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An Evaluation of GABAB Receptors on Modulating Neuroinflammation in a Non-Transgenic Animal Model of Alzheimer's Disease

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AN EVALUATION OF $GABA_B$ RECEPTORS ON MODULATING NEUROINFLAMMATION IN A NON-TRANSGENIC ANIMAL MODEL OF ALZHEIMER'S DISEASE

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ABSTRACT

An Evaluation of $GABA_B$ Receptors on Modulating Neuroinflammation in a Non-Transgenic Animal Model of Alzheimer's Disease

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Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive memory loss and distinct neuropathological hallmarks, including amyloid beta plaques (Aβ) and neurofibrillary tau tangles (NFT). Although the etiology remains to be discovered, several risk factors exist that significantly contribute to developing AD. Diabetes is one of the major risk factors associated with AD and is characterized by disrupted insulin signaling that may contribute to or exacerbate AD pathologies. Furthermore, both disorders result in increased neuroinflammation. Considerable evidence has demonstrated that a chronic inflammatory response, in particular chronic microglia activation, promotes Aβ production as well as the hyperphosphorylation of tau through the sustained release and increased levels of several pro-inflammatory cytokines. These data make understanding the mechanisms driving the inflammatory response and treatment of the inflammation an important target in AD research. In addition to aberrant microglia functioning, the loss of a number of aspects of GABAergic signaling, including $GABA_B$ receptors, have been reported in clinical AD populations and animal models of AD. As microglia express functional $GABA_B$ receptors and activation on microglia appear to reduce their activity, GABA signaling may result in a decrease in pro-inflammatory cytokine production. Therefore, the purpose of this study is to investigate the role of $GABA_B$ in

neuroinflammation encompassing to AD pathogenesis using a non-transgenic animal model related to diabetes. Using a low-dose schedule of streptozotocin (STZ) administration to induce a sustained hyperglycemic state, we treated with animals with a $GABA_B$ receptor agonist (baclofen) to reduce activated microglia and pro-inflammatory effects. We found that STZ administration led to significantly increased blood glucose levels, memory impairments in the novel object recognition task, hyperphosphorylated tau, increased activated microglia, and pro-inflammatory cytokines. Treatment with baclofen ameliorated the above changes induced by STZ. Therefore, $GABA_B$ receptors play a role in modulating microglia function and neuroinflammation.

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CHAPTER 1

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that affects up to 11-16 million people worldwide (Alzheimer's Association, 2016). AD diagnoses is increasing at an alarming rate due to the rapid aging of the global population. Someone in the United States will develop AD every 66 seconds. By 2050, this rate of development is expected to raise to every 33 seconds (Alzheimer's Association, 2016). The cost of AD on family members, caregivers, and/or nursing home fees result in a financial burden of approximately $221 billion (Alzheimer's Association, 2016). Clinical symptoms of AD include the progressive memory loss and decline in cognitive functions, which may lead to behavioral alterations including anxiety, psychosis, and confusion (Cummings *et al.*, 2008). The pathological hallmarks of AD are amyloid β (Aβ) plaques and neurofibrillary tau tangles (NFT) which eventually lead to neuronal loss (Glenner and Wong, 1984; Grundke-Iqbal *et al.*, 1986).

According to the amyloid hypothesis of AD, the progression of the disorder is speculated to begin with the gradual accumulation and aggregation of Aβ peptides leading to a molecular and cellular cascade that eventually results in synaptic alterations, microglial activation, and insoluble tau helical filaments (Haass and Selkoe, 2007). Genetic factors are associated with the development of AD pathologies; however, 95-99% of AD cases (referred to as sporadic AD) are not accounted for by genetics alone (Alzheimer's Association, 2016). Risk factors including advanced age and Type 2 diabetes mellitus have been associated with the development of sporadic AD (Haan, 2006). Approximately 80% of patients with AD have a form of insulin dysregulation and patients with diabetes are twice as likely to develop AD (Ott *et al.*, 1996; Janson *et al.*, 2004).

Neuroinflammation is associated with both AD and diabetes (Srodulski *et al.*, 2014). While inflammation in the brain can play a beneficial role in reducing AD pathologies during the early stages of the disorder, chronic neuroinflammation has been shown to exacerbate Aβ aggregation and plaque formation as well as increased tau hyperphosphorylation (Glass *et al.*, 2010; Rubio-Perez and Morillas-Ruiz, 2012). Microglia, the main driver of the immune response in the brain, are found in abundance clustered near Aβ plaques in AD brains (Rezai-Zadeh *et al.*, 2011; Jay *et al.*, 2015). In response to injury or stressors, these immune cells release pro-inflammatory cytokines. When left unchecked, continued release of pro-inflammatory cytokines can contribute to pathology and disease progression. However, the mechanisms that contribute to the sustained, chronic inflammation in AD are not entirely understood.

One possible method of regulating or suppressing microglial activation may be through endogenous inhibitory neurotransmitter activity. Gamma-aminobutryic acid (GABA) is the main inhibitory neurotransmitter in the brain and recent evidence suggests that GABAergic signaling undergoes profound pathological changes in AD resulting in decreased neurotransmission and receptor expression (Iwakiri *et al.*, 2005; Yanfang Li *et al.*, 2016). The loss of function due to reduction of the neuronal expression of $GABA_B$ receptor could have detrimental effects, as these receptor subtypes play a role in oscillatory activity associated with memory and cognitive functioning (Brown *et al.*, 2007). Interestingly, microglia also express functional $GABA_B$ receptors (Kuhn *et al.*, 2004). In a cell culture study, the administration of a $GABA_B$ receptor agonist, baclofen, attenuated the release of pro-inflammatory cytokines from microglia after an immune challenge (Kuhn *et al.*, 2004). Therefore, it is interesting to speculate the neuroinflammatory effects of $GABA_B$ receptor activation on AD pathologies.

The purpose of this study is to evaluate the role of $GABA_B$ receptors as it relates to neuroinflammation in an animal model utilizing risk factor diabetes mellitus to recapitulate aspects of AD pathology. Streptozotocin (STZ) is commonly used in research to disrupt insulin production and altered insulin signaling that results in similar pathologies with sporadic AD, including neuroinflammation. In this experiment, we found that a low-dose (40 mg/kg), staggered administration schedule resulted in sustained, elevated blood glucose levels (indicative of insulin dysregulation). After STZ administration, a subset of animals received the $GABA_B$ receptor agonist, baclofen, twice a day for two weeks with the goal of reducing neuroinflammation for a sustained period of time. Following which, learning and memory was assessed in two tasks commonly used in AD rodent models (novel object recognition and cued and contextual fear conditioning). The results demonstrated that baclofen ameliorated the STZ-induced memory impairments in the novel object recognition task while no significant learning impairments were observed in cued and contextual fear conditioning. Baclofen administration reduced neuroinflammatory markers and rescued protein changes associated with AD that were altered with the STZ administration. These data suggest that $GABA_B$ receptors can modulate microglia function and neuroinflammation that can rescue memory impairments and AD pathological markers.

CHAPTER 2

REVIEW OF RELATED MATERIAL

Alzheimer's Disease Neuropathological Hypotheses

Alzheimer's disease (AD) is characterized clinically by a progressive decline in memory and cognitive functions. Initial clinical diagnosis is determined by a physician using neurological and clinical tests as well as blood and brain imaging. These symptoms vary among individuals; however, the most common initial symptom is the progressive inability to remember new information due to the hippocampal region being the first area affected in this disorder (Padurariu *et al.*, 2012). The core clinical symptoms of AD include memory loss that disrupts daily life, challenges in problem solving or planning, difficulty to completing familiar tasks, trouble understanding visual images and spatial relationships, increase anxiety, agitation, sleep disturbances, etc. (Alzheimer's Association, 2016). Confirmation of diagnosis is made postmortem by the examination of senile amyloid-β (Aβ) plaques and neurofibrillary tau tangles (NSTs) in the brain, particularly in the hippocampus. These neuropathological hallmarks of the disorder lead to neuronal cell loss that occur before any noticeable clinical symptoms (Mattson, 2008). The initial cause of the pathological symptoms is unknown; however, researchers speculate that the accumulation of Aβ followed by the deposition of NFTs triggers the onset of synaptic and neuronal dysfunction and subsequent neuronal loss (Hardy and Higgins, 1992).

Aβ plaques are extracellular structures composed of amyloid β peptides 40-42 amino acids in length. Aβ peptides are generated by proteolytic cleavage of β-amyloid precursor protein (APP) and are normal products of APP metabolism that occurs throughout life (Hardy and Selkoe, 2002). APP is a single-transmembrane, receptor-like protein found ubiquitously in neuronal and non-neuronal cells and has also been discovered circulating in extracellular fluids like cerebrospinal

fluid (CSF) and plasma (Haass *et al.*, 1992; Seubert *et al.*, 1992; Shoji *et al.*, 1992; Busciglio *et al.*, 1993). Processing of APP is mediated by two membrane-bound proteases, α-secretase or β-secretase (also known as β-site APP-cleaving enzyme (BACE); (Haass, 2004). Following BACE cleavage, a special type of protease complex that is mediated by γ-secretase along with obligatory presenilin-1 (PS1) or PS2, nicastrin, anterior pharynx-defective 1 (APH1), and presenilin enhancer 2 (PEN2) makes an intramembrane scission to APP (Wolfe *et al.*, 1999; Steiner *et al.*, 2000; Kimberly *et al.*, 2003; Takasugi *et al.*, 2003; Edbauer *et al.*, 2003; Haass, 2004). There are variable sites at which this complex cuts but it is the final cut at the γ-site that releases Aβ into biological fluids. Variability with the γ-cut can occur and lead to Aβ amino acids 38, 40, or 42, with the Aβ42 peptide having a readiness to self-aggregate leading to the pathogenicity of Aβ (Borchelt *et al.*, 1996).

Several lines of evidence suggest aberrant APP processing in AD. One of the first indications of the role of APP in AD comes from individuals with Down syndrome. Young adults with Down syndrome display the clinical and histopathological signs of AD, including plaques containing Aβ and NFT (KE Wisniewski *et al.*, 1985). These individuals have a third copy of chromosome 21, the location where the *APP* gene is located, prompting researchers to investigate mutations of APP in AD (Holtzman *et al.*, 1996). It was subsequently found that all known mutations linked to familial AD, or early on-set AD, affect APP processing or the propensity of Aβ aggregation (Heppner *et al.*, 2015). For example, certain mutations in the genes that encode the presenilin proteins favor the cleavage of APP by γ-secretases (Citron *et al.*, 1992; X-D Cai *et al.*, 1993; Suzuki *et al.*, 1994) and separate mutations in APP result in a high self-aggregation of Aβ into amyloid fibrils (T Wisniewski *et al.*, 1991). These mutations result in toxic amyloidgenic

Aβ plaques consisting mostly of Aβ42, as observed by transgenic mice and cell lines harboring human APP mutations (LaFerla and KN Green, 2012; Heppner *et al.*, 2015).

Aβ can coexist as monomers, oligomers, protofibrils, fibrils and Aβ plaques with varying levels of pathogenic impact. Soluble oligomers are Aβ assemblies that can bind to other macromolecules or cell membranes and become insoluble. Oligomers can aggregate to form protofibrils then fibrils which are the basis of plaques. Differences exist in aggregation between the soluble oligomers Aβ40 and Aβ42. Due to the extended length in amino acid number of Aβ42 and conformational freedom of its N termini, Aβ42 forms less compact fibrils compared to the more compact form resulting from Aβ40 (Roychaudhuri *et al.*, 2009). Fibrils are aggregates of Aβ with a β structure that make up insoluble plaques found in AD (Cavallucci *et al.*, 2012). Surprisingly, plaque number does not correlate significantly with neuronal death and cognitive impairment (McLean *et al.*, 1999), whereas soluble Aβ oligomers appear to be more detrimental (Haass and Selkoe, 2007).

Previous, somewhat crude methods of analyzing Aβ plaque deposition in postmortem brain tissue of AD patients used two-dimensional plaque counts that can miss small, heterogeneous Aβ-assembly forms listed above. More precise methods using specific Aβ enzyme-linked-immuno-sorbent assays (ELISAs), along with western blotting and mass spectrometry, allows for a multifactorial analysis of Aβ quality and quantity that can be further correlated with cognitive measures. Soluble Aβ correlates better with cognitive deficits compared to plaque counts (Lue *et al.*, 1999; McLean *et al.*, 1999; Jun Wang *et al.*, 1999; Näslund *et al.*, 2000; Haass and Selkoe, 2007). It is difficult to deduce whether large accumulation of insoluble Aβ contribute solely to neuronal injury, as they are surrounded by a number of smaller, diffusible oligomers (Haass and

Selkoe, 2007). Soluble, low-number oligomers can inhibit hippocampal long-term potentiation (LTP) as observed in cell cultures (Townsend *et al.*, 2006) and interfere with memory of learned behavior in wake, behaving rats (Cleary *et al.*, 2005). Therefore, soluble, low-number oligomers are considered more toxic and detrimental than plaques in AD.

Several risk factors associated with sporadic AD and Aβ aggregation have recently been discovered. Evidence from genome-wide association studies (GWAS) confirm expression of the ε4 allele of the *APOE* gene with increased risk of sporadic AD (Harold *et al.*, 2009; Lambert *et al.*, 2009). The *APOE* gene is found in three alleles: ε2 (8.4% frequency), ε3 (77.9% frequency), and ε4 (13.7% frequency; (Farrer *et al.*, 1997). Increased frequency of AD and lower age of onset is associated with *APOE* ε4, with approximately 40% of AD patient as carriers of two ε4 alleles (Corder *et al.*, 1993; Rebeck *et al.*, 1993; Farrer *et al.*, 1997). Apolipoprotien E protein (ApoE) is found in liver and macrophages where it regulates cholesterol metabolism and lipid homeostasis (Mahley and Rall, 2000). ApoE4 carriers have an increased risk of developing atherosclerosis, coronary heart disease, and stroke (Mahley and Rall, 2000; Lahoz *et al.*, 2001). In the central nervous system (CNS), ApoE (produced mainly by astrocytes) transports cholesterol to neurons through ApoE receptors (Bu, 2009). Several studies in humans and rodent models provide evidence that Aβ are modulated by the ApoE isoform (with ε4 having increased plaque load and ε2 being protective), which suggests its role in metabolism and aggregation of Aβ (Reiman *et al.*, 2009; Bales *et al.*, 2009; Castellano *et al.*, 2011). For instance, in APP transgenic mice, ApoE4 is less efficient at Aβ clearance (Castellano *et al.*, 2011). Furthermore, ApoE deficient mice are able to clear Aβ more effectively compared to control mice (DeMattos *et al.*, 2004). Although studies provide clear evidence that ApoE plays a critical role in mediating deposition and clearance of Aβ levels, the mechanisms in doing so remained to be discovered.

The other core pathological feature of AD is neurofibrillary tau tangles (NFT). Tau is a microtubule-associated protein that plays an important role in microtubule assembly and stabilization, necessary for neuronal morphology and structure, transportation of vesicles and organelles, and an anchor for enzymes (Yipeng Wang and E Mandelkow, 2015). The structure of tau has a proline-rich region with two domains on each end: a basic assembly domain on the C-terminal and an acidic projection domain on the N-terminal (Mukrasch *et al.*, 2009). The opposing charges on opposite ends of tau are crucial for interactions between microtubules as well as for internal folding and aggregation (E-M Mandelkow and E Mandelkow, 2012).

An important characteristic of tau is the large number of potential phosphorylation sites. Maintaining balance between phosphorylation and dephosphorylation under physiological conditions is critical (Johnson and Stoothoff, 2004). Under normal conditions, tau phosphorylation promotes microtubule assembly and axonal transport (Johnson and Stoothoff, 2004). Several phosphatases interact with tau to reverse phosphorylation, including protein phosphatase 1 (PP1), PP2A, PP2B, and PPC (Tian and Jianzhi Wang, 2002). The conversion of normal tau into paired helical filaments (PHF, the main component of NFT) occurs when specific combinations of Ser/Thr kinases (cdk5/GSK3 and calcium calmodulin kinase II/GSK3β) hyperphosphorylate within the proline-rich segment of tau (KL Rosenberg *et al.*, 2008). This state can be reversed by PP2A, which has the ability to dephosphorylate tau, preventing its assembly into PHFs, and allowing it to bind back to microtubules. Alternatively, if different combinations of protein kinases rephosphorylate tau, then the PHF will lead to NFT formation associated with AD (Jian Zhi Wang *et al.*, 2007). Hyperphosphorylated tau is resistant to degradation and prone to aggregation leading to NFT.

An addition to hyperphosphorylated tau, NFT are composed of truncated tau proteins that exhibit different conformational properties compared to normal tau. Truncated tau can result in abnormal microtubule assemblies leading to neuronal toxicity (Zilka *et al.*, 2006). Normal posttranslational modifications involve the cleavage of tau by caspases and calpain. (Jian Zhi Wang *et al.*, 2007). However, tau can become prone to hyperphosphorylation when abnormal cleaving events occur. One suggestion is that when tau is cleaved at its N-terminus by calpain, it becomes susceptible for caspase to cleave tau at its C-terminus resulting in toxic truncated tau (Jian Zhi Wang *et al.*, 2007). In AD brain tissue, caspases associated with these tau cleavage events (caspase 8[23] and caspase9[24]) are co-localized with NFT (Rohn *et al.*, 2002). The development of these altered posttranslational modification events leading to truncated tau remain to be discovered.

There are no clear answers to describe the interaction between NFT and Aβ. Furthermore, mutations and abnormalities in tau are not specific to AD. Tau is located on chromosome 17 and specific mutations in this gene (FTDP-17) are linked to frontotemporal dementia with parkinsonism resulting in abnormally phosphorylated tau pathology with no amyloid pathology (Baker *et al.*, 1997; Hutton *et al.*, 1998; Spillantini *et al.*, 1998; Poorkaj *et al.*, 1998). However, studies have observed indications of one pathology influencing the other. Specifically, in mice overexpressing a mutant form of tau commonly used to study tauopathies (a general term for neurodegenerative disorders that involve the aggregation of tau protein into NFT), injection of Aβ42 peptides result in elevated hyperphosphorylated tau (Gotz *et al.*, 2001). Analogously, AD mice expressing mutations in both APP and tau display tau pathology earlier compared to mice with just the tau mutation (Lewis *et al.*, 2001; M Pérez *et al.*, 2005; Ribé *et al.*, 2005; Terwel *et al.*, 2008).

One possible link between NFT and Aβ points to the actions of a protein kinase called glycogen synthase kinase 3 beta (GSK3β). GSK3β impairs the ability of tau to both bind and stabilize microtubules (Johnson and Hartigan, 1999; Cho and Johnson, 2004). Tau becomes hyperphosphorylated in GSK3β-overexpressing mice (Engel *et al.*, 2006). PS1 mutation (one of the mutations in familial AD associated with altered Aβ processing) can increase GSK3β activity and its association with motor proteins on microtubules that interact with tau, leading to dissociation of tau with microtubules and the possibility to aggregate (Pigino *et al.*, 2003). Various AD transgenic animal models and *in vitro* studies (targeting either mutations in APP, tau, or both) display increased GSK3β activity (Takashima *et al.*, 1996; 1998; Ishizawa *et al.*, 2003; Billings *et al.*, 2007; Terwel *et al.*, 2008). *In vivo* and *in vitro* AD model studies also demonstrate that inhibition of GSK3β attenuates APP processing and reduces hypersphosphorylated tau (Phiel *et al.*, 2003; Noble *et al.*, 2005). A more recent study compared the interaction of single nucleotide polymorphisms (SNPs) associated with the *GSK3β* gene with postmortem AD pathologies and found interactions between SNPs and both Aβ plaques and NFT (Hohman *et al.*, 2016). The authors suggest that Aβ and NFT progression may be independent and that GSK3β activity is the point at which the pathologies intersect.

The progression of NFT and hyperphosphorylated tau in AD follows a distinct neurological pattern that begins in the entorhinal cortex (Braak stages I-II), spreads the limbic and adjoining neocortex (stages III-IV), then to the neocortex including the secondary and primary fields (stages V-VI) (H Braak and E Braak, 1991; Bancher *et al.*, 1993; H Braak and E Braak, 1997; H Braak *et al.*, 2006). The degree of NFT formation in postmortem AD brains and the progression through the Braak staging is strongly correlated with increasing memory loss and dementia (H Braak and E Braak, 1991; 1997). Since the limbic system, particularly the hippocampus, is involved in

memory systems, Braak staging correlates the memory deficits associated with AD. Other pathologies follow a similar pattern of neurological pattern referred to as Braak staging, including Aβ plaques, reactive microglia, and cholinergic loss (Serrano-Pozo *et al.*, 2011). However, compared to Aβ oligomers and plaques, some researchers believe that the NFT are the main source of cognitive decline in patients with AD (Wilcock and Esiri, 1982; Delaère *et al.*, 1989; Arriagada *et al.*, 1992; Duyckaerts *et al.*, 1997; Gómez-Isla *et al.*, 1997; Delacourte *et al.*, 2002; Giannakopoulos *et al.*, 2003; Guillozet *et al.*, 2003; Bretteville and Planel, 2008). Animal models with tau mutations corroborate these findings, demonstrating significant impairment in learning and memory and altered hippocampal synaptic plasticity (Polydoro *et al.*, 2009; Hoover *et al.*, 2010; Van der Jeugd *et al.*, 2011; Burnouf *et al.*, 2012).

Lastly, cholinergic cell loss is commonly found in postmortem analysis of AD brains and is the another core feature of the disorder. The pattern of cell loss follows a similar progression to the Braak staging discussed previously (Beach *et al.*, 2000). Additional evidence towards disruptions in acetylcholine signaling are associated with the reduction in acetyltransferase (the enzyme that modulates production of acetylcholine) and acetylcholinesterase (the enzyme that degrades acetylcholine) (Davies, 1979). It is unclear how cholinergic changes are brought about and has been the focus of considerable research. Most of the currently available drug therapies for AD target the cholinergic system by inhibiting acetylcholinesterase. Although these drugs can slow the progression of symptoms in patients with mild cases of AD, they do not halt the disorder (Rogers *et al.*, 1998; Rösler *et al.*, 1999; Birks, 2006).

Many transgenic mouse models of AD exist to help researchers understand the disease progression. They have been exceedingly useful in highlighting signaling mechanisms and protein interactions related to the disorder; however, no single transgenic model represents all aspects of

the disease spectrum. The most common class of transgenic mice are those overexpressing human mutations in APP. These animals typically develop Aβ plaques composed of Aβ42 between 6-9 months of age (LaFerla and KN Green, 2012). They also develop memory impairments that can be observed before the appearance of plaques, suggesting that Aβ may be mediating cognitive decline (LaFerla and KN Green, 2012). Based on data from transgenic mice, researchers discovered that Aβ oligomeric species may play a larger role in the pathogenicity of AD (Haass and Selkoe, 2007). Interestingly, NFT are not observed in APP-overexpressing mice, yet some display hyperphosphorylated tau (Götz *et al.*, 2007). Several reasons may account for the lack of NFT, including rodent tau having a different structure that prevents it from accumulating into NFT or another possibly is that the rodent's lifespan is not long enough to allow for the development of NFT which takes decades in humans. To account for the lack of NFT, multigenic mice were created that have mutated human tau in addition to overexpression APP (Lewis *et al.*, 2001; Oddo *et al.*, 2003). Sophisticated transgenic mouse models have since been generated, incorporating a variety of known genetic mutations to mirror AD pathologies. In addition to the core pathologies, most of these models exhibit cognitive decline and increased neuroinflammation (LaFerla and KN Green, 2012). They have revealed discoveries of potential disease progression and interactions not previously considered.

Translational issues exist with the vast majority of transgenic models used to recapitulate AD pathologies, since nearly all are mutations associated with APP processing or tau (or both). However, these mutations reflect those found in familial AD and not what is observed with sporadic AD, which is far more prevalent. Although useful in advancing our understanding of the disorder, concerns arise with using transgenic AD models to examine new therapeutics or targets slated for the heterogeneous human AD population and may contribute to the lack of consistency

between results in preclinical trials and human clinical trials (LaFerla and KN Green, 2012). Using animal models that exhibit known risk factors associated sporadic AD to examine drug therapies would be a beneficial translational approach.

Diabetes Risk Factor

Individuals with diabetes have an increased risk of developing AD (Brands *et al.*, 2005; Biessels *et al.*, 2006). Diabetes is a metabolic disorder that results in hyperglycemia and insulin dysregulation. Currently, 80% of patients diagnosed with AD have impairments in glucose tolerance or have been diagnosed with diabetes (Janson *et al.*, 2004). Approximately 23.6 million people in the United States are affected by diabetes, with this number predicted to increase to over 29 million by 2050 (Centers for Disease Control, 2014). Between 5-10% of these individuals have Type 1 diabetes, characterized by hyperglycemia and insulin deficiency. Whereas the most common form is Type 2 diabetes, accounting for 90-95% of cases and is associated with hyperinsulinemia and insulin resistance. Mild to severe cognitive impairments are associated with both forms of diabetes, including memory impairments and attention (Strachan *et al.*, 1997; Awad *et al.*, 2004). Individuals with Type 2 diabetes are twice as likely to develop AD later in life compared to the normal population (Janson *et al.*, 2004), while Type 1 diabetes patients have an 80% chance of being diagnosed with AD (Whitmer *et al.*, 2015). Due to the heterogeneity of diabetes symptoms, it is difficult to determine which component contributes to the risk associated with developing AD.

Evidence exists that suggest insulin resistance may be a contributing factor in AD. Insulin is produced by pancreatic beta cells and regulates glucose metabolism in the periphery (Woods *et al.*, 1985; Saltiel and Kahn, 2001). Early studies of brain glucose metabolism considered the brain as insulin insensitive, since the uptake of glucose by the CNS is not dependent on insulin. The

transportation of glucose into neurons and glia relies on GLUT3 and GLUT1 receptors (McEwen and Reagan, 2004; Gray *et al.*, 2014). However, it has since been discovered that CNS insulin levels regulate overall energy homeostasis as well as control of food intake, metabolic rate, and energy expenditure (Schwartz *et al.*, 1992; Chavez *et al.*, 1995). In addition, insulin concentration in the brain is independent of circulating peripheral insulin (Havrankova *et al.*, 1979), yet insulin readily crosses the blood brain barrier via an insulin receptor-mediated transport process (Baskin *et al.*, 1987; Baura *et al.*, 1993; Banks, Jaspan, Huang, *et al.*, 1997; Banks, Jaspan, and Kastin, 1997). Evidence suggests that insulin is produced locally in the brain, as insulin CNS concentrations are 10-100 times higher compared to plasma levels (Havrankova, Roth, *et al.*, 1978); however, further investigations are needed to elucidate the mechanisms of its local production.

Receptors for insulin are selectively distributed in the brain, with the highest density found in the olfactory bulb, hippocampus, amygdala, cerebral cortex, and hypothalamus (Havrankova, Schmechel, *et al.*, 1978; Havrankova, Roth, *et al.*, 1978; Baskin *et al.*, 1987; Unger *et al.*, 1991). Both neurons and astrocytes express insulin receptors located at the synapses (Abbott *et al.*, 1999). Due to their abundance in the hippocampus and areas of the medial temporal cortex, insulin and insulin receptors play a role in modulating synaptic activity, LTP, and memory (Baskin *et al.*, 1988; W-Q Zhao and Alkon, 2001). For instance, insulin influences cell membrane expression of *N*-methyl-$_{D}$-aspartate (NMDA) receptors, which are highly abundant in these regions and are critical for LTP and synaptic plasticity (Skeberdis *et al.*, 2001).

Memory enhancement after insulin administration has been observed in several studies. Rodent studies have found enhanced memory performance in the Morris water maze and passive-avoidance task after intracerebroventricular insulin infusions (Park *et al.*, 2000; Haj-ali *et al.*,

2009). In healthy humans, intranasal and intravenous administration of insulin enhances story recall and improvement in cognitive flexibility (Kern *et al.*, 1999; Craft *et al.*, 1999; Fehm *et al.*, 2000). Similarly, studies demonstrate that insulin receptor expression can be modulated by learning. In one study, insulin signaling molecules and the amount of insulin receptor mRNA in the hippocampus was increased in rats trained in the Morris water maze compared to untrained rats (W Zhao *et al.*, 1999).

In AD, low concentrations of insulin and an increase number of insulin receptors are observed compared to age-matched controls (Frölich *et al.*, 1999; Hoyer, 2002; Craft and Watson, 2004). Dysregulation of cellular processes related to insulin are thought to contribute to both AD and type 2 diabetes, including glucose and cholesterol metabolism, ApoE processing, and second messenger signaling (Janson *et al.*, 2004; Martins *et al.*, 2006; Moreira *et al.*, 2007; W-Q Zhao and Townsend, 2009). It is further speculated that insulin dysregulation in AD exacerbates the formation of Aβ plaques and NFT (Sims-Robinson *et al.*, 2010). One theory suggests that altered insulin signaling results in chronic oxidative metabolism and increased acidosis in the Golgi apparatus and endoplasmic reticulum that alter APP metabolism, resulting in a favorable atmosphere for the accumulation of Aβ (Frölich *et al.*, 1999; Hoyer, 2002).

Insulin and Aβ are both degraded by insulin-degrading enzyme (IDE) in healthy neurons and microglia. In type 2 diabetes, insulin resistance results in elevated insulin levels potentially leading to competition between Aβ and insulin for IDE (Gasparini and H Xu, 2003). Brain tissue from AD patients display significantly lower amounts of IDE mRNA compared to age-matched controls (A Pérez *et al.*, 2000; Cook *et al.*, 2003; Farris *et al.*, 2003). Thus, a deficiency in IDE processing may contribute to pathologies in AD and diabetes. In a study examining the effects of insulin on Aβ42 levels in CSF of healthy older adults, researchers found that intravenous insulin

administration led to an increase of Aβ42 in the CSF (Watson *et al.*, 2003). As mentioned previously, insulin infusions lead to enhanced memory abilities but this effect was decreased in older individuals with the greater increase in CSF Aβ42 concentrations. The clearance mechanisms of Aβ42, potentially via IDE, may be disrupted in older adults as a consequence of age and the sustained elevation of Aβ42 may affect memory (Craft and Watson, 2004). Insulin sensitizers may aid in alleviating competition between insulin and Aβ with IDE by increasing the sensitivity of insulin receptors (Pedersen *et al.*, 2006). AD transgenic mice treated with the insulin sensitizer, rosiglitazone, had reduced Aβ42 levels and improvement in spatial learning and memory (Pedersen *et al.*, 2006). A preliminary study using rosiglitazone in AD patients resulted in better cognitive measures after treatment versus before; however, no change or decline in Aβ42 levels were observed (Watson *et al.*, 2005). Together, insulin dysregulation influences Aβ levels potentially via IDE.

In vitro and *in vivo* studies demonstrate that insulin regulates tau phosphorylation (M Hong and VM-Y Lee, 1997; Lesort and Johnson, 2000; Schubert *et al.*, 2003; Cheng *et al.*, 2005). Conversely, hyperinsulinemia occurs following tau hyperphosphorylation in rat brains (Freude *et al.*, 2005). Furthermore, mice deficient in insulin receptor substrate-2 (Schubert *et al.*, 2003; 2004) or the neuronal insulin receptor gene (Schubert *et al.*, 2004) result in increased tau phosphorylation and NFT. Intranasal insulin has been shown to ameliorate tau hyperphosphorylation in a rodent model of type 2 diabetes (Yang *et al.*, 2013). Despite observing changes in phosphorylated states of tau in culture and animal models of diabetes, little is known about diabetes and tau pathogenesis.

Mitogen-activated protein kinase (MAPK) and Akt-signaling pathways (both implicated in AD pathogenesis) are cellular mechanisms activated after insulin receptor signaling. MAPK is ubiquitous kinase that regulates cell proliferation and cell death (Pearson *et al.*, 2001). MAPK

immunoreactivity and expression is increased in AD brains compared to normal controls (Hensley *et al.*, 1999) and is correlated with Aβ plaques and NFT (Hensley *et al.*, 1999; Munoz and Ammit, 2010). Further, studies have demonstrated its involvement in tau phosphorylation, neuroinflammation, and synaptic plasticity (Munoz and Ammit, 2010). Akt signaling mediates cell proliferation and protein synthesis (Brazil and Hemmings, 2001; Tremblay and Giguère, 2008). Through Akt signaling, insulin receptor activation via insulin leads to GSK3β inactivation. Changes in glucose and insulin concentrations in the hippocampus and cortex influences GSK3β activity (Clodfelder-Miller *et al.*, 2005). During insulin resistance, GSK3β is dephosphorylated and, thus, activated (Clodfelder-Miller *et al.*, 2006); (Balaraman *et al.*, 2006). Activation of GSK3β can further perpetuate insulin resistance by reducing glucose clearance (J Lee and Kim, 2007), increased PS1 activity leading to elevation in Aβ production (Phiel *et al.*, 2003), and hyperphosphorylated tau (Balaraman *et al.*, 2006).

The association between AD and diabetes is particularly strong among *APOE* ε4 carriers (Kuusisto *et al.*, 1997; Peila *et al.*, 2002; Irie *et al.*, 2008; Matsuzaki *et al.*, 2010). For example, ApoE4 carriers with type 2 diabetes have a five-fold risk of developing AD compared with individuals who are not ApoE4 carriers and do not have diabetes (Haan, 2006). The prevalence of Aβ plaques and NFT are greater in patients with diabetes who have ApoE4 and insulin dysregulation in some patients with sporadic AD to has been linked to the *APOE* genotype (Peila *et al.*, 2002).

Clinical trials examining effect of intranasal insulin as potential therapy in early AD are ongoing. Insulin can bypass the blood-brain barrier and enter the CSF via intranasal administration (Born *et al.*, 2002). Studies on older adults with AD or mild cognitive impairment showed significant improvement in memory with both low and high doses of intranasal insulin that

persisted two months after the end of treatment (Craft, 2012; Yarchoan and Arnold, 2014). Although acute intranasal insulin treatment shows promise with cognitive functioning, long-term studies are imperative to ensure that hyperinsulinemic conditions do not promote further insulin resistance in AD patients.

A commonly used method to study diabetes and AD pathologies in rodents uses the administration of a compound called streptozotocin (STZ; 2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose). STZ is selective for glucose transporter 2 (GLUT2) located mainly on insulin producing pancreatic beta cells and results in alkylation and methylation of DNA leading to apoptosis (Murata *et al.*, 1999; Szkudelski, 2001). Permanent diabetes results when high doses (100-200 mg/kg, intraperitoneal) are administered to rodents, that results in little to no insulin production and high mortality rate among subjects (Szkudelski, 2001; Grünblatt *et al.*, 2007). Alternatively, multiple low to moderate doses of STZ (30-60 mg/kg, intraperitoneal) results in insulin resistance by maintaining insulin-immunoreactive cells in the pancreas (Ito *et al.*, 1999). In this animal model of type 1 diabetes, animals display learning deficits (Stranahan *et al.*, 2008), increased GSK3β activity (Jope and Johnson, 2004), increased tau phosphorylation, increase Aβ levels, and neuroinflammation (Mensah-Brown *et al.*, 2005; Jolivalt *et al.*, 2008). Two routes of administration are common using STZ: peripheral administration that targets pancreatic beta cells and intracerebroventricular (ICV) infusions that results in reduced expression of insulin receptors in the brain (Nazem *et al.*, 2015). Both models develop learning and memory impairments in a variety of tasks (including Morris water maze, novel object recognition, and Barnes maze), AD pathologies, and elevated neuroinflammation (Šalković-Petrišić *et al.*, 2011; Nazem *et al.*, 2015; Murtishaw *et al.*, 2016; Murtishaw *et al.,* in review). When STZ is administered to APP transgenic mice, induction of diabetes exacerbated AD symptoms including learning deficits, increased tau

phosphorylation, increase number of Aβ plaques, increased GSK3β activity, and decreased insulin receptor activity (Jolivalt *et al.*, 2010). Thus, STZ administration provides researchers with a translational approach to examine sporadic AD that encompasses the risk factor diabetes.

A strong link exists between AD and diabetes, yet both disorders occur on a spectrum and exhibit heterogeneity in their symptomology. A feature related to both disorders that may play a role in exacerbating AD pathologies is chronic neuroinflammation. Determining the relationship between AD, diabetes, and neuroinflammation is vital to providing effective treatment in AD.

Neuroinflammation Risk Factor

Inflammation is a complex process that occurs in response to injury, infections, or threats that restores the body back to normal physiology. The CNS has a specialized immune system due to the protective blood-brain barrier and this system involves complex orchestrations across many neuronal and non-neuronal cell types (Burda and Sofroniew, 2014). The principal responders to damage to the CNS are glial cells (primarily microglia, oligodendrocytes, and astrocytes), which preserve homeostasis to allow neurons to function normally. An alteration in glial cell function can greatly impact neuronal synchrony and overall CNS function. Reactive gliosis, the nonspecific reaction glial cells to damage in the CNS, can take on different forms depending on the type of injury, insult, or even disease state (Sofroniew and Vinters, 2009; Burda and Sofroniew, 2014). Various cells types are involved in reactive gliosis that respond in different ways to an array of insults. For instance, acute insults initiate tissue replacement and would repair, while disperse and chronic diseases trigger progressive tissue changes (Burda and Sofroniew, 2014). Neuroinflammation is a state of chronic, sustained inflammatory response which can persist long after the initial injury (Z Cai *et al.*, 2014).

Reactive gliosis and subsequent sustained neuroinflammation is a dynamic, complicated process in neurodegenerative disorders. Although many cells types and factors are involved, microglia have been the primary focus in AD inflammation research, as their function appears to be dysregulated in the disorder. Microglia serve as the main resident immune cells, making up 10 to 15% of total cells in the CNS. These phagocytic macrophages circulate or "survey" the environment where they are uniformly distributed and provide signals that influences astrocytes and neurons (Z Cai *et al.*, 2014). During normal, physiological conditions, microglia exhibit a deactivated or ramified state where they release anti-inflammatory cytokines and neurotrophic factors and regulate synaptic plasticity (Streit, 2002). They also participate in removing debris from non-neuronal apoptotic cell death (Schafer and Stevens, 2015).

In response to injury or invasion, adenosine triphosphate dependent mechanism attracts microglia to the site of injury where the microglia initiate repair functions (Davalos *et al.*, 2005; Haynes *et al.*, 2006). They switch to a reactivate (or primed) state and change their chemical and morphological structure. Their normally protracted filopodia that allows them to monitor synaptic activity retract and they take on more of an amoeboid structure, compromising microglial regulation of network homeostasis (Van Eldik *et al.*, 2016). They become fully phagocytic and release a variety of factors including pro-inflammatory cytokines, free radicals, and neurotoxins (Wierzba-Bobrowicz *et al.*, 2002). The types of cytokines released and alterations of gene expression classify microglia generally as classically activated (M1) or alternatively activated (M2). M1-polarized microglia are poor phagocytes that release pro-inflammatory cytokines, including tumor necrosis factor - α (TNFα), interleukin (IL)-1, IL-6, IL-12, IL-18, nitric oxide, and prostaglandins (Malm *et al.*, 2015). Alternatively, high phagocytosis capabilities and secretion

of anti-inflammatory cytokines, such as IL-10, IL-4, IL-13, and transforming growth factor-β, are a feature of M2-polarized microglia (Malm *et al.*, 2015).

Aβ, NFT, and neuronal cell loss are perhaps stimulants of microglia in the AD brain. In the presence of Aβ, microglia release chemokines (specifically C-C chemokine ligand 2) (Boddeke *et al.*, 1999), which attract other microglia. In AD patients and transgenic AD mice, levels of C-C chemokine ligand 2 (CCL2) are increased (Oddo *et al.*, 2003; Jankowsky *et al.*, 2004; Janelsins *et al.*, 2005) and mice that do not express its receptor (CCR2) have microglial impairments in being recruited to the site of Aβ plaques as well as higher levels of Aβ (Khoury *et al.*, 2007; Naert and Rivest, 2011). A variety of cell surface recognition receptors allow microglia to detect fibrillary forms of Aβ, including Toll-like receptors (TLRs) 2, 4, and 6, cluster of differentiation (CD) 14, CD 36, A1 scavenger receptors (SCARA1), and class B2 scavenger receptors (Coraci *et al.*, 2002; Khoury *et al.*, 2003; Frenkel *et al.*, 2013). After stimulation of these receptors, various signal transduction pathways are activated that lead to nuclear factor kappa B (NF-κB)-dependent transcription of pro-inflammatory cytokines , reactive oxygen species, and phagocytosis (DR McDonald *et al.*, 1998; Bamberger *et al.*, 2003; Alarcón *et al.*, 2005; Fang *et al.*, 2010; Heneka *et al.*, 2012). The pathophysiology of diabetes also implicates increased phosphorylation of NF-κB and subsequent increase in pro-inflammatory cytokines (Negi *et al.*, 2010; Yirmiya and Goshen, 2011; Datusalia and SS Sharma, 2014). At this point in the signaling pathway, these responses can have both advantageous and deleterious effects. For example, enhanced expression of SCARA1 aids to clear Aβ (Frenkel *et al.*, 2013). Conversely, mediates pro-inflammatory production, of which has detrimental effects.

Enhanced expression of pro-inflammatory cytokines have been found in AD and diabetes brain and CSF, including TNFα, IL-6, IL-10, and IL-1β (Blum-Degen *et al.*, 1995; Tarkowski *et*

al., 2002; Mrak and Griffin, 2005b; H Jiang *et al.*, 2011; Yirmiya and Goshen, 2011; Datusalia and SS Sharma, 2014). Chronic released of pro-inflammatory cytokines by microglia increase the production of Aβ by favoring the toxic cleavage of APP towards γ- and β- secretase (Blasko *et al.*, 2000; HS Hong *et al.*, 2003; Liao *et al.*, 2004; Malm *et al.*, 2015). Moreover, chronic inflammation reduces the levels of IDE and the phagocytic capability of microglia, further perpetuating Aβ accumulation (Chung *et al.*, 1999; Koenigsknecht-Talboo and Landreth, 2005; Rezai-Zadeh *et al.*, 2011). Reactive microglia are initially beneficial to the system in response to a harmful stimulus; however, chronic reactive responses may amplify destructive effects.

While the removal of these toxins is initially advantageous, a wealth of evidence demonstrates that the upregulation of certain immune system components may result in further neurodegeneration more destructive than the initial pathogenic stimulants (Akiyama *et al.*, 2000). AD is a very slow process that may span 20 years between initial Aβ accumulation to the appearance of cognitive deficits (Villemagne *et al.*, 2013). Increased pro-inflammatory cytokines can increase Aβ deposition and deficits in learning and memory (Games *et al.*, 1995; Wyss-Coray and Mucke, 2002; Guo *et al.*, 2002; Wyss-Coray, 2006). In a study examining P301S mutant human tau transgenic mice, activated microglia were detected early in neurodegeneration link tau abnormalities and microglia (Yoshiyama *et al.*, 2007). Activation of inflammatory markers (TNF-α and monocyte chemoattractant protein-1) was observed in triple transgenic (overexpressing APP, PS1, and tau mutations) AD mice as early as three months of age and occur alongside the accumulation of Aβ (Janelsins *et al.*, 2005). In cell culture studies, IL-6 and IL-1 amplify levels of tau hyperphosphorylation and NFT (Yuekui Li *et al.*, 2003; Quintanilla *et al.*, 2004; Saez *et al.*, 2004). As mentioned in the previous chapter, non-transgenic AD models using the diabetes risk factor display increased neuroinflammation. Animals given STZ exhibited increased IL-1 and

TNF-α in addition to microglial activation (Prickaerts *et al.*, 1999; Y Chen *et al.*, 2013; Murtishaw *et al.*, 2016). Therefore, neuroinflammation enhances AD pathologies in postmortem brain tissue and animal models of AD.

Inflammation associated with AD was initially thought to occur during end stages of the disorder and did not contribute to the progression of symptoms. However, data from genetic, preclinical, and bioinformatics studies reveal that the immune system not only accompanies but contributes to AD symptoms (Zhang *et al.*, 2013). A current hypothesis implicates microglia as the main facilitator in neuroinflammation that contributes to and progresses AD pathology (Zheng *et al.*, 2010). The most compelling research to date that links microglia with AD progression comes from genome-wide association studies (GWAS) in a which rare variants of several genes associated with microglia has been identified as a risk factors for sporadic AD (Guerreiro *et al.*, 2013; Jonsson *et al.*, 2013; Bertram *et al.*, 2013; Benitez *et al.*, 2013; Ruiz *et al.*, 2013; Slattery *et al.*, 2014). A rare missense mutation associated with TREM2, a transmembrane receptor found in various tissue macrophages including microglia and bone marrow-derived macrophages, is one such risk factor (Daws *et al.*, 2001; Paloneva *et al.*, 2002; Schmid *et al.*, 2002). Overexpression of TREM2 on microglia *in vitro* increases its ability to clear Aβ (Melchior *et al.*, 2010; T Jiang *et al.*, 2014) and attenuate the release of pro-inflammatory cytokines (K Takahashi *et al.*, 2005; Turnbull *et al.*, 2006; Hamerman *et al.*, 2006). Microglia are commonly found near Aβ plaques in AD mouse models; however, in TREM2-deficient mice, microglia are absent from Aβ plaques (Ulrich *et al.*, 2014; Yaming Wang *et al.*, 2015). Additional studies found that TREM2 reduces microglial phagocytic function and pro-inflammatory response in the presence of Aβ (Hickman and Khoury, 2014; Kleinberger *et al.*, 2014). Another risk factor for AD discovered from GWAS is associated with increased CD33 expression in microglia and monocytes (Van Eldik *et al.*, 2016). The

increased expression of CD33 is suggested to promote Aβ42 accumulation (Griciuc *et al.*, 2013; Bradshaw *et al.*, 2013; Malik *et al.*, 2013). As discussed in previous chapters, the ApoE4 is another risk factor associated with AD discovered through GWAS. Studies in microglia provide evidence that ApoE4 is less efficient at promoting enzyme-mediated clearance of Aβ compared to ApoE3 (Q Jiang *et al.*, 2008). Independent of Aβ, ApoE4 activate an inflammatory response that leads to the breakdown of the blood-brain barrier and leakage of microvasculature that releases toxic proteins into the brain (Bell *et al.*, 2012). These risk factors provide a crucial link between microglia, Aβ clearance, and sporadic AD.

Early epidemiological studies found that individuals with arthritis had a lower rate of developing AD and this observation has since been correlated with the use of nonsteroidal anti-inflammatory drugs (NSAIDs) (McGeer *et al.*, 1990). Follow-up studies have reported that there is a 50% reduction in the risk of developing AD for those who are long-term users of NSAIDs (Wyss-Coray, 2006). The main targets of NSAIDs are cyclooxygenase (COX-1) and COX-2 where they act by inhibiting key inducers of inflammation (prostaglandins and thromboxanes) (Wyss-Coray, 2006). With respect to AD, alternative targets of NSAIDs have been examined including peroxisome proliferator-activated receptor-γ (PPAR-γ) and the presenilins. In cell culture, PPAR-γ reduces Aβ levels by inhibiting the activity of β-secretase cleaving enzyme 1 (BACE1) promotor, which the enzyme that metabolizes APP into the pathogenic form (Sastre *et al.*, 2006). Studies using cell culture and APP transgenic mice have found reductions in Aβ levels with different NSAIDs that have varying affinity for COX and alternative targets (Weggen *et al.*, 2001; Eriksen *et al.*, 2003; Y Takahashi *et al.*, 2003; Lleó *et al.*, 2004; Beher *et al.*, 2004; Gasparini *et al.*, 2004). One particular NSAIDs (*R*-flurbiprofen or Flurizan™) did exceptionally well in Phase I and II trials, where it slowed functional and cognitive decline in AD patients for up to 21 months of

treatment yet failed to show any beneficial changes in several Phase III trials (RC Green *et al.*, 2009). Overall, these studies suggest that the downstream targets of NSAIDs may be acting upon Aβ or Aβ precursors but not necessarily on directly reducing inflammation that is chronically activated in AD.

As mentioned, microglia respond to and proliferate towards chemokines. The soluble form of a chemokine, called fractalkine or CX3CL1, mediates microglial chemoattraction (Maciejewski-Lenior *et al.*, 1999). Exogenous application of CX3CL1 results in increased microglial proliferation, as they express the fractalkine receptor CX3CR1 (Hatori *et al.*, 2002). Insufficient signaling of CX3CL1/R1 leads to an enhanced microglial inflammatory response, as demonstrated by mice lacking functional CX3CR1 receptors (Cardona *et al.*, 2006). For example, APP/PS1 mice lacking functional CX3CR1 showed increased levels of TNFα and IL-1β (S Lee *et al.*, 2017). Methods to reduce chronic microglia proliferation in AD models would be useful to understanding the disorder. In particular, baclofen (a $GABA_B$ receptor agonist) has been found to reduce pro-inflammatory cytokine release in peripheral leucocytes that express CX3CR1 (Duthey *et al.*, 2010) and microglia cell cultures (Kuhn *et al.*, 2004). Not only can $GABA_B$ receptors lead to inhibition of microglia via the neurotransmitter GABA but it has suggested that they can also interfere with microglial chemotaxis through chemokine receptors (Duthey *et al.*, 2010). Therefore, outlining the role of $GABA_B$ inhibition on microglia may aid in our understanding of neuroinflammation in AD.

$GABA_B$ Signaling in Alzheimer's Disease and Neuroinflammation

Multiple neurotransmitter systems are implicated in the progression of AD, including acetylcholine, dopamine, glutamate, monoaminergic systems, and GABA (Francis *et al.*, 1999;

Iwakiri *et al.*, 2005; Yanfang Li *et al.*, 2016). However, GABA signaling on microglia may serve a role in reducing neuroinflammation and subsequently halt the progression of AD symptoms.

Gamma-aminobutryic acid (GABA) the main inhibitory neurotransmitter in the brain and is synthesized from glutamate in neurons expressing glutamic acid decarboxylase (GAD). Two main classes of GABA receptor systems exist: $GABA_A$ and $GABA_B$. $GABA_A$ receptors are ionotropic and permeable to chloride. Activation of these receptors leads to a quick-onset hyperpolarization (Macdonald and Olsen, 1994). $GABA_B$ receptors are metabotropic G_i/G_o coupled receptors located pre- and postsynaptically (Bettler *et al.*, 2004), where they function to regulate ion channels by either activating potassium channels or inhibiting calcium channels (Gassmann and Bettler, 2012). Furthermore, $GABA_B$ receptor modulates GABA and glutamate neurotransmitter release and reduces depolarization induced by excitatory neurotransmitters (Bettler *et al.*, 2004). In neurons, $GABA_B$ plays a large role in regulating oscillatory activity necessary for cognition and learning and memory (Gaiarsa *et al.*, 2011).

A growing body of literature illustrates the role of GABA in regulating inflammatory responses and suggests that GABA induces a neuroprotective phenotype in microglia (Mead *et al.*, 2012). Microglia express functional $GABA_B$ receptors in culture and *in vivo* (Kuhn *et al.*, 2004; Liu *et al.*, 2016). Studies demonstrate that microglia can modulate pro-inflammatory cytokine release in response to GABA concentrations. For example, $GABA_B$ receptor agonists activate outward rectifying potassium channel conductance in microglia and reduce the release of pro-inflammatory cytokines IL-6 and IL-12p40 (Kuhn *et al.*, 2004). Furthermore, $GABA_B$ receptors are increased on microglia in response to injury (Kuhn *et al.*, 2004). As mentioned in the previous chapter, $GABA_B$ receptors might be able to alter chemotaxis of microglia. For example, baclofen reduces the migration of CX3CR1 containing peripheral blood monocytes towards CX3CL1 by

70% (Duthey *et al.*, 2010). CX3CR1 are also G protein coupled receptors. It is proposed that the G_i protein associated with $GABA_B$ receptors may interfere and inhibit the function of pro-inflammatory CX3CR1 linked to the G_i signaling pathway through heterologous desensitization (Duthey *et al.*, 2010). Taken together, $GABA_B$ receptors may serve to regulate microglia activity in times of stress.

In AD, GABAergic signaling undergoes profound pathological changes in AD resulting in decreased neurotransmission and neuronal receptor expression (Iwakiri *et al.*, 2005; Yanfang Li *et al.*, 2016). Conflicting data exist over the altered expression of various $GABA_A$ receptor subtypes in brain samples from AD patients (Yuan and Shan, 2014). However, a correlation exists between the reduction $GABA_B$ receptor expression and AD pathologies in AD brains (Iwakiri *et al.*, 2005).

Together these data suggest that a reduction in GABAergic tone in AD may compromise an important anti-inflammatory pathway via $GABA_B$ receptor function on microglia and possibly exacerbate progression of AD. As of yet, there are no studies outlining the effect of $GABA_B$ receptors on microglia in AD.

Experimental Hypotheses and Implications

The purpose of this study is to examine the role of $GABA_B$ receptors on neuroinflammation in a rodent model of sporadic AD that encompasses the diabetes risk factor. A considerable amount of data exists examining AD pathologies in transgenic rodent models. However, these models recapitulate genetic manipulations that are only observed in familial AD patients (1-5% of AD population). Inducing a diabetic-like state in an animal model that displays AD pathologies and neuroinflammation is a valid, translational approach in examining mechanisms of the disorder.

Since it has been demonstrated that $GABA_B$ receptor activation on microglia reduces pro-inflammatory cytokines and neuroinflammation, we propose to investigate their role in a diabetes

animal model of AD examining behavior and brain tissue. To induce a diabetic-like state, we administered STZ at 40 mg/kg (intraperitoneal) to C57BL/6J mice using a staggered protocol similar to the schedule of administration from our laboratory that results in sustained, elevated blood glucose levels with zero mortality (Murtishaw *et al*., in review). When mice reached a group average of 250 mg/dL blood glucose level (indicative of a diabetic state; (Atkinson, 2011), the $GABA_B$ receptor agonist (baclofen, 2 mg/kg, intraperitoneal) administration began. This dose was selected based on previous behavioral experiments in our laboratory as well as studies indicating that baclofen can induce lethargy, significantly decrease muscle tone, and alter eating behavior at higher doses (Patel and Ebenezer, 2010; Heaney *et al.*, 2012; Heaney and Kinney, 2016). Baclofen was administered for two weeks, twice a day to ensure that the drug is chronically activating $GABA_B$ receptors. It should be noted that pancreatic beta cells contain $GABA_B$ receptors which are involved in insulin production and studies demonstrate that baclofen produces an increase in insulin release in these cells (Brice *et al.*, 2002). Although baclofen may enhance the efficiency of remaining pancreas beta cells, it cannot reverse the effects of STZ-induced pancreatic beta cell death.

Behavioral tests commenced after a drug washout period to make certain that active baclofen will not contributing to behavior. The open field test was performed to examine anxiety phenotypes. To assess learning and memory, exploratory behavior in the novel objection recognition test (NOR) was measured. Animal models of AD consistently show deficits in exploring novel objects in this task, suggesting learning and memory deficits (Antunes and Biala, 2012; Murtishaw *et al.*, 2016; Murtishaw *et al*., in review). To investigate associative learning, freezing behavior was measured in the cued and contextual fear conditioning test. Components of

this task can reveal hippocampal dysfunction (Maren *et al.*, 2013), the first region of neuronal loss in AD (Padurariu *et al.*, 2012). Finally, nociceptive differences were tested in the tail flick test.

To investigate protein and mRNA changes in the hippocampus and cortex consistent with those seen in AD and neuroinflammation, protocols including western blotting, immunohistochemistry, and RT-PCR were performed on brain tissue. Targets outlined in Table 1 were analyzed via western blotting and involve AD related pathological targets (phosphorylated tau and Aβ oligomers), a major protein involved in insulin signaling (IDE), and $GABA_B$ receptor subunits. Activated microglia and microvascular hemorrhages associated with AD were assessed via immunohistochemistry. Pro-inflammatory and anti-inflammatory cytokine (outlined in Table 2) mRNA expression were measured with RT-PCR.

Hypothesis 1:

Administration of STZ will lead to behavioral and biochemical changes associated with AD.

Implications for Hypothesis 1: If administration of STZ leads to behavioral and biochemical changes associated with AD, then these data highlight the link between insulin dysregulation and its contribution to the progression of AD pathology.

Hypothesis 2:

Chronic activation of $GABA_B$ receptors (via baclofen administration) in a diabetes model will attenuate neuroinflammation leading to a rescue in behavioral and biochemical changes associated with AD.

Implications for Hypothesis 2: If deficits in behavior and changes related to AD pathology and neuroinflammation in brain tissue induced by STZ administration are rescued by chronic baclofen administration, these data would suggest that

$GABA_B$ receptor activation modulates neuroinflammatory processes.

CHAPTER 3

MATERIALS AND METHODS

Subjects

Sample size to determine the number of subjects was calculated *a prior* using power analysis software, G Power (Faul *et al.*, 2007). Using previously collected data our laboratory (Murtishaw *et al.,* in review) with power was set at 0.80 and $\alpha = 0.05$ (two-tailed), sample size was calculated at n = 4 per treatment group. To sufficient power for each of the three tissue analyses, n = 12 per treatment group was determined (n = 48 total animals used).

C57BL/6J mice (Jackson Laboratory) were housed six per cage by treatment group (STZ or non-STZ). The mouse colony room was on a 12:12 light/dark cycle and mice had access to standard chow and water available ad libitum. Behavioral tests were conducted during the light cycle. All procedures were approved by the University of Nevada, Las Vegas Institutional Animal Care and Use Committee and were performed in accordance with NIH guidelines for the care and use of laboratory animals.

Drugs Treatments

Mice were randomly assigned to STZ, STZ + baclofen, baclofen alone, or control group (n = 12 per group). STZ (Sigma-Aldrich) was prepared immediately before use by dissolving in filtered 0.1 M sodium citrate buffer (pH 4.5) for a final concentration of 4 mg/mL, as STZ is pharmacologically active for 15 minutes before a fresh batch needs to be prepared (Schein *et al.*, 1973). Baclofen (Sigma-Aldrich) was dissolved in pharmaceutical grade saline (.9% NaCl) for a final concentration of 0.2 mg/mL.

Apparatus

Open Field and Novel Object Recognition Chamber

Plexiglas chambers (37 cm L x 37 cm W x 37 cm H) with white interior was used for both the open field and the novel object recognition tasks. Objects for the novel object recognition task include a yellow semicircle (7.5 cm D x 3.5 cm H), green rectangular pyramid (7.5 cm L x 7.5 cm W x 7.5 cm H), red pyramid (8 cm L x 7 cm W x 6 cm H), and blue semi cylinder (7.5 cm L x 7.5 cm W x 3.3 cm H). Noldus EthoVision XT (Version 11.5) measured the velocity, time spent in the perimeter (10 centimeters from the wall), and amount time investigating objects (calculated when the animal's nose is four centimeters from the object).

Fear Conditioning Chambers

For training and contextual day, two Freeze Monitor chambers (San Diego Instruments) measuring 25.4 cm (L) x 25.4 cm (W) x 19.05 cm (H) with stainless steel grid floors and Plexiglas walls was used. At the end of each session, chambers were cleaned with 50% Formula 409® (Chlorox). For cued fear, two chambers measuring 43.18 cm (L) x 12.7 cm (W) x 26.67 cm (H) with opaque walls and an added scent of vanilla was used to ensure a different visual and olfactory context. After each session, a solution of 10% ethanol was used to clean the chambers. To operate the chambers, they were connected to a computer running Freeze Monitor software (San Diego Instruments) and freezing behavior were recorded using Noldus EthoVision XT (Version 11.5) automated software.

Tail Flick

Water in a 100 mL beaker was heated and maintained at 48 degrees Celsius on a heat plate. A Sony Handycam was used to record behavior during the task and independent observers blind

to treatment groups measured the amount of time it took for the animal to flick the distal ¼ portion of their tail out of the hot water bath.

Drug Administration

Induction of Diabetes

Freshly prepared STZ was administered via intraperitoneal injection at a volume of 0.1 mL/10 g to achieve a concentration of 40 mg/kg. Control mice were administered vehicle (citrate buffer) via intraperitoneal injection at a volume of 0.1 mL/10 g. Continual monitoring of blood glucose levels during STZ injections determined the timing and number of administrations, along with data from previous work in our laboratory (Murtishaw *et al*., in review). Animals were injected on days 1, 2, 3, 14, 15, 35, and 44 (the first day of STZ injections is Day 1). After STZ or citrate buffer vehicle administration, all animals were given 10% Ensure® (Abbott Laboratories) mixed in their water for 24 hours to counteract initial hypoglycemia due to insulin release from destroyed pancreatic beta cells (Szkudelski, 2001).

During the experiment, blood glucose levels were monitored to confirm that the animals reached an elevated and sustained diabetic state. To measure blood glucose levels, lateral tail vein blood was collected after two hours of fasting. While gently restraining the animal, the withdrawal site was cleaned with alcohol. Using a sterile scalpel blade, lateral tail vein was nicked to obtain a small droplet of blood. AlphaTrak® Blood Glucose Monitoring System measured blood glucose levels. After the blood sample is obtained, gentle pressure was applied to the tail to stop the bleeding. Baseline measurements were collected a week before STZ injections. Measurements of blood glucose and weights were taken twice a week after injections begin. A reading of 250 mg/dL is considered hyperglycemic and equivalent to a diabetic state in mice (Atkinson, 2011). Baclofen treatments began when the STZ average blood glucose levels reached 250 mg/dL.

Baclofen treatment

Mice received either baclofen (0.2 mg/mL) or saline vehicle at an injection volume of 0.1 mL/g (intraperitoneal) to achieve a final concentration of 2 mg/kg. Injections were given twice daily (10 hours apart) for two weeks to maintain consistent activation of $GABA_B$ receptors throughout treatment. During the baclofen treatment, blood glucose was monitored twice a week.

Behavioral Testing

All behavioral tests were performed in a testing room separate from the colony room. Unless otherwise noted, behavior was tracked using an automated tracking system (Noldus EthoVision, Version 11.5). Behavioral testing began 36-hours after final baclofen injection to ensure complete metabolism of the drug.

Open Field Task

To assess anxiety phenotypes, behavior in the open field task was examined. Animals were be taken from the colony room and individually placed in the open field chambers located in a separate testing room. They were allowed to explore the chambers for five minutes while their activity (velocity, time spent in walls, and time spent in center) was recorded via the tracking system. At the end of the trial, animals were removed from the chambers and placed back in their home cage in the colony room. Chambers were cleaned with 10% ethanol solution before the next session.

Novel Object Recognition (NOR)

NOR is a widely used model to investigate memory alterations using a rodent's innate exploratory behavior (Antunes and Biala, 2011). Twenty-four hours following the open field task, novel object recognition was performed. On Day 1, a pair of identical objects (either yellow semicircles, green rectangular pyramids, red pyramids, or blue semi cylinders were used and

counterbalanced across subjects) were placed in two corners of each chamber. Animals were given five minutes to explore the chamber and objects while the tracking system recorded the amount of time spent with each object. On Day 2 (twenty-four hours later), one of the objects from Day 1 was replaced with a new object (novel object). During the five-minute trial, the tracking system measured the amount of time spent with the familiar and the novel object. Animals were removed at the end of the session and placed back in their home cage. Chambers were cleaned with 10% ethanol solution after each trial on each day.

Cued and Contextual Fear Conditioning

To assess associative learning abilities, animals were trained and tested in a fear conditioning task. Twenty-four hours following Day 2 of novel object recognition, trace fear conditioning training began. Animals were individually placed in a testing chamber attached to the Freeze Monitor system. After two minutes of acclimatization in the testing chamber, a 2.9 kHz 88 dB tone conditioned stimulus (CS) was presented for 30 second. At the cessation of the tone, a 4 second delay occurred before the administration of a 1 second 0.3 mA foot shock (unconditioned stimulus; US). A total of four CS-US pairings was presented and separated by a two-minute interval. Freezing behavior was monitored during the first and last two minutes of the trial using the automated tracking system. After the session, animals were taken back to their home cage and the chamber was cleaned with 50% Formula 409® (Chlorox) solution.

Cued fear conditioning took place in an altered context chamber twenty-four hours after training. Animals were individually placed in a chamber and freezing behavior was continuously monitor by the automated tracking system. After two minutes in the chamber, the original CS tone was presented for 30 s every two minutes for a total of four presentations. At the end of the trial,

animals were taken back to their home cage and the chambers cleaned with 10% ethanol solution to ensure a different olfactory cue than on training day.

Contextual fear took place in the original training chamber twenty-four hours following cued fear. Animals were individually placed in the chambers and allowed to explore for ten minutes without any CS or US presentations. Freezing behavior was continually monitored by the automated tracking system and data binned into two minute intervals. Following the session, animals were placed back in their home cage and the chamber was cleaned with 50% Formula 409® (Chlorox) solution.

Tail Flick

To assess differences in nociception, the tail flick procedure was performed twenty-four hours after the last day of fear conditioning. Animals were taken into a separate room with a beaker of hot water (48 degrees Celsius). The last one-fourth portion of each animal's tail was placed in the hot water bath and the latency with which the animal flicks its tail out of the hot water was recorded.

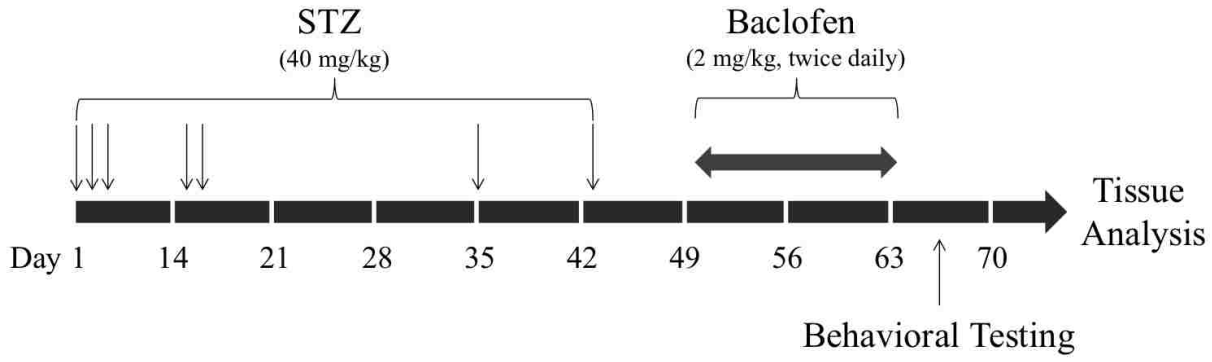

Figure 1 Timeline of Experiment

Tissue Examination

Tissue collection

Animals were randomized within treatment groups for RT-PCR, western blotting, and immunohistochemistry tissue processing prior to tissue collection (n = 4 per procedure per treatment group). All animals will be humanely euthanized with carbon dioxide asphyxiation prior to transcardial perfusion of ice cold physiological saline. For RT-PCR and western blotting, brains were rapidly removed, the hippocampus and cortex dissected out, and flash frozen with liquid nitrogen before being stored in -80 degrees Celsius. For immunohistochemistry, 4% paraformaldehyde (PFA) solution was perfused following ice cold saline, whole brains were removed, and placed in to a vial of additional 4% PFA.

SDS-PAGE Western Blotting

To examine protein expression of various targets outlined in Table 1, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) Western blotting procedure was performed. Hippocampal and cortex tissue was homogenized using Bio-Plex® Cell Lysis Kit (Bio-Rad), POLYTRON® homogenizer (Kinematica), a 24-hour -80-degree Celsius freeze/thaw, and sonication (Sonifer SFX150, VWR). Following sonication, samples were centrifuged at 4500 x g for 15 minutes and supernatant removed. Protein concentration was determined using Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific). Samples (20 μg) were separated on 10% SDS-PAGE gels and electro-transferred onto PVDF membranes (Immunobilon-FL, 0.45 micron; Millipore). Following blocking with Odyssey Blocking Buffer (LI-COR), membranes were probed with primary antibodies (see Table 1).

After overnight incubation of primary antibodies on a shaker in 4 degree Celsius and subsequent washes, membranes were probed with fluorescence-based secondary antibodies (LI-COR). After incubation and washes, membranes were imaged and analyzed using Odyssey® Infrared Imaging System (LI-COR) running Image Studio Software® (LI-COR). All proteins of interest were normalized to β-actin with the exception of the phosphorylated proteins (pTau and pGSK3β) which was normalized to the total protein (Tau and GSK3β, respectively).

Table 1 SDS-Page Western Blotting Antibodies

Antibody	Description
β-actin (1:20000; ProteinTech)	Control antibody probed on the same membrane has proteins of interest as a loading control and will be used to normalize due to its stability across treatment manipulations.
IDE (Insulin degrading enzyme; 1:1000; Abcam)	Degrades Aβ and insulin.
$GABA_BR1$ (1:1000; Cell Signaling Technology)	Obligatory $GABA_B$ receptor subunit that binds ligands. Antibody detects both pre- and post-synaptic isoforms of the receptor (1a and 1b).
$GABA_BR2$ (1:750; Cell Signaling Technology)	Obligatory $GABA_B$ receptor subunit coupled to G proteins.
GSK3β (1:1000; Cell Signaling Technology)	Kinase involved in phosphorylating tau.
Phosphorylated GSK3β (1:1000; Cell Signaling Technology)	Inactive form of GSK3β.
Aβ oligomers (1:1000; Abcam)	Detects total oligomeric species of Aβ.
Tau (1:1000; Abcam)	Detects total tau protein levels.
Phosphorylated Tau (Serine 396; 1:1000; Santa Cruz Biotech)	Detects levels of tau phosphorylated at serine 396 and will be compared against total tau levels (pTau/Tau).

Immunohistochemistry

Cortex and hippocampal coronal sections (15 μm thick) was sectioned on a cryostat (Hacker-Bright OTF5000) and stored free floating in 1xPBS at 4 degrees Celsius in plastic 12-well plates.

For staining to examine activated microglia, a procedure using the Iba1 antibody and 3,3'-Diaminobenzidine tetrahydrochloride counterstain was used. Sections were blocked in 5% normal goat serum for 45 minutes then incubated overnight at 4 degrees Celsius in Iba1 antibody (Wako). The following day, sections were washed in 1xPBS (5 x 5 minutes) and incubated for 30 minutes with diluted biotinylated secondary antibody (ABC Kit; Vector Labs). After another 5 x 5 minute washes in 1xPBS, sections were incubated for 30 minutes with VECTASTAIN® ABC Reagent (ABC Kit; Vector Labs). Following a set of 5 x 5 minute washes, sections were stained with DAB (Sigma-Aldrich) diluted in 1xPBS and 0.03% hydrogen peroxide until color develops. Sections were immediately be washed in 1xPBS, mounted on slides, and cover-slipped.

To examine microhemorrhages, the Prussian blue staining procedure was followed. Sections from the hippocampus and cortex were slide mounted and air dried overnight. The following day, sections were briefly rehydrated in water for 30 seconds, followed by incubation in freshly prepared 5% potassium ferrocyanide (Sigma Aldrich) and 5% hydrochloride acid (Sigma Aldrich) for 30 minutes. Following 5 x 5 minute washes in water, sections were counter stained in filtered 1% nuclear fast red solution for 5 minutes. Following 3 x 1 minute washes in water, sections were quickly dehydrated in succession of two dips in 95% ethanol, 100% ethanol, then xylene. Sections were then immediately covered-slipped.

Images at 20x objective were taken of the cortex and hippocampus using a Zeiss Axioskop II Plus microscope (Carl Zeiss MicroImaging, Inc.). Two independent experimenters blind to the

treatment groups counted the cells expressing the Iba1 from the DAB counterstain and, separately, the microhemorrhages from the Prussian blue staining were counted.

RT-PCR

To examine messenger RNA (mRNA) expression of pro- and anti-inflammatory cytokines associated with neuroinflammation (see Table 2) in hippocampal and cortex tissue, reverse transcriptase polymerase chain reaction (RT-PCR) was performed. mRNA was extracted from hippocampal and cortex tissue using RNeasy Mini Kit (Qiagen). mRNA concentration and quality was determined using a full spectrum spectrophotometer (NanoDrop 1000). Equal concentrations of mRNA per sample were reverse transcribed using QuantiNova Reverse Transcription Kit (Qiagen) in triplicates on Bio-Rad® C1000 Touch Thermal Cycler. Each sample of cDNA triplicate were run in triplicate with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) using Bio-Rad® CFX96 Real-Time PCR Detection System. The thermal cycling protocol was followed according to the recommended master mix instructions and as follows: 30 seconds at 95 degrees Celsius (polymerase activation and DNA denaturation) then amplification consisting of 15 seconds at 95 degrees Celsius (denaturation) and 30 seconds at 60 degrees Celsius (annealing/extension with plate read) for 40 cycles. Melt-curve analysis were performed following amplification at 65-95 degrees Celsius with a 0.5 degree increment every 5 seconds.

Table 2 RT-PCR Primer Targets

Target	Accession Number	Description
β-actin	NM_007393.5	Control/normalizing housekeeping gene (Stephens *et al.*, 2011).
GAPDH (glyceraldehyde 3-phosphate dehydrogenase)	NM_008084.3	Control/normalizing housekeeping gene (Stephens *et al.*, 2011).
HPRT1 (hypoxanthine guanine phosphoribosyl transferase 1)	NM_013556.2	Control/normalizing housekeeping gene (Stephens *et al.*, 2011).
TNF α (tumor necrosis factor alpha)	NM_013693.3	Pro-inflammatory cytokine released by microglia and suggested to be elevated in AD (Bhaskar *et al.*, 2014).
IL-6	NM_031168.2	Pro-inflammatory cytokine released by glial cells and elevated in AD (Hüll *et al.*, 1996).
IL-1β	NM_001513.1	Pro-inflammatory cytokine released by glial cells, elevated in AD, and implicated in vascular dementia (V Sharma, 2011).
IL-1α	NM_010554.4	Pro-inflammatory cytokine released by glial cells and elevated in AD (Rainero *et al.*, 2004).
IL-10	NM_010548.2	Anti-inflammatory cytokine released by glial cells. Reduction in IL-10 attenuates AD pathology (Guillot-Sestier *et al.*, 2015)

Statistical Analyses

Differences in blood glucose and body weights were analyzed by one-way between subjects analysis of variance (ANOVA) with group as the factor.

Open field data using time spent (in seconds) in the border was analyzed by one-way between subjects ANOVA with group as the factor.

Time spent investigating objects in Day 1 NOR was analyzed by one-way between subjects ANOVA with group as the factor. Time spent investigating the novel object over total time spent

investigating both novel and familiar objects in Day 2 NOR was compared using a Student's *t*-test against chance (50%) for each treatment and control group.

Time spent freezing in each day of CCF was analyzed by one-way between subjects ANOVA with group as the factor. Specifically, on CCF Training Day, only the first 120 seconds and the last 120 seconds was analyzed. Further, a Student's *t*-test for each treatment and control group was performed comparing freezing during the first 120 seconds versus the last 120 seconds. On CCF Cued Day, the trial was divided into the following portions to analyze differences in freezing across the session: first 120 seconds, during cue 1, post cue 1, during cue 2, post cue 2, during cue 3, post cue 3, during due 4, and last 120 seconds. Finally, on CCF Contextual Day, freezing during two minute bins was analyzed.

Western blotting data was analyzed by initially normalizing the protein of interest band to the control band (β-actin) or the phosphorylated form of the protein to the total protein (pTau/Tau or pGSK3β/GSK3β). Following normalization, proportion to control was determined by averaging all of the normalized control samples per membrane and setting the treatment subjects values over the averaged control values. Finally, the proportion to control for each sample was analyzed by one-way between subjects ANOVA with group as the factor.

Immunohistochemistry cell counts were analyzed by one-way between subjects ANOVA with group as the factor.

RT-PCR data were analyzed using threshold cycle value (Ct) normalized to housekeeping genes. Differences in the change of Ct (ΔCt) for experimental groups and control conditions were examined. ΔCt were analyzed by one-way between subjects ANOVA with group as the factor.

Tukey post-hoc comparisons of treatment groups was performed following any significant ANOVA to determine group significance.

CHAPTER 4

RESULTS

Induction of Diabetes

Blood Glucose Levels

Confirmation of a diabetic state was made measuring blood glucose levels before STZ administration and twice a week after the first injection. The initial injection schedule was based on data from our laboratory (40 mg/kg STZ on day 1, 2, 3, 14, and 15; Murtishaw et al., in review) and additional two injections were required to achieve a group average of 250 mg/dL (Figure 2). Before the start of the STZ injections, the four groups had equivalent blood glucose measurements ($F_{(3,44)} = 0.8587$, $p = 0.4696$). Significant increase blood glucose levels for both groups receiving STZ compared to controls began on Post Injection Day 17 ($F_{(3,44)} = 13.612$, $p = 0.000$; Tukey post-hoc analysis: Control versus STZ, $p = 0.000$; Control versus STZ Bac, $p = 0.001$). Both the STZ and STZ Bac group had significantly increased blood glucose levels across days before the beginning of the baclofen injections (Post Injection Day 6 through Post Injection Day 48; $F_{(3,44)} = 43.386$, $p = 0.000$; Tukey post-hoc analysis: Control versus STZ, $p = 0.000$; Control versus STZ Bac, $p = 0.000$). The baclofen injections resulted in a significant decrease in blood glucose levels in the STZ versus STZ Bac group ($F_{(3,44)} = 118.283$; STZ versus STZ Bac, $p = 0.000$). However, the both the STZ and STZ Bac groups were significantly elevated compared to controls ($F_{(3,44)} = 118.283$, $p = 0.000$; Tukey post-hoc analysis: Control versus STZ, $p = 0.000$; Control versus STZ Bac, $p = 0.000$). Notably, no differences were observed between the controls and baclofen alone group (Controls versus Bac, $p = 0.952$). After the baclofen injections, blood glucose levels for both STZ administered groups remained elevated compared to saline ($F_{(3,44)} = 94.495$, $p = 0.000$; Tukey post-hoc analysis: Control versus STZ, $p = 0.000$; Control versus STZ Bac, $p = 0.000$). Although

significantly elevated compared to saline, the STZ Bac group was significantly decreased compared to the STZ alone group (Tukey post-hoc analysis: p = 0.019). In short, STZ administration led to significantly elevated blood glucose measurements across days. Baclofen was able to decrease measurements in the STZ animals, suggesting its actions enhancing pancreatic beta cell function. However, the group averages for STZ Bac were still elevated compared to controls and baclofen alone.

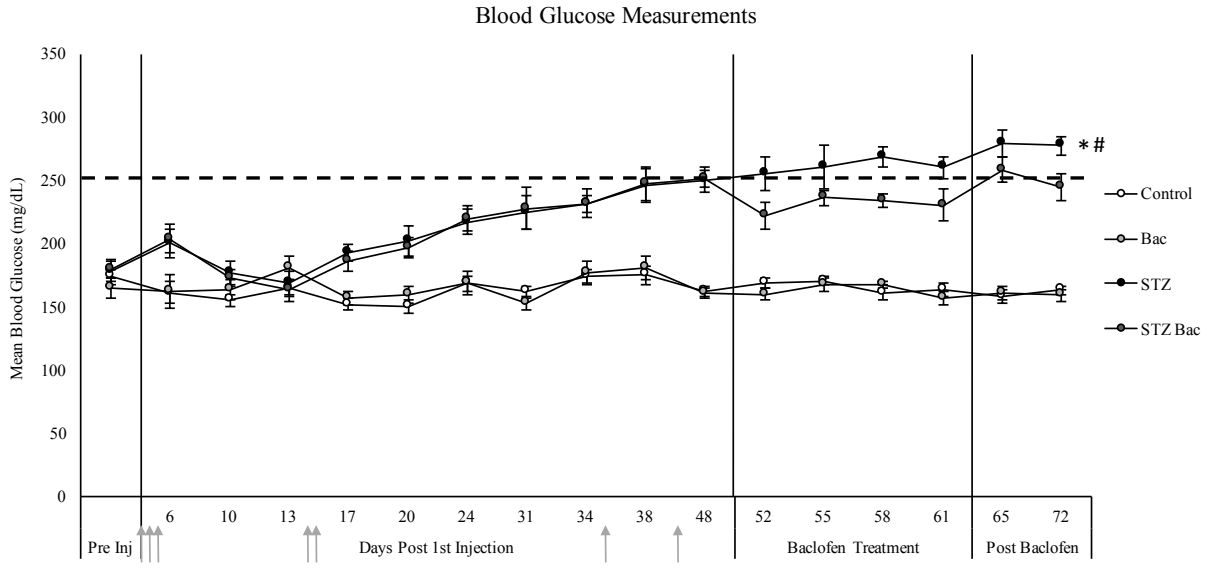

Figure 2 Blood Glucose Measurements. Mean blood glucose levels (± SEM) were significantly increased due to STZ administration. * = significantly different compared to controls ($p < 0.05$). # = significantly different compared to STZ Bac ($p < 0.05$).

Body Weight

Body weights were recorded throughout the experiment to observe changes due to drug treatment. No differences were seen between groups with mean baseline body weight before injections began (Figure 3; $F_{(3,44)} = 0.302$, $p = 0.824$). Similarly, no effect of treatment on body weights were observed during the STZ administration ($F_{(3,44)} = 0.281$, $p = 0.839$), during baclofen

injections ($F_{(3,44)} = 0.533$, $p = 0.662$) , or at the completion of the experiment ($F_{(3,44)} = 0.524$, $p = 0.668$). Therefore, the drug treatments in this experiment did not influence body weight.

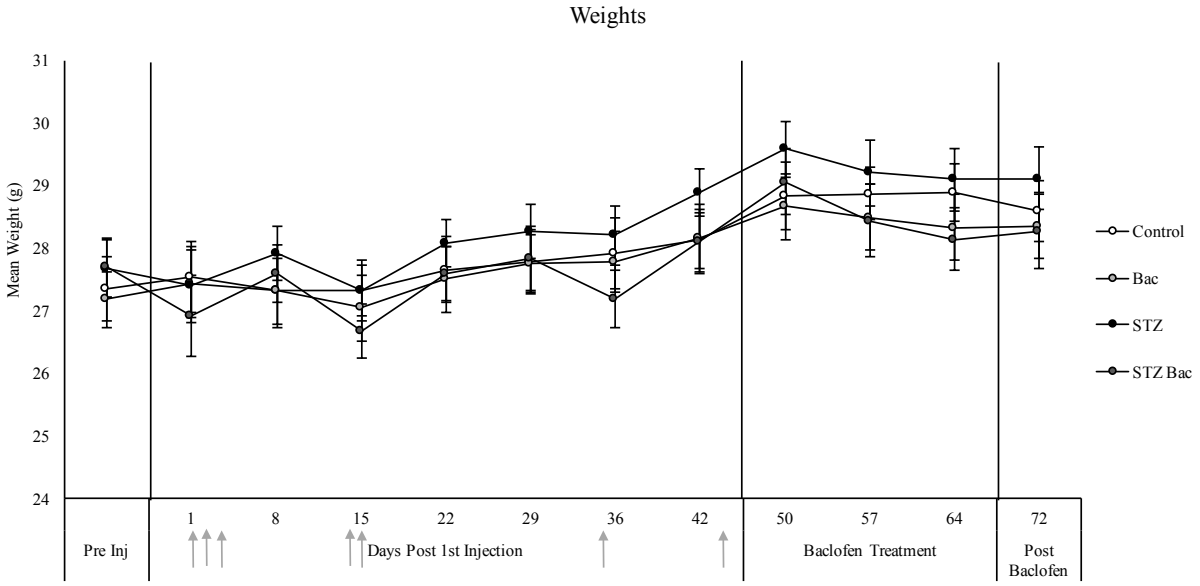

Figure 3 Body Weight Measurements. No significant differences in body weights (± SEM) were observed across the experiment.

Behavioral Testing

Open Field

The open field test was performed to assess anxiety phenotypes or locomotor changes that may manifest due to treatment. Time spent in the perimeter of the chamber was measured as well as velocity and distance travelled during the five-minute session. Although the averages for velocity (Figure 4B; $F_{(3,44)} = 0.483$, $p = 0.696$) and total distance travelled (Figure 4C; $F_{(3,44)} = 0.482$, $p = 0.697$) are not statistically significant between treatment groups compared to controls , the STZ group displayed a trend towards significant increase in time spent in the perimeter of the chamber (Figure 4A; $F_{(3,44)} = 2.716$, $p = 0.056$).

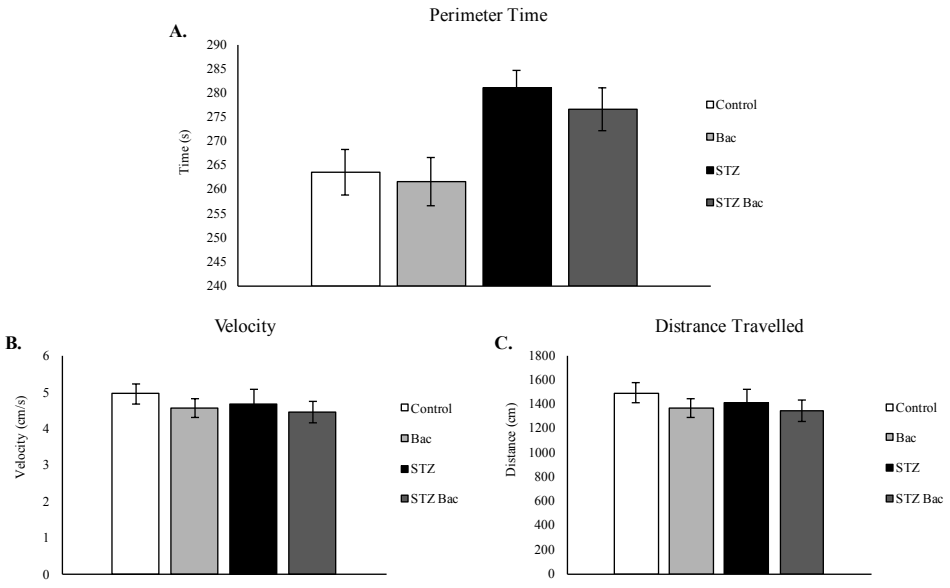

Figure 4 Open Field Data. No significant differences were observed for mean time spent (± SEM) in the perimeter of the chamber (**A**), velocity measured during the trial (**B**), or distance travelled (**C**).

Novel Object Recognition

To evaluate learning and memory differences between treatment groups, the NOR test was performed. This task utilizes the rodents' innate preference for novelty to measure memory abilities. On Day 1 of NOR, groups spent equivalent percent time with both of the identical objects (Figure 5A; $F_{(3,44)} = 1.094$, $p = 0.362$) and displayed no differences in velocity (Figure 5B; $F_{(3,44)} = 0.805$, $p = 0.498$) and distance travelled (Figure 5C; $F_{(3,44)} = 0.805$, $p = 0.498$).

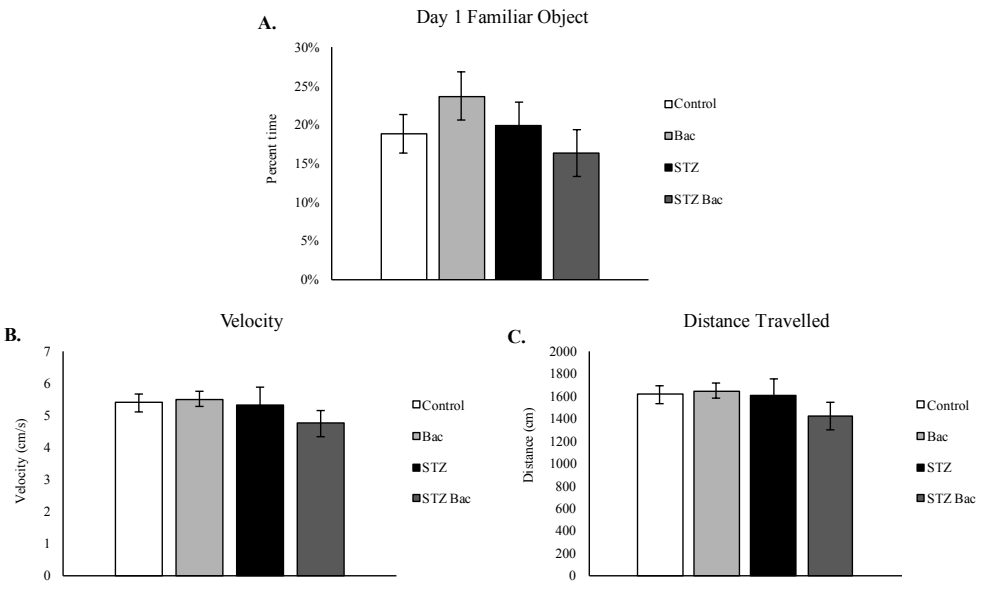

Figure 5 Day 1 Novel Object Recognition Data. **A** No differences were observed in percent time exploring both objects (± SEM). Mean velocity (± SEM; B) and distance travelled (± SEM; C) were equivalent between groups.

On the following day, the novel object was introduced. Control and baclofen-treated (Bac) animals spent more time with the novel object, as revealed by a significant discrimination index for each group (Figure 6A; Control: $t_{(11)} = 2.572$, $p = 0.026$; Bac: $t_{(11)} = 4.551$, $p = 0.001$). The STZ group spent equal time with both objects ($t_{(11)} = 0.345$, $p = 0.737$), indicating a lack of object recognition. Treatment with baclofen reversed this deficit, similar to what was observed in the control group and baclofen alone (STZ Bac: $t_{(11)} = 2.529$, $p = 0.028$). No differences observed in velocity (Figure 6B; $F_{(3,44)} = 0.598$, $p = 0.62$) and distance travelled (Figure 6C; $F_{(3,44)} = 0.568$, $p = 0.639$). Therefore, insulin dysregulation induced by STZ administrations led to memory impairments that was attenuated by baclofen treatments.

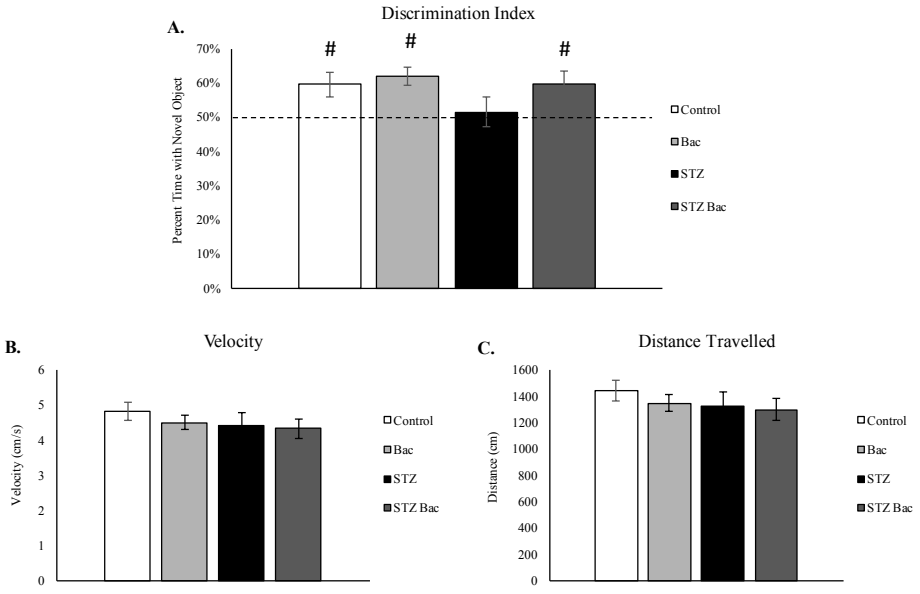

Figure 6 Day 2 Novel Object Recognition Data. **A** Control, Bac, and STZ Bac spent equivalently more time with the novel object than the familiar object. The STZ group spent equal time with the novel and familiar objects. No differences were found with average velocity (± SEM; **B**) and distance travelled (± SEM; **C**). # = significantly greater than chance levels ($p < 0.05$).

Cued and Contextual Fear Conditioning

Associative fear learning was assessed in the CCF task. During training on Day 1, freezing behavior was measured during the first 120 seconds (before the four CS-US pairings; Pre CS-US) and the last 120 seconds (after the four CS-US pairings; Post CS-US). No differences in freezing was observed between the treatment groups (Figure 7; Pre CS-US: $F_{(3,44)} = 1.233$, $p = 0.309$; Post CS-US: $F_{(3,44)} = 0.447$, $p = 0.72$). The amount of freezing significantly increased from Pre CS-US to Post CS-US within each treatment group (Figure 7; Control: $t_{(11)} = 14.895$, $p = 0.000$; Bac: $t_{(11)} = 9.603$, $p = 0.000$; STZ: $t_{(11)} = 11.27$, $p = 0.000$; STZ Bac: $t_{(11)} = 15.722$, $p = 0.000$), indicating that the CS-US pairings elicited fear behavior equally between all groups.

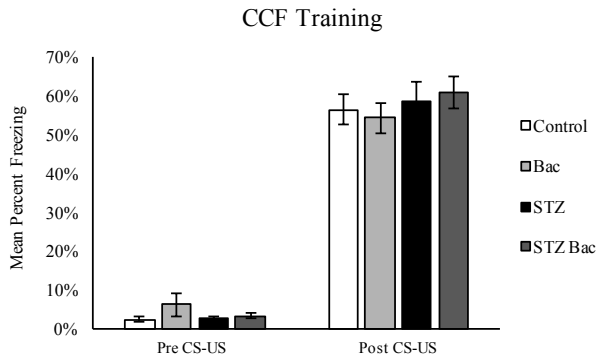

Figure 7 CCF Training Data. Mean percent freezing (± SEM) during the first 120 seconds before the CS-US pairings (Pre CS-US) and the last 120 seconds following the four CS-US pairings (Post CS-US). No differences were found between groups for both portions of the trial. Significantly increased freezing within all groups was observed comparing the Pre CS-US with Post CS-US ($p < 0.05$).

To examine if a learned association to the cue was made, the cue was presented in the altered context (Cued Fear) the following day. Freezing was measured before the presentation of the cue (Pre CS), during the condition stimuli (CS 1, CS 2, CS 3, and CS 4), and after the presentations of the cues (Post CS). No differences in freezing was observed during the first 120 seconds before the presentation of the first cue (Figure 8; $F_{(3,44)} = 0.965$, $p = 0.418$), indicating a lack of fear response to the altered context. Across the entire session, the STZ group displayed a significant increase in freezing during the cues compared controls (Figure 8; repeated measures ANOVA across cues, $F_{(1,22)} = 4.87$, $p = 0.038$). Specifically, post-hoc analysis revealed that STZ displayed significant freezing during CS 2 ($F_{(3,44)} = 4.405$, $p = 0.009$; Tukey post-hoc analysis: Control versus STZ, $p = 0.009$).

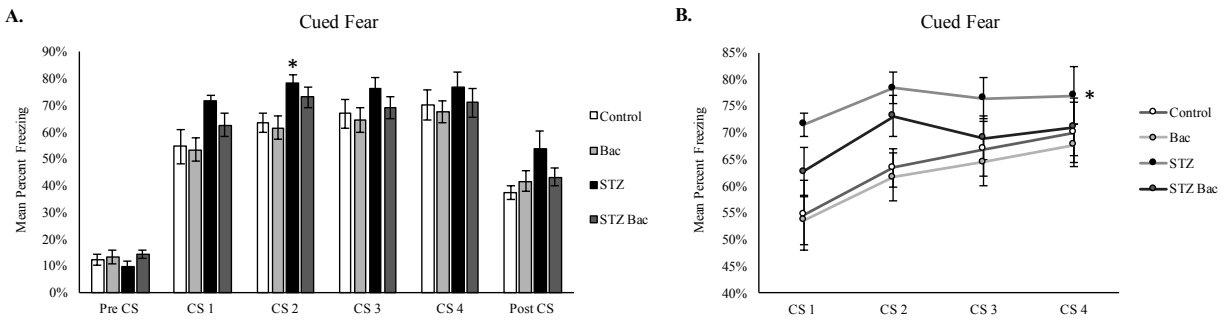

Figure 8 CCF Cued Fear Data. Mean percent freezing (± SEM) in the Cued Fear portion of CCF. **A.** Freezing before, during, and after cue presentations (CS 1, CS 2, CS 3, and CS 4). No differences in freezing before the presentations of cues. STZ displayed elevated freezing during the cues using repeated measures analysis (**B.**). * = significantly different ($p < 0.05$) compared to controls.

To test for a learned contextual fear association, the animals were placed back into the original training chamber and freezing behavior was measured. Across the ten minute session, no differences in freezing behavior was observed between groups (Figure 9; $F_{(3,44)} = 0.346$, $p = 0.792$), suggesting a lack of difference in the learned association to the context.

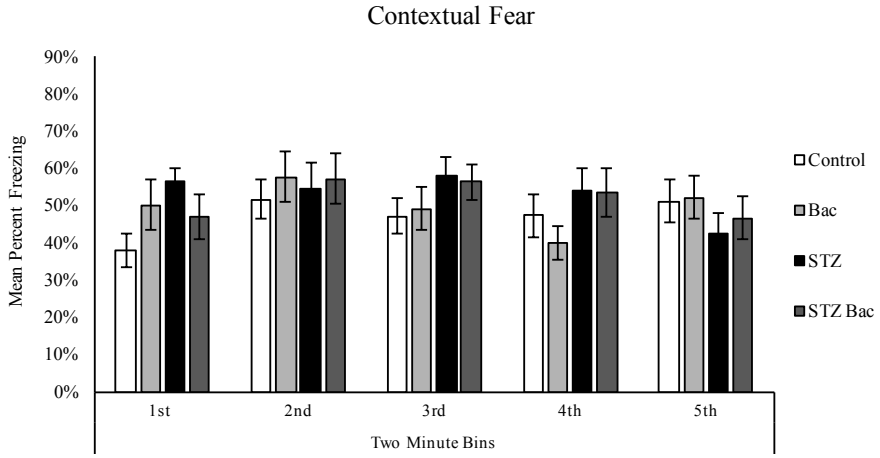

Figure 9 CCF Contextual Fear Data. Mean percent freezing (± SEM) in the original context in which the animals were shocked No differences in freezing between treatment groups was observed.

Tail Flick

The CCF task relies on equivalent nociceptive responses to acquire the learn association. Nociceptive differences were assessed using the tail flick task. All groups had equivalent latencies responded to the hot water bath (Figure 10; $F_{(3,44)} = 0.134$, $p = 0.939$); therefore, pain threshold differences can be ruled out as a variable in CCF mean freezing levels.

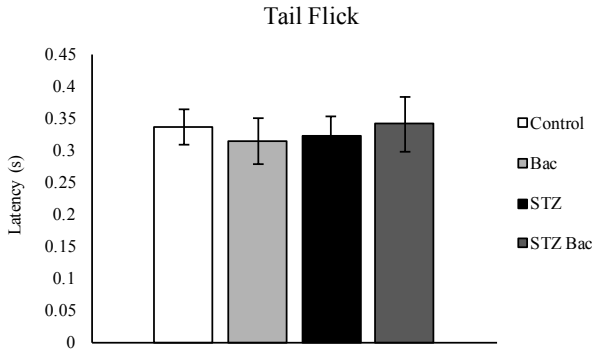

Figure 10 Tail Flick Data. No significant differences were observed between treatment groups in mean latency (± SEM) to remove the tail from a hot water bath.

Tissue Examination

SDS-Page Western Blotting

To examine protein changes due to effect of treatments, various AD pathology, insulin dysregulation, and $GABA_B$ receptor targets (Table 1) were examined.

Protein levels of phosphorylated tau (pTau) was examined in the hippocampus and the cortex. A significant increase in pTau in proportion to total tau was observed in the hippocampus tissue of the STZ group (Figure 11A; $F_{(3,26)} = 7.329$, $p = 0.001$; Tukey post-hoc analysis: Control versus STZ, $p = 0.028$) while no changes were observed for total tau in the hippocampus ($F_{(3,28)} =$

0.332, $p = 0.802$). Cortex protein levels of pTau were unchanged between treatment groups (Figure 11B; $F_{(3,28)} = 1.694$, $p = 0.191$), consistent with the progression of AD pathologies.

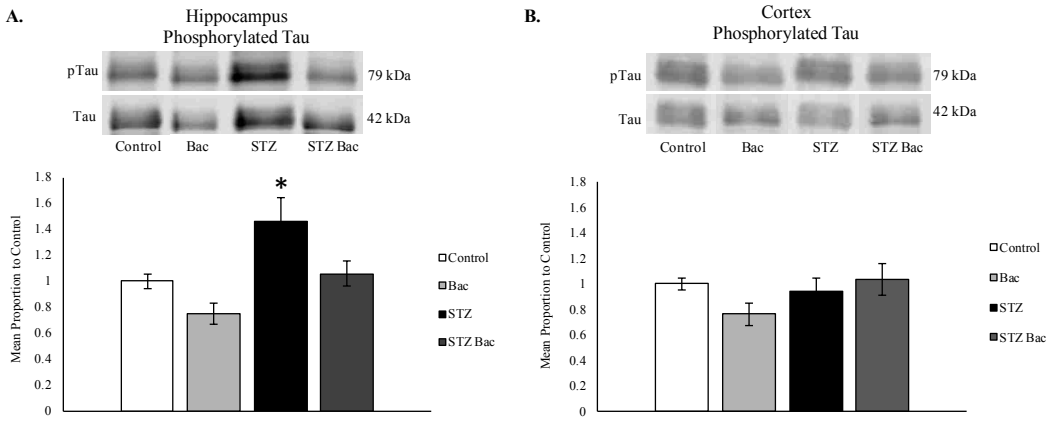

Figure 11 Western Blotting Data: Phosphorylated Tau/Tau. **A.** Representative images of the western blot for phosphorylated Tau/Tau (pTau/Tau) in the hippocampus. STZ group displayed a significant increase in protein levels compared to controls. **B.** Representative images of the western blots for pTau/Tau in the cortex. No significant differences were observed between treatment groups. * = significantly different from controls, $p < 0.05$.

To examine a major target in a potential mechanism of increased tau phosphorylation, GSK3β protein levels were analyzed in the hippocampus and the cortex. Phosphorylated GSK3β is the inactive form of the kinase and is inhibited from phosphorylating tau (Llorens-Martin *et al.*, 2014). In this analysis, the ratio of phosphorylated GSK3β (pGSK3β) to GSK3β were compared. In the hippocampus, no significant differences were observed between treatment groups (Figure 12A; $F_{(3,28)} = 2.523$, $p = 0.078$). However, the baclofen group displayed a trend in reduced pGSK3β/GSK3β (Tukey post hoc analysis: Control versus Bac, $p = 0.076$). No differences were seen in overall hippocampal GSK3β protein levels ($F_{(3,28)} = 0.614$, $p = 0.612$). The cortex tissue did not reveal significant differences in pGSK3β between treatment groups (Figure 12B; $F_{(3,28)} = 0.634$, $p = 0.599$). Therefore, GSK3β levels were unchanged in this AD model.

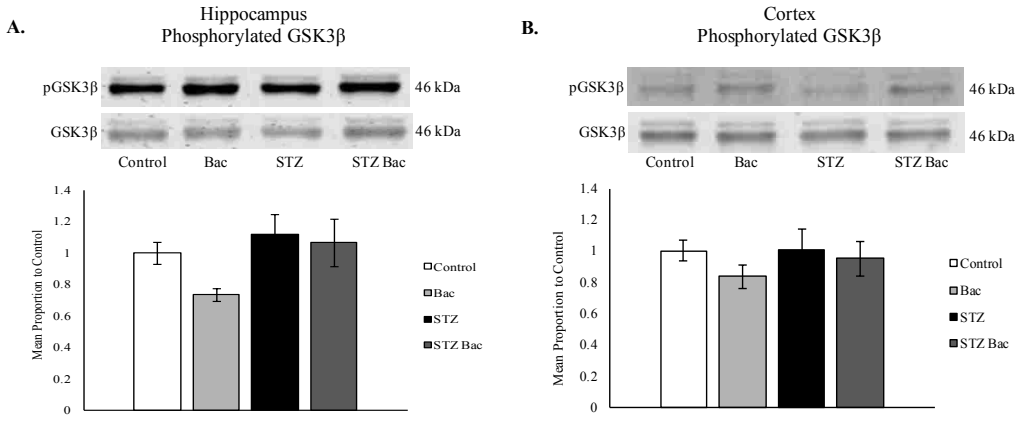

Figure 12 Western Blotting Data: GSK3β. **A.** Representative images of the western blot in the hippocampus. No significant differences were observed in the mean proportion to control (± SEM) between treatment groups. **B.** Representative images of the western blot in the cortex. Similarly, no significant differences were found between treatment groups.

In addition to pTau, another major pathology of AD are increased levels of total Aβ oligomers. However, in this model, no changes in Aβ oligomer levels with treatment of STZ in the hippocampus (Figure 13; $F_{(3, 28)} = 1.305$, $p = 0.292$). Since no changes were found in the hippocampus, Aβ oligomer levels in the cortex were not analyzed.

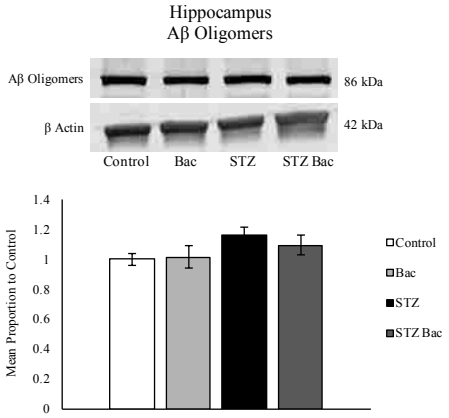

Figure 13 Western Blotting Data: Aβ Oligomers. Representative images of the western blot. No differences in protein levels for Aβ Oligomers were found in the hippocampus.

Insulin degrading enzyme (IDE) protein levels were analyzed in the hippocampus and cortex to detect a mechanism of altered insulin dysregulation in AD. No changes were observed in either the hippocampus (Figure 14A; $F_{(3,28)} = 0.144$, $p = 0.933$) or the cortex (Figure 14B; $F_{(3,28)} = 1.76$), $p = 0.178$). Therefore, the mechanism of clearance of insulin and Aβ in this model were unchanged.

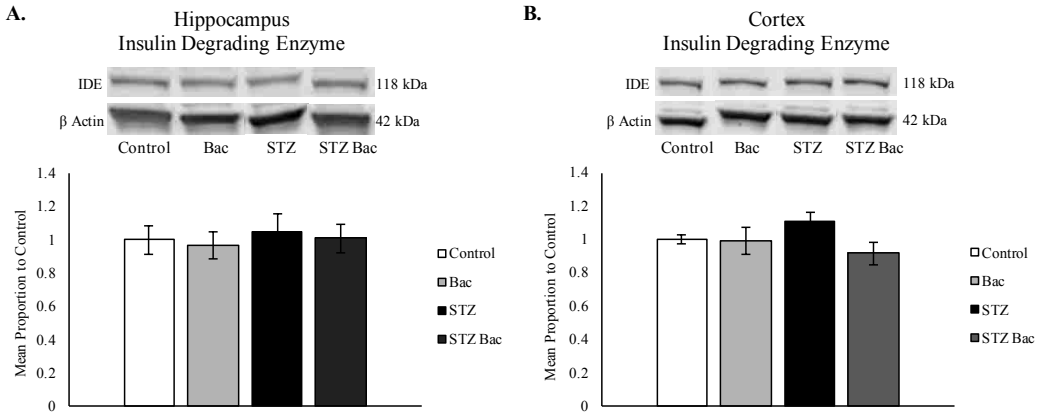

Figure 14 Western Blotting Data: IDE in Hippocampus and Cortex. **A.** Representative images of the western blot in the hippocampus. No significant differences were observed in the mean proportion to control (± SEM) between treatment groups. **B.** Representative images of the western blot in the cortex. Similarly, no significant differences were found between treatment groups.

$GABA_B$ receptor subunit protein levels were analyzed to see if the baclofen treatments resulted in receptor expression alterations. The obligatory $GABA_BR2$ protein levels were assessed in the hippocampus and cortex. However, no changes were found in either brain region (Hippocampus: Figure 15A; $F_{(3,28)} = 1.803$, $p = 0.170$; Cortex: Figure 15B; $F_{(3,28)} = 0.009$, $p = 0.999$). Similarly, no differences were found in the two isoforms of GABAB1 receptor in the

hippocampus (Figure 15C; $GABA_BR1a$: $F_{(3,28)} = 1.442$, $p = 0.252$; $GABA_BR1b$: $F_{(3,28)} = 0.112$, $p = 0.952$). Baclofen administration did not alter overall $GABA_B$ receptor levels.

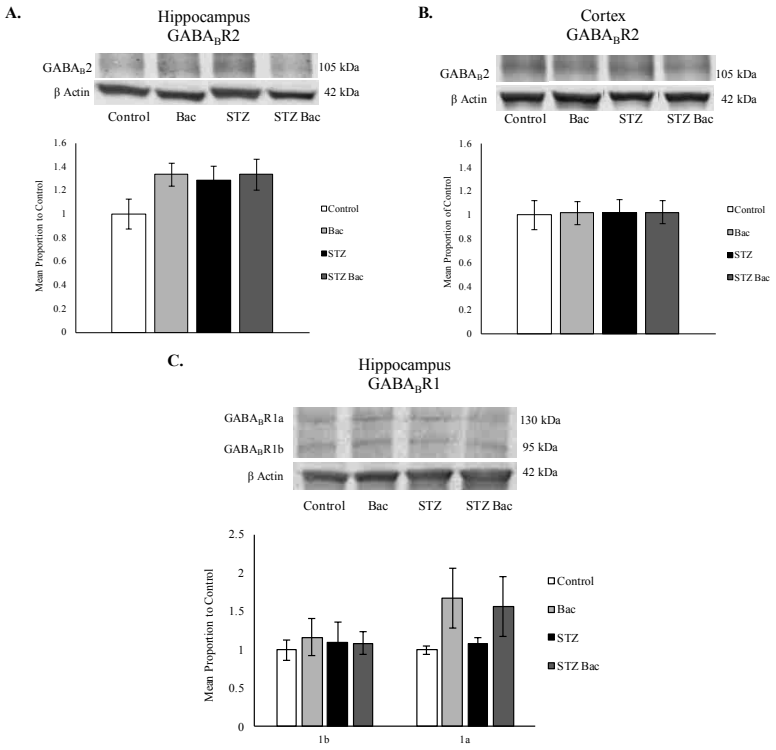

Figure 15 Western Blotting Data: $GABA_BR2$ and $GABA_BR1$. **A.** Representative images of the $GABA_BR2$ western blot in the hippocampus. No significant differences were observed in the mean proportion to control (± SEM) between treatment groups. **B.** Representative images of the $GABA_BR2$ western blot in the cortex. Similarly, no significant differences were found between treatment groups. **C.** Representative images of the $GABA_BR1$ western blot in the hippocampus. No differences were observed between treatment group for either isoform of the receptor.

Immunohistochemistry

Protein levels for phagocytic microglia (or reactive microglia) and histological staining for microvasculature hemorrhages were analyzed using immunohistochemistry.

Iba1 protein is specific for reactive microglia. In the hippocampus, a significant increase in the number of reactive microglia was observed for the STZ group (Figure 16A; $F_{(3,183)} = 3.998$,

p = 0.009; Tukey post-hoc analysis: Control versus STZ, p = 0.005). In the cortex tissue, a significant increase was found between the STZ group receiving baclofen and the baclofen alone group (Figure 16B; $F_{(3,187)}$ = 3.168, p = 0.026; Tukey post-hoc analysis: Bac versus STZ Bac, p = 0.05). The STZ group was not significantly different from controls in the cortex (Control versus STZ, p = 0.343). These results suggest that STZ administration leads to reactive microglia in the hippocampus and elevated, yet not significant numbers in the cortex. The combination of STZ and baclofen treatments leads to increase number of Iba1 positive microglia in the cortex compared to baclofen alone.

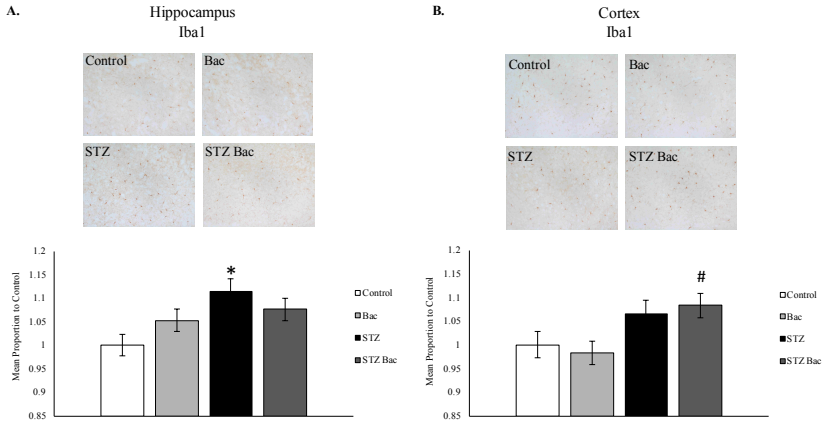

Figure 16 Immunohistochemistry Data: Iba1. **A.** Representative images of Iba1 positive microglia in the hippocampus. The STZ group had significantly more Iba1 microglia versus controls. **B.** Representative images of Iba1 positive microglia in the cortex. The STZ Bac group displayed an increase number of Iba1 positive microglia compared to the Bac group. * = significantly different from control, $p < 0.05$. # = significantly different from Bac, $p < 0.05$.

Microvascular hemorrhages are found in AD and diabetic patients. As revealed by Prussian blue staining, the number of microvascular hemorrhages were not statistical significant between treatment groups in the hippocampus (Figure 17A; $F_{(3,43)}$ = 0.217, p = 0.189) and in the cortex (Figure 17B; $F_{(3,44)}$ = 1.034, p = 0.387). Based on these results, STZ treatment did not result in microhemorrhages during the time frame of this experiment.

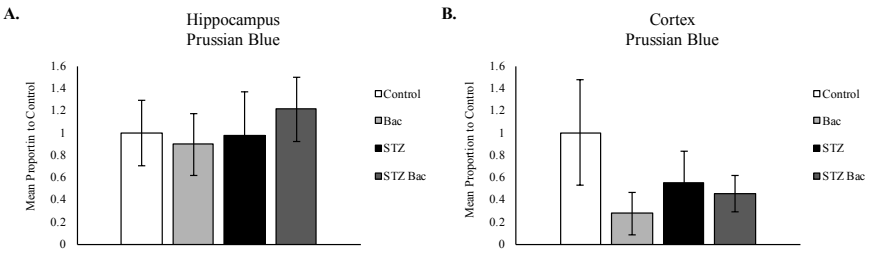

Figure 17 Immunohistochemistry Data: Prussian blue. No changes were observed between treatment groups in the number of microhemorrhages in the hippocampus (**A.**) or the cortex (**B.**).

RT-PCR

Cytokine mRNA levels associated with AD were measured using RT-PCR. IL-1β is a pro-inflammatory cytokine that is increased in AD patients. In this experiment, IL-1β RNA expression was significantly increased in the STZ group in the hippocampus (Figure 18A; $F_{(3,44)} = 10.1928$, $p = 0.000$; Tukey post-hoc analysis: Control versus STZ, $p = 0.000$). The STZ group displayed significantly reduced RNA expression in the cortex compared to the baclofen group, but not the control group (Figure 18B; $F_{(3,44)} = 5.834$, $p = 0.0019$; Tukey post-hoc analysis: Control versus STZ, $p = 0.1407$; Bac versus STZ, $p = 0.0009$). These results demonstrate that STZ administration is able modulate pro-inflammatory cytokine levels in a region-specific manner.

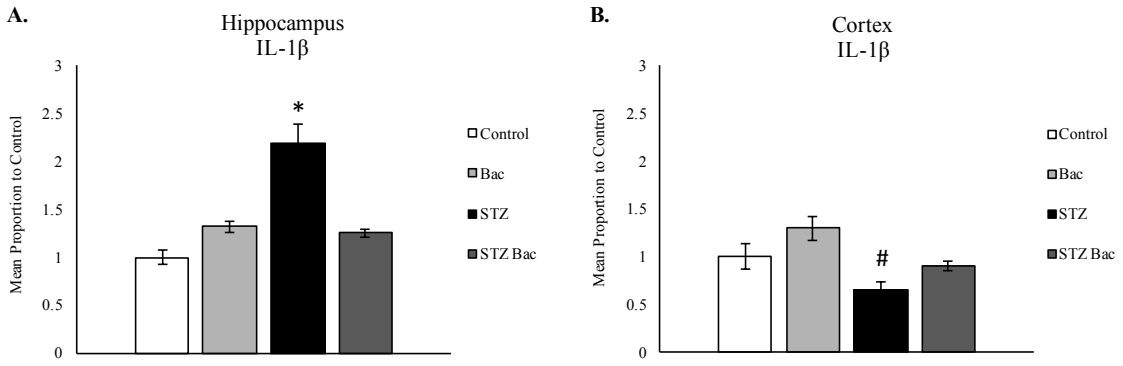

Figure 18 RT-PCR Data: IL-1β. **A.** The STZ group displayed significantly increased levels of IL-1β RNA in the hippocampus compared to controls. **B.** The STZ group had significantly reduced IL-1β RNA compared to the baclofen alone group. * = statistically significant versus controls, $p < 0.05$. # = statistically significantly versus baclofen, $p < 0.05$.

IL-10 anti-inflammatory RNA levels were examined in the hippocampus and cortex. The STZ group displayed a significant increase in IL-10 RNA levels in the hippocampus (Figure 19A; $F_{(3,44)} = 11.8987$, $p = 0.000$; Tukey post-hoc analysis: Control versus STZ, $p = 0.000$). In the cortex, the baclofen group displayed significantly elevated RNA levels compared to controls while no changes were found in the STZ group (Figure 19B; $F_{(3,44)} = 15.8617$, $p = 0.000$; Tukey post-hoc analysis: Control versus Bac, $p = 0.000$).

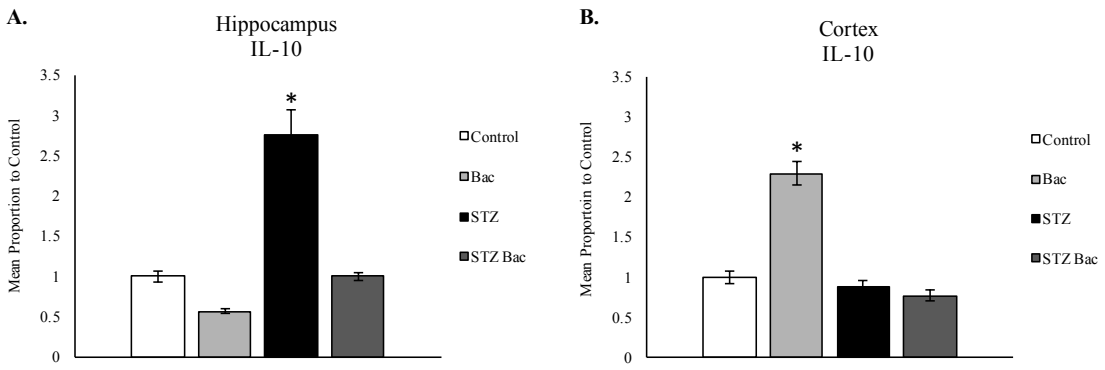

Figure 19 RT-PCR Data: IL-10. **A.** The STZ group displayed significantly increased levels of IL-10 RNA in the hippocampus compared to controls. **B.** The baclofen group revealed significantly elevated IL-10 RNA expression compared to the controls. * = statistically significant versus controls, $p < 0.05$.

Other pro-inflammatory markers were assessed in the hippocampus. Both groups given STZ (STZ and STZ Bac) displayed a significant decrease in TNF α RNA levels (Figure 20A; $F_{(3,44)} = 9.3223$, $p = 0.0001$; Tukey post-hoc analysis: Control versus STZ, $p = 0.0003$; Control versus STZ Bac, $p = 0.0039$). The baclofen group exhibited a significant increase with both IL-1α (Figure 20B; $F_{(3,44)} = 3.0885$, $p = 0.0367$; Tukey post-hoc analysis: Control versus Bac, $p = 0.0362$) and IL-6 (Figure 20C; $F_{(3,44)} = 3.3211$, $p = 0.0282$; Control versus Bac, $p = 0.0311$) in the hippocampus. Both the STZ administration and, separately, the $GABA_B$ agonist influences pro-inflammatory markers.

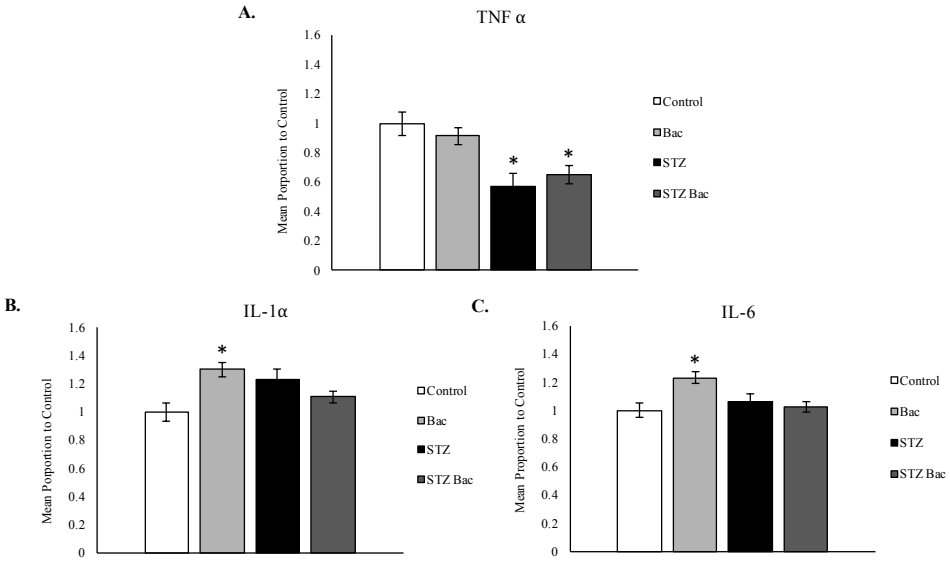

Figure 20 RT-PCR Data: TNF α, IL-1α, and IL-6. **A.** The STZ and STZ Bac group displayed significantly lower RNA expression levels of TNF α in the hippocampus compared to controls. **B.** The baclofen group revealed significantly elevated IL-1α RNA expression compared to the controls in the hippocampus. **C.** The baclofen group show elevated IL-6 RNA levels in the hippocampus. * = statistically significant versus controls, $p < 0.05$.

CHAPTER 5

DISCUSSION

The purpose of this study is to examine the role of $GABA_B$ receptors on neuroinflammation and AD pathology in a diabetes rodent model. We found that inducing hyperglycemia using the compound STZ resulted in behavioral, biochemical, and inflammatory changes similar to what is observed in other AD rodent models and in the AD patient population. Further, we found that administration of a $GABA_B$ receptor agonist (baclofen) attenuated the AD-related behavior deficits and pathologies induced by STZ.

Analysis of a diabetic-like state during STZ injections was made by measuring blood glucose levels. STZ is capable of producing mild to severe diabetes depending on dosage and schedule of administration (Deeds *et al.*, 2011). A single, high doses of STZ (100-200 mg/kg IP) leads to a severe hyperglycemic state, with excessively high blood glucose levels (>400 mg/dL) occurring rapidly in animals and a 70±7% mortality rate (Lu *et al.*, 1998; Ito *et al.*, 1999; Hayashi *et al.*, 2006; Bloch *et al.*, 2006). The choice of a low-dose (40 mg/kg IP), staggered protocol used in this experiment reflects our concerns over the permanent destruction of pancreatic beta cells that would result in little to no production of insulin does not result in AD pathologies. STZ-induced diabetes can be highly variable and a clear, standard protocol does not exist in the literature (Deeds *et al.*, 2011). Variability in response to STZ occurs between mouse strains and also within subgroups (age, vendor, and even inbred strain) of the same genetic background, complicating the STZ administration protocol (Gurley *et al.*, 2006; Deeds *et al.*, 2011). A low-dose, staggered protocol allows researchers to monitor the level blood glucose to determine if another STZ administration is necessary throughout the experiment and to avoid irreversible and extensive toxicity. STZ has acute effects (24-48 hours) and longer term effects (up to two weeks) on

pancreatic beta cells (Deeds *et al.*, 2011). Immediately after administration, the destruction of pancreatic beta cells results in excessive insulin release and hypoglycemia (Szkudelski, 2001). The drop in insulin production can be observed by the increase in blood glucose levels. Some of the surviving cells begin to die within two weeks after STZ due to infiltration of lymphocytes, further increasing blood glucose levels (Like and Rossini, 1976; OBrien *et al.*, 1996). The pattern can be observed by a gradual increase of blood glucose levels over time that eventually plateaus (Figure 2). In this experiment, our goal was to achieve a group average of 250 mg/dL to avoid mortality that can occur with significantly elevated hyperglycemia. After seven intermittent injections on days 1, 2, 3, 14, 15, 35, and 44, blood glucose levels reached an average of 250 mg/dL with zero mortality rate among our animals.

Administration of the $GABA_B$ receptor agonist, baclofen, had an interesting effect on blood glucose levels. Pancreatic beta cells produce and respond to the neurotransmitter GABA, where it plays a role in regulating insulin secretion (Wan *et al.*, 2015). In addition to beta cells, the pancreas contains alpha cells that increases insulin secretion in response to GABA (Brice *et al.*, 2002). Although the signaling mechanism of GABA on beta and alpha cells is not fully elucidated, a proposed mechanism exists. For example, it is thought that beta cells release insulin in response to increasing glucose levels, which activates insulin receptors on alpha cells. This leads to the translocation and subsequent upregulation of $GABA_A$ receptors on the alpha cell surface that increases membrane depolarization resulting in the inhibition of glucagon release (E Xu *et al.*, 2006). Glucagon is a hormone that has the opposite function of insulin in that can increase glucose levels in the blood. This pathway has suggested to be disrupted in diabetic patients that results in unsuppressed glucagon secretion (Wan *et al.*, 2015).

Pancreatic beta cells respond differently compared to alpha cells in the presence of GABA, where GABA results in membrane depolarization and subsequent release of insulin (Dong *et al.*, 2006; Braun *et al.*, 2010; Soltani *et al.*, 2011). However, studies investigating $GABA_B$ receptor modulation on insulin release have shown conflicting results. For example, $GABA_B$ receptors suppress insulin release in the presence of high glucose concentrations (over 180 mg/dL) (Gu *et al.*, 1993; Brice *et al.*, 2002) but have no effect on lower glucose levels (Brice *et al.*, 2002; Wan *et al.*, 2015). In addition, pre-treating non-obese diabetic mice (transgenic mice that develop diabetes without the weight gain) with baclofen delays the onset of diabetes and increases beta cell proliferation (Beales *et al.*, 1995). Conversely, mice lacking $GABA_B$ receptors (specifically, $GABA_B$R1 subunit) exhibit increased pancreatic insulin release compared to wildtype controls (Szkudelski, 2001). In our experiment, activation of pancreatic $GABA_B$ receptors via baclofen appeared to have a slightly beneficial effect. After two days of the baclofen treatment, the STZ Bac group displayed significantly decreased levels compared to the STZ group (see Figure 2). Importantly, the reported decrease in blood glucose levels was still significantly increased compared to the control and baclofen alone groups. Therefore, the STZ Bac displayed significantly elevated blood glucose levels despite the influence of pancreatic $GABA_B$ receptors.

The memory impairments in NOR exhibited by the STZ group are consistent with those reported in AD patients and preclinical rodent models (Görtz *et al.*, 2008; Ambrée *et al.*, 2009). The NOR task relies on proper functioning of the hippocampus, in that hippocampal lesions result in impairments in discrimination between familiar and novel objects (Antunes and Biala, 2012). In AD, the entorhinal cortex and hippocampal formation are primarily affected in the initial stages of the disease (H Braak and E Braak, 1991; 1997). Insulin deficiency produces impairments in hippocampal synaptic plasticity and neurogenesis that underlie cognitive deficits (Stranahan *et al.*,

2008; Murtishaw et al, in review; Prickaerts *et al.*, 1999; Y Chen *et al.*, 2013; Jabbarpour *et al.*, 2014). Treatment with baclofen for two weeks prior to testing was able to reverse the STZ-induced deficits. As no differences between baclofen alone and control group were found, this suggests that the action of baclofen may be mediated through the attenuation of STZ-induced neuroinflammation and phosphorylated tau also found in this study.

Conditioned fear learning was assessed in this model using CCF. The neurological mechanisms of fear conditioning are well studied and highly conserved across species (LeDoux, 1994). Our experiment used a variation in CCF training protocol called trace conditioning, where there is a time interval separating the offset of the tone and the onset of the shock. This protocol increases the level of difficulty in associating of the auditory cue and the contextual environment with the shock. Lesion studies demonstrate that the cued fear association portion of this task (responding to the cue alone in an altered context from which subjects were shocked) is dependent on both the hippocampus and amygdala, while the context portion of the task (responding the environment in which they were shocked without cue presentations) depends primarly on the hippocampus (Solomon and Vander Schaaf, 1986; Moyer *et al.*, 1990; Sutherland and RJ McDonald, 1990; C Chen *et al.*, 1996). In AD, amygdalar-hippocampal communication is disrupted, resulting in impaired acquired conditioned fear response (Hamann *et al.*, 2002). Preclinical AD models also show fear response deficits in CCF (Webster *et al.*, 2014) (Kilgore *et al.*, 2010; Hanna *et al.*, 2012). In our study, animals exhibited equal freezing behavior after the presentation of the conditioned stimulus (CS) and unconditioned stimulus (US) pairings during the training session. In the Cued Fear session, the STZ group displayed an increase in freezing behavior compared to controls across the presentation of the cues. This increase in freezing may reflect an anxiolytic phenotype. Similar to what was observed in the open field test, where the data

hint at anxiety-like behavior with the STZ group, in that these animals had a nonsignificant trend towards more time in the perimeter of the chamber. Studies have reported that metabolic disorders and STZ-induced diabetes can lead to increased reactivity of the HPA axis, which results in hypersensitivity and the inability to effectively shut off the stress response (Scribner *et al.*, 1991; Magariños and McEwen, 2000; Ikeda *et al.*, 2015). Changes in pain response were also evaluated using the tail flick test and no differences were observed. Interestingly, all groups showed equivalent freezing behavior in the Contextual Fear portion, the session that has the most hippocampal contribution. It is possible that cue and the context are too salient to detect a difference between groups, even with the more challenging trace conditioning training protocol.

Several protein targets associated with AD were analyzed in the hippocampus and cortex of animals in this experiment to understand the molecular mechanisms linking diabetes and the effect of baclofen administration. No changes in Aβ oligomers were observed between treatment groups. This is not surprising as the metabolism of APP in rodents is different compared to humans and they do not develop toxic Aβ oligomers. Typically, transgenic animals expressing human APP or direct infusion of Aβ42 peptides into the brain in non-transgenic rodent models display significant changes in brain Aβ oligomers. Along with the lack of changes in Aβ, no differences IDE protein levels were found between treatment groups. Even though STZ results in insulin dysfunction, IDE may not be affected without the competition of elevated Aβ. However, region specific changes with phosphorylated tau were found in the STZ group. The STZ group had significantly increased phosphorylated tau in the hippocampus that was attenuated by the baclofen administration. Increased levels of phosphorylated tau correlate with cognitive and memory deficits, similar to what was observed in NOR with the STZ group. The lack of change with phosphorylated tau in the cortex is consistent with Braak staging in AD, with NFT beginning in

the hippocampus initially then eventually appearing in the cortex in late stages of the disorder (H Braak and E Braak, 1991; 1997; H Braak *et al.*, 2006). As mentioned previously, NFTs (composed of hyperphosphorylated tau) have high correlation with poor memory performance in AD patients and in preclinical models (H Braak and E Braak, 1991; Arriagada *et al.*, 1992; Bancher *et al.*, 1993; H Braak and E Braak, 1997; Guillozet *et al.*, 2003; SantaCruz, 2005). For example, in a study using a preclinical AD model with tau mutations, untreated transgenic mice displayed significant deficits in the NOR task while administration of a phosphorylated tau antibody ameliorated the impairment (Sankaranarayanan *et al.*, 2015). To examine a potential mechanism of increased phosphorylated tau associated with AD, we analyzed GSKβ levels in the hippocampus and cortex. Over-activity of GSKβ can lead to increases in phosphorylated tau, memory impairments, and Aβ oligomer production (Hooper *et al.*, 2007). However, no changes were observed in GSKβ protein levels in hippocampus or cortex between treatment groups. According to several AD hypotheses, Aβ peptides indirectly leads GSK3β activity which then all contribute to phosphorylated tau (Terwel *et al.*, 2008; Dewachter *et al.*, 2009; Kremer *et al.*, 2011). Therefore, hyperphosphorylated tau may have occurred through increased inflammation in this study rather than through altered GSK3β signaling that was attenuated by reduced inflammation via $GABA_B$ receptor activation.

Neuroinflammation and neuroinflammatory markers were examined in this experiment, as they are a characteristic of diabetes and AD pathology (Mrak and Griffin, 2005a). Microglia constantly survey the environment and receive signals from surrounding cells. Brain tissue injury, invading pathogens, and pathological conditions associated with neurological disorders can cause microglia to be reactive, a state in which they surround and attempt to clear the debris. In addition, microglia change their morphology, upregulate cell-surface receptors, and release cytokines, chemokines, and other factors with the goal of repairing and restoring the area to homeostasis

(Solito and Sastre, 2012). While this response proves to have immediate beneficial effects in the brain, sustained neuroinflammation due to pathological conditions, such as those seen in AD, can lead to damage and accumulation of the pathogen that initially triggered the response. Our hypothesis was that administration of STZ would lead to an increased number of reactive microglia, elevated pro-inflammatory cytokines and an increase in anti-inflammatory cytokines, as neuroinflammation has been demonstrated to produce cognitive deficits in similar studies using STZ (Biessels *et al.*, 1998; 2007; Jabbarpour *et al.*, 2014).

Our results showed that the STZ group displayed an increased number of reactive microglia and elevated IL-1β pro-inflammatory cytokine RNA levels in the hippocampus but not the cortex. Microglia and IL-1β can influence the phosphorylation of tau. Consistent with data from our study, elevated expression IL-1β leads to increased phosphorylated tau (Yuekui Li *et al.*, 2003; Gorlovoy *et al.*, 2009) and overexpression of IL-1β in the hippocampus of non-APP mice lead to memory impairments (Moore *et al.*, 2009; Hein *et al.*, 2010; DC Lee *et al.*, 2013). Treatment with baclofen reduced the number of reactive microglia, expression of IL-1β, and phosphorylated tau induced by STZ back to control and baclofen alone levels in the hippocampus. Therefore, it appears that the reactive microglia and the pro-inflammatory cytokine IL-1β in the STZ be involved in the hyperphosphorylated tau and behavioral deficits that are all reversed by $GABA_B$ activation.

Our results also found increased expression of IL-10 RNA in the hippocampus of the STZ group, while the baclofen alone group had significant elevated RNA expression of IL-10 in the cortex. IL-10 is an anti-inflammatory cytokine and is a key player in controlling the immune response in the brain (Wyss-Coray and Mucke, 2002; Williams *et al.*, 2004; Ming O Li and Flavell, 2008). Studies have reported increased levels of IL-10 protein and RNA in preclinical models of AD (Apelt and Schliebs, 2001; Heneka and OBanion, 2007) while no correlation in AD patient

tissue has been found (Apelt and Schliebs, 2001). Increases in anti-inflammatory markers may be attempting to counteract actions of reactive microglia and pro-inflammatory cytokines. The STZ group showed a region-specific elevation with IL-10 that correlates with the region-specific changes in reactive microglia, phosphorylated tau, and IL-1β data that is also reversed by the administration of baclofen. Conversely, the region-specific changes with the baclofen alone group is puzzling. However, one should keep in mind that neuroinflammatory processes are highly interactive and do not occur in isolation. Amplification of one mediator leads to a dampening of another which all interact and influence different inflammatory pathways. Therefore, elevation of IL-10 RNA in the cortex requires further elucidation.

TNFα is another common pro-inflammatory cytokine associated with AD and diabetes examined in this study. We found a significant decrease in TNFα RNA expression levels in both the STZ and STZ Bac group in the hippocampus. In AD patients and preclinical models, as well as diabetic patients and STZ preclinical models, TNFα is significantly elevated (Dickson *et al.*, 1993; Benzing *et al.*, 1999; Limb *et al.*, 1999; Mehlhorn *et al.*, 2000; Carmo *et al.*, 2000; Krady *et al.*, 2005; Gezen-Ak *et al.*, 2013), where it is found to be a mediator of acute and chronic inflammation and activated by Aβ-induced cytotoxicity in AD (PB Rosenberg, 2005). However, researchers have suggested that TNFα levels may wax and wane during different stages of AD. For example, a study examining brain tissue of AD patients found lower TNFα levels in the cortex and hippocampus of AD patients compared to healthy age-matched controls (Lanzrein *et al.*, 1998) that correlated with a previous study that found lower TNFα serum levels (Cacabelos *et al.*, 1994). Low levels of TNFα in AD may be indicative of a dysfunctional inflammatory process (Lanzrein *et al.*, 1998; Gezen-Ak *et al.*, 2013). In addition, high levels of IL-10 can inhibit the synthesis of TNFα (PB Rosenberg, 2005), which correlates with our findings in the STZ group. Whether

elevated IL-10 RNA expression in the STZ group is inhibiting TNFα or if there is altered neuroflammatory processes induced by STZ is unclear in this experiment. However, the reduction in TNFα in the STZ group was not rescued by the baclofen treatment, suggesting that $GABA_B$ activation was not able to modulate this cytokine.

IL-6 RNA levels were increased in the hippocampus of the baclofen alone group. IL-6 is detectable at low levels in healthy adults and significantly elevated under pathological conditions (Vallières and Rivest, 1997). Although IL-6 can add to detrimental AD pathology, it does exhibit immunosuppressive and anti-inflammatory properties under certain conditions. Several studies have found that IL-6 regulates neuronal survival and function (Gadient and Otten, 1997; Gruol and Nelson, 1997; Campbell, 1998; Feng *et al.*, 2015). Specifically, one study found that baclofen attenuated lipopolysaccharide-induced increase in IL-6 in microglia cell culture. Further, the same study showed that baclofen alone dose-dependently reduced IL-6 released by microglia (Kuhn *et al.*, 2004). Similarly, the baclofen alone group displayed increases in the pro-inflammatory IL-1α in the hippocampus. Although IL-β and IL-1α bind to the same receptor, little is discussed about the role of IL-1α in AD or diabetes. Therefore, the increases in IL-6 and IL-1α levels after $GABA_B$ receptor activation require further evaluation.

Administration of baclofen attenuated the STZ-induced levels of several neuroinflammatory markers and memory deficits. Reactive microglia increase their expression of $GABA_B$ receptors (Kuhn *et al.*, 2004), suggesting that they play a role in regulating neuroinflammation. Microglia $GABA_B$ receptors attenuate the release of lipopolysaccharide-induced IL-6 but does not influence TNFα release (Kuhn *et al.*, 2004). We examined $GABA_B$ receptor subunit protein expression in the hippocampus and cortex in the treatment groups. Although no differences were found, there appears to be a trend towards an increase in both of the

groups who received baclofen (Bac and STZ Bac) for the $GABA_BR1a$ isoform in the hippocampus. However, the homogenized tissue examined was not specific to microglia and detects neuronal $GABA_B$ receptor levels as well. Alterations in neuronal $GABA_B$ receptor levels could disrupt the synchrony and network of systems within the brain and have deleterious implications on learning and memory (Heaney and Kinney, 2016). If the treatments in this experiment altered the levels of microglial $GABA_B$ receptors, using the western blot procedure would not be sensitive enough to detect changes. Given the data we have with baclofen administration in STZ animals (rescue in memory impairment, phosphorylated tau, reactive microglia number, IL-1β, and IL-10), we can indirectly infer that $GABA_B$ receptors play a role in modulating microglia function.

Overall, this experiment demonstrates that administration of STZ leads to select AD pathologies and microglia-induced neuroinflammation that are ameliorated by chronic activation of $GABA_B$ receptors. Further studies are required to outline mechanism by which STZ leads to AD-related behavior and protein changes in the brain. For instance, examining insulin receptor number and resistance would be beneficial in stating how much insulin dysregulation contributes to the neuroinflammation with our STZ model. With regards to future studies using baclofen in a STZ model, intracerebroventricular infusions instead of systemic administration should be a consideration to bypass the effect baclofen had on pancreatic beta cells. Limitations to the present study include a direct link to $GABA_B$ specifically on microglia. Using flow cytometry that selects for specific proteins on microglia would be advantageous in providing a direct link to the current study. Further, evaluating the effects of STZ administration in a mouse model that lack $GABA_B$ receptors specifically on microglia can shed light on immune functions. By crossing CX3CR1 mice (CX3CR1 are receptors expressed only by microglia in the brain (Cardona *et al.*, 2006)) with Cre/Lox inducible $GABA_BR1^{lox511/lox511}$ mice, we can induce the inactivation of $GABA_B$ receptors

on microglia. We could then evaluate behavior and biochemical changes after STZ administration in these animals to elucidate the role of $GABA_B$ receptors on microglia. Even though questions remain to be answered regarding how $GABA_B$ receptors are involved in the neuroinflammatory response, this study provides data that demonstrates $GABA_B$ receptor activation attenuates neuroinflammatory markers and subsequent AD pathologies.

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Zhao W-Q, and Alkon DL (2001) Role of insulin and insulin receptor in learning and memory. *Molecular and Cellular Endocrinology* **177**:125–134.

Zhao W-Q, and Townsend M (2009) Insulin resistance and amyloidogenesis as common molecular foundation for type 2 diabetes and Alzheimer's disease. *BBA - Molecular Basis of Disease* **1792**:482–496.

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CURRICULUM VITAE

Monica M. Bolton

Contact Information:

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Education:

University of Nevada, Las Vegas, 2010-Present
Ph.D. in Experimental Psychology, Neuroscience Area (Defense Date: January 17, 2017)
Dissertation Title: "An Evaluation of $GABA_B$ Receptors on Modulating Neuroinflammation in a Non-Transgenic Animal Model of Alzheimer's Disease"

Medical Science Liaison Society, 2016
Candidate training for medical science liaison positions in the pharmaceutical industry.

University of Nevada, Las Vegas, 2013
M.A. in Experimental Psychology, Neuroscience Area
Thesis Title: "Alterations of NMDA and $GABA_B$ Receptor Function in Development: A Potential Animal Model of Schizophrenia"

University of Nevada, Las Vegas, 2009
B.S. in Biological Sciences, concentration in Pre-Medicine
B.A. in Psychology

Teaching Experience:

Academic Success Center Bridge Instructor: Summers 2014 and 2015
University of Nevada, Las Vegas
Instructor incoming college freshman in remedial math course topics to prepare them for the math placement exam with the goal to be placed into the appropriate math course for their major.

Psychology of Learning (PSY 420): Fall 2014 – Spring 2015
University of Nevada, Las Vegas
Instructor of two sections of upper level undergraduate psychology course. Responsible for formatting class lectures and course outline, generating assignments and exams, lecturing material, and mentoring students.

Introduction to Psychology (PSY 101): Fall 2013 – Spring 2015
University of Nevada, Las Vegas
Instructor for two sections of undergraduate psychology course per semester. Responsible for creating lecture material, lecturing, making quizzes/exams,

implementing assignments and research projects, mentoring students, and grading.

Research Mentor Experience:

Mentor for Undergraduate Research in Nevada INBRE (IDeA Network of Biomedical Research Excellence) Undergraduate Research Opportunities Program: Summer 2012-2015

Mentored undergraduate students receiving the summer Nevada INBRE grant. Students received hands-on laboratory experience in behavioral neuroscience.

Mentor for Undergraduate Research in EPSCoR/IDeA Undergraduate Research Opportunities Program: Spring 2015

Mentored undergraduate student receiving NSF grant to conduct research in a STEM based laboratory.

Mentor for UNLV McNair Scholars Summer Research Institute: (Summer 2013-2014)

Mentored undergraduate students receiving McNair Scholarship awards. Students received hands-on laboratory experience in behavioral neuroscience.

Publications:

Murtishaw AS, Heaney CF, **Bolton MM**, Sabbagh JJ, Langhardt, MA, Kinney JW (2016). Effect of acute lipopolysaccharide-induced inflammation in intracerebroventricular-streptozotocin injected rats. Neuropharmacology 101: 110-122.

Bolton MM, Heaney CF, Murtishaw AS, Magcalas CM, Kinney JW (2015). Postnatal alterations in $GABA_B$ receptor tone produce sensorimotor gating deficits and protein level differences in adulthood. International Journal of Developmental Neuroscience 14: 17-27.

Sabbagh JJ, Murtishaw AS, **Bolton MM**, Heaney CF, Langhardt M, Kinney JW (2013). Chronic ketamine produces altered distribution of parvalbumin-positive cells in the hippocampus of adult rats. Neuroscience Letters 550: 69-74.

Heaney CF, **Bolton MM**, Murtishaw AS, Sabbagh JJ, Magcalas CM, Kinney JW (2012). Baclofen administration alters fear extinction and GABAergic protein levels. Neurobiology of Learning and Memory 98(3): 261-71.

Sabbagh JJ, Heaney CF, **Bolton MM**, Murtishaw AS, Kinney JW (in press, 2012). Efficacy of acute versus chronic administration of an NMDA receptor antagonist to induce an animal model of schizophrenia. Physiology of Behavior 107(3): 355-63.

Sabbagh JJ, Heaney CF, **Bolton MM**, Murtishaw AS, Ure JA, Kinney JW (2012). Administration of donepezil does not rescue galanin-induced spatial learning

deficits. International Journal of Neuroscience 122(12): 742-7.

Bolton MM, Heaney CF, Sabbagh JJ, Murtishaw AS, Kinney JW (2012). Deficits in Emotional Learning and Memory in an Animal Model of Schizophrenia. Behavioral Brain Research 233(1): 35-44.

Publications in Review

Murtishaw AS, **Bolton MM**, Belmonte KC, Kinney JW (submitted). Dementia-related pathologies following low-dose, staggered administration of streptozotocin. Psychopharmacology.

Bolton MM, Murtishaw AS, Heaney CF, Langhardt MA, Kinney JW (in prep). Interactions of ketamine administration and mTOR signaling on parvalbumin positive neurons. Translational Psychiatry.

Bolton MM, Murtishaw AS, Heaney CF, Langhardt MA, Kinney JW (submitted). Evaluation of ketamine-induced changes in spatial working memory and GABAergic systems. Progress in Neuropsychopharmacology and Biological Psychiatry.

Heaney CF, **Bolton MM**, Murtishaw AS, Langhardt MA, Kinney JW (in review). Dose response effects of $GABA_B$ ligands on spatial learning and memory. Learning and Memory.

Hensleigh E, Murtishaw A, Treat MD, Bolton MM, Heaney CF, Kinney JW, van Breukelen, F (in review). Torpor does not influence spatial memory in hibernating golden-mantled ground squirrels. Learning and Behavior.

Presentations:

Bolton MM. An evaluation of $GABA_B$ receptors in modulating neuroinflammation. Talk given at the COBRE CNTN 1st Annual Meeting at the Cleveland Clinic Lou Ruvo Center for Brain Health in Las Vegas, NV, 2016.

Bolton MM, Murtishaw AS, Salazar AM, Calvin, KN, Nagele, RF, Bergman HO, Kinney JW. An evaluation of $GABA_B$ receptors in modulating neuroinflammation. Poster presented at the Society for Neuroscience in San Diego, CA, 2016.

Bolton MM, Heaney CF, Murtishaw AS, Langhardt MA, Calvin KN, Kinney JW. Interactions of Ketamine Administration and mTOR Signaling on Parvalbumin Positive Neurons. Poster presented at AAPS National Biotechnology Conference in Boston, MA, 2016.

Bolton MM, Heaney CF, Murtishaw AS, Langhardt MA, Calvin KN, Kinney JW. Interactions of Ketamine Administration and mTOR Signaling on Parvalbumin Positive Neurons. Poster presented at Society for Neuroscience in Chicago, IL, 2015.

Bolton MM, Heaney CF, Murtishaw AS, Langhardt MA, Kinney JW. Interactions of behavioral training and ketamine administration on changes in parvalbumin positive neurons. Poster presented at the International Behavioral Neuroscience Society Annual Meeting in Victoria, British Columbia, 2015.

Bolton MM, Heaney CF, Murtishaw AS, Langhardt MA, Kinney JW. Interactions of behavioral training and ketamine administration on changes in parvalbumin positive neurons. Talk given at UNLV Graduate and Professional Student Association Research Forum, 2015.

Bolton MM, Heaney CF, Murtishaw AS, Langhardt MA, Kinney JW. Interactions of behavioral training and ketamine administration on changes in parvalbumin positive neurons. Poster presented at the American Chemical Society in Henderson, NV, 2014.

Bolton MM, Heaney CF, Murtishaw AS, Langhardt MA, Kinney JW. Interactions of behavioral training and ketamine administration on changes in parvalbumin positive neurons. Poster presented at Society for Neuroscience annual meeting Washington DC, 2014.

Bolton MM, Heaney CF, Murtishaw AS, Kinney JW. Developmental alteration of $GABA_B$ receptor function results in behavioral deficits in adulthood. Poster presented at The Sierra Nevada Chapter for Society for Neuroscience 5th Annual Research Symposium in Reno, NV 2013.

Bolton MM, Heaney CF, Murtishaw AS, Kinney JW. 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.

Bolton MM, Heaney CF, Sabbagh JJ, Murtishaw AS, Magcalas CM, Kinney JW. Comparison of postnatal ketamine dosage on behavioral deficits in adulthood. Presentation at the University of Nevada, Las Vegas Graduate and Professional Student Association Research Forum 2013.

Bolton MM, Heaney CF, Sabbagh JJ, Murtishaw AS, Magcalas CM, Kinney JW. Comparison of postnatal ketamine dosage on behavioral deficits in adulthood. Poster presented at The Sierra Nevada Chapter for Society for Neuroscience 4th Annual Research Symposium in Reno, NV 2012.

Bolton MM, Heaney CF, Sabbagh JJ, Murtishaw AS, Magcalas CM, Kinney JW. Comparison of postnatal ketamine dosage on behavioral deficits in adulthood.

Poster presented at Society for Neuroscience annual meeting New Orleans, LA 2012.

Bolton MM, Heaney CF, Sabbagh JJ, Murtishaw AS, Kinney JW (2011, November). Comparison of an Adult and Developmental Animal Model of Schizophrenia. Poster presented at Society for Neuroscience annual meeting in Washington, D.C.

Invited Speaking Engagements

Association for Psychological Science Student Caucus Invited Panelist: May 2014. Invited to speak at a panel during the Association for Psychological Science (APS) national conference. The panel is organized by the APS Student Caucus and titled "The Naked Truth Part II: Surviving Graduate School."

Psi Chi International Honor Society in Psychology, UNLV Local Chapter, Invited Panelist: January 2015: Invited to speak on a panel during the Psi Chi UNLV Local Undergraduate Chapter meeting. The panel consisted of current graduate students discussing their experience getting into graduate school and life in graduate school.

Awards/Grants:

Doctoral Graduate Research Assistantship: Fall 2015 – Spring 2016 ($15,500). Examination of biomarkers and novel treatments in Alzheimer's disease.

Patricia Sastaunak Scholarship: Fall 2015 – Spring 2016 ($2,500). Competitive university-wide scholarship awarded to graduate students who have demonstrated substantial academic accomplishments.

University of Nevada, Las Vegas Graduate Professional Student Association Travel Award: Summer 2015 ($450). University-wide travel grant to aid students with funding to present research at national conferences. This award was given to fund travel to the International Behavioral Neuroscience Society Annual Meeting in Victoria, B.C., Canada.

Outstanding Presentation Award, University of Nevada, Las Vegas Graduate and Professional Student Association Annual Research Forum: Spring 2015.

University of Nevada, Las Vegas Graduate Professional Student Association Travel Award: Fall 2014 ($800). University-wide travel grant to aid students with funding to present research at national conferences. This award was given to fund travel to the Society for Neuroscience conference in Washington, D.C.

University of Nevada, Las Vegas Graduate Professional Student Association Travel Award: Summer 2014 ($200). University-wide travel grant to aid students with funding to present research at national conferences. This award was given to fund travel to the Association for Psychological Sciences conference in San Francisco, CA.

Outstanding Presentation Award, University of Nevada, Las Vegas Graduate and Professional Student Association Annual Research Forum: Spring 2014.

Patricia Sastaunak Scholarship: Fall 2013 – Spring 2014 ($2,500). Competitive university-wide scholarship awarded to graduate students who have demonstrated substantial academic accomplishments.

University of Nevada, Las Vegas Graduate Professional Student Association Travel Award: Fall 2013 ($400). University-wide travel grant to aid students with funding to present research at national conferences. This award was given to fund travel to the Society for Neuroscience conference in San Diego, CA.

Patricia Sastaunak Scholarship: Fall 2012 – Spring 2013 ($2,500). Competitive university-wide scholarship awarded to graduate students who have demonstrated substantial academic accomplishments.

University of Nevada, Las Vegas Graduate Professional Student Association Travel Award: Fall 2012 ($450). University-wide travel grant to aid students with funding to present research at national conferences. This award was given to fund travel to Society for Neuroscience conference in New Orleans, LA.

Outstanding Presentation Award, University of Nevada, Las Vegas Graduate and Professional Student Association Annual Research Forum: Spring 2012.

University of Nevada, Las Vegas Graduate Professional Student Association Travel Award: Fall 2011 ($350). University-wide travel grant to aid students with funding to present research at national conferences. This award was given to fund travel to the Society for Neuroscience conference in Washington, D.C.

University Memberships:

University of Nevada, Las Vegas Psychology Department Experimental Graduate Student Committee President: Fall 2012 – Spring 2013. Student-elected position to act as the liaison between the psychology department faculty and graduate students. Responsibilities include running committee meetings, attend faculty meetings, write faculty meeting minutes, and organize Interview Day activities for potential incoming graduate students.

University of Nevada, Las Vegas Psychology Department Experimental Graduate Student Committee Secretary: Fall 2011 – Spring 2012. Student-elected position to document details of the committee meetings. Responsibilities include attending committee meetings and taking notes, writing and e-mailing meeting minutes to the department, communicate with other graduate students in the department regarding activities, and organizing the end of the year party and other events.

Graduate Neuroscience Association, Co-Founder and Committee Member: Fall 2011 to present. An association intended to inform graduate students about current research in the field of neuroscience.

Neuroscience Journal Club, Co-Founder and Secretary: Fall 2010 – present. An organization formed to educate undergraduate students about the field of neuroscience, how to read and analyze scientific articles, and organize events in the community.

Professional Memberships:

Medical Science Liaison Society member since 2016
American Association of Pharmaceutical Sciences member since 2015
National Association of Professional Women member since 2015
International Behavioral Neuroscience Society member since 2013
Association for Psychological Science member since 2013
Society for Neuroscience, Sierra Nevada Chapter member since 2010
Society for Neuroscience member since 2009

Service:

International Behavioral Neuroscience Society, Student Councilor Elect 2017

Communications and Media Committee Member for the International Behavioral Neuroscience Society: January 2015 to present. Recruit members from all over the world to join the organization, advertise the society on social media, maintain social media platforms, and interview professionals in the field of behavioral neuroscience to promote the organization.
International Student Representative Nominee for the International Behavioral Neuroscience Society: June 2015. Nominated as a candidate to represent students (graduate and post-doctoral) from all over the world on the International Behavioral Neuroscience Society advisory council.
Psi Chi International Honor Society in Psychology, UNLV Local Chapter Research Forum Judge: April 2015. Neuroscience judge for the university undergraduate Psi Chi chapter.
UNLV College of Sciences Science Fair Judge: March 2015. Behavioral and Social Science judge for a regional high school science fair.
Las Vegas Brain Bee Organizer and Judge: February 2015. Appointed as the Logistics Coordinator and one of three judges for the Las Vegas Brain Bee. Participants were local high school students demonstrating their knowledge in neuroscience. The winner from this competition was sent to the National Brain Bee.
Brain Education Week, Head Coordinator for the Clark County School District: Fall 2014. Work with representatives from the Clark County School District to incorporate Brain Awareness presentations and knowledge regarding the brain and nervous system into school curriculum starting in the 2014-2015 school year.
Brain Safety Initiative of the International Behavioral Neuroscience Society, Volunteer Head Coordinator: June 2014. Initiated the first annual philanthropy event

for the International Behavioral Neuroscience Society where we raised over $1000 to donate to the local community (Clark County School District's Safe Routes to School Program). In addition, I implemented and organized an educational outreach event in a local school (Wright Elementary School) where we educated the students on brain facts and safety.

Las Vegas Brain Bee Organizer: February 2014. Appointed as the Logistics Coordinator for the Las Vegas Brain Bee. Participants were local high school students demonstrating their knowledge in neuroscience. The winner from this competition was sent to the National Brain Bee.

Nevada Brain Bee Association, Co-Founder and Board Member: Fall 2013 – present. Under the umbrella of the International Brain Bee Association, the Nevada Brain Bee Association held the Inaugural Las Vegas Brain Bee in February 2014. This annual event is for high school students to demonstrate their knowledge of the brain and compete for a spot at the National Brain Bee to represent the state of Nevada. The winner at our Inaugural Las Vegas Brain Bee took third place overall in the National Brain Bee competition in March 2014, putting Las Vegas on the map for neuroscience education.

Brain Awareness Campaign, Head Coordinator at UNLV: Fall 2012 – present. The Brain Awareness educates the public and promotes brain science at local schools and community centers. Funding for these events provided by the Sierra Nevada Chapter of Society for Neuroscience ($200 in 2013 and $650 in 2014).

APS Mentorship Program: Fall 2010 – present. The APS Mentorship Program helps undergraduate students with career plans and future goals by pairing them with a graduate student to form a peer-mentor relationship.