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An *in vitro* Model of Alzheimer's Disease Using Streptozotocin and Glucose to Test the Ability of Metformin and Insulin to Alleviate Neurodegeneration

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By

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Abstract

Alzheimer's disease (AD), a neurodegenerative disease, leads to a decline in memory and other mental abilities severe enough to interfere with everyday activity. Recent research has found increased risk of developing AD in the presence of Type 2 Diabetes Mellitus (T2DM). Increased blood glucose levels have been positively correlated with accelerated cognitive decline, and growing evidence suggest defective insulin signaling pathways within the AD brain. In this study, embryonic cortical neurons were cultured and treated with streptozotocin (STZ) and high levels of glucose in order to produce a neurodegenerative model of AD. MTS assays were used to assess cell viability. Combination of glucose and STZ led to significantly more toxicity than in conditions of only STZ ($p < 0.05$). Next, metformin and insulin, two agents currently used to combat high glucose levels in diabetic patients, were used separately and in conjunction to test their abilities in relieving neuronal toxicity. As expected, insulin, which is thought to act as a growth factor, led to a significant decrease in toxicity in all experiments ($p < 0.05$). Administration of a 24 hour metformin pre-treatment to 130 mM glucose led to significantly less neuronal toxicity than without a pre-treatment ($p = 0.012$). Overall, there were no consistent observable effects of STZ or Metformin. Future experiments will aim to determine the mechanisms of action of metformin, STZ, and how metformin pre-treatment is able to exert its beneficial effects.

Commonly Used Abbreviations

2xTg-AD (Mutations in APP and PS1)
3xTg-AD (Mutations in APP, PS1, and MAPT)
(Aβ) Amyloid beta
(AD) Alzheimer's disease
(AMPK) Adenosine monophosphate kinase
(ApoE) Apolipoprotein E
(APP) Amyloid Precursor Protein
(AβO) Amyloid beta Oligomers
(BACE1) β-secretase
(BBB) Blood Brain Barrier
(CNS) Central Nervous System
(CSF) Cerebrospinal fluid
(GM) Growth media
(GSK3) Glycogen synthase kinase
(IFGM) Insulin free growth media
(IGF) Insulin-like growth factor
(IR) Insulin receptor
(IRS-1) Insulin receptor substrate-1
(MAPT) Microtubule associated protein tau
(NFT) Neurofibrillary tangle
(PI3K) Phosphoinositide 3-kinase
(PP2A) Protein phosphatase 2A
(PS 1, 2) Presenilin 1, 2
(ROS) Reactive oxygen species
(STZ) Streptozotocin
(T2DM) Type 2 Diabetes Mellitus

Table of Contents **Page Number**

Introduction

Overview

Over the years, the term dementia has been assigned to describe a collection of symptoms including a decline in memory and other mental abilities severe enough to interfere with everyday tasks (Alzheimer's Association 2014). Several types of dementia include: vascular dementia, resulting in cognitive decline thought to be caused by blocked or reduced blood flow to the brain; Lewy body dementia resulting in cognitive decline, hallucinations and sleep disturbances thought to be caused by abnormal aggregations of alpha-synuclein protein; and the most common type of dementia, Alzheimer's disease (AD), accounting for 63% of all cases (Alzheimer's Association 2014). These types of dementia are often referred to as neurodegenerative diseases, leading to a progressive and irreversible loss of neurons.

Recent research has shown the prevalence of Type 2 Diabetes Mellitus (T2DM) increases with age (Umegaki 2012). Further, studies have shown T2DM is a risk factor for dementia, specifically AD (Vignini et al. 2013; Umegaki 2012). The link between these two diseases may lie within disrupted insulin signaling pathways, decreased glucose metabolism, and insulin resistance (Butterfield, Domenico, and Barone 2014; De Felice, Lourenco, and Ferreira 2014; Vignini et al. 2013).

This study aimed to create a model of neurodegeneration using streptozotocin and high levels of glucose to mimic neuronal loss at levels similar to what is seen in AD. Initially, the T2DM drug metformin along with exogenous application of insulin, were tested *in vitro* in primary cortical neuronal cultures for their ability to relieve

neurodegeneration. Later experiments looked to determine a time of metformin pre-treatment that would lead to the greatest decrease in neuronal toxicity seen under conditions of glucose and/or streptozotocin.

Alzheimer's Disease overview

AD is officially listed as the sixth-leading cause of death in the United States and continues to grow as the population ages (Alzheimer's Association 2014). While other leading causes of death, such as stroke and heart disease have been on the decline, AD related deaths have been dramatically increasing (Alzheimer's Association 2014). Currently, AD affects over five million Americans, and over 35 million worldwide, making it an enormous health issue (Selkoe 2012). These numbers are expected to continue to grow as the population ages and other causes of death in late life continue to recede. Of persons aged 65 years and older, the disease has been estimated to affect 10-13% of the population, with the rate increasing exponentially with older age to 19% among 75-84 year olds, and nearly 47% among those 85 years and older (Han and Han 2014). The Alzheimer's Association states that the financial and emotional costs for care of AD patients will continue to rise in the absence of a therapeutic method to slow or stop the disease. Given the prevalence of dementia and AD, the developments of treatment and prevention strategies are crucial.

Prevention of AD, as with other diseases, depends primarily on the understanding of its pathology. The first steps toward uncovering this disease began over a century ago when Alois Alzheimer began observing his patient, Auguste D. In his description of Auguste's symptoms, he included progressive cognitive impairment, focal symptoms,

hallucinations and delusions (Maurer, Volk, and Gerbaldo 1997). Upon her death, Alzheimer was able to study the structure of her brain during autopsy. During examination, he described the prevalence of extracellular amyloid plaques and intraneuronal neurofibrillary tangles (Maurer, Volk, and Gerbaldo 1997). These two initial discoveries have become the two main hallmarks associated with Alzheimer's disease today.

One of the main difficulties associated with AD is that it cannot be diagnosed with certainty until a post-mortem examination. In most cases, by the time a probable AD diagnosis is determined the disease has been present in the individual for several years (Alzheimer's Association 2014). Through the course of the disease, an individual's progression from mild AD to moderate and then to severe AD can occur at different rates. Typically the stages begin with subtle cognitive deficits such as forgetting a name, then progress with detectable functional deficits along with increased cognitive deficits, and finally these symptoms culminate as clinical dementia (Kozauer and Katz 2013). The most common symptoms begin with difficulty in remembering new information; this is due to disruption in neuronal function originating in places involved with forming memories, such as the hippocampus. As neuronal loss spreads to other cortical areas, a wider range of symptoms including mood changes, trouble understanding spatial relationships, and problems with speaking or writing will arise (Alzheimer's Association 2011). In late stage AD, patients will often become bed-bound and reliant on around the clock care. The disease is associated with a median survival of 8-10 years after diagnosis,

and is considered a terminal illness, with death being the most typical result of the disease (Leuzy and Gauthier 2012).

In addition to the main two hallmarks of AD, neuroimaging of the hippocampus and cortical areas have shown increased brain atrophy. This brain shrinkage is accompanied with a decrease in cerebral metabolism, as seen in fluorodeoxyglucose-positron emission tomography (FDG-PET) scans (Gili et al. 2010). One study quantified volumes of cortical gray matter in the brains of 33 AD patients during autopsy (Mouton et al. 1998). Prior to patient death, researchers implemented the Mini Mental State Examination (MMSE), which has been determined to be a valid and reliable 30-point examination testing cognitive abilities. Cortical atrophy was 20-25% greater in AD brains when compared to controls, and there was a strong correlation between MMSE performance and cortical volume loss. Brain atrophy, along with amyloid plaques and neurofibrillary tangles throughout the brain, may be some of the factors involved in the cognitive decline seen in AD.

The symptoms arising from these pathologies include loss of short-term memory, difficulties in executing everyday activities, withdrawal from social life, and a decline in spatial reasoning and language (Zhao et al. 2014). Though there are no current pharmacological treatments to prevent these symptoms, some researchers have developed cognitive training tasks, such as face-name association and auditory-verbal tasks, specifically aimed at cognitive functions supporting the accomplishment of everyday tasks and independent living (Hosseini, Kramer, and Kesler 2014). This method of

improving cognitive performance in the early stages of AD can serve as a non-pharmacological, cost-effective option in the meantime.

Alzheimer's Disease Pathology

There are two main types of AD classified by the time they begin developing in an individual. The first type of AD, an inheritable early onset form, is autosomal dominant and appears in a younger age, accounting for approximately 1% of all cases (Alzheimer's Association 2014). The causative factors of this form of AD are inheritable mutations in the genes of presenilin 1 (PS1), presenilin 2 (PS2) and amyloid precursor protein (APP) (Selkoe 2012; Ewers et al. 2011). The second type, late onset AD, is the more common form and accounts for the majority of AD cases, though it is less well understood compared to the early-onset form. The strongest genetic risk factor of this late onset form is the presence of the apolipoprotein E (ApoE) ε4 allele, on chromosome 19 (Ewers et al. 2011). However, a mutation in the ApoE ε4 allele is not necessary to increase the risk of developing AD (Zhao et al. 2014). Along with age and genetics, other suspected risk factors include cardiovascular diseases, high cholesterol, type 2 diabetes, high blood pressure, physical inactivity and head trauma/ traumatic brain injury (Alzheimer's Association 2014).

Mutations in AD-causing genes directly affect the generation of amyloid beta (Aβ) protein by altering APP or the protease that cleaves this substrate (Hettich 2014). The prevalence of the plaque phenotype has lead to strong evidence for the amyloid hypothesis, which states that AD arises in part from a chronic imbalance between Aβ production and Aβ clearance in the brain (Najem et al. 2014). Support for this hypothesis

originated when cloning of presenilins showed direct relation to amyloid production, and presenilin mutations, in PS1 and PS2, increased the production of amyloid beta (Hardy and Selkoe 2002). However, some researchers are questioning aspects of AD, particularly the toxicity of Aβ compared to Aβ oligomers, suggesting the oligomers are more toxic (LaFerla 2010; De Felice, Lourenco, and Ferreira 2014; Oddo et al. 2005). It is important to note that Aβ can exist in several states (monomeric, oligomeric, fibrillar) and it is unknown which form of Aβ is, or if all are, responsible for the observed pathology (LaFerla 2010).

The extracellular plaque deposits of the Aβ peptide are known as the first hallmark of AD. The APP gene encodes for the β-amyloid precursor protein, which functions in brain development and various biological processes during adulthood (Selkoe 2012). Amyloid precursor protein (APP) is cleaved by β-secretase (BACE1) and γ-secretase, resulting in the formation of Aβ peptides. Cleaving of APP will result in a shorter chain of either 40 amino acids, the most abundant form, or a chain of 42 amino acids, the longer and less common form (Hubin et al. 2014). Aβ has been linked to synaptic dysfunction, neuronal connectivity disruption, and neuronal death in a brain region-specific manner (Murphy and LeVine 2010). It is also associated with the formation of reactive oxygen species (ROS), nitrogen species, induction of calcium-dependent excitotoxicity, and impairments of cellular respiration (Butterfield, Domenico, and Barone 2014).

In its 42 amino acid chain, Aβ is more likely to aggregate and lead to plaques (Hubin et al. 2014). Aβ has also been shown to interact with pathways regulating the

phosphorylation of the microtubule-associated protein tau, resulting in hyperphosphorylation that leads to an accumulation of neurofibrillary tangles (NFT). Specifically, *in vitro* models using neuronal cell lines, primary hippocampal and cortical neuron cultures have shown tau alterations and tau phosphorylation when synthetic Aβ was applied to cultures (Stancu et al. 2014). A similar study was able to provide evidence showing that Aβ oligomers, prepared either *in vitro* or extracted from AD brains stimulated tau hyperphosphorylation (De Felice et al. 2008). Hippocampal neurons were treated with different concentrations of Aβ oligomers or Aβ fibrils, after which western blotting and immunocytochemistry assays were performed. Results from this experiment showed high levels of tau phosphorylation at Threonine (Thr) 231, which can be used to distinguish between AD and non-AD subjects which do not have high levels of tau phosphorylation at Thr231; the amount of tau phosphorylation was verified by quantitative immunofluorescence microscopy (De Felice et al. 2008). Though these studies, as well as others, have shown some evidence suggesting a link between the Aβ and tau pathology, the exact role of Aβ's involvement in AD is still unclear and debated. Questions regarding how Aβ may lead to neurodegeneration, why Aβ clearance is impaired in AD, and why Aβ amounts cannot be correlated with severity of AD, are still being researched.

Starting in 1985, immunocytochemical and biochemical analyses of NFTs showed tangles were composed of microtubule-associated protein tau (Selkoe 2001). Tau expression is high in regions of the brain involved in memory consolidation such as the hippocampus, which is also important in the developing brain (Hampel et al. 2010). In its

normal state, phosphorylated tau protein will stabilize axonal microtubules, aid in neurite growth, transport axoplasm, and promote axonal and synaptic plasticity in the developing brain (Zhao et al. 2014; Hampel et al. 2010). However, under abnormal conditions, such as those seen with AD, tau may undergo hyperphosphorylation. Experiments involving neurofibrillary degeneration show the amount of brain regions affected by tau are positively correlated with progression of the disease (Delacourte et al. 1999). In the hyperphosphorylated state, tau will detach from microtubules leading to cytoskeletal collapse. Hyperphosphorylation of tau is thought to lead to the destabilization of microtubules, and eventually impairments in axonal transport, as well as neuronal dysfunction (Hampel et al. 2010; LaFerla 2010). Mandelkow and colleagues (2003) showed the capability of tau to reduce net anterograde transport of vesicles and cell organelles, away from the neuronal cell body to the synapse, by blocking microtubule tracks. Consequently, this may lead to inadequate nutrient flow to synapses and synaptic dysfunction.

While the exact role of Aβ and NFT's in the development of AD is unclear, recent research has shown there may be a link between the two. One study, done by Oddo and colleagues in 2005, looked at the interaction between Aβ oligomers and tau pathology in an *in vivo* model using 3xTg-AD (mutations in APP, PS1, and MAPT (microtubule associated protein tau)) mice. Oddo and colleagues (2005) found there was co-localization of the Aβ oligomers (suspected to be the most toxic form of Aβ) with early somatodendritic tau pathology, but not late hyperphosphorylated tau. This co-localization pattern may be indicative of Aβ oligomers contributing to the development of tau

pathology, however this is not certain. Further, an injection of an Aβ oligomeric-specific antibody into the hippocampus of 12-month-old 3xTg-AD mice led to the removal of Aβ oligomers and clearance of early tau pathology; uninjected mice and vehicle injected mice showed no such clearance (Oddo et al. 2005). This research led to the hypothesis that the development of the tau pathology lies downstream of Aβ in the neurodegenerative cascade, indicating it may be triggered as a consequence of the Aβ pathology (LaFerla 2010). These results suggest a link between the main to hallmarks of AD, however, until it is determined whether there is a definite connection, therapeutic intervention for the disease should be aimed at halting both pathologies.

Though these two pathologies have shown their extensive role in AD, there are yet many other pathways suspected to play a role in the development of this disease. There is a growing body of evidence pointing toward defective insulin signaling pathways as a cause, or a risk factor, for developing AD. Disruption of the insulin pathway may contribute to the development of AD pathology. As a result, an emerging field of Alzheimer's research is looking at the relationship between AD and Diabetes Mellitus (De Felice, Lourenco, and Ferreira 2014; Vignini et al. 2013; de la Monte and Wands 2008).

Insulin signaling – The link between AD and T2DM

Neurons have a constantly high glucose demand in order to carry out regular brain functions, and cannot adapt to irregular glucose uptake under the influence of insulin (Tomlinson and Gardiner 2008). Under diabetic conditions of hyperglycemia, an excess of glucose in the bloodstream, neuronal glucose levels can increase up to fourfold; if

these levels stay persistently high, intracellular glucose metabolism leads to neuronal damage, known as glucose neurotoxicity (Tomlinson and Gardiner 2008). There are several suggested molecular mechanisms of glucose neurotoxicity: one is glucose-driven oxidative stress, which occurs through a combination of free-radical generation, while another is via intracellular signals, which activate MAP kinases. The negative effects of elevated glucose levels result in neuronal conduction abnormalities, impaired axonal regeneration, and altered ion fluxes (Tomlinson and Gardiner 2008). Under normal conditions, homeostatic mechanisms prevent potentially high glucose concentrations from accumulating by directing glucose into stores located in muscle, fat and liver tissue. The hormone insulin is primarily involved in normalizing rising blood glucose levels by signaling cells to take up glucose and store it as glycogen; because of this, insulin is vital to maintaining energy homeostasis within the body (Spielman and Klegeris 2014). When insulin is present, it is able to activate the GLUT4 glucose transporter. This activation allows GLUT4 to translocate to the cell membrane where it will allow for the uptake of glucose molecules.

Due to its role in energy homeostasis, insulin and insulin receptors (IRs) are found in high concentrations within the hypothalamus and hippocampus (Ghasemi et al. 2013). In recent years, it has become evident that insulin is able to cross the blood brain barrier (BBB), via an active transport mechanism, and exert its effects on neurons and other cells of the brain (though some insulin is also produced locally within the brain). One recent review done by Banks and colleagues (2012), focused on the relationship between insulin, glucose, the central nervous system (CNS), and the BBB. Initially it was thought

that the CNS was an insulin - insensitive tissue; however, it is now suspected that insulin is able to cross the BBB through a saturable transport system, involving saturation of insulin binding sites on endothelial cells which may represent transporters (Banks, Owen, and Erickson 2012; Vignini et al. 2013). Several research groups have tested this hypothesis by radioactively labeling insulin and confirming its ability to cross the BBB via a saturable mechanism (one of the main ways for peptides in the periphery to enter the brain).

One of the main diseases associated with insulin is diabetes mellitus. Type 1 diabetes mellitus (T1DM) is associated with hypoglycemia, low levels of glucose in the bloodstream, and an inability to produce insulin (Cnop et al. 2005). Typically, T1DM appears at an early age, is not associated with obesity, and requires daily insulin injections in order to utilize glucose. On the other hand, T2DM appears at a later age and can be associated with obesity. People with T2DM are able to produce insulin, however, there is an inadequate response to insulin known as insulin resistance (Fernandez-Real and Pickup 2012).

Within the CNS, insulin is expected to play a different role compared to its function in the periphery. Here, insulin seems to act as a growth factor by promoting synaptogenesis and nerve growth, which may be why insulin resistance is suspected to lead to cognitive decline (Nelson et al. 2008). CNS insulin may be playing an important role in learning and memory, promotion of cellular growth, differentiation, and at high concentrations may be involved in inflammatory activity (Spielman and Klegeris 2014; Butterfield, Domenico, and Barone 2014). Several behavioral studies have shown that

rats trained in the Morris water maze have altered IR patterns in the CA1 and CA3 regions of the hippocampus further suggesting insulin's role in memory. Under normal conditions, insulin in the brain may promote cell survival by inhibiting apoptosis-inducing peptides and enhancing neurite growth, synapse formation, and promoting normal mitochondrial function (Spielman and Klegeris 2014). In the absence of insulin or with a loss of insulin sensitivity, many pathways are disrupted, including the depolarization of mitochondria leading to excess ROS (Spielman and Klegeris 2014). Thus, disorders involving insulin pathway dysfunction are being studied in relation to neurodegenerative diseases.

The relationship between AD and Type 2 Diabetes Mellitus (T2DM) was first hypothesized in the Rotterdam study when Ott and colleagues showed that T2DM almost doubled the risk of dementia, particularly AD (Vignini et al. 2013). A subsequent study, done over the course of 9 years, examined a dementia-free cohort of human subjects with diabetes or borderline diabetes for the development of dementia (Xu et al. 2009). Results indicated that well controlled diabetes, defined as blood glucose levels of <7.8 mmol/l, was not significantly related to dementia risk (Xu et al. 2009). Borderline diabetes, defined as having blood glucose levels of 7.8-11.0 mmol/l, was related to an increased risk of dementia and AD (Xu et al. 2009). Uncontrolled diabetes, defined as having blood glucose levels >11.0 mmol/l, was associated with an increased risk of vascular and degenerative dementia (Xu et al. 2009). These results indicate controlling blood glucose levels might alleviate the detrimental effect of diabetes on dementia, and furthermore that glucose dysregulation may be involved in neurodegeneration.

Several other studies looking at the relationship between AD and elevated glucose levels, impaired glucose tolerance, and diabetes, have shown similar findings of an increased risk of developing AD with the presence of T2DM (Yarchoan and Arnold 2014). In 2013, Crane and colleagues showed blood glucose levels positively associated with accelerated cognitive decline, even without clinically diagnosed diabetes. This particular study was interested in determining whether higher glucose levels increased the risk of dementia in people with and without diabetes. Approximately 2,581 dementia-free participants, baseline age of 76 years old, were chosen and evaluated at 2-year intervals for development of dementia; other risk factors assessed include glucose levels, diabetes, and ApoE genotype (Crane et al. 2013). During the 6.8 year follow up period, dementia developed in 25.4% of the participants. In participants with diabetes and the highest glucose levels, risk for dementia was increased. For participants without diabetes, but glucose with levels between 95 mg/dl - 115 mg/dl, an increase in the average glucose level was correlated to an increase in Hazard Ratio for dementia, $p = 0.01$. Those with diabetes, defined as having glucose levels between 150 mg/dl - 190 mg/dl, had a similar increase in Hazard Ratio for dementia, $p = 0.002$. Results point toward higher glucose levels contributing to an increase risk of dementia through chronic hyperglycemia and insulin resistance (Crane et al. 2013). This long-term study allowed for the examination of how high glucose levels, even when not high enough to be diagnosed as diabetes, are associated with an increased risk of dementia. Evidence from this, and similar studies, suggests that brains of AD patients show cellular insulin resistance and insulin insufficiency (Yarchoan and Arnold 2014; de la Monte and Wands 2008).

The insulin signaling pathways are conserved in virtually all cell types that express the insulin receptor (IR), including human neurons (Yarchoan and Arnold 2014) [Figure 1]. When insulin binds to its cell surface IR, it activates intrinsic IR tyrosine kinase activity (De Felice, Lourenco, and Ferreira 2014). Once the IR is phosphorylated at the tyrosine residues, intracellular-signaling pathways lead to the activation of insulin receptor substrate-1 (IRS-1). This in turn leads to the activation of downstream effectors, such as phosphoinositide 3-kinase (PI3K). PI3K activates the Serine/Threonine kinase (PDK-1), which phosphorylates and activates AKT. AKT inhibits constitutively active glycogen synthase kinase-3 (GSK-3). Given that insulin receptors are widely expressed in the hippocampus, and insulin's involvement in learning and memory, it is possible that a disruption in insulin receptor signaling may lead to impairment in cognitive function.

In a normal brain, phosphorylation of insulin receptor-β subunit, IRS-1, AKT, and other insulin signaling proteins increases robustly with the presence of insulin; however, this signaling response is blunted in the AD brain (Yarchoan and Arnold 2014). Initial discoveries leading to the determination of diminished insulin signaling in AD brains came from post-mortem examination of abnormalities in the expression of genes encoding for insulin, IGF-1 (insulin-like growth factor), IRs and downstream signaling (de la Monte and Wands 2008). This is now referred to as insulin resistance, and is defined as inadequate response to insulin by target cells due to a down-regulated expression of IR, IGF-1 receptor, and IRS proteins (Butterfield, Domenico, and Barone 2014).

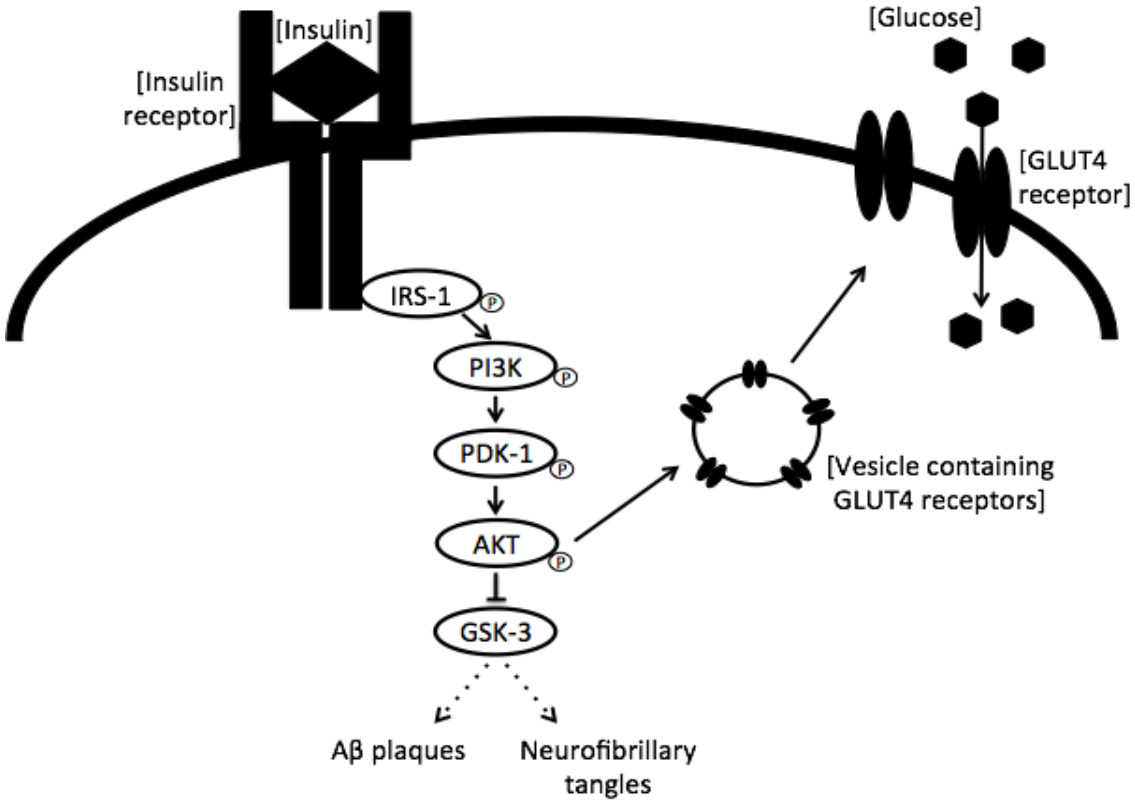


Figure 1. Insulin Signaling Pathway. The insulin receptor is composed of two extracellular α subunits and two transmembrane β subunits. Binding of insulin to the α subunit leads to a conformational change leading to autophosphorylation of tyrosine residues in the β subunit. Receptor activation leads to phosphorylation of tyrosine residues on the insulin receptor substrate 1 (IRS-1) protein, which then phosphorylates phosphoinositide 3-kinase (PI3K). PI3K phosphorylates PIP2 molecules, converting them to PIP3. PIP3 is then able to lead to the downstream phosphorylation of phosphoinositide-dependent kinase-1 (PDK-1). PDK-1 phosphorylates AKT, which leads to both the inhibition of GSK-3, and translocation of a vesicle containing the GLUT4 transporter to the cell membrane. The GLUT4 transporter then allows glucose to enter the cell. GSK-3, when not inhibited by AKT, is hypothesized to play a role in the development of AD pathology.

A hypothesis surrounding this research states hyperinsulinemia, meaning there are excessive levels of insulin in the blood relative to the amount of glucose, may induce brain insulin resistance by causing a reduction in IR expression and receptor kinase activity, promoting the development of Aβ and tau pathology. Additionally, insulin resistance may promote Aβ by increasing its secretion and preventing its degradation. Conversely, it may be that insulin resistance arises as a result of Aβ, These hypotheses came about due to the ability of Aβ to directly bind to the IR and because Aβ has been shown to activate GSK-3 (Yarchoan and Arnold 2014; Butterfield, Domenico, and Barone 2014).

The relationship between insulin abnormalities and AD has been studied looking closely at the pathologies involved in both. A study done by Wang and colleagues in 2010 evaluated the effect of insulin deficiency on APP processing and Aβ generation using a 2xTg (mutations in APP and PS1) mouse model treated with streptozotocin (a drug discussed in subsequent sections). Results indicated insulin deficiency reduced IR phosphorylation, promoting APP processing and therefore accelerating development of amyloid plaques (Wang et al. 2010). *In vitro* studies have also suggested that insulin may modulate Aβ release and degradation of the APP fragment (Craft and Watson 2004). This study, by Craft and Watson, examined differences of Aβ in human cerebrospinal fluid (CSF) 120 minutes after intravenous infusion of insulin or saline. The infusion of insulin led to an increase in Aβ concentrations, most apparently seen in older individuals. It was hypothesized that in younger individuals, there was an effective clearance of Aβ, which became ineffective in the older group (Craft and Watson 2004). These results may link

back toward the amyloid hypothesis stating that AD arises, in part, due to an imbalance between Aβ production and clearance.

There are hypotheses suggesting that the development of AD hallmarks, through abnormal insulin signaling, may be occurring via an increase in GSK-3 activity (Yarchoan and Arnold 2014). Under normal conditions, insulin stimulation will activate the signaling cascade IR/IRSs/PI3K leading to the phosphorylation of AKT, and thereby inhibiting GSK-3 activity [Figure 1]. However, under disrupted insulin signaling pathways, the inhibition of GSK-3 by AKT is removed. Under these conditions, GSK3 activity is linked to both hallmarks of AD: it is one of the main kinases involved in tau hyperphosphorylation, and it modulates the metabolism of Aβ (Ghasemi et al. 2013). Initial studies looking for tau kinases associated with microtubules found that GSK3 (initially called tau kinase I) was able to modify several sites of the tau protein in NFTs (Avila, Wandosell, and Hernandez 2010). This tau-site-specific phosphorylation was found to be present during the development of paired helical filaments found in tangles. A *Drosophila melanogaster* model of AD, with an overexpression of human wild-type tau, showed neurodegeneration and abnormal filaments (Avila, Wandosell, and Hernandez 2010). Replication in a mouse model using mutant GSK3 showed a reduction in brain volume and an increase in tau phosphorylation (Avila, Wandosell, and Hernandez 2010). These results suggest disruptive insulin signaling may lead to hyperphosphorylation of tau, leading to tangles in the brain, and ultimately the progression of AD pathology.

Experiments have also been done looking at the involvement of GSK3 in the production of Aβ. Specifically, GSK3α has been shown to regulate APP cleavage, resulting in increased production of Aβ (Hooper, Killick, and Lovestone 2008). Exposure of neurons to Aβ increased GSK3β activity through inhibition of PI3K signaling. GSK-3β phosphorylates PS1 at serine 353 and 357 residues, leading to an increase in the amount of Aβ-42, which aggregate into plaques (Maesako et al. 2012). The previously mentioned Aβ oligomers have also been shown to play a role in the activation of GSK-3β (Maesako et al. 2012; Jimenez et al. 2011). Other evidence has suggested that GSK3 may be able to modulate the generation and response to Aβ through interactions with PSs, specifically modifying localization and function of PS1; these experiments have shown the ability of PS1 to activate PI3K, therefore inhibiting GSK3 activity and tau hyperphosphorylation (Avila, Wandosell, and Hernandez 2010).

Though a definite mechanism of action has not been determined as of yet, it is expected that a disruption in insulin signaling allows GSK-3β to phosphorylate IR and IRS-1, leading to further impairment of insulin signaling (Ghasemi 2013). When GSK-3 activity is no longer inhibited, GSK-3β abnormally phosphorylates tau (Maesako et al. 2012). Further research into degenerative insulin pathway signaling is necessary to elucidate the precise mechanism by which it promotes AD.

Though the exact link between degenerative insulin signaling pathways and AD remains unclear, several hypotheses have suggested the main linking feature of the two is insulin resistance (De Felice, Lourenco, and Ferreira 2014; Yarchoan and Arnold 2014; Vignini et al. 2013). At the molecular level, insulin resistance is shown to occur when

IRS-1 is phosphorylated at inhibitory serine residues leading to its dissociation from the IR, blocking downstream insulin signaling (De Felice, Lourenco, and Ferreira 2014). Serine phosphorylation is common to both AD and diabetes. Looking to uncover the mechanism of insulin resistance in AD, Bomfim and colleagues (2012) examined brain tissue from AD patients and saw elevated levels of IRS-1pSer, analogous to what occurs in peripheral tissue of diabetes patients. In the periphery, it was observed that aberrant tumor necrosis factor–α (TNF-α) signaling lead to the activation of the stress kinase c-Jun N-terminal kinase (JNK), which phosphorylates IRS-1 at serine residues, blocking downstream insulin signaling. In primary rat hippocampal neuronal cultures treated with Aβ oligomers, it was found, through western blot analysis, that Aβ oligomers (AβO) activated the JNK/TNF- α pathway and induced IRS-1 phosphorylation at multiple serine residues (Bomfim et al. 2012). These findings indicate that there may be a similar pathway leading to insulin resistance in the periphery of diabetes patients and in the brains of AD patients, pointing at a linking factor between the two diseases.

In order to produce insulin resistance and diabetes like conditions in *in vivo* models, many research groups have used streptozotocin (STZ).

Streptozotocin to mimic T2DM conditions

Streptozotocin (STZ) is a nitrosamide methylnitrosourea linked to the C2 position of D glucose, which when metabolized is able to cause DNA damage through generation of reactive oxygen species, hydrogen peroxide and nitric oxide (de la Monte and Wands 2008). It functions mainly as an alkylating agent that damages insulin producing cells and insulin receptors, thereby causing insulin depletion (Yarchoan and Arnold 2014; Devi et

al. 2012). Because of this, STZ is typically used to generate diabetes-like conditions, making it a viable option to study the link between insulin resistance and AD. When exposed to intracerebral STZ, rats show impaired cognitive function and histopathological features of AD, including neurodegeneration, gliosis, abnormal GSK-3 activation and an increase in NFT and Aβ plaques (Yarchoan and Arnold 2014).

A recent study compared the most commonly used 3xTg mouse model of AD to a mouse model generated via an intracerebroventricular (icv) administration of STZ, the icv-STZ model. The icv-STZ model showed impairments in short-term memory, and in encoding and remembering spatial coordinates during the Morris water maze assessment (Chen et al. 2013). Compared to the 3xTg-AD model, there was a greater amount of neuroinflammation in the hippocampus of the icv-STZ model. Importantly, there was dysregulation in the brain insulin-signaling pathway of both models of AD. In the icv-STZ model, there was an upregulation of IRS-1 and PI3K, while PDK1 was downregulated (Chen et al. 2013). Overall, the icv-STZ model has been shown to be a suitable model for AD in regards to the insulin-resistant brain state (Chen et al. 2013; Salkovic-Petrisic et al. 2009).

Testing this further, a study made use of the icv-STZ model of AD and demonstrated that the insulin-resistant brain state is accompanied with mitochondrial abnormalities. Carried out on three-month-old Wistar rats, STZ administration showed a significant increase in hippocampal Aβ levels and hippocampal hyperphosphorylated tau protein levels (Correia et al. 2013). Within brain mitochondria, there were disruptions in ATP content, and a decrease in mitochondrial transmembrane potential, as well as an

increase in hydrogen peroxide levels (Correia et al. 2013). These mitochondrial abnormalities are an additional source of oxidative stress, which has been implicated in the pathology of both diabetes and AD (Butterfield, Domenico, and Barone 2014).

The use of STZ *in vitro,* will allow for greater examination of the pathologies linking Alzheimer's disease to diabetes. In 2008, de la Monte and colleagues looked specifically to connect the effects of icv-STZ to AD by characterizing neuropathology, molecular pathology, and abnormalities in gene expression related to insulin signaling. Administration of 50 mg/kg STZ, via intracerebral injection, to rats resulted in atrophied brains, with evidence of neurodegeneration, activated GSK-3β, phospho-tau and Aβ (de la Monte and Wands 2008). These adverse effects were all related to reduced expression of genes relating to insulin signaling, and have also been detected in AD brains. The disruptions in insulin signaling suggest it would be beneficial to use STZ *in vitro* to closely assess the underlying mechanisms.

Several studies implemented techniques looking at the effect of STZ within the brain. One such study examined the dendritic morphology of pyramidal neurons in the prefrontal cortex, occipital cortex, and hippocampus (Martinez-Tellez, Gomez-Villalobos, and Flores 2005). Brains were removed and analyzed using the Golgi-Cox stain; rats with STZ induced-DM showed a decrease in the dendritic length of pyramidal cells in the previously mentioned regions. The CA1 hippocampus region was the most affected with a 58% reduction in dendritic spines. A similar study made use of cresyl violet staining of brain sections to look at surviving neurons in the hippocampus (Pamidi and Nayak 2014). Similar results showed the CA1 region had a significant decrease in

surviving neurons as compared to normal controls. These results suggest using STZ to mimic DM conditions may affect regions within the brain involved in cognitive disorders, giving further insight into AD.

Having hippocampal degeneration upwards of 50% further strengthens the notion that STZ may be used as a drug not only to induce diabetes, but also AD. In addition, STZ, depending on its dose and route of administration, is able to reduce circulating insulin levels by 40-80% (Arroba et al. 2007). These characteristics of STZ, along with its ability to effect a wide range of cell populations, further suggest it may be used to study the link between AD and T2DM. For this research, I plan on combining STZ along with high levels of glucose to produce an *in vitro* model of Alzheimer's disease.

Metformin activity

Metformin, a biguanide, has been used as a drug therapy for the management of T2DM for over a decade in the US (Hundal and Inzucchi 2003). The blood glucose-lowering actions of metformin result primarily from amelioration of insulin resistance. In the liver, metformin works to reduce hepatic glucose output, due to a reduction in the rate of gluconeogenesis, with a small effect on glycogenolysis (Scarpello and Howlett 2008). Rodent studies have shown the ability of metformin to cross the BBB, however these results have not yet been confirmed in human studies (Yarchoan and Arnold 2014). Metformin has been shown to play a role in the activation of the enzyme adenosine monophosphate kinase (AMPK) to increase GLUT4, a glucose transporter, translocation in muscle and fat and reduce gluconeogenesis in liver (Scarpello and Howlett 2008). Several studies have suggested and supported the idea that the ability of metformin to

activate AMPK is necessary for the decrease in glucose production and the increase in fatty acid oxidation in hepatocytes (Zhou et al. 2001; Zou et al. 2004).

The supposed main modes of metformin action involve suppressing endogenous glucose production, countering insulin resistance, increasing glucose uptake, and activating adenosine monophosphate kinase (AMPK) (Scarpello and Howlett 2008; Chen et al. 2009; Hundal and Inzucchi 2003). Because metformin lowers glucose levels without increasing insulin secretion it has been considered an "insulin sensitizer" (Hundal and Inzucchi 2003). Recent studies have confirmed metformin's role in suppressant effects of glycation and oxidative stress (Scarpello and Howlett 2008). While the molecular mechanism of metformin is not yet understood, several have been proposed: activation of AMPK and inhibition of mTOR, inhibition of glucagon-induced elevation of cAMP and activation of PKA, and inhibition of the mitochondrial respiratory chain (Butterfield, Domenico, and Barone 2014). Studies using hepatocytes and mitochondria, found the hepatic glucose suppression ability of metformin is accompanied by inhibition of complex I in the mitochondrial electron transport chain; this is done via an unknown mechanism (Rena, Pearson, and Sakamoto 2013). Results have also indicated the magnitude of glucose production inhibition is correlated to the amount of inhibition of the respiratory chain. One hypothesized mechanism of action is as followed: as metformin inhibits the mitochondrial respiratory chain, a deficit in energy production will occur. This will lead to cells reducing their consumption of energy, leading to an increase in AMP concentration and then an increase in AMPK activity. This will lead to inhibition

of gluconeogenesis, which may contribute to the metabolic and therapeutic responses to metformin (Rena, Pearson, and Sakamoto 2013).

Much of the current research surrounding metformin is trying to delve deeper into its mechanism of action, particularly through the AMPK pathway. A major target capable of mediating a decrease in hepatic glucose production and an increase in skeletal myocyte glucose uptake via metformin, is AMPK, a multi-subunit enzyme recognized as a major regulator of lipid biosynthetic pathways (Zhou et al. 2001). AMPK has been shown to play a role in fatty acid oxidation, muscle glucose uptake, and expression of cAMP-stimulated gluconeogenic genes; also chronic activation of AMPK may induce the expression of GLUT4 (Zhou et al. 2001).

In primary cultured hepatocytes from Sprague Dawley rats, metformin activated AMPK in a concentration and time-dependent manner. After a 1-hour treatment, 500 μM metformin was required to significantly activate AMPK; however after 7 hours, 50 μM was sufficient for activation (Zhou et al. 2001). Overtime, the amount of metformin required for AMPK activation may decrease due to a build up of the drug. Further, through the use of an AMPK inhibitor, it was revealed that AMPK activation is required for the inhibition of hepatocyte glucose production by metformin. These effects were also assessed *in vivo* in Sprague Dawley rats. Rats were orally dosed with metformin or vehicle (water) for 5 days, starved for 20 hours, and then fed for 2 hours, upon which tissue and blood samples were obtained for analysis. The effect of metformin was analyzed specifically during re-feed conditions, during which hepatic lipid synthesis should have been dramatic (Zhou et al. 2001). Results suggested hepatic fatty acid

oxidation was induced in metformin-treated rats, along with decreases in plasma insulin. This study indicated the primary target of metformin activation as well as downstream results of AMPK phosphorylation.

A recent study looked at the effect of metformin on primary cultured neurons, a human cell line of AD and *in vivo* in mice (Hettich et al. 2014). The primary neurons were treated with or without 2.5 mM metformin for a 24-hour period, after which BACE1 activity was analyzed using a fluorometric β-secretase activity assay (Hettich et al. 2014). Compared to controls, metformin was able to significantly reduce BACE1 activity; this may have been accomplished through decreased BACE1 messenger RNA (mRNA), decreased protein levels or decreased BACE1 enzyme activity. The use of western blots revealed that BACE1 protein levels were significantly reduced; however, polymerase chain reaction (PCR) revealed BACE1 mRNA levels did not change. The experiments were replicated in an *in vivo* setting in mice for 2 weeks with 5 g/l metformin in drinking water. Results showed significantly reduce BACE1 protein levels, as tested through whole brain lysates on western blots. In addition, metformin was also shown to reduce Aβ formation, by reducing the APP cleavage products of BACE1 (Hettich et al. 2014). Another study done in 2009 by Chen and colleagues, in mouse primary cortical cultures, showed similar results in that metformin had a sensitizing effect on insulin, enhancing the anti-Aβ effect of insulin and therefore reducing Aβ generation.

In addition to affecting the first hallmark of AD, metformin has also been shown to play a role in tau pathology. One of the major tau phosphatases in the brain, protein phosphatase 2A (PP2A), has been shown to be reduced in activity and expression in AD

patients (Kickstein et al. 2010). This study looked at the ability of metformin to act on tau phosphorylation through the mechanistic target of rapamycin (mTOR) and PP2A signaling pathway using primary cortical neurons from the brains of wildtype and transgenic embryos. Because PP2A activity is regulated by mTOR, the group looked to inhibit mTOR activity, therefore increasing PP2A activity and reducing tau phosphorylation (Kickstein et al. 2010). After treatment of cortical neurons, western blot analyses indicated metformin-induced dephosphorylation of tau after 2-4 hours, and that dephosphorylation increased with time. Next, wanting to confirm that metformin requires PP2A activity to regulate the tau dephosphorylation, the group used an okadaic acid (OA) treatment to block PP2A activity. The OA treatment was able to entirely block the tau dephosphorylation potency of metformin, indicating PP2A must be an important mediator in the observed beneficial effects (Kickstein et al. 2010). Overall, the results indicated PP2A is regulated by mTOR, but it is still not completely clear whether metformin inhibits mTOR activity or activates PP2A activity. These results, in conjunction with the aforementioned, suggest the diabetic drug metformin may be used as a potential treatment for AD in combating both amyloid beta plaques and neurofibrillary tangles.

Present study

The initial component of this study was to produce a model of neurodegeneration using STZ and high levels of glucose to aid in understanding AD mechanisms. Concentration curves were used to determine dosages of glucose and STZ, which when used in conjunction would result in cell viability of approximately 50%. This

neurodegeneration model of AD is expected to work by causing glucose neurotoxicity and damage to the insulin signaling pathway. After damaging the existing insulin pathways with STZ, exogenous insulin was applied to cell cultures. We hypothesized application of insulin would help rescue neuronal survival by reducing extracellular glucose levels, and raise cell viability under the AD model back to control conditions.

These steps led to the proposed metformin study to determine whether the T2DM drug used *in vitro* would be able to alleviate neurodegeneration caused through the glucose/streptozotocin AD model. Initial experiments sought to gain familiarity with metformin: testing various concentrations, determining if high concentrations would produce toxicity, and looking to see how cell viability would compare between control conditions and metformin conditions. After initial experiments, we hypothesized that metformin applied at an experimentally established time point, might be able to aid in neuronal survival through preventing neural toxicity caused by high glucose levels and damaged insulin signaling pathways. Applying metformin at an earlier time point may allow cells to transcribe sufficient GLUT4 receptor proteins in order to prepare for high levels of exogenous glucose application.

Materials and Methods

Primary cortical neuron cultures

To prepare 96-well and 24-well plates for cell culture, poly-L-lysine was applied overnight; poly-L-lysine allows the cells (neurons) to adhere to the plate surface. Plates were subsequently washed three times with Hank's Balanced Buffer Solution (HBSS) to

remove poly-L-lysine residue. Next, plating media, prepared using neurobasal media (Gibco, REF: 21103-049), Penicillin Streptomycin (Gibco, REF: 15140-122), and Fetal Bovine Serum (FBS) (Gibco, REF: 16000-044), was applied to each plate before placing it into an incubator (37°C, 5% CO_2).

A pregnant female Sprague Dawley rat was sacrificed using a CO_2 chamber when fetuses reached embryonic day 17. Fetuses were then surgically removed, decapitated, and placed into dishes containing HBSS over ice. Next, frontal lobes were removed from each brain, and meninges were stripped and discarded. Lobes were dissociated using forceps in a fresh dish containing HBSS. Brain tissue was placed into a trypsin solution (Sigma Aldrich, REF: T4424) for five minutes to aid in further breaking down of tissue. Trypsin was removed by performing two HBSS washes for three-minute cycles. Next, the tissue was placed into a 15-mL conical tube containing 3 mL plating media and dissociated with a flame polished glass pipette until no visible clumps remained. Neuronal cultures were plated at a concentration of 1×10^6 cells/well in the poly-L lysine treated 96-well plates.

Growth media (GM), prepared with neurobasal media, b27 supplement (Gibco, REF: 17504-044), and Penicillin- Streptomycin, was used to feed neurons and replenish nutrients necessary for survival. Every other day, approximately 50% of the media was removed from each well and replaced with fresh, room temperature growth media. By removing only half of the media any beneficial factors secreted by neurons during growth were maintained. Neurons were grown for two weeks prior to experimentation (Table 1).

Table 1. Timeline of experimental procedure.

Day 1	Day 3	Day 5	Day 8	Day 10	Day 12	Day 14	Day 15
Dissection of SD rat, plating of 96-well plate. Fed with growth media.	Plates fed with growth media.	Plates fed with growth media.	Plates fed with growth media.	Plates fed with growth media.	Plates fed with growth media.	2-week-old cells stimulated for 24-hour period on 96-well plates.	MTS assay performed on 96-well plates.

Neuronal observations

Observations of plates were done under a compound light microscope to determine neural growth and changes in axons and dendrites. Most observations were done on feeding days and most importantly on days before stimulation to ensure proper growth and development of neurons.

MTS assay

MTS cell viability assays were performed to assess the cell survival rates for all experiments done on 96-well plates. An MTS assay (Promega, REF: G358B) is a chromogenic assay involving the metabolism of the compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) -2-(4-sulfophenyl)-2H tetrazolium (MTS). The reaction takes place in living cells when mitochondrial reductase enzymes are active. Initially, the MTS dye appears yellow, and once metabolized by living cells, the dye will turn a dark purple color. After a 1-3 hour incubation period, the plate is placed in a plate reader and optical intensity of each well is read at 490 nm. Readings are converted to percent

survival, as each condition is standardized to the growth media control condition, allowing for across the plate comparison of conditions.

Glucose concentration curve, Experiment 1

Initial experiments were done to determine concentrations of glucose to mimic the stress conditions seen in AD. Glucose, 150 mM, was prepared with 2.25 grams anhydrous dextrose (EMD Chemicals Inc., REF: DXO145-1) dissolved in 100 mL insulin free growth media (IFGM), prepared with neurobasal, Penicillin Streptomycin, and b27 supplement minus insulin (Gibco, REF: A18956-01); pH was maintained between 7.1-7.3. Concentrations of glucose between 100 mM - 150 mM, prepared by diluting 150 mM glucose in IFGM were tested under a 24-hour stimulation period and 48-hour period. Conditions were then tested with 100 nM aqueous insulin (Sigma Aldrich, REF: SLBF4191), and without insulin, to determine if insulin is able to alleviate toxicity caused by glucose. Experiments were performed on 96-well plates, and cell viability was assessed through MTS assay.

Metformin concentration curve, Experiment 2

A concentration curve was generated to test metformin (Fluka Analytical, REF: PHR1084) concentrations between 400 - 700 μM. A control amount of glucose, 130 mM was used for all conditions, as well as a control amount of insulin, 100 nm; metformin was added at the same time as all variables excepted during metformin pre-treatment (Experiment 7). The concentrations of metformin increased at 100 μM increments and stimulations occurred over a 24-hour time period. Cell viability was determined through MTS assay.

Model of neurodegeneration, Experiments 3 and 4

After two weeks of cell growth, STZ was tested at concentrations of 100 μM, 200 μM and 500 μM, with and without glucose. The main aim was to establish a model of neurodegeneration using a combination of STZ and glucose resulting in approximately 50% cell viability as observed through MTS assay. Once the concentration of STZ was established (100 μM), experiments were performed to test the ability of insulin alone to aid in cell survival in the presence of glucose and STZ (Experiment 3). Next, the model of neurodegeneration tested the ability of both Metformin and insulin, alone and conjunction, to aid in cell survival (Experiment 4). Cell survival was determined through MTS assays.

Metformin with STZ or Glucose, Experiments 5 and 6

In order to gain a better understanding of metformin's capabilities, experiments were performed looking at metformin with STZ as the only stressor, (Experiment 5). Next, experiments looked at metformin with glucose as the only stressor, (Experiment 6). Insulin (100 nM) was present various conditions during both experiments. Cell survival was determined through MTS assays.

Metformin pre-treatment, Experiment 7

After performing the previous experiments and determining the concentrations of glucose (130 mM), STZ (100 μM) and Metformin (400 μM), the next step was to determine a time where application of Metformin would lead to the greatest amount of cell survival. Time points of Metformin pre-treatment were: 24 hours prior, 12 hours prior, 5 hours prior, 3 hours prior, and directly prior to adding STZ/glucose stressors.

Experiments also tested the difference in cell survival between a 24 hour pre-treatment with Metformin and direct addition of Metformin before adding stressors. Stressors were applied for a 24-hour stimulation period, post pre-treatment. Cell viability was determined through MTS assay.

Statistical analysis

All data was analyzed using the Statistical Package for the Social Sciences (SPSS). Graphs were made using Microsoft Excel and error bars represent the standard error of the mean. Independent samples t-tests and multifactorial ANOVAs were the two main statistical analyses that were used.

Results

Experiment 1, Figure 2 – Glucose concentration curve

Initial experiments were carried out to establish a concentration of glucose in the absence of insulin to produce a model of neurodegeneration. These concentrations were tested at both a 24-hour period stimulation and a 48-hour period stimulation, [Figure 2]. Cell viability was assessed through an MTS assay.

A 1-way ANOVA looking at cell viability during the 24 hours time point concentrations of glucose (100 mM, 130 mM, 150 mM, 200 mM) showed a significant difference between groups ($p < 0.0005$), [Figure 2]. There was a main effect of insulin; cell viability in all conditions containing insulin was significantly higher than in those without insulin ($p < 0.0005$). There was also a main effect of glucose; all conditions with glucose had significantly lower cell viability than conditions without glucose ($p < 0.0005$). A Least Significant Difference (LSD) post hoc test showed cell viability with

100 mM glucose (*Mean ± Standard error of the mean;* 0.88 ± 0.04) was significantly higher than with 200 mM glucose (0.47; 0.11), ($p < 0.0005$). LSD post hoc test showed cell viability in the 130 mM glucose condition (0.51 ± 0.06) was significantly higher than the 200 mM condition (0.47 ± 0.11), ($p = 0.003$).

Next, a 1-way ANOVA looking at cell viability during the 48 hour time point for concentrations of glucose (100 mM, 130 mM, 150 mM, 200 mM) showed a significant difference between groups, ($p < 0.0005$), [Figure 2]. There was a main effect of insulin; cell viability in all conditions containing insulin was significantly higher than in those without insulin ($p < 0.0005$). There was also a main effect of glucose; all conditions with glucose had significantly lower cell viability than conditions without glucose ($p < 0.0005$). There was no significant difference in cell viability between concentrations of glucose ($p > 0.05$).

Finally, a 2-way ANOVA was performed to compare the concentrations of glucose (100 mM, 130 mM, 150 mM, 200 mM) between the 24 hour and 48 hour treatment times. There was no significant difference between the two time points, ($p = 0.102$), [Figure 2].

Based on the ability of 130 mM glucose to result in approximately 50% cell viability both in the 48 hour and 24 hour time points, it was chosen as the concentration for subsequent experiments. Also, the 24 hour treatment time was chosen because there was no significant difference in viability between the two time points.

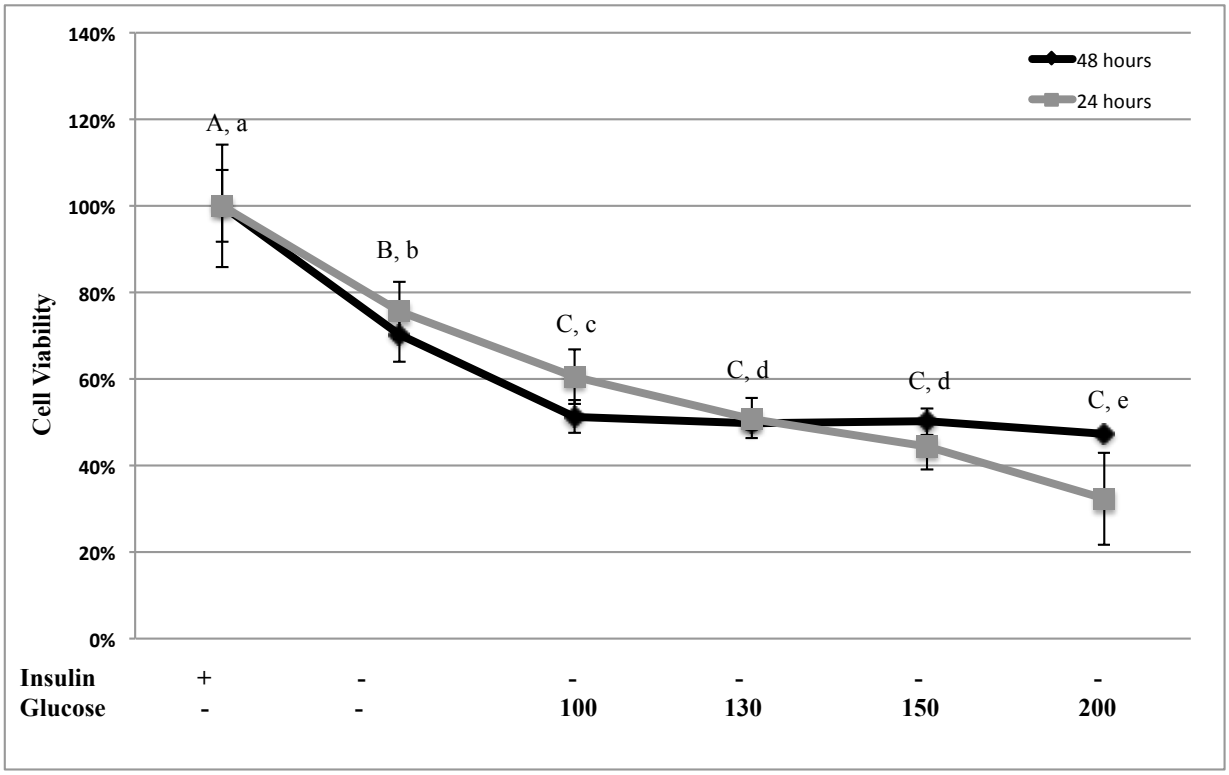


Figure 2. Glucose Concentration Curve in Insulin Free Growth Media. Glucose conditions measured in mM. Cell viability was measured through MTS assay; absorbance read at 490 nm. All conditions standardized to the initial control condition of growth media containing insulin. Error bars represent +/- standard error of the mean (SEM). Statistical significance ($p < 0.05$) indicated by difference in letter; capital letters represent 48 hour treatments, lower case letters represent 24 hour treatments. ($N \geq 12$ total experimental replications).

Experiment 2*,* Figure 3 – Metformin concentration curve

Experiments were done to determine a concentration of metformin, which would produce no cell death as compared to control conditions, [Figure 3]. A one-way ANOVA was performed to test difference between the 5 conditions of metformin, 0 μM metformin, 400 μM metformin, 500 μM metformin, 600 μM metformin and 700 μM metformin on cell survival. Insulin containing conditions had greater cell viability than those without insulin ($p < 0.0005$). Glucose containing conditions had significantly lower cell viability than those without glucose, ($p < 0.0005$).

There was a statistically significant difference between groups, ($p = 0.000$). LSD post hoc showed the condition of 400 μM metformin was significantly higher than 500 μM metformin ($p = 0.02$), 600 μM metformin ($p = 0.005$), and 700 μM metformin ($p = 0.001$), [Figure 3]. The 400 μM metformin condition was chosen because no significant decrease in cell viability was observed compared to the condition of 130 μM glucose without metformin.

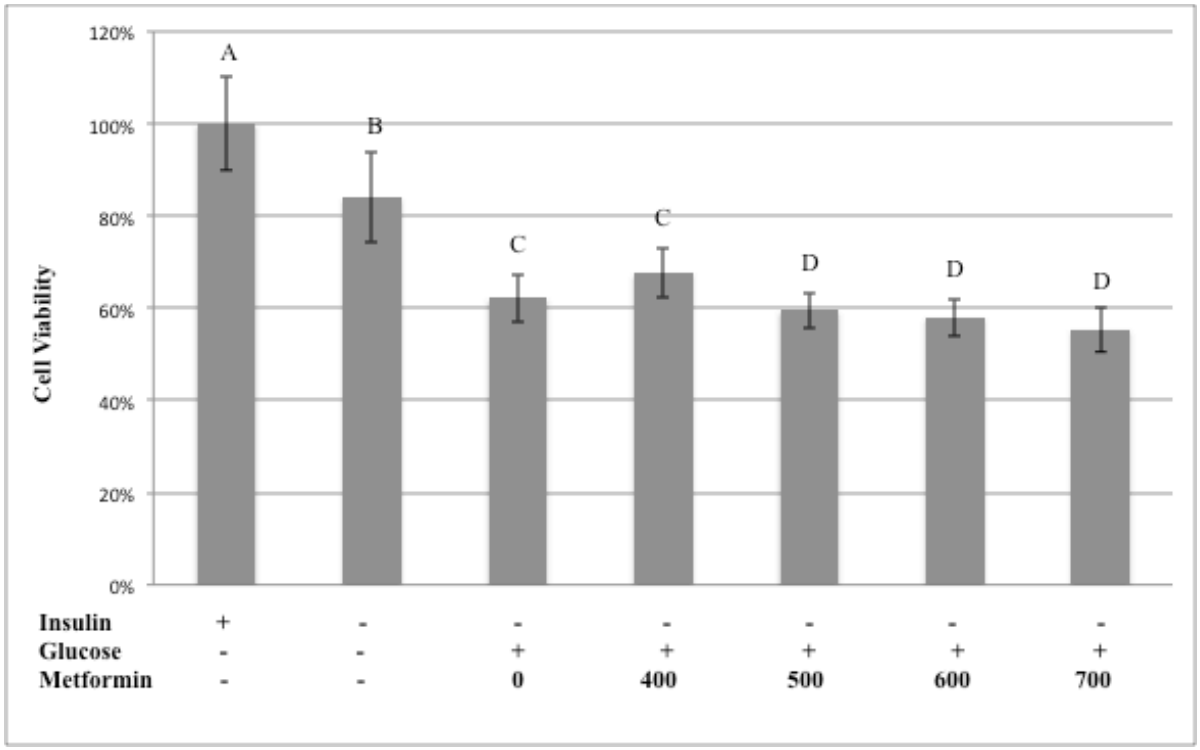


Figure 3. Metformin Concentration Curve with 130 mM Glucose. Metformin conditions measured in μM. Cell viability was measured through MTS assay; absorbance read at 490 nm. All conditions standardized to the initial control condition of growth media containing insulin. Statistical significance ($p < 0.05$) indicated by difference in letter. Error bars represent +/- SEM. ($N \geq 50$ replications per condition).

Experiment 3, Figure 4 – Insulin/Glucose/STZ

After a concentration of glucose (130 mM) was chosen, experiments were conducted to find a suitable amount of STZ. Based on previous research, done by Rachel Masia and Katelyn Cusmano in the Knowles lab, the STZ concentration curve tested 100 μM STZ, 200 μM, and 500 μM STZ in order to select 100 μM STZ as the most appropriate concentration. Glucose and STZ were then tested in conjunction to produce a diabetic model of AD [Figure 4]. Neuronal toxicity was analyzed using MTS assay.

A 2 (insulin conditions) x 2 (STZ conditions) x 2 (glucose conditions) 3 way ANOVA was performed [Figure 4]. There was a main effect of insulin; cell viability in conditions with insulin (0.79 ± 0.56) is significantly higher than those without insulin (0.62 ± 0.42), ($p \leq 0.0005$). There was a main effect of glucose; cell viability in conditions containing glucose (0.60 ± 0.42) was significantly lower than conditions without glucose (0.85 ± 0.58), ($p \leq 0.0005$). There was a trend seen with STZ; cell viability in conditions without STZ (0.77 ± 0.55) was higher than conditions with STZ (0.69 ± 0.46), ($p = 0.088$).

There was no significant interaction between insulin, glucose, or STZ ($p > 0.05$), so no further interpretation of results could be completed [Figure 4].

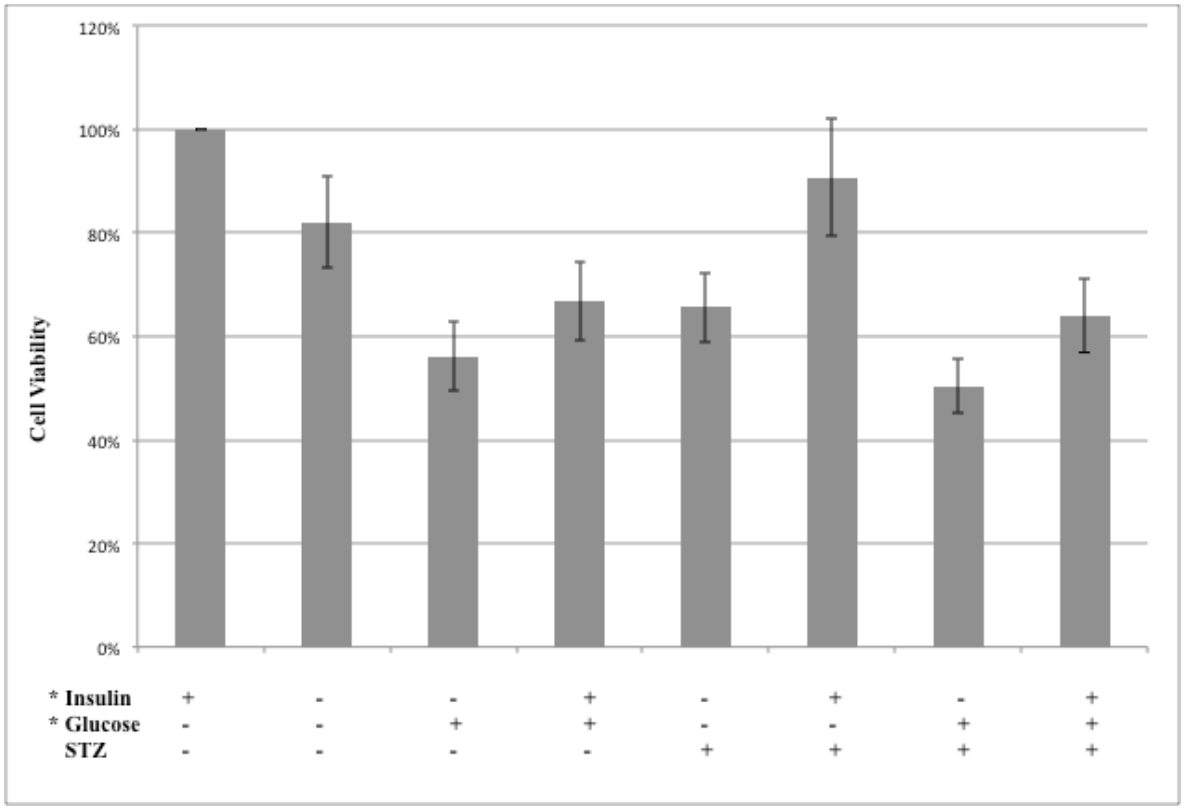
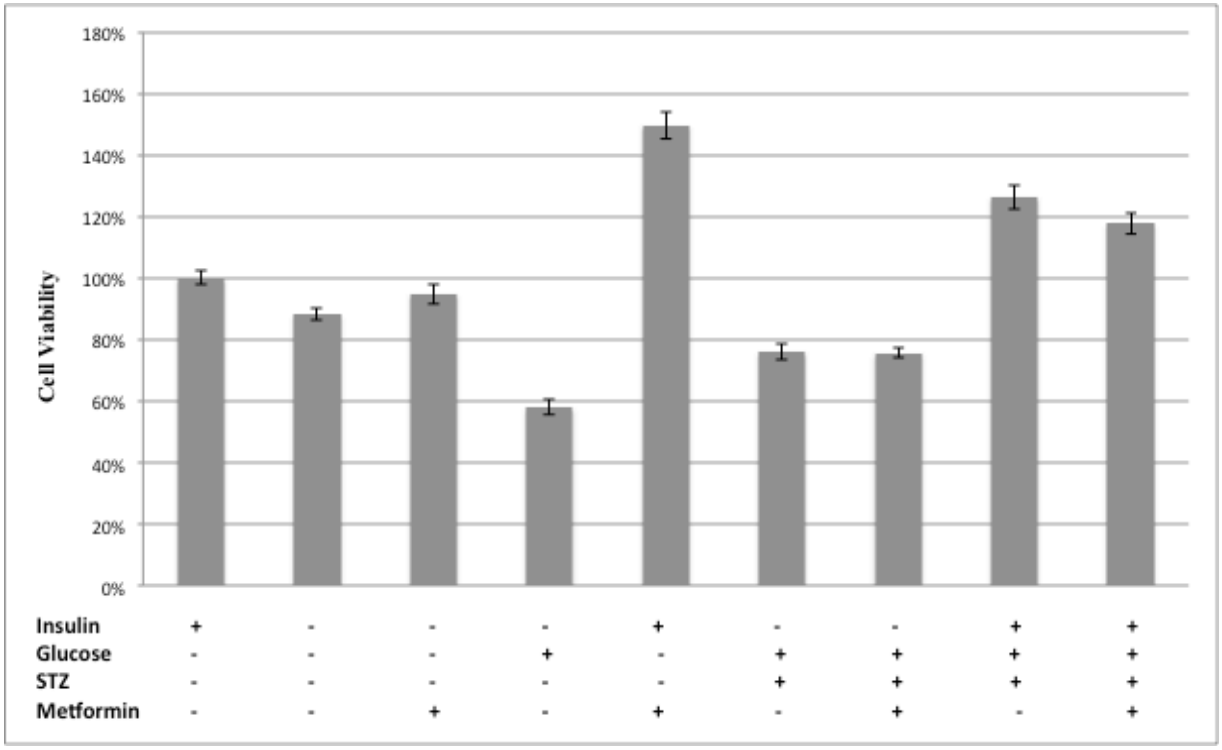


Figure 4. The Effect of 100 µM Streptozotocin With or Without 130 mM Glucose in Combination With or Without 100 nm Aqueous Insulin On Cell Viability in Cortical Neuronal Culture. Cell viability was measured through MTS assay; absorbance read at 490 nm. All conditions standardized to the initial control condition of growth media containing insulin. Error bars represent +/- SEM. ($N \geq 50$ replications per condition). * Signifies a main effect, $p < 0.05$.

Experiment 4, Figure 5A and 5B - Insulin/Glucose/STZ/Metformin

Next, we looked at cell viability in glucose and STZ conditions, with metformin, insulin, or with a combination of both. The goal was to determine whether metformin and/or insulin were able to decrease neuronal toxicity in the presence of glucose and/or STZ. Not all factorials were represented to perform a multifactorial ANOVA. A one-way ANOVA showed significant differences in cell viability between conditions ($p \leq 0.0005$) [Figure 5A]. LSD post-hoc analysis was used to look more closely at different conditions. There was a significant difference between insulin/metformin compared to all conditions except insulin/glucose/STZ, and insulin/glucose/STZ/metformin [Figure 5B]. There was a significant decrease in cell toxicity with the addition of insulin to the glucose/STZ condition, ($p = 0.001$) [Figure 5B]. There was also a significant decrease in cell toxicity when insulin was added to the condition of glucose/STZ/metformin, suggesting beneficial effects of insulin, and not metformin ($p \leq 0.0005$) [Figure 5B]. Addition of STZ to glucose did not exacerbate cell toxicity ($p > 0.05$), nor did addition of metformin to glucose/STZ lessen cell toxicity, ($p > 0.05$) [Figure 5B].

5A.



5B.

	I	C	M	G	I/M	G/S	G/S/M	I/G/S	I/G/S/M
I		-	-	0.03*	0.01*	-	0.08^	0.06^	-
C	-		-	-	0.001*	-	-	0.006*	0.028*
M	-	-		0.067^	0.006*	-	-	0.038*	-
G	0.03*	-	0.067^		0.000*	-	-	0.000*	0.001*
I/M	0.01*	0.001*	0.006*	0.000*		0.000*	0.000*	-	0.07^
G/S	-	-	-	-	0.000*		-	0.001*	0.004*
G/S/M	0.08^	-	-	-	0.000*	-		0.000*	0.000*
I/G/S	0.06^	0.006*	0.038*	0.000*	-	0.001*	0.000*		-
I/G/S/M	-	0.028*	-	0.001*	0.07^	0.004*	0.000*	-	

Figure 5A. The Effect of 100 µM Streptozotocin, With and Without 130 mM Glucose, With and Without 100 nm Insulin on Cell Viability, Separate and in Combination With a 400 µM Metformin Treatment. Cell viability was measured through MTS assay; absorbance read at 490 nm. All conditions standardized to the initial control condition of growth media containing insulin. Error bars represent +/- SEM. ($N \geq 23$ replications per condition).

Figure 5B. Pairwise comparisons based on Figure 5A.

I = Insulin; C= Control, no insulin/glucose/STZ/metformin; M= Metformin; G = Glucose; S= STZ. * Signifies significant difference ($p < 0.05$). ^ Signifies trend, ($p = 0.05$-0.09).

Experiment 5, Figure 6 – Insulin/STZ/Metformin

In order to look more closely at the effect of metformin on neuronal survival, experiments were done using STZ as the only stressor [Figure 6]. MTS assays were performed to assess cell viability. A 2 (insulin conditions) x 2 (STZ conditions) x 2 (metformin conditions) 3-way ANOVA was performed to compare main effects of variables. There was a main effect of insulin; cell viability in conditions containing insulin was significantly higher than in those without insulin ($p \leq 0.0005$). There was a main effect of STZ; cell viability in conditions containing STZ was significantly higher than in those without STZ ($p = 0.003$). An unexpected trend was seen with metformin; cell viability in metformin conditions was lower than viability without metformin ($p = 0.064$).

There was a significant interaction between insulin and metformin ($p = 0.015$), Figure 6. For conditions containing metformin, cell viability with insulin (1.11 ± 0.06) was significantly higher without insulin (0.88 ± 0.05) ($p \leq 0.0005$). For conditions containing insulin, cell viability with metformin (1.11 ± 0.06) was significantly lower without metformin (1.39 ± 0.11) ($p = 0.002$). There was a significant interaction between insulin and STZ ($p = 0.014$) [Figure 6]. For conditions with STZ, cell viability without insulin (0.87 ± 0.05) was significantly lower than with insulin (1.74 ± 0.16) ($p \leq 0.0005$). Thus, there was a beneficial effect of insulin, as previously seen, however there was no beneficial effect of metformin.

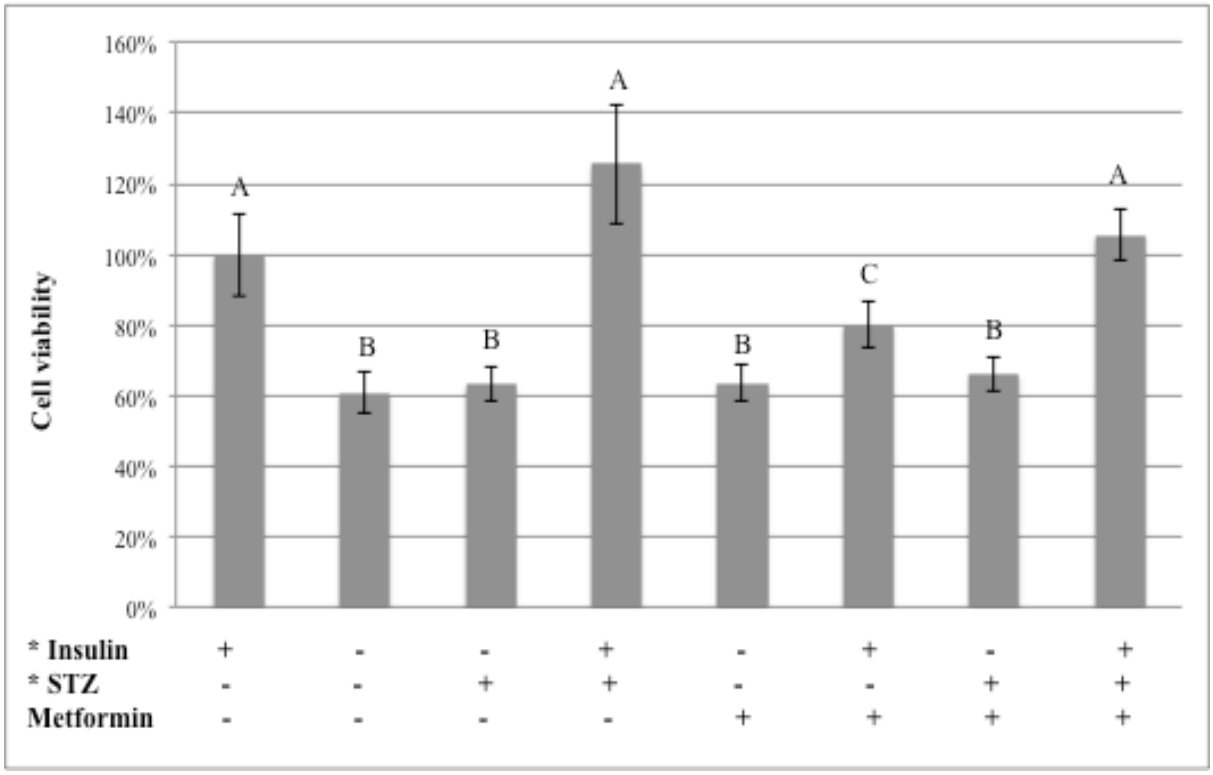


Figure 6. The Effect of 100 µM Streptozotocin on Cell Viability in Cortical Neuronal Culture With and Without Treatments of 100 nm Insulin and 400 µM Metformin. Cell viability was measured through MTS assay; absorbance read at 490 nm. All conditions standardized to the initial control condition of growth media containing insulin. Error bars represent +/- SEM. ($N \geq 48$ replications per condition). Statistical significance ($p < 0.05$) indicated by difference in letter. * Signifies a main effect, $p < 0.05$.

Experiment 6, Figure 7 – Insulin/Glucose/Metformin

Next, statistical tests were done to look at the effectiveness of metformin and insulin against glucose. A 2 (insulin conditions) x 2 (glucose conditions) x 2 (metformin conditions) 3-way ANOVA was performed to look at main effects [Figure 7]. As seen previously, there was a main effect of insulin; cell viability in conditions containing insulin was significantly higher than in those without insulin ($p \leq 0.0005$). There was a main effect of metformin; cell viability in metformin conditions was lower than viability without metformin ($p = 0.049$). There was no main effect of glucose ($p > 0.05$).

There was a significant interaction between insulin and metformin ($p = 0.009$) [Figure 7]. For conditions containing metformin, cell viability with insulin (1.02 ± 0.05) was significantly higher than without insulin (0.82 ± 0.04) ($p \leq 0.0005$). For conditions containing insulin, cell viability without metformin (1.36 ± 0.05) was significantly higher than with metformin (1.02 ± 0.05) ($p = 0.04$).

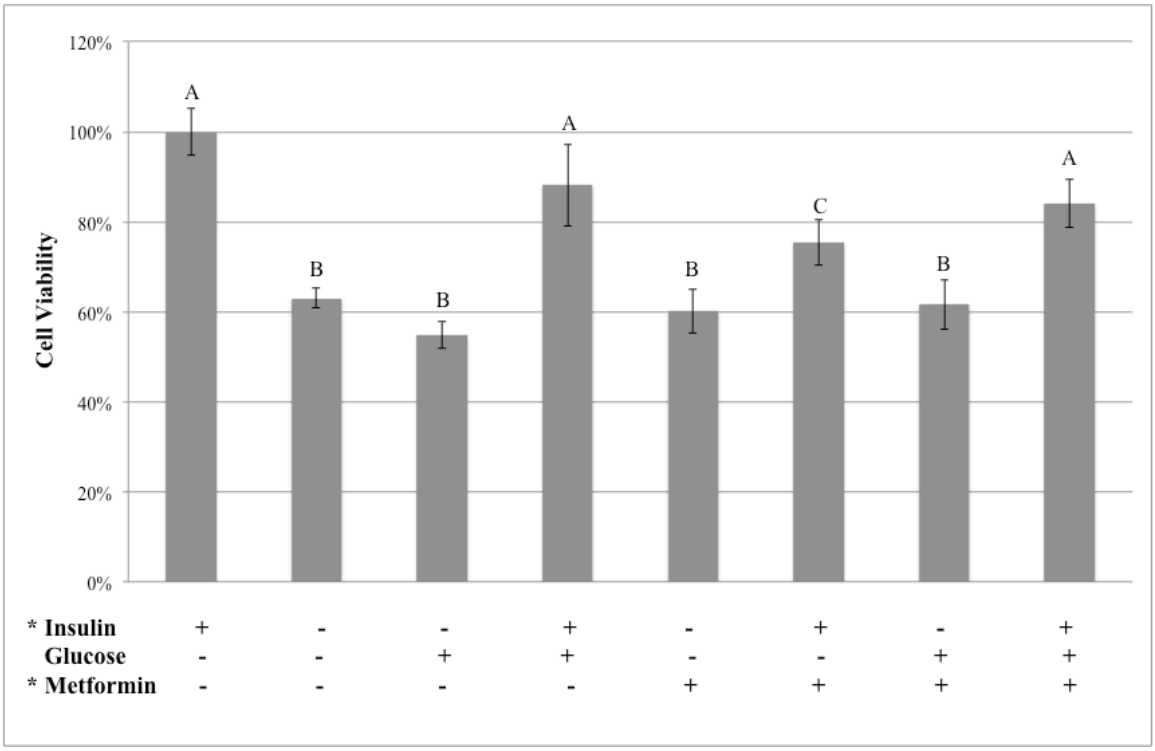


Figure 7. The Effect of 130 mM Glucose on Cell Viability in Cortical Neuronal Culture With and Without Treatments of 100 nm Insulin and 400 µM Metformin. Cell viability was measured through MTS assay; absorbance read at 490 nm. All conditions standardized to the initial control condition of growth media containing insulin. Error bars represent +/- SEM. ($N \geq 48$ replications per condition). Statistical significance ($p < 0.05$) indicated by difference in letter. * Signifies a main effect, $p < 0.05$.

Experiment 7, Figure 8 – Metformin pre-treatment

Several time points were chosen to use for a Metformin pre-treatment; however time points over a 24 hour pre-treatment were leading to increased cell death (data not shown here). It was essential to start with a pre-treatment time point that was not showing any increased cell toxicity, thus the 24-hour pretreatment was chosen for this experiment. Conditions were tested with and without a 24-hour pre-treatment of Metformin to determine if there was a decrease in neuronal toxicity [Figure 8].

Independent samples t-tests were performed comparing conditions with and without a 24 hour Metformin pre-treatment shown in Figure 8. First, cell viability for the 130 mM glucose condition (0.59 ± 0.07) was compared to 130 mM glucose with a Metformin pre-treatment (0.82 ± 0.03) With the Metformin pre-treatment there was a significant decrease in neuronal toxicity, ($p = 0.01$). There was no significant difference in cell viability for the condition of glucose/STZ compared to glucose/STZ with a pre-treatment, ($p > 0.05$). Finally, cell viability for the condition of glucose/STZ/insulin (0.84 ± 0.07) was significantly higher than the condition of glucose/STZ/insulin with a Metformin pre-treatment (0.69 ± 0.02), ($p = 0.002$).

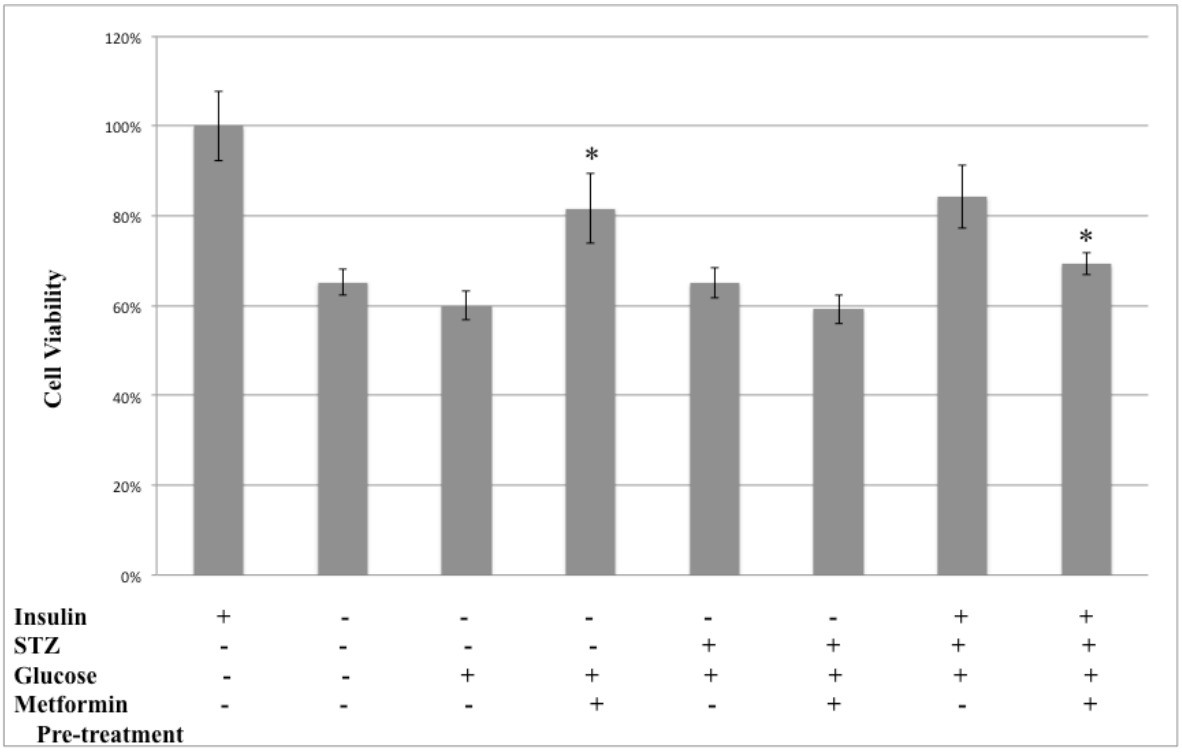


Figure 8. Conditions of 130 mM Glucose With and Without 100 µM Streptozotocin and 100 nm Insulin, Treated With and Without 24-hour Metformin Pre-treatment. Cell viability was measured through MTS assay; absorbance read at 490 nm. All conditions standardized to the initial control condition of growth media containing insulin. Error bars represent +/- SEM. ($N \geq 12$ total experimental replications).* Signifies significant difference compared to the same condition without metformin pre-treatment, $p < 0.05$.

Discussion

The initial aim of this research was to establish a model of neurodegeneration using high concentrations of glucose in combination with STZ. All subsequent experiments were aimed at determining whether or not insulin and metformin, alone and conjunction, would be able to decrease neuronal toxicity. The final aims of this research was to determine a pre-treatment time point of metformin application that would be able to significantly reduce neuronal toxicity compared to conditions without the pre-treatment.

Development of the glucose/STZ model of neurodegeneration

Research done by Papa and colleagues in 1995 examined dendritic spine development, using confocal laser scanning microscopy and immunocytochemistry, in *in vitro* hippocampal neuronal cultures of embryonic day 19 Wistar rats. Dendritic spines are areas that typically receive input from excitatory synapses; they function as important signaling systems essential for synaptic function and plasticity modulated by sensory experience (Nimchinsky, Sabatini, and Svoboda 2002). At 1 week in culture, spines resembled long filopodia without synaptic contacts (Papa et al. 1995). Closer to 3 weeks in culture, spines were closer to what is seen *in vivo*; they were filled with filamentous material and formed asymmetric synapses. Studies looking at spines have shown decreases in dendritic length and dendritic spine density with increased age (Nimchinsky, Sabatini, and Svoboda 2002; Papa et al. 1995). While there is normal spine loss, known as "dendritic pruning," recent research has shown dendritic spine loss in areas in close

proximity to Aβ plaques in AD patients; this may, or may not, contribute to cognitive decline and other AD pathology (Bittner et al. 2012).

An additional study done by Jin and colleagues (2011) looked at the difference between experimentation with hippocampal neurons from embryonic day 18 rats, cultured for 7 days versus 18 days. Experiments focused on examining Aβ dimers and cytoskeletal changes. With a 7-day growth period, cultures had not reached a maturation point that would show neurotoxicity with the presence of synthetic Aβ (Jin et al. 2011). Western blots of lysates from hippocampal neurons of 18 days growth, found significant expression of mature tau compared to the 7 days growth. Results indicated it was beneficial, and possible, to grow the neuronal cultures for an 18 day period prior to experimentation to develop a mature model system.

Based on the findings that a longer growth period of neuronal cultures would lead to maturation, increases in dendritic length, and spine density, we determined that neuronal cultures would be grown for two weeks in an incubator at 37˚C with 5% CO_2. At this point in growth, the cultures would most closely represent a mature culture system with well-developed axons and dendrites, similar to what would be seen in the brain of AD patient. Observations under a light microscope close to two weeks of growth showed neurons with dense processes (data not shown). Before this time point, the cultures would be more representative of a younger immature brain with fewer synaptic connections. This was directly seen under a light microscope with neuronal cultures of several days in age having one soma with very few branching processes.

The goal of this experiment was to produce a model of neurodegeneration that used high levels of glucose in combination with STZ to reduce neuronal viability by 50%. The 50% survival point was chosen as a middle point where neuronal cultures would have detectable degeneration compared to the control conditions, however this level of toxicity could still be affected by application of therapeutic agents. The aim was to produce an amount of neurodegeneration that was not toxic to the point of no return. This model would allow for the examination of pathologies linking T2DM and neurodegeneration.

As mentioned, this main linking feature is thought to be insulin resistance, the inadequate response to insulin by target cells (De Felice, Lourenco, and Ferreira 2014; Yarchoan and Arnold 2014; Vignini et al. 2013). Cell viability of all experiments was measured through MTS assay. This assay allowed us to determine which cells were still living and able to metabolize the yellow tetrazolium dye. The control condition of growth media was set to 100% cell survival; this condition contained no toxins and therefore theoretically no toxicity should have been observed. All other conditions were standardized to this control condition.

The first group of experiments (Experiment 1) [Figure 2], were done testing high glucose concentrations leading to measurable cell death. The control condition of growth media contains 25 mM glucose, an amount that will allow cells to carry on essential processes such as metabolism. Concentrations higher than this, 100 mM - 200 mM [Figure 2], were tested at 24 hour and 48 hour treatment times. The 48 hour concentration curve resulted in an approximately 50% cell viability for all concentrations of glucose,

while the 24 hour curve resulted in a typical curve where the highest concentration (200 mM) was the most toxic, approximately 36% cell viability. It may be that the 48 hour stimulation with glucose is too long of a time point to provide differences in toxicity based on concentration. 130 mM glucose was chosen because of the overlap in cell viability during both the 48 hour and 24 hour stimulation, approximately 50% cell viability. The control condition of growth media (25 mM glucose) could be thought of as a normal glucose level necessary for survival. Increasing this concentration, even briefly for 24 hours, lead to severe neurotoxicity [Figure 2]. This may parallel the way in which high glucose levels in the body can affect cells and neurons, eventually leading to the cognitive decline seen in T2DM.

As neuronal glucose levels increase, signs of glucose neurotoxicity become very apparent. Consistently high intracellular glucose metabolism leads to neuronal damage, known as glucose neurotoxicity (Tomlinson and Gardiner 2008). Two suspected molecular mechanisms of glucose neurotoxicity include: glucose-driven oxidative stress, which occurs through a combination of free-radical generation, and intracellular signals activating MAP kinases (Tomilson and Gardiner 2008). The negative effects of elevated glucose levels result in neuronal conduction abnormalities, impaired axonal regeneration, and altered ion fluxes (Tomlinson and Gardiner 2008). Under normal conditions, insulin is able to prevent glucose accumulation in high levels by activating the GLUT4 transporter. It is possible that under high glucose conditions, undamaged mitochondria are occupied with attempting to metabolize the large amount glucose and are unable to metabolize the tetrazolium found in MTS dye.

In all experiments, the addition of 100 nm insulin led to a significant decrease in neuronal toxicity as seen in experiments 1-8. As previously mentioned, insulin is suggested to act as a growth factor by promoting synaptogenesis and nerve growth (Nelson et al. 2008). Insulin, when added to toxic glucose concentrations, may aid in glucose uptake and also promote neuronal growth and survival. Additionally, it has been suggested that insulin and some neurotrophic factors, primarily nerve growth factor (NGF), have similar structures, cellular actions, early signaling events and receptor structure/function (Pittenger and Vinik 2003). These similarities further suggest why insulin is able to have beneficial effect on cell viability when added to high levels of glucose.

The next step in the development of the neurodegeneration model of AD was to add STZ to the glucose, (Experiment 3) [Figure 4]. Preliminary STZ concentration curves were performed testing the conditions of 100 μM, 200 μM, 500 μM. Based on the method protocols of previous lab members, and because there was no detectable difference in toxicity for the three conditions, 100 μM STZ was chosen. Compared to the insulin free condition, STZ alone lead to approximately 25% increase in neuronal toxicity; trend observed [Figure 4]. However, results of STZ, as seen in Figures 5 and 6, were not consistent through experimentation.

STZ is most commonly used in rat models to produce diabetic conditions. When administered through an intracerebroventricular injection to male Wistar rats, STZ was shown to cause brain mitochondrial abnormalities; weeks later, rats showed cognitive decline (Correia et al. 2013). When STZ was administered neonatally in an acute

fashion, STZ resulted in T1DM through destruction of pancreatic β-cells (Pittenger and Vinik 2008). Our use of STZ is expected to cause toxicity by negatively influencing mitochondria, likely through ROS production. Future experiments may want to use ELISA assays to quantify and compare levels of glucose in cultures treated with and without STZ. It is hypothesized that neuronal cultures treated with STZ will be unable to take up exogenous application of glucose, therefore these cultures will have a higher amount of glucose present in the media for a longer amount of time leading to glucose neurotoxicity, as compared to cultures without STZ treatment.

When neuronal cultures were treated with a combination of STZ/glucose, resulting in cell viability of approximately 50%, there was significantly more toxicity as compared to the STZ condition itself, cell viability of approximately 66%; however there was no significant difference between STZ/glucose and the glucose condition, (Experiment 3) [Figure 4]. This may indicate that the majority of the toxicity seen is occurring via glucose excitotoxicity. Additionally, high levels of glucose and STZ are both able to exert their effects through production of reactive oxygen species; therefore, the two toxicity-producing pathways might be overlapping. Though *in vivo* studies STZ has been shown to specifically destroy insulin producing cells, it is likely that in *in vitro* STZ is having toxic effects through production of ROS.

In these experiments, STZ and high levels of glucose were used at the same time point; thus, it is possible that STZ did not have an adequate amount of time to exert its effects. Future studies may look to apply STZ several days before glucose in order to allow both stressors to produce toxicity separately through their suspected modes of

action. It would be advantageous for future studies to look at reactive oxygen species production, through use of a dichlorodihydrofluorescein diacetate assay, under glucose conditions, STZ conditions, and under conditions of both glucose and STZ to see how the production of reactive oxygen species compares.

The glucose and STZ condition resulted in approximately 50% cell viability as predicted, Figure 4. In a typical AD patient, the 50% loss of neurons is seen over many years of having the disease (Mayeux and Sano 1999); however, in this model of neurodegeneration, the 50% loss is seen over merely 24 hours. Thus, while the amount of neuronal loss is accurate, the time span is not. To improve this model, neurodegeneration could be caused at a slower rate to reflect what is normally seen in AD patients. It would also be worthwhile to see how having a gap in time between the application of glucose/STZ and metformin/insulin affects cell viability. As seen in Figure 4, the addition of insulin to the glucose and glucose/STZ condition was able to significantly improve cell viability; similar results also seen in experiments 4, 5, and 6.

There has been evidence showing loss of neurotrophic support, through insulin pathway damage, leads to pro-apoptotic signals involving mitochondrial membrane depolarization and cytochrome C release (Huang et al. 2003). In one particular *in vitro* study, mitochondrial polarization was observed in sensory neurons cultured from rats with STZ-induced diabetes treated with and without insulin. Mitochondrial membrane potential was analyzed using R123 fluorescence and carbonyl cyanide m-chlorophenylhydrozone (CCCP), used to collapse the proton gradient and inner membrane potential (Huang et al. 2003). Results showed insulin was able to prevent

deficits in mitochondrial membrane potential. The mitochondrial membrane potential in sensory neurons from diabetic rats treated with insulin matched control levels, while conditions without insulin had a significantly lower level of mitochondrial polarization. The ability of insulin to prevent depolarization may indicate why damage to the insulin pathway leads to apoptotic signals and cytochrome C release.

The toxicity resulting from STZ administration was almost completely blocked with the addition of insulin (Experiment 3) [Figure 4]; toxicity of STZ was completely blocked with the addition of insulin for (Experiment 5) [Figure 6]. Several studies have shown that in STZ treated rats, insulin application is able to prevent diabetes-induced deficits in mitochondrial inner membrane potential (Huang et al. 2003; Srinivasan, Stevens, and Wiley 2000). It is likely that insulin is able to prevent mitochondrial depolarization and halt apoptotic signaling in this *in vitro* model of AD, therefore reducing the amount cell death.

Once the neurodegeneration model of AD was developed, all subsequent experiments focused on Metformin intervention. Continuing with this model in the future, it would be beneficial to begin introduce components that would strengthen the AD component. Many recent studies have looked at the addition of Aβ oligomers in models of AD (Oddo et al. 2005; De Felice et al. 2008; LaFerla 2010). Specifically, one group has shown the ability of Aβ oligomers to directly induce tau hyperphosphorylation and neurodegeneration (Jin et al. 2011). The use of oligomers could allow us to produce both AD hallmarks *in vitro* to further, and more accurately, study the relation between T2DM.

Metformin intervention

Before starting experiments testing the effectiveness of metformin, it was necessary to determine a concentration that would not add toxic effects to the cultures. Studies using Metformin in *in vitro* experiments have typically used concentrations between 400 μM and 3.2 mM (Gupta, Bisht, and Dey 2011; Zhou et al. 2001). A past lab member has used the 400 μM concentration of Metformin (Cusmano 2014). For these reasons, it seemed most appropriate to use test concentrations on the lower end of the scale, between 400 μM and 700 μM (Experiment 2) [Figure 3]. Metformin concentrations were tested directly with 130 mM glucose in order to ensure that the metformin concentration did not produce additional toxicity compared to the 130 mM glucose concentration. Overall, the only concentration of Metformin that resulted in significantly more cell viability than the other conditions was 400 μM, paralleling the concentration that was chosen previously in the Knowles lab.

After the model of AD had been established using glucose and STZ [Figure 4], we looked to test these conditions in the presence of metformin to determine if metformin could further decrease toxicity [Figure 5]. There was no observable beneficial effect of metformin on cell viability. While insulin was able to relieve toxicity seen in insulin free conditions, metformin alone was not able to positively affect viability. When metformin was added in conjunction with insulin, cell viability increased to upwards of 140%, (Experiment 4) [Figure 5]. Metformin at this time point only seemed to have a slight beneficial effect in the presence of insulin, however these results were not always consistent. Because metformin is thought to be acting as an “insulin sensitizer,” it may be

indirectly affecting glucose levels by affecting the coupling of insulin to its receptor, thereby initiating the insulin signaling cascade. In (Experiments 5 and 6) [Figure 6 and 7], there was a negative effect of metformin, conditions with metformin had decreased cell viability compared to conditions without metformin. While the initial metformin concentration curve showed metformin at higher concentrations might be stressful for neurons, it is possible that through interaction with one of the other three substances (glucose, STZ or insulin) 400 μM metformin also led to toxic effects; however, the exact mechanism remains unclear.

The experiments performed with the conditions listed in Figure 5, were difficult to analyze because not all conditions were represented. There were many inconsistencies in the results seen in Figure 5 compared to those seen in Figure 6 and 7. For example, in Figure 5, the condition of insulin and metformin had significantly greater cell viability compared to the condition of insulin without metformin. However, in Figures 6 and 7, the condition of insulin without metformin had greater cell viability than insulin with metformin. Moving forward we wanted to separate conditions to look closely at what was occurring with metformin in the presence of STZ, (Experiment 5) [Figure 6] and glucose, (Experiment 6) [Figure 7] separately.

First, it seemed most appropriate to look at how metformin is able to affect toxicity seen in the presences of STZ. With these experiments [Figure 6], it became even more apparent that a decrease in toxicity was only observed in the presence of insulin. Addition of metformin again had no beneficial effect on the cell viability for any of the conditions. STZ is typically used in *in vivo* studies to produce diabetic conditions,

including insulin resistance (Correia et al. 2011); thus, it was hypothesized that if in *in vitro* neuronal cultures STZ works in a parallel manner, the diabetes drug metformin would have some beneficial effect on toxicity. Overall, there were no consistent results seen with only STZ administration, (Experiment 3) [Figure 4], (Experiment 4) [Figure 5], Experiment 5 [Figure 6]. It is necessary to gain a better understanding of how STZ exerts its effects *in vitro* compared to *in vivo* to move forward with experimentation.

As mentioned previously, STZ is thought to damage DNA and insulin producing cells/receptors, through production of ROS, hydrogen peroxide and nitric oxide (Yarchoan and Arnold 2014). Therefore, it is plausible that metformin may be able to target ROS production in conditions containing STZ. Moving forward it would be beneficial to move away from using MTS assays testing cell viability, and instead move toward specific assays testing the supposed mechanisms of metformin activation. Particularly, the OxiSelect In Vitro ROS/RNS Assay (Cell Biolabs, Inc.) is able to measure total ROS, reactive nitrogen species, hydrogen peroxide, and nitric oxide, and would be an ideal test to observe how metformin affects ROS production abilities of STZ. It is also necessary to test out STZ application several days prior to metformin treatment in order to more accurately assess how metformin is able to combat insulin resistance.

Though there was no consistent observable effect on cell viability, it is possible that metformin is affecting another aspect of cellular health. Specifically, one of the main hypotheses surrounding metformin's mechanism of action is its ability to decrease oxidative stress and apoptotic signals. It is possible that application at the same time point

as glucose/STZ does not allow for metformin to affect these processes. Thus, benefits from reducing oxidative stress and apoptotic signaling might need additional time, possibly through a metformin pre-treatment to become observable (Scarpello and Howlett 2008).

Next, we looked at cell viability in glucose conditions with and without metformin and insulin (Experiment 6) [Figure 7]. The American Diabetes Association, European Association for the Study of Diabetes, and International Diabetes Federation place metformin as a first-line therapy for T2DM. Metformin is used in diabetic patients because of its proposed ability to ameliorate insulin resistance, decrease hyperinsulinemia and glucose toxicity (Scarpello and Howlett 2008). Therefore, it was expected that in the presence of high glucose levels (130 mM), metformin may able to decrease the amount of neuronal toxicity. This was not seen [Figure 7], and the only significant decrease in toxicity was with the addition of insulin. Also, there was no main effect of glucose in these experiments, [Figure 7]. This could be due to cell viability under the insulin free media condition (63%) being lower than previously seen.

It is important to note that typically individuals that are diagnosed with T2DM will be administered metformin as a treatment option. These people already have high glucose levels and insulin resistance; metformin is prescribed down stream to the appearance of T2DM pathologies, hypothesized to control glucose levels, reduce gluconeogenesis, and reduce hepatic glucose output (Scarpello and Howlett 2008). In comparison, during this study, metformin was applied at the same time as glucose and STZ, making this model distinctly different from what is seen *in vivo*. Further, glucose

levels and gluconeogenesis within the brain are at much lower levels compared to glucose levels in the periphery (Dunn-Meynell et al. 2009). Thus, in the brain metformin may be exerting its effects in a different manner compared to the periphery.

Additionally, it is possible that the concentration of glucose was too high for metformin to have a beneficial effect. Moreover, the dose of metformin may have been too low compared to the amount of metformin typically used in human (500-1500 mg/day) and rat *in vivo* models of diabetes (400 μM - 3.2 mM). There was also an instance when 130 mM glucose was prepared and used on cells without checking the pH. Typically, the pH of 130 mM glucose prepared in insulin free growth media is approximately 8.0. During initial preparation the pH is adjusted to the range of 7.1-7.4 with the addition of 0.1 M HCL. In several conditions however, this was not done and may have resulted in unfavorable conditions for neuronal growth. These challenges may have made it more difficult to see positive effects of metformin.

Next, metformin pre-treatment was tested in a final attempt to decrease neuronal toxicity with metformin intervention, (Experiment 7). Initially a wide range of time points, 7 days, 5 days, 3 days and 1 day, of 400 μM metformin were chosen and applied to cultures in the absence of any toxins. The results showed a decrease in cell viability with metformin pre-treatment in all time points except 1 day (data not shown). These times were eliminated as options because we did not want metformin to add any toxicity in the presence of glucose and STZ. Prior to stimulation with glucose, STZ and insulin, certain conditions were given a 24-hour pre-treatment of metformin [Figure 8]. The main findings of these experiments showed that with the 24-hour pre-treatment, there was

significantly less toxicity seen in the presence of 130 mM glucose, 82% cell viability, compared to the condition without the pre-treatment, 60% cell viability. Although, there was no beneficial effect of the metformin pre-treatment for the glucose/STZ condition, and there was a decrease in cell viability with the metformin pre-treatment in the insulin/glucose/STZ condition [Figure 8].

Future experiments could focus on how a metformin pre-treatment was able to decrease neuronal toxicity. However, there are many supposed mechanisms through which metformin affects pathways necessary for inducing or inhibiting protein expression. It was hypothesized that the metformin pre-treatment may be able to promote expression of necessary proteins in order to prepare the neuronal cultures for the 130 mM glucose that would be applied in the following day. I am primarily interested in the mechanism by which metformin activates the AMPK pathway.

Experiments have shown 500 μM metformin treatment, in media containing 100 nM insulin, in primary cultured hepatocytes lead to a significantly activated AMPK (Zhou et al. 2001). A chronic activation of AMPK may induce the expression of GLUT4. As shown in Figure 1, GLUT4 is a glucose transporter that translocates from the cytoplasm to the cell membrane in the presence of insulin. Because the condition in which a 24-hour metformin pre-treatment was able to decrease toxicity in the presence of 130 mM glucose and absence of insulin [Figure 8], it may be that metformin is able to play a role in the activation of GLUT4 receptor proteins. Thus, a metformin pre-treatment may give cells enough time to induce GLUT4 expression and prepare receptors to bring

in extracellular glucose. Future experiments using western blots would allow us to test protein levels of GLUT4 before and after the addition of metformin.

From these set of experiments, we have learned concentrations of glucose, STZ, insulin and metformin, which can be used moving forward. Therefore, I would suggest taking these conditions and immediately starting experiments looking at indices of cellular health other than cell viability. However, to mimic *in vivo* metformin conditions the concentration should be raised. It will be interesting to see how results of JC-1 mitochondrial membrane potential assays compare and contrast to the results presented above. Additionally, there were approximately 12 24-well plates that could not be analyzed due to the lack of time. These plates could have provided beneficial information on how glucose, STZ, insulin, and metformin affect microtubule stability.

Additionally, there were many crucial plates that were lost due to unforeseen circumstances. Several times the incubator ran out of CO_2 compromising the health of neuronal cultures and leading to very low levels in cell viability, which could not be used for experimentation. Also, twice in the past semester we received Sprague Dawley rats that were not pregnant. This significantly decreased the amount of replications that were planned for experiments 5,6, and 7.

Conclusions

The glucose/STZ model of neurodegeneration worked as expected and resulted in approximately 50% neuronal toxicity in most conditions; although effects of glucose and STZ were not additive. To make this model more representative of AD, it would be beneficial to add in Aβ. This would allow us to look at how Aβ interacts with the insulin

signaling pathway; whether exposure of neurons to Aβ increases GSK-3β levels through inhibition of PI3K signaling (Maeseko et al. 2012); and if also if metformin is able to decrease Aβ levels by reducing the APP cleavage products of BACE1 (Hettich et al. 2014). In all experiments insulin was able to relieve neuronal toxicity in the presence of glucose and/or STZ. Moving forward it will be interesting to examine how exactly insulin is exerting its beneficial effects in this *in vitro* model.

The main findings of this study revealed that a metformin and STZ did not have consistent measurable effects, while insulin was able to relieve toxicity in all conditions. It is necessary to study metformin and STZ closely to determine their method of action, and how to alter conditions to get reproducible and consistent baseline results. Further, we saw that metformin pre-treatment is able to relieve neuronal toxicity in the presence of high levels of glucose, under insulin free conditions. It is necessary to examine aspects of the metformin pre-treatment more closely. Specifically, it will be important to figure out whether not the pre-treatment has an effect on AMPK phosphorylation, GLUT4 levels, endogenous glucose levels, and ROS levels. It may be worthwhile to continue from this point and test different concentrations of metformin pre-treatment with 130 mM glucose to see if there are other concentrations that will work better at reducing neuronal toxicity. Overall, further experimentation with metformin pre-treatments will help us gain a better and more complete understanding of metformin's capabilities.

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