Dedication:

This work is dedicated to the family, friends, and mentors who have helped me achieve my goals. I could not have gotten to where I am today without your support.

Drew University

College of Liberal Arts

The Effect of Metformin and Insulin on Neuronal Degeneration in a Glucose/Streptozotocin Model of Alzheimer's Disease

A Thesis in Neuroscience

by

Katelyn Cusmano

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Abstract

Alzheimer's disease (AD) is a debilitating disorder characterized by dementia and loss of functioning in daily activities. Recent epidemiological studies have suggested a link between type two diabetes mellitus (TD2M) and AD. TD2M is a condition in which the body is resistant to insulin causing a buildup of glucose in the body, a situation that has been thought to occur in the brain in patients with AD. Metformin is one current treatment for TD2M and is thought to help regulate glucose levels. If there is a link between TD2M and AD then Metformin could be a treatment for AD by alleviating insulin resistance in the brain, preventing oxidative stress on neurons, and avoiding neurodegeneration. In this study, fetal rat cortical neurons were cultured and treated with high levels of glucose and streptozotocin in the absence of insulin to create oxidative stress of the neurons similar to that in patients with both AD and TD2M. Insulin and Metformin were added in an attempt to rescue the neurons as measured by cell viability and by the stability of microtubules. While there were measurable decreases in response to the cell stressors, which were partially protected by insulin, Metformin had no measurable protective effect. Future experiments will focus on changing the dose and time course of Metformin exposure to see whether there are conditions that improve cellular response.

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Introduction

Overview

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that currently affects over five million people in the United States and is responsible for approximately 500,000 deaths per year (Alzheimer's Disease Association 2013). The cause of Alzheimer's disease is unknown although it is thought that two hallmarks of the disease, amyloid beta plaques and neurofibrillary tangles, may play a role. Insulin signaling could contribute to this pathology through pathways regulating amyloid beta plaque and neurofibrillary tangle formation, like the glycogen synthase kinase 3β pathway. Recent studies have suggested a connection between insulin deficiency in type two diabetes mellitus (TD2M) and the progression of Alzheimer's disease (Corriea et al. 2011; De la Monte and Wands 2008). This project investigates whether an *in vitro* model of AD using elevated glucose levels and streptozotocin can cause neuronal degradation. It further studies the potential of insulin and the TD2M drug Metformin to alleviate the toxicity caused by glucose and streptozotocin.

History of Alzheimer's disease

AD was first described by Alois Alzheimer in 1906 (Strassnig and Ganguli 2005). He presented a case study of a patient, Auguste D., who showed a slow loss of memory function, as well as changes in personality and decreased language ability. However, for approximately sixty years after this discovery, AD was typically only associated with the rare cases of presenile dementia. It was not until 1976 when AD became recognized as one of the most common forms of dementia (Katzman 1976). Today, AD is currently cited by the Center for

Disease Control and Prevention as the sixth leading cause of death in the United States (Hoyert and Xu 2012).

AD is a progressive neurodegenerative disorder that currently affects over five million people in the United States (Alzheimer's Disease Association 2013). As such, it is the most prevalent form of dementia, compromising from 60-80% of all dementia cases (Aker et al. 2010). AD most often afflicts the elderly, although it is not limited to the aging population. Due to this, the prevalence of people living with AD will continue to increase as the baby boomer generation ages (Corriea et al. 2011). A report published by the Alzheimer's Disease Association states that someone in the United States is diagnosed with AD every sixty-eight seconds, and the prevalence of AD in the United States is projected to double by the year 2050 (Alzheimer's Disease Association 2013). There is currently no cure for the disease. Therefore, these projections point to the importance of research into the causes and treatment of AD in order to prevent the drastic increase in those afflicted with AD in the future.

Over the course of the disease, cell death within the brain can reach approximately 50% (Mayeux and Sano 1999). At this point, patients have a difficult time with basic functions including dressing, remembering their own name and names of family members, and taking medications (Mayeux and Sano 1999). Neuronal degradation begins in areas like the hippocampus and spreads throughout much of the brain including areas in the frontal and temporal cortex (Mayeux and Sano 1999; Mattson 2004). Most patients die from secondary health ailments including poor nutrition and bacterial infections (Akter et al. 2010).

There is currently no way to definitively diagnose AD until autopsy, when one can look for hallmarks indicating the presence of the disease. However, there are criteria that suggest probable AD. These symptoms include problems with memory, difficulty with solving problems, lack of accidents or injuries consistent with acute brain damage, and oftentimes personality changes (Mayeux and Sano 1999). The changes within the brain are broken down in autopsy into stages based on differences in neurofibrillary tangles, known as Braak stages (Braak and Braak 1995). In stages one and two, tangles are found primarily in the transentorhinal cortex. In stages three and four, tangles form in the hippocampus and other limbic areas. Stages five and six have extensive tangle formation in the neocortex. When other diseases and disorders have been ruled out and the criteria are met a patient may be diagnosed with probable AD.

Economic impact

Not only is the rise in AD prevalence a public health problem, but it also has significant financial implications (Weiler 1987). As AD is a progressive disease, patients often live up to ten years after onset of early symptoms (Mayeux and Sano 1999). As a result, patients with AD typically need long term care by the late stages of the disease, consisting of in home care nurses or nursing homes. This type of intensive care costs families, insurers, and the government billions of dollars (Weiler 1987).

It is has been estimated that between hospital and hospice care, the cost of taking care of patients in the United States with AD was approximately $183 billion in 2011, and that number continues to increase as the cost of healthcare and the number of people affected with the disease increase (Shearer et al. 2012). Within the United States, it is one of the most

costly medical conditions, ranking third behind cancer and heart disease (Alzheimer's Disease Association 2013). AD will continue to impact a multitude of people both physically and financially if a cure is not found.

Pathology of AD

The cause of AD is unknown, although it is thought that two hallmarks of the disease, amyloid beta plaques and neurofibrillary tangles, play a causal role (Corriea et al. 2011). Amyloid precursor protein (APP) is a protein in cells that is cut by gamma secretase into a smaller chain of either forty or forty two amino acid monomers (Riek et al. 2001). Normally APP is cut at a length of forty monomers and fewer at the forty two monomer chain, since the latter is considered more toxic to cells in the body (Riek et al. 2001). When excess amyloid beta 42 is cut, these proteins accumulate in clumps in the brain of AD patients, forming plaques (Riek et al. 2001). While the direct role of plaques is still being determined, one effect of the accumulation of amyloid beta is the activation of microglia and the immune response, leading to the release of nitric acid and cytokines (Hardy and Selkoe 2002). In the process of this activation it is possible that neurons in the surrounding areas are also damaged.

There are many theories about the potential effect of plaques on the brain, including neuronal stress and ultimately cell death (Riek et al. 2001). However, the exact role that plaques play in AD is currently unknown. One study using a passive avoidance task in mice found that the injection of amyloid beta oligomers led to a decrease in avoidance in the task. The authors suggested that amyloid beta aggregation may lead to decreased synaptic density

and difficulties with memory consolidation, a process that may also play a role in AD (Freier et al. 2011). In this way, plaques could contribute to memory loss.

Neurofibrillary tangles are caused by a dysfunction in tau protein. In healthy cells tau binds to microtubules within the cell, stabilizing the cytoskeleton and maintaining cell structure (Feinstein and Wilson 2005). The dynamic nature of microtubules is important for cell survival. Hyperphosphorylated tau protein leads to a break down in the cytoskeleton of neurons due to a decrease in interaction between microtubules and tau. This makes it difficult, if not impossible, for the neurons to function via communication with other neurons and often leads to cell death. In contrast with plaques, the number of tangles present in the brain has been shown to be positively correlated with progression of the disease (Schonheit, Zarski, and Ohm 2004).

Current research suggests a possible interaction between mechanistic pathways leading to plaques and those leading to tangles. Jin et al. (2011) purified amyloid beta dimers from human AD brains and exposed primary hippocampal neurons from fetal rats to the dimers. Western blots and immunhistological tests showed increased tau phosphorylation. This suggests that the addition of amyloid beta oligomers can lead to microtubule breakdown due to tau hyperphosphorylation. The exact mechanism behind this breakdown is currently unknown, although research is investigating this potential link. However, the number of tangles in the brain of an AD patient is typically associated with the number of years with the disease while no such correlation can be made for amyloid beta plaques (Schonheit, Zarski, and Ohm 2004). Because of this, there are likely some differences in the mechanistic pathway even if there could be an interaction.

While the exact cause of the disease is unknown, there are potential pathways implicated in the development of AD, including the insulin pathway. Recent studies suggest that TD2M could be a potential risk factor for the development of AD (De la Monte and Wands 2008). Because of this, the insulin pathway could play a major role in the development of AD.

Insulin Functioning in the Brain

The processes that occur within the brain require a substantial amount of energy, requiring significant glucose metabolism and oxygen consumption within the brain (Clarke and Sokoloff 1999). Over 20% of glucose in the body is used by the brain (Attwell and Laughlin 2001) and additionally approximately 156 μmol/100g brain tissue of oxygen is utilized every minute (Clarke and Sokoloff 1999). Because of the significant energy expenditure, mitochondria, organelles in the cell responsible for energy production, play an especially significant role in the brain. Because the brain utilizes the most energy of any organ within the body, disruptions in mitochondrial functioning can have significant consequences within the brain. There are three primary components of mitochondrial functioning that could contribute to AD pathology including the production of ATP, the production of reactive oxygen species (ROS), and the initiation of the apoptotic pathway (Moreira et al. 2007).

Insulin is a hormone that is critical for regulation of cellular glucose. Insulin binding to the α subunit of insulin receptors in the cell membrane leads to cross phosphorylation of the β subunit of the receptor (figure 1; Berg et al 2012). Once activated, insulin-receptor substrates (IRS) bind to sites on the receptor and are phosphorylated. IRS anchors

phosphoinositide 3-kinase (PI3K) to the membrane, initiating the addition of a phosphoryl group to phosphatidylinositol 4,5-bisphosphate (PIP_2) producing (PIP_3). PIP_3 dependent protein kinase (PDK1) binds to PIP_3, activating the kinase which in turn activates additional proteins including protein kinase B (Akt). Akt phosphorylates proteins responsible for the translocation of glucose transporter 4 (Glut 4) to the cell membrane, allowing for the intake of glucose.

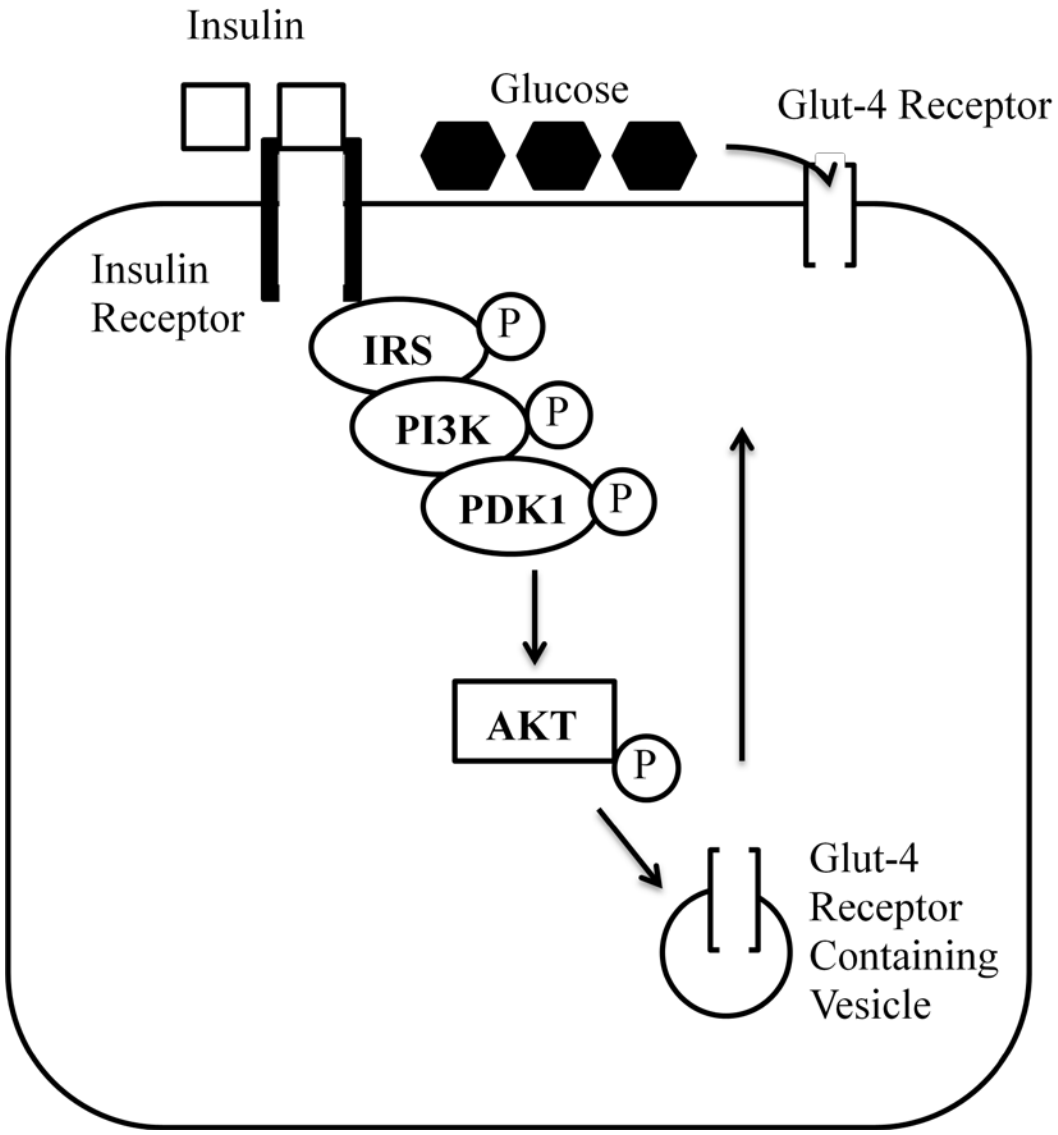

Figure 1: The binding of insulin to insulin receptors activates insulin receptor tyrosine kinase, anchoring phosphoinositide 3-kinase (PI3K) to the membrane where it phosphorylates PIP_2 to PIP_3. PIP_3 activates phosphoinositide-dependent kinase-1 (PDK1). PDK1 phosphorylates protein kinase B (AKT) that translocates a vesicle containing the Glut-4 receptor to the plasma membrane leading to the uptake of glucose.

The insulin pathway plays a significant role in the function of learning and memory as well as synaptic plasticity. Stranahan et al. (2008) fed rats diets high in fat, glucose, and high-fructose corn syrup over a period of eight months. The rats fed these diets exhibited signs of insulin resistance including high fasting glucose levels. Performance on the Morris Water Maze significantly decreased in rats with the altered diet in comparison to control rats, indicating problems with spatial learning and memory. In this way, possible deficits within the insulin pathway are tied to decreases in cognitive functioning. Furthermore, when stimulated, CA1 hippocampal neurons of rats with the high fat, high glucose diets showed reduced long term potentiation (LTP) suggesting a decrease in synaptic plasticity in this region (Stranahan et al. 2008). This research suggests that changes within the brain associated with insulin pathway deficits could be tied to deficits in aspects of neuronal functioning typically associated with learning and memory.

AD patients exhibit decreased insulin levels in the brain that correlate with decreased cognitive functioning in humans (Laron 2009). Insulin's role in glucose uptake decreases glucose metabolism in the brains of patients with AD (Mosconi 2005). There are also differences in insulin gene expression in AD. Jackson et al. (2013) used an $APP^{swe}Psen^{de9}$ mouse strain, a model typically used to represent AD, to study the genomic changes between an AD model and the normal brain. They found changes in gene expression in the insulin signaling pathway, specifically downregulation of gene expression of *Irs4* for an insulin receptor substrate protein, IRS4. De la Monte and Wands (2008) examined this even further by measuring insulin gene expression at different Braak stages in frontal lobe tissue. As Braak stages progressed, there were reductions in insulin, IGF-1, and IGF-2 receptor gene

expression. Changes in insulin levels and gene expression implicate the insulin pathway as a contributing factor to AD.

Glycogen Synthase Kinase-3B

Insulin has been shown to regulate the formation of both neurofibrillary tangles and amyloid beta plaques through the glycogen-synthase kinase 3β (GSK-3β) pathway, indicating a potential link between dysfunction in insulin signaling leading to AD pathology (Takashima 2006). Insulin binding to insulin receptors within cell membranes results in the cross phosphorylation of the insulin receptor and the binding of IRS. IRS anchors PI3K to the membrane and leads to the downstream activation of the protein kinase B (Akt) (figure 2). Akt inhibits GSK-3β which then inhibits activation of the apoptotic pathway, phosphorylation of tau, and regulation of the amyloid beta levels (Takashima 2006). Decreased insulin signaling increases the GSK 3β activity, causing increased hyperphosphorylation of tau, and ultimately the formation of tangles. In addition, the insulin and GSK 3β pathways have been linked to the formation of plaques (Maesako et al. 2011). Dysfunction in insulin signaling could lead to increased activation of GSK 3β and thus increased accumulation of amyloid beta 42 leading to plaque formation within the brain.

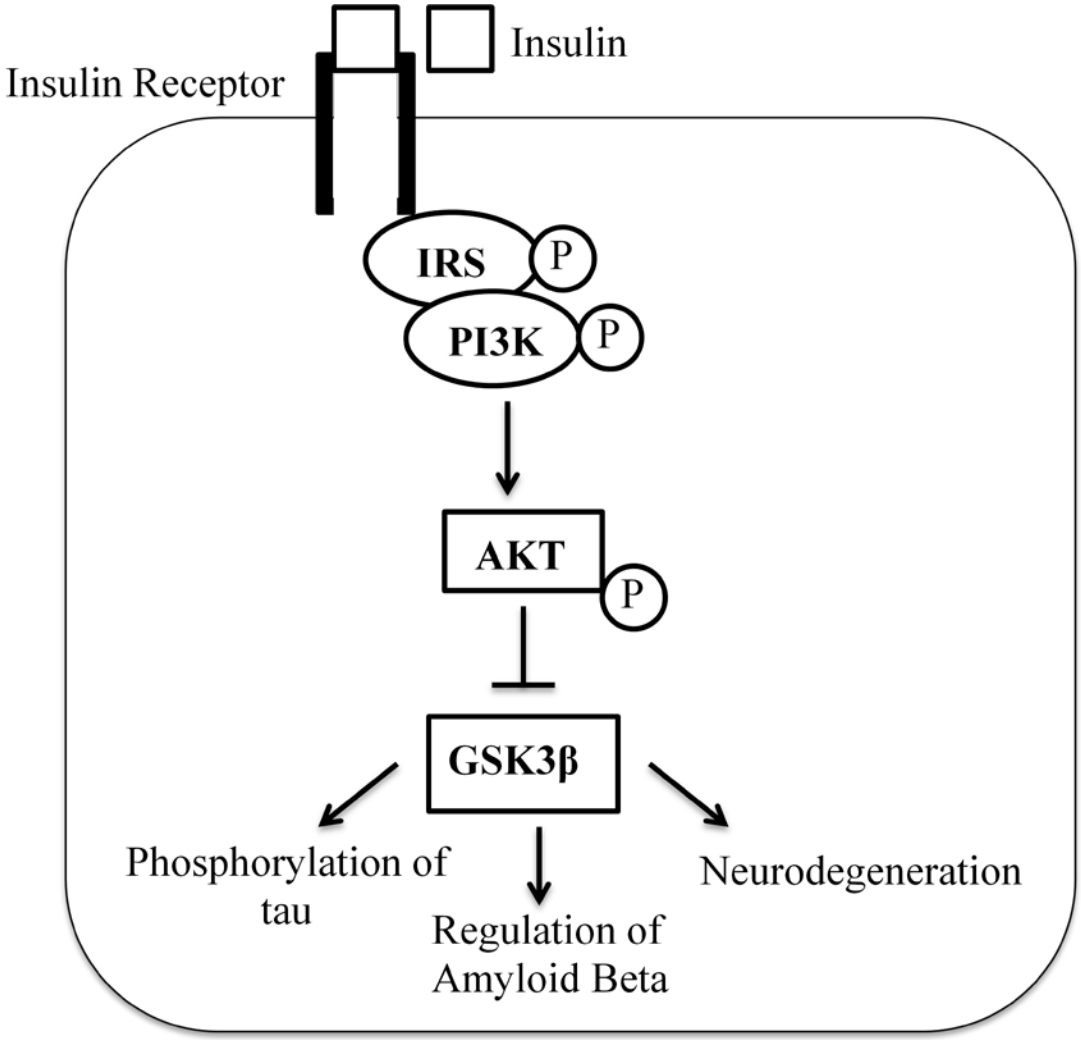

Figure 2: Insulin binding leads to dimerization of the insulin receptor, activating PI3K. PI3K phosphorylates AKT, inhibiting GSK3β. When active, GSK3β is responsible for the phosphorylation of tau and activating pathways that regulate amyloid beta.

AD Model

Streptozotocin

Streptozotocin is a nitrosourea compound from *Streptomyces griseus* (Wei et al. 2003) that is often used as a model for diabetes due to cytotoxic effects on beta cells (Lester et al. 2006). Streptozotocin functions by producing reactive oxygen species (ROS) (De la Monte and Wands 2008). ROS are oxygen molecules that contain unpaired electrons, making them much more reactive than oxygen molecules containing paired electrons (Halliwell 1989). In normal functioning cells, the production of ROS is minimized by cytochrome c oxidase which is responsible for containing ROS within the electron transport chain. However, when released these ROS lead to DNA damage through interactions with the purine and pyrimidine bases that make up DNA leading to mutations and even breaks within DNA strands (Cox et al. 2012). These disruptions can have serious implications for the cell and eventually lead to apoptosis.

Streptozotocin is used to model TD2M in *in vivo* models because it is able to cross the cell membrane of cells that produce insulin, known as islet cells (De la Monte and Wands 2008). Within these cells, streptozotocin has been shown to lead to the production of ROS. The production of ROS can lead to damage such as DNA mutations in islet cells that can cause diabetes-like pathology.

Streptozotocin has been used as a TD2M model in neuronal culture to study neuronal morphology (Martinez-Tellez et al. 2005). They administered streptozotocin to Sprague Dawley rats and after sixteen weeks removed and stained the brains using a Golgi-Cox

methodology. There were significant changes within the dendrites of pyramidal neurons including a decrease in both the length of dendrites as well as the number of spines. These changes occurred in the prefrontal cortex, the hippocampus, and the occipital cortex. This suggests that treatment with streptozotocin can cause significant changes in the neuronal structure within the brain that could lead to neuronal degradation.

Recent studies have suggested a connection between TD2M and AD (Messier, Claude, Teutenberg 2005; De la Monte and Wands 2008). Diabetics are more likely than the general population to develop AD (De la Monte and Wands 2008). It has further been suggested that insulin resistance similar to that seen in TD2M mellitus is present in the brain with some researchers calling AD “Type Three Diabetes” (Messier, Claude, Teutenberg 2005; De la Monte and Wands 2008).

If AD is a form of diabetes, there will be an accumulation of extracellular glucose in the brain due to insulin resistance. Extremely high glucose levels can lead to cell death (Wang et al. 2010). Correia et al. (2011) showed that streptozotocin can be administered to rats to create a brain state that is insulin resistant similar to what is hypothesized to occur in AD. This insulin resistance leads to memory loss and problems with cognitive functioning in rodents much like the symptoms of an AD patient. This means that a model based on streptozotocin may be useful to determine possible treatments for the disease.

Previous experiments have demonstrated that insulin can protect neurons from neurodegeneration due to streptozotocin. De la Monte and Wands (2008) demonstrated that the insulin resistant state created through the use of streptozotocin can be alleviated through the use of insulin treatments similar to those used to treat TD2M. They further postulated that

AD is another form of diabetes specific to the brain. Most of the current experiments on the subject use *in vivo* rat models after exposure to a drug to create Alzheimer's like models. The rats are assessed in performing behavioral tests before and after administration of therapeutic agents in an attempt to test the memory loss symptoms. However, very few cortical cell culture models have been performed. An advantage of using *in vitro* assessments is that it allows for a direct observation of the effect of insulin signaling on primary neuronal cultures that were stimulated with glucose and streptozotocin. An increase in insulin signaling may decrease the build-up of extracellular glucose in turn minimizing oxidative stress. For this reason, an *in vitro* model will be used in this research as opposed to the *in vivo* model used in most studies. I will combine streptozotocin with high levels of glucose similar to that postulated to be present in the brain in AD.

Metformin

A current treatment for TD2M is the drug Metformin (Turner et al. 1999). Metformin is a biguanide oral medication that has been shown to decrease gluconeogenesis, as well as potentiate the effect of insulin in the periphery of patients with TD2M (Klip and Leiter 1990). Metformin has been shown to decrease plasma glucose levels by as much as 30% (Kirpichnikov, McFarlane, Sowers 2002). Some studies have reported that this occurs through an increase in insulin binding to glucose transporters in cell membranes. However, the exact mechanism is still being studied (Klip and Leiter 1990). Most studies point to a mechanism involving an increase in glucose transport, primarily through GLUT 4 transporters, a glucose transporter also found in the brain that is insulin sensitive (Klip and Leiter 1990).

Metformin activates 5’ AMP-activated protein kinase (AMPK) in the body (Fryer, Parbu-Patel, Carling 2002). Studies suggest that Metformin decreases TD2M symptoms through the activation of AMPK and may have glucose lowering effects. This is due to an increase in muscle glucose uptake and a decrease in hepatic glucose production which occurs through the inhibition of gluconeogenesis (Kirpichnikov, McFarlane, Sowers 2002). In addition, there is evidence that Metformin also decreases the oxidation of fatty acids (Kirpichnikov, McFarlane, Sowers 2002). This is significant as oxidation of fatty acids, such as those found in visceral adipose tissue, often leads to gluconeogenesis and the production of glucose, thereby increasing glucose concentrations. Decreased oxidation leads to a decrease in hepatic glucose levels.

Metformin in the brain

Metformin was previously used in a TD2M model to look at changes in the dentate gyrus in a rat model (Hwang et al 2010). Zucker diabetic fatty (ZDF) rats, which are used as a model of TD2M, were used in comparison to Zucker diabetic control (ZLC) rats. They determined that there were elevated blood glucose levels in the ZDF rats in comparison to the ZLC rats. In addition, these elevated levels were reduced by the addition of Metformin, although not to the level of the control. Metformin treatment also attenuated the decrease in neuronal cell proliferation.

It is still unknown whether Metformin can cross the blood-brain barrier or if it can be modified to cross the blood-brain barrier (El Mir et al. 2007). Recent research has shown that in addition to its effectiveness as a TD2M treatment, this drug may also have neuroprotective effects in a model using prolonged chronic insulin exposure to create insulin resistance in a

mouse neuroblastoma cell line (Gupta, Bisht, Dey 2011). The investigators found that this insulin resistance also led to impaired glucose uptake. Further, extracellular signal-regulated kinases (ERK1 and ERK2), involved in the activation of apoptosis within the cell, showed increased activation under insulin resistant conditions. In addition, there was an approximately 30% increase of secreted levels of amyloid beta 42 within these neurons. The addition of Metformin showed enhanced AMPK Thr172 phosphorylation, suggesting that Metformin affected neurons by activating AMPK as has been previously suggested in the periphery. Amyloid beta 42 levels decreased by 42% after Metformin treatment and ERK activation decreased. These findings suggest that Metformin may decrease neuronal death through the insulin signaling pathway. It also suggests that through this pathway Metformin can improve pathologies considered to play a role in AD, like increased amyloid beta 42 levels.

Another proposed mechanism of neuroprotection involves inhibiting mitochondrial processes leading to apoptosis. El Mir et al. (2007) found that Metformin prevented cell death from the antitumor drug etoposide in a dose dependent manner. The authors suggest that this neural protection occurs by decreasing permeability transition pore (PTP) opening and therefore decreasing the release of cytochrome c, the release of which typically triggers the apoptotic pathway. Other studies found similar results concerning the inhibition of apoptosis. It has also been suggested that this occurs through the repression of caspase 9 which decreases the cleavage of caspase 3 (Ullah et al. 2012). Caspase 3 typically cleaves poly ADP-ribose polymerase 1 (PARP-1) which leads to the cleavage of other proteins that progress the cell through the apoptotic pathway. Metformin has also been shown to increase

the expression of Bcl-2, a protein shown to decrease apoptotic signals (Ullah et al. 2012). Furthermore, Metformin decreases Bax expression, a gene responsible for increasing apoptotic signaling (Ullah et al. 2012). It has also been suggested that Metformin has the potential to protect against neuronal degradation by attenuating elevated amyloid beta 42 levels in db/db mice, as well as attenuating the increase in the tau kinase JNK functioning in db/db mice models (Li et al. 2002). Due to its use as a diabetes treatment, as well as its neuroprotective effects, current and future research will examine the possibility of Metformin alone and in conjunction with insulin as a possible method to alleviate neuronal toxicity.

Present Study

The first component of this project was establishing a cell culture model showing neuronal toxicity through potential players in the insulin pathway. High levels of glucose were hypothesized to cause neuronal toxicity. Because of its known toxic effects on cells, streptozotocin was also predicted to have neurotoxic effects. Following the application of each agent in isolation, an AD model was established by combining streptozotocin and high levels of glucose. The combination of streptozotocin and glucose was predicted to cause toxicity and neuronal degeneration through oxidative stress within the cells.

Once a model was established, the role of insulin was studied. It was hypothesized that insulin deprivation causes neuronal toxicity and that the application of insulin would decrease oxidative stress on neurons. If this is the case, then neuronal death in an AD model combining streptozotocin and glucose was hypothesized to reduce with the addition of insulin.

After gaining a better understanding of the effect of insulin on cell viability this project investigated whether the TD2M drug Metformin, independently and in conjunction with insulin, could have rescue effects on cells and prevent neural degeneration. It was hypothesized that if Metformin decreased apoptotic signals in cells this would prevent neural degeneration in an AD model combining streptozotocin and glucose.

Materials and Methods

Primary Cortical Neuron Cultures

Twenty four and ninety six well clear plates were prepared using poly-L-lysine overnight and then washing three times with Hank's Balanced Salt Solution (HBSS) to remove poly-L-lysine residue. Poly-L-lysine provides an adhesive surface for neurons to attach to during plating. We cultured primary neurons through dissection of an E17 pregnant Sprague Dawley female rat. Then we removed the fetuses, separated their heads, harvested their brains and dissociated the brain tissue in a solution of trypsin for five minutes.

Tissue was then washed in HBSS two times in order to remove any excess trypsin. Tissue was dissociated by a flame tipped pipette in plating media (neurobasal media, Penicillin Streptomycin, Fetal Bovine Serum). We plated my primary neuronal cultures at a concentration of 1 x 10^5 cells/well in clear 96 well plates for use with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assays.

Cells were cared for with growth media containing Glutamax (neurobasal media, Penicillin Streptomycin, b27 supplement, Glutamax) for the first five days in an incubator

(37°C, 5% CO_2). After five days, growth media without Glutamax was used. Cells were allowed to grow for two weeks before testing began. Neuronal health was maintained with replacement of half the growth media present in each well every other day. This provided the neurons with a new supply of necessary nutrients while keeping many of the neurotrophic factors released by the neurons to further neuronal development.

Table 1: Timeline of experimental procedure

Day 1	Day 3	Day 5	Day 7	Day 9	Day 11	Day 13	Day 14	Day 16
Dissection-24 and 96 well plates made. Growth media with Glutamax used.	All cells fed with growth media containing Glutamax.	All cells fed with growth media without Glutamax.	1-week cells stimulated, 2-week cells fed.	1-week cells MTS assay run, 2-week cells fed.	2-week cells fed.	2-week cells fed.	2-week cells stimulated, 24-well plate fixed and stained.	2-week cells MTS assay.

Treatment Conditions

Glucose Concentration Tests

After one or two weeks we added 100 microliters aqueous insulin free glucose of increasing concentrations (75-150 mM) to the wells in order to stress the cells similarly to the oxidative stress on neurons seen in AD. Half of the conditions were co-treated with insulin (100 nM) in order to assess whether insulin can rescue neurons stressed from glucose. Cells were stimulated in an incubator for forty eight hours prior to data collection.

Streptozotocin Tests

After one or two weeks we added 100 microliters of various combinations of solutions containing streptozotocin (STZ), glucose, and insulin (table 1). Glucose concentrations tested included 125 and 150 mM glucose. Streptozotocin concentrations include 100 μM and 1 mM.

The combination of glucose and streptozotocin was used in order to stress the cells similarly to the possible oxidative stress on neurons seen in AD if AD is in part due to insulin resistance within the brain. Neurons were co-treated with insulin (100 nM) in order to measure whether insulin can rescue neurons stressed from glucose and streptozotocin. Cells were stimulated for forty eight hours.

Metformin Tests

In order to determine Metformin concentrations that are not toxic to cells we performed a concentration curve with concentrations ranging from 0-1000 μM in 100 μM increments in standard growth media and insulin free growth media.

After two weeks we performed tests identical to the streptozotocin tests but with the addition of 400 μM Metformin. Neurons were co-treated with insulin (100 nM) in order to measure whether insulin can rescue neurons stressed from glucose. Cells were stimulated for forty eight hours. Glucose concentrations tested included 125 and 150 mM glucose. Streptozotocin concentrations include 100 μM and 1 mM.

MTS Assay

After a 48 hour treatment of glucose and insulin, we measured cell viability using a dye called 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS). We added 20 microliters to each well. When first added, the dye is bright yellow in color. As the neurons metabolize the dye the color turns a dark purple, allowing less light through the well. The more functioning, healthy neurons in each well, the more dye will be metabolized. A plate reader read the plates at 490 nm after four hours of incubation which then provided absorbance readings for the amount of light that went through each well. Results suggested which wells have more neurons that metabolized dye and which suffered significant cell death. Absorbance readings were converted to percent cell survival by standardizing each condition to the control which allowed for comparison across plates.

Cell Staining and Fluorescence

We fixed cells in the 24 well plate using 4% paraformaldeyde for twenty minutes. Cultures were then washed in phosphate buffered saline (PBS) three times for three minutes apiece. 0.5% Triton-100 was applied to cells for 10 minutes in order to create pores in the neuronal membranes. After one wash with PBS we added primary monoclonal anti-acetylated tubulin antibody produced in mice for one hour. After an hour, we removed the primary antibody and washed cells three times with PBS before adding the secondary anti-mouse IgG fragment-Cy3 antibody produced in sheep. We incubated cells for one hour following application of the secondary antibody and washed cells three times with PBS. We then visualized cells under a florescent microscope and measured mean intensity and percent area coverage.

Statistical Treatment of Data

All graphs were made in Excel. We evaluated data using statistical testing. A multifactorial ANOVA test allowed me to identify main effects and interactions. Tukey post hoc tests were run on all significant effects. All statistical tests were completed using Statistical Package for the Social Sciences (SPSS).

Results

In order to establish the model of neural degeneration that would be used for all subsequent experiments we first tested increasing levels of glucose with and without insulin. Cell viability was measured using an MTS assay. Toxicity levels varied when two week fetal rat neurons were treated for 48 hours with increasing concentrations of glucose with and without 100 nm insulin (figure 3). In a 2x5 2-way ANOVA there were main effects of insulin ($p=0.001$) and glucose ($p=0.000$) but no interaction between insulin and glucose ($p=0.301$). At 25 mM glucose (the amount of glucose in standard growth media), there was no toxicity observed (figure 3). In addition, at 100 mM glucose with insulin there was little measureable cell death ($p=0.423$). At 150 mM glucose, there were toxic effects in the no insulin condition in comparison to the control ($p=0.034$), but not in the insulin containing condition ($p=0.240$). In addition, at 175 mM glucose, there was decreased neuronal cell viability in the insulin deprived condition ($p=0.005$) in comparison to all other glucose concentrations that increased with the addition of insulin.

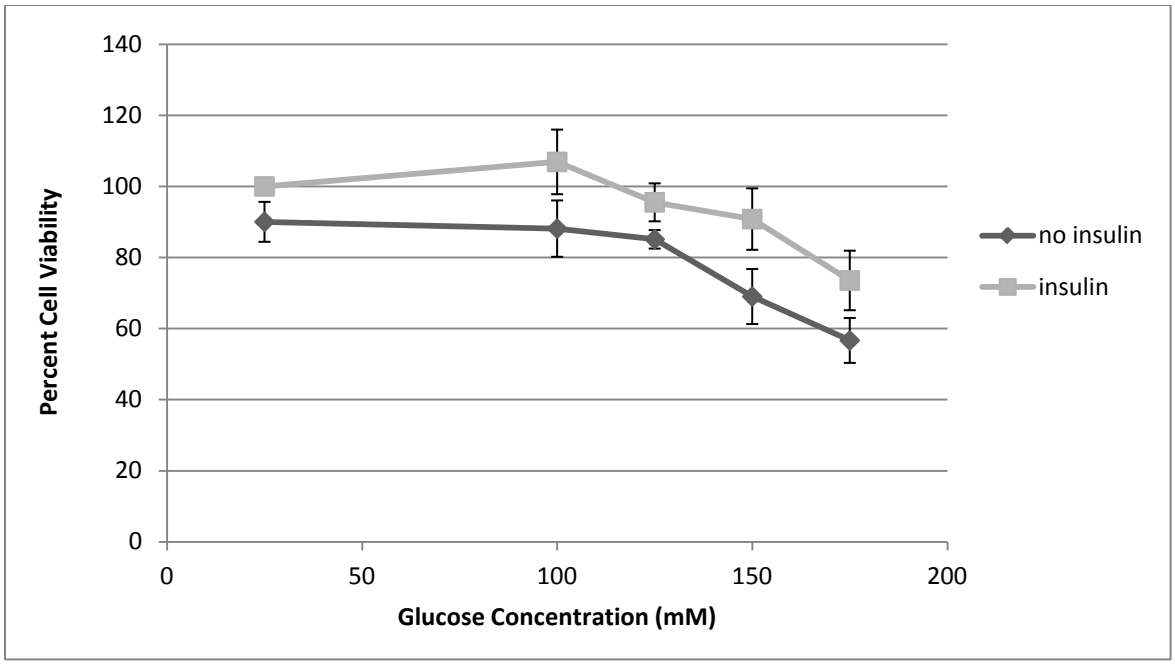

Figure 3: The percent cell viability as measured by an MTS assay of two week fetal rat neurons stimulated for forty eight hours with increasing concentrations of glucose with and without insulin. Data are mean +/- SEM. (N=5) Main effect of insulin and main effect of glucose $p < 0.05$.

Once levels of glucose toxicity were determined a concentration curve for Metformin was performed in growth media with and without insulin to determine concentrations of Metformin that would not cause neuronal toxicity. The range of concentrations tested was chosen based on previous research (El Mir et al. 2007; Ullah et al. 2012). An MTS assay was performed to assess cell viability. In insulin containing growth media, there was no effect of Metformin ($p=0.304$) in concentrations ranging from 100 μM to 1 mM (Figure 4). In insulin free media, there was an effect of Metformin ($p = 0.02$). There was an increase in cell viability between the 0 mM Metformin in insulin free media and 200, 500, 600, and 750 μM Metformin ($p=0.02$, $p=0.01$, $p=0.01$, $p=0.03$).

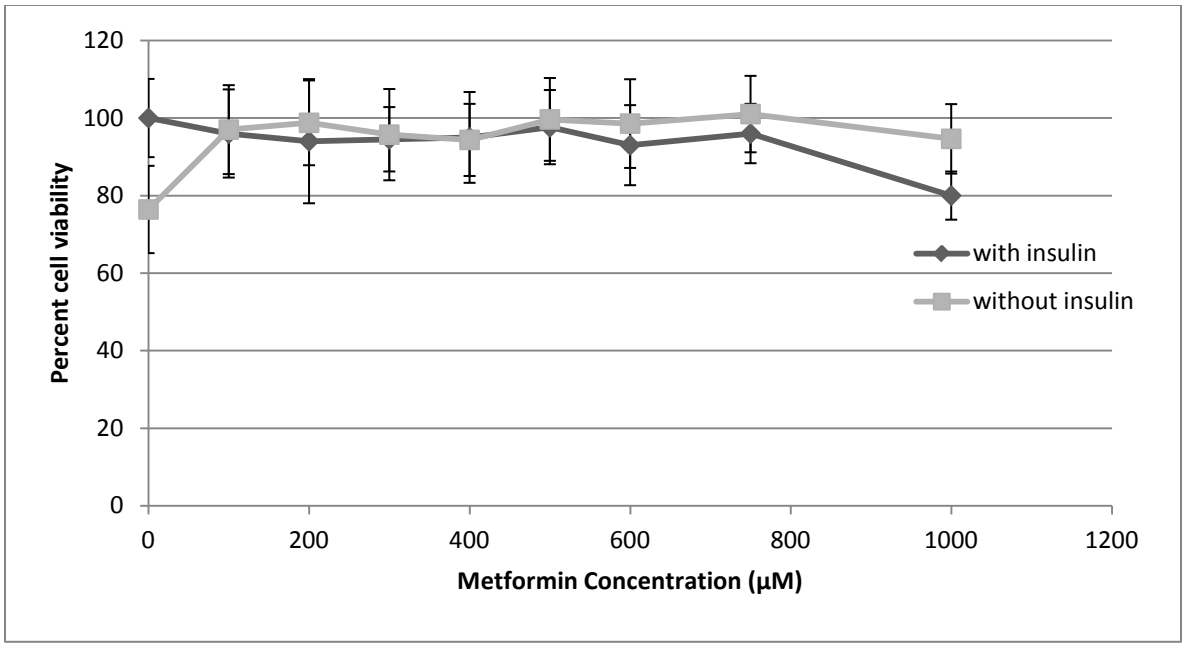

Figure 4: Cell viability as measured by an MTS assay of Metformin concentrations ranging from 0 to 1 mM in insulin containing growth media with and without insulin. Data are mean +/- SEM. (N=6)

400μM Metformin was chosen for subsequent experiments because it did not cause neuronal toxicity and would provide a mid-range assessment of the impact of Metformin. Metformin at this concentration was used with high levels of glucose to determine the ability of Metformin to protect cells against the toxic effect of glucose. Cellular health was assessed using an acetylated tubulin stain to visualize stable microtubules. From this stain percent area coverage of stable microtubules, a potential indicator of neuronal health, was measured. A 2x2 2-way ANOVA was performed on assays of percent area coverage of acetylated tubulin stain. In measures of percent area coverage of microtubules there was a main effect of glucose ($p = 0.002$; figure 5). There was a decrease between control conditions (growth media containing 25 mM glucose) and those containing 125 mM glucose. There was no effect of Metformin on percent area coverage ($p=0.31$).

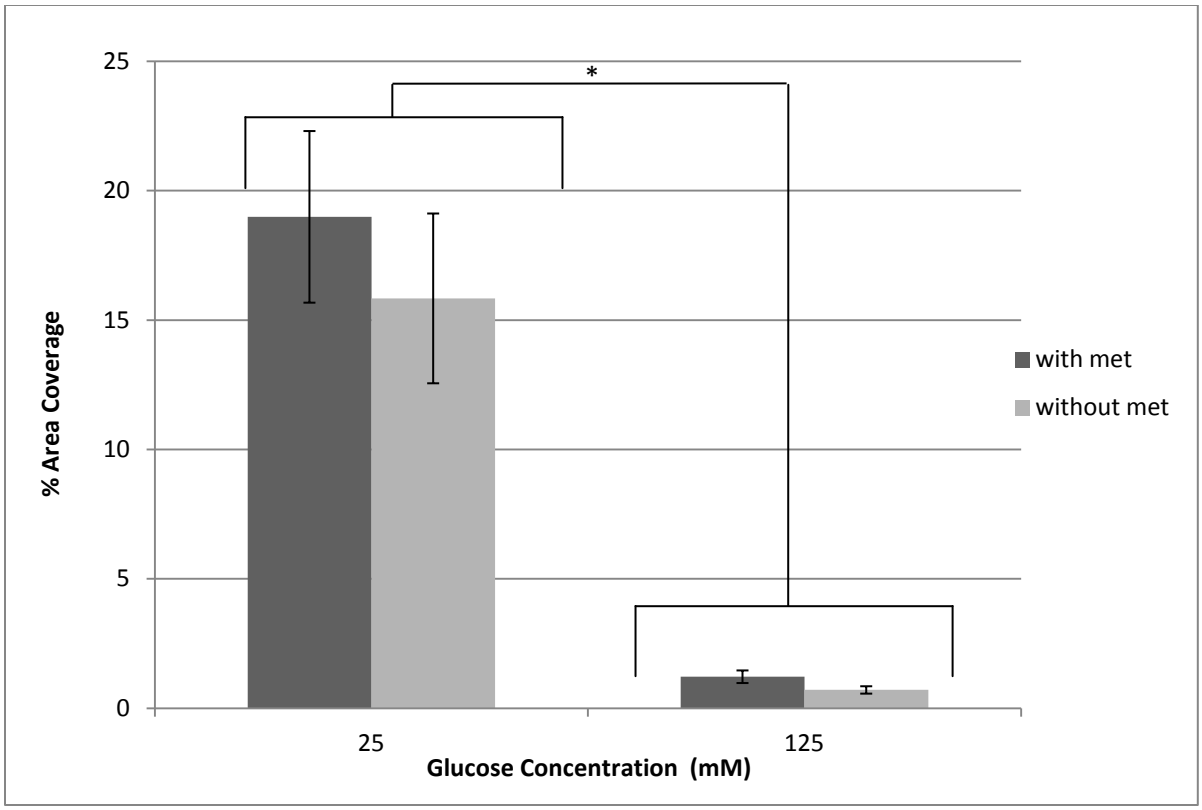

Figure 5: The percent area coverage of tubulin stain in control (25 mM) and high (125 mM) glucose concentrations with and without 400 μM Metformin. Error is standard error of the mean. (N = 16). * = p < 0.05.

In addition to percent area coverage, other measures were used to evaluate the potential of Metformin to protect against the toxic effects of elevated glucose levels. Cellular health was assessed using measures of the mean intensity of the acetylated tubulin stain. A 2x2 2-way ANOVA showed that there was a main effect of glucose ($p = 0.00$; figure 6). Control conditions with growth media containing 25 mM glucose had a greater mean intensity of stain than those containing 125 mM glucose. There was no main effect of Metformin ($p = 0.15$).

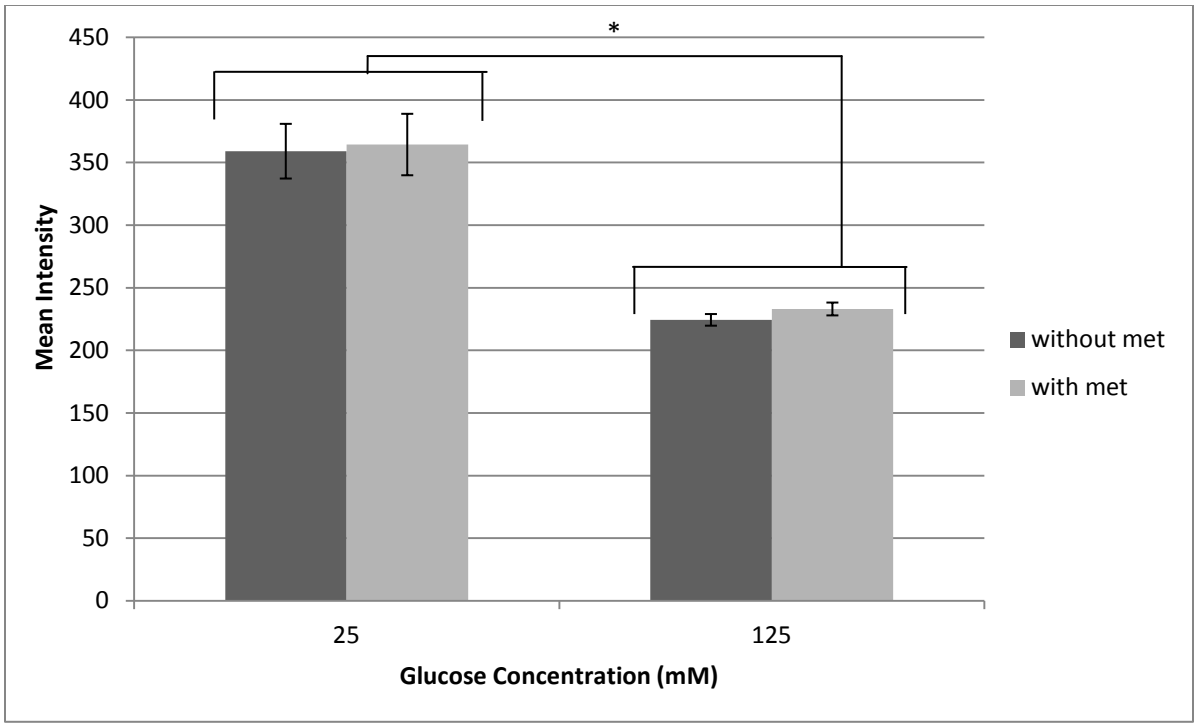

Figure 6: The average intensity of tubulin stain of microtubules in control (25 mM) and high (125 mM) glucose concentrations with and without 400 μM Metformin of an acetylated tubulin stain. Main effect of glucose (p =0.00. Data are mean +/- SEM. (N = 16) * = p < 0.05

Once neuronal toxicity due to high glucose levels was established, neuronal health was tested under conditions containing a combination of glucose and STZ to create a more accurate model of diabetic-like conditions within cell culture. Cellular health was assessed using an MTS assay. When streptozotocin was added to the glucose/insulin combination in two week fetal rat neurons (figure 7) in growth media containing 25 mM glucose, a 2x2x2 3-way ANOVA showed a main effect of both insulin, glucose, and STZ ($p<0.05$). The insulin deprived conditions had more cell death than those conditions containing insulin ($p=0.00$). Glucose containing conditions had lower percent cell survival than conditions without glucose ($p=0.01$), and STZ containing conditions had lower percent cell survival than conditions without STZ ($p=0.025$).

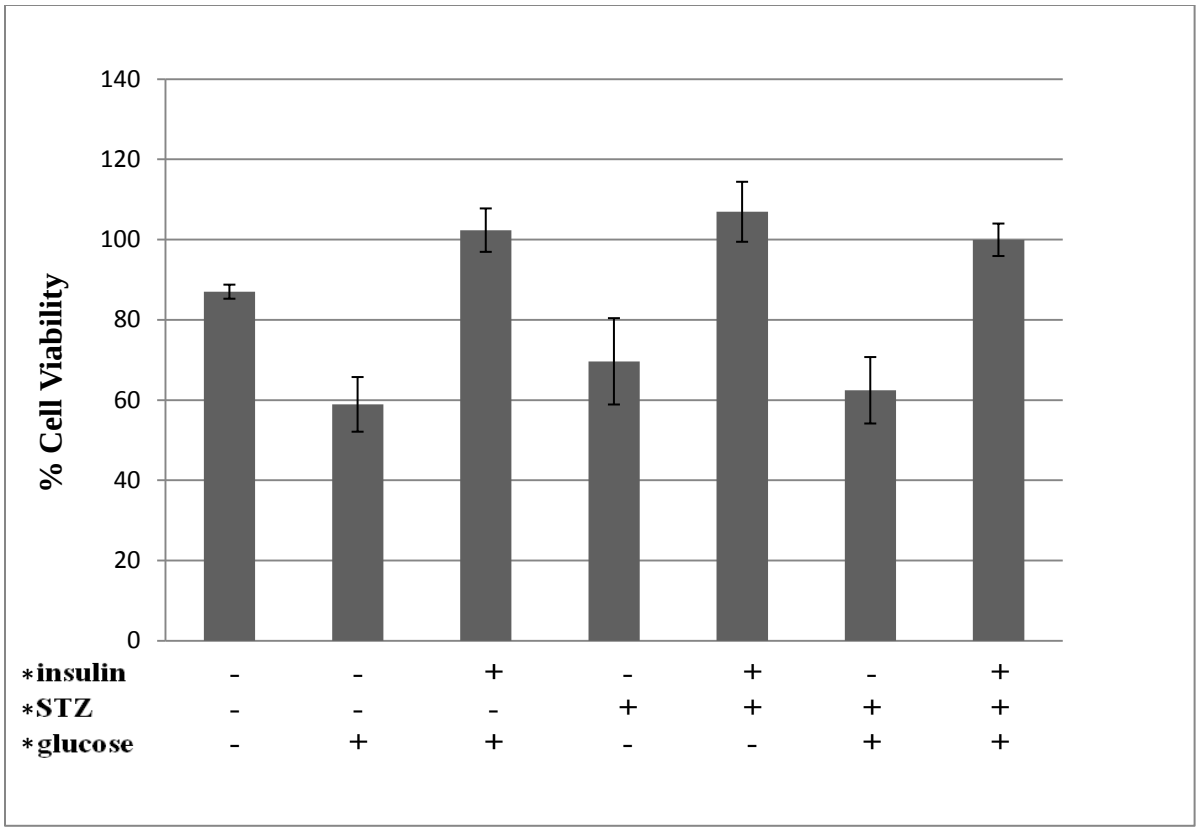

Figure 7: Effect of 100 mM glucose and 100 µM Streptozotocin (STZ) separate and in combination on neuronal toxicity with and without insulin treatment (100 nM) in two week fetal rat neurons. Percent cell viability obtained from an MTS assay. Data are mean +/- SEM. (N = 10). Percent cell viability standardized to growth media condition containing insulin. * = main effect, $p < 0.05$.

The toxicity caused by the combination of glucose and STZ created a model of neuronal degeneration in diabetic-like conditions. Metformin was added to this model to examine its potential in rescuing neurons from the toxicity of these stressors. Cellular health was measured using an MTS assay of cell viability. Overall, there was a main effect of insulin ($p=0.00$; figure 8). Conditions containing insulin had higher percent cell viability than conditions without insulin suggesting that insulin deficiency can be toxic to cells. There were also main effects of glucose and STZ ($p=0.00$ and $p=0.005$). There was no effect of Metformin ($p=0.19$).

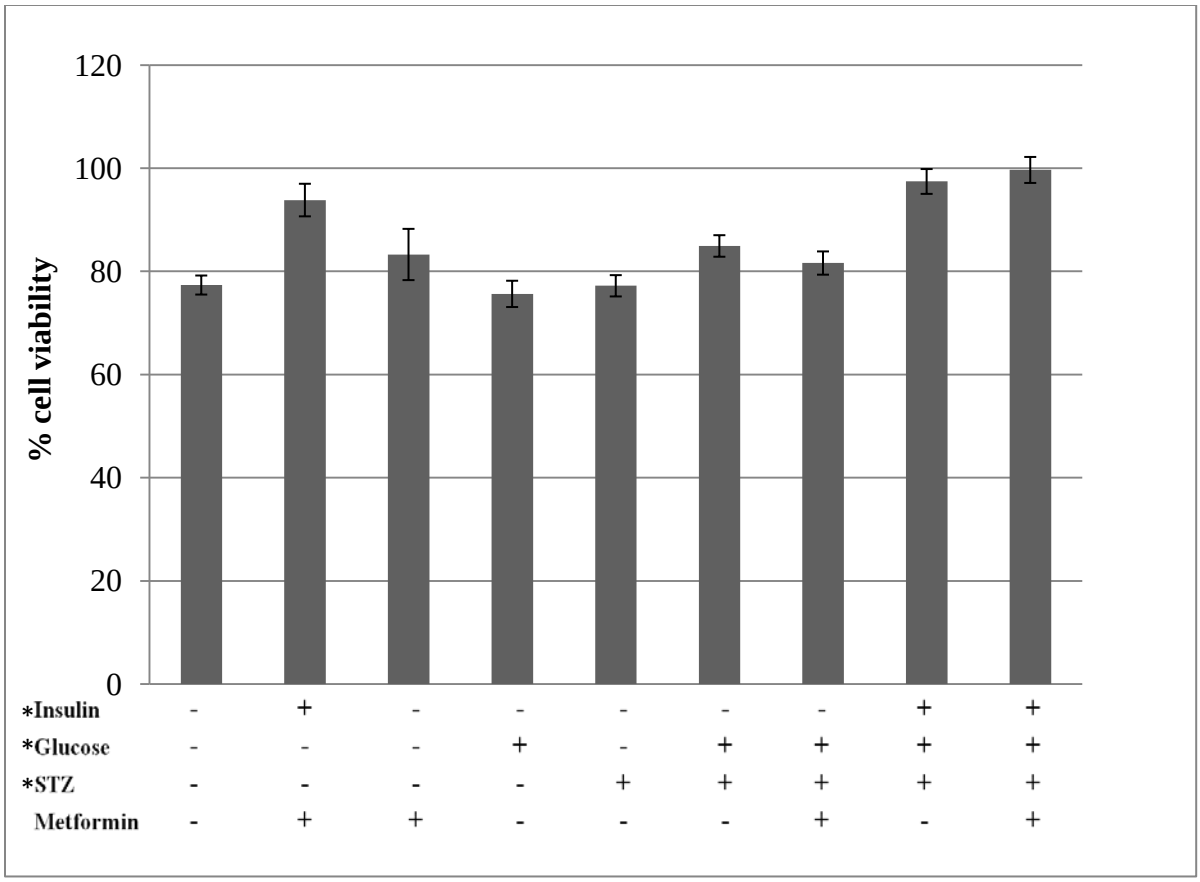

Figure 8: Effect of glucose and streptozotocin separate and in combination on percent cell viability with and without insulin treatment (100 nM) and with and without the addition of 400 µM Metformin in two week fetal rat neurons. Percent cell viability obtained through MTS assay. (N = 10). Data are mean +/- SEM. Main effect of insulin, glucose, and STZ ($p<0.05$). Percent cell viability standardized to growth media condition containing insulin. * = main effect, $p < 0.05$

Discussion

Model of Degeneration

The first goal of this research was to isolate a model of AD using high levels of extracellular glucose and STZ. We chose to focus on disruptions in the insulin pathway because problems in the insulin pathway in the brain have been implicated in AD including a correlation between decreased insulin levels and decreased cognitive functioning (Laron 2009). In conjunction with changes in insulin levels, a link between TD2M and AD could suggest an insulin resistant brain state in AD (De la Monte and Wands 2008).

Insulin's role in glucose transporter translocation suggests that creating a cell culture model using high levels of glucose could mirror some of the effects of irregular functioning of the insulin pathway. In order to test this we first studied the effect of high levels of glucose on neuronal health (figure 3). By adding additional glucose to growth media it was possible to see the effect of high levels of extracellular glucose on overall cell viability (figure 3) and it was hypothesized that high levels of glucose would lead to neural degeneration. To assess changes in neuronal health we used an MTS assay to measure cell. Cell viability provides a general measure of the number and health of the neurons in culture and serves as an overall comparison of cellular health across conditions.

Control conditions contained low levels of extracellular glucose (25 mM). Cells in the body, including neurons, need glucose to provide the energy necessary to survive and maintain function (Berg et al. 2012). However, toxicity was seen in conditions with high glucose conditions, including 150 and 175 mM (figure 3), which decreased cell viability to

close to 60%. This suggests that a buildup of extracellular glucose, as would occur in a problem with insulin signaling within the brain, can produce toxic conditions severe enough to cause neuronal death. A buildup of extracellular glucose could lead to increased intracellular glucose uptake, thereby producing oxidative stress within the cell (Tomlinson and Gardiner 2008). One proposed mechanism for this is through the mitogen-activated protein kinase (MAP kinase) pathway (Tomlinson and Gardiner 2008). High extracellular glucose levels can lead to intracellular changes in pathways including activating the MAP kinase pathway (Tomlinson and Gardiner 2008). MAP kinase is involved in regulating pathways including cellular growth and apoptosis (Berg et al. 2012). The activation of MAP kinase by elevated extracellular glucose levels could lead to the downstream activation of the apoptotic pathway and cellular toxicity. Oxidative stress can lead to the formation of reactive oxygen species which can cause structural damage within the cell and lead to the induction of the apoptotic pathway (Moreira et al. 2007; Berg et al. 2012). Furthermore, once activated, oxidative stress can be responsible for the progression of the apoptotic pathway by causing the release of cytochrome c, leading to cell death (Wang 2001). It is possible that cell death seen at the high levels of glucose including 150 mM and 175 mM could be working through the induction of the apoptotic pathway.

Glucose toxicity was shown to be mitigated in conditions containing insulin at the low and moderate glucose concentrations (figure 3). However, at extremely high concentrations of glucose, namely 175 mM, insulin was no longer effective at preventing the toxic effects. It is likely that at these high concentrations the effect on neuronal health is severe and the cellular pathways are disrupted to a point where the addition of insulin does

not have a sufficient effect on the improvement of cell health. In this case, mitochondria may be producing reactive oxygen species at a rate that cannot be protected against (Tomlinson and Gardiner 2008) or the strength of the signal leading into the apoptotic pathway may be too robust to overcome. To test these hypotheses in the future, assays testing mitochondrial health, such as a JC1 mitochondrial dye which measures mitochondrial membrane potential could be used. This technique could be used to measure the overall percentage of healthy mitochondria in each condition.

In addition, STZ, a drug often used as a model for TD2M, could cause neuronal death through production of ROS. STZ destroys pancreatic β-cells and has been used to model diabetes in both *in vivo* and *in vitro* studies (De la Monte and Wands 2008). Administering STZ directly into the brain via intracerebroventricular injection (ICV) injection simulates this insulin resistance seen in AD brains and has been used as a model of AD in past literature (De la Monte and Wands 2008). Our laboratory has been studying the effects of STZ on isolated rat cortical neurons as a potential *in vitro* model of AD. It was predicted that a cell culture model combining glucose and STZ would decrease cell viability.

Once we were able to identify extracellular glucose concentrations that showed toxicity and those toxic conditions that had the potential to be reversed we were able to use these concentrations in additional studies. The next step was to study the effect of the high glucose concentrations in conjunction with STZ (figure 7). STZ is often used in biological models because it causes disruptions in the insulin pathway similar to those in TD2M. Treatment with STZ has been shown to lead to the production of ROS which can lead to destruction of insulin producing cells (Like and Rossini 1976). In addition, when

administered to rats, STZ has been shown to create an insulin resistant brain state in conjunction with memory loss and cognitive problems (Correia et al. 2011). Previous research in the lab of Dr. Roger Knowles has suggested that there are still many components within the insulin pathway that need to be tested to determine if STZ causes insulin resistance and where in the insulin pathway this occurs (Rachel Masia, Honors Thesis 2013). Past work showed that in isolation, a STZ concentration of 100μM produced neuronal toxicity and was chosen for use in this study based on past research (Rachel Masia, Honors Thesis 2013).

In addition, STZ caused approximately 20% cell death in two week old neurons (figure 7). However, there was not increased toxicity when glucose and STZ were combined. This indicates that glucose and STZ could produce toxic effects through similar pathways within the brain. If elevated glucose levels are causing the production of ROS or leading to the activation of MAP kinase and STZ causes toxicity in the same manner, than there would not be an additive toxic effect on neurons. It is also possible that one of the compounds is working at a maximal effect, decreasing neuronal viability to a point where no additional opportunity for the other compound to exert an effect. Another explanation could be that one compound is working upstream of the other, preventing the ability of the other compound to contribute to cell viability downstream. A better understanding of how STZ exerts its effects in the brain would provide more insight into this issue. In the body, STZ enters pancreatic β cells through glucose transporters and causes DNA damage, leading to β cell apoptosis (Szkudelski 2001). However, the mechanism of action in the brain is unknown, and future studies will attempt to identify the STZ pathway of neurodegeneration.

The effect of the presence and absence of insulin was also considered in the models of toxicity using glucose and the combination of glucose and STZ. There was an effect of insulin in both models (figures 1 and 7). The absence of insulin produced toxic effects that were rescued by the addition of insulin. Because of the many functions of insulin within neurons, without insulin, toxicity could be caused by dysfunction in glucose transport regulation, GSK-3β regulation, and/or oxidative stress (Berg et al. 2012; Maesako et al. 2011; Moreira 2007). To further assess these alternatives, a molecular probe for reactive oxygen species, such as dichlorodihydrofluorescin diacetate (DCF) could be used to measure ROS levels (Lee et al. 1999). In addition, a western blot could be performed to assess relative amounts of GSK-3β present (Taylor et al. 2013).

Because there is toxicity in the glucose/STZ model under insulin free conditions (figure 7) this model was used to assess treatments used for TD2M. The glucose/STZ model provided levels of cell viability that were lowered, but not to the point that was un-recoverable. This model was used with the addition of Metformin with and without insulin (figure 8) to assess the potential of Metformin to prevent neural degeneration.

This AD model is unique in that it approaches neuronal toxicity through dysfunction in insulin pathways. Other models for AD have been used in the literature including glutamate and amyloid beta. Glutamate is an excitatory neurotransmitter in the brain (Kandel et al. 2010) that in excess can produce toxic effects. Research has suggested that there is lower glutamate reuptake in AD patients which could cause excitotoxicity and contribute to the development of the AD model of glutamate (Hynd et al. 2004). Amyloid beta is another common model of AD because aggregates of amyloid beta form plaques in the brain of AD

patients (Riek et al. 2001). However, because the cause of AD is unknown and the exact contribution of glutamate and amyloid beta is still to be determined, the glucose/STZ model of AD provides a new approach to neurodegeneration in AD.

Metformin Intervention

It was predicted that the TD2M drug Metformin could further alleviate cell toxicity caused by glucose and streptozotocin by potentiating the effect of insulin within the cells. Metformin is a drug currently used to treat TD2M. However, its potential as an aid in the prevention of neural degeneration has been suggested in recent literature (Gupta, Bisht, Dey 2011). Concentrations of Metformin used in cell culture have differed drastically ranging fro m 1 0 0μM (El Mir et al. 2007) to 3.2 mM (Gupta, Bisht, Dey 2011). We chose concentrations of Metformin that were not toxic. We did this in growth media as well as insulin free media (figure 4) and tested concentrations ranging from 50 μM to 1 mM. There was no toxicity seen at these concentrations. We chose to continue using 400 μM Metformin as it was in the range that did not appear toxic to neuronal health.

Our data show that Metformin did not reduce toxicity caused by glucose and glucose/STZ. An anti-acetylated tubulin stain provided information on neurite growth and stability. Acetylated tubulin is present in stable microtubules (Palazzo et al. 2003) and visualization with this stain allowed for measures of area coverage of neurites (figure 5) as well as intensity of stain (figure 6). Neurons that are breaking down due to apoptosis and poor neuronal health will likely have less acetylated tubulin than healthy neurons (Schulze et al. 1987). 125 mM glucose significantly decreased percent area coverage by 16% and mean intensity of stain by 125 (figures 5 and 6). A decrease in percent area coverage could be

indicative of neural degeneration as stable microtubules break down (Palazzo et al. 2003). In addition, a decrease in mean intensity of stain suggests decreased presence of acetylated tubulin and unstable microtubules which could also indicate neuronal toxicity (Palazzo et al. 2003). At 400 μM Metformin did not rescue cells from glucose toxicity as there was no difference in percent area coverage and mean intensity of the anti-acetylated tubulin stain (figures 5 and 6). However, there was an unexpectedly high decrease in percent area coverage due to glucose (figure 5) which could indicate that the cells were extremely sensitive to glucose. Future experiments could use lower concentrations of glucose to examine any potential rescue effects of Metformin on microtubule stability. Measurements of microtubule stability are important because a treatment that can increase the cell viability but does not have an effect on the cells' ability to develop neurites and communicate would not be helpful.

In measures of cell viability, there was also no difference in conditions with and without Metformin (figure 8). While there was a decrease in conditions without insulin, these effects were rescued by the addition of insulin but not the addition of Metformin. Metformin also did not alleviate toxic effects due to STZ and glucose. Because we used a concentration of Metformin lower than suggested in the literature, we could use higher concentrations of Metformin in future studies to see whether it is more effective at protecting against neuronal degeneration. At higher concentrations Metformin could decrease cytochrome c release as was suggested by El Mir et al (2007) and decrease apoptotic signaling.

There were challenges encountered over the course of the experiment that may have contributed to the lack of effect on neuronal health by Metformin. In order to promote

neuronal growth, cells were maintained for the first five days in growth media containing Glutamax. Glutamax helps stabilize the neuronal environment by decreasing ammonia production and maintaining baseline levels of L-glutamine in the media (Life Technologies 2011). This will allow cells to remain healthy and functioning for longer periods of time. However, once we began using Glutamax in growth media the neurons grew too confluent and concentrations of glucose that were previously toxic no longer showed toxicity. Because of this we had to slightly alter our model, using slightly higher concentrations of glucose or using the higher end of most of our glucose concentration curves (mostly 175 mM glucose) as well as by cutting the concentration of cells plated in half.

In addition, we had problems with contamination of cell culture. While we supplement our growth media with Penicillin Streptomycin, this does not prevent against all bacteria and fungi. All cells are cultured and maintained in sterile conditions and all media and solutions filtered before treatment. However, it is still possible for neuronal health to be compromised by bacteria despite using aseptic techniques, and we did lose some plates that were intended for use with STZ alone and STZ and Metformin. The information from these plates would have filled critical gaps in our knowledge.

Furthermore, we did at times have trouble with our cells developing sufficiently. There were times when there were few neurons and those that were there had minimal development of neurites. One hypothesis could be the Penicillin Streptomycin that we use in our media to prevent against bacterial infection is having toxic effects on the neuronal cultures. Studies have demonstrated that these antibiotics could actually prevent neuronal development by causing mitochondria damage within the cells by altering the

electrophysiological prosperities of the neurons (Bahrami and Janahmadi 2013). Future experiments could test similar conditions as those in this study without the use of antibiotics or with significantly less antibiotics.

It is also possible that Metformin does not protect against neuronal degeneration caused by glucose/STZ or that the measures we used to assess differences in neuronal health did not capture other changes that may have been present. In addition, acetylated tubulin is not necessarily a measure of neuronal health as overly acetylated tubulin might mean that a cell is locked into place and exhibits decreased plasticity which is also not beneficial for the cell. There are other assays we could use to assess neuronal health. One avenue could be to test mitochondrial health of neurons using a JC1 dye. As mitochondria are the powerhouse for the cell, poorly functioning mitochondria could indicate unhealthy neurons. Radioactive labels could be used to assess differences in ROS production (Lee et al. 1999). In addition, it is possible that the 48 hour time course used was not effective but a longer time course with Metformin treatment could provide a reduction in neural toxicity from glucose and STZ.

Alterations in experimental design could provide additional information on the effect of glucose, STZ, and Metformin on neuronal health. In preliminarily studies (data not included) with glucose, streptozotocin, and insulin we originally did not see toxicity in the insulin free growth media condition. To examine whether this was due to no toxicity in insulin deprived conditions or just a lack of removing all of the growth media containing insulin, we began washing plates with insulin free growth media before stimulation. This assured that no growth media with insulin remained in wells during stimulation. After this change, we saw

approximately 15% cell death due to insulin deprivation. I would like to repeat my initial glucose/insulin experiments following this procedural change.

One way to improve insight into the insulin pathway within the brain as well as the mechanism of Metformin would be to measure endogenous insulin concentrations. While we tried to control for the amount of insulin added to each condition by using insulin free media and adding in a controlled amount of insulin (100 nM) this does not take into account that the neurons are secreting their own insulin. It would be helpful in the future to measure this endogenous insulin concentration through an enzyme-linked immunosorbent assay (ELISA) for more precise measures of insulin concentration within each condition.

Furthermore, it would be beneficial to determine through which pathway Metformin is functioning. One way to test this would be by using an insulin receptor antagonist. One potential antagonist is S169, which has been found to cause hyperglycemia and decreased glucose uptake in Sprague-Dawley rats (Vikram and Jena 2010). If any effect of Metformin is reversed this suggests that Metformin is working through a pathway involving insulin receptors. If however, there is no change in the action of Metformin, then it is likely another pathway is involved in the functioning of Metformin.

It is also possible that a glucose/STZ *in vitro* model of AD may not capture the potential benefits of Metformin. Current *in vitro* models of AD utilizing glutamate or amyloid beta may be able to reflect changes in neuronal health due to Metformin that were not visible in a model using glucose/STZ. In addition, studying the effect of Metformin in an *in vitro* model shows the direct effect of Metformin on neuronal health. However, it is possible that by isolating neurons, information on changes that occur within the body that can have an impact

on the brain could be lost. Experiments using an *in vivo* model would provide information on how Metformin effects the organism as a whole or changes in cellular health that can only be seen in interactions between the body and brain.

Conclusions

The glucose/STZ model did produce significant decreases in neuronal health. Therefore, its continued use as a potential model of cell death due to problems in insulin signaling remains possible. In addition, insulin free conditions produced significant decreases in cell viability indicating the importance of the presence of insulin and the insulin pathway on neuronal health.

Metformin's potential to prevent neuronal degeneration in an AD model is still in question. At 400 μM, Metformin does not appear to be effective in reducing the cell death in a model of AD using high glucose concentrations and STZ. However, many studies using Metformin have used higher concentrations of the drug. Therefore, further experiments could consider the potential of higher concentrations of Metformin or extended pretreatment time on the ability to protect against neuronal degeneration. In addition, the effect of Metformin on neuronal health in other models of AD could be studied. This includes other *in vitro* models like those utilizing glutamate and/or amyloid beta as well as *in vivo* models.

In addition, isolating the pathway and mechanism of action of Metformin in the brain would provide more insight into its potential in the prevention of neural degeneration. Therefore, while my results do not suggest that at one specific concentration of Metformin

the drug is effective at reducing neuronal toxicity, the potential of the drug still stands as additional concentrations should be considered before further conclusions are made.

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