Dedication:

This work is dedicated to my grandfather, Ali Miraoui (RIP: August 2012), who shared my love for science, and to every patient and caregiver facing the daily struggles and difficulties of Alzheimer's Disease.

Drew University

College of Liberal Arts

Effects of Methylene Blue on Nerve Cell Viability in an Alzheimer's Disease Hyperglycemic *In vitro* Model

A Thesis in Biology:

by

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Abstract

Alzheimer's disease (AD) is a neurodegenerative disease pronounced by memory impairment as well as a loss of one's cognitive abilities. Type 2 Diabetes (T2DM) is a metabolic disorder marked by abnormal glucose regulation and decrease in insulin signaling. This study examines the effects of a T2DM-like state pathways, including hyperglycemic and insulin-free conditions, on neurodegeneration using an *in vitro* embryonic nerve cell culture model. Results suggest high glucose (150 mM) levels and insulin deprivation independently induce neurodegeneration, while a combined effect of two times more neurodegeneration was observed when both conditions were present. This thesis also examines the effect of a potential AD drug called Methylene Blue (MB) on preventing neurodegeneration induced by hyperglycemic and insulin deprived conditions. Results suggest MB independently has no neurotoxic effects on nerve cells at a low dose of 100nM. In insulin deprived conditions, MB had no neuroprotective effect, while in insulin conditions MB showed potential signs of reducing neurodegeneration through an insulin-mediated pathway. The effects of MB on neuronal health and microtubule stability in the presence of high glucose and insulin deprivation was also examined in a preliminary study using immunostaining. Neuritic intensity and neurite counts were used to quantitatively measure the effects of MB on neuronal microtubule stability, while fluorescent images and stain brightness were used to qualitatively measure MB's effects on microtubule stability. An increase in neurite counts and neuritic intensity were

observed in MB conditions; however, no conclusions can be made due to the limited amount of data. Similarly, no conclusions could be made on qualitative observations of increased dendritic branching and axon lengths in MB containing conditions, due to limited data. Besides running more replicates of the experiments conducted in this study to further support my hypotheses, other future experiments will focus on measuring the effects of MB on a more specific neurodegenerative model of AD using hyperglycemic and insulin-free conditions along with AD pathology to induce neurodegeneration.

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INTRODUCTION

In this thesis, two major questions were investigated using an *in vitro* embryonic cell culture model. The first is "Can a Type 2 Diabetes Mellitus (T2DM) disease state, such as high glucose and insulin deprivation, induce Alzheimer's Disease (AD)-like neurodegeneration. The second is if high glucose levels and insulin-deprivation can induce neurodegeneration, can a potential AD drug called Methylene Blue (MB) prevent this neurodegeneration from occurring. Even though, this is not a clinical study, our results may have clinical implications in the future diagnosis of AD. If T2DM disease pathways induce neurodegeneration and MB reduces nerve cell death in these conditions, these preliminary results could have the future potential to change the way we test AD drugs. Instead of recruiting a diverse group of participants, researchers could test potential AD drugs, like MB, on a smaller and more specific population of drug trial participants. This study does not prove nor disprove the causative effect of T2DM in AD or other alternative pathways involving AD and metabolic disorders, rather it recognizes T2DM as playing a contributing factor in neurodegeneration, a hallmark of AD.

Alzheimer's Disease (AD) Overview

Alzheimer's Disease (AD) is the most common form of dementia in the United States and is one of the most fatal and irreversible diseases today (Alzheimer's Association (AA) 2014, AA 2015). It is estimated that in 2010, 600,000 people died from AD in the United States alone, and in 2015, this number rose to approximately

700,000 deaths (AA 2015). Moreover, 5.3 million Americans are estimated to be living with AD today; 95% of whom are above the age of 65 (AA 2015). By 2050, this number is expected to grow to 13.8 million, suggesting an incidence of one person developing the disease every 33 seconds (AA 2014, Sperling et al., 2011). This growth in AD cases is hypothesized to correspond with increased life expectancy in recent decades due to advancements in medicine (WHO 2014). Rapid drug discovery along with easier access to medical facilities have improved peoples' life expectancy; therefore more people are living beyond the age of 65 and are at an increased risk for AD.

AD is a progressive neurodegenerative disease that is pronounced by memory impairment as well as a loss of one's cognitive abilities. Neurons, along with glial cells, are the primary cells involved in brain signaling and function. Unlike other types of cells, neurons do not undergo cellular division or mitosis (Purves et al., 2001). Furthermore, these cells are made up of dendrites, which receive signals from other nerve cells, a cell body called the soma, and an axon, which propagates the signals to a post synaptic terminal and across a gap junction called a synapse (Knolbloch and Mansuy 2008). If abnormal physiological or molecular changes occur in the brain and in neuronal synaptic signaling, detrimental impacts to cognition and memory are hypothesized to result. These impacts can lead to severe AD-like symptoms such as lack of judgment and planning abilities, confusion, lack of comprehension, misplacing items, anxiety, depression, severe memory loss, and in very late stages of the disease motor skill deficiencies and trouble swallowing (AA

2014, Knolbloch and Mansuy 2008). Additionally, patients can live nearly a decade with severe symptoms resulting not only in emotional burdens on patient caregivers and families, but financial burdens as well. Thus, the estimated cost for AD patient care in 2014 was estimated to be a staggering $100 billion annually (AA 2014). This number is expected to grow with increased mortality rates across the globe.

The cause of AD symptoms and the development of the disease are unknown, but some scientists hypothesize two hallmarks of the disease, plaques and tangles, might be playing a causal role (Freir et al., 2011, Da Roucha-Soto et al., 2012, Knowles et al., 1999). Still, there is very little direct evidence to support this hypothesis as plaque and tangle pathologies are difficult to identify in AD patients. Plaques can only be identified during later stages of the disease using PET scans, after protein dyes label and bind to Aβ. Tangles, on the other hand, can only be identified after an AD patient has passed, by performing autopsy procedures (Kadir et al., 2012). Therefore, this makes it very difficult for researchers to find concrete evidence supporting a potential relationship between plaque and tangle pathologies and the development, as well as progression, of AD.

Hallmarks of AD

Neurodegeneration

AD is marked by a gradual loss of neurons in specific brain regions, such as the hippocampus, as the disease progresses (Kitamura and Inokuchi 2014). Some

scientists hypothesize pathophysiological processes involving plaques and tangles might be contributing to this trend of nerve cell death (Freir et al., 2011, Kadir et al., 2012). Others hypothesize abnormalities in cellular signaling and processing such as mitochondrial dysfunction, insulin signaling or inflammation might play an active role in neurotoxicity and neurodegeneration (Scott and Hunter 1965, Debling et al., 2006, Chen et al., 2008, Zhao et al., 2009, DeFelice et al., 2014). Additionally, some recent literature suggests other diseases, including metabolic disorders like T2DM, as contributing factors in neuroinflammation and ultimately nerve cell death (Boyle et al., 2006, Kadir et al., 2012, Lourenco et al., 2013).

Plaque Pathology

Today, the cause of AD remains unknown, but some scientists hypothesize pathophysiological processes might play a key role in the development and progression of the disease. One of the two major pathologies suggested to play such a role is plaque pathology. Plaques are circular deposits made up of Amyloid Beta (Aβ) aggregates that form around neurons (Knowles 2004, Freir et al., 2011). Aβ is a peptide normally produced in the brain through β, γ-secretase cleavage of Amyloid Precursor Proteins (APP) (De Felice et al., 2009). Interestingly monomeric Aβ is not believed to be toxic to neurons (DeFelice et al., 2009), but aggregates of the peptide are hypothesized to lead to disruptions in neuronal signaling and ultimately cell death (Freir et al., 2011). Furthermore, many scientists hypothesize γ-secretase cleavage of C99, a peptide fragment that is produced by β secretase cleavage of APP,

can happen at two separate sites giving rise to amyloid beta peptides that are either 40 amino acids long or 42 amino acids long (Kun et al., 2010, Columbo et al., 2013). Aβ-42 is hypothesized to form aggregates that can lead to the development of plaque formation, while Aβ-40 has been associated with the monomeric form of the peptide that is normally produced in the brain (Alberdi et al., 2010). Even though the total amount of Aβ being produced is constant in all individuals, the ratio of Aβ-42 versus Aβ-40 produced may differ between healthy individuals and AD patients.

Moreover, there are various hypotheses concerning the role of Aβ aggregates in AD. Some literature supports the Aβ cascade hypothesis, which suggests Aβ plays a primary role in the cascade of events associated with the progression of AD (Tayeb et al., 2012, Auld et al., 2002). In a number of studies, this pathological process has been suggested to cause the deterioration of a neurotransmitter known as acetylcholine, resulting in cognitive deficits associated with the disease. Additionally, some AD literature suggests Aβ oligomers may be acting as acetylcholine receptor agonists, preventing other peptides and kinases from binding to the receptors (Fodero et al., 2004). Fodero and colleagues found an overall decrease in acetylcholine neurotransmitters in cholinergic neurons of AD brains; however, they found higher levels of localized acetylcholine around amyloid plaques, suggesting the peptide may be regulating the release of this neurochemical (2004). Alternatively, other literature suggests the activation of M1 G-protein coupled muscarinic acetylcholine receptors, which play a vital role in short term memory processing in the hippocampus, can stimulate non-amlydiogenic APP

production, reducing the amount of Aβ being produced (Patel and Jhamandas 2012, Buxbaum et al., 1992). This hypothesis suggests some types of acetylcholine receptors might be able to counteract possible Aβ-induced neuretic damage by reducing the levels of peptide being produced.

Other studies hypothesize Aβ might be interacting with other types of neurotransmitters, receptors, or kinases. Some literature suggests glycogen synthase kinase-3-beta (GSK-3β) might play a key role in mediating Aβ-induced neuretic damage (DaRocha-Souto et al., 2012). GSK-3β overactivity is suggested to increase Aβ-oligomer production and tau hyperphosphorylation, leading to neuroinflammation, toxicity, and cell death (DaRocha-Souto et al., 2012, Hooper et al., 2008, Jope at al., 2007). Moreover, in an *in vivo* study, Da Rocha and colleagues suggested increased levels of Aβ could also have a negative effect on GSK-3β, by potentially extending the kinase's activity; therefore, leading to a measurable decrease in dendritic spine density (2012). This study also suggests the relationship between Aβ-oligomers and GSK-3β might involve a negative feedback mechanism that may be contributing to cognitive impairment in AD. Moreover, other literature hypothesizes inhibition of GSK-3β activity through phosphorylation reduces Aβ-induced neuronal toxicity through a CREB mediated pathway (DaRocha-Souto et al., 2012). CREB gene expression is hypothesized to be important for cognitive function and is suggested to decrease in the presence of high levels of GSK-3β activity and Aβ oligomers (DaRocha-Souto et al., 2012). Therefore, when GSK-3β activity is partially inhibited, CREB gene expression is suggested to increase, leading to reduced risks

for Aβ-induced neuronal toxicity. Still, the main mechanism of action for this pathway remains unknown and is under investigation (Ryan and Pimplikar 2005).

Another hypothesis presented in the AD literature suggests glutamate receptors called NMDA receptors as play a vital role in Aβ-induced neuronal loss. In one particular study, 8-10 day old cortical cell cultures were exposed to Aβ-42 oligomers in the presence and absence of NMDA antagonists (Alberdi et al., 2010). NMDA antagonists bind to NMDA receptors preventing glutamate neurotransmitters from binding and calcium from entering the cell (Alberdie et al., 2010). Thus, the researchers hypothesized in the absence of NMDA antagonists, high levels of Aβ-42 bind to these glutamate receptors, resulting in Ca^{2+} ion channels remaining open for prolonged periods of time. Therefore, this is suggested to cause a rush of Ca^{2+} to enter the nerve cell, resulting in neuritic cell death and ultimately apoptotic cell death (Alberdi et al., 2010). However, in the presence of NMDA antagonists, the researchers found a corresponding decrease in neuronal loss and hypothesized this occurred because fewer Aβ oligomers were able to bind to the glutamate receptors. Moreover, various studies suggest Aβ type, such as Aβ-40 versus Aβ-42, is relevant and can be vital to determining whether or not plaque deposits do in fact play a causal role in AD (Alberdi et al., 2010, Manelli et al., 2007).

Even though some *in vitro* and *in vivo* studies involving treatments which target plaque pathology appear to be promising, none have passed clinical drug trials. Moreover, plaque deposits spread in an irregular manner and have been suggested to increase even in non-dementia patients due to aging (Schonheit et al.,

2004). Additionally, various clinical tests run on human subjects have shown many individuals living with plaque deposits in their brains who do not show signs of dementia or AD (Rogers and Morrison 1985, Terry et al., 1991). Furthermore, Wischik and colleagues ran biochemical experiments and found there was a 76% overlap in the amount of Aβ in normal elderly individuals and in AD patients in late stages of the disease (2014). PET imaging markers have also indicated a weak correlation between Aβ aggregates and AD. Therefore, many scientists hypothesize Aβ levels do not differentiate normal aging from progressive AD (Wischik et al., 2014, Knowles et al., 1999, Kadir et al., 2012, De Kosky and Scheff 1990, Prohovnik et al., 2006, Freir et al., 2011).

Tau Pathology

Neurofibrillary tangles (NFT) are the second major pathology of AD and are believed to directly correlate with the progression of the disease (Knowles et al., 1999, Kadir et al., 2012, Freir et al., 2011). NFTs are located in the soma and axons of neurites (Wischik et al., 2014) and are suggested to follow *grosso* modo, a classified type of spreading described by Braak (Schonheit et al., 2004). This makes it easier for scientists to identify tangle growth patterns in comparison to plaque patterns. However, tangles do pose one particularly challenging dilemma. When and where these aggregated fibrils develop over the course of AD is still unknown. This is mainly due to the fact that tangles can only be identified by physically performing brain autopsies. Thus, in order to identify intermediates linking NFTs to AD, many

scientists are interested in investigating the molecular makeup and mechanisms of action of these fibrils (Jin et al., 2011, Schonheit et al., 2004, Feinstein and Wilson 2005).

NFTs are predominantly made up of tau protein and fibers known as paired helical filaments (PHF). Tau is a microtubule associated protein (MAP) that binds to tubulin in microtubules and directly regulates their growth and retraction (Iqbal et al., 2010, Friedhoff et al., 2000, Feinstein and Wilson 2005). Even though this protein is commonly recognized for its role in stabilizing microtubules, it also has important functions within the neuron including anchoring kinases and phosphatases, advancing axonal growth, and signal amplification (Iqbal et al., 2000). Moreover, tau has six known isoforms, which are encoded by a single gene on the 17th chromosome (Iqbal et al., 2010). Changes in tau function are hypothesized to lead to downstream effects associated with AD. Aggregates of this protein are hypothesized to produce severe consequences that are associated with the progressive decline seen in AD. Tau can undergo hyperphosphorylation and is suggested to form aggregates within the neuron called tangles (Jin et al., 2011, Schonheit et al., 2004, Feinstein et al., 2005). Nevertheless, there are multiple hypotheses concerning the relationship between tau pathology and the progression of AD.

Tau has been hypothesized to either undergo a gain, loss, or change in function resulting in the progression of AD. (Condon et al., 2012) A gain of function hypothesis indicated in a recent study suggested genetic or environmental factors

cause tau to bind less to microtubules and to form aggregates of abnormal cytotoxic tangles that then cause cell death (Feinstein and Wilson 2005). The function gained by tau in this hypothesis is aggregation, which results in neuronal loss. On the other hand, loss of function tau hypotheses have suggested genetic and environmental factors in fact result in tau being unable to regulate microtubules properly so that these microtubules become highly unstabilized and cannot perform their normal cell function, ultimately causing cell death (Feinstein and Wilson 2005). The loss of function was the tau's normal ability to properly regulate the shrinking and growing of microtubules. The final hypothesis concerning tau is a combination of the two previous hypotheses in that it suggests a change in function as a genetic or environmental factor causes the tau to bind less to the microtubules and aggregate to form cytotoxic tangles, which in turn means there is less tau being used to stabilize microtubules, resulting in overactive microtubules that cause cell death.

Potential Risk Factors for AD

The primary risk factor for developing AD is aging (AA 2014, Columbo et al., 2013, Atamna and Kumar 2010, Iqbal et al., 2010, Manelli et al., 2007, Riley et al., 2005, Rojas et al., 2011). After the age of 65, the risk of developing AD doubles every five years and by the age of 85 the risk for AD is nearly 50% (AA 2014). However, aging, as the primary risk factor for AD provides very little information when running clinical tests on possible disease-modifying treatments. The most transparent and obvious reason being that everyone, in fact, ages. Therefore,

participants in a typical AD drug trial are usually very diverse. In most cases the only similarity between them is that they are above the age of 65, giving rise to many variables that could affect the results of these clinical trials. Thus, if the drug abruptly fails, the results are found as inconclusive, and AD researchers are faced with the overwhelming dilemma of trying to determine which underlying variables may be involved in the drug failure. What if we could selectively target a smaller population of individuals, like diabetic patients, when testing a potential AD drug? Many researchers are investigating this possibility by analyzing the effects of potential genetic and environmental risk factors for AD. The three main genetic risk factors for early onset AD include mutations in Amyloid Precursor Protein (APP), Presinillin 1 (PS1), and Presnillin 2 (PS2) (Wang et al., 2004, NIH 2011), while the genetic risk factor for late onset AD is suggested to be the allele of the Apolipoprotein E4 gene (ApoE4) on chromosome 19 (Manelli et al., 2007, Tai et al., 2014, Spell et al., 2004, Belinson and Michaelson 2009). Potential environmental risk factors for AD include low education attainment (Riley et al., 2005, Gatz et al., 2001), improper diet and exercise (Um et al., 2011), inflammation, an overactive immune system, mitochondrial dysfunction (Boumezbour et al., 2010, Eckert et al., 2012), and increased levels of reactive oxygen species (ROS) (Callaway et al., 2004). Other potential risk factors include metabolic disorders such as Type 2 Diabetes Melliltus (T2DM) (De Felice and Ferreira 2014, Brands et al., 2006) and other forms of dementia such as mild cognitive impairment (MCI) (Boyle et al., 2006, Kadir et al., 2012).

Type 2 Diabetes Mellitus (T2DM)

According to the Centers for Disease Control and Prevention (CDC) National Diabetes Statistic, T2DM is reportedly the seventh leading cause of death in America and accounts for approximately 90-95% of all diabetes cases (2014). T2DM is characterized by insulin resistance, which is an abnormality that occurs in insulin signaling (Dineley et al., 2014). Insulin is a hormone normally found in the body that is not only essential for glucose uptake, but also metabolic homeostasis, preventing chronic inflammation, and increasing mitochondrial stability (Dineley et al., 2014). Moreover, insulin is produced in response to a rise in glucose levels, which usually occurs in response to food consumption. Thus, when a person eats food most of it gets converted by liver cells into a monosaccharide called glucose (Nussey and Whitehead 2001). Glucose passes through the gut wall and goes directly into the blood stream where it can be used by nerve cells to support cellular function. This includes producing energy through glycolysis, converting glucose to a 'stored energy' form called glycogen, or increasing overall mitochondrial function (Nussey and Whitehead 2001, Cheng et al., 2010). As blood glucose levels begin to rise, islets of cells in the pancreas begin to produce the vital hormone called insulin. Insulin binds to insulin receptors (IR), which activate tyrosine kinases that in turn phosphorylate adaptor proteins (De Felice et al., 2014). These adaptor proteins play an important role in restoring energy metabolism and homeostasis in cells. Inhibition of IR due to neuroinflammation or stress is hypothesized to ultimately lead to insulin resistance and corresponding cognitive decline (Chen et al., 2008,

Zhao et al., 2009, DeFelice et al., 2014, DeFelice and Ferreira 2014, Solas et al., 2013, Steen et al., 2005). In various clinical trials, an increase in insulin was associated with lower scores on cognitive performance tests (Stolk et al., 1997). Stolk and colleagues conducted the Rotterdam study, in which 5,510 subjects above the age of 55 were given high doses of insulin. Furthermore, cognitive performances were assessed using a Mini Mental State Examination. The researchers hypothesized there was a direct correlation between high insulin levels and lower cognitive scores that could also be gender specific (Stolk et al., 1997). Their results indicated that high levels of insulin were associated with cognitive deficits only in women, and no cognitive decline was observed in men. Similarly, in a recent cohort study, diabetic patients above the age of 70, were assessed for cognitive impairment according to the following cognitive tests: Telephone Interview of Cognitive Status (TICS), East Boston Memory Test (EBMT), and Verbal Fluency (VF) (Debling et al., 2006). The researchers hypothesized diabetes could either directly influence cognitive function hyperglycemia and insulin resistance or indirectly influence cognition through microangiopathy of the brain (Debling et al., 2006). The results indicated diabetic patients had lower mean scores below the 25th percentile in all cognitive exams in comparison to non-diabetic participants. Thus, many researchers today believe metabolic dysfunction and signs of cognitive impairment might be linked through insulin resistance. Moreover, statistics also appear to support this hypothesis, with over 80% of individuals diagnosed with AD being T2DM patients as well (Zhao et al., 2009). Nevertheless, researchers are not only interested in

determining whether or not abnormal insulin signaling is the major link between the two diseases, but how this occurs and what are the underlying mechanisms at play.

Potential Causes of Insulin Resistance

Some studies suggest high blood glucose levels give rise to an increase in pro-inflammatory cytokines, like Tumor Necrosis Factor Alpha (TNFα), leading to IR inhibition and ultimately insulin resistance (Dineley et al., 2014, Cheng et al., 2010, De Felice and Ferreira 2014). However, other studies suggest aging plays a primary role in producing higher levels of TNFα, lower levels of glucocorticoids, and Aβ induced pathways all leading to loss of insulin sensitivity (De Felice et al., 2014, Zhao et al.,. 2009, Chen et al., 2008, Solas et al., 2013). In one particular study APPswe/PS1 transgenic mice were injected with anti-Aβ neutralizing antibody, while wild type (WT) mice were injected with high levels of Aβ-42, in order to determine whether AD-like pathology could induce insulin resistance and metabolic dysfunction (Zhang et al., 2013). The researchers found that WT mice developed insulin resistance in cells located in the inner lining of the liver called hepatocytes. Furthermore, the researchers hypothesized the insulin resistance occurred due to Aβ42 induced JAK/STAT3/SOCS-1 signaling (Zhang et al., 2013). SOCS-1 is a cytokine suppressor and insulin inhibitor that becomes activated as result of STAT3 proteins binding to JAK kinase receptors (Zhang et al., 2012, Zhang et al., 2013). Furthermore, improved insulin sensitivity and corresponding lower levels of

JAK/STAT3/SOCS-1 signaling were observed in the transgenic mice, suggesting a strong relationship between AD pathology and insulin resistance (Zhang et al., 2013). Moreover, supporting evidence was provided for this hypothesis in an earlier study conducted by Zhang and colleagues (2012). However, instead of using an *in vivo* model, the researchers used an *in vitro* hepatic cell culture model. Results were found to be conclusive and supported the JAK/STAT3/SOCS-1 cascade hypothesis. On the other hand, other studies hypothesize Aβ may work through a pro-inflammatory pathway leading to insulin resistance (DeFelice et al., 2014, De Felice and Ferreira 2014, Zhao et al., 2009). Zhao and colleagues hypothesize an increase in Aβ aggregates leads to an increase in TNFα, which in turn leads to inhibition of IRs and ultimately, insulin resistance (2009). Moreover, in a recent *in vivo* study, potential intermediates were hypothesized to link TNFα induced neuroinflammation with cognitive decline (Lourenco et al., 2013). Lourenco and colleagues used both *in vivo* and *in vitro* animal models to investigate this relationship further (2013). In the hippocampi of transgenic APP/PS1 mice injected with high levels of Amyloid Beta Oligomers (AβO), higher levels of double-stranded RNA-dependent protein kinase (PKR) were present compared to Wild Type (WT) mice. PKR is believed to affect pro-inflammatory pathways and lead to a rise in eukaryotic translation initiation factor 2α (elF2α), which is hypothesized to produce IRS-1 inhibition and insulin resistance. (Lourenco et al., 2013). Furthermore, *in vitro* results indicated Aβ produced high levels of elF2α in dendrites and cell bodies of hippocampi neurons. Moreover, the researchers hypothesized TNFα plays a vital

role in PKR activation and believed rises in TNFα would stimulate higher levels of PKR, which would lead to increased elF2α phosphorylation and induce insulin resistance. Thus, additional supporting evidence for this hypothesis was observed when researchers treated Aβ-stimulated hippocampal neurons with a TNFα receptor antibody called Infliximab. Infliximab was found to reduce PKR and corresponding elF2α levels in the presence of Aβ, therefore potentially reducing IR inhibition and insulin resistance. Consequently, this study suggests an overlap between pro-inflammatory pathways, AD pathology, and PKR/elF2α signaling pathways (Lourenco et al., 2013).

Effect of Insulin Resistance on Cognition

Along with clinical trials, many independent studies have been conducted investigating the role of insulin in promoting cognitive function. Some studies hypothesize insulin is an important regulator of neuronal physiology and function. Thus, in one *in vivo* study, IRs were suggested to play an important role in specifically maintaining synaptic density, dendritic plasticity, and circuit function in optic nerve cells (Chiu et al., 2008). The researchers first transfected the nerve cells with dominant negative-insulin receptor (dnIR), then measured the neuronal responses to natural light in live *Xenopus tadpoles*. Results, based on electromicroscopy data, indicated that transfected neurons contained fewer synapses and responded less to light intensity and stimulation. Furthermore, when running multiphoton time lapse imaging analysis, the researchers also observed a

loss in dendritic plasticity in cells with inhibited IRs. This was hypothesized to produce brain circuit dysfunction. Consequently, the researchers concluded insulin was a valuable player in neuronal function and stability. Alternatively, other studies hypothesize insulin resistance might give rise to an inflammatory mediated insulin pathway that could be linked to progressive cognitive decline in AD. The researchers used an *in vivo* aged mouse model in order to analyze the effects of neuroinflammation on memory in the hippocampus (Chen et al., 2008). They hypothesized that because aging leads to higher levels of neuroinflammation, this would in turn give rise to cytokines that would have neurotoxic effects on memory, leading to a faster decline in cognitive abilities (Chen et al., 2008). Another study ran glucose tolerance assessments on AD and control individuals at the baseline and two year period marks. They did this in order to determine insulin and glucose areas under the curve (AUC). Furthermore, the researchers hypothesized an increase in cognitive decline would correspond with a decrease in insulin levels (Burns et al., 2012). Moreover, the study suggests an AD pathology-associated mechanism in which insulin regulates the amount of Aβ being produced and tau being phosphorylated through its influence on GSK3β function (Burns et al., 2012).

Nevertheless, this study examines the effects of hyperglycemic conditions and insulin deprivation, both being T2DM disease pathways, on neurodegeneration, a hallmark of AD. This is based on previous literature suggesting low insulin (Burns et al. 2012) and hyperglycemic conditions (Debling et al. 2006) may lead to cognitive deficits

Treatments

Currently, there is no cure for Alzheimer's Disease (AD), but a few drugs on the market today claim to decrease symptoms associated with the disease. Memantine, an NMDA antagonist, and Avagestat, a γ-secretase inhibitor, target Aβ-induced neurotoxicity. Rivastigmine and Donepezil, cholinesterase inhibitors, target enzymatic hydrolysis of acetylcholine neurotransmitters, while Metformin, a T2DM drug, targets kinase activity. Many of these drugs are suggested to decrease AD associated symptoms, but their therapeutic effects are short and temporary, lasting just a few months (Tayeb et al., 2012). Moreover, all of these drugs cannot reverse neurodegeneration and cell death, but they merely slow down the progression of the disease for a period of time (Bales et al., 2006, Tayeb et al., 2012).

Memantine and Avagestat target Aβ-induced neuronal toxicity. Memantine acts as an NMDA antagonist by blocking Aβ peptides from binding to glutamate receptors; therefore resulting in reduced Ca^{2+} overload and less apoptotic nerve cell death. Some studies suggest memantine has low-to-moderate affinity for NMDA receptors, resulting in less nerve cell excitotoxicity (Klyubin et al., 2011). Moreover, memantine has also been suggested to improve spatial learning in AD transgenic mouse models due to its low-moderate affinity (Minkeviciene et al., 2004). Alternatively, other studies hypothesize memantine can have adverse effects on memory and cognition (Creeley et al., 2006). In an *in vivo* study, memantine produced disruptions in memory retention and locomotor activity in adult rats at low doses of 10 mg/kg and even 5 mg/kg (Creeley et al., 2006). Moreover, other

studies suggest NMDA antagonists trigger apoptotic cellular pathways in brain regions commonly affected by AD like the hippocampus (Hardingham et al., 2002, Ikonomidou and Turski 2004). Avagestat, on the other hand, is a γ-secretase inhibitor (GSI) and acts by preventing proteolytic cleavage of APP fragments in order to reduce Aβ peptide production and related neurotoxicity. However, similar to memantine, avagestat has been found to have adverse effects. Gamma secretases are important not only for Aβ production, but also for Notch protein signaling. Notch transmembrane protein signaling is suggested to be important for gastrointestinal (GI) tract, thymus, and spleen activity as well as skin and hair health (Tong et al., 2012). Therefore, GSIs can have adverse effects by inhibiting γ-secretase Notch cleavage, resulting in toxic side effects to major organs and overall cellular function (Tong et al., 2012, Milano et al., 2004, Wong et al., 2004).

Cholinesterase inhibitors (ChEI), such as Rivastigmine and Donepezil, are suggested to slow mental decline in people with mild to moderate AD by preventing the acetycholinesterase enzyme from hydrolyzing acetylcholine neurotransmitters (Bales et al., 2006). Neurotransmitters are important for nerve cell to cell signaling; therefore altering the amount of acetylcholine in the brain can have major consequences not only on neuronal health, but cognition, memory, and behavior. In an *in vivo* study, ChEIs were hypothesized to reduce behavioral deficits in 9-month-old transgenic mice (Tg2576) that over expressed APP and developed Aβ plaque deposits, as a model for AD (Dong et al., 2005). Additionally, another study examined the effects of Donepezil on a 72 year old chronic dialysis AD patient

(Suwata et al., 2002). Suwata and colleagues suggested the drug improved psychiatric health within just a few months of therapy (2002). However, even though symptomatic improvements have been observed when administering ChEI drugs to AD patients, the overall efficacy of these drugs is still questionable (Tayeb et al., 2012, Raschetti et al., 2005). ChEIs tend to have a limited therapeutic window of just a few months and can neither stop nor reverse neurodegeneration and toxicity in AD (Bales et al., 2006, Tayeb et al., 2012). In one study, scientists performed a cohort study investigating the effectiveness of ChEIs in mild to moderate AD patients (Raschetti et al., 2005). After testing multiple types of ChEIs, like Rivistagmine, Raschetti and colleagues found limited improvements in patient cognitive scores on mini-mental state examinations (MMSE) over a 9 month therapy period. Therefore, the researchers hypothesized ChEIs have a "modest" effect on AD patients, since AD symptoms were only relived temporarily and only in a fraction of the overall population of participants (Raschetti et al., 2005).

Since T2DM is a hypothesized potential risk factor for AD, many research labs have been investigating the effects of a T2DM drug, called Metformin, on neuronal viability and plasticity as well as cognition and memory in AD (Wang et al., 2012, Gupta et al., 2011, Li et al., 2012). Wang and colleagues hypothesized metformin could trigger stem cell differentiation into neurons and improve spatial memory, all through a PKC-CBP pathway. The researchers suggest metformin triggers neurogenesis by activating a kinase called AMP-activated protein kinase (AMPK). AMPK activation results in a cascade of other kinases becoming activated,

including protein kinase C. Moreover, protein kinase C activates the transcriptional co activator CBP through phosphorylation, and CBP promotes stem cells to differentiate into neurons. Additionally, other studies suggest metformin combined with vitamin C supplements can reduce cognitive impairment in T2DM patients (Moore et al., 2013). Nevertheless, metformin's mechanism of action is still under investigation. In one recent study, metformin activated AMPK cascade pathways resulted in cognitive dysfunction in male rats but therapeutic cognitive effects in female rats (Di Tacchio et al., 2014); therefore, suggesting gender based therapy as a potential focus for metformin treatment.

Thus, symptomatic treatments of AD have shown modest signs of slowing the progression of the disease, but have limited therapeutic windows. Moreover, much of the literature is divided concerning the therapeutic effects of AD symptomatic treatments, treatments that target AD symptoms like depression, in both *in vivo* and *in vitro* experiments and in clinical trials. Nonetheless, drugs like metformin used to treat metabolic disorders like T2DM have shown promising signs of improving cognition and memory through an AMPK activation cascade and stem cell differentiation. Similarly, in this project the therapeutic effect of another promising drug, called Methylene Blue (MB), is investigated using an AD T2DM model.

Methylene Blue (MB)

MB Overview

Methylthioninium Chloride (MTC), also known as methylene blue (MB), is a fairly inexpensive redox agent that belongs to the phenothiazinium family (Paban et al., 2014). This blue dye is readily available and is notably one of the most versatile agents today, paving the way for many advancements in modern science including chemotherapy, photosensitization (Rojas et al., 2011), and ifosfamide-induced encephalopathy (Paban et al., 2014). Nevertheless, it is wildly recognized for its robust staining abilities and therapeutic effects as a treatment for methemoglobonemia (Paban et al., 2014), schizophrenia, and malaria (Rojas et al., 2011).

In 1886, a German professor named Dr. Paul Ehrlich discovered the cellular staining properties of MB (Nobel Lectures 1901-1921). He not only succeeded in specifically targeting nerve cell tissue using MB but also proved this could be done using a live *in vivo* rat model for the first time (Wrubel et al., 2007, Nobel Lectures 1901-1921). Ehrlich's discovery was revolutionary and fueled over 100 years of MB staining in laboratories across the globe (Rojas et al., 2011). Consequently, in 1978 Martinez Jr. came across another unique property of MB. Martinez discovered that at a particularly low dose (1 mg/kg), MB showed signs of improving cognition and memory retention in mice's inhibitory avoidance responses (Martinez Jr. et al., 1978). However, at a high dose (50 mg/kg) it showed reverse effects and produced anterograde amnesia. Martinez and his colleagues hypothesized this occurred

because MB can convert methemoglobin into hemoglobin at a low dose, resulting in increased memory retention (Martinez et al., 1978). But at a high dose, Martinez believed MB underwent a reverse mechanism. Similarly, in a later study MB was found to completely restore memory retention when administered at a low dose (1 mg/kg) in rats with sodium azide, an inhibitor of cytochrome oxidase (Callaway et al., 2002). Nevertheless, Martinez also discovered a time dependent attribute to MB's effect on memory retention. When mice were given a high dose IP injection of MB fifteen minutes prior to inhibitory avoidance training, it caused anterograde amnesia. But, when the drug was given 5 or 30 minutes prior to training it did not result in this same effect (Martinez et al., 1978). Thus, these studies provided potential insights concerning the time course and dose effects of MB, as well as the metabolic and therapeutic effects of MB in the brain. MB proved to be a multifaceted agent that could treat a wide array of diseases and disorders, and in 2008 it showed signs of having even more novel properties.

In 2008 Professor Claude Wischik, co-founder of the pharmaceutical company TauRx, made a breakthrough announcement at the International Conference of Alzheimer's Disease (ICAD) in Chicago, Illinois (Wischik et al., 2008). Wischik declared he found the "miracle cure" for Alzheimer's Disease and it was called Rember. Rember is a modified form of MB and is believed to target the aggregation of Tau protein; therefore, preventing the formation of neurofibrillary tangles (Wischik et al., 2008). At the ICAD conference, Wischik announced Rember had successfully passed Phase 2 Drug trials and reduced the progression of AD by

90% in just two years (Wischik et al., 2008). How was this possible and what underlying mechanisms led to such results? MB's main mechanism of action is still unknown and is under investigation, but various studies present potential hypotheses to answer this question. In an *in vitro* study, Wischik et al., (1996) hypothesized MB acts as a tau inhibitor and prevents tau proteins from undergoing dimerization. This, in turn, prevents the tau proteins from aggregating and forming paired helical filaments (PHF), which are the fibers that lead to the formation of tangles. Moreover, Wischik's novel discovery proved to have great potential as most AD drugs tend to fail during Phase 2 clinical tests, but Rember passed with flying colors and was approved by the FDA for Phase 3 drug trials in 2012. On September 22nd, 2014, TauRx announced it had completed recruitment for its Phase III drug trial with nearly 700 patients diagnosed with mild to moderate AD participating in the study. The company believes its studies will near completion in 2016.

Thus, due to Rember's successful clinical tests, MB shows promising signs as a potential therapeutic treatment for AD. MB is suggested to be a suitable AD drug candidate due to its adept qualities in accessing the brain. For one thing, MB is able to cross the blood brain barrier (Atamna and Kumar 2010, Peter et al., 2000), a necessary component for most cognitive treatments. MB also has a low redox potential of 11 mV, allowing it to easily undergo reduction or oxidation when necessary (Atamna and Kumar 2010). This is advantageous because some skeptics believe the blue color of the dye creates a bias in placebo based clinical trials and *in vivo* experimental models. If a rat is given the drug diluted in saline, it might refuse

to drink the liquid because of the blue pigmentation of drug. However, this concern can be alleviated as MB becomes colorless when converted to its oxidized form called leucomethylene blue (MBH_2). MBH_2 can become re-oxidized back to MB (Atamna and Kumar 2010), allowing cells to re-metabolize the agent. Furthermore another valuable characteristic of MB is its high solubility in water and organic solvents, as well as its high permeability through multiple membranes, allowing it to enter various parts of the cell including mitochondria (Atamna and Kumar 2010).

Potential Mechanisms of MB

The underlying effects of MB are still under investigation, but AD literature presents possible hypotheses concerning the agent's main mechanism of action. Wischik and colleagues hypothesize MB acts as a tau inhibitor by binding to tau substrates and preventing tau-tau binding dimerization (Wischik et al., 2014, Wischik et al., 2010, Wischik et al., 1996). Therefore, the researchers suggest tau undergoes a gain of function mutation in which an environmental risk factor causes it to hyperphosphorylate. This is hypothesized to result in conformational changes to tubulin binding sites, thus tau fragments disattach from microtubules and become suspended. The suspended tau fragments are suggested to then bind to each other at tau-tau binding domains leading to dimerization and ultimately, neurotoxicity (Figure 1). Hence, MB acts to prevent suspended tau fragments from forming aggregates, and this is suggested to contribute to reduced neuronal loss (Wischik et al., 2014, Wischik et al., 2010). But, it is important to note that MB's role

as a tau inhibitor can only have potential therapeutic effects in AD if tau-tau binding and dimerization is in fact the cause of neurotoxicity, which we do not know to be true. Some studies suggest tau does not undergo a gain of function mutation, but rather a loss of function mutation (Feinstein and Wilson 2005). Feinstein and colleagues suggest an environmental factor causes tau to lose its ability to properly regulate microtubules, resulting in abnormal microtubule growth and retraction. Moreover, the researchers suggest unregulated microtubules as being the primary cause of neurotoxicity as opposed to the tau protein. Consequently, MB would not have therapeutic effects as a tau inhibitor if neurodegeneration is induced by microtubule instability as opposed to hyperphosphorylated tau.

An alternative hypothesis regarding the MB mechanism of action suggests the agent acts as an electron carrier in nerve cell mitochondria, giving rise to improved mitochondrial respiration, overall neural health, and metabolism (Zhang et al., 2006, Rojas et al., 2011). Because the primary risk factor in AD is aging and mitochondrial activity has been found to decline with age (Sullivan and Brown 2005), some researchers hypothesize decreased mitochondrial activity could give rise to AD associated cognitive deficits and memory impairment (Boumezbour et al., 2010, Eckert et al., 2012). Callaway and colleagues found MB could restore spatial memory retention in rats by acting as an electron carrier in the inner mitochondrial membrane and activating cytochrome c oxidases (complex IV) (2002). In the presence of cytochrome oxidase inhibitors, MB was suggested to improve mitochondrial function by trapping leaking electrons and bypassing blocked points

of electron flow, leading to complex IV mitochondrial activation. Complex IV activation results in oxygen molecules getting reduced into water molecules, giving rise to an increase in cellular respiration and mitochondrial function (Callaway et al., 2002, Scott and Hunter 1966).

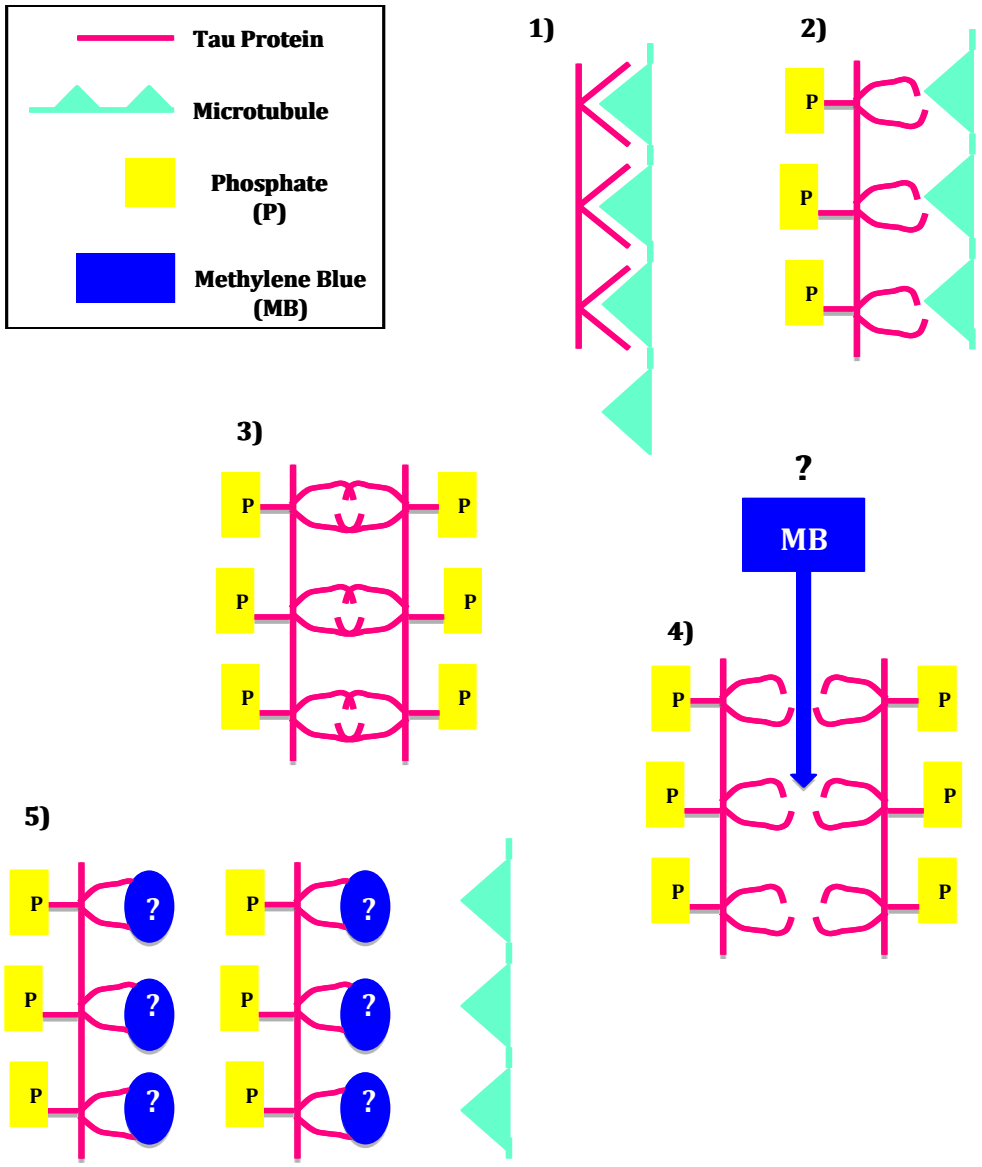

Figure 1: Potential mechanism of Methylene Blue (MB) as a tau inhibitor. 1) Tau protein stabilizes microtubule growth and retraction by binding to tubulin. 2) Environmental or genetic risk factors results in tau hyperphoshphorylation leading to conformational changes in protein filament. 3) Tau hyperphosphorylation leads to tau-tau dimerization and formation of intracellular tangles, resulting in induced neurotoxicity. 4) MB treatment acts as a tau-tau dimerization inhibitor through an unknown mechanism of action. 5) Tau protein filaments can no longer dimerize nor bind to microtubules, reducing tangle-induced nerve cell death.

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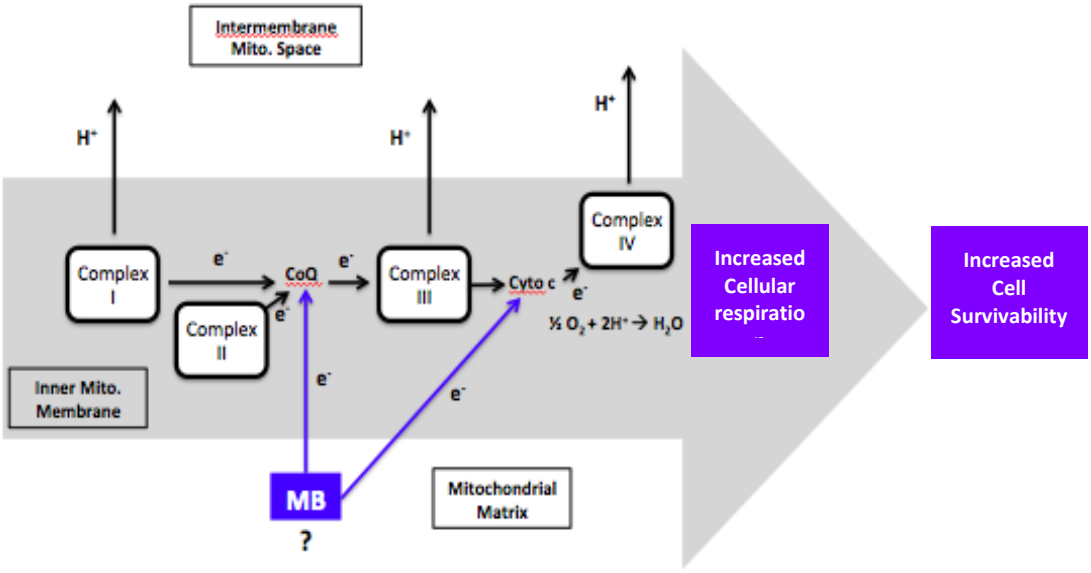

Figure 2: Potential mechanism of Methylene Blue (MB) as an electron carrier in mitochondria, resulting in increased metabolism and cellular respiration (Rojas et al., 2011). In this model, MB carries and donates electrons to cytochrome Q and cytochrome c oxidase, leading to the activation of complex IV. Complex IV activation causes oxygen and hydrogen molecules to be reduced into water molecules, resulting in increased cellular respiration and ultimately increased nerve cell viability.

Present Study

The overarching goal of this thesis was to begin investigating the potential relationship between AD and T2DM and the therapeutic effects of MB. The first major aim of this thesis was to establish a neurodegenerative model using hyperglycemic and/or insulin deprivation conditions. Based on previous literature, I hypothesized T2DM mechanisms, such as hyperglycemia and insulin resistance, will have a detrimental effect on nerve cell viability and lead to cell death (Chiu et al., 2008, Debling et al., 2006, Burns et al., 2012). We tested this hypothesis using an *in vitro* embryonic nerve cell culture model in which neurons were stimulated with or without insulin in the presence or absence of high glucose (150 mM). Hyperglycemic conditions with insulin deprivation were used to simulate T2DM disease mechanisms. An MTS assay was used to determine approximate average cell counts in each condition.

Studying the effects of diabetes-like conditions on neurodegeneration, a hallmark of AD, may be an important step in future diagnosis of AD. The biggest risk factor for AD is aging (AA 2014, Columbo et al., 2013, Atamna and Kumar 2010, Iqbal et al., 2010, Manelli et al., 2007, Riley et al., 2005, Rojas et al., 2011); however this provides very little information when running clinical trials on potential AD drugs. Due to the large amount of subject diversity in AD clinical trials, if a potential AD drug fails, which is the case more than 99% of the time (Cummings et al. 2014), researchers are faced with the overwhelming dilemma of trying to identify underlying variables that might have contributed to the drug failure. However, if we

could identify a specific population of individuals at risk for AD, this might mean fewer confounding variables, the development of AD drugs that target pathways relevant to that particular population, and an increased likelihood of the drug to work. Moreover, even though this is a preliminary study that is just beginning to investigate the effects of high glucose and insulin deprivation on nerve cell viability, future experiments might be able to provide potential evidence supporting the clinical implications of our hypothesis.

Once a neurodegenerative model using diabetic-like conditions is established, the second major aim of this thesis is to analyze the effects of a potential AD drug called Methylene Blue (MB) on preventing neurodegeneration caused by high glucose and insulin deprivation. We hypothesize MB will prevent neurodegeneration induced by hyperglycemic and/or insulin deprivation conditions. Literature suggests MB has therapeutic effects by increasing mitochondrial activity (Zhang et al., 2006, Rojas et al., 2011) or by acting as a tau inhibitor (Wischik et al., 1996, 2010, 2014). If MB is successful in reducing neurodegeneration, we hypothesize it may have future clinical implications by potentially reducing the progression of AD by allowing neurons to survive longer under conditions in which there is reduced insulin signaling and deregulation of glucose transport. If so, then future clinical trials may enroll subjects who have been identified as having T2DM as well as being in the early stages of AD in order to see if MB can slow down the progression of the disease.

We also conducted a preliminary pilot study investigating the effects of insulin, high glucose (150 mM), and MB on cell health and microtubule stability. Neurons need stabilized microtubules to maintain their long axonal and dendritic projections (Ruiz-Canada 2004). If cells are alive, but don't have the ability to maintain the complex neuronal network necessary for cognition following MB treatment, then treatments like MB would not be very effective.

MATERIALS & METHODS

Cell Culture Model

Rat embryonic cortical neurons were used a cell culture model of diabetic brain conditions. The model used in this study is a general model that could be used to not only represent Type 2 Diabetes (T2DM) and Alzheimer's Disease (AD), but other types of metabolic and neurodegenerative diseases. This thesis presents a small sample of successful experiments conducted using this model and excludes many experiments that were performed but could not be included due to culture contamination and other confounding variables.

Cell Culture Plate Preparation

Clear 96 well and 24 well plates (Costar) were used for the experiments in this study. The plates were coated with poly-L-lysine hydrobromide (SIGMA-Aldrich, Ref #: P6282), a solution that creates a "glue-like" film allowing cells to adhere to the bottom of each well. The poly-L-lysine was purchased as a lyophilized

powder (5 mg) that was then diluted using 50 mL of sterilized H_2O. Plates were incubated with poly-L-lysine at room temperature for a minimum of 24 hours before the excess poly-L-lysine was removed and the wells were washed with Hank's Balanced Salt Solution (HBSS). During washing, enough HBSS to coat the bottom of each well was applied before plates were left to incubate at room temperature for 15 minutes. This step was repeated two more times. After plates were washed using HBSS, 100 μL of Plating Media (PM) was applied to each well in each 96-well plate while 500 μL of PM was applied to each well in each 24 well plate. Plating Media (PM) is a solution that serves as a temporary environment for cells to reside in during dissection. The solution is prepared by adding 50 mL of Fetal Bovine Serum (FBS) (Gibco, Ref #: 10099-141) and 1 mL Penicillin/Streptomycin Antibiotic (Gibco, Ref #: 15140-122) into 500 mL Neurobasal Medium (has 25mM Glucose; Gibco, Ref #: 21103-049). Cell culture plates were incubated at 37°C, 5% CO_2, for a minimum of 24 hours prior to dissection.

Table 1: Experimental Procedure Timeline

Day 1	**Day 3-15**	**Day 16**	**Day 18**	**Day 19**
Dissection and plating of embryonic nerve cells	Cells fed every 2-3 days by aspirating half GM and adding new GM.	48-hour nerve cell stimulation with appropriate conditions	Data Collection Assays (MTS, Fixation and Staining) are run.	Dark Room-Immunocytochemistry data analysis

Dissection

An eighteen day (E18) pregnant rat was sacrificed by being placed into a CO_2 chamber. A T-section incision was made to her belly and the embryos were removed. One embryo was typically obtained for each experiment. The embryos were then decapitated and the heads were placed into a 50 mm Petri dish filled with HBSS. The embryonic heads were dissected using forceps to anchor the head in place, while another pair of forceps was used to peel away the excess peripheral tissue surrounding the brain. Once the brain hemispheres became visible, forceps were used to carefully extract and transfer the brain into a second 50 mm Petri dish containing HBSS. The two frontal hemispheres were then isolated from the cerebellum and midbrain regions. A microscope was used to identify membranous layers of blood vessels called meninges. Meninges are toxic if left on fetal cell cultures (Masia 2013), therefore it was important that they were removed during the dissection process. Once all of the meninges were removed, the two frontal hemispheres were transferred to a final HBSS dish before being gently teased apart into smaller pieces. The fragments of cortical hemisphere were transferred into 3 mL of 0.25% Porcine trypsin solution (SIGMA-Aldrich, Ref #: 59429C) in a sterile laminar flow hood and incubated in a water bath (37°C) for five minutes. Trypsin is a protease that is used to breakdown portions of the extracellular matrix and connective tissue in order to allow for easier dissociation of cortical membranes. After incubation in trypsin, the cells were then washed in HBSS (3 mL) twice for three minutes each. Once washes were completed, the cells were transferred into 4

mL of PM solution and dissociated by pipetting up and down with a glass pipette 50 times or until no more tissue clumps were visible. Prior to this step, the glass pipette was passed through a Bunsen Burner flame to make cellular tissue dissociation easier. Cells were then counted using a hemocytometer and plated at 1 x 10^5 cells/mL in PM. Cell cultures were incubated at 37°C for approximately one hour before PM was replaced with Growth Media (GM). GM solution was prepared by adding 10 mL of B-27 Supplement (Gibco, Ref #: 17504-044) and 1 mL Penicillin/Streptomycin Antibiotic (Gibco, Ref #: 15140-122) into 500 mL Neurobasal Medium containing L-Glutamine (Gibco, Ref #: 21103-049). According to a private correspondence with Gibco, B-27 media supplement contains a dose of 100 nM insulin. No cells were plated in the outermost wells of each 96 well plate, and instead only GM or PM was applied to those wells. This was done to ensure minimal evaporation occurred in wells containing cells, which would be countered by the solution in the outermost wells. Cells were fed every two days with GM and were incubated (37°C) for approximately two weeks prior to stimulation.

Neuronal Stimulation

Cells were stimulated two weeks post dissection to allow time for neurite maturity and synaptic formation. Previous experiments have shown that one-week embryonic neuronal cell cultures are less sensitive to lower MB conditions (Figure 3); therefore cultures were incubated for approximately two weeks in subsequent experiments to maximize drug sensitivity. Nerve cells were stimulated with the

following conditions: control (GM), GM with MB (100 nM), Insulin-Free (IF) GM with and without MB (100 nM), high glucose (150 mM) with and without MB (100 nM), and IF 150 mM glucose with and without MB (100 nM). Therefore, a total of eight conditions were prepared for each cell culture plate. A MB dose of 100 nM was selected as an appropriate neuroprotective stimulant according to previous experimental data (Figure 3) and supporting experiments (La Clair 2010). A high glucose concentration of 150 mM was selected as an appropriate neurotoxic stimulant according to previous experiments (Masia 2013, Mourad 2013).

The control condition contained only normal GM, which was prepared as described above. Insulin-free GM conditions were prepared by making normal GM, replacing B-27 Supplement (Gibco, Ref #: 17504-044) with B-27 Minus Insulin Supplement (Gibco, Ref #: A1895601). High glucose (150 mM) conditions were prepared by diluting dextrose (EMD Chemicals Inc), a form of glucose, in GM in order to make a stock solution. Insulin-free high glucose (150 mM) was prepared following the same procedure as above, except IF GM instead of normal GM was used to dilute the dextrose. All solutions were prepared no longer than a day prior to cell stimulation and were stored at 4°C. Solutions were incubated in a water bath (37°C) prior to usage.

Methylene Blue (MB) was stored at 4°C and diluted to 100 nM from a stock solution of 1 mM using normal GM. A multistep dilution process was implemented to ensure proper dissociation of the dye and included diluting the stock solution (1 mM) to 10 μM then from 10 μM to 100 nM. The MB concentration was chosen

according to the literature (Atamna et al., 2008, Rojas et al., 2011, Scott and Hunter 1965) and previous experiments (La Clair 2010).

Immunocytochemistry

After a 48 hour stimulation, cells in 24 well cultures were fixed using 4% paraformaldehyde in phosphate buffered saline (PBS) (SIGMA-Aldrich, Ref #: 79382) for 20 minutes at room temperature. Paraformaldehyde is used to fix cells in order to preserve cellular morphology. The fixed cells were washed in PBS solution three times in order to remove any excess paraformaldehyde. This PBS solution was prepared by dissolving one PBS tablet in 200mL of sterilized H_2O. The cells were then incubated with 0.5% Triton X-100 (SIGMA-Aldrich, Ref #: T8532) solution for ten minutes at room temperature. The Triton solution was prepared by dissolving 1 mL Triton x-100 Electrophoresis reagent (SIGMA-Aldrich, Ref #: T8532) in 200 mL of PBS solution. After 10 minutes, the Triton solution was removed and plates were briefly washed with PBS solution. A primary mouse antibody, monoclonal anti-acetylated tubulin clone 6-11B-1 (SIGMA-Aldrich, Ref #: T6793), was then incubated on cells for 1 hour at room temperature on an orbital shaker. The primary antibody binds to alpha-tubulin, which makes up the microtubules in cells. Plates were placed on an orbital shaker for one hour to ensure the antibody was completely distributed to all cells. The cells were then washed with PBS three additional times before a secondary antibody, Polyclonal Anti-Mouse IgG $F(ab)_2$ fragment-Cy3 produced in sheep (SIGMA-Aldrich, Ref #: C2181), was applied. The secondary antibody binds to

the primary antibody and is conjugated to a Cy3 (green) fluorophore, which allows for detection of cellular structures under a fluorescent microscope. The secondary antibody was left on for a minimum of one hour before plates were washed with PBS, sealed with parafilm, and refrigerated at 4°C.

Immunofluorescence Microscopy

Fluorescent images of cells in 24 well culture plates were analyzed using an inverted fluorescent microscope. NIS Elements Advanced Research Microscope Imaging Software was used to analyze cellular morphology, neurite counts, and neuritic intensity using Regions of Interest (ROI) tools. Neuritic intensity measures the brightness of the stain absorbed by the nerve cells. A consistent exposure time of 5 seconds was set for all images. Quantitative analysis of images included measuring neuritic counts and intensity. Neuritic counts corresponding with increasing distances (microns) from nerve cell bodies were analyzed by selecting a circle icon. This circle icon creates rings centered on the cell bodies that each increase with diameter. The first ring has a radius of 50 microns from the cell bodies, the second ring 100 microns, and the third ring 150 microns. The number of neurite projections at each of these distances was counted. Neuritic intensity at 150 microns was also measured. This was determined by selecting “Draw Polygonal ROI” and tracing the area of each neurite that crossed the perimeter of the 150 micron ring. Qualitative analysis of fluorescent images was also performed by

observing brightness of tubulin staining, relative lengths of axons, and density of dendritic branching.

MTS Cell Viability Assay

After a 48 hour stimulation, tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)(Cell Titer 96 Aqueous One Solution of Cell Proliferation Assay by Promega, Ref #: G3582) was used to measure cell viability. Tetrazolium salts such as MTS (Huang et al., 2004, Riss et al., 2013), get internalized by cells and tautomerize into a purple/brown insoluble product by mitochondria in live cells. After incubating at 37°C for three hours, a resulting color change occurs in the wells based on the proportion of cells that are viable. Wells with high cellular viability appear brown in color, while wells with lower cell viability appear yellow, indicating no insoluble product was formed. Cellular absorbances were measured using Microplate Manager 5.2.1 Endpoint Protocol Bio Rad Build 106 Reader Model 680 at a wavelength of 490 nm. Percent viability was calculated by normalizing the control absorbance mean value to 100%. Percent viability of the remaining conditions was calculated by dividing each condition mean value by the control mean value, then multiplying by 100%. An MTS assay is a useful cell viability test used to assess the average percentage of cells that survived in stimulation conditions versus control conditions, yet it can have some limitations. For example, some cells stop respiring when they are under stress, therefore an MTS assay would not count these cells as

'alive' due to their lack of being able to tautomerize the dye, which happens in the mitochondria. Another limitation of MTS assays is that the MTS tautomerization depends on mitochondrial activity, while MB dye is hypothesized to also affect mitochondrial respiration. Therefore, it is possible that in MB-containing conditions, high cell viability absorbances could be attributed to confounding MB effects on the mitochondrial tautomerization of the MTS dye. Nonetheless, an MTS assay is still a quick and efficient way of determining the average cell counts per well, but using other types of cell viability assays in the future to support these data might also be beneficial.

Statistical Analysis

Microsoft Excel was used to create all figures and tables. Percent cell viability was computed using mean data values. Standard errors were computed using raw data values in Excel and were supported using the software program Statistical Package for Social Sciences (SPSS). One Way ANOVA were performed using SPSS and were used to identify statistically significant interactions between groups. P-values lower than 0.05 were considered to be statistically significant.

RESULTS

In order to find an optimal concentration of MB to use in culture, I used a glutamate Alzheimer's model to test the effects of low versus high doses of Methylene Blue (MB) on percent cell viability **(Figure 3)**. Percent viability was measured in one-week nerve cells stimulated with high glutamate (20 mM), low MB

(100 nM) and high MB (1mM). The results demonstrate reduced viability (loss of 45%) in nerve cells stimulated with high glutamate (25 mM) ($p < 0.0001$) and high MB (25mM) (loss of 27%, $p < 0.0001$). Alternatively in low MB (100 nm) conditions, a 10% decrease in cell viability was observed, but this decrease was not statistically significant ($p = 0.293$). Low (loss of 51%, $p < 0.0001$) versus high MB (loss of 56%, $p < 0.0001$) concentrations in the presence of glutamate did not appear to prevent glutamate-induced neurodegeneration from occurring. A One-Way ANOVA was used to compare groups to the control condition (GM). **(Figure 3)**. We expected to see glutamate-induced neurodegeneration in the absence of low MB, and reduced glutamate effects in the presence of low MB. We hypothesize high MB conditions would lead to increased neuronal toxicity in the presence of glutamate (Martinez Jr. et al., 1978).

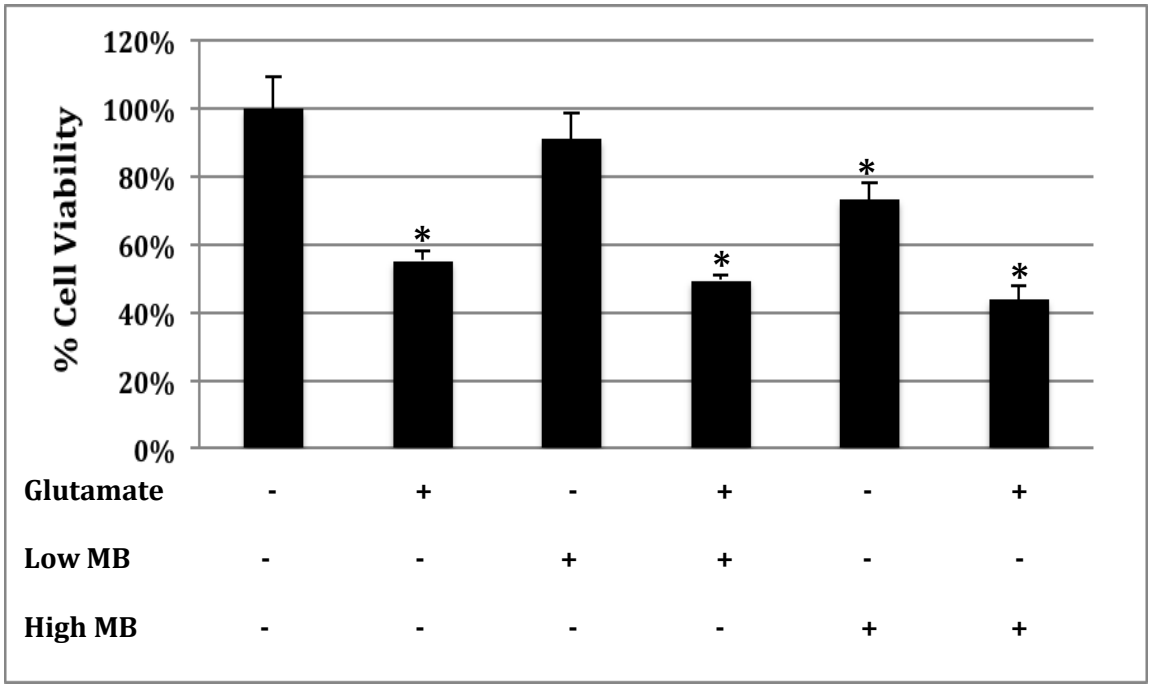

Figure 3: Percent viability for one-week old nerve cells stimulated with high glutamate, low MB (100 nM), and high MB (1 mM) conditions. The control condition contained only normal GM (25 mM glucose), serving as the baseline at 100%. Percent viability data were standardized to the control value for all treatment conditions. All conditions contained insulin. Data are mean +/- SEM. (N= 116 wells, 2 plates).

*** = main effect, $p < 0.05$**

After determining a non-toxic dose of MB that could be used to stimulate nerve cells in subsequent experiments, previous data collected by fellow colleagues (Masia 2013) was analyzed in order to select a high concentration of glucose. Masia and colleagues data suggested 150 mM glucose induced neurodegeneration in embryonic neuronal cultures (2013). Therefore, a concentration of 150 mM glucose was used to model diabetic hyperglycemic conditions in the absence and presence of insulin.

In order to begin to analyze the relationship between Type 2 diabetes and Alzheimer's disease along with the effects of MB on diabetic brain conditions, 16-day old embryonic nerve cells were stimulated with high glucose (150 mM), insulin, and MB (100 nM) **(Figure 4)**. Percent nerve cell viabilities were calculated using data collected from MTS Assays. Nerve cells stimulated with insulin and MB in the absence of high glucose (150 mM) ($p=0.446$; cell viability 116%) and the presence of high glucose ($p= 0.673$, cell viability 109%) showed no signs of neuronal toxicity relative to the control. MB conditions in the presence of insulin with high glucose were not statistically different ($p= 0.182$) from independent high glucose conditions. Nerve cells in an insulin deprived environment showed 21% neurotoxicity ($p=0.007$) in the absence of high glucose and 37% neurotoxicity ($p=0.000$) in the presence of high glucose conditions. In addition, 24.16% neurotoxicity was observed in nerve cells stimulated with high glucose (150 mM) in the presence of insulin ($p=0.046$). MB had no neuroprotective effect against neurotoxicity observed in insulin-free normal ($p=0.047$) and 150 mM glucose-containing ($p=0.673$)

conditions. **(Figure 4)**. We expected to see neurodegeneration in conditions containing high glucose and in insulin-free conditions. This hypothesis was supported. We also expected to see MB have no neurotoxic effects, which was also observed in our experimental results, and to reduce neurodegeneration which our results neither prove nor disprove.

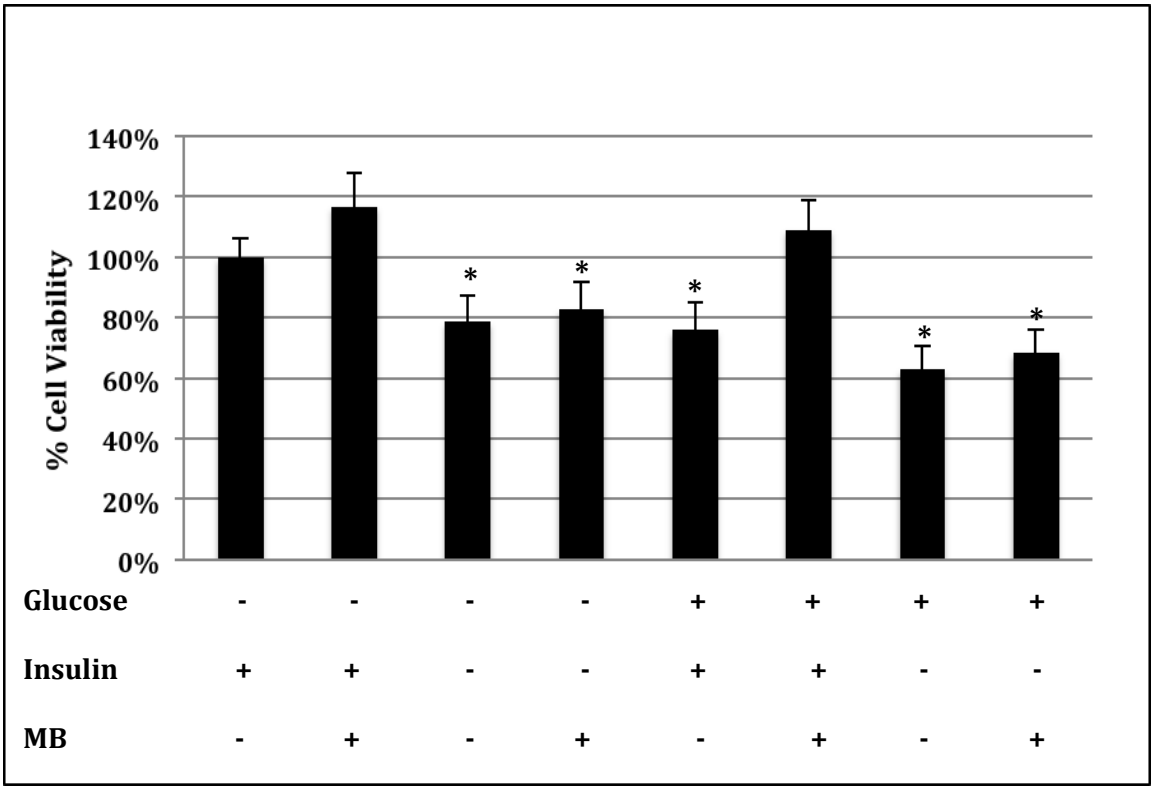

Figure 4: Percent cell viability for 16-day old nerve cells following stimulation with insulin, high glucose (150 mM) and MB (100 nM). The control of these experiments was normal GM (25 mM glucose) containing insulin. Percent viability values in treatment conditions were normalized to the control condition, which served as a baseline at 100%. Data are mean +/- SEM. (N=463 well, 8 plates).
*** = main effect, $p < 0.05$**

A pilot study was conducted using immunocytochemistry techniques to analyze the morphology and microtubule stability of 16-day old embryonic nerve cell cultures stimulated with glucose (150 mM), insulin, and MB (100 nM). Monoclonal-anti-acetylated tubulin primary antibodies and anti-mouse IgG $F(ab)_2$ fragment-Cy3 secondary antibodies were used to identify nerve cells through fluorescent antibody binding to tubulin. A quantitative analysis was performed by measuring the neuritic density 50, 100, and 150 microns away from nerve cell bodies **(Figure 5)**. We expected to see an increase in microtubule stability in conditions containing MB relative to the control. According to our results, all conditions showed a decrease in neurites corresponding with increasing distance. MB with insulin stimulated conditions showed an increase in the number of neurites from 2 to 10 when compared to the control condition. In insulin-deprived conditions, a corresponding decrease in neurite counts was observed in comparison to the control. Insulin-free conditions containing MB and all conditions stimulated with glucose (150 mM) showed no neuretic growth projecting from the nerve cell bodies.

Neuretic intensity stability at a distance of 150 microns from nerve cell bodies was analyzed as a quantitative measure of microtubule and cytoskeletal stability **(Figure 6)**. An increase in neuretic intensity was observed in MB with insulin stimulated conditions in comparison to the control condition **(Figure 6)**. In insulin deprived conditions, a decrease in neuretic intensity to approximately 100 microns was observed in comparison to the control condition. No neuritic

projections, therefore no neuritic intensity, was observed in insulin-free conditions containing MB or in hyperglycemic conditions. Sample fluorescent images of immunocytochemistry data were used as a qualitative measure of nerve cell morphology and microtubule stability **(Figure 7).** Nerve cells stimulated with MB in the presence of insulin (B.) had brighter tubulin staining, longer axons, and a higher density of dendritic branching, when compared to the control condition (A.) **(Figure 7).** A decrease in staining brightness and dendritic branching was observed in insulin deprived (C, D, G, H) and glucose (150 mM) conditions (E, F, G, H) **(Figure 7)**.

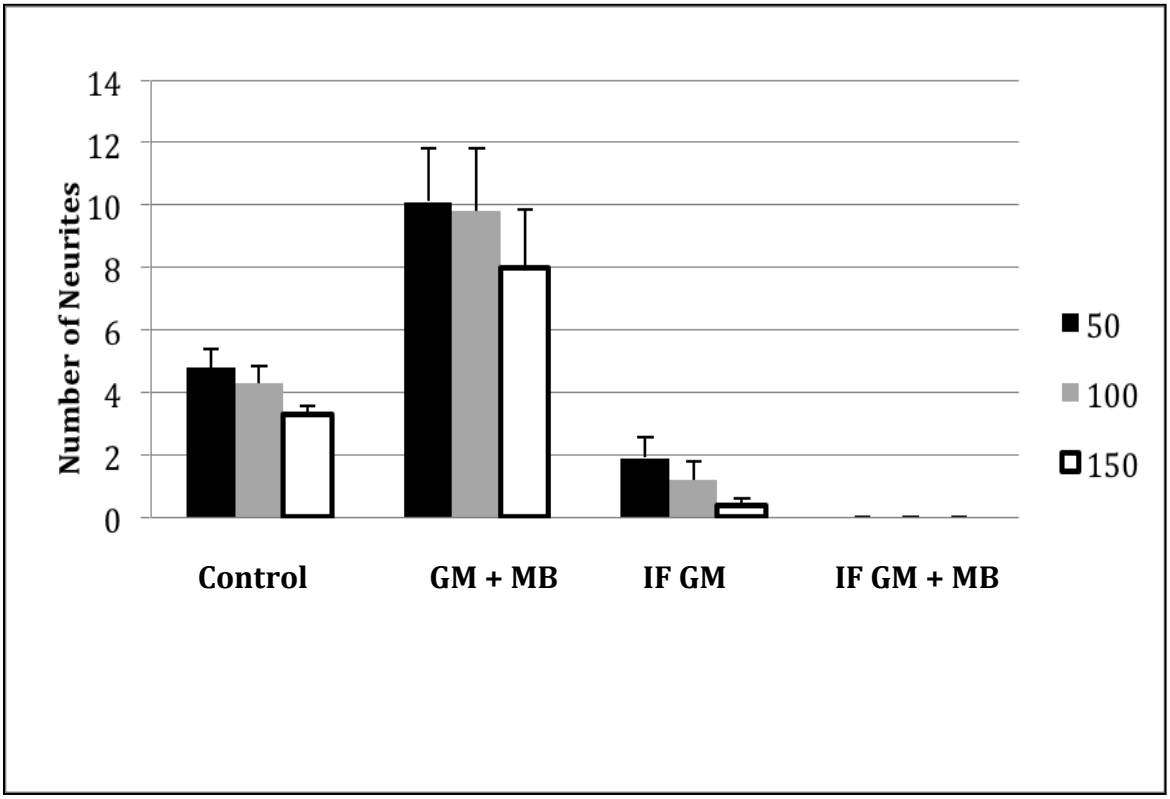

Figure 5: Pilot study demonstrating the effects of glucose, insulin, and MB on the number of neurites growing 50, 100, and 150 microns from neuronal cell bodies. Conditions containing high glucose concentrations (150 mM) in the presence and absence of insulin and MB are not depicted in the following graph due to no neuritic growth observed at all distances (50, 100, 150 microns) from the neuronal cell bodies. Only one plate was used for these results out of a total of 20 experiments due to contamination. (N= 24 wells, 1 plate).

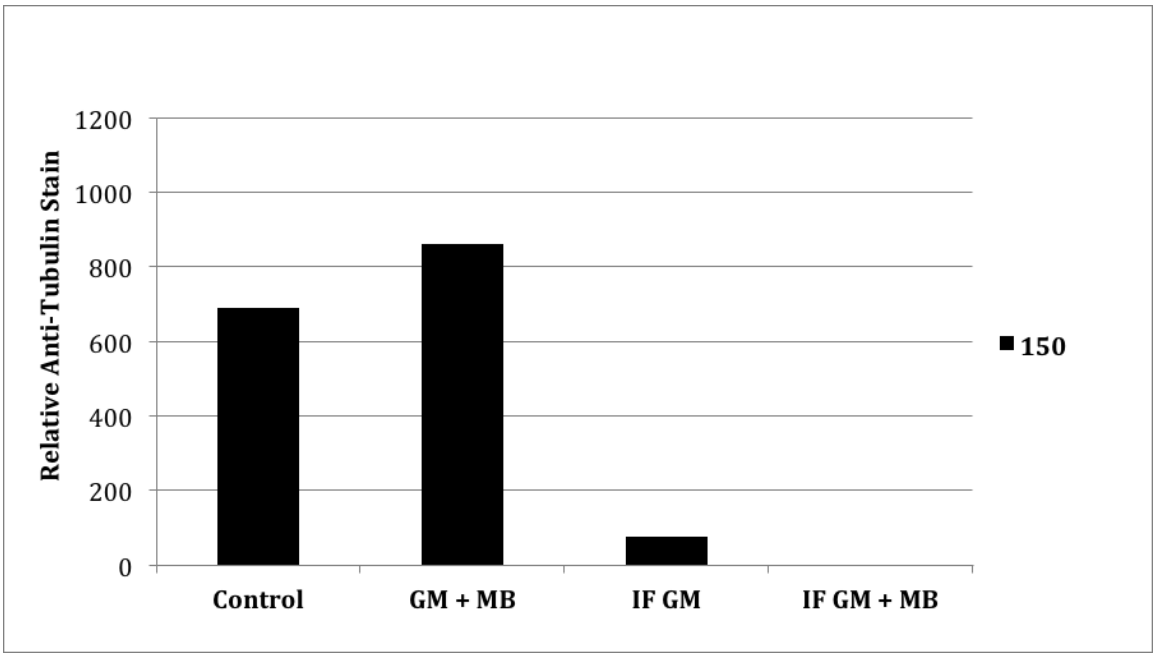

Figure 6: Pilot study demonstrating the effects of glucose, insulin, and MB on relative anti-tubulin staining at 150 microns away from nerve cell bodies. Conditions containing high glucose concentrations (150mM) in the presence and absence of insulin and MB are not depicted in the following graph due to no neuritic growth observed.

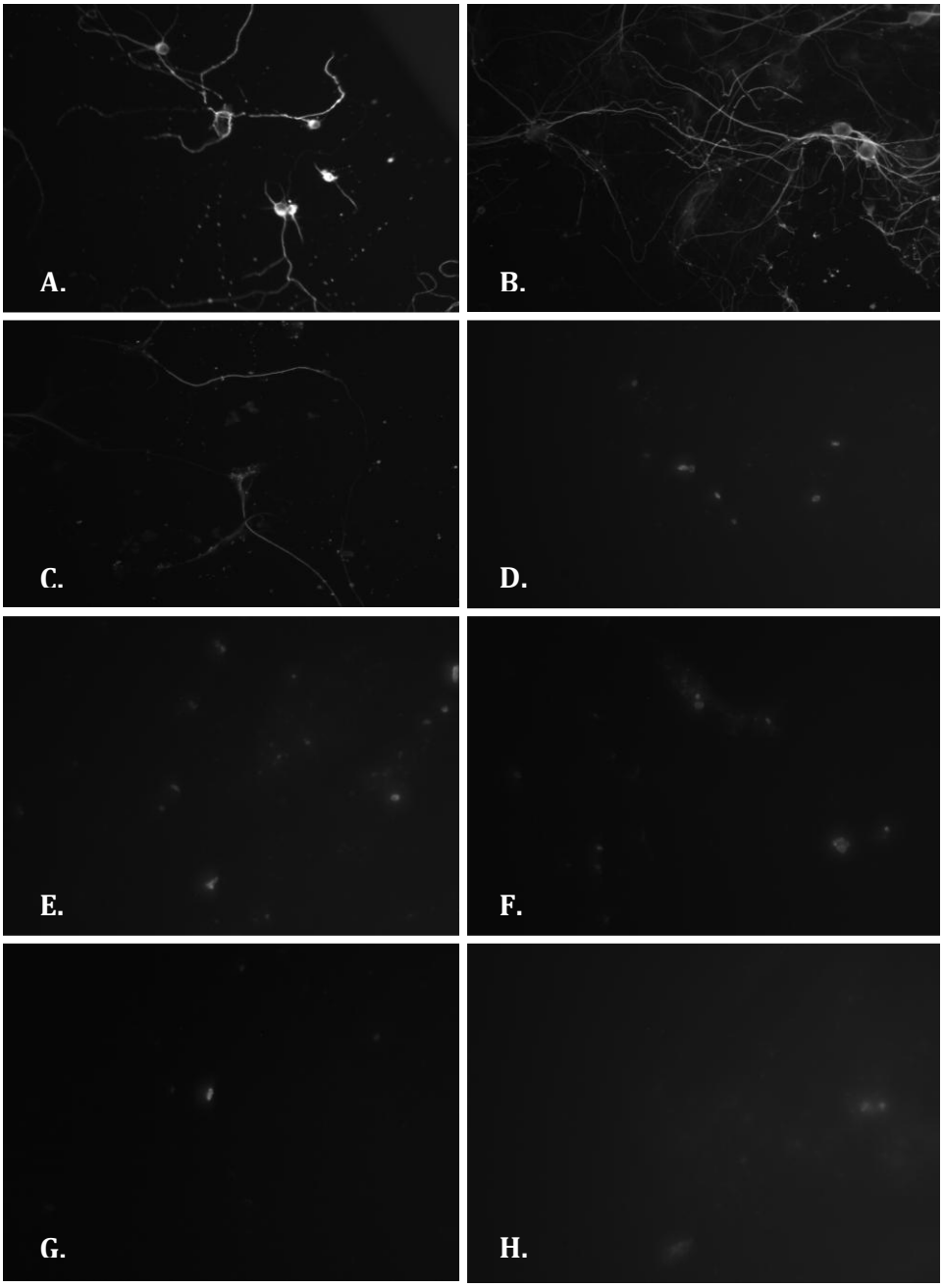

Figure 7: Pilot study sample fluorescent images of acetylated tubulin binding in 16-day old nerve cells stimulated with insulin, high glucose (150 mM), and MB (100 nM) conditions in a 24-well plate. A. Control condition: contained normal GM (25 mM glucose). **B.** GM with MB condition. **C.** Insulin free (IF) GM condition. **D.** IF with MB condition. **E.** High glucose (150 mM) in the presence of insulin condition. **F.** High glucose with insulin and MB condition. **G.** IF high glucose (150 mM) condition. **H.** IF high glucose with MB condition.

DISCUSSION

Overarching Hypothesis

One of the major aims of this thesis was to begin investigating the potential relationship between AD and T2DM. My overarching hypothesis is that T2DM mechanisms, such as hyperglycemia and insulin resistance, will have a detrimental effect on nerve cell viability and lead to cell death. One of the major hallmarks of AD is neurodegeneration; therefore if this hypothesis is correct, I expect to see a corresponding decrease in nerve cell viability with high levels of glucose and a similar trend in insulin deprived conditions. Moreover, this is a preliminary study that has only begun to test one of the major hypothesis linking AD to T2DM, but other hypotheses may also exist. Alternative hypotheses might suggest a reverse relationship between AD and T2DM, in which AD might be a risk factor for metabolic disorders (Zhao et al., 2009, De Felice et al., 2014, De Felice and Ferreira 2014, Lourenco et al., 2013). Other viable hypotheses might suggest an autonomous risk factor, such as aging (Chen et al., 2008) or inflammation (DeFelice et al., 2014, De Felice and Ferreira 2014, Zhao et al., 2009, Lourenco et al., 2013), that might lead to T2DM and AD pathways that are independent of one another. Therefore, this hypothesis would suggest AD and T2DM are not linked to one another. This study supports the hypothesis that T2DM hyperglycemic and insulin deprived conditions lead to neurodegeneration, but does not directly prove nor disprove the hypothesis that T2DM plays a causative role in AD. In the future, this overarching hypothesis could be more fully supported by performing other types of experiments that could

involve looking at the effects of hyperglycemic conditions on aged mice or running an epidemiological study examining physiologic brain changes in diabetic patients versus placebo groups. Additionally, future experiments could be conducted to disprove alternative hypotheses in order to more fully support our hypothesis. Still, it is important to note that AD is a very complex disease that can be caused by a combination of disease mechanisms and risk factors. Our study aims to investigate one of these potential pathways using hyperglycemic and insulin deprived conditions to create a neurodegenerative model of AD.

The second major aim of this thesis was to analyze the effects of a potential AD drug called Methylene Blue (MB) on preventing neurodegeneration caused by high glucose and insulin deprivation. If MB is successful in reducing neurodegeneration, this would suggest it as a possible treatment for AD that could reduce the progression of the disease. However, this would not necessarily suggest MB could prevent AD from occurring altogether, due to the multifaceted nature and complexity of this disease. MB is currently undergoing phase 3 clinical trials as a potential AD drug and is hypothesized to reduce neurotoxicity by increasing mitochondrial activity (Zhang et al., 2006, Rojas et al., 2011) or by acting as a tau inhibitor (Wischik et al., 1996, 2010, 2014). This thesis investigates the novel hypothesis that MB may have therapeutic effects and reduce neurodegeneration through an alternative metabolic pathway involving insulin and/or glucose. Strong evidence found in current literature suggests the deregulation of glucose levels and loss of insulin signaling plays an important role in neurodegeneration in AD brains

and can even reduce the detrimental effects of AD pathology (Carvalho et al., 2013 , Willette et al., 2014). Therefore, if MB can reduce this neurodegeneration from occurring through a glucose or insulin-mediated pathway, this might also indirectly lead to other downstream effects, such as reductions in plaque deposits (Willette et al., 2014), that can also help reduce the progression of AD. Future experiments could be useful in identifying MB's potential mechanism of action through an insulin or glucose mediated pathway. One future experiment might involve measuring insulin receptor activity in response to MB cellular stimulation, while another experiment might involve measuring rates of glucose-ligand binding to nerve cell surface receptors. Still, by investigating whether or not MB can reduce neurodegeneration induced by hyperglycemic and insulin-deprived conditions, the results in this study can be important in potentially proposing alternative treatment strategies for people at risk for AD.

Model Analysis

An *in vitro* cell culture model was used to analyze the effects of glucose and insulin on nerve cell viability. Hyperglycemic conditions were modeled using high glucose concentrations of 150 mM and were combined with insulin deprivation conditions to model T2DM. We decided to test each variable independently to determine whether neurotoxicity was attributed to high glucose or insulin deprivation before analyzing the combined effect of hyperglycemic conditions in the absence of insulin. In conditions containing high glucose (150 mM) in the presence

of insulin, nerve cells responded with approximately 25% neurotoxicity ($p= 0.046$) when compared to control conditions (Figure 4). This suggests high glucose levels are a contributing factor in neurodegeneration (Farmer et al., 2012). We decided to then measure cell viability in nerve cells stimulated with insulin-free GM. Similarly to high glucose conditions, insulin-free conditions led to a reduction in nerve cell viability of approximately 24% ($p= 0.007$) relative to the control. Moreover, even though no exogenous insulin was added to these cultures, there remains the possibility that nerve cells could still be producing low levels of insulin in culture. Therefore, observed neurotoxicity in the total absence of insulin could in fact be greater than 25% cell death. Our results provide evidence suggesting insulin deprivation and high glucose conditions can each independently be contributing factors in neurotoxicity and support out first hypothesis.

After analyzing the independent effects of high glucose and insulin deprivation, we then analyzed the combined effect of these two conditions on nerve cell viability. According to percent viability results, high glucose (150 mM) with no exogenous insulin had a significant neurotoxic effect (loss of 38%; $p < 0.0001$) on nerve cell cultures when compared to the control (Figure 4). This suggests high glucose levels and insulin deprivation conditions can induce a combined effect that leads to nearly 2 times more neurodegeneration than the effects of each variable independently. Moreover, in future experiments we would be interested in further investigating the effects of these variables on nerve cell viability by running a dose response experiment for insulin versus glucose. We would measure the affects of

very low doses of insulin, such as 1 nM and 10 nM, on cell viability to identify the particular dose at which neurodegeneration begins to progress. We would also try to investigate the effects of high insulin doses, such as 1 μM and 2 μM, on cell viability. According to one study, very high levels of insulin can lead to cognitive deficits (Stolk et al., 1997), suggesting insulin has a specific therapeutic window before it begins to show signs of neurotoxic effects. Similarly, we would also like to determine the therapeutic window of glucose and to measure the effects of hypoglycemic conditions on cell viability, as some literature suggests very low glucose levels can lead to cognitive impairment (Kodl and Seaquist 2008).

Other studies have also supported our hypothesis that hyperglycemic conditions in an insulin-deprived environment lead to neurodegeneration (Chiu et al., 2008, Debling et al., 2006, Burns et al., 2012). In one particular study, researchers found evidence suggesting insulin receptors help maintain synaptic density, dendritic plasticity, and circuit function in optic nerve cells (Chiu et al., 2008). This study provides useful ideas for future experiments where we could investigate the underlying mechanisms that link insulin and insulin receptor activity with neurodegeneration. This future experiment might further provide supporting evidence for our hypothesis. Moreover, in another study, Burns and colleagues ran glucose and insulin tolerance assessments on AD patients and hypothesized a decrease in insulin levels and corresponding increase in blood glucose levels would lead to cognitive deficits (2012). According to their results, the researchers hypothesized cognitive deficits were downstream of an insulin mediated pathway

that affects GSK3β activity and function (Burns et al., 2012). In future experiments, we might try to measure GSK3β gene expression in cultures that have been stimulated with insulin to identify potential mechanisms involved in insulin induced neurodegeneration. Furthermore, because GSK3β activity has been hypothesized to lead to AD pathology (DaRocha-Souto et al., 2012), it would be interesting to see if cultures stimulated with Aβ and insulin would demonstrate a combined effect that leads to even greater neurodegeneration. This might provide supporting evidence suggesting diabetes-like conditions do not only have direct effects that lead to neurodegeneration, but may also indirectly induce AD pathology.

An *in vitro* embryonic cortical nerve cell culture model was used to simulate neurodegeneration in AD by manipulating glucose levels and insulin. This model has many benefits, but it could also be improved in the future. One of the benefits of this model is that it allows you to manipulate and control experimental conditions and independent variables. In this study, we were able to manipulate cell culture conditions by stimulating nerve cells with specific doses of glucose and methylene blue (MB), along with manipulating nerve cell environmental conditions by using insulin free versus insulin rich media supplement. An exact dose of glucose was used to stimulate nerve cells over a specific period of time. In the future, we would like to use *in vivo* models that further support our *in vitro* results. Using an *in vivo* model in the future along with our current model might be advantageous as it would allow us to measure the effects of glucose and insulin in a natural brain environment. Because glucose is normally transported via the blood to the brain,

using an *in vivo* model might allow us to measure the effects of aged and insulin-deprived mice and corresponding increases in glucose levels on cognition and brain activity. Furthermore, in our model cells are stimulated with a high dose of glucose (25 mM) relative to normal human physiologic brain levels. In the human brain, glucose is transported in small increments continuously as a person eats. In the future, we could attempt to stimulate nerve cells with low doses of glucose in variable amounts over a long period of time to better simulate normal glucose production in the human brain. However, an *in vitro* model can also pose its own challenges. For one thing, because environmental conditions are artificial, they can only be used as approximated models of neuronal cellular response to glucose and insulin in normal human brains or AD and T2DM patient brains.

It is also important to note that AD is a long term neurodegenerative disease that progressively worsens over a period of many years and decades, while this study uses a model that measures short term effects of glucose, insulin, and MB over 48 hours. Therefore, cells might be sensitized in response to these conditions over a few hours, but become habituated to long-term stimulant exposure. In the future, we would like to test this hypothesis and to determine whether or not cells undergo habituation when stimulated with insulin, glucose, or MB over 48 hours versus 72 hours or maybe even a week. Moreover, using a short-term model might also affect potential clinical implications of this study. Successful results observed in our experiments might not necessarily mean successful results will be observed in AD or T2DM patients treated with a low dose of MB. But these results provide important

data that can have future clinical implications after further experiments are conducted. By asking whether T2DM-related disease pathways lead to neurodegeneration, we open the door for other researchers to entertain the prospect of testing potential AD drugs on a smaller and more specific population of study participants, like T2DM patients. In the case of MB, this dye could potentially be useful when tested in T2DM patients that have abnormal glucose levels, but normal to high insulin levels.

Effect of MB

The second major goal of this study was to analyze the effects of a potential AD drug called Methylene Blue (MB) on preventing neurodegeneration caused by high glucose and insulin deprivation. In order to begin investigating this hypothesis, we first needed to measure the effective dose of MB in embryonic neuronal culture. This was essential to identify a non-toxic dose of MB that could have potential therapeutic effects on nerve cells (Martinez et al., 1978, Callaway et al., 2002). In a pilot study, one-week nerve cells were stimulated with high glutamate (20 mM) and either low MB (100 nM) or high MB (1 mM) conditions over a 48 hour period before overall cell counts were measured using an MTS assay (Figure 3). A high dose of glutamate (20 mM) was selected as a neurotoxic stimulant according to previous literature (Masia 2013, Alberdi et al. 2010). Results indicated cells stimulated with high glutamate (20 mM) had 45% ($p = 0.000$) neurodegeneration relative to the control. This provided supporting evidence for previous literature suggesting high

levels of glutamate lead to neurotoxicity (Alberdi et al., 2010). In high MB conditions, neurodegeneration was observed in the absence of glutamate (27%) and presence of glutamate (56%), supporting literature suggesting MB can be toxic at high concentrations (Martinez Jr. et al., 1978). A combined effect of high MB and glutamate was observed (loss 56%), leading to nearly 2 times more neurodegeneration than when each variable was present independently (Figure 3).

At low doses, MB appeared to have 10% neurotoxicity relative to the control, but statistical analysis confirmed this decrease in cell viability was not significant ($p = 0.293$)(Figure 3). In the presence of glutamate (20 mm), low MB ($p < 0.0001$) did not prevent neurodegeneration (51% loss) from occurring when compared to independent glutamate conditions (loss of 45%, $p < 0.0001$). We did not expect this to occur but hypothesize low MB did not prevent glutamate-induced neurodegeneration due to a lack of cellular sensitization. According to a study conducted by Jin and colleagues, 7-day-old nerve cell cultures are less sensitive to stimulants than are older nerve cells (2011). Therefore, in subsequent experiments we decided to stimulate nerve cells at two weeks or older in order to increase neuronal sensitivity to MB. We hypothesized low MB (100 nM) would potentially have therapeutic effects on the older nerve cells leading to reduced neurodegeneration (Figure 3).

After determining the dose dependency of MB and testing its effects in a glutamate AD model, we focused on analyzing the potential effects of the blue dye on nerve cells stimulated with diabetic-like conditions (Figure 4). This is a novel

question that has never been tested before and could potentially suggest a new MB mechanism of action that could be further investigated in future studies. Sixteen-day old nerve cells were stimulated with insulin, glucose (150 mM), and MB (Figure 4). Results indicated cells stimulated with MB in the presence of insulin had no neurotoxic effects ($p = 0.446$, 116% cell viability) and higher levels of percent cell viability relative to the control. However, statistical data analysis indicated this condition ($p=0.446$) was not significantly different from the control condition. Moreover, the large variability in the data made it hard to distinguish if modest fluctuations in the data were due to chance or to experimental conditions. Similarly, nerve cells stimulated with MB and high glucose (150 mM) ($p=0.673$, 109% cell viability) in the presence of insulin had a 9% increase in cell viability relative to the control, but this also was not statistically signficant. Moreover, because nerve cells do not divide (Purves et al., 2001), we do not think higher percent cell viabilities are a product of nerve cell proliferation or division. Rather, if the higher viability in MB conditions with insulin and high glucose was due to experimental conditions and not chance, we hypothesize this might be attributed to slight toxicity in the control condition. One of the limitations of our *in vitro* model was maintaining environmental conditions during the 16-day period of nerve cell culture incubation. Fluctuations in incubator CO_2 levels might have occurred due to CO_2 tank failures or due to excessive opening and closing of the incubator door when feeding cultures every 2 days. This could have also affected the mitochondrial genesis of the cells. Moreover, potential glial cells present in our cell cultures could have also

contributed to the higher viability in MB containing conditions versus control conditions, because glial cells, unlike neurons, can divide. However, one could also argue that embryonic cortical hemispheres may have lower levels of glial cell density, therefore glial proliferation might not have had a significant impact on cell viability. Moreover, the loss of neurons in control conditions might mimic overall stressful conditions that might be occurring in aged individuals who are at risk of developing AD and that embryonic cortical hemispheres have very low levels of glial cells. In the future, we could stimulate the nerve cells for a longer period of time with MB to determine if MB can continue to protect the neurons from potential *in* vitro related stress. We could also try using glutamax, which provides extra nutrients for nerve cells, making them less susceptible to stress and strictly monitoring Co_2 tank levels.

When comparing MB conditions in the presence of insulin and high glucose to independent high glucose conditions, statistical data suggests these conditions are not different from one another ($p = 0.182$). However, when comparing each of these conditions separately to the control, independent high glucose conditions are significantly different ($p = 0.046$) from the control while MB in the presence of insulin and high glucose conditions are not ($p = 0.673$). Therefore, we cannot fully prove not disprove that MB prevents neurodegeneration from occurring in high glucose conditions in the presence of insulin. Our second hypothesis is neither supported nor unsupported as the statistical data is unclear. We think variability in the raw data values collected from a total of eight plates that were stimulated with

varying conditions of insulin, high glucose (150 mM), and MB (100 nM) might have contributed to the ambiguity in statistical data. In future studies, we would like to replicate our experiments (Figure 4) in order to acquire statistical evidence that supports our second hypothesis. Furthermore, according to our observed results we hypothesize MB may have therapeutic effects through an insulin-mediated pathway. Statistical and experimental data analysis support the hypothesis that low MB (100 nM) is a non-toxic dose in our *in vitro* model, but further experiments would need to be conducted to support our hypothesis that MB prevents hyperglycemic-induced neurodegeneration and that MB potentially has an insulin-mediated mechanism of action. Although, no literature suggests MB reduces neurodegeneration through an insulin mediated pathway, supporting evidence from our insulin-deprivation results further led us to form this hypothesis. In insulin-deprived conditions, MB had no neuroprotective effects in both the absence of high glucose ($p = 0.047$) and presence of high glucose ($p < 0.000$) (Figure 4). Based on these results and MB's effects in insulin-containing conditions, we hypothesize MB can potentially prevent neurodegeneration from occurring in the presence of insulin and has no neurprotective effects in the absence of insulin.

Other future studies investigating the effects of MB on preventing neurodegeneration induced by hyperglycemic conditions could involve using other types of cell viability assays besides MTS assays. MTS assays are beneficial in that they are a quick method used to determine average cell counts in stimulated conditions. However, like most scientific tools and methods, MTS assays do have

some limitations. The first limitation is that they may not take into account live cells that are no longer respiring (Riss et al., 2013, Huang et al., 2004). MTS assays rely on cellular mitochondrial activity in order to determine average live cell counts. Respiring cells that are able to tautomerize the dye into a soluble product are counted as being alive, while cells that have stopped respiring are not. However, it is important to note that MTS assays are used to determine average cell counts per well in a 96 well plate. Therefore, if analyzing MB conditions in the presence of high glucose or any other condition, it is possible some of the cells in that well stopped their mitochondrial respiration due to stress, but the high percent viability value (119%)(Figure 4) suggests most of the cells in that condition were still respiring and were therefore included in the MTS data as live cells. Another potential limitation of MTS Assays that may or may not have influenced data measuring the effects of MB conditions in the presence or absence of insulin could be that MB increased mitochondrial respiration leading to higher cell counts in the MTS data. Previous literature suggests MB may influence mitochondrial respiration by acting as an electron carrier (Miraoui Figure 2; Zhang et al., 2006, Rojas et al., 2011). This may have an impact on MTS assay results, since MTS dye is also converted by cellular mitochondria. Therefore, cell viability data in MB- containing conditions could be exaggerated and cell counts might in fact be lower in insulin-containing and insulin-deprived conditions. Furthermore, we hypothesize that even if MB increased mitochondrial respiration leading to higher MTS assay cell counts, insulin-deprived conditions containing MB were so much lower than insulin present

conditions with MB that even after removing confounding variables, the results could still suggest the same trends observed in our study (Figure 4). We do not have supporting evidence to prove nor disprove this hypothesis, but would be open to using other types of cell viability assays to further investigate this hypothesis. Additionally, these cell viability assays could be used to further support our first hypothesis suggesting insulin-free and high glucose conditions induce neurodegeneration. We could also investigate other ways in which MB, high glucose, and insulin-free conditions affect nerve cells besides nerve cell counts, such as examining the effects of these conditions on neuronal morphology and microtubule stability. We decided to begin investigating these effects by conducting a preliminary pilot study using immunocytochemistry techniques.

A pilot study was conducted to investigate the effects of MB (100 nM) on neuronal health, as measured by microtubule stability. This study only included results gathered from one 24-well nerve cell culture plate, as all other neuronal cultures died due to contamination. Due to the limited amount of data available, no statistical analysis was performed on these results, but the data were still included as a potential experiment to be further investigated and replicated in the future.

Neurons need stabilized microtubules to maintain their long axonal and dendritic projections (Ruiz-Canada 2004). If cells are alive but don't have the ability to maintain the complex neuronal network necessary for cognition, then treatments like those with MB would not be very effective. In this study, a 24 well plate was quantitatively and qualitatively analyzed using immunostaining and fluorescence

techniques. Cells were stimulated over a 48 hour period with the same conditions as in Figure 4 and stained using a primary tubulin monoclonal mouse antibody and a secondary anti-mouse antibody. An immunfluorescence microscope was then used to quantitatively analyze microtubule stability by measuring neurite counts and neuritic intensity (Figure 5,6). Additionally, a qualitative analysis of microtubules was performed by analyzing cell morphology, including stain brightness, axonal lengths, and dendritic branching (Figure 7). According to quantitative results, neuritic intensity and neurite counts increased in MB conditions relative to the control condition. This suggests MB can potentially improve microtubule stability by affecting neuritic intensity and counts, which might mean these nerve cells are less susceptible to neurodegeneration and cell death. Future experiments replicating this pilot study could potentially provide evidence supporting this hypothesis.

Qualitative observations of cell morphology showed an increase in dendritic branching, stain brightness, and axon lengths in conditions containing MB relative to the control condition. Brightness is a measure of the amount of binding antibody. Therefore, if more antigen (tubulin) is present then more antibody binding will occur and the brighter the stain will be. Our results suggest MB may potentially lead to increased tubulin production in microtubules; therefore increased antigen/antibody binding and a brighter stain. Moreover, increased dendritic branching and axonal lengths have been hypothesized to give rise to improved synaptic signaling and neuronal signaling (Rusakov et al., 1996), leading to higher levels of nerve cell viability. Therefore, observed increases in dendritic branching

and axonal lengths in MB containing conditions may suggest MB indirectly leads to increased neuronal signaling and synapse formation (Figure 7).

In hyperglycemic conditions and insulin-deprived conditions, no immunofluorescence results were measured. We hypothesize this was due to bacterial contamination that may have contributed to the cell death in those conditions. In future studies, we would like to replicate these conditions using the same *in vitro* model in order to find supporting evidence for our two major hypotheses and to investigate the effects of diabetic-like conditions on microtubule stability and overall neuronal health.

Future Experiments

In the future we would like to replicate the experiments conducted in this study (Figure 4-7) to more fully support our two major hypotheses: the first hypothesis being that hyperglycemic and insulin-deprived conditions induce neurodegeneration, and the second hypothesis being that MB prevents neurodegeneration in these diabetic-like conditions. Other future experiments might involve measuring the interaction between AD and Type 2 Diabetes pathways by using AD-related marker like Aβ aggregates or hyperphosphorylated tau in the absence and presence of diabetes-like conditions to induce neurodegeneration. We would also like to measure the effects of MB on neurodegeneration in these conditions. Moreover, another future experiment might involve measuring the combined effects of MB and an insulin-sensitizing drug (Li et al., 2012), like

Metformin, in insulin-deprived conditions to determine whether this may or may not reduce neurodegeneration. This future experiment might support our hypothesis that MB may reduce neurodegeneration through an insulin-mediated pathway. We would also like to use other cell viability assays and research techniques to reduce limitations faced during this study and to provide further evidence supporting our hypotheses. In this study, we are able to conclude that high glucose (150 mM) levels and insulin-deprivation can each independently induce neurodegeneration and that MB may have potential effects in reducing neurodegeneration induced by these diabetic-like conditions possibly through an insulin-mediated pathway. Our data also suggests MB may improve neuronal microtubule stability and nerve cell health.

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