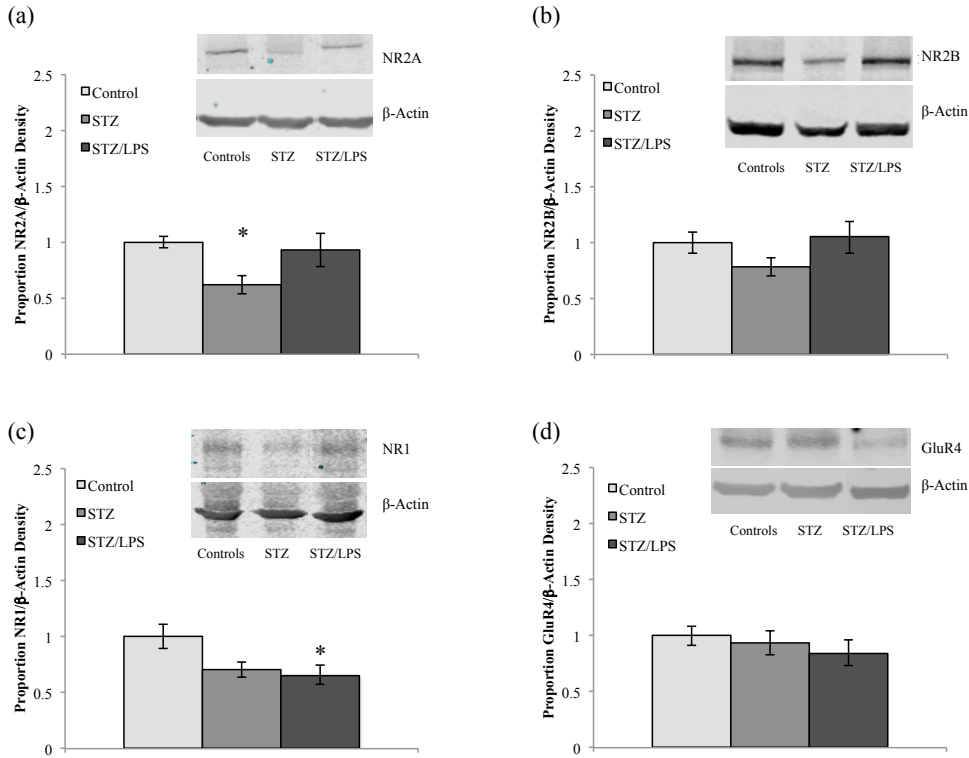


Figure 10 Western Blot Data: Glutamatergic Related Proteins. (a) Proportion (\pm SEM) and representative blot of NR2A receptor subunit. Expression of NR2A in STZ was significantly reduced. (b) Proportion (\pm SEM) and representative blot of NR2B receptor subunits. No significant differences were observed. (c) Proportion (\pm SEM) and representative blot of NR1 receptor subunit. Expression of NR1 in STZ/LPS was significantly reduced. (d) Proportion (\pm SEM) and representative blot of GluR4. No significant differences were observed. * = significantly different that controls ($p<0.05$).

Protein levels of multiple specific receptor subtypes related to glutamatergic signaling were also analyzed in an effort to determine connections between the behavioral and biochemical changes. The evaluation of the NR2A subunit of NMDA receptors revealed a significant reduction in STZ ($F_{2,27}=4.751$, $p<0.05$; Tukey post-hoc: controls vs STZ $p<0.05$, see figure 10a). There was also a significant decrease of the NR1 receptor subunit in the hippocampus for STZ/LPS ($F_{2,27}=4.192$, $p<0.05$; Tukey post-hoc

Controls vs STZ/LPS $p < 0.05$, see figure 10b). No differences were observed in hippocampal NR2B subunit levels ($F_{2,27} = 1.637$, $p > 0.05$, see figure 10c). Additionally, the AMPA receptor subunit GluR4 did not show any differences between groups ($F_{2,27} = 0.324$, $p > 0.05$, see figure 10d).

Examinations of multiple receptors and proteins related to GABAergic signaling were conducted. Examination of the GABA_{B1a} subunit revealed a significant increase in the STZ group ($F_{2,30} = 3.813$, $p < 0.05$; Tukey post-hoc: controls vs STZ $p < 0.05$, see figure 11a). Levels of GABA_{B1b} were also significantly elevated for the STZ group ($F_{2,30} = 3.656$, $p < 0.05$; Tukey post-hoc: controls vs STZ $p < 0.05$, see figure 11a). Additionally, Tukey post-hoc revealed that GABA_{B1b} levels were significantly different between STZ and STZ/LPS (Tukey post-hoc: STZ vs STZ/LPS $p < 0.05$, see figure 11a). Evaluation of the GABA_{B2} receptor did not reveal any significant differences in any of the groups, despite a trend towards an increase in the STZ group ($F_{2,27} = 3.081$, $p > 0.05$, see figure 11b). No differences were found in levels of GIRK2, an inwardly-rectifying potassium channel regulated by GABA_B ($F_{2,27} = 0.436$, $p > 0.05$, see figure 11d). Similarly, no differences were detected in GAD65 levels ($F_{2,27} = 0.537$, $p > 0.05$, see figure 11c).

Several inflammatory markers were also investigated to determine if there were any lasting changes to the immune response. Examination of interleukin-6 did not yield any significant differences ($F_{2,27} = 1.224$, $p > 0.05$, see figure 12a). The evaluation of tumor necrosis factor receptor 1 (TNFR1), a receptor for TNF- α , did not reveal any differences between treatment groups ($F_{2,27} = 0.334$, $p = 0.719$, see 12b).

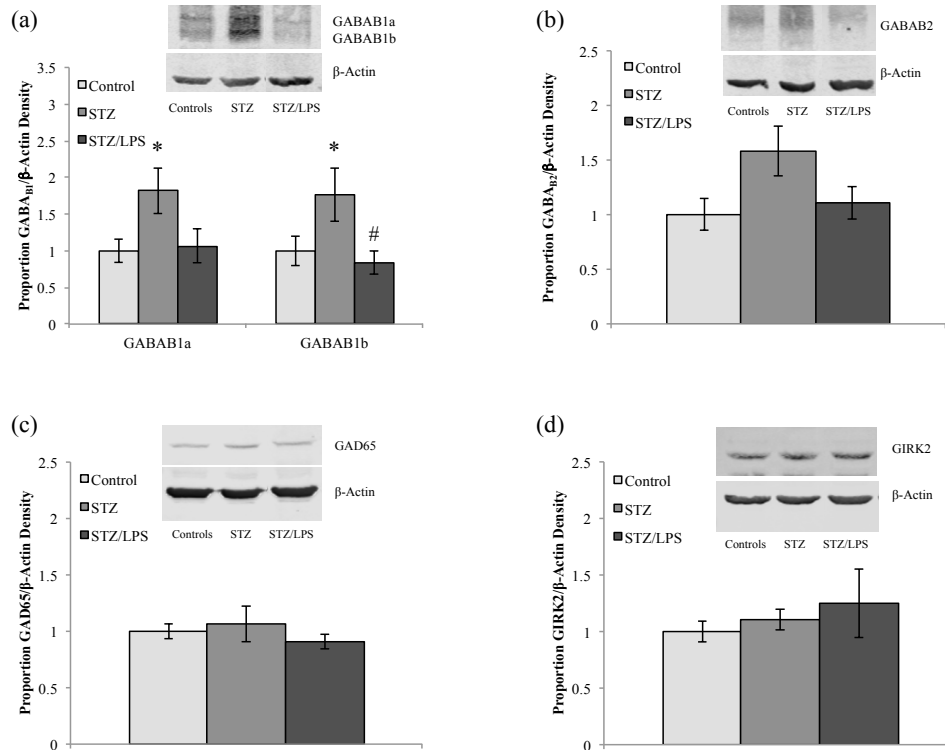


Figure 11 Western Blot Data: GABAergic Related Proteins. (a) Proportion (\pm SEM) and representative blot of GABA_{B1a} and GABA_{B1b} receptor subunits. There was a significant elevation of GABA_{B1a} and GABA_{B1b} in STZ. Additionally, expression of GABA_{B1b} was significantly different between STZ and STZ/LPS. (b) Proportion (\pm SEM) and representative blot of GABA_{B2}. A non-significant increase was observed for STZ. (c) Proportion (\pm SEM) and representative blot of GAD65. No significant differences were observed. (d) Proportion (\pm SEM) and representative blot of GIRK2. No significant differences were observed. * = significantly different than controls ($p < 0.05$). # = STZ significantly different than STZ/LPS ($p < 0.05$).

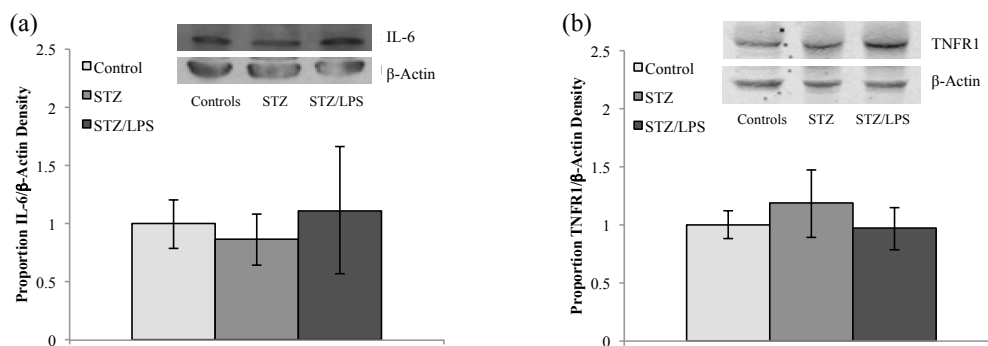


Figure 12 Western Blot Data: Inflammatory Related Proteins. (a) Proportion (\pm SEM) and representative blot of IL-6. No significant differences were observed. (b) Proportion (\pm SEM) and representative blot of TNFR1. No significant differences were observed.

CHAPTER 5

DISCUSSION, CONCLUSION, AND FUTURE DIRECTIONS

The above study was designed to investigate the effects of an acute inflammatory response in a diabetic model of sporadic Alzheimer's disease. Specifically, we found that the infusion of STZ-ICV (Hypothesis 1) led to an impairment in non-spatial (NOR) and spatial (MWM) learning, alterations in several pathological hallmarks of AD, and disruptions to both glutamatergic and GABAergic signaling. Further, we hypothesized that a one-time administration of LPS to STZ-ICV animals (STZ/LPS) would further exacerbate the STZ-induced deficits (Hypothesis 2), however, we saw a subtle improvement in spatial learning, a reduction in A β levels compared to the diabetic alone model, and restoration of several NMDA and GABA receptor subunits.

The diabetic model of AD utilizes streptozotocin, a diabetogenic compound. It has been reported that STZ-ICV disrupts glucose utilization through an insulin-dependent mechanism and targets myelin in the cerebral cortex and hippocampus³⁰², leading to memory and protein changes similar to those found in sAD. In this study, STZ induced both learning and memory deficits, as well as an increase in several pathological features of AD without any differences in weight.

Temperatures and weights were tracked following LPS injection to determine if an immune response was induced. Indeed, following LPS administration, temperatures were significantly elevated in both LPS-treated groups (LPS and STZ/LPS) for the first 48 hours and then began to return to baseline after 72 hours. One week later, temperatures had completely returned to baseline levels. This temperature data supports other studies showing that following LPS administration, fever peaks between 6-8 hours and then

gradually returns to baseline^{373,374}. Additionally, body weights decreased slightly in the STZ/LPS treatment group immediately following LPS but began to return to normal after a week, completely returning to normal body weights by the end of the experiment. Literature supports that LPS has a pyrogenic effect, attributable weight loss, and induces the release of numerous proinflammatory cytokines, including TNF- α , IL-1 β and IL-6^{375,376}. More importantly, peripheral LPS induces neuroinflammation by increasing brain cytokine levels of TNF- α , IL-1 β and IL-6³⁷⁷⁻³⁸⁰ and activating microglia^{381,382}. We can thus assume, based upon the weight loss and increased body temperatures following LPS administration, cytokine levels were elevated in both plasma and in the brain and that microglia were activated throughout the brain.

Animals were tested in the open field for locomotor deficits and anxiety-like behavior. Because no differences were observed in this task, any differences observed in the learning and memory tasks (NOR and MWM) cannot be attributed to differences in either exploratory behavior or motoric abilities.

The same testing arena used for OF was also used for NOR, the first learning and memory task. On the first day of NOR, also referred to as the familiarization phase, the subject was placed in the arena with two identical objects (A+A or B+B) and allowed to freely explore for 5 minutes. No differences were observed in exploration time, indicating that no treatment group showed a preference for one object over the other which would suggest a bias towards one side of the chamber (place preference). Additionally, regardless of which objects were presented during Day 1 (A+A or B+B) there was no difference in total time spent investigating the objects (object preference). Twenty-four hours later, animals were placed back in the arena with one old object and one novel

object (A+B). STZ and STZ/LPS exhibited significant reductions in their preference for exploring the novel object. NOR is one way of testing recognition memory in rodents, which has been shown to be mostly dependent upon the perirhinal cortex and hippocampus^{383,384}. Previous work demonstrates that the perihinal cortex initially encodes basic information about familiarity or novelty of an object but decays relatively quickly, while the hippocampus encodes object memory through information about the experience of interacting with the object and maintains strong novel object preference after long delays³⁸³.

Studies have shown that NMDA receptor 1 subunit (NR1) knockout-mice in the CA1 region of the hippocampus significantly impaired object recognition³⁸⁵. While analysis only revealed a strong trend rather than a significant reduction of NR1 in STZ and a significant reduction in STZ/LPS, NR1 levels in the hippocampus was one of the few receptor subunits (GABAergic or glutamatergic) that showed large alterations in both STZ and STZ/LPS. This reduction in hippocampal NR1 protein levels might explain the NOR impairments seen in this experiment by both STZ and STZ/LPS. In this non-spatial, hippocampal-dependent task, both STZ and STZ/LPS failed to investigate the novel object more than the familiar object, suggesting that the one-time immune activation did not play a significant role in improving or further impairing learning in this simple task. Deficits in object recognition is commonly seen in patients with AD³⁸⁶ and used to validate transgenic models of AD³⁸⁷⁻³⁸⁹.

Spatial learning and memory results from the Morris water maze yielded interesting findings. During the hidden platform training, the animals learn to locate and navigate towards a platform located just beneath the surface of the water. In this experiment, STZ

and STZ/LPS had significantly longer latencies to locate the hidden platform indicating that both groups experienced impairments in spatial learning. Furthermore, path length data suggests that the STZ/LPS animals may have taken a more direct route in their search strategy as the significant impairment seen in latency is no longer evident. There are multiple search strategies that an animal can adopt; the most efficient search strategy is to immediately orient to the extramaze cues and swim directly to the hidden platform. Another strategy, albeit less efficient, is for the subject to swim the perimeter of the maze until a specific location or cue is found and then orient itself toward the hidden platform³⁹⁰. Indeed, we see that the STZ group had significantly elevated thigmotaxis, whereas the STZ/LPS-treated animals did not explore the perimeter of the maze while orienting themselves to the same degree.

Even more compelling than the hidden platform latency impairments for both STZ and STZ/LPS are the probe trial data. In two different measures of probe performance, time spent in target quadrant and annulus crossings, STZ does not produce a statistically significant selective search, further indicating impairment in this spatial learning task. However, in both probe trial measures STZ/LPS demonstrated a selective search, performing at equivalent levels to the control animals. The difference between the two groups could be due to the difference in search strategies as previously discussed. STZ demonstrated a search strategy of swimming along the perimeter slightly longer than the other groups before finally locating the target location. Thigmotaxis, the amount of time spent swimming along the perimeter of the maze, can be measured to look for anxiety-like behavior in this task. However, open field data demonstrated no differences in overall anxiety or exploratory behavior, allowing us to interpret the increased perimeter

swimming time simply as a difference in search strategy^{391,392}.

It is not entirely clear why in two hippocampal-dependent tasks, one non-spatial (NOR) and one spatial (MWM), the simpler non-spatial task revealed deficits in both STZ and STZ/LPS, but a subtle, yet significant, improvement for STZ/LPS in the more difficult spatial task, while the STZ did not experience that same benefit. It is possible that in the more complex MWM task, considerably more brain regions than just the hippocampus, including the amygdala, thalamus, locus coeruleus, basal forebrain, are recruited in order to carry out this cognitive task³⁹³.

One possible mechanism that may underlie the learning impairments discussed above is the differing presence of A β oligomers between treatment groups. As mentioned above in the introduction, research regarding the role of A β in AD has focused primarily on the presence of large aggregated amyloid plaques. Recent research, however, suggests that early AD memory loss can be better explained by the presence of small, soluble forms of A β including monomers, dimers, and smaller oligomers³⁹⁴. Central administration of oligomeric A β results in MWM deficits in both hidden training and the probe trial³⁹⁵. Lesne and colleagues found that infusion of A β 12mer (which they term A β *56) did not affect subjects ability to find the hidden platform in the MWM across days¹⁰⁷. Furthermore, they found that infusion of A β 12mer two hours prior to the probe trial resulted in impairments in probe performance. We saw an overall increase in total A β oligomers in both STZ and STZ/LPS that may have impaired performance during the hidden training for both groups. Additionally, we saw an elevation of certain oligomers only in STZ, including 10mer and 22mer, which may give rise to the probe performance deficits seen only in STZ and not in STZ/LPS.

Additionally, NMDA receptors have been extensively studied because of their critical role in spatial learning. NMDA receptors are composed of subunits NR1, NR2 and NR3. Functional NMDA receptors contain one NR1 subunit and another one of several possible subunits, including NR2 (2A, 2B, 2C or 2D) or NR3. In the above experiment, there is a marked decrease in the NR2A receptor subunit in STZ animals. The use of NMDA receptor antagonists results in deficits in both hidden training and probe trial performance^{396,397}. The reduction of NMDA receptor subunit NR2A in the STZ group could be a possible explanation for the impaired hidden and probe performance, especially due to the fact that STZ/LPS did not experience a decrease in NR2A levels and demonstrated a selective probe search. Furthermore, A β oligomers initially disrupt NMDA receptor function but ultimately results in the removal of NMDA receptors from dendrites^{398,399}. Specifically, the NR1 subunit is a necessary component to the receptor complex or necessary for the assembly of the receptor complex that binds oligomers and allows for dendritic targeting of neurons^{400,401}. NMDA receptors aren't themselves thought to bind A β oligomers but rather act as a key player in the process, thus the endocytosis of NMDA receptors is likely an attempt to avoid further oligomeric targeting of the dendrites. In the above study, STZ treatment results in an elevation of oligomeric species, which likely targets dendrites via NMDA receptors, followed by the compensatory reduction in NMDA receptors, which ultimately manifests as cognitive disruptions in hippocampal-dependent learning tasks.

Postmortem studies of AD brains have revealed an increase in the GABA_{B1a} receptor subunit during the Braak stages III and IV⁴⁰². Braak staging was first described in 1991 and is used to describe the degree and distribution of NFTs in AD brains. Braak

found that NFTs followed a characteristic pattern of distribution. In stages I and II, NFTs are limited to the transentorhinal cortex. Stages III and IV are marked by NFTs in the entorhinal cortex and limbic system. In the final stages, V and VI, NFTs are found throughout all cortical areas. Iwakiri and colleagues ⁴⁰² found that the increase in GABA_{B1a} subunits during Braak III and IV was transient, followed by reductions in GABA_{B1a} in the later Braak stages. These alterations in GABA_{B1a} suggest that GABA_{B1a} expression changes with progression of NFTs in the hippocampus and cortex, contributing to the hippocampal circuitry dysfunction seen in AD patients. The elevated levels of GABA_{B1a} exhibited in the STZ animals may mirror this transient increase of GABAergic tone seen in post-mortem AD brains.

It has been hypothesized that decreased function of NMDA receptors reduces activation of GABAergic neurons, lowering overall inhibitory drive, and thus leading to the symptoms seen in schizophrenia ⁴⁰³. Building off of this hypothesis, it is entirely possible that the decrease in NMDA receptors found on GABAergic neurons are resulting in less activation of these inhibitory neurons. Neurons downstream from the GABAergic interneurons have not yet been affected like the GABAergic neurons that express the NMDA receptors and thus increase GABA receptors to compensate for a decrease in GABAergic tone. As previously discussed, oligomeric A β initially causes an increase in NMDA activity that ultimately leads to a decrease in NMDA receptor subunits ³⁹⁸. Further, over-activation of excitatory amino acid receptors, namely NMDA receptors, leads to a compensatory activation of inhibitory tone in an attempt to counter the excessive excitatory activity ⁴⁰². Thus, the increase in GABAergic signaling in the STZ-treated animals could be a result of increased A β oligomers, which would first increase

NMDA activity causing the compensatory activation of inhibitory tone before internalizing the NMDA receptors. It is possible that the time course of the experiment captured the brief window of increased compensatory GABAergic tone before being downregulated following internalization of NMDA receptors, which might be the same small, transient window that Iwakiri and colleagues found elevated GABA_{B1a} in the earlier Braak stages prior to reductions in GABA_{B1a} levels.

It is possible that the increase in GABA_B receptors seen in the STZ may be due to an entirely different mechanism that hasn't been explored yet. It has been shown that an increase in TNF α leads to the endocytosis of GABA_A receptors and an overall decrease in inhibitory synaptic strength⁴⁰⁴. IL-1 β has been shown to reduce GABA inhibition in the hippocampus, though these studies utilized patch clamp recordings and didn't investigate whether GABA receptors were being removed from the synapse^{405,406}. LPS triggers the release of several cytokines, including both IL-1 β and TNF α , which may lead to the decrease of GABA receptor subunits (elevated due to STZ-treatment), bringing expressed levels back to levels that mirror controls in the STZ/LPS group.

We found elevated levels of tau phosphorylated at site Threonine 231 in both STZ-treated groups. This finding is consistent with other STZ studies demonstrating an increase in pTau levels at numerous phosphorylation sites^{278,304,305}. Additionally, LPS has been shown to increase the amount of phosphorylated tau through numerous kinases including GSK-3 β and cdk5^{28,29,368}. The amount of phosphorylated tau following STZ did not seem to be affected by subsequent treatment with LPS, suggesting that microglia activation played no role in either the rescue nor the exacerbation of tau pathology.

Additionally, we were interested to see if either STZ-treatment, alone or with

subsequent treatment with LPS, led to long-lasting changes in immune-related markers. We looked at levels of IL-6, a proinflammatory cytokine, which is released by LPS-induced activation of microglia cells and repeatedly reported elevated in patients with AD. No changes were noted in IL-6 levels in any treatment groups. We also investigated levels of TNFR1, one of two possible receptors for TNF α . TNFR1 promotes the activation of transcription factor nuclear factor- κ B (NF- κ B), which in turn promotes further production of proinflammatory cytokines⁴⁰⁷. We found no differences in expression of TNFR1. Thus, the immune activation via LPS was indeed transient and did not cause long-lasting changes to these markers of immune function.

In the above studies, we hypothesized that an acute inflammatory response would be sufficient to mount a microglia response resulting in exacerbated behavioral and pathological changes when combined with STZ. As has been thoroughly described above, we did not see further deficits in the STZ/LPS but rather a subtle rescue in spatial learning during the MWM probe, significantly less elevated A β oligomers, and restorations of the alterations of several NMDA- and GABA-receptor subunits that were changed in the STZ group. Lowered levels of A β oligomers following immune activation may initially seem counterintuitive to the neuroinflammation hypothesis that an inflammatory response exacerbates and progresses AD pathologies, including A β products. However, we know that an acute inflammatory response in the brain, when microglia are activated and then return to a resting state, is beneficial and plays a neuroprotective role. Indeed, numerous studies demonstrate that activated microglia will phagocytose and degrade A β through activation of TLRs (TLR2, TLR4, and TLR9)⁴⁰⁸⁻⁴¹⁰. Furthermore, inactive TLR4 in APP^{swe}/PSEN1^{dE9} mice exhibited increased

hippocampal A β burden than mice with an intact TLR4 gene ⁴¹⁰. Intrahippocampal infusion of LPS into APP+PS1 transgenic mice resulted in activated microglia and resulted in a reduction of A β load in the hippocampus ^{411,412}. Quinn and colleagues ⁴¹³ demonstrated that seven days following intraperitoneal injection of LPS resulted in dramatic reductions of amyloid deposition in the cortex of Tg2576 mice (transgenic mice that carry the APP Swedish mutation). In the present study, it is clear that the overall elevation of total oligomers is markedly less when TLR4 is activated by LPS following STZ-treatment, presumably through microglia-mediated phagocytosis.

Our study set out to examine the role that insulin signaling perturbation plays in the development of AD-like deficits, both separately and in tandem with an inflammatory response, as neuroinflammation is implicated in accelerating the disease. Rather than an exacerbation of cognitive deficits and pathological markers, we saw benefits to spatial learning and several proteins that were restored following an acute inflammatory response in the STZ-treated animals. This finding, while initially surprising, compliments existing literature suggesting a brief immune activation that is quickly attenuated can play a beneficial role by activating microglia that can remove and degrade A β peptides. To our knowledge, this is one of only a few studies to investigate an immune response in the STZ-model, as it pertains to AD. These results help elucidate the role that the immune response may play in the pathogenesis of AD and pave the way for future studies to better understand the interplay of microglia and immune-related proteins with the disrupted insulin signaling seen in patients with DM. The interplay of inflammation in the brain and DM may elucidate common pathways and proteins between the two risk factors that, in turn, may lead to studies investigating potential therapeutic options.

Additional studies are necessary to determine when an inflammatory response shifts from being neuroprotective, as seen in the present study, to a neurotoxic role. Examination of chronic LPS injections following STZ-treatment may shed light on when this shift may occur, allowing us to create a timeline of beneficial microglia activation versus detrimental microglia activity. Additionally, a chronic LPS study would benefit from collecting whole brains and probing for activated microglia, by using an antibody for Iba1, to determine the extent of microglia activation that is occurring and in what regions of the brain.

Furthermore, limitations to the present study include the lack of data regarding insulin signaling. We cannot rule out that microglia activation may have partially restored insulin signaling or blocked some activity of STZ on insulin receptor signaling. Recent evidence suggests that fractalkine, a CXC3 chemokine that mediates interactions between neurons and microglia, plays an essential role in insulin signaling⁴¹⁴. In future studies, examination of proteins related to insulin signaling should be included to support or rule out this possibility. Additionally, we found no differences in immune markers several weeks following the initial LPS treatment. Thus, it would be useful in a future study to remove brains shortly after the LPS administration to determine what cytokines and other immune related proteins, including fractalkines, were altered during that brief period that may have played a beneficial role.

Of particular interest for possible future studies would be to examine DM as a risk factor in a more translational approach by administering STZ systemically rather than ICV. Peripheral STZ targets insulin producing cells in the pancreas and might be a better model of Type 1 DM. Furthermore, Type II DM is more often implicated as a risk factor

than T1DM and can be induced by feeding rats high-fat chow. While both of these approaches would be extremely useful in creating models that can be easily translated to the human population, disrupting insulin signaling directly in the brain (STZ-ICV) allows for the elucidation of the role that brain insulin plays in normal functioning and when disrupted, its role in AD. Regardless of the route that insulin disruption is created (high-fat diet, peripheral STZ, STZ-ICV), understanding the intricate relationship that the nervous system has with the immune system is critical to our understating of AD and other neurological disorders.

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- peptide. *Brain*. 2005;128(8):1778–1789.
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Curriculum Vitae
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Professional Information:

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Education:

Ph.D. student in Experimental Psychology, Neuroscience emphasis
(2011 – present; expected date of graduation: May 2016).
University of Nevada, Las Vegas, Las Vegas, NV 89154

B.A. Psychology with Biology minor (May 2011).
University of Nevada, Las Vegas, Las Vegas, NV 89154

Teaching Experience

- Psychology 101 (Fall 2013, 2 courses; Spring 2014, 2 courses)
 - Full responsibility for all aspects of the course, including preparing lectures, giving lectures, grading, and holding office hours. *Class size: 35*

Research Skills/Techniques:

- Extensive experience with the appropriate care and use of animal subjects, including rats, mice, and ground squirrels;
- Skilled in research design, implementation, and data analysis in animal learning;
- Expertise in several behavioral tasks with rodents including the Morris water maze, Barnes maze, radial arm maze, cued and contextual fear conditioning, acoustic startle, pre-pulse inhibition, open field, novel object recognition, odor recognition, tail flick nociception task, and general screening to examine basic sensory function and reflexes;
- Experienced in rodent breeding and sexing;
- Experienced in aseptic surgical techniques;
- Proficient in stereotaxic surgical procedures, including chronic cannula placement and osmotic mini-pump implantation in rats;
- Experienced in multiple wound closure techniques associated with stereotaxic surgery, including suture closure and dental acrylic application;
- Considerable expertise in neural tissue collection, including transcardiac perfusion and dissection of specific brain structures;

- Experienced in the frozen sectioning of neural tissue for histological analyses;
- Extensive experience utilizing immunohistochemistry techniques, including the use of DAB and immunofluorescence;
- Proficient in light and fluorescent microscopy, including conventional and confocal microscopes;
- Expertise in tissue homogenization and protein concentration assays;
- Skilled in western blotting and gel electrophoresis techniques;
- Trained on multiple western blot imaging techniques, including the Typhoon variable mode imaging system, the Odyssey IR Imaging system, and the UVP imaging system;
- Trained in labeling techniques for demonstration of neurogenesis utilizing BrdU;
- Experienced in programming of software for behavioral testing;
- Proficient with ELISA technique and interpretation;
- Expertise in annotative work, including detailed annotation of the central nervous system.

Research Experience

- Aug. 2011-Present: Doctoral student and graduate assistant in a behavioral neuroscience laboratory under the direction of Dr. Jefferson Kinney. Our lab primarily investigates the neurobiology of learning and memory with an emphasis on the biological basis of neurological/psychological diseases such as Alzheimer's disease and schizophrenia.
- Jan. 2009-July 2011: Undergraduate research assistant in a behavioral neuroscience laboratory under the direction of Dr. Jefferson Kinney.
- Summer 2007: Research assistant for the FACE (Free-Air-Carbon dioxide-Emission) project under the supervision of Dr. Stan Smith and Dr. Dene Charlet. FACE project was a joint effort between the Department of Energy and UNLV, to study the effects of carbon dioxide levels on the desert ecosystem.

Publications:

Murtishaw, A.S., Heaney, C.H., Bolton, M.M., Sabbagh, J.J., Langhardt, M.A., and Kinney, J.W. An acute inflammatory response improves learning and memory deficits and reduces pathological markers in a diabetic animal model of Alzheimer's disease. *Submitted to Neurobiology of Aging*, May 2014. (Impact factor=6.166)

Hensleigh, E., **Murtishaw, A.S.**, Treat, M., Heaney, C.H., Bolton, M.M., Sabbagh, J.J., Kinney, J.W., and van Bruekelen, F. The effect of torpor on spatial memory in ground squirrels (*Spermophilus lateralis*) throughout a hibernation season. *Submitted to Animal Behavior*, May 2014. (Impact factor=3.405)

Bolton, M.M., Heaney, C.F., **Murtishaw, A.S.**, and Kinney, J.W., Magcalas, C.M., and Kinney, J.W. Postnatal alterations in GABA_B receptor tone produce behavioral deficits and protein level differences in adulthood. *Submitted to Developmental Neuroscience*, June 2014. (Impact factor=3.41)

Sabbagh, J.J., **Murtishaw, A.S.**, Bolton, M.M., Heaney, C.F., Langhardt, M.A., and Kinney, J.W. Chronic ketamine produces altered distribution of parvalbumin-positive cells in the hippocampus of adult rats. *Neuroscience Letters* 2013; 550: 69-74. (Impact factor=2.146)

Bolton, M.M., Heaney, C.F., Sabbagh, J.J., **Murtishaw, A.S.**, and Kinney, J.W. Deficits in emotional learning and memory in an animal model of schizophrenia. *Behavioral Brain Research* 2012; 233(1):35-44. (Impact factor=3.327)

Heaney, C.F., Bolton, M.M., **Murtishaw, A.S.**, Sabbagh, J.J., Magcalas, C.M., and Kinney, J.W. Baclofen administration alters fear extinction and GABAergic protein levels. *Neurobiology of Learning and Memory* 2012; 98(3):261-71. (Impact factor=3.860)

Sabbagh, J.J., Heaney, C.F., Bolton, M.M., **Murtishaw, A.S.**, Ure, J.A., and Kinney, J.W. Differences in the effects of galanin and donepezil following changes in cholinergic tone. *International Journal of Neuroscience* 2012; 122(12):742-7. (Impact factor=1.216)

Sabbagh, J.J., Heaney, C.F., Bolton, M.M., **Murtishaw, A.S.** and Kinney, J.W. Examination of spatial learning deficits in an animal model of schizophrenia. *Physiology and Behavior* 2012; 107(3):355-63. (Impact factor=3.160)

Poster Presentations:

Murtishaw, A.S., Heaney, C.F., Bolton, M.M., Belmonte, K.D., Hagins, P., Langhardt, M.A., and Kinney, J.W. Chronic inflammation in a diabetic model of Alzheimer's disease. Poster at the International Behavioral Neuroscience Society annual meeting Las Vegas, NV, Summer 2014.

Belmonte, K.D., **Murtishaw, A.S.**, Heaney, C.F., Bolton, M.M., Kinney, J.W. An acute inflammatory response in a diabetic model of Alzheimer's disease. Poster presented at the Nevada IDeA Network of Biomedical Research Excellence Undergraduate Research Opportunity Program Poster Symposium, Summer 2013.

Belmonte, K.D., **Murtishaw, A.S.**, Heaney, C.F., Bolton, M.M., Kinney, J.W. An acute inflammatory response in a diabetic model of Alzheimer's disease. Poster presented at the McNair Scholars Summer Research Program Poster Symposium, Fall 2013.

- Bolton, M.M., Heaney, C.F., **Murtishaw, A.S.**, Langhardt, M.A., Kinney, J.W.
Developmental alteration of GABA_B receptor function results in behavioral deficits in adulthood. Poster presented at Society for Neuroscience annual meeting San Diego, CA 2013.
- Heaney, C.F., Bolton, M.M., **Murtishaw, A.S.**, Kinney, J.W. The effects of baclofen and phaclofen on performance in the Morris water maze. Poster presented at Society for Neuroscience annual meeting San Diego, CA 2013.
- Langhardt, M.A., Bolton, M.M., Heaney, C.F., **Murtishaw, A.S.**, Nagls, S., Kinney, J.W.
Evaluation of ketamine-induced changes in spatial working memory and GABAergic systems. Poster presented at Society for Neuroscience annual meeting San Diego, CA 2013.
- Langhardt M.A., Bolton, M.M., **Murtishaw, A.S.**, Heaney, C.F., Kinney, J.W. Ketamine Induced Deficits in Working Memory with Relevance to Schizophrenia. Oral presentation at the 21st Annual University of California, Berkeley McNair Scholars Symposium, August 2013.
- Langhardt, M.A., Bolton, M.M., Heaney, C.F., **Murtishaw, A.S.**, Kinney, J.W. (2013)
Ketamine-induced deficits in working memory with relevance to schizophrenia. Poster presented at the 2013 UNLV McNair Scholars Research Symposium.
- Murtishaw, A.S.**, Heaney, C.F., Bolton, M.M., Langhardt, M.A, Belmont, K.C.D, Kinney, J.W. An acute LPS-induced inflammatory response in a diabetic model of Alzheimer's disease. Poster presented at Society for Neuroscience annual meeting San Diego, CA 2013.
- Bolton, M.M., Heaney, C.F., Sabbagh, J.J., **Murtishaw, A.S.**, Magcalas, C.M., Kinney, J.W.
Comparison of postnatal ketamine dosage on behavioral deficits in adulthood. Poster presented at Society for Neuroscience annual meeting New Orleans, LA 2012.
- Heaney, C.F., Bolton, M.M., **Murtishaw, A.S.**, Sabbagh, J.J, Magcalas, C.M., Kinney, J.W.
Changes in GABA_B receptor tone in development produce behavioral deficits in adulthood. Poster presented at Society for Neuroscience annual meeting New Orleans, LA 2012.
- Magcalas, C.M., Heaney, C.F., Bolton, M.M., **Murtishaw, A.S.**, Sabbagh, J.J., and Kinney, J.W. Alterations in GABA_B in development produce behavioral and protein changes in adulthood. Poster presented at the Undergraduate Research Opportunity Program session, (Nevada InBRE), UNLV August 2012.

Murtishaw, A.S., Sabbagh, J.J., Heaney, C.F., Bolton, M.M., Magcalas, C.M., Langhardt, M.A., Kinney, J.W. Ketamine-induced behavioral impairments and alterations in hippocampal GABAergic neuron distribution. Poster presented at Society for Neuroscience annual meeting New Orleans, LA 2012.

Sabbagh, J.J., **Murtishaw, A.S.**, Heaney, C.F., Bolton, M.M., Magcalas, C.M., Kinney, J.W. Chronic calcium dysregulation produces cognitive deficits and biochemical changes relevant to Alzheimer's disease. Poster presented at Society for Neuroscience annual meeting New Orleans, LA 2012.

Bolton, M.M., Heaney, C.F., Sabbagh, J.J., **Murtishaw, A.S.**, Kinney, J.W. Comparison of an adult and developmental animal model of schizophrenia. Poster presented at Society for Neuroscience annual meeting Washington, D.C. 2011.

Heaney, C.F., Bolton, M.M., **Murtishaw, A.S.**, Sabbagh, J.J., Kinney, J.W. An investigation of the effects of alterations in GABAB receptor function on learning and memory. Poster presented at Society for Neuroscience annual meeting Washington, D.C. 2011.

Sabbagh, J.J., Bolton, M.M., Heaney, C.F., **Murtishaw, A.S.**, Kinney, J.W. Deficits in emotional learning and memory in an animal model of schizophrenia. Poster presented at Society for Neuroscience annual meeting Washington, D.C. 2011.

Heaney, C.F., Sabbagh, J.J., Bolton, M.M., **Murtishaw, A.S.**, Santa-Ana, I., Kinney, J.W. An investigation of alterations in GABAergic tone in an animal model of schizophrenia. Poster presented at Society for Neuroscience annual meeting San Diego, CA 2010.

Sabbagh, J.J., Heaney, C.F., Bolton, M.M., **Murtishaw, A.S.**, Ure, J.A., Kinney, J.W. Donepezil and galanin interactions in learning and memory and a model of cholinergic loss. Poster presented at Society for Neuroscience annual meeting, San Diego, CA 2010.

Oral Presentations:

Murtishaw, A.S. Chronic inflammation in a diabetic model of Alzheimer's disease. Department of Psychology Research Highlights. University of Nevada, Las Vegas, March 2014.

Murtishaw, A.S. LPS-induced chronic inflammation in a model of sporadic Alzheimer's disease. GPSA Research Forum, University of Nevada, Las Vegas, March 2014

Murtishaw, A.S. Ketamine-induced behavioral impairments and alterations in hippocampal GABAergic neuron distribution. GPSA Research Forum, University of Nevada, Las Vegas, May 2013.

Murtishaw, A.S. Acute inflammation in a diabetic model of Alzheimer's disease. Department of Psychology Proseminar, University of Nevada, Las Vegas, February, 2013.

Murtishaw, A.S. GABAergic alteration and behavioral impairments from ketamine: A possible mechanism for efficacy in treatment resistant depression? Department of Psychology Proseminar, University of Nevada, Las Vegas, May 2013.

Service:

- Organizing committee for first annual Las Vegas Brain Bee (Spring 2014).
- Honors Thesis Committee Member for Krystal Belmonte (Spring 2014-Fall 2014).
- Mentor for undergraduate recipients of Nevada IDEa Network of Biomedical Research Excellence Undergraduate Research Opportunity Program (Summer 2013, Krystal Belmonte; Summer 2014, Patrick Hagins).
- Mentor for undergraduate McNair Scholars (Summer 2013, Krystal Belmonte).
- Head of Organizing Committee for LGBT Social to be held at Society for Neuroscience 2014 Annual Conference (Fall 2013-Present).
- Founding member and Board member of Nevada Brain Bee Association (Fall 2013-Current).
- Serve as a member of Q:UNLV. Q:UNLV is a council steered by UNLV's Vice President to promote diversity and inclusion for the LGBTQ staff and faculty at UNLV (2012-current).
- Organized outreach program as part of Society for Neuroscience Brain Awareness week of presentations elementary schools across Las Vegas (2012, 2013, 2014).
- Supervision and training of undergraduate research assistants on several techniques, including the Morris water maze, radial arm maze, Barnes maze, cued and contextual fear conditioning, acoustic startle, neural tissue collection, cryostat sectioning, western blotting, ELISA, and immunohistochemistry.
- Founding member of the University of Nevada, Las Vegas Graduate Neuroscience Association (2011 – present).
- Teaching and guidance of undergraduate students as a graduate student member of the University of Nevada, Las Vegas Neuroscience Journal Club (2009-2011).

Awards:

- International Behavioral Neuroscience Society 2014 Conference Presentation Winner, 2nd place.

- Awarded a travel grant from the University of Las Vegas Graduate and Professional Student Association to attend and present at the 2014 Society for Neuroscience annual conference in New Orleans, LA.
- Awarded a travel grant from the University of Las Vegas Graduate and Professional Student Association to attend and present at the 2014 Society for International Behavioral Neuroscience Society in Las Vegas, NV.
- Dean's Graduate Research Stipend Award (Summer 2014)
- UNLV GPSA 2014 Research Forum Outstanding Presentation, 2nd place.
- Awarded a travel grant from the University of Las Vegas Graduate and Professional Student Association to attend and present at the 2013 Society for Neuroscience annual conference in San Diego, CA.
- Awarded a travel grant from the University of Las Vegas Graduate and Professional Student Association to attend and present at the 2012 Society for Neuroscience conference in New Orleans, LA.

Memberships in Professional and Scientific Societies:

- Member of International Society of Neuroimmunology (2014 – Current)
- Member of PsychoNeuroImmunology Research Society (2014 – Current)
- Member of Alzheimer's Association International Society to Advance Alzheimer's Research and Treatment (2014 – Current)
- Member of National Organization of Gay and Lesbian Scientists and Technical Professionals (2013 – Current)
- Member of International Behavioral Neuroscience Society (2013 – Current)
- Member of Sierra Nevada chapter of the Society for Neuroscience (2009 – Current).
- Member of Society for Neuroscience (2008 – Current);

Professional References

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