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An Evaluation of Alzheimer's Disease-related Pathology in Two Different Models of Diabetes in Immune-challenged Mice

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AN EVALUATION OF ALZHEIMER'S DISEASE-RELATED PATHOLOGY IN TWO DIFFERENT MODELS OF DIABETES IN IMMUNE-CHALLENGED MICE

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ABSTRACT

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Obesity, type 2 diabetes mellitus (T2DM), and metabolic syndrome are related disorders with wide-ranging and devastating effects that can be observed throughout the body. One important and understudied organ of damage is the brain. Clinical and epidemiological studies have found that T2DM, and more specifically hyperinsulinemia, significantly increases the risk of cognitive decline and increases the likelihood of Alzheimer's disease (AD) and other forms of dementia in the elderly. Insulin has slightly different functions in the peripheral body than in the central nervous system and the dysregulation of these functions may contribute to the onset and progression of late-life neurodegenerative disease. These experiments were designed to investigate cognitive function and AD-related disease pathology in two different models of diabetes, one model resulting from a diabetogenic compound that selectively targets insulin-producing pancreatic β-cells and the other model based on diet-induced obesity. Additionally, these diabetic models were combined with a genetic mouse model of inflammation to explore the compounding effects of multiple AD risk factors. We found that diabetic-status, regardless of whether it was drug- or diet-induced, resulted in profound impairments in learning and memory and subtle alterations to AD-related histopathology within the hippocampus. Additionally, impairments were most dramatic in male mice; whereas females appeared to be more resistant to metabolic disturbances.

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CHAPTER 1

INTRODUCTION

The United States Census Bureau projects that our population will grow by nearly 25% to over 400 million people by the year 2050 and that 1 in 5 Americans will be over the age of 65 (Colby & Ortman, 2015). As our elderly population continues to grow, Type-2 diabetes mellitus (T2DM) and Alzheimer's disease (AD) are also increasing at alarming rates. Both of these diseases are chronic and complicated and ultimately lead to devastating outcomes. The Center for Disease Control and Prevention rank both of these diseases in the top ten causes of death and these rankings climb higher within the elderly population (CDC, 2014; Tschanz et al., 2004).

Diabetes mellitus (DM) is a complex metabolic disorder characterized by hyperglycemia, disturbed insulin signaling, and is associated with microvascular and macrovascular complications, including cardiovascular disease, nephropathy, retinopathy, and neuropathy (Li & Hölscher, 2007). Current world-wide prevalence rates of DM indicate that 415 million people are diagnosed with the disease (IDF, 2015). In the United States alone, current estimates indicate that nearly 29 million, or 9.3% of the population, are diagnosed with diabetes. Conservative estimates suggest that another 86 million have prediabetes, which is often defined as an intermediate state of increased blood glucose, placing them at high risk for developing diabetes (CDC, 2014). Type-1 diabetes (T1DM) accounts for about 5-10% of DM cases and is associated with hyperglycemia, deficient insulin production, and cognitive deficits with varying degree of severity depending upon the age of onset of diabetes, the degree of glycemic control, and the duration of the disease (Brands, Biessels, de Haan, Kappelle, & Kessels, 2005). Type-2 diabetes (T2DM) is the predominant form of the disease, accounting for 90-95% of cases and is

characterized by hyperglycemia, insulin resistance, and is associated with obesity, hypertension, hypercholesterolemia, and hyperlipidemia (Kloppenborg, van den Berg, Kappelle, & Biessels, 2008). Cognitive impairments in learning and memory, mental flexibility, and executive functioning are commonly associated with T2DM (Awad, Gagnon, & Messier, 2004; Strachan, Deary, Ewing, & Frier, 1997).

AD is the most common form of dementia, accounting for nearly 80% of cases (Alzheimer's Association, 2016). In 2016, there are an estimated 5.4 million AD patients in the USA and nearly 46.8 million worldwide and this number is expected to reach 131.5 million by 2050 (Alzheimer's Association, 2016; IDF, 2017). The greatest risk factor for developing AD is increased age: 11% of individuals over the age of 65 have AD and that number increases to 32% for individuals over the age of 85 (Alzheimer's Association, 2016) AD is a neurodegenerative disorder marked by key symptoms such as a progressive decline in memory, impairments in speech, language, spatial orientation, and disturbances in sensorimotor systems (Martins et al., 2006). In addition to severe cognitive impairments, the core pathological hallmarks of AD include β-amyloid (Aβ) plaques, neurofibrillary tangles, neuronal loss, and neuroinflammation (Alzheimer, Stelzmann, Schnitzlein, & Murtagh, 1995; Selkoe, 2000). The exact pathological etiology of AD is unknown but prevailing theories are based on the build-up of Aβ peptides into neurotoxic soluble Aβ oligomers and insoluble Aβ plaques or the accumulation of neurofibrillary tangles (Selkoe, 2000; Small & Duff, 2008). Familial AD has been linked to a number of mutations that exist in three genes (*amyloid precursor protein* (APP), *presenilin-1*, and *presenilin-2*) that lead to an early-onset of AD (<65 years of age); however, these mutations only account for 1-5% of total AD cases (Ridha et al., 2006). The vast majority of AD cases are

sporadic in origin and are less clearly defined by a single mutation but rather some combination of many different genetic, environmental, and behavioral risk factors (Pedersen, Gatz, Berg, & Johansson, 2004). There are a number of risk factors that have been identified for developing late-onset AD and include increased age, presence of APOE-ε4, and cardiovascular disease (Corder et al., 1993; Cummings, 2004). APOE-ε4 has been shown to greatly compound and increases the effect of other important risk factors of late-onset AD, including hyperinsulinemia and T2DM (Luchsinger, Tang, Shea, & Mayeux, 2004; Peila et al., 2002).

In addition to the above neuropathological hallmarks, AD is characterized by vascular lesions, glucose intolerance, adiposity, hypertension, hyperglycemia, hyperinsulinemia, and insulin resistance, which are all symptoms also associated with T2DM (Haan, 2006). A study conducted by researchers at the Mayo Clinic found that a staggering 80% of AD patients either had diagnosed T2DM or impaired fasting glucose, indicating rampant metabolic disturbances (Janson et al., 2004). A key focus of recent years has been expanding our understanding in the role of insulin signaling in normal brain functions and the manner in which abnormalities to insulin signaling and metabolism contribute to disorders of aging, particularly Alzheimer's disease.

CHAPTER 2

REVIEW OF RELATED MATERIAL

Type 2 Diabetes and Alzheimer's Disease

The clinical association between T2DM and Alzheimer's disease is well established, as well as with other neurodegenerative disorders, including vascular dementia and Parkinson's disease (Biessels, Staekenborg, Brunner, Brayne, & Scheltens, 2006; Brands et al., 2005; Janson et al., 2004). Numerous cross-sectional studies have shown that the percentage of T2DM among AD patients is significantly greater than age-matched non-AD controls (Kuusisto et al., 1997; Ott et al., 1996; Stewart & Liolitsa, 1999). Longitudinal studies demonstrate that T2DM more than doubles the risk of AD when compared to non-diabetic control groups (Arvanitakis, Wilson, Bienias, Evans, & Bennett, 2004; Grodstein et al., 2001; Leibson et al., 1997; Ott et al., 1999; Peila et al., 2002). Additionally, higher incidence rates of AD is observed among those who have suffered from T2DM for longer than 5 years (Leibson et al., 1997)

It is important to note that T2DM is not a disease that is exclusively associated with the elderly. Despite higher prevalence rates in older aged individuals, T2DM is being diagnosed more frequently in young people and the number of cases appear to parallel the rise in childhood obesity (Freedman, Mei, Srinivasan, Berenson, & Dietz, 2007; Weiss et al., 2004). The number of individuals diagnosed with DM is expected to surge to 592 million by 2035 with diagnoses occurring earlier in life (IDF, 2015). This could be especially problematic since the longer the duration of DM in an individual, the more profound the cognitive impairments and the greater the risk of developing AD (Leibson et al., 1997). Obese adolescents with T2DM score significantly lower than healthy-age matched controls on a number of cognitive measures,

including verbal memory, executive function, and psychomotor efficiency (Yau et al., 2010). Even in the absence of T2DM, individuals that exhibit impairments in glucose metabolism and increased serum insulin are at higher risk for mild cognitive impairment, suggesting an important contribution of improper glycemic control and hyperinsulinemia to cognitive decline (Stolk et al., 1997; Yaffe, Blackwell, Whitmer, Krueger, & Barrett Connor, 2006).

The Honolulu Asia Aging study, a cohort study of ethnic Japanese males with AD, found that comorbidity with T2DM caused a higher number of neurofibrillary tangles, amyloid plaques, and cerebral amyloid angiopathy (Peila et al., 2002). The association between T2DM and AD is particularly strong among individuals who possess the APOE ε4 allele, as those with T2DM and are also APOE ε4 carriers have twice the risk of developing AD compared to non-diabetic APOE ε4 carriers (Peila et al., 2002). Additionally, although the brains from patients with T2DM frequently exhibit amyloid deposition, this amyloid deposition is markedly greater in those with both T2DM and the APOE ε4 genotype (Messier, 2003; Peila et al., 2002). T2DM poses an increased risk for developing AD on its own but that risk is greatly exacerbated in those carrying an APOE ε4 allele, which can be found in nearly half of all AD patients (Alzheimer's Association, 2016).

T2DM is a multi-faceted disease with many contributing risk factors and several key hallmarks, including hyperglycemia and hyperinsulinemia, but research is beginning to suggest that hyperinsulinemia and insulin dysregulation may be the biggest factor in cognitive decline and onset of dementia. Numerous studies have demonstrated that serum levels of insulin of AD with no diagnosis of T2DM or any other metabolic disease are elevated compared to controls (Carantoni et al., 2000; Fujisawa, Sasaki, & Akiyama, 1991; Kuusisto et al., 1997). Despite

elevated peripheral insulin levels, individuals diagnosed with Alzheimer's disease exhibit marked reductions in insulin receptor expression in the brain (Frölich et al., 1998). Post-mortem brains of patients with either AD or T2DM show decreased levels and activity in several components of the insulin-AKT signaling pathway and this signaling deficiency is significantly more severe in individuals with both AD and T2DM (Liu, Grundke-Iqbal, & Gong, 2011). Recently, it has been demonstrated that higher insulin resistance in late middle-aged adults results in a greater correlation with increased amyloid plaque deposition in frontal and temporal areas of the brain with no diagnosis of AD or cognitive impairment (Willette et al., 2015). The pattern of amyloidosis in these cognitively healthy but insulin resistant individuals reflects a deposition pattern that closely mirrors early AD progression. Disruptions to insulin signaling may make neurons more susceptible to metabolic stress, which would accelerated neuronal dysfunction. Increased density of amyloid plaque deposition positively correlates with the duration of T2DM in post-mortem brains (Janson et al., 2004).

Several components within the insulin signaling pathway have been implicated in the onset and progression of Alzheimer's disease, namely the influence on tau phosphorylation and APP metabolism, which will be discussed below.

Insulin Signaling and Insulin Resistance

Sine the discovery of insulin in 1921, the signaling mechanisms and biological effects of insulin have been widely studied in the classical insulin target organs of the periphery, namely skeletal muscle, fat, and the liver, especially in regards to glucose uptake, gene expression, and cellular proliferation (Schwartz & Porte, 2005; Wilcox, 2005).

The insulin receptor is a hetero-tetrameric receptor that consists of two extracellular α-subunits that bind insulin and two transmembrane β-subunits that lead to intracellular tyrosine kinase activity. Following insulin binding to the α-subunit, the β-subunits are activated leading to autophosphorylation of the receptor complex. Once the insulin receptor complex is activated, insulin receptor substrate (IRS) proteins are phosphorylated leading to the activation of phosphoinositide-3 kinase (PI3K). P13K, in turn, typically activates one of two major signaling pathways. The first being the mitogen-associated protein kinase (MAPK) pathway or the other the Akt pathway. While both pathways are vital for cellular differentiation and growth, Akt signaling is also involved with protein synthesis and plays a key role in the phosphorylation and subsequent inhibition of GSK-3β (Wilcox, 2005). The inhibition of GSK-3β leads to an increase in glycogen synthesis in the periphery and prevents this kinase from phosphorylating tau proteins within the brain, which will be detailed in a later section (Hooper, Killick, & Lovestone, 2008).

Insulin plays a crucial role in glucose homeostasis through the regulation of hepatic glucose production and glucose uptake into various tissues and managing this balance. The vast majority of the body, with the exception of the brain, relies on insulin to transport glucose from the bloodstream into the cells of peripheral target tissues. Insulin binding has been shown to regulate glucose transport through coordinating the translocation of glucose transporter 4 (GLUT4) into the cellular membrane in adipocytes and myocytes (Huang & Czech, 2007). GLUT4 is the major insulin-mediated glucose transporter in peripheral tissues. Under normal conditions, insulin activation of the P13K-Akt pathway leads to the phosphorylation of AS160, which is directly responsible for the rapid and precise insertion of GLUT4 from intracellular stores into the membrane (Kim & Feldman, 2012). Decreased association of the P13K regulatory

p85 and IRS leads to impaired Akt signaling and impaired GLUT4 insertion is the primary cause of insulin resistance and has been reported in obese individuals, patients with T2DM, and in both genetically obese and high-fat fed animals (Asano et al., 2007; Kim & Feldman, 2012).

Hyperinsulinemia precedes the classic hyperglycemic state by many years in the majority of T2DM cases, resulting in insulin receptor insensitivity and a defect of insulin signal transduction due to chronic overstimulation (Weyer, Hanson, Tataranni, Bogardus, & Pratley, 2000). Despite hyperinsulinemia being considered the primary cause of T2DM onset, hyperinsulinemia is present in most diagnosed cases of T2DM, but not all cases, particularly those in late-stage T2DM (Laakso, 1993). The loss of hyperinsulinemia as T2DM progresses may be explained by the profound pancreatic β-cell death that occurs in later stages of the disease as β-cells become exhausted from producing increasingly larger amounts of insulin to compensate for insulin receptor insensitivity and an apoptotic response to glucose toxicity (Jörns, Tiedge, Ziv, Shafrir, & Lenzen, 2002; Robertson & Harmon, 2006).

Hyperglycemia is generally considered the major cause for the development of diabetic end-organ damage and common complications, including diabetic neuropathy and retinopathy (Gispen & Biessels, 2000). The toxic effects of hyperglycemia ultimately trigger several metabolic and molecular cascades that can lead to progressive neuronal dysfunction. Long-term exposure to hyperglycemia leads to abnormalities in cerebral capillaries, such as a thickening of the basement membrane, an increase in reactive oxygen species, and advanced glycation of important structural proteins, leading to ischemia of the brain and subcortical white-matter lesions (Mankovsky, Metzger, Molitch, & Biller, 1996). White matter lesions within the frontal lobe are associated with cognitive impairments and are common in healthy elderly adults but the

severity and prevalence is strongly increased in patients with vascular risk factors, T2DM, and among those with dementia (Manschot et al., 2006; Pantoni et al., 1999). In addition to β-amyloid deposition in the brain parenchyma, Aβ accumulates along the blood vessels in AD brains contributing to endothelial dysfunction and leading to cerebral amyloid angiopathy (Grinberg, Korczyn, & Heinsen, 2012). Impaired integrity of the blood-brain-barrier has been reported in both human AD studies and in animal models of AD (Blennow et al., 1990; Skoog et al., 1998). The combination of a compromised blood-brain-barrier due to glucose toxicity and the detrimental effects of dysregulated insulin signaling is particularly problematic in disease progression.

Insulin resistance, regardless of disease origin (obesity, T2DM, metabolic disease), is exceedingly common in older adults, with current estimates suggesting that over half of individuals over the age of 60 are affected with some notable degree of insulin resistance (Craft, 2005). Chronic peripheral hyperinsulinemia ultimately results in down-regulation of brain insulin uptake and long-term reduction in brain insulin levels affecting cognition and increasing inflammation in the CNS (Baura et al., 1996; Craft et al., 1998; Fishel et al., 2005).

Insulin Signaling in the Brain

The brain was long thought to be an "insulin insensitive organ" until insulin receptors were first localized in the CNS in 1978 and while not completely insulin-independent, the brain is certainly insulin-responsive (Belfiore, Frasca, Pandini, Sciacca, & Vigneri, 2009; Havrankova, Roth, & Brownstein, 1978). Insulin receptors are widely distributed throughout the brain with the highest concentrations in specific regions such as the olfactory bulb, the hypothalamus, frontal cortex, and areas within the hippocampus (Havrankova et al., 1978; van Houten, Posner,

Kopriwa, & Brawer, 1979). Insulin receptors are located on synapses in both neurons and astrocytes, with a much higher concentration on neuronal post-synaptic densities (Abbott, Wells, & Fallon, 1999). Despite considerable homology in insulin receptors located in the periphery and in the brain, there are some differences between them. Brain insulin receptors located within the brain are smaller in overall size due to smaller α-subunits and, unlike peripheral receptors, are not internalized or desensitized after insulin binding (Heidenreich, Zahniser, Berhanu, Brandenburg, & Olefsky, 1983).

Interestingly, there does appear to be regional differences in insulin receptor density between embryonic brains and mature brains, implying a developmental role for insulin. For example, a developing brain undergoing rapid neurogenesis has extremely high levels of insulin receptors in the thalamus, the caudate-putamen, and in numerous discrete nuclei located in the mesencephalon and brainstem but these areas express relatively low levels of insulin receptors in an adult brain (Kar, Chabot, & Quirion, 1993). The brain of the *Drosophila* fruit fly contains clusters of neurons that co-express four insulin genes — two are homologous to mouse and human insulin and two genes are dissimilar and found only in *Drosophila* — that upon ablation results in undergrowth, developmental delays, and lethality (Rulifson, Kim, & Nusse, 2002). Entirely knocking out insulin in mice does not immediately result in glycosuria at birth but they do rapidly develop classical symptoms of DM with ketoacidosis leading to death within just 48 hours (Duvillié et al., 1997).

Insulin, primarily produced by the pancreas, must cross the blood-brain barrier to initiate signaling within the CNS. Plasma insulin circulates at levels nearly one hundred times greater than are observed in the cerebrospinal fluid (Woods, Seeley, Baskin, & Schwartz, 2003). The

rate of insulin passage from plasma to brain interstitial fluid is tightly regulated with specific, selective active transport across the blood-brain-barrier (Baura et al., 1996; Pardridge, 1986). Insulin receptors located on the luminal surface of capillary endothelial cells are internalized into the cell as part of a membrane-bound vesicle upon insulin binding, which is then transported across the cell and exocytosed to release insulin into the brain interstitial fluid (Woods et al., 2003). Studies have shown that peripheral administration of insulin leads to an increase in insulin levels within the CNS although less than 1% of peripheral insulin crosses the blood-brain barrier (Margolis & Altszuler, 1967; Woods, Lotter, McKay, & Porte, 1979). Despite the majority of insulin in the CNS being transported across the blood-brain-barrier, some evidence indicates that a small percentage of CNS insulin levels can be produced neurons within the brain (Devaskar et al., 1994; Schechter, Holtzclaw, Sadiq, Kahn, & Devaskar, 1988). It has been posited that these insulin producing brain cells and pancreatic-β cells evolved from the same common ancestral insulin-producing neuron (Rulifson et al., 2002).

Recent evidence suggests that insulin resistant individuals experience impaired transport of insulin from the periphery into the CNS, suggesting that receptor mediated transport of insulin across the blood-brain-barrier is also subject to similar regulatory mechanisms occurring in peripheral insulin resistance (Heni et al., 2014). Insulin resistance occurring at the blood-brain-barrier can lead to the inadequate levels of brain insulin seen in AD (Craft et al., 1998). Supporting this relationship between insulin resistance and impaired transport across the blood-brain-barrier, it has been well-documented that exercise and weight loss can reverse insulin resistance occurring in the peripheral body in T2DM and obesity (Gurley, Griesel, & Olson, 2016; Kennedy et al., 1999; O'Gorman et al., 2006), but newer evidence that a decrease in body

fat correlates with increased brain insulin sensitivity suggests that a similar restoration is occurring to insulin transport across the blood-brain-barrier and insulin receptors within the brain (Tschritter et al., 2012).

The mammalian brain depends on glucose as its main source of energy by consuming nearly 20% of all glucose-derived energy in the body despite accounting for only ~2% of total body weight (Mergenthaler, Lindauer, Dienel, & Meisel, 2013). The largest potion of energy consumed by the brain is for neuronal communication and information processing in the form of action potentials, post-synaptic potentials, and the maintenance of ion gradients to maintain the resting membrane potential (Howarth, Gleeson, & Attwell, 2012; Ivannikov, Sugimori, & Llinás, 2010). Glucose entry into the brain is primarily driven by the large blood-to-brain concentration gradient that facilitates movement across the endothelial membrane via GLUT1 transporters. GLUT1 is also highly expressed on astrocytes, microglia, and oligodendrocytes, facilitating glucose uptake into the glial cells. Neurons express GLUT3, which has a higher transport rate than GLUT1, ensuring that neurons have a sufficient glucose supply under a variety of glucose levels and varying activity state demands (Dienel, 2012). It is important to note that both GLUT1 and GLUT3, the primary glucose transporters in the brain, are insulin-independent and rely on concentration gradients to drive the movement of glucose from the extracellular milieu into the cell (Simpson, Carruthers, & Vannucci, 2007). Additionally, astrocytes are large consumers of glucose and other brain metabolites and serve a key role in helping neurons meet their energy demands by converting glucose to lactate and shuttling lactate to neurons as a supplemental fuel source (Pellerin & Magistretti, 2011). The astrocyte-to-neuron lactate shuffle is intimately linked to glutamate uptake which drives glycolysis and glutamate recycling in astrocytes in order to

provide glutamine and lactate to neurons and appears to play a crucial role in long-term memory formation, particularly during consolidation (Newman, Korol, & Gold, 2011; Suzuki et al., 2011).

Although neurodegenerative diseases have not typically been considered to be caused by a disturbed metabolism, numerous studies demonstrate that bioenergetic defects are emerging as important pathophysiological mechanisms (Kapogiannis & Mattson, 2011). One of the earliest signs of AD is altered cerebral blood flow and a reduction in brain glucose metabolism (Hoyer, 2004). The expression of GLUT1 and GLUT3 glucose transporters is reduced in AD brains within discrete regions such as the temporal and parietal lobes compared to control subjects (Simpson, Chundu, Davies-Hill, Honer, & Davies, 1994). In a mouse model of AD overexpressing human APP, the expression of GLUT1 is reduced both at the blood-brain-barrier and in astrocytes, resulting in impaired glucose transport and reduced astrocytic-derived lactate during neuronal activation (Merlini, Meyer, Ulmann-Schuler, & Nitsch, 2011).

Despite insulin not playing a major role in glucose uptake within the brain, insulin still plays a key modulatory role in energy homeostasis. Insulin, along with leptin and several other neuropeptides and neurotransmitters, target the hypothalamus to create a complex and tightly regulated network (Leibowitz & Wortley, 2004). The administration of insulin directly into the brain has an anorexigenic effect; whereas, the inhibition of insulin signaling yields an orexigenic effect (Carvalheira et al., 2003; Obici, Feng, Karkanias, Baskin, & Rossetti, 2002; Woods et al., 1979). Intracerebroventricular administration of insulin leads to an upregulation of α-melanocyte stimulating hormone (α-MSH), an anorexigenic peptide; additionally, this insulin-induced reduction in food intake can be prevented with an α-MSH antagonist (Benoit et al., 2002).

Insulin activation of the PI3K pathway leads to activation of pATP-dependent potassium channels (K_{ATP}) channels in both the pancreas and within the brain, in regions like the hypothalamus and hippocampus (Khan, Goforth, Zhang, & Satin, 2001; Niswender et al., 2003). Within the pancreas, this insulin-induced K_{ATP} hyperpolarization acts as a negative feedback in β-cells to cease insulin production (Khan et al., 2001). In the hypothalamus, K_{ATP} channel activation is thought to serve as a molecular endpoint after activation of insulin and leptin receptors, which both inactivate the same hypothalamic glucose-response neurons via the PI3K pathway (Spanswick, Smith, Mirshamsi, Routh, & Ashford, 2000).

Insulin not only plays a key role in the inhibition of food intake energy expenditure but also directly activates dopaminergic neurons within the mesolimbic pathway (Figlewicz et al., 2007). Mice with a brain specific knockout of insulin receptor exhibit an increase in monoamine oxidase A and B, resulting in an increased turnover of dopamine and depressive-like behavior (Kleinridders et al., 2015). Insulin has been shown to play a modulatory role in neurotransmission by promoting NMDA glutamate receptor phosphorylation that increases the opening of Ca^{2+} channels, influencing AMPA receptor internalization, and recruiting post-synaptic GABA receptors (Wan, Xiong, Man, Ackerley, & Braunton, 1997). Intranasal insulin administration improves verbal memory and selective attention in healthy humans under normal glycemic conditions (Kern et al., 2001). Younger individuals with diabetes do not typically exhibit severe evidence of learning and memory impairments; however, subjects with T2DM over 60 years of age perform significantly worse than age-matched non-diabetic controls in a number of cognitive tasks, including memory recall and processing speed (R. Kumar, Looi, & Raphael, 2009; Ruis et al., 2009; C. M. Ryan & Geckle, 2000). A recent study found that the

incidence of mild-cognitive impairment was much higher in elderly patients with T2DM than non-diabetic subjects (Gao et al., 2017). Additionally, T2DM-related cognitive deficits appear to more pronounced the longer the duration of the disease, suggesting cumulative damage as the disease progresses due to prolonged exposure the devastating effects of neuronal insulin signaling perturbations (Hazari, Reddy, Uzma, & Kumar, 2015).

Insulin Signaling and Tau Pathology

Tau is expressed abundantly throughout the central nervous system. Tau is located primarily within the axons of neurons is associated with microtubules. The carboxy-terminus of tau is characterized by a series of repeats that help determine the ability of tau to stabilize and facilitate the polymerization of microtubules (Gustke, Trinczek, Biernat, & Mandelkow, 1994; Weingarten, Lockwood, Hwo, & Kirschner, 1975). The ability of tau to bind to and promote the stabilization of microtubules is regulated by phosphorylation around this microtubule binding carboxy-terminal domain with over 30 potential phosphorylation sites (Avila, Lucas, Perez, & Hernandez, 2004; Sergeant et al., 2008). A number of Ser/Thr kinases and phosphatases are responsible for the regulation of tau phosphorylation, including GSK-3β and protein phosphatase 2A (PP2A). GSK-3β and PP2A are considered two of the most important proteins involved with regulating tau phosphorylation (Hooper et al., 2008; Planel, Yasutake, Fujita, & Ishiguro, 2001). Hyperphosphorylated tau is the principal component of the paired helical filaments that make up the core of the neurofibrillary tangles that are associated with AD (Grundke-Iqbal et al., 1986; Iqbal, Zaidi, Bancher, & Grundke-Iqbal, 1994).

Hyperphosphorylation of tau is a complex interaction of increased kinase activity and decreased phosphatase activity. GSK-3β is the kinase that has emerged as the most important

kinase related to AD pathology. GSK-3β is capable of phosphorylating tau on virtually all known phosphorylation sites and is consistently upregulated in the brains of AD patients (Lovestone et al., 1994; Muñoz-Montaño, Moreno, Avila, & Diaz-Nido, 1997). Insulin receptor activation of Akt typically results in phosphorylation of GSK-3β, which prevents GSK-3β from phosphorylating tau proteins but when insulin resistance occurs Akt is unable to inhibit GSK-3β, which results in rampant tau hyperphosphorylation (Taniguchi, Emanuelli, & Kahn, 2006). Tau dysfunction is not only due to the over activity of kinases that add phosphate groups but the inability of phosphatases to dephosphorylate tau at an appropriate level.

Ser/Thr phosphatases, the main type of phosphatases in the brain, can be sorted into five main types (PP1, PP2A, PP2B, PP2C, and PP5) based upon their specificities (Liu, Grundke-Iqbal, Iqbal, & Gong, 2005). PP2A is the major tau phosphatase, accounting for over 70% of total phosphatase activity occurring within the brain (Liu et al., 2005). PP2A has a much stronger affinity than other phosphatases to dephosphorylate PHF and restore the microtubule assembly-promoting ability of tau (Wang, Gong, Zaidi, Grundke-Iqbal, & Iqbal, 1995).

Patients with AD have been shown to have reduced PP2A mRNA expression in the hippocampus and PP2A protein levels to be selectively decreased in AD-affected regions, including the frontal and temporal regions, but not in other areas that are not typically associated with AD (Sontag et al., 2004). Streptozotocin (STZ) is a toxin produced by *Streptomycetes achromogenes* and is commonly used to model DM. This toxin targets pancreatic β-cells by entering through the glucose transporter GLUT2 where it alkylates DNA, which leads to cell death (Delaney et al., 1995). While STZ is often utilized to induce insulin deficiencies that are widely associated with T1DM, there is evidence to suggest that smaller doses of STZ can lead to

a diabetic state in between T1DM and T2DM, similar to the β-cell exhaustion seen in the later stages of T2DM (Reed et al., 2000; Srinivasan, Viswanad, Asrat, Kaul, & Ramarao, 2005). Increased tau phosphorylation following STZ administration in rodents has been robustly demonstrated at a number of phosphorylation sites (Clodfelder-Miller, Zmijewska, Johnson, & Jope, 2006; Murtishaw et al., 2016; Qu et al., 2011). An increasing number of studies are emerging to investigate the role that spontaneous T2DM has on AD pathogenesis, including the use of transgenic mice and diet-induced obesity. Obese mice with mutations in the leptin receptor, termed db/db mice, develop hyperglycemia, hyperinsulinemia, numerous epitopes of hyperphosphorylated tau, and impairments in spatial learning (Kim, Backus, Oh, Hayes, & Feldman, 2009; Li, Deng, Sheng, & Zuo, 2012). Diet-induced obesity in the 3xTg mouse model of AD given access to high-fat chow results in aggravated tau pathology, further indicating a critical role between T2DM and tau pathology (Julien et al., 2010).

Insulin Signaling and Amyloid Pathology

A key feature of AD is the altered expression and improper processing of APP that accumulates Aβ peptides into neurotoxic oligomers and amyloid plaques. The APP protein is a type 1 integral membrane with a large extracellular domain, a hydrophobic transmembrane domain, and a short C-terminus intracellular domain and is critical for neuronal synaptogenesis, synapse remodeling, and neurite outgrowth (Zhang, Ma, Zhang, & Xu, 2012; Zheng & Koo, 2006). APP metabolism can follow two distinct pathways: the nonamyloidgenic pathway or the amyloidgenic pathway. In the nonamyloidgenic pathway, APP is cleaved by α-secretase, which releases an extracellular neurotrophic NH_2-terminal soluble fragment (sAPP) and an intracellular COOH-terminal fragment (CTFα) that does not generate Aβ peptides (Allinson, Parkin, Turner,

& Hooper, 2003). In the amyloidgenic pathway, APP is cleaved by β-secretase resulting in a soluble intracellular domain (sAPPβ) that is then further cleaved by γ-secretase to form an APP intracellular domain (AICD) and the amyloidgenic Aβ peptide, which then aggregates and fibrillates to form amyloid plaques within the brain (Buoso, Lanni, Schettini, Govoni, & Racchi, 2010; Zhang et al., 2012). Numerous studies now show that insulin signaling is capable of regulating key steps in the amyloid cascade and has a profound impact on the aggregation of Aβ within the brain (Pandini et al., 2013).

As previously discussed, GSK3 is key signaling molecule downstream of AKT, which can come in two forms: GSK3α and GSK3β (Takashima, 2006). GSK3α increases Aβ production by promoting the stimulation of γ-secretase cleavage of APP along the amyloidgenic pathway, increasing Aβ burden throughout the brain (Muyllaert et al., 2008). Normally Akt phosphorylation leads to the phosphorylation of GSK3α, holding it in a fairly inactive state; however, impaired insulin signaling reduces Akt phosphorylation leading to reduced phosphorylation of GSK3α, thus promoting its activation (Kim, Sullivan, Backus, & Feldman, 2011). It has also been shown that Akt can inhibit APP trafficking and the secretion of Aβ peptides, demonstrating that Akt signal is vital for both the amyloid and tau pathologies seen in AD (Shineman, Dain, Kim, & Lee, 2009).

Insulin increases the presence of insulin-degrading enzyme (IDE), which is the main enzyme responsible for insulin degradation (Hari, Shii, & Roth, 1987). IDE regulated downstream of insulin receptor and is upregulated through the activation of the PI3K-Akt pathway, serving in a classical negative feedback capacity (Zhao et al., 2004). IDE plays a major role in catabolic regulation by degrading a number of short peptides in addition to insulin,

including insulin-like growth factors I and II, amylin, as well as Aβ peptides (Qiu & Folstein, 2006). Additionally, under physiological conditions, microglia have been shown to secrete high levels of extracellular IDE that degrades the extracellular Aβ that has been secreted by neurons (Qiu et al., 1998). The ability of Aβ to degrade IDE has been shown to be lower in AD brains (Pérez, Morelli, Cresto, & Castaño, 2000). Additionally, hippocampal levels of IDE are significantly lower in AD patients compared to controls (Cook et al., 2003). Elevated levels of insulin induce Aβ accumulation due to the competition of increasing levels of Aβ for IDE, leading to a self-propagating cycle and, ultimately, once brain insulin levels have decreased because of insulin resistance at the blood-brain-barrier, IDE production is drastically reduced leading to further Aβ accumulation (Craft et al., 1998; Fishel et al., 2005).

The GK rat, an animal model of T2DM, expresses a partial loss-of-function IDE mutation that results in a 30% reduction in insulin degradation and increased Aβ accumulation (Farris et al., 2004). In mice with complete deletions of IDE, Aβ levels in the brain are dramatically increased, further suggesting a critical relationship between IDE activity and Aβ clearance (Farris et al., 2003; Miller et al., 2003). APP transgenic mice that were also bred to overexpress human IDE experienced a significant reduction in Aβ levels, an almost complete prevention of amyloid plaque deposition, and a rescue of premature death often associated with mice expressing *APP* mutations (Leissring et al., 2003).

Interestingly, soluble Aβ can bind to the insulin receptors in neurons located within the hippocampus and prevent autophosphorylation of the insulin receptor from occurring and thus blocking the downsttream activation of the PI3K/Akt pathway (Townsend, Mehta, & Selkoe, 2007). In addition to the loss of Akt signaling, Aβ and Aβ-derived diffusible ligands (ADDLs)

binding to insulin receptors results in a massive downregulation of surface insulin receptors resulting in increased loss of synaptic spines and inhibition of long-term potentiation, which could be mitigated by increasing the amount of available insulin (De Felice et al., 2009; X. Liu et al., 2014). PDK, a key regulatory protein in the PI3K-Akt pathway, is prevented from interacting with Akt by intracellular Aβ interference further inhibiting Akt activation (Frisardi et al., 2010).

Rats given unlimited access to fructose-containing water leads to insulin resistance and results in increased amyloidgenic pathway processing of APP through an increase in expression of β-secretase and stimulation of γ-secretase activity, as well as a decreased level of IDE, resulting in enhanced Aβ production and aggregation (Luo et al., 2011). APPswe/PS1dE9 mice fed a high-fat diet for 23 weeks displayed severe hyperinsulinemia, an increase in Aβ levels, and significantly elevated amyloid plaque deposition (Ramos-Rodriguez et al., 2014). Insulin depletion has been shown to increase hippocampal tau hyperphosphorylation of APP transgenic mice, which typically display excessive production of β-amyloid peptides and subsequent Aβ plaques, further demonstrating potentially linked pathways between the amyloid and tau pathologies (Jolivalt et al., 2010).

Deposition of β-amyloid in pancreatic islet cells and in the brain is a shared pathology between T2DM and AD (Beeler, Riederer, Waeber, & Abderrahmani, 2009). Amyloid in the pancreas is produced by pancreatic β-cells and co-released with insulin (S. E. Kahn, D'Alessio, Schwartz, & Fujimoto, 1990). The islet amyloid peptide is cleaved from islet amyloid polypeptide (IAPP), which shares a 90% structural similarity with APP in the brain (Cooper et al., 1987). Mice that overexpress IAPP develop Aβ plaques and tau tangles in β-cells leading to β-cell dysfunction and develop DM; the targeted disruption of IAPP improves glucose tolerance

and enhances insulin secretion (Gebre-Medhin et al., 1998; Janson et al., 1996). Patients with T2DM exhibit amyloid plaques and tau tangles in pancreatic islets, suggesting common pathophysiological mechanisms in AD and T2DM (Miklossy et al., 2010).

As discussed above, decreased levels of PP2A or increased inhibition of PP2A is typically associated with the tau-related pathology associated with AD by failing to dephosphorylate tau proteins at a healthy rate; however, evidence suggests that PP2A may also play a role in amyloid pathology. PP2A has been shown to dephosphorylate APP at Thr-668 and that PP2A can be inactivated using okadaic acid, which increases phosphorylation of APP resulting in increased production and secretion of both sAPPα and sAPPβ (Sontag et al., 2007). Increased APP phosphorylation at Thr-668 has been implicated in an increase in the amyloidgenic processing of APP and Aβ production (Ando, Iijima, Elliott, Kirino, & Suzuki, 2001). Further supporting the link between tau and amyloid pathologies, neurons cultured from Tau $^{-/-}$ mice are protected from Aβ induced neuronal death and cytotoxicity, suggesting tau is crucial for the neurotoxic effects of Aβ (Rapoport, Dawson, Binder, Vitek, & Ferreira, 2002). This observation is supported by post-mortem studies that show that amyloid deposition is diffuse and widespread and doesn't correlate well with cognitive decline, whereas tau pathology tends to be progressive in nature, following a distinct sequential pattern, and strongly correlates with the degree of dementia observed in clinical patients and in memory loss associated with AD (E. Braak et al., 1999; H. Braak & Braak, 1991; 1997).

Experimental Hypotheses and Implications

Insulin resistance is one of the core features of metabolic syndrome, which is a clustering of diseases that include T2DM and obesity. Until recently, the research related to insulin

resistance was targeted towards peripheral tissue such as muscle and adipose tissue; however, a more recent focus on insulin resistance within the nervous system suggests that the brain and the blood-brain-barrier is susceptible to the damaging effects of insulin resistance. Insulin resistance in T2DM has been correlated with an increase in risk for developing AD through the damaging effects of Aβ and tau-related toxicity on neurons. Recent evidence supports the notion that AD, or at least some cases of AD, might be a slow-progressing metabolic disease occurring within the CNS, and an increasing weight of evidence demonstrates this intricate link between insulin resistance and AD. Individuals with T2DM and obesity are at a much higher risk for the development of AD. Additionally, AD patients often develop insulin resistance and hyperglycemia. Insulin resistance, due to an impairment in insulin signaling, is a common pathway between T2DM and AD, representing a key link. Insulin plays a key role in Aβ and tau regulation. In turn, Aβ has profoundly negative effects on insulin signaling,

The purpose of this study is to examine the role that insulin disturbances play in the pathogenesis of AD. A considerable amount of data exists regarding the onset and progression AD pathology in transgenic models utilizing known mutations causing familiar AD; however, these only represent roughly 1-5% of the human population and don't accurately reflect the disease population. Inducing a diabetic-like state in an animal model that displays AD pathology is a valid, translational approach in examining mechanisms associated with sporadic AD.

This experiment is to examine the role of two different types of insulin perturbations on dementia-related pathology. To induce a diabetic-like state in Cohort 1, mice were injected with STZ at 45 mg/kg (intraperitoneal) using a staggered protocol similar to previous work in our laboratory (Murtishaw et al., 2018). One major difference in this project, compared to our

previous work, is to extend the timeline between STZ administration and behavioral testing. Our laboratory has previously tested typically subjects within 6 weeks of STZ injections; however, we wanted to see if an extended 6 months of extended hyperglycemia resulted in similar or worse deficits to a shorter timeline. In Cohort 2, mice were placed on a high-fat diet for 6 months to induce obesity and insulin resistance. Additionally, both treatments were administered in both C57BL/6J and CX3CR1$^{-/-}$ mice to explore the interaction of multiple risk factors associated with AD. As mentioned previously, neuroinflammation is a core feature of AD and serves to rapidly progress the disease pathology. CX3CR1 is the obligate receptor for the CX3CL1 chemokine and is found almost exclusively on microglia within the brain. CX3CR1$^{-/-}$ mice have a green-fluorescent protein inserted into the CX3CL1 binding site, leading to mice that are lacking a functional CX3CR1 and consequently experience elevated neuroinflammation. Please see Table 1 below for a complete list of experimental groups in each cohort.

Behavioral tests commenced 6 months following onset of treatment. The open field was performed to access anxiety-like behavior. To assess basic learning and memory, exploratory behavior was measured in the novel object recognition (NOR) task. The Barnes maze task was utilized to evaluate deficits in spatial learning and memory. To investigate protein changes related to AD pathology, Western blotting was conducted on hippocampal tissue. Specifically, proteins of interest focused on targets associated with tau pathology and insulin signaling. Tau pathology was primarily focused on because mice don't naturally form amyloid plaques and these mice lacked any inserted transgenes related to amyloid pathology. Microvascular hemorrhages associated with AD were analyzed via immunohistochemistry.

Hypothesis 1:

Administration of STZ will lead to behavioral and biochemical changes associated with AD following prolonged exposure to hyperglycemia. These STZ-induced alterations will be more pronounced in CX3CR1$^{gfp/gfp}$ animals.

Implications for Hypothesis 1: If administration of STZ leads to enhanced AD pathology in CX3CR1$^{gfp/gfp}$, then these data highlight the link between insulin perturbation and inflammation.

Hypothesis 2:

Obesity-induced insulin resistance will lead to behavioral and biochemical changes associated with AD. Insulin resistance will be more pronounced in animals with enhanced neuroinflammation (CX3CR1$^{gfp/gfp}$ mice).

Implications for Hypothesis 2: If obesity-related insulin alterations leads to AD-pathology, then these data would suggest that HFD could be a potential model to recapitulate sporadic AD in order to better represent our clinical population. Additionally, if obesity and inflammation interact to exacerbate deficits in behavior and changes related to AD, these data would suggest an additive effect between these multiple AD risk factors.

Table 1 Experimental Cohorts

Cohort 1 (STZ)		Cohort 2 (HFD)	
Males	Females	Males	Females
Controls (n=12)	Controls (n=12)	Controls (n=12)	Controls (n=12)
STZ (n=12)	STZ (n=12)	HFD (n=12)	HFD (n=12)
$CX3CR1^{-/-}$ (n=12)	$CX3CR1^{-/-}$ (n=12)	$CX3CR1^{-/-}$ (n=12)	$CX3CR1^{-/-}$ (n=12)
$CX3CR1^{-/-}$ + STZ (n=12)	$CX3CR1^{-/-}$ + STZ (n=12)	$CX3CR1^{-/-}$+ HFD (n=12)	$CX3CR1^{-/-}$+ HFD (n=12)

Figure 1 Cohort 1 Timeline

Figure 2 Cohort 2 Timeline

CHAPTER 3

MATERIALS AND METHODS

Subjects

The number of subjects needed was calculated using G Power (Faul, Erdfelder, Lang, & Buchner, 2007). With power set at 0.80 and $\alpha = 0.05$ (two-tailed), sample size was determined to be a minimum of n = 10 per treatment group for behavioral testing and n = 4 for tissue analysis, by using data previously collected in our laboratory (Murtishaw et al., 2018).). To provide sufficient power for this experiment and to account for the occasional loss of an animal, n = 12 per treatment group was utilized in each cohort.

Male and female C57BL/6J and CX3CR1$^{tm`Litt/J}$ mice (referred to as CX3CR1$^{-/-}$) were housed six per cage and were separated by sex, genotype, and treatment group. The colony room was set to a 12:12 light:dark cycle and all behavioral tests were conducted within the light cycle. Mice had access to chow and water *ad libitum*, with the exception of a six hour fasting period prior to blood glucose readings wherein food was removed. Animals still had access to water during the fasting period and food was returned immediately following blood glucose monitoring. All procedures performed in this experiment were approved by the University of Nevada, Las Vegas Institutional Animal Care and Use Committee and conducted in accordance with the NIH guidelines for the care and use of laboratory animals.

Treatment Groups

Animals in Cohort 1 were randomly assigned to vehicle or STZ groups (n = 12 per group), resulting in four treatment groups for both males and females: Control, STZ, CX3CR1$^{-/-}$, and CX3CR1$^{-/-}$+STZ. Streptozotocin (Sigma-Aldrich) was prepared fresh prior to administration

by dissolving the streptozotocin in 0.1 M sodium citrate buffer (pH 4.5) for a final concentration of 4.5 mg/mL. Following a 6-hour fast, animals were injected with STZ via intraperitoneal injection at a volume of 0.1 mL/10 g to achieve a final concentration of 45 mg/kg. Because STZ is pharmacologically active for only 15 minutes (Schein, Kahn, Gorden, Wells, & Devita, 1973), all injections were administered within 10 minutes of mixing before preparing a fresh batch. Control mice were administered vehicle (citrate buffer) via intraperitoneal injection at a volume of 0.1 mL/10 g. Based upon previous work in our laboratory, animals were injected on days 1, 2, 3, 14, 15, 16 (Murtishaw et al., 2018). Streptozotocin is known to cause an initial state of potentially lethal hypoglycemia due to destroyed pancreatic β-cells releasing large amounts of insulin (Szkudelski, 2001). To counteract this hypoglycemia, all animals were given 5% Ensure® mixed in their water for 24 hours.

Animals in Cohort 2 were randomly assigned to normal chow or high-fat chow groups (n = 12 per group), resulting in four treatment groups for both males and females: Control, HFD, $CX3CR1^{-/-}$, and $CX3CR1^{-/-}$+HFD. A standard high-fat chow with 60 kcal% fat (D12452, Research Diets) and corresponding control chow with 10 kcal% fat (D12451J, Research Diets) were utilized in this experiment. Experimental chow was maintained throughout the entire duration of the experiment.

Animals were 3 months old at the beginning of treatment (STZ injections or HFD chow). Blood glucose levels were monitored throughout the experiment to confirm hyperglycemia in animals by collecting lateral tail vein blood after a six hour fast. The withdrawal site was cleaned with alcohol while gently restraining the animal, followed by a nick to the lateral tail vein with a sterile blade to obtain a small drop of blood. Blood glucose levels were read using an

AlphaTrak® Blood Glucose Monitoring System. Baseline measurements of blood glucose and weights were collected one day prior to the beginning of treatment and then obtained once a month for the duration of the experiment, which lasted for a total of 6 months.

Behavioral Testing

Open Field

To assess mobility and any potential anxious behavior, the open field task was utilized to monitor behavior. A Plexiglass chamber (37 cm L x 37 cm W x 37 cm H) with white interior was used. Animals were removed from the colony room, brought to the dedicated testing room, and placed in the open field chamber. Subjects were allowed to freely explore for 5 minutes while their activity was recorded via the tracking system. Animals were removed from the arena at the end of the trial and placed back in their home cage located in in the colony room. Chambers were thoroughly cleaned with 10% ethanol between each session. Data collected included total distance travelled and time spent in perimeter of the arena.

Novel Object Recognition

Novel object recognition was used to investigate memory capitalizing on a rodent's innate exploratory behavior and preference for novelty items (Antunes & Biala, 2011). The novel object recognition was performed in the same chamber used for open field and began twenty-four hours following the open field task. On the first day of testing, animals received a randomized pair of identical objects that were placed in opposing corners of the arena. Potential objects included: yellow cylinders, red circles, or black squares. Each object was similar in size and had been previously tested to ensure similar saliency. During the five-minute trial, animals were allowed to freely explore while the tracking system measured amount of time investigating

each object (calculated as the animal's nose being less than 4 centimeters from the object) and speed. Following the trial completion, animals were placed back in their home cage in the colony room. The chamber and object were cleaned with 10% ethanol between each trial.

The second day of Novel Object Recognition was performed twenty-four hours later. Animals were placed back in the chamber with one of the original objects from Day 1 testing along with a new object (novel object). During the 5-minute trial, the tracking system measured the amount of time spent with both the familiar and novel objects as well as speed. Animals were returned back to their home cage following the completion of the trial. Chambers and objects were cleaned with 10% ethanol solution between every session to remove any olfactory bias.

Barnes Maze

The Barnes maze was utilized to assess cognitive deficits in spatial learning and memory. The maze, made of white, circular plastic (92 cm diameter) with a black false bottom, was purchased from Maze Engineers (Glenview, IL). Twenty holes (5 cm diameter) were spaced evenly along the perimeter of the maze with a small black plastic escape chamber and a small black plastic ramp were placed under the target hole. A black curtain with was placed two foot away from the edge of the maze and contained seven distinct visuo-spatial cues utilizing different colors, shapes, and patterns. Bright lights placed around the maze and a loud tone (86 dB, 4 kHz) were used to motivate the animals to find the escape hole.

Animals were randomly assigned to one of three possible different escape locations to avoid any possible spatial bias. The Barnes maze protocol consisted of three distinct phases: habituation, training, and probe. A single habituation trial consisted of the animal placed under a large glass container overlapping the escape chamber and nearby area for three minutes and

allowed to freely enter the hole. Animals were gently guided into the hole after three minutes had elapsed and given two minutes in the dark escape chamber with the tone turned off. Training phase to assess learning began twenty-four hours later and lasted for four days. During each day of the training days, animals received four individual trials separated by fifteen minutes. Each trial the animal was placed in the center of the maze under a black container for 15 seconds. After the container was removed, subjects were allowed to freely explore the maze until entering the escape chamber or until three minutes had elapsed, upon which animals were gently lead to the target hole. Animals were rewarded with one-minute in the dark escape chamber without the tone and then placed back in home cage until next trial. The probe trial was conducted twenty-four hours after the final training day to assess memory. During the probe trial, the escape chamber was removed and animals were allowed to freely explore the maze for a total of three minutes. Throughout the experiment, trials were recorded by an overhead tracking system (Ethovision), which tracked latency, speed, and distance. Errors were manually scored by researchers blind to treatment group. The maze was thoroughly cleaned with 70% ethanol between every trial to remove any potential scent cues.

Tissue Examination

Tissue Collection

Animals were humanely euthanized via carbon dioxide asphyxiation. Immediately following carbon dioxide asphyxiation, subjects had blood drawn via cardiac puncture to be used in an insulin ELISA. Following the blood collection, brains were rapidly removed, the hippocampus dissected out, until finally the tissue was rapidly frozen using liquid nitrogen. Tissue was kept at -80˚ Celsius until further processing.

ELISA

Blood was collected in capillary microvettes (Kent Scientific) during euthanization, immediately centrifuged (4500 x g) at 4˚ Celsius for 10 minutes, and plasma was then carefully removed. Plasma insulin levels were determined by using an Insulin ELISA (Alpco), following manufacturer instructions. Briefly, standards, controls, and samples were added to the microplate, along with the included working strength conjugate, and incubated for 2 hours at room temperature. Wells were then washed 6 times, TMB Substrate added, and incubated for 15 minutes at room temperature. Following the final incubation, stop solution was added and the plate was read at 450 nm.

SDS-PAGE Western Blotting

Western blotting was performed to examine protein level expression related to tau pathology and insulin signaling within the hippocampus. Hippocampal tissue was homogenized using the Bio-Plex® Cell Lysis Kit (Bio-Rad) and a number of techniques to rupture cells including an initial homogenization with a POLYTRON ® homogenizer (Kinematica), followed by a 24-hour freeze-thaw cycle at -80˚ Celsius, and finally by sonication (Sonifer SFX, VWR). Following the final sonication step, supernatant was collected following centrifugation at 4500 x g for 15 minutes. Utilizing a Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific), protein concentrations for each sample was calculated. 20 μg of each sample were loaded onto a 10% SDS-PAGE gel to separate proteins and then transferred onto a PVDF membrane (Immunobilon-FL, 0.45 micron; Millipore). Membranes were blocked using Odyssey Blocking Buffer (LI-COR) and then probed with primary antibodies overnight at 4˚ Celsius (AKT, 1:1000, Abcam; β-Actin, 1:20,000, ProteinTech; Insulin degrading enzyme, 1:1,000, Abcam; GSK3β, 1:1,000, Cell

Signaling; pAKT, 1:1000, Abcam; pGSK3β, 1:1,000, Cell Signaling; phospho-insulin receptor, 1:1000, Abcam; phospho-Tau Serine202, 1:1,000, Santa Cruz; phospho-Tau Threonine231, 1:1000, Abcam; phospho-Tau Serine396, 1:1,000, Santa Cruz; phospho-Tau Serine404, 1:1000, Santa Cruz; pan-Tau, 1:10,000, Abcam).

The following day, membranes were probed with a fluorescence-based secondary antibodies based upon host species of primary antibody (LI-COR). Following secondary antibody incubation and washes, each membrane was imaged using the Odyssey® Infrared Imaging System (LI-COR) . All obtained images were analyzed utilizing Image Studio Software® (LI-COR). Each protein of interest was normalized to an appropriate housekeeping protein: β-actin, AKT, or GSK3β.

Statistical Analyses

Differences in blood glucose, body weights, and Barnes maze hidden training (latency, errors, speed) were analyzed by repeated measures analysis of variance (ANOVA) with group as the factor. Open field data (total distance travelled and perimeter time), novel object recognition day 1 data (time spent investigating objects and speed), novel object recognition day 2 speed, Barnes maze probe data (number of nose pokes into holes, speed), and tissue analyses (ELISA, Western blot) were analyzed by one-way between subjects ANOVA with group as the factor.

For NOR Day 2, a performance index for novelty preference over the familiar object and was compared against chance (50%) using a Student's *t*-test for each group. Similarly, time spent in target quadrant during the Barnes maze probe trial was compared against chance (25%) using a Student's *t*-test for each group.

Following a significant ANOVA, a Tukey post-hoc comparison of groups was performed to determine points of significance. Within each cohort, males and females were analyzed separately.

CHAPTER 4

RESULTS

Metabolic Measures

Body Weight

Body weights were monitored throughout the course of the experiment to observe any changes due to drug treatment or diet.

In Cohort 1, baseline weights established prior to administration of STZ did not differ between groups ($F_{3,44} = 0.418$, $p = 0.741$; Figure 3a). Across the experiment, statistical analysis revealed a significant ANOVA for Cohort 1 males ($F_{3,44} = 4.657$, $p = 0.007$); however, post-hoc analysis did not reveal any significant differences against controls (Figure 3a). The significant difference was driven by the weights of CX3CR1$^{-/-}$ animals being significantly greater than STZ ($p = 0.005$) and CX3CR1$^{-/-}$ + STZ ($p = 0.04$).

In Cohort 1 females, there was no difference in baseline weights ($F_{3,44} = 0.928$, $p = 0.435$; Figure 3a). Across the experiment, CX3CR1$^{-/-}$ females demonstrated significantly higher weights ($F_{3,44} = 6.841$, $p = 0.001$; Tukey post-hoc: controls vs CX3CR1$^{-/-}$ $p = 0.026$; figure 3a).

In Cohort 2, there was no difference in baseline weights for males prior to beginning high-fat chow ($F_{3,44} = 2.703$, $p = 0.057$; Figure 3b). Across the course of the experiment, both high-fat diet groups exhibited significantly greater weights compared to controls ($F_{3,44} = 110.823$, $p < 0.001$; Tukey post-hoc: controls vs HFD $p < 0.001$, controls vs CX3CR1$^{-/-}$+HFD $p < 0.001$; Figure 3b). Additionally, Tukey post-hoc revealed that CX3CR1$^{-/-}$+HFD did not gain as much weight compared to HFD-alone throughout the experiment ($p = 0.048$).

Weights in Cohort 2 females did not differ at baseline ($F_{3,44} = 0.409$, $p = 0.747$; Figure 2b) but were significantly different across the experiment ($F_{3,44} = 58.474$, $p < 0.001$; Figure 2b) with both HFD groups weighing significantly more than controls (Tukey post-hoc: controls vs HFD $p < 0.001$, controls vs CX3CR1$^{-/-}$+HFD $p < 0.001$).

Blood Glucose Levels

Blood glucose levels were monitored prior to the start of STZ injections or before beginning HFD at three months of age and then monitored monthly over the course of the experiment.

In Cohort 1 males, no differences were observed in baseline blood glucose levels ($F_{3,44} = 0.418$, $p = 0.741$; Figure 4a). Following STZ injections, a significant difference arose in blood glucose across the remaining experiment with both STZ-treated groups exhibiting elevated blood glucose ($F_{3,44} = 82.274$, $p < 0.001$; Tukey post-hoc: controls vs STZ $p < 0.001$, controls vs CX3CR1$^{-/-}$+STZ $p < 0.001$; Figure 4a). Additionally, blood glucose in CX3CR1$^{-/-}$+STZ males was significantly greater compared to STZ-alone animals (Tukey post-hoc: $p < 0.001$).

In Cohort 1 females, there was no significant difference in baseline weights ($F_{3,44} = 0.928$, $p = 0.435$; Figure 4a). Though noticeably lower than males following injections, blood glucose in STZ and CX3CR1$^{-/-}$+STZ females were still significantly elevated across the experiment ($F_{3,44} = 50.061$, $p < 0.001$; Tukey post-hoc: controls vs STZ $p < 0.001$, controls vs CX3CR1$^{-/-}$+STZ $p < 0.001$; Figure 4a).

In Cohort 2 males, there were no differences observed in baseline blood glucose levels ($F_{3,44} = 0.791$, $p = 0.506$; Figure 4b). Following the commencement of high-fat chow, blood glucose levels were significantly elevated in all treatment groups ($F_{3,44} = 75.158$, $p < 0.001$;

Tukey post-hoc: controls vs HFD $p < 0.001$, controls vs CX3CR1$^{-/-}$ $p = 0.002$, controls vs CX3CR1$^{-/-}$+HFD $p < 0.001$; Figure 4b).

Cohort 2 females did not differ in blood glucose at baseline ($F_{3,44} = 1.44$, $p = 0.245$, Figure 4b); however, blood glucose levels were significantly elevated in all treatment groups across the experiment ($F_{3,44} = 64.168$, $p < 0.001$; Tukey post-hoc: controls vs HFD $p < 0.001$, controls vs CX3CR1$^{-/-}$ $p = 0.044$, controls vs CX3CR1$^{-/-}$+HFD $p < 0.001$; Figure 4b).

Plasma Insulin Levels

To further evaluate the diabetic status of treatment groups, plasma insulin levels were measured at the end of the experiment.

In Cohort 1 males, insulin levels were significantly reduced in both STZ-treated groups ($F_{3,16} = 4.727$, $p = 0.015$; Tukey post-hoc: controls vs STZ $p = 0.042$, controls vs CX3CR1$^{-/-}$ +STZ $p = 0.035$; Figure 5a). No differences were observed in the insulin levels in Cohort 1 females ($F_{3,16} = 1.875$; $p = 0.174$; Figure 5a).

In Cohort 2 males, high-fat chow led to significantly greater plasma insulin levels ($F_{3,16} = 11.252$, $p < 0.001$: Tukey post-hoc: controls vs HFD $p = 0.028$, controls vs CX3CR1$^{-/-}$+HFD $p = 0.001$; Figure 5b). Similarly in Cohort 2 females, high-fat chow resulted in a large increase in plasma insulin ($F_{3,16} = 13.252$, $p < 0.001$: Tukey post-hoc: controls vs HFD $p = 0.009$, controls vs CX3CR1$^{-/-}$+HFD $p < 0.001$; Figure 5b).

Behavioral Testing

Open Field

The open field task was performed to monitor potential differences in exploratory behavior and anxiety-like phenotypes by measuring total distance travelled throughout the arena and the proportion time spent in the perimeter of the arena.

Cohort 1 males did not demonstrate any differences in open field on total distance travelled ($F_{3,44} = 0.22$, $p = 0.882$; Figure 6a) or proportion of time spent in the perimeter of the arena ($F_{3,44} = 0.22$, $p = 0.292$; Figure 6c).

No differences were observed in Cohort 1 females on total distance travelled ($F_{3,44} = 1.102$, $p = 0.359$; Figure 6a) or in the proportion time spent in the perimeter of the maze ($F_{3,44} = 1.922$, $p = 0.14$; Figure 6c).

Cohort 2 males travelled equivalently throughout the open field arena ($F_{3,44} = 1.117$, $p = 0.352$; Figure 6b) and did not differ in proportion time spent in perimeter ($F_{3,44} = 0.803$, $p = 0.499$; Figure 6d).

Cohort 2 females did not show differences in distance travelled ($F_{3,44} = 0.849$, $p = 0.475$; Figure 6b) or proportion time spent in the border of the arena ($F_{3,44} = 0.571$, $p = 0.637$; Figure 6d).

Novel Object Recognition

The novel object recognition task was performed as a test of basic learning and memory. Animals are innately drawn to novelty items in their environment and difficulties identifying objects as new or familiar is a common deficit in various forms of dementia. This task takes advantage of a rodent's innate preference for novelty items and is considered a gold-standard test in Alzheimer's disease research.

On NOR Day 1, animals were presented with identical objects. Males in Cohort 1 spent equivalent times investigating the two identical objects ($F_{3,44} = 0.624$, $p = 0.604$; Figure 7a) and moved around the arena at a comparable speed ($F_{3,44} = 0.21$; $p = 0.889$; Figure 7c).

No differences were observed in Cohort 1 females on time spent investigating the two objects ($F_{3,44} = 0.75$, $p = 0.529$; Figure 7a) or on speed ($F_{3,44} = 1.185$, $p = 0.326$; Figure 7c).

Cohort 2 males did not exhibit any significant differences on NOR Day 1 in the time spent investigating objects ($F_{3,44} = 0.177$, $p = 0.912$; Figure 7b) or in the speed at which they moved around the chamber ($F_{3,44} = 1.176$, $p = 0.33$; Figure 7d). No differences were observed in Cohort 2 females on time spent investigating objects ($F_{3,44} = 0.976$, $p = 0.412$; Figure 7b) or speed ($F_{3,44} = 1.902$, $p = 0.143$; Figure 7d).

On NOR Day 2, animals were presented with an object that they had previously encountered on Day 1 and a novel object presented during Day 2 for the first time. A performance index was created to demonstrate the novelty preference and compared against chance, which was set at 0.5 as chance predicts that animals will spend equivalent times with both objects.

In Cohort 1 males, controls ($t_{11} = 3.691$, $p = 0.004$; Figure 8a) and CX3CR1$^{-/-}$ ($t_{11} = 2.366$, $p = 0.037$; Figure 8a) spent significantly more time with the novel object, whereas STZ and CX3CR1$^{-/-}$+STZ groups failed to demonstrate a novelty preference ($t_{11} = 1.275$, $p = 0.229$ and $t_{11} = -.356$, $p = 0.728$, respectively; Figure 8a). Cohort 1 males moved at equivalent speed throughout the task ($F_{3,44} = 0.231$, $p = 0.874$; Figure 8c).

Females in Cohort 1 mirrored the Cohort males with controls and CX3CR1$^{-/-}$ demonstrating a significant novelty preferences ($t_{11} = 3.529$, $p = 0.005$ and $t_{11} = 3.322$, $p = 0.007$,

respectively; Figure 8a) and the STZ and CX3CR1$^{-/-}$+STZ groups failing to demonstrate a novelty preference (t_{11} = 0.625, p = 0.546 and t_{11} = -.69, p = 0.504, respectively; Figure 8a). No differences were observed in speed in Cohort 1 females ($F_{3,44}$ = 1.628, p = 0.197; Figure 8c).

In Cohort 2 males, all groups with the exception of CX3CR1$^{-/-}$+HFD demonstrated novelty preferences (controls t_{11} = 4.393, p = 0.001, HFD t_{11} = 0.847, p = 0.416, CX3CR1$^{-/-}$ t_{11} = 2.748, p = 0.019, and CX3CR1$^{-/-}$+HFD t_{11} = 2.327, p = 0.04; Figure 8b). No differences were observed in Cohort 2 males on speed ($F_{3,44}$ = 0.347, p = 0.791; Figure 8d).

In Cohort 2 females, controls and CX3CR1$^{-/-}$ groups demonstrated a novelty preference (t_{11} = 3.583, p = 0.005 and t_{11} = 2.496, p = 0.032, respectively; Figure 8b). HFD and CX3CR1$^{-/-}$ +HFD females failed to demonstrate a novelty preference (t_{11} = 1.382, p = 0.195 and t_{11} = 0.557, p = 0.589, respectively; Figure 8b). No differences were observed in speed ($F_{3,44}$ = 0.635, p = 0.597; Figure 8d) in Cohort 2 females.

Barnes Maze, Hidden Training

The Barnes maze was performed to measure any potential differences in spatial learning and learning. This task utilized bright lights and a loud tone, which are both aversive to rodents, to encourage the animal to seek out the hidden dark chamber placed under one of the holes. During the four training days, the latency to find the escape hole was measured, along with errors and the speed at which the subject moved across the maze.

In Cohort 1 males, the STZ and CX3CR1$^{-/-}$+STZ groups took significantly more time to locate the escape chamber ($F_{3,188}$ = 7.153, p < 0.001; Tukey post-hoc: controls vs STZ p = 0.012, controls vs CX3CR1$^{-/-}$+STZ p < 0.001; Figure 9a). These same male STZ and CX3CR1$^{-/-}$+STZ groups also committed significantly more errors than the other groups in Cohort 1 ($F_{3,188}$ = 7.653,

$p < 0.001$; Tukey post-hoc: controls vs STZ $p = 0.011$, controls vs CX3CR1$^{-/-}$+STZ $p < 0.001$; Figure 9c). No differences were observed in speed ($F_{3,188} = 1.963$, $p = 0.121$; Figure 9e).

In Cohort 1 females, statistical analysis revealed an overall significant difference in latency to find the escape chamber ($F_{3,188} = 2.894$, $p = 0.037$; Figure 9a); however, no groups significantly differed from the control group with CX3CR1$^{-/-}$+STZ failing to reach significance in the post-hoc analysis ($p = 0.053$). Female mice in both the STZ and CX3CR1$^{-/-}$+STZ groups made significantly more errors across the experiment ($F_{3,188} = 5.648$, $p < 0.001$; Tukey post-hoc: controls vs STZ $p = 0.005$, controls vs CX3CR1$^{-/-}$+STZ $p = 0.017$; Figure 9c). No differences were observed in speed ($F_{3,188} = 1.723$, $p = 0.164$; Figure 9e).

In Cohort 2 males, significantly longer latencies to find the escape chamber arose for the HFD alone group ($F_{3,188} = 6.981$, $p < 0.001$; Tukey post-hoc: controls vs HFD $p = 0.005$; Figure 9b). Only the HFD alone group displayed significant differences in the amount of errors committed during training days ($F_{3,188} = 9.212$, $p < 0.001$; Tukey post-hoc: controls vs HFD $p < 0.001$; Figure 9d). No significant differences were observed in speed ($F_{3,188} = 2.083$, $p = 0.104$; Figure 9f).

In Cohort 2 females, only CX3CR1$^{-/-}$+HFD displayed significantly longer latencies ($F_{3,188} = 7.43$, $p < 0.001$; Tukey post-hoc: controls vs CX3CR1$^{-/-}$HFD $p < 0.001$; Figure 9b). Similarly, only CX3CR1$^{-/-}$+HFD females committed significantly more errors compared to controls ($F_{3,188} = 6.881$, $p < 0.001$; Tukey post-hoc: controls vs CX3CR1$^{-/-}$+HFD $p < 0.001$; Figure 9d). No differences were observed in speed ($F_{3,188} = 0.626$, $p = 0.599$; Figure 9f).

Barnes Maze Probe Trial

During the Barnes maze probe trial, the hidden escape chamber was removed twenty-four hours after the final hidden training day. The amount of time that the animal spent in the target area and the number of nose pokes into the escape hole was measured.

In Cohort 1 males, control and CX3CR1$^{-/-}$ mice displayed selective searches for the escape chamber by spending significantly more time in the target quadrant than chance predicts (controls $t_{11} = 5.598$, $p < 0.001$; CX3CR1$^{-/-}$ $t_{11} = 3.425$, $p = 0.006$; Figure 10a). Both STZ-treated groups failed to demonstrate a significant selective search (STZ $t_{11} = 2.13$, $p = 0.416$; CX3CR1$^{-/-}$ +STZ $t_{11} = 1.974$, $p = 0.074$; Figure 10a). No significant differences were observed in Cohort 1 males in the number of nose pokes into the escape hole ($F_{3,44} = 2.268$; $p = 0.094$; Figure 10c). No differences were observed in speed ($F_{3,44} = 0.557$; $p = 0.646$; Figure 10e).

In Cohort 1 females, all groups demonstrated a selective search by spending significantly more time in the target quadrant (controls $t_{11} = 4.382$, $p = 0.001$; STZ $t_{11} = 3.469$, $p = 0.005$; CX3CR1$^{-/-}$ $t_{11} = 5.968$, $p < 0.001$; CX3CR1$^{-/-}$+STZ $t_{11} = 5.598$, $p < 0.001$; Figure 10a). No significant differences were observed in number of target hole pokes ($F_{3,44} = 1.357$, $p = 0.268$; Figure 10c) or in speed ($F_{3,44} = 1.201$; $p = 0.32$; Figure 10e).

In Cohort 2 males, all groups demonstrated selective searches for the target quadrant (controls $t_{11} = 9.413$, $p < 0.001$; HFD $t_{11} = 2.4897$, $p = 0.015$; CX3CR1$^{-/-}$ $t_{11} = 3.852$, $p = 0.003$; CX3CR1$^{-/-}$+HFD $t_{11} = 3.174$, $p = 0.009$; Figure 10b). Only the HFD alone group demonstrated significantly fewer nose pokes into the target hole ($F_{3,44} = 3.104$, $p = 0.036$; Tukey post-hoc: controls vs HFD $p = 0.035$; Figure 10d). No difference was observed in speed ($F_{3,44} = 0.453$, $p = 0.715$; Figure 10f).

In Cohort 2 females, only CX3CR1$^{-/-}$+HFD failed to display a selective search (controls $t_{11} = 5.57$, $p < 0.001$; HFD $t_{11} = 4.135$, $p = 0.002$; CX3CR1$^{-/-}$ $t_{11} = 4.26$, $p = 0.001$; CX3CR1$^{-/-}$ +HFD $t_{11} = 2.161$, $p = 0.054$; Figure 10b). Similarly, only the CX3CR1$^{-/-}$+HFD group had significantly fewer nose pokes into the target hole ($F_{3,44} = 9.851$, $p < 0.001$; Tukey post-hoc: controls vs CX3CR1$^{-/-}$+HFD $p = 0.035$; Figure 10d). No difference was observed in speed ($F_{3,44} = 1.28$, $p = 0.293$; Figure 10f).

Tissue Analysis

Western Blotting: Phosphorylated and Total Tau

Western blotting was conducted to examine differences in protein expression between treatment groups in two broad categories: the first being phosphorylated tau and the second being proteins associated with insulin signaling. Four different epitopes of phosphorylated tau (serine 396, serine 404, serine 202, and threonine 231) are commonly found to be hyperphosphorylated in patients with Alzheimer's disease, and to a lesser degree, in patients with T2DM. Total tau levels were also analyzed to see if there were any gross abnormalities to pan-tau, which could indicate excessive cellular death.

In Cohort 1 males, both groups treated with STZ (STZ alone and CX3CR1$^{-/-}$+STZ) had significant elevations in tau phosphorylated at site serine 396 ($F_{3,28} = 5.148$, $p = 0.006$; Tukey post-hoc: controls vs STZ $p = 0.008$, controls vs CX3CR1$^{-/-}$+STZ $p = 0.036$; Figure 11a). Only STZ males displayed significant elevations in tau phosphorylated at site serine 404 ($F_{3,28} = 3.17$, $p = 0.029$; Tukey post-hoc: controls vs STZ $p = 0.029$; Figure 11c). No differences were observed in phosphorylated tau at site serine 202 ($F_{3,28} = 2.921$, $p = 0.054$; Figure 12a),

phosphorylated tau at site threonine 231 ($F_{3,28}$ = 1.67, p = 0.199; Figure 12c), and no differences were detected in total tau ($F_{3,28}$ = 1.073, p = 0.377; Figure 12e).

In Cohort 1 females, no signification differences were observed in tau phosphorylated at sites serine 396 ($F_{3,28}$ = 1.994, p = 0.138; Figure 11a), serine 404 ($F_{3,28}$ = 0.711, p = 0.553; Figure 11c), serine 202 ($F_{3,28}$ = 0.536, p = 0.662; Figure 12a), serine 202 ($F_{3,28}$ = 0.306, p = 0.821; Figure 12c), or in total tau ($F_{3,28}$ = 0.558, p = 0.647; Figure 12e).

In Cohort 2 males, there was a significant increase in phosphorylated tau at site serine 396 in th:e HFD group ($F_{3,28}$ = 3.056, p = 0.045; Tukey post-hoc: controls vs HFD p = 0.048; Figure 11b). Tau phosphorylated at site serine 404 ($F_{3,28}$ = 0.251, p = 0.86; Figure 11d), site serine 202 ($F_{3,28}$ = 1.124, p = 0.356; Figure 12b), and site threonine 231 ($F_{3,28}$ = 0.887, p = 0.46; Figure 12d) remained unchanged. No differences were observed in levels of total tau ($F_{3,28}$ = 0.468, p = 0.707; Figure 12f),

Cohort 2 females did not display any significant differences in any phosphorylated tau epitopes: serine 396 ($F_{3,28}$ = 0.652, p = 0.588; Figure 11b), serine 404 ($F_{3,28}$ = 1.106, p = 0.363; Figure 11d), serine 202 ($F_{3,28}$ = 0778, p = 0.523; Figure 12b), threonine 231 ($F_{3,28}$ = 1.861, p = 0.159; Figure 12d) or in total tau ($F_{3,28}$ = 0.135, p = 0.938; Figure 12f).

Western Blotting: Insulin Signaling-related Proteins

Several proteins associated with insulin signaling were evaluated to determine if any components within this pathway were altered and could serve as a potential pathway for increased tau phosphorylation. Analyzed proteins included: insulin degrading enzyme, pAKT, pGSK3β, and cdk5.

In Cohort 1 males, a significant decrease in levels of insulin degrading enzyme were observed in both the CX3CR1$^{-/-}$ and CX3CR1$^{-/-}$+STZ groups ($F_{3,28}$ = 5.623, p = 0.004; Tukey post-hoc: controls vs CX3CR1$^{-/-}$ p = 0.004, controls vs CX3CR1$^{-/-}$+STZ p = 0.014; Figure 13a). No significant differences were observed in levels of pAKT ($F_{3,28}$ = 1.867, p = 0.159; Figure 13c). Levels of pGSK3β remained unchanged between the groups ($F_{3,28}$ = 0.28, p = 0.839; Figure 14a). No differences were detected in cdk5 ($F_{3,28}$ = 1.81, p = 0.169; Figure 14c).

In Cohort 1 females, there was a significant decrease in insulin degrading enzyme both the CX3CR1$^{-/-}$ and CX3CR1$^{-/-}$+STZ groups ($F_{3,28}$ = 8.796, p < 0.001; Tukey post-hoc: controls vs CX3CR1$^{-/-}$ p < 0.001, controls vs CX3CR1$^{-/-}$+STZ p = 0.015; Figure 13a). No differences were observed in levels of pAKT ($F_{3,28}$ = 1.933, p = 0.147; Figure 13c), pGSK3β ($F_{3,28}$ = 1.151, p = 0.346; Figure 14a), or cdk5 ($F_{3,28}$ = 0.443, p = 0.724; Figure 14c).

In Cohort 2 males, a significant decrease in insulin degrading enzyme was observed in both the CX3CR1$^{-/-}$ and CX3CR1$^{-/-}$+HFD groups ($F_{3,28}$ = 21.099, p < 0.001; Tukey post-hoc: controls vs CX3CR1$^{-/-}$ p < 0.001, controls vs CX3CR1$^{-/-}$+HFD p < 0.001; Figure 13b). Statistical analysis did not reveal any significant differences in levels of pAKT ($F_{3,28}$ = 0.442, p = 0.725; Figure 13d). A reduction in pGSK3β was observed in the HFD and CX3CR1$^{-/-}$+HFD groups ($F_{3,28}$ = 5.388, p = 0.005; Tukey post-hoc: controls vs HFD p = 0.004, controls vs CX3CR1$^{-/-}$ +HFD p = 0.039; Figure 14b). No differences were observed in cdk5 levels ($F_{3,28}$ = 1.45, p = 0.249; Figure 14d).

In Cohort 2 females, insulin degrading enzyme was found to be significantly reduced in both the CX3CR1$^{-/-}$ and CX3CR1$^{-/-}$+HFD groups ($F_{3,28}$ = 41.0741, p < 0.001; Tukey post-hoc: controls vs CX3CR1$^{-/-}$ p < 0.001, controls vs CX3CR1$^{-/-}$+HFD p < 0.001; Figure 13b). No

differences were observed in pAKT ($F_{3,28}$ = 0.791, p = 0.509; Figure 13d), pGSK3β ($F_{3,28}$ = 1.981, p = 0.14; Figure 14b), or in cdk5 ($F_{3,28}$ = 1.861, p = 0.159; Figure 14d).

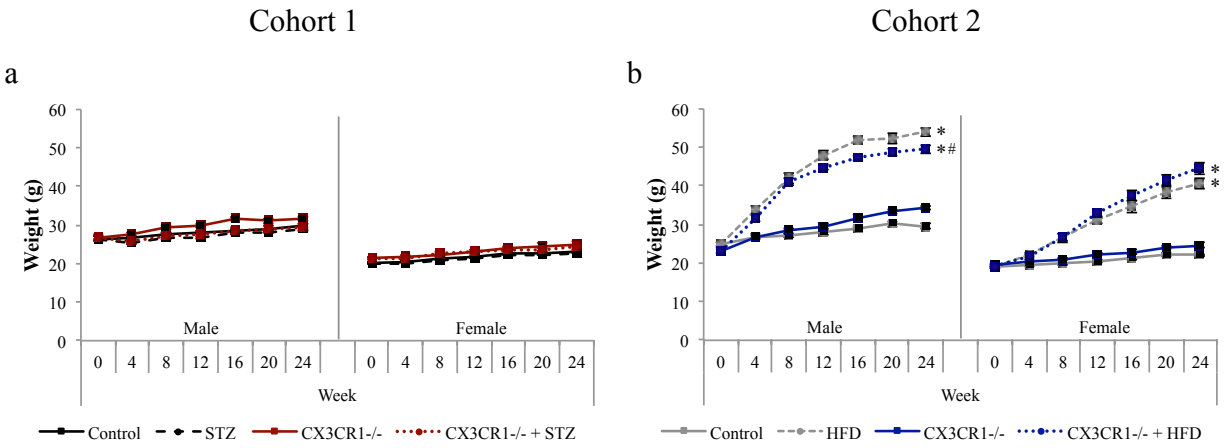

Figure 3 Weights. **a)** STZ did not result in significant differences in body weight (±SEM) in Cohort 1 males or females. **b)** HFD significantly increased body weights (±SEM) in both males and females across the experiment. * = $p < 0.05$ against controls; # = $p < 0.05$ HFD versus CX3CR1$^{-/-}$+HFD

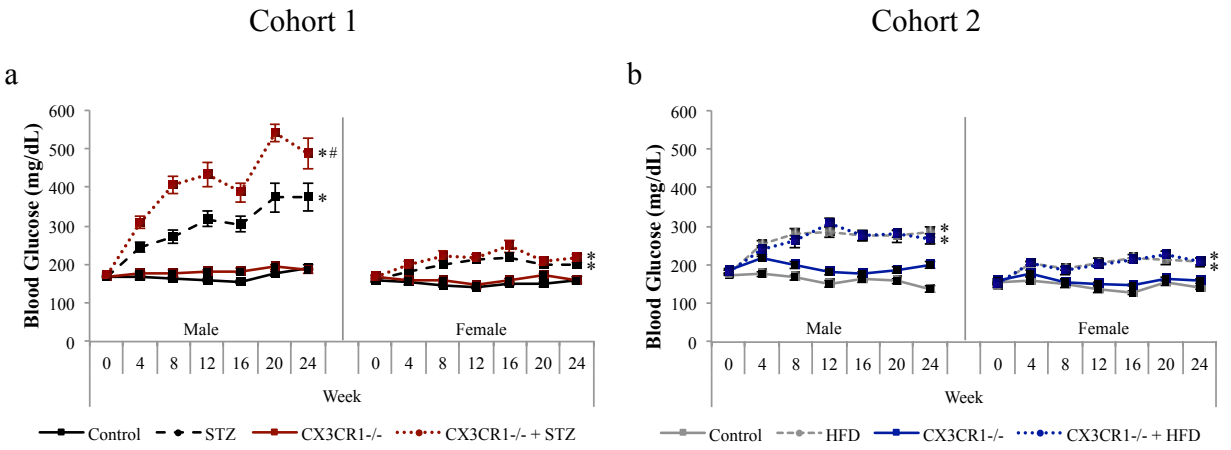

Figure 4 Blood glucose. **a)** STZ resulted in significantly increased blood glucose levels (±SEM) in both males and females. **b)** HFD significantly increased blood glucose (±SEM) in both males and females across the experiment. * = $p < 0.05$ against controls; # = $p < 0.05$ STZ versus CX3CR1$^{-/-}$+STZ

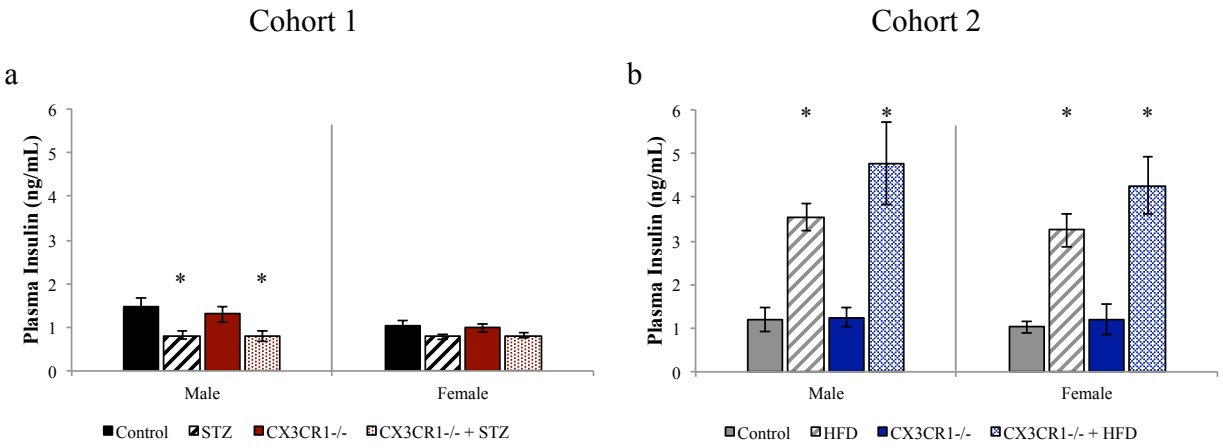

Figure 5 Plasma insulin. **a)** STZ resulted in significantly decreased plasma insulin (±SEM) in males. **b)** HFD significantly increased plasma insulin (±SEM) in both males and females. * = $p < 0.05$ against controls.

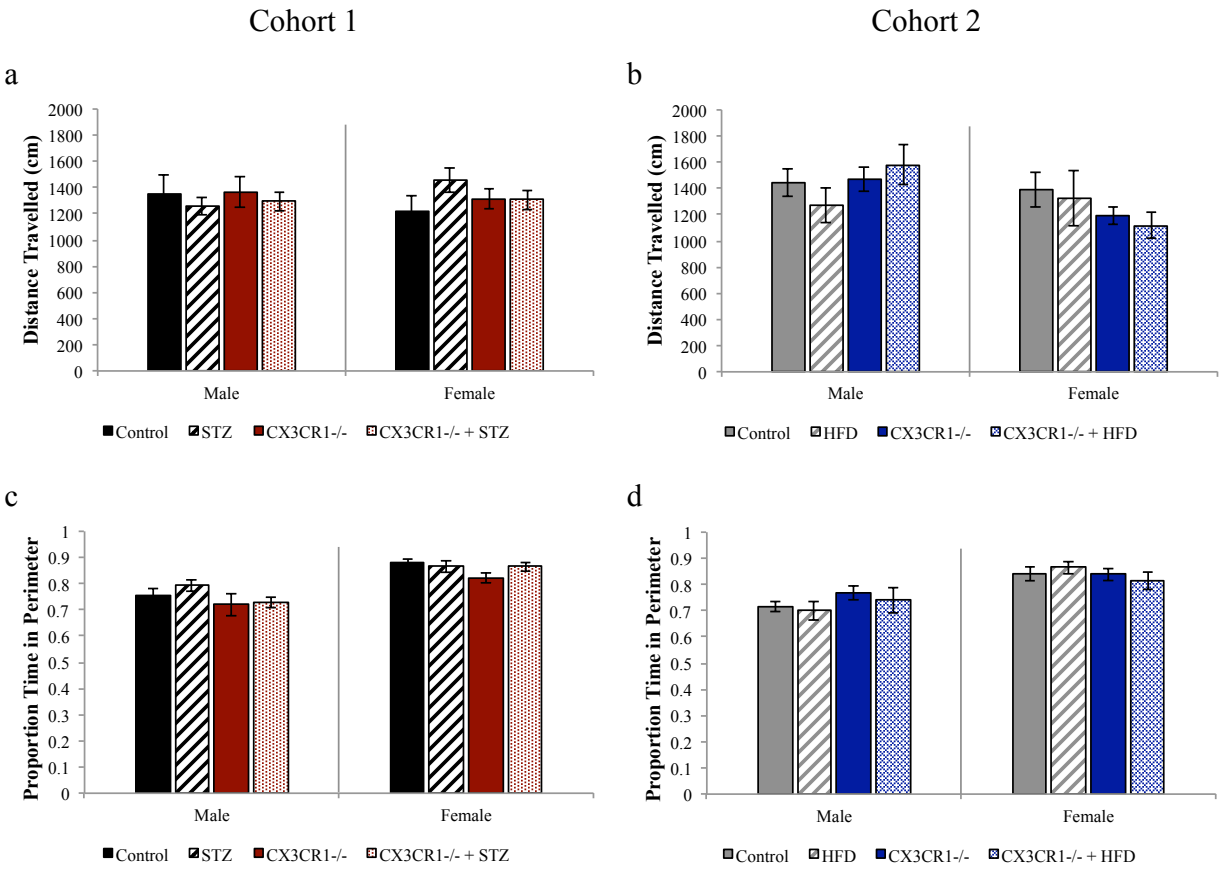

Figure 6 Open Field. No differences were observed in distance travelled (**a, b;** ±SEM) or time spent in the perimeter of the arena (**c, d;** ±SEM) during the open field in either cohort.

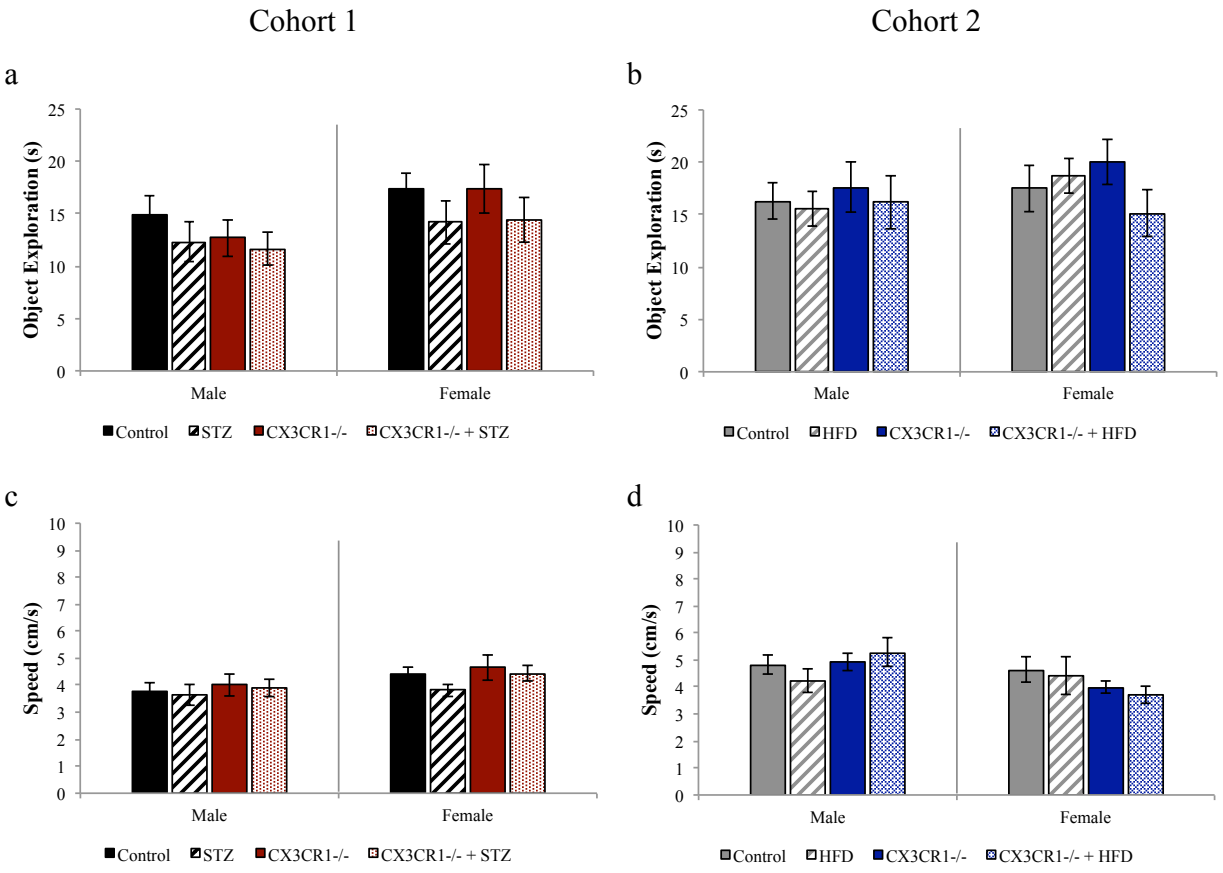

Figure 7 Novel Object Recognition, Day 1. No differences were observed in time spent investigating identical objects (**a, b;** ±SEM) or speed (**c, d;** ±SEM) in either cohort.

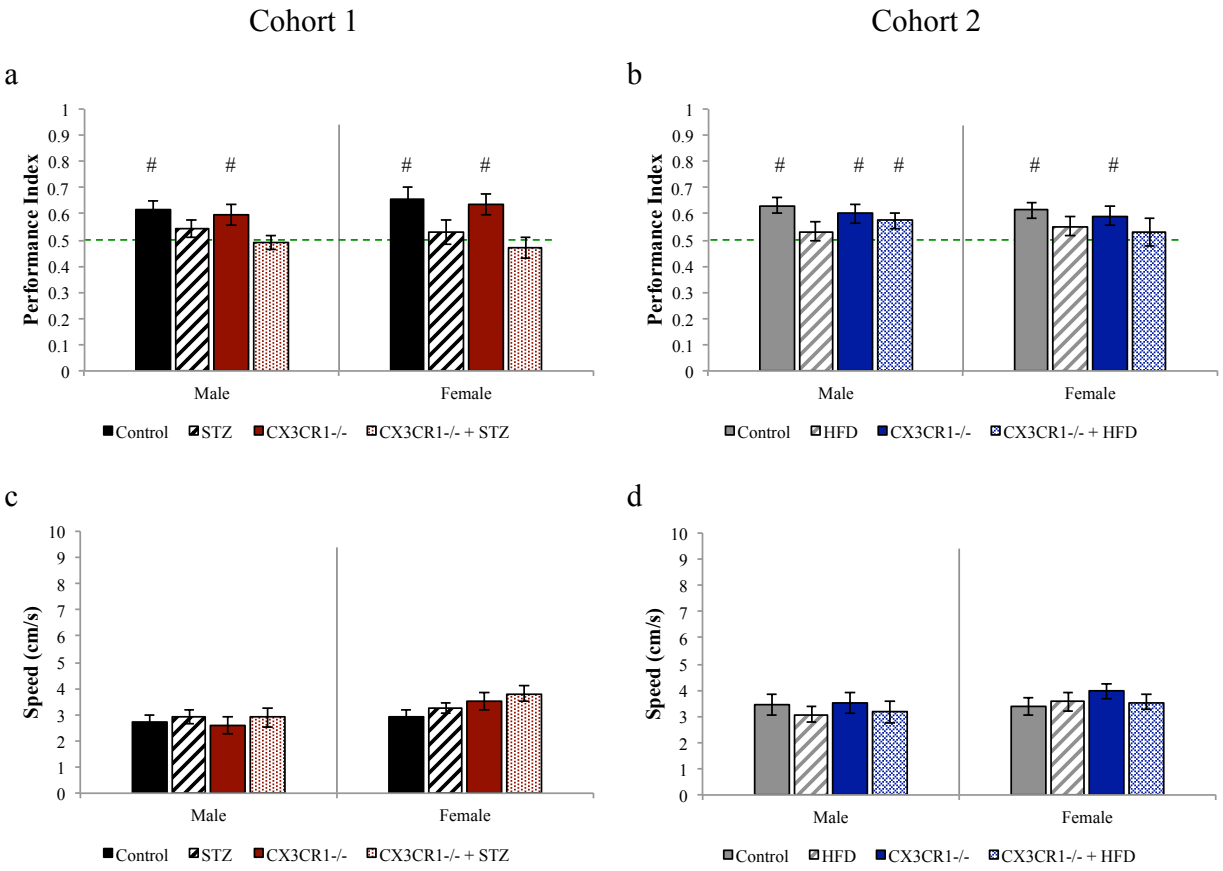

Figure 8 Novel Object Recognition, Day 2. **a)** Controls and CX3CR1$^{-/-}$ in Cohort 1 (males and females) spent significantly more time with the novel object. STZ and CX3CR1$^{-/-}$+STZ males and females did not display a novelty preference. **b)** In Cohort 2, controls (male and female), CX3CR1$^{-/-}$ (male and female), and CX3CR1$^{-/-}$+HFD (males) spent significantly more time with the novel object; whereas HFD (males and females) and CX3CR1$^{-/-}$+HFD (females) spent less time with the novel object. No differences were observed in speed in any groups (**c, d;** ±SEM). # = significantly greater than chance ($p < 0.05$)

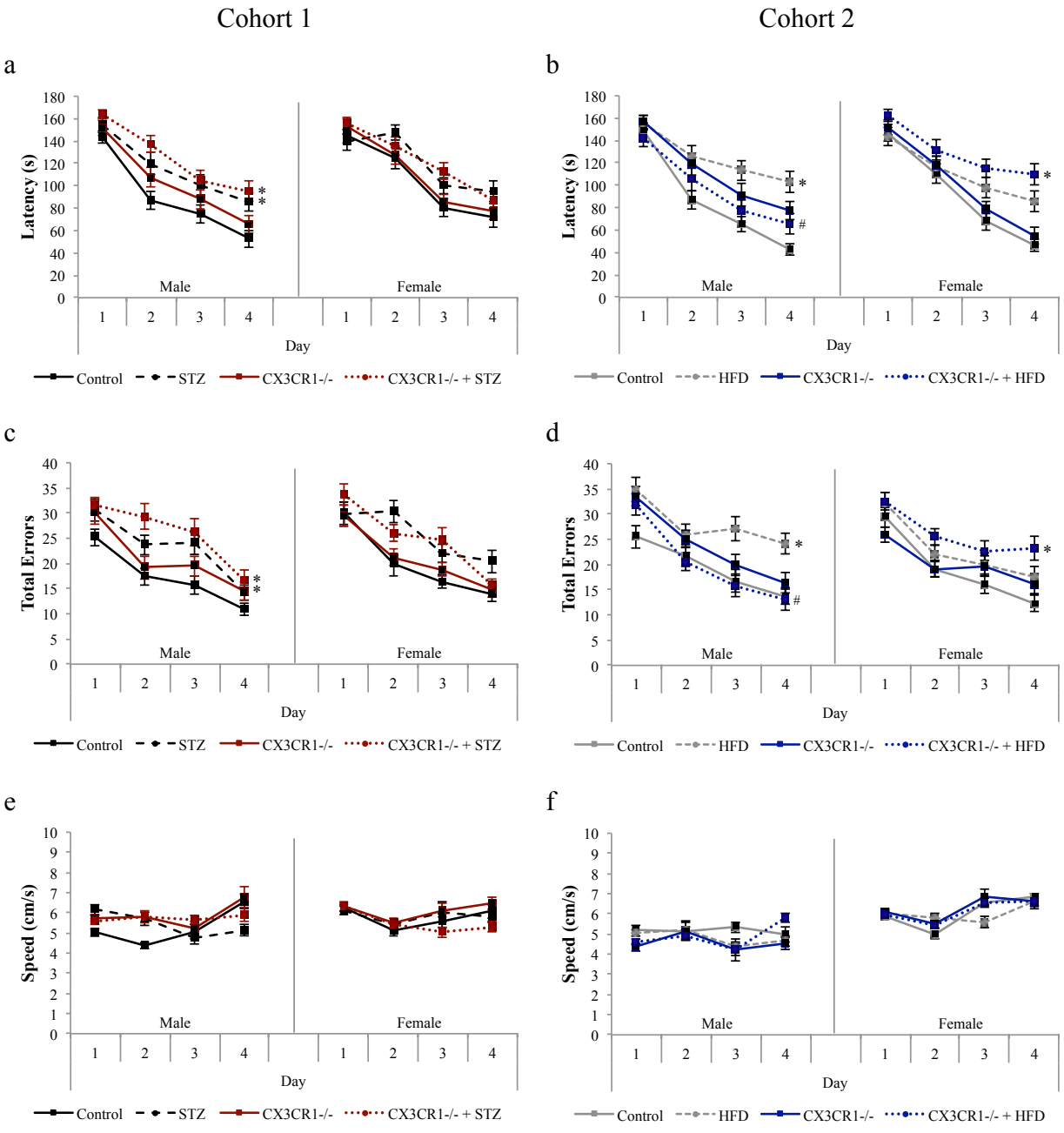

Figure 9 Barnes Maze, Hidden Training. STZ and CX3CR1$^{-/-}$+STZ males had significantly longer latencies (**a**, ±SEM) and errors (**c**, ±SEM**).** In Cohort 2, male HFD and female CX3CR1$^{-/-}$ +HFD took longer to find the escape chamber (**b**, ±SEM) and committed more errors (**d**, ±SEM). No differences were observed in speed in any groups (**e, f;** ±SEM). * = $p < 0.05$ against controls; # = $p < 0.05$ HFD versus CX3CR1$^{-/-}$+HFD.

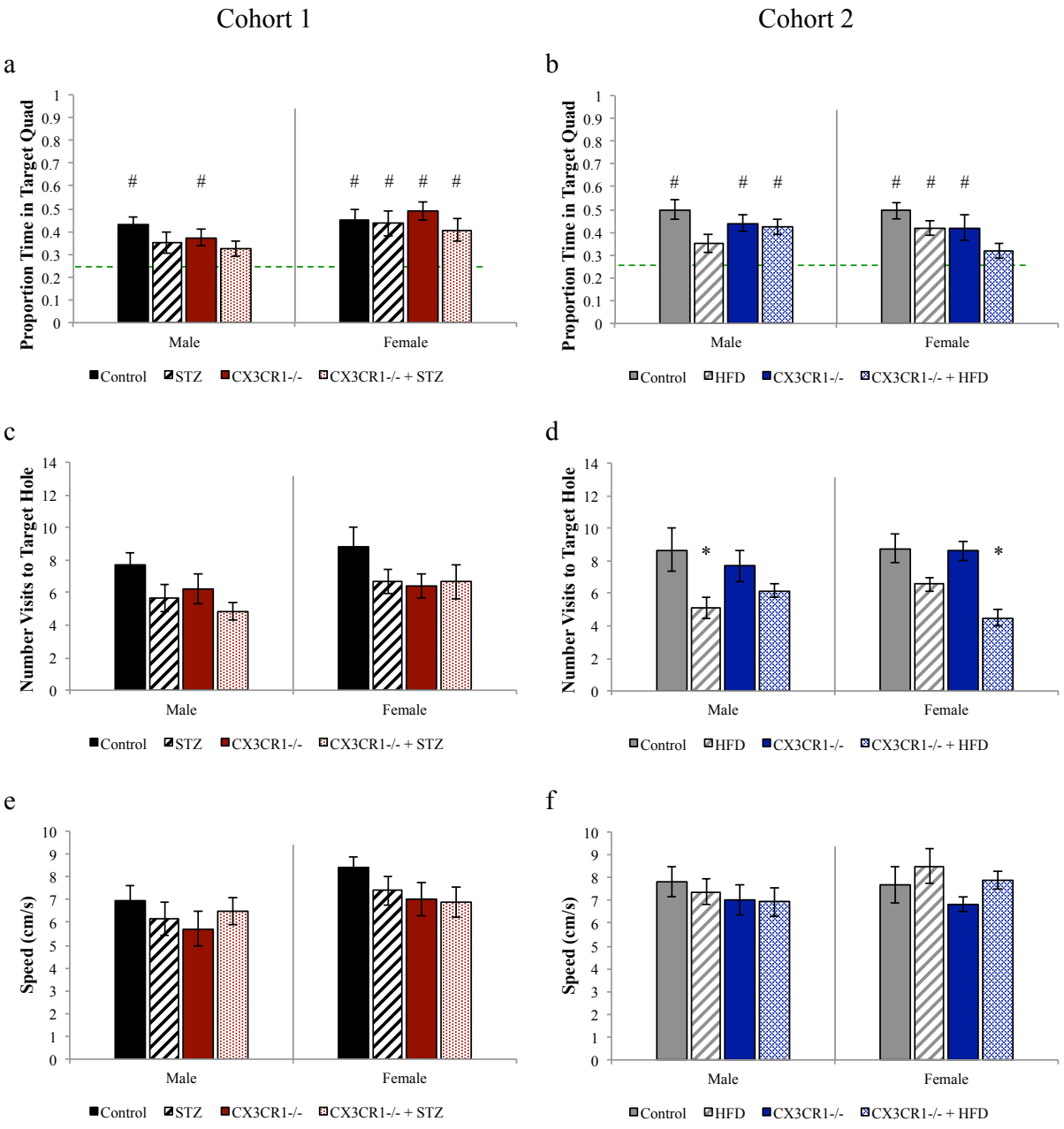

Figure 10 Barnes Maze, Probe Trial. **a)** STZ and CX3CR1$^{-/-}$+STZ males did not spend significantly more time in the target quadrant than chance would predict; all other groups displayed a selective search **b)** In Cohort 2, all groups, except for HFD males and CX3CR1$^{-/-}$ +HFD females, displayed selective searches. **c)** No differences in target hole pokes in Cohort 1 (±SEM). **d)** Male HFD and female CX3CR1$^{-/-}$+HFD groups performed significantly fewer target hole pokes (±SEM). No differences were observed in speed in any groups (**e, f;** ±SEM). * = $p < 0.05$ against controls; # = significantly greater than chance ($p < 0.05$).

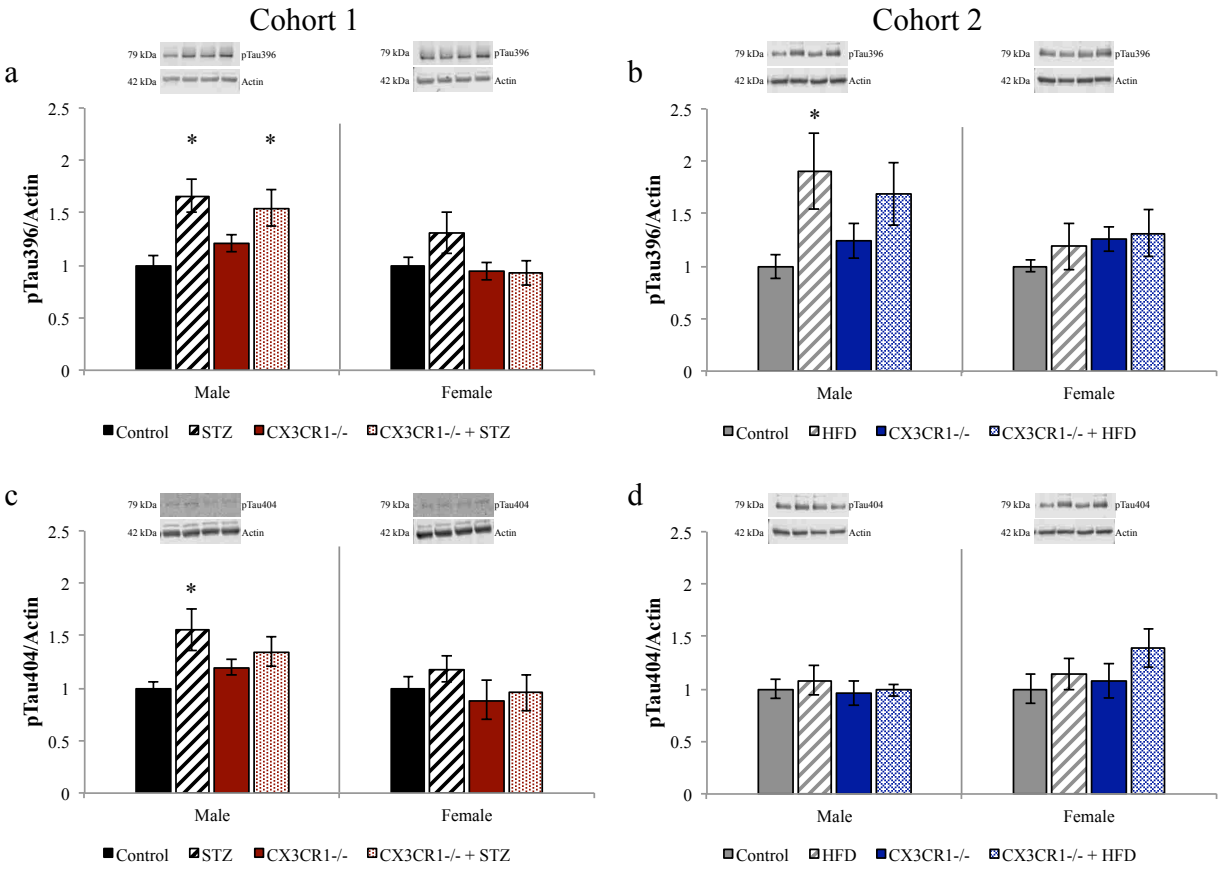

Figure 11 Western Blot: pTau396 and pTau404. **a)** STZ and CX3CR1$^{-/-}$+STZ males had significantly elevated pTau396 (±SEM). **b**) Male HFD mice displayed elevated levels of pTau396 (±SEM). **c)** STZ resulted in increased pTau404 in males. **d)** No differences were observed in pTau404 levels (±SEM) in Cohort 2. * = $p < 0.05$ against controls.

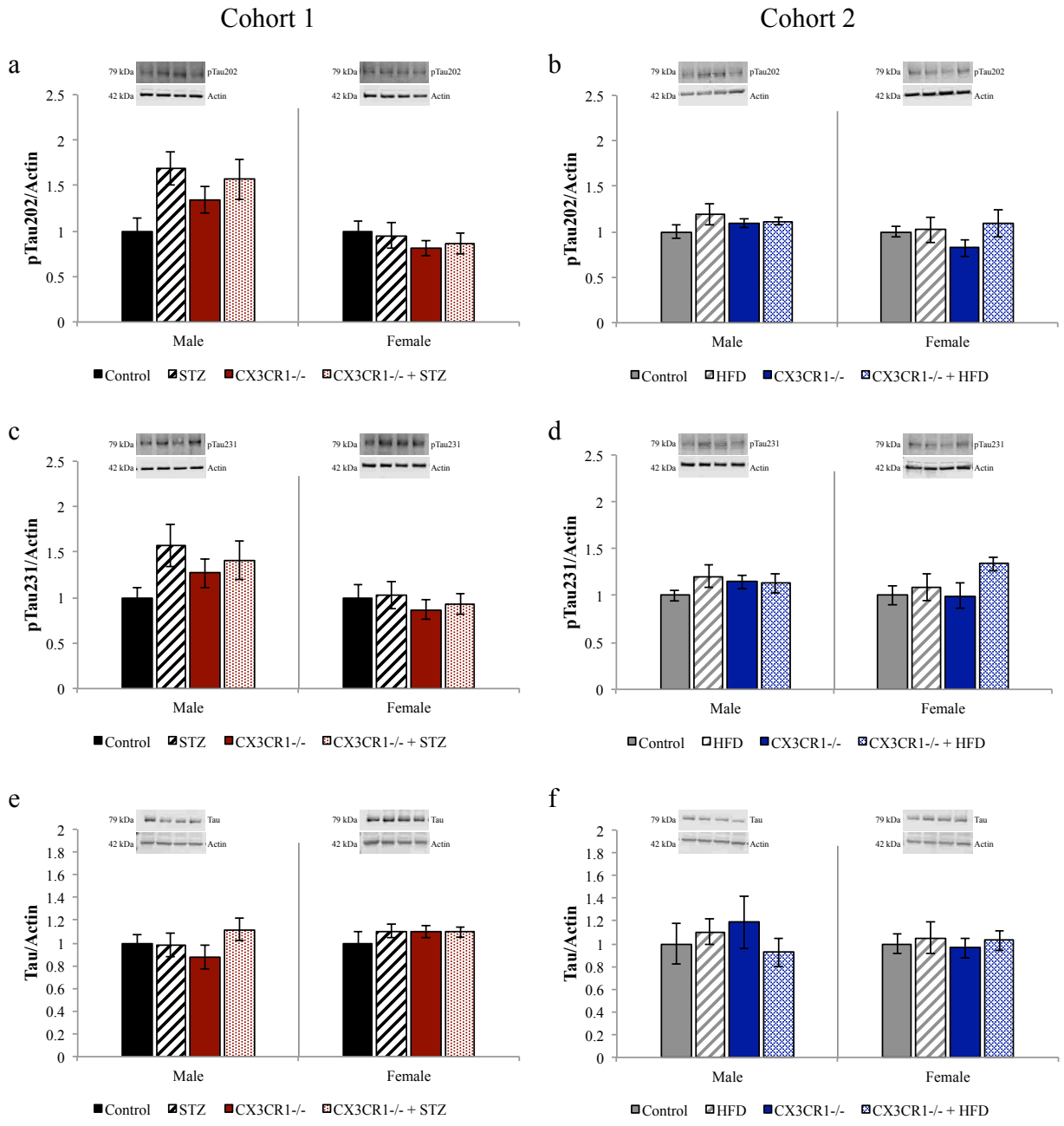

Figure 12 Western Blot: pTau202, pTau404, Total Tau. No differences were observed in pTau202 (**a, b**; ±SEM**)**, pTau231 (**c, d**, ±SEM) or total tau **(e, f**, ±SEM**)** in either cohort.

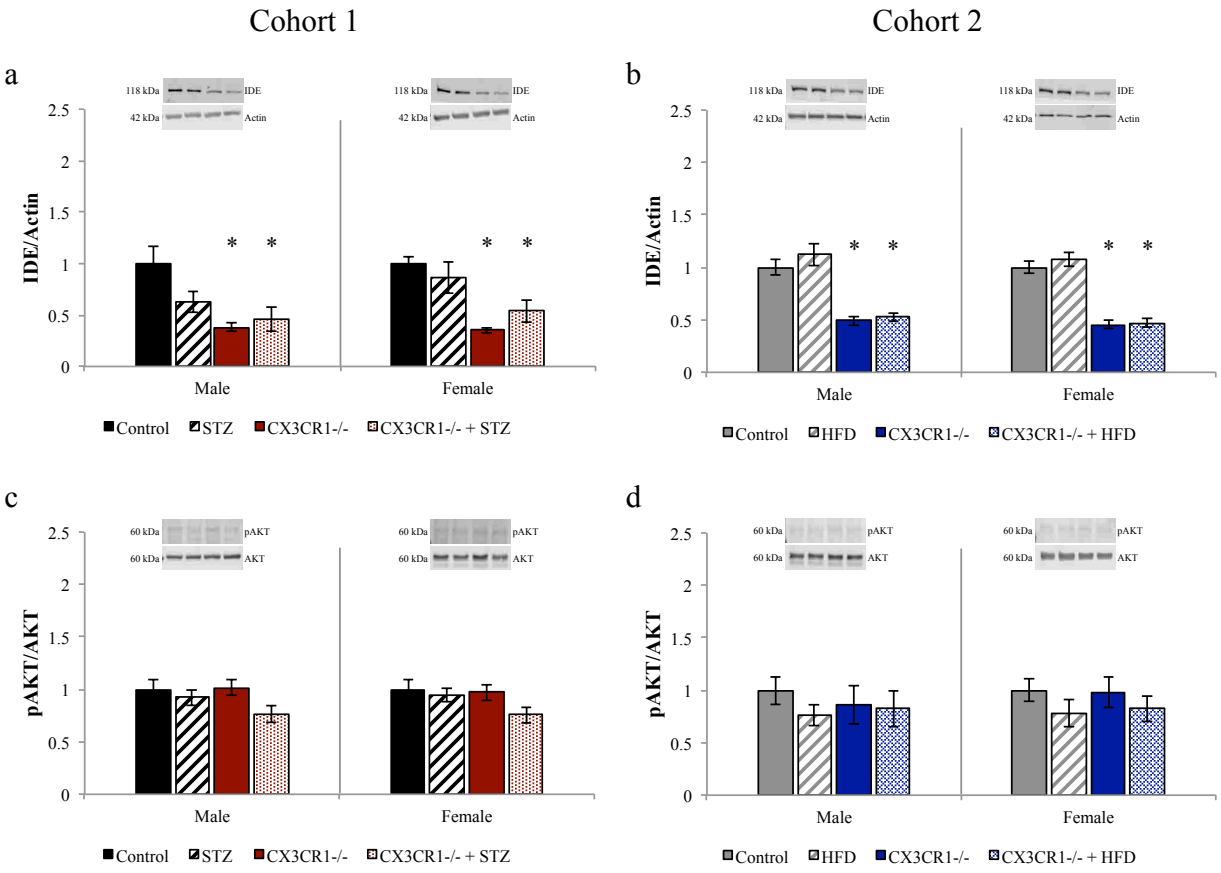

Figure 13 Western Blot: IDE and pAKT. Significant reductions were observed in all CX3CR1$^{-/-}$ groups, regardless of DM status (**a, b**; ±SEM). No significant differences were observed in pAKT in either cohort (**c, d**; ±SEM). * = $p < 0.05$.

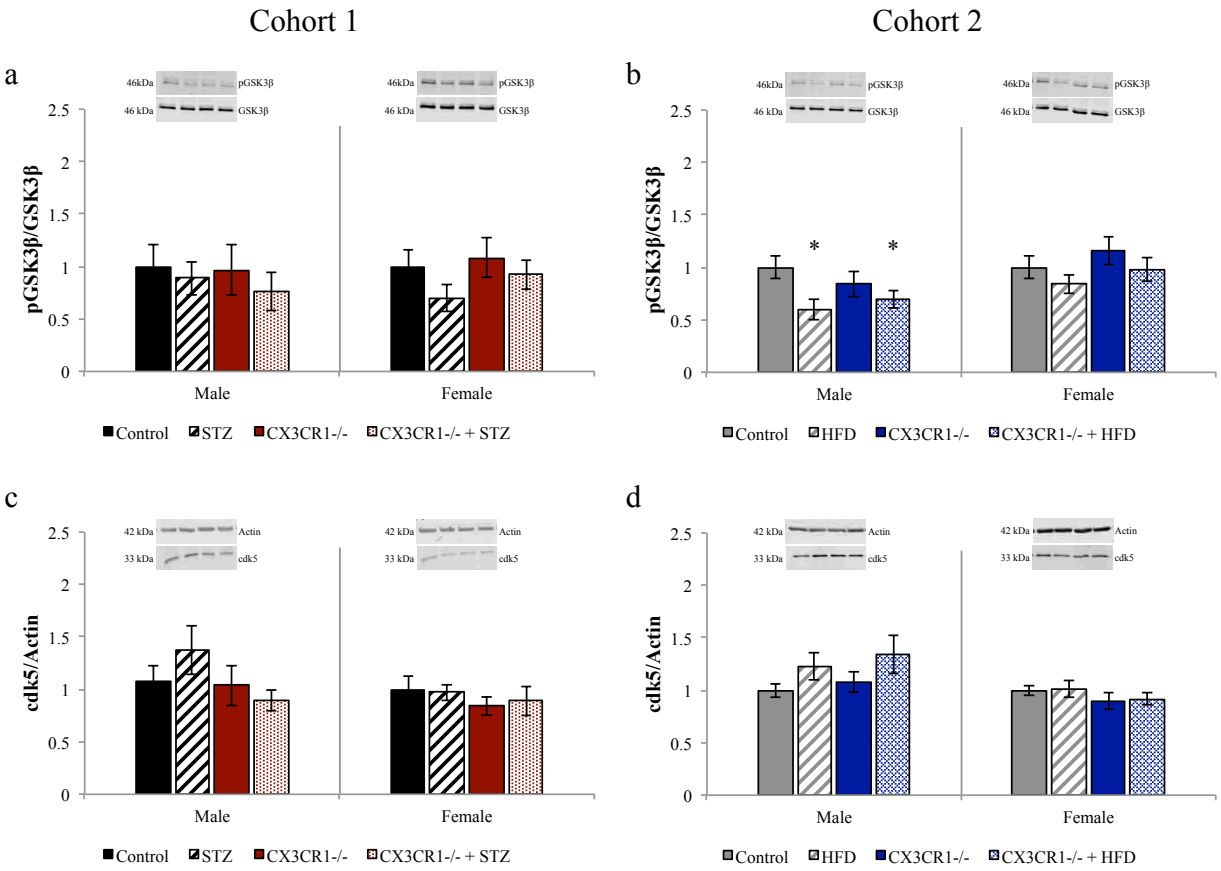

Figure 14 Western Blot: pGSKβ and cdk5. **a)** No significant changes in pGSKβ (±SEM) in Cohort 1. **b)** HFD and CX3CR1$^{-/-}$+HFD males experienced reduced pGSKβ levels (±SEM). Levels of cdk5 remained unchanged in all groups in both cohorts (**c, d**; ±SEM). * = $p < 0.05$.

CHAPTER 5

CONCLUSION

The above studies were designed to investigate the effects of two different models of diabetes and their interactions within an established model of inflammation on alterations related to Alzheimer's disease. Specifically we evaluated learning and memory and tau-related pathology. We found that both models (Cohort 1 = STZ and Cohort 2 = HFD) led to impairments in learning and memory, as evidenced by deficits in both the novel object recognition and Barnes maze tasks. We hypothesized that these outcomes would be worsened when combined with the CX3CR1$^{-/-}$ mouse model of inflammation. While we generally saw equivalent or worse outcomes in male and female CX3CR1$^{-/-}$+STZ compared to STZ alone, we surprisingly observed that HFD led to slightly better cognitive outcomes when combined with CX3CR1$^{-/-}$ mouse model, which will be discussed more below.

In these experiments, we utilized streptozotocin to induce diabetes in Cohort 1. Our lab has previously demonstrated that a similar protocol to the one used in this experiment resulted in a sustained state of hyperglycemia, which we were able to replicate in this study (Murtishaw et al., 2018). Interestingly, males in the CX3CR1+STZ groupd had significantly greater elevated blood glucose than the STZ-alone males, indicating an additive effect between genotype and STZ. Both groups of females administered STZ also experienced hyperglycemia, regardless of genotype. The elevation in blood glucose was much more subdued in female mice when contrasted with the more severe elevation observed in the males. This same pattern, of males experiencing much higher blood glucose than females, was observed in the high-fat diet cohorts and blood glucose never reached the levels observed in the STZ cohort. Female mice have been

found to be more resistant to metabolic challenges than males, including the use of both streptozotocin and high-fat diet (Elias et al., 1994; Pettersson, Waldén, Carlsson, Jansson, & Phillipson, 2012). It is interesting to note that the majority of research conducted on diabetes in rodents is primarily performed on male mice due to female mice exhibiting less pronounced disturbed metabolic phenotypes. However, these differences can also be seen in the human population. Type-1 diabetes is predominated by males and can be as disproportionate as 1:7 in populations that arise from a European origin (Gale & Gillespie, 2001). Additionally, there appears to be a reversal in prevalence depending upon stage of reproductive life with more diabetic men prior to puberty and more women are diagnosed after menopause (Wild, Roglic, Green, Sicree, & King, 2004).

A major point of these experiments was to evaluate whether our low-dose, staggered streptozotocin protocol resulted in sustained metabolic changes as far out as six months after the initial injections rather than just six weeks. These data suggest that our streptozotocin protocol is sufficient to result in a state of hyperglycemia that lasts for a significant amount of time. Six months following the injections, blood glucose levels were still elevated in all groups that had received streptozotocin. It is possible that those elevated levels might eventually begin to return to normal levels if the pancreas is able to regenerate enough pancreatic β-cells but it is evident from these data that six months is not enough time for sufficient pancreatic β-cell regeneration, at least not enough to begin to lower blood glucose.

In these studies, administration of streptozotocin led to significant decreases in plasma insulin in male mice but not in female mice; whereas circulating insulin was dramatically increased in all groups fed a high-fat diet, regardless of genotype or sex. These data are

consistent with observations in other STZ studies, including our own lab, showing that reducing the number of pancreatic β-cells will result in lowered levels of plasma insulin (Murtishaw et al., 2018). Diet-induced obesity has been shown to increase circulating insulin levels in response to increasingly elevated blood glucose and that the pancreas releases more insulin to divert rising glucose levels from the bloodstream into target tissue, which ultimately leads to the insulin resistance commonly observed in T2DM (Winzell & Ahrén, 2004).

Memory impairments observed in the novel object recognition task are consistent with those that are observed in patients with AD and rodent models of AD. The novel object recognition task relies on the entorhinal cortex and the hippocampus (Antunes & Biala, 2011; Reger, Hovda, & Giza, 2009). These two regions are areas of the brain that appear to be the most vulnerable in early stages of AD (Braak & Braak, 1991). Lesions to the hippocampus have been shown to impair the ability of an animal to discriminate between an object that is familiar and an object that is novel (Antunes & Biala, 2011). Animals were tested in the open field arena for deficits in locomotor activity and anxiety-like phenotypes but because no differences were observed in these parameters the deficits in learning and memory are not likely due to differences in mobility or anxious behavior. Animals across all groups, in both cohorts, explored objects equivalently and moved around the arena comparably on the first day of novel object training. Diabetic-status, regardless of STZ or HFD-origin, resulted in reductions in preference for the novel object. Interestingly, we did not see novel object deficits in male CX3CR1$^{-/-}$+HFD mice, whereas HFD-alone led to impaired novelty recognition. This same pattern of learning deficits occurring in HFD males but not CX3CR1$^{-/-}$+HFD males was also observed in the Barnes maze, which will be discussed below.

Animals were also tested in the Barnes maze task, a more complex hippocampal-dependent task, to assess spatial learning and memory. In Cohort 1, both male groups treated with streptozotocin took longer to find the hidden escape chamber, committed significantly more errors, and spent less time in the target quadrant during the probe trial. Females treated with streptozotocin exhibited no differences in any measure of the Barnes maze. Interestingly, HFD males in Cohort 2 exhibited deficits in spatial learning, whereas CX3CR1$^{-/-}$+HFD did not demonstrate any such impairment. In fact, CX3CR1$^{-/-}$+HFD males performed comparable to controls and even slightly better than their CX3CR1$^{-/-}$ littermates on certain aspects of the Barnes maze. Others have found that CX3CR1$^{-/-}$ mice have impaired hippocampal-learning in the Morris water maze, another task to measure spatial learning (Rogers et al., 2011). These same CX3CR1$^{-/-}$ mice were shown to have significantly reduced hippocampal-dependent long-term potentiation compared to controls (Rogers et al., 2011).

Perhaps of most interest, recent reports have demonstrated that the CX3CL1/CX3CR1 signaling system is a novel regulatory mechanism on the production of insulin secretion and pancreatic β-cell function (Lee et al., 2013). Lee et al. found that the removal of functional CX3CR1 leads to metabolic disturbances, including hyperglycemia, reduced insulin production, and changes in pancreatic β-cells morphology. It is plausible that if CX3CR1$^{-/-}$ mice are already experiencing an abnormal metabolic profile resulting in altered glucose metabolism that the introduction of a high-fat diet led to an alternative fuel source that inadvertently resulted in better cognitive outcomes. Ketones, in particular acetoacetate and β-hydroxybutyrate, can be relied upon as an alternative fuel source for the brain by converting β-hydroxybutyrate to acetoacetate, acetoacetate to acetoacetyl CoA, and acetoacetyl CoA to acetyl CoA, which can then be used in

the citric acid cycle as an energy source (Cunnane et al., 2011). The lack of cognitive impairments in the CX3CR1$^{-/-}$+HFD male mice was an unexpected finding and contrary to our initial hypothesis that outcomes would be worse than in HFD mice alone. No measures were taken to monitor ketone levels during the course of the experiment or other metabolic parameters that might elucidate shifts in carbohydrate or lipid metabolism, as this was outside the scope of these experiments. Future studies should investigate this more thoroughly, including an exploration as to why this benefit was observed in male but not female CX3CR1$^{-/-}$+HFD animals.

A number of proteins associated with AD-related tau pathology and insulin signaling were investigated in hippocampal tissue within these cohorts. Significantly increased levels of phosphorylated tau were observed only in male mice treated with streptozotocin (both STZ alone and CX3CR1$^{-/-}$+STZ) and in male mice fed HFD alone. No significant changes in phosphorylated tau were observed in CX3CR1$^{-/-}$+HFD male mice or any of the female groups. Neurofibrillary tangles, which are composed of hyperphosphorylated tau, are highly associated with cognitive deficits in patients with AD and are commonly observed in AD preclinical models (Braak & Braak, 1991; Shi et al., 2011). We analyzed two major kinases, GSK3β and cdk5, to evaluate potential mechanisms associated with the increase in phosphorylated tau at sites Serine 396 and Serine 404. Only males on high-fat chow exhibited significantly altered levels of pGSK3β with no streptozotocin-treated groups exhibiting the same changes. Given the large number of potential kinases that can phosphorylate tau, it is possible that another kinase is significantly changed in the streptozotocin model or that small changes in numerous kinases contribute to an overall increase in tau phosphorylation and that these small alterations, while

large enough to be biologically significant, are not large enough to be statistically significant. Additionally, there could be significant alterations in phosphatases, the proteins that remove phosphate groups from tau to restore tau's affinity for microtubules, which results in the same outcome of tau hyperphosphorylation. Phosphatases were not analyzed in these cohorts and future experiments should include an analysis of both kinases and phosphatases to get a better understanding of the dynamic nature of tau phosphorylation and dephosphorylation in the presence of metabolic disturbances.

One of the most striking results in these experiments was the finding that CX3CR1$^{-/-}$ mice, regardless of diabetic status, displayed significant reductions in insulin degrading enzyme compared to wildtype mice. As far as we can tell, we are the first to report that insulin degrading enzyme is significantly reduced in the hippocampus of CX3CR1$^{-/-}$ mice. As mentioned above, Lee et al. (2014) demonstrated that CX3CR1$^{-/-}$ mice exhibited metabolic disturbances but did not measure metabolic alterations in the brain and did not evaluate insulin degrading enzyme in any tissue. Our data fit within their findings of CX3CL1/CX3CR1 as a novel regulatory pathway in insulin production and that CX3CR1 deficiency could lead to alterations in insulin production and therefore reductions in insulin degrading enzyme. More research is needed to understand the role that the CX3CL1/CX3CR1 pathway plays in metabolic homeostasis both in the periphery and within the CNS.

Given the role of insulin resistance and insulin signaling perturbations in the pathogenesis of AD, it is possible that interventions currently approved for T2DM may also be useful in the treatment of AD. Insulin treatment reduces the development of tau pathology through an enhanced regulation of GSK-3β as numerous studies have demonstrated that insulin

administration mitigate tau phosphorylation following STZ administration (Jolivalt et al., 2008; Planel et al., 2007). However, the route of insulin delivery may be especially important in our clinical AD population due to chronic hyperinsulinemia in the peripheral tissues and hypoinsulinemia in the brain. In fact, several studies have shown that T2DM patients who regularly take insulin as a form of treatment are at even greater risk for AD than individuals with T2DM who either take no medications or utilize other forms of T2DM medication, by as much as fourfold (Huang et al., 2014; Ott et al., 1999). Intranasal insulin appears to be a viable long-term treatment option since intranasal administration appears to quickly elevate levels of insulin within the brain by crossing perivascular channels and axonal pathways in olfactory and trigeminal areas without affecting peripheral insulin levels (Reger et al., 2006; Thorne, Pronk, Padmanabhan, & Frey, 2004). Intranasal insulin administration has shown some therapeutic promise results improving recall, attention, and several other cognitive measures in patients with AD and mild cognitive impairment (Craft et al., 2012; Reger et al., 2008).

Thiazolidinedioenes (TZDs), rosiglitazone, and pioglitazone are ligands for peroxisome proliferator-activated receptors (PPARs), a family of nuclear receptors involved in the regulation of gene transcription associated with lipid and glucose metabolism (Gryguek-Gorniak, 2014). TZDs are commonly used as an anti-diabetic drug due to their beneficial effects on glucose homeostasis by increasing insulin sensitivity. In several AD mouse models, long-term administration of rosiglitazone resulted in enhanced clearance of Aβ, a reduction in amyloid plaques, decreased tau phosphorylation, and improved cognitive functions (Escribano et al., 2010; Yu et al., 2015). Additionally, rosiglitazone reduced tau phosphorylation in Otsuka Long Evans Tokushima Fatty rats, a model of spontaneously occurring T2DM, further implicating

insulin signaling in the formation of AD-related pathologies(Yoon et al., 2010). The use of rosiglitazone in human clinical trials has had mixed results. One study found that six months of treatment with rosiglitazone improved recall and selective attention in patients with mild AD compared to age-matched controls, while another found that rosiglitazone improved scores on the Alzheimer's Disease Assessment Scale-Cognitive subscale but only in individuals with APOEε4 negative genotypes (Risner et al., 2006; Watson, Cholerton, Reger, & Baker, 2005). Another large multicenter trial found no evidence that rosiglitazone resulted in any meaningful cognitive or global functional improvements in AD patients (Tzimopoulou et al., 2010).

Memantine, one of the few drugs approved by the Food and Drug Administration for clinical use of AD, is known to be a non-competitive, moderate affinity antagonist of the NMDA receptor and is used to relieve excessive glutamate noise in order to ameliorate glutamate excitotoxicity and slow the progression of neuronal death (Chen & Lipton, 2006; Lipton, 2004). Much more recently, Memantine was demonstrated to act on hippocampal K_{ATP} to promote CaMKII activity in the brains of an *APP* transgenic AD model to enhance LTP and improved blood glucose levels in the *ob/ob* diabetic mouse model, suggesting that Memantine could be potentially advantageous in treating mild to moderate AD patients with diabetes (Moriguchi et al., 2016).

The power of preventative measures should not be overlooked in the research frenzy to treat AD. A staggering 90% of all T2DM cases are estimated to be entirely preventable through modifications to diet, activity levels, and simple behavioral modifications (Hu et al., 2001). Previous estimates have suggested that nearly half of all AD cases are attributable to modifiable risk factors (smoking, physical inactivity, depression, hypertension, diabetes, and obesity) and

that a 10-25% reduction in these risks could reduce the number of AD cases in the United States by 500,000 and nearly 1-3 million cases world-wide (Barnes & Yaffe, 2011).

A recent study found that adherence to a Mediterranean-diet, increased physical activity, and a lower BMI correlated with lower levels of amyloid deposition and tau tangles in patients with mild cognitive impairment (Merrill et al., 2016). Neuropsychological examinations in healthy elderly adults, 60-75 years of age, indicate that beginning a regular aerobic program improved performance in cognitive tasks that rely heavily on frontal lobe function when compared to age-matched controls who did not exercise regularly (Kramer et al., 1999). In a randomized control study with elderly adults, those who began a regular exercise program experienced an increase in hippocampal BDNF levels, an increase in hippocampal volume, and improvements to spatial memory compared to the non-exercising control group who experienced significant loss in hippocampal volume (Erickson et al., 2011). A small series of case studies recently conducted at the Mary S. Easton Center for Alzheimer's Disease Research at UCLA found that a twenty-five point behavioral modification program intended to improve metabolic performance, including items such as a low-grain diet, intermittent fasting, optimized sleep, daily exercise, and the addition of resveratrol and Axona (a prescription medical food specifically for AD) to the diet, significantly improved cognitive functions in 9 out of the 10 test subjects, warranting the need for more extensive, controlled clinical trial on the importance of diet and lifestyle interventions (Bredesen, 2014). Indeed, in a large double-blind randomized controlled study with multi-domain intervention, including exercise and dietary guidance, elderly patients in the multi-domain intervention experienced significant improvements across several cognitive domains (Ngandu et al., 2015).

The benefits of studies that include exercise should not come as a surprise since it is well-known that aerobic exercise and resistance training improves insulin sensitivity and dramatically increases GLUT4 expression and mobilization through both insulin–dependent and insulin-independent pathways in peripheral tissues (Lehnen, 2013). Rats that were fed a high-fat diet for 16 weeks and given access to running wheels had improved memory, restored insulin sensitivity, and increased hippocampal BDNF levels compared to high-fat diet rats not given regular exercise (Noble et al., 2014). Recent evidence suggests that exercise can also restore insulin sensitivity at the blood-brain-barrier leading to an increase in brain insulin levels similar to the effect of exercise on the reversal of insulin resistance that has now been documented both in peripheral tissues and in the CNS (Biessels & Reagan, 2015; Tschritter et al., 2012).

Interestingly, recent evidence suggests that the insulin-dependent GLUT4 transporter can be found in low levels on neurons within several brain regions, including the cortex and hippocampus, where energy demands are high and can be rapidly inserted in the typical insulin-dependent manner but also in an insulin-independent manner following neuronal activity, similar to GLUT4 insertion in muscle tissue following exercise (Ashrafi, Wu, Farrell, & Ryan, 2017). Decreased glucose utilization in the brains of AD patients is consistently noted as one of the first symptoms in early stages of the disease (Hoyer, 2004; Simpson et al., 1994). The brain shows an incredible amount of metabolic flexibility by being able to easily utilize not only glucose and lactate but also ketones as fuel (Mergenthaler et al., 2013). Recent work involving PET imaging has demonstrated that while key regions of the brain involved with AD pathology experience decreased uptake of glucose, those same areas showed no impairments with ketone uptake, suggesting that supplying the brain with ketones might be a viable complementary strategy to

counteract metabolic imbalances within the brain (Castellano et al., 2014). Axona or AC-1202, the prescription medical food used in the UCLA study mentioned above, is a proprietary food replacement high in medium chain triglycerides from which ketone bodies are easily produced (Ashrafi et al., 2017; Cunnane et al., 2016). As expected, AC-1202 significantly elevated serum ketone levels without further modification to an existing diet and resulted in significant cognitive improvements in patients with mild AD compared to the placebo controls (Henderson et al., 2009). AC-1204 is the pharmaceutical follow-up to AC-1202 and enrollment has just completed in a large-scale Phase 3 clinical trial. Providing an alternative energy source to a glucose-starved brain in the form of ketones shows consistent improvements in cognitive measures but still needs to be thoroughly evaluated on other pathological markers of the disease. Given the cognitive benefits observed in the male CX3CR1$^{-/-}$+HFD mice, an interesting follow-up study would be to investigate the benefits of a ketogenic diet or AC-1202 in the metabolically challenged CX3CR1$^{-/-}$ mouse model or in other preclinical AD models.

There have been no new treatments approved for AD in over 12 years and the failure rate of clinical trials in potential new drugs is 99.6% in drug trials conducted since 2002 (Cummings, Morstorf, & Zhong, 2014). Due to the absence of effective treatments that permanently halt or reverse AD progression, it is certainly warranted to demand increased scrutiny for effective preventative measures, particularly in regards to well-established risk factors, such as insulin resistance and T2DM.

Insulin resistance is the core pathogenic feature of metabolic syndrome, a cluster of diseases that include T2DM and obesity. Until recently, the research related to insulin resistance was targeted towards peripheral tissue such as muscle and adipose tissue; however, a more recent

focus on insulin resistance within the nervous system suggests that the brain and the blood-brain-barrier is susceptible to the damaging effects of insulin resistance. Insulin resistance observed in T2DM has been demonstrated to correlate with an increased risk in the development of AD by making brain cells more susceptible to the damaging effects of Aβ and tau-related toxicity. Recent evidence supports the notion that AD, or at least some cases of AD, may be a slow-progressing brain metabolic disease, with an increasing weight of evidence demonstrating an intricate link between insulin resistance and AD. Individuals with obesity and T2DM are at an increased risk for dementia. Patients with dementia, particularly with AD, commonly develop insulin resistance and hyperglycemia. Insulin resistance is a common pathway between T2DM and AD. Insulin signaling plays a key role in Aβ and tau regulation. In turn, Aβ has profoundly negative effects on insulin signaling.

Despite the increased scrutiny regarding insulin resistance in the nervous system, there is still a paucity of data to come to definitive conclusions regarding the exact role that insulin resistance plays in various disease states considering that metabolic disease is often accompanied with hyperinsulinemia, hyperglycemia, hyperlipidemia and inflammation. Additionally, a number of genetic and environmental risk factors, including a lack of exercise, increased BMI, smoking, unmanaged stress, and even aging can effect the development and persistence of insulin resistance (Sesti, 2006). The effects of T2DM on the brain are most pronounced in the elderly, suggesting that the aging brain is more susceptible to the effects of DM, likely due to the fact that numerous processes that would normally mitigate the toxic effects of hyperglycemia, oxidative stress, advanced glycation end-products are all impacted in an aged brain (Biessels et

al., 2002). Further studies are needed to understand these various contributions, as well as potential therapeutic targets.

Overall, these experiments demonstrate that perturbations to insulin, whether through streptozotocin or high-fat diet, have dramatic impacts on learning and memory. Profound cognitive deficits were observed in recognition memory and spatial learning in male diabetic mice; whereas female mice were much less likely to experience comparable cognitive deficits. The data collected from the tissue analyses suggest that these metabolic disruptions do not necessarily result in dramatic histopathological changes related to AD. Both streptozotocin and high-fat diet models are excellent routes of inducing diabetes in rodents but do not appear to be sufficient to induce overt AD-like alterations in the brain on the cellular level, at least on multiple targets on their own. These diabetic models could be useful tools when combined with other AD risk factors or AD-related mututations to investigate the synergistic effect of multiple risk factors. These experiments attempted to combine the diabetic risk associated with AD with an inflammation model to explore this cumulative risk; however, because CX3CR1$^{-/-}$ mice have recently been shown to already have preexisting metabolic alterations, the unexpected and contrary results between the streptozotocin and HFD cohorts further complicates this pathway of inquiry. It is possible that utilizing a different route of inflammation, such as administration of lipopolysaccharide or PolyI:C to activate toll-like receptors, could be more beneficial in investigations into the combined risk of diabetes and inflammation.

The results from these experiments suggest that both cohorts represent a good model of DM. The streptozotocin model resulted in dramatic elevations in blood glucose levels and modest reductions in insulin; whereas, the high-fat diet model resulted in more subdued

elevations in blood glucose levels but marked increases in circulating insulin levels. These different diabetic models can allow the researcher the opportunity to investigate the contributions of severe hyperglycemia following streptozotocin versus the dramatic hyperinsulinemia associated with diet-induced obesity on cognitive outcomes and other alterations within the brain. It is thought that hyperglycemia and hyperinsulinemia contribute differently to AD pathogenesis and these two different models could help elucidate their individual contributions. It is difficult to separate out their individual impact though as one is rarely present without the other in most models of DM.

Continued research to further elucidate the exact role and mechanisms by which insulin resistance can contribute to the development and progression of AD-related pathology is imperative to developing therapeutic interventions. Research continues to support the notion that high peripheral insulin levels and peripheral insulin resistance modulate cognition and AD-related pathologies within the brain. Given the global obesity epidemic, there is a mounting need to understand the relationship between insulin resistance, cognitive impairments, and the long-term term damage occurring within the nervous system. The model of insulin resistance and metabolic impairments contributing to the pathogenesis of AD is only one possible etiology in a disease with a myriad of potential etiologies and may not apply to all AD patients; however, further knowledge in this area might yield promising therapeutic interventions and preventative measures that will be particularly useful in a subpopulation of AD patients.

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Yu, Y., Li, X., Blanchard, J., Li, Y., Iqbal, K., Liu, F., & Gong, C.-X. (2015). Insulin sensitizers improve learning and attenuate tau hyperphosphorylation and neuroinflammation in 3xTg-AD mice. *Journal of Neural Transmission (Vienna, Austria : 1996)*, *122*(4), 593–606.

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Andrew S. Murtishaw
Curriculum Vitae

CONTACT INFORMATION

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EDUCATION

Ph.D. Experimental Psychology, Neuroscience; August 2014 – August 2018
University of Nevada, Las Vegas
Dissertation Title: "An Evaluation of Alzheimer's Disease-related Pathology in Two Different Mouse Models of Diabetes in Immune-challenged Mice"
Advisor: Jefferson Kinney, Ph.D.

M.A. Experimental Psychology, Neuroscience; August 2011 – May 2014
University of Nevada, Las Vegas
Thesis Title: "Effect of Acute LPS-induced Immune Activation on Brain Insulin Signaling Disruption in a Diabetic Model of Alzheimer's Disease"
Advisor: Jefferson Kinney, Ph.D.

B.A. Psychology with Biology minor; May 2011
University of Nevada, Las Vegas

AWARDS/HONORS/NOMINATIONS

University of Nevada, Las Vegas Graduate and Professional Student 2014 Research Forum Oral Presentation Winner, 1st place, May 2017 ($300)
Graduate students awarded travel and research grants, approximately 200 total presenters, are invited to present their research at a university wide research forum. GPSA awards cash prizes for top presentations.

University of Nevada, Las Vegas Psychology Department Summer Research Scholarship, Summer 2017 ($3,000)
One of five graduate students within the psychology department awarded summer research scholarship to further dissertation project.

University of Nevada, Las Vegas Patricia Sastaunik Scholarship, 2016-2017 ($2,500)

University of Nevada, Las Vegas Summer Session Scholarship, 2016 ($2,000)
This competitive, merit-based scholarship is awarded to graduate students to continue their research during the summer semester.

University of Nevada, Las Vegas Graduate and Professional Student Association Travel Grant, June 2016 ($500)
University-wide grant available to aid students to travel and present research at conferences. Awarded a grant to attend and present at the 2015 International Behavioral Neuroscience Society conference in Budapest, Hungary.

University of Nevada, Las Vegas Outstanding Graduate Student Teaching Award, 1st Place, Spring 2016 ($2,500)
Highly competitive campus-wide teaching award given to a graduate student. Nominated as the candidate representing the College of Liberal Arts and then awarded first-place from amongst all college nominees.

University of Nevada, Las Vegas Edward Lovinger Scholarship, Spring 2015 ($1,500)
Awarded to a Psychology graduate student for outstanding achievements in their graduate work.

University of Nevada, Las Vegas Foundation Board of Trustees Fellowship, Fall 2015–Spring 2017 ($60,000)
The most prestigious fellowship awarded by UNLV. This highly competitive university-wide award is granted to one doctoral student in the final two years of their dissertation research. Finalists undergo a review and interview with a committee panel. Award includes a $30,000 stipend per academic year, full tuition remission, and covers student health insurance.

University of Nevada, Las Vegas Summer Scholarship, Summer 2015 ($2,000)
This competitive, merit-based scholarship is awarded to graduate students to continue their research during the summer semester.

International Behavioral Neuroscience Society (IBNS) Travel Award, June 2015 ($700)
Competitive travel grant awarded by IBNS to graduate students to offset the cost to attend and present their research at the IBNS annual conference held in Victoria, Canada in June 2015.

University of Nevada, Las Vegas Graduate and Professional Student Association Travel Grant, June 2015 ($250)
University-wide grant available to aid students to travel and present research at conferences. Awarded a grant to attend and present at the 2015 International Behavioral Neuroscience Society conference in Victoria, Canada.

American Chemical Society 2014 Southern Nevada Section Research Competition Winner, 1st Place, November 2014 ($400)
Annual ACS research competition for regional graduate students; winners receive a cash prize.

University of Nevada, Las Vegas Graduate and Professional Student Association Travel Grant, November 2014 ($1,000)
University-wide grant available to aid students to travel and present research at conferences. Awarded a grant to attend and present at the 2014 Society for Neuroscience conference in Washington, D.C

International Behavioral Neuroscience Society 2014 Conference Presentation Winner, 2nd Place, June 2014 ($305)
Presentation competition amongst all IBNS graduate students presenters, approximately 80 total. Winners receive waived registration for the 2015 conference, valued at $305.

University of Nevada, Las Vegas Dean's Graduate Stipend Award, Summer 2014 ($3,000)
College of Liberal Arts competitive summer stipend awarded to five students in recognition of their academic and research productivity. Three students from each department within the college may be nominated each year.

University of Nevada, Las Vegas Graduate and Professional Student Association Travel Grant, June 2014 ($285)
University-wide grant to aid students to travel to conferences to present their research. Awarded a grant to cover the cost of conference registration for the 2014 International Behavioral Neuroscience Society annual conference in Las Vegas, NV (June 2014).

University of Nevada, Las Vegas Graduate and Professional Student 2014 Research Forum Oral Presentation Winner, 2nd place, May 2014 ($150)
Graduate students awarded travel and research grants, approximately 150 total presenters, are invited to present their research at a university wide research forum. GPSA awards cash prizes for top presentations.

University of Nevada, Las Vegas Graduate and Professional Student Association Travel Grant, November 2013 ($325)
University-wide grant to aid students to travel to conferences to present their research. Awarded a travel grant to offset part of the cost to attend and present at the 2013 Society for Neuroscience annual conference in San Diego, CA.

University of Nevada, Las Vegas Graduate and Professional Student Association Travel Grant, October 2012 ($500)
Awarded a travel grant to attend and present at the 2012 Society for Neuroscience conference in New Orleans, LA.

TEACHING EXPERIENCE

Physiology of Psychology (Neurobiology)
Department of Psychology, University of Nevada, Las Vegas
- Fall 2014, 2 courses; Spring 2015, 2 courses
- Full responsibility for all aspects of the course, including preparing lectures, giving lectures, grading, conducting sheep brain dissection lab, and holding office hours. Additional emphasis on reading and understanding peer-reviewed journal articles and writing their own science communications. *Each class size: 40 students*

Introductory Psychology
Department of Psychology, University of Nevada, Las Vegas
- Fall 2013, 2 courses; Spring 2014, 2 courses

- Full responsibility for all aspects of the course, including preparing lectures, giving lectures, grading, and holding office hours. *Each class size: 35 students*

RESEARCH EXPERIENCE

UNLV Neurobiology of Disease and Behavior Laboratory, Aug. 2011–August 2018
University of Nevada, Las Vegas; Advisor: Jefferson Kinney, Ph.D.
Doctoral student, senior researcher, and laboratory manager. Our lab primarily investigates the neurobiology of learning and memory with an emphasis on the biological basis of neurological/psychological diseases such as Alzheimer's disease and schizophrenia. Details of specific research projects listed below. As a graduate student, responsibilities include proposing, planning, and conducting experiments. Experiments include a variety of techniques including brain surgery, behavioral tasks of learning and memory, and biological assays to look at harvested brain tissue.

Nevada Institute for Children's Research & Policy, May 2015–August 2015
Graduate level research assistant for the Partnership in Community Health (PICH), a CDC-funded grant, with the Southern Nevada Health District. PICH aimed to increase public access to smoke-free multi-housing properties and venues and to increase the access to mobile health management tools for patients with Type-II Diabetes Mellitus.

UNLV Behavioral Neuroscience Laboratory, Jan. 2009–July 2011
University of Nevada, Las Vegas; Supervisor: Jefferson Kinney, Ph.D.
Undergraduate research assistant in a behavioral neuroscience laboratory under the direction of Dr. Jefferson Kinney. Primary responsibilities included running animals in behavioral tasks of learning and memory and biological assays on harvested brain tissue.

UNLV Immunotoxicology Research and Core Laboratory, Spring 2011
University of Nevada, Las Vegas; Supervisors: Deborah Keil, Ph.D
Assisted with Nellis Dunes Dust Project, a study on the immunotoxic effects of inhaling dust from the Nellis Dunes recreation area in Southern Nevada.

Nevada Desert Research Center, Summer 2007
University of Nevada, Las Vegas; Supervisors: Stan Smith, Ph.D. & Dene Charlet, Ph.D.
Undergraduate research assistant for the FACE (Free-Air-Carbon dioxide-Emission) project. The FACE project was a joint effort between the Department of Energy and UNLV to study the effects of carbon dioxide levels on the desert ecosystem.

RESEARCH SKILLS

Behavioral Techniques
- Extensive experience with the appropriate care and use of animal subjects, including rats, mice, and ground squirrels
- Skilled in research design, implementation, and data analysis in animal learning
- Expertise in several behavioral tasks with rodents including the Morris water maze, Barnes maze,

radial arm maze, cued and contextual fear conditioning, acoustic startle, pre-pulse inhibition, open field, novel object recognition, odor recognition, tail flick nociception task
- Skilled in a rodent blood collection procedures including tail vein and retro-orbital eye
- Experienced in rodent breeding and sexing
- Proficient in psychopharmacology experimental design, implementation, and analysis
- Skilled in a multi-tiered behavioral phenotyping procedure to examine transgenic and knockout mice. The strategy includes an initial examination of neurological reflexes and progresses to more complex behaviors
- Skilled in handling hibernating species, and bringing them in and out of torpor
- Experience in monitoring various bodily parameters including blood glucose levels and body temperatures
- Skilled in multiple routes of drug administration including intraperitoneal, subcutaneous, oral gavage, intravenous, and intracranial.

Bench Techniques
- Experienced in aseptic surgical techniques
- Proficient in stereotaxic surgical procedures, including chronic cannula placement and osmotic mini-pump implantation in rats
- Experienced in multiple wound closure techniques associated with stereotaxic surgery, including suture closure and dental acrylic application
- Considerable expertise in neural tissue collection, including transcardiac perfusion and dissection of specific brain structures
- Experienced in the frozen and fixed sectioning of neural tissue for histological analyses
- Extensive experience utilizing immunohistochemistry techniques, including the use of DAB and immunofluorescence
- Proficient in light and fluorescent microscopy, including conventional and confocal microscopes
- Expertise in tissue homogenization and protein concentration assays
- Skilled in western blotting and gel electrophoresis techniques
- Extensive experience on multiple western blot imaging techniques, including the Typhoon variable mode imaging system, the Odyssey IR Imaging system, and the UVP imaging system
- Trained in labeling techniques for demonstration of neurogenesis utilizing BrdU
- Experienced in programming of software for behavioral testing
- Proficient with ELISA technique and interpretation
- Skilled in RT-PCR and qPCR including end-point and real-time
- Trained in basic genotyping techniques

PEER-REVIEWED PUBLICATIONS

9. **Murtishaw AS,** Heaney CF, Bolton MM, Belmonte KCD, Langhardt MA, Kinney JW. Intermittent streptozotocin administration induces behavioral and pathological features relevant to Alzheimer's disease and vascular dementia. Neuropharmacology 2018 (Accepted).

8. Baer M, Klemetson B, Scott D, **Murtishaw AS**, Navalta JW, Kinney JW, Landers MR. The effects of fatigue on balance in individuals with Parkinson's disease: influence of medication and Brain-derived neurotrophic factor genotype. Journal of Neurologic Physical Therapy 2018.

7. **Murtishaw AS**, Heaney CF, Bolton MM, Langhardt MA, Kinney JW**.** An acute inflammatory response improves learning and memory deficits and reduces pathological markers in a diabetes animal model of Alzheimer's disease. Neuropharmacology 2016; 101:110–122.

6. Bolton MM, Heaney CF, **Murtishaw AS,** Sabbagh JJ, Magcalas CM, Kinney JW. Postnatal alterations in $GABA_B$ receptor tone produce sensorimotor gating and protein level differences in adulthood. International Journal of Developmental Neuroscience 2015; 41:17–27.

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

4. Bolton MM, Heaney CF, Sabbagh JJ, **Murtishaw AS,** Kinney JW. Deficits in emotional learning and memory in an animal model of schizophrenia. Behavioral Brain Research 2012; 233(1):35–44.

3. Heaney CF, Bolton MM, **Murtishaw AS,** Sabbagh JJ, Magcalas CM, Kinney JW. Baclofen administration alters fear extinction and GABAergic protein levels. Neurobiology of Learning and Memory 2012; 98(3):261–271.

2. Sabbagh JJ, Heaney CF, Bolton MM, **Murtishaw AS,** Ure JA, Kinney JW. Differences in the effects of galanin and donepezil following changes in cholinergic tone. International Journal of Neuroscience 2012; 122(12):742–747.

1. Sabbagh JJ, Heaney CF, Bolton MM, **Murtishaw AS,** Kinney JW. Examination of spatial learning deficits in an animal model of schizophrenia. Physiology and Behavior 2012; 107(3):355–363.

MANUSCRIPTS IN PREPARATION

5. **Murtishaw AS,** Bolton MM, Boren AJ, Toughlian JE, Ortiz AO, Bergman HO, Tran CM, Salazar AM, Kinney JW. Differential effects of two diabetic models, high-fat diet and streptozotocin, on tau-related pathology in CX3CR1 knockout mice (Preparation in progress).

4. Kinney JW, Bemiller S, **Murtishaw AS**, Leisgang A, Salazar AM, Lamb B. Inflammatory markers in Alzheimer's disease (Submitted to Alzheimer's & Dementia: Translational Research & Clinical Interventions).

3. Bolton MM, **Murtishaw AS**, Heaney CF, Langhardt MA, Kinney JW. Evaluation of ketamine-induced changes in spatial working memory and GABAergic systems. Submitted to Neuropsychopharmacology and Biological Psychiatry.

2. Bolton MM, **Murtishaw AS**, Heaney CF, Langhardt MA, Kinney JW. Interactions of ketamine administration and mTOR signaling on parvalbumin positive neurons. Submitted to International Journal of Neuroscience.

1. *Hensleigh E, ***Murtishaw AS,** Treat M, Heaney CF, Bolton MM, Sabbagh JJ, Kinney JW, van Bruekelen F. The effect of torpor on spatial memory in ground squirrels (Spermophilus lateralis) throughout a hibernation season. Submitted to Animal Behavior. *=Co-First Authors

POSTER AND PLATFORM PRESENTATIONS

61. **Murtishaw AS,** Bolton MM, Boren AJ, Toughlian JE, Ortiz AO, Bergman HO, Tran CM, Salazar AM, Kinney JW. Effect of hyperglycemia in the APP/PS1 mouse model of Alzheimer's disease. Society for Neuroscience annual meeting. Washington, D.C., November 2017.

60. **Murtishaw AS,** Bolton MM, Boren AJ, Salazar AM, Toughlian JE, Ortiz AA, Kinney JW. High-fat diet induced insulin disruption in CX3CR1 knockout mice on dementia-related pathology. Society for Neuroscience annual meeting. Washington, D.C., November 2017.

59. Salazar AM, **Murtishaw AS,** Bolton MM, Kinney JW. Neuromodulators of inflammation in mouse administered with exogenous soluble gp130. Society for Neuroscience annual meeting. Washington, D.C., November 2017.

58. **Murtishaw AS,** Bolton MM, Boren AJ, Salazar AM, Toughlian JE, Ortiz AA, Kinney JW. Alterations of high-fat diet in CX3CR1 knockout mice on neuroinflammation, metabolic markers, and Alzheimer's disease-related pathology. Society for the Study of Ingestive Behavior annual conference. Montreal, Canada, July 2017.

57. Baer M, Klemetson B, Scott D, Navalta J, **Murtishaw AS**, Kinney JW, Landers M. The effects of fatigue on balance in individuals with Parkinson's disease: influence of medication and brain-derived neurotrophic factor genotype. American Physical Therapy Association, Combined Sections Meeting annual conference. San Antonio, Texas, February 2017.

56. **Murtishaw AS,** Bolton MM, Boren AJ, Kinney JW. The effects of insulin impairments in CX3CR1 knockout mice on dementia-related pathology and neuroinflammation. Society for Neuroscience annual meeting. San Diego, CA, November 2016.

55. Bolton MM, **Murtishaw AS,** Salazar AM, Calvin KN, Nagele RF, Bergman HO, Kinney JW. An evaluation of GABA-B receptors in modulation neuroinflammation. Society for Neuroscience annual meeting. San Diego, CA, November 2016.

54. **Murtishaw AS,** Bolton MM, Boren AJ, Kinney JW. GABA-B receptor modulation in a model of chronic inflammation. Southern Nevada COBRE 1st Annual meeting. Las Vegas, NV. October 2016.

53. **Murtishaw AS.** Central and peripheral disruptions to insulin signaling on behavior and pathology related to dementia. UNLV Neuroscience Journal Club. Las Vegas, NV, July 2016 [Oral presentation].

52. **Murtishaw AS**, Bolton MM, Heaney CF, Langhardt MA, Belmonte KCD, Boren, AJ, Calvin KN, Kinney JW. Effects of GABA-B receptor modulation in a model of chronic inflammation. International Behavioral Neuroscience Society annual meeting. Budapest, Hungary, June 2016.

51. **Murtishaw AS.** Understanding the role of diabetes in the development of Alzheimer's disease. UNLV Graduate and Professional Student Association Research Forum. Las Vegas, NV, March 2016 [Oral presentation].

50. **Murtishaw AS**, Heaney CF, Bolton MM, Belmonte KCD, Langhardt MA, Calvin KN, Boren AJ, Kinney JW. A novel administration of systemic streptozotocin leads to alterations relevant to vascular dementia and Alzheimer's disease. Society for Neuroscience annual meeting. Chicago, IL, October 2015.

49. Bolton MM, Heaney CF, **Murtishaw AS**, Langhardt MA, Kinney JW. Interactions of ketamine administration and mTOR signaling on parvalbumin-positive neurons. Society for Neuroscience annual meeting. Chicago, IL, October 2015.

48. Bolton MM, Heaney CF, **Murtishaw AS**, Kinney JW. Interactions of behavioral training and ketamine administration on changes in parvalbumin-positive neurons. International Behavioral Neuroscience Society annual meeting. Victoria, British Columbia, Canada, June 2015.

47. **Murtishaw AS**, Heaney CF, Bolton MM, Belmonte KCD, Langhardt MA, Kinney JW. An evaluation of peripheral insulin disruption on behavior, phosphorylated tau levels, and microglia activity. International Behavioral Neuroscience Society annual meeting. Victoria, British Columbia, Canada, June 2015.

46. **Murtishaw AS.** Type-II Diabetes Mellitus & Alzheimer's disease. Invited speaker. University of Phoenix Fourth Annual Research and Scholarship Symposium. Las Vegas, Nevada, May 2015 [Oral presentation].

45. Bolton MM, Heaney CF, **Murtishaw AS,** Langhardt MA, Kinney JW. Ketamine administration on changes in parvalbumin neurons in various behavioral measures. UNLV Graduate and Professional Student Association Research Forum. Las Vegas, NV, March 2015.

44. Heaney CF, Bolton MM, **Murtishaw AS,** Kinney JW. $GABA_B$ ligand dose-dependent changes in spatial learning and hippocampal GABAergic and plasticity proteins. UNLV Graduate and Professional Student Association Research Forum. Las Vegas, NV, March 2015.

43. **Murtishaw AS**. Insulin signaling disruption within the brain: Relevance to Alzheimer's disease. UNLV Graduate and Professional Student Association Research Forum. Las Vegas, NV, March 2015 [Oral presentation].

42. Bolton MM, Heaney CF, **Murtishaw AS,** Langhardt MA, Kinney JW. Interactions of behavioral training and ketamine administration on changes in parvalbumin neurons. American Chemical Society Southern Nevada Section Annual Research Competition. Henderson, NV, November 2014.

41. Heaney CF, Bolton MM, **Murtishaw AS,** Kinney JW. $GABA_B$ ligand dose-dependent changes in spatial learning and hippocampal GABAergic and plasticity proteins. American Chemical Society Southern Nevada Section Annual Research Competition. Henderson, NV, November 2014.

40. **Murtishaw AS,** Heaney CF, Bolton MM, Belmonte KCD, Hagins PM, Langhardt MA, Kinney JW. An investigation of insulin receptor disruption and chronic inflammation as risk factors of Alzheimer's disease. American Chemical Society Southern Nevada Section Annual Research Competition. Henderson, NV, November 2014.

39. Bolton MM, Heaney CF, **Murtishaw AS,** Langhardt MA, Kinney JW. Interactions of behavioral training and ketamine administration on changes in parvalbumin neurons. Society for Neuroscience annual meeting. Washington, D.C.,November 2014.

38. Heaney CF, Bolton MM, **Murtishaw AS,** Kinney JW. $GABA_B$ ligand dose-dependent changes in spatial learning and hippocampal GABAergic and plasticity proteins. Society for Neuroscience annual meeting. Washington, D.C., November 2014.

37. Langhardt MA, **Murtishaw AS**, Heaney CF, Bolton MM, Belmonte KC., Hagins PM, Kinney JW. Facilitation of $GABA_B$ receptor function modulates chronic inflammatory effects. Society for Neuroscience annual meeting. Washington, D.C., November 2014.

36. **Murtishaw AS,** Heaney CF, Bolton MM, Belmonte KCD, Hagins PM, Langhardt MA, Kinney JW. Chronic inflammation and insulin signaling perturbations in a diabetic model of Alzheimer's disease. Society for Neuroscience annual meeting. Washington, D.C., November 2014.

35. Heaney CF, Bolton MM, **Murtishaw AS,** Kinney JW. Evaluation of multiple doses of $GABA_B$ ligands on learning and memory. GABAergic Signaling in Health & Disease, Neuropharmacology 24^{th} annual meeting. Pentagon City, VA, November 2014.

34. Hagins PM, **Murtishaw AS,** Heaney CF, Bolton MM, Belmonte KCD, Langhardt MA, Kinney JW. Chronic inflammation in a diabetic model of Alzheimer's disease. Nevada IDeA Network of Biomedical Research Excellence Undergraduate Research Opportunity Program Poster Symposium. Las Vegas, NV, August 2014.

33. **Murtishaw AS,** Heaney CF, Bolton MM, Belmonte KCD, Hagins PM, Langhardt MA, Kinney, J.W. Chronic inflammation in a diabetic model of Alzheimer's disease. International Behavioral Neuroscience Society annual meeting. Las Vegas, NV, June 2014.

32. Bolton MM, Heaney CF, **Murtishaw AS**, Kinney JW. Developmental Alteration of $GABA_B$ Receptor Function Results in Behavioral Deficits in Adulthood. UNLV Graduate and Professional Student Association Research Forum. Las Vegas, NV, May 2014.

31. Heaney CF, Bolton MM, **Murtishaw AS**, Kinney JW. The effects of baclofen and phaclofen on performance in the Morris water maze. UNLV Graduate and Professional Student Association Research Forum. Las Vegas, NV, May 2014.

30. **Murtishaw AS.** Chronic inflammation in a diabetic model of Alzheimer's disease. UNLV Department of Psychology Research Highlights. Las Vegas, Nevada, March 2014 [Oral presentation].

29. **Murtishaw AS.** LPS-induced chronic inflammation in a model of sporadic Alzheimer's disease. UNLV Graduate and Professional Student Association Research Forum. Las Vegas, NV, March 2014 [Oral presentation].

28. Bolton MM, Heaney CF, **Murtishaw AS,** Langhardt MA, Kinney JW. Developmental alteration of $GABA_B$ receptor function results in behavioral deficits in adulthood. Society for Neuroscience annual meeting. San Diego, CA, November 2013.

27. Heaney CF, Bolton MM, **Murtishaw AS,** Kinney JW. The effects of baclofen and phaclofen on performance in the Morris water maze. Society for Neuroscience annual meeting. San Diego, CA, November 2013.

26. Langhardt MA, Bolton MM, Heaney CF, **Murtishaw AS,** Nagls S, Kinney JW. Evaluation of ketamine-induced changes in spatial working memory and GABAergic systems. Society for Neuroscience annual meeting. San Diego, CA, November 2013.

25. **Murtishaw AS,** Heaney CF, Bolton MM, Langhardt MA, Belmont KCD, Kinney JW. An acute LPS-induced inflammatory response in a diabetic model of Alzheimer's disease. Society for Neuroscience annual meeting. San Diego, CA, October 2013.

24. Belmonte KCD, **Murtishaw AS**, Heaney CF, Bolton MM, Kinney JW. An acute inflammatory response in a diabetic model of Alzheimer's disease. McNair Scholars Research Program Poster Symposium. Las Vegas, NV, October 2013.

23. Langhardt MA**,** Bolton MM, Heaney CF, **Murtishaw AS**, Kinney JW. Ketamine-induced deficits in working memory with relevance to schizophrenia. UNLV McNair Scholars Research Symposium. Las Vegas, NV, October 2013.

22. Belmonte KCD, **Murtishaw AS**, Heaney CF, Bolton MM, Kinney JW. An acute inflammatory response in a diabetic model of Alzheimer's disease. Nevada IdeA Network of Biomedical Research Excellence Undergraduate Research Opportunity Program Poster Symposium. Las Vegas, NV, August 2013.

21. Langhardt MA, Bolton MM, **Murtishaw AS**, Heaney CF, Kinney JW. Ketamine Induced Deficits in Working Memory with Relevance to Schizophrenia. University of California, Berkeley 21st Annual McNair Scholars Symposium. Berkeley, CA, August 2013.

20. Bolton MM, Heaney CF, Sabbagh JJ, **Murtishaw AS,** Magcalas CM, Kinney J.W. Comparison of postnatal ketamine dosage on behavioral deficits in adulthood. UNLV Graduate and Professional Student Association Research Forum. Las Vegas, NV, May 2013.

19. Heaney CF, Bolton MM, **Murtishaw AS**, Sabbagh JJ, Magcalas CM, Kinney JW. Changes in $GABA_B$ tone in development produces behavioral deficits in adulthood. UNLV Graduate and Professional Student Association Research Forum. Las Vegas, NV, May 2013.

18. **Murtishaw AS.** Ketamine-induced behavioral impairments and alterations in hippocampal GABAergic neuron distribution. UNLV Graduate and Professional Student Association Research Forum. Las Vegas, NV, May 2013 [Oral presentation].

17. **Murtishaw AS.** Acute inflammation in a diabetic model of Alzheimer's disease. UNLV Department of Psychology Proseminar. Las Vegas, Nevada, February 2013 [Oral presentation].

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

15. Heaney CF, Bolton MM, **Murtishaw AS**, Sabbagh JJ, Magcalas CM, Kinney JW. Changes in $GABA_B$ receptor tone in development produces behavioral deficits in adulthood. Sierra Nevada Chapter for Society for Neuroscience 4th Annual Research Symposium. Reno, NV, November 2012.

14. Bolton MM, Heaney CF, Sabbagh JJ, **Murtishaw AS**, Magcalas CM, Kinney JW. Comparison of postnatal ketamine dosage on behavioral deficits in adulthood. Society for Neuroscience annual meeting. New Orleans, LA, October 2012.

13. Heaney CF, Bolton MM, **Murtishaw AS**, Sabbagh JJ, Magcalas CM, Kinney JW. Changes in GABAB receptor tone in development produce behavioral deficits in adulthood. Society for Neuroscience annual meeting. New Orleans, LA, October 2012.

12. **Murtishaw AS**, Sabbagh JJ, Heaney CF, Bolton MM, Magcalas CM, Langhardt MA, Kinney JW. Ketamine-induced behavioral impairments and alterations in hippocampal GABAergic neuron distribution. Society for Neuroscience annual meeting. New Orleans, LA, October 2012.

11. Sabbagh JJ, **Murtishaw AS**, Heaney CF, Bolton MM, Magcalas CM, Kinney JW. Chronic calcium dysregulation produces cognitive deficits and biochemical changes relevant to Alzheimer's disease. Society for Neuroscience annual meeting. New Orleans, LA, October 2012.

10. Magcalas CM, Heaney CF, Bolton MM, **Murtishaw AS**, Sabbagh JJ, Kinney JW. Alterations in $GABA_B$ in development produce behavioral and protein changes in adulthood. Nevada IdeA Network of Biomedical Research Excellence Undergraduate Research Opportunity Program Poster Symposium. Las Vegas, NV, August 2012.

9. Bolton MM, Heaney CF, Sabbagh JJ, **Murtishaw AS**, Kinney JW. Comparison of an adult and developmental animal model of schizophrenia. UNLV Graduate and Professional Student Association Research Forum. Las Vegas, NV, May 2012.

8. Heaney CF, Bolton MM, **Murtishaw AS**, Sabbagh JJ, Magcalas CM, Kinney JW. An investigation of the effects of alterations of $GABA_B$ receptor function on learning and memory. UNLV Graduate and Professional Student Association Research Forum. Las Vegas, NV, May 2012.

7. **Murtishaw AS.** GABAergic alteration and behavioral impairments from ketamine: A possible mechanism for treatment resistant depression. UNLV Department of Psychology Proseminar. Las Vegas, Nevada, May 2012 [Oral presentation].

6. Bolton MM, Heaney CF, Sabbagh, JJ, **Murtishaw AS**, Kinney JW. Comparison of an adult and developmental animal model of schizophrenia. Society for Neuroscience annual meeting. Washington, D.C., 2011.

5. Heaney CF, Sabbagh JJ, Bolton MM, **Murtishaw AS**, Santa-Ana I, Kinney JW. An investigation of alterations in GABAergic tone in an animal model of schizophrenia. UNLV Graduate and Professional Student Association Research Forum. Las Vegas, NV, May 2011.

4. Heaney CF, Bolton MM, **Murtishaw AS**, Sabbagh JJ, Kinney JW. An investigation of the effects of alterations in GABAB receptor function on learning and memory. Society for Neuroscience annual meeting. Washington, D.C., November 2011.

3. Sabbagh JJ, Bolton MM, Heaney CF, **Murtishaw, AS**, Kinney JW. Deficits in emotional learning and memory in an animal model of schizophrenia. Society for Neuroscience annual meeting. Washington, D.C., November 2011.

2. Heaney CF, Sabbagh JJ, Bolton MM, **Murtishaw AS**, Santa-Ana I, Kinney JW. An investigation of alterations in GABAergic tone in an animal model of schizophrenia. Society for Neuroscience annual meeting. San Diego, CA, November 2010.

1. Sabbagh JJ, Heaney CF, Bolton MM, **Murtishaw AS**, Ure JA, Kinney JW. Donepezil and galanin interactions in learning and memory and a model of cholinergic loss. Society for Neuroscience annual meeting. San Diego, CA, November 2010.

GRANT SUBMISSIONS, SIGNFICICANT CONTRIBUTIONS

NIH P20 Center of Biomedical Research Excellence (COBRE)
"Center for Neurodegeneration and Translational Neuroscience"
PI: Jeffrey Cummings, M.D.; Project 3 Director: Jefferson Kinney, Ph.D.
Funded Fall 2015, $11 million grant

Faculty Opportunity Awards Program, University of Nevada, Las Vegas
"Evaluation of biomarkers in Alzheimer's disease animal models and clinical populations"
PI: Jefferson Kinney, Ph.D.
Not funded.

McKnight Endowment Fund for Neuroscience, Memory and Cognitive Disorder Award "An Investigation of Neuroinflammation in a Diabetic Model of Alzheimer's Disease"

PI: Jefferson Kinney, Ph.D.
Not funded.

SERVICE/OUTREACH

UNLV Best Teaching Practices Expo Committee Member & Judge, Jan. 2017
Asked to serve as a judge and on the planning advisory board for the first annual best teaching practices expo designed to highlight innovative teaching methods of various full-time and part-time instructors at UNLV.

SfN LGBT Social, Nov. 2017, Nov. 2016, Oct. 2015, Nov. 2014,
Sole organizer of an SfN LGBT Social held at the Society for Neuroscience Annual Conferences in 2014, 2015, and 2016. This event serves as a networking platform to bring LGBT neuroscientists together from across the globe to share research. This social was regularly attended by ~300 LGBT neuroscientists.

UNLV Psychology Department Research Panel, Sept. 2016
Invited to participate on a panel discussing experience regarding research and graduate school for newly admitted graduate students within the UNLV Psychology Department.

Graduate Student Success Panel, Aug. 2016.
Invited to participate in a university-wide panel for incoming graduate students on successful strategies to meet the various demands of graduate school.

Graduate Student Research Panel, Oct. 2015
Invited to participate on a panel discussing experience regarding research and graduate school for undergraduate students seeking advice on graduate school and for fellow graduate students.

IBNS Communications Committee, Dec. 2014–Present
The Communications Committee of the International Behavioral Neuroscience Society disseminates accurate and timely information concerning research in the field of behavioral neuroscience, to the scientific community, funding agencies, legislative authorities, and the general public.

IBNS Newsletter Guest Editor, Dec. 2014–Feb. 2015
Edited and contributed to the quarterly newsletter for members of the International Behavioral Neuroscience Society.

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Guest Lecturer and Science Consultant LAW 728 Bioethics, August 2014 – December 2014.
UNLV Boyd School of Law. Las Vegas, Nevada

Las Vegas Brain Bee, Feb. 2014–Present
Along with 6 other board members of the Nevada Brain Bee, we organized the first annual Las Vegas Brain Bee in 2014 and subsequent Brain Bees in 2015, 2016, and 2017. Funding was secured to send the winner to National Brain Bee in Baltimore, MD.

UNLV Honors Thesis Committee Member: Krystal Belmonte, 2014
Thesis title: "Diabetic model of Alzheimer's disease via intraperitoneal induction."

UROP Mentor: Patrick Hagins, Summer 2014
Served as mentor for Patrick Hagins, recipient of Nevada IDeA Network of Biomedical Research Excellence Undergraduate Research Opportunity Program.

IBNS Local Organizing Committee, Fall 2013–Summer 2014
Served as a member of the Local Organizing Committee for the International Behavioral Neuroscience Society 2014 Conference held in Las Vegas, NV. IBNS sponsored a Brain Safety Initiative, which included a brain awareness event at Wright Elementary and a fundraiser donation to Clark County School District for the Safe Routes to School Program to purchase bike helmets for underprivileged children.

UROP Mentor: Krystal Belmonte, Summer 2014
Served as mentor for Krystal Belmonte, recipient of Nevada IDeA Network of Biomedical Research Excellence Undergraduate Research Opportunity Program & McNair Scholar Summer Research Award.

McNair Mentor: Krystal Belmonte, Summer 2014
Served as mentor for Krystal Belmonte, recipient of McNair Scholar Summer Research Award.

Nevada Brain Bee Association, Fall 2013–Present
Founding member and Board member of the Nevada Brain Bee Association. NBBA is 501(c)(3) Non-profit organization founded in 2013. NBAA is a regional division of the International Brain Bee, which is a worldwide neuroscience competition for high school students.

Guest lecturer PSY 403 (Physiology of Psychology): Mechanisms of learning and memory.
UNLV, Las Vegas, Nevada, June 2013.

McNair Mentor: Michael Langhardt, Summer 2013
Served as mentor for Michael Langhardt, recipient of McNair Scholar Summer Research Award.

Experimental Student Council, **Neuroscience Emphasis Representative,** 2012–2016
ESC serves as a liaison between the graduate students and the faculty in the Experimental Psychology Department.

Q:UNLV, 2012–Present
Serve as a member of Q:UNLV. Q:UNLV is a council steered by UNLV's Vice-President to promote diversity and inclusion for the LGBTQ staff and faculty at UNLV.

Brain Awareness Week, 2012–Present
Organize local outreach programs as part of The Dana Foundation's Brain Awareness Week to promote brain safety and neuroscience awareness at numerous elementary schools in Las Vegas, Searchlight, Pahrump, and across Southern Nevada.

UNLV Graduate Neuroscience Association, 2011–Present

Founding member of GNA. GNA meets monthly to discuss recent advances and publications in neuroscience.

UNLV Neuroscience Journal Club, 2009–Present
As a graduate student member of the NJC my main focus is primarily to teach undergraduate students how to read and understand journal articles.

Anatomy & Physiology Tutor, 2008–2011
Tutored students in BIOL 348 (Human Anatomy) and BIOL 223 (Anatomy & Physiology I).

PROFESSIONAL MEMBERSHIPS

Society for the Study of Ingestive Behavior, 2015–Present
Alzheimer's Association, Desert Southwest Chapter, 2014–Present
Alzheimer's Association International Society to Advance Alzheimer's Research and Treatment, 2013–Present
National Organization of Gay and Lesbian Scientists and Technical Professionals, 2013–Present
International Behavioral Neuroscience Society, 2013–Present
Sierra Nevada Chapter of the Society for Neuroscience, 2009–Present
Society for Neuroscience, 2008–Present