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Evaluating the Effects of Methylene Blue on the Growth of Axons and the Health of Primary Neuronal Cell Cultures; Implications in Alzheimer Disease Research

A Thesis in Biology

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ABSTRACT

Alzheimer disease (AD) is a neurodegenerative illness that causes a gradual decline in memory and significantly affects cognitive functioning. One compound that may aid AD patients is methylene blue (MB). MB is an inexpensive dye that has been known to cross the blood-brain barrier (Stack et al. 2014; Jiang et al. 2015) and has been safely used in humans (Naylor et al. 1986). Previously, a derivative of MB, Rember, was utilized in a phase II clinical drug trial, where individuals who were administered MB demonstrated less of a cognitive decline in comparison to the placebo group. While the exact mechanism of action of MB in relation to AD remains unknown, it is possible that MB may be working as an anti-tau aggregation drug (Hosokawa et al. 2012) or an energy-enhancing compound (Shen et al. 2013). With this in mind, the overarching goal of this study was to provide insight into MB's effects in an *in vitro* primary neuronal cell culture using embryonic rat neurons, while placing particular emphasis on the growth of axons. We first assessed whether nanomolar (nM) concentrations of MB produced neurotoxic effects in primary neurons. Results indicated that doses up to 200 nM MB had no neurotoxic effects. Next, we observed tau, a protein that drives the formation of axons (Wang and Liu 2008), in neurons treated with the same previous doses of MB, and we detected various growth rates of neurons *in vitro*. As part of this aim, we raised the question if MB interacts with the axonal protein tau, would those interactions alter the development of axons? Based on our findings, there was no inhibition of axonal growth, and 100 nM MB led to a significant increase in axonal lengths. With this enhancement property of MB eluded, axonal lengths were distributed into frequencies, and we

observed an increase in long axons and a decrease in short axons with 100 nM MB. This effect was more pronounced with a 24-hour MB stimulation as opposed to a 48-hour MB stimulation. We also categorized axonal lengths into long axons (>75 microns), and we determined that there was a significant difference between axonal lengths and doses with both 24-hour and 48-hour exposure periods. An increasing impact of dose was seen with doses up to 100 nM MB. Because axon outgrowth is an energy intensive function, we examined ATP levels as a possible explanation for the enhancement property of 100 nM MB on axon lengths. We detected a significant increase in ATP levels with a 48-hour exposure to MB but not with a 24-hour exposure. In a pilot study, we analyzed potential neuroprotective effects of MB against glutamate excitotoxicity. Little to no attenuation was seen with MB. While we did not directly test whether MB became bound to phosphorylated tau, because axon outgrowth was not inhibited, tau's function in elongating the axon did not appear to be disrupted. This study has implications for future AD research by highlighting MB's ability to serve as a cognitive enhancing drug.

TABLE OF CONTENTS

ACRONYMS AND ABBREVIATIONS....1
INTRODUCTION....2
Alzheimer Disease (AD) Overview....2
Hallmarks of AD....4
Amyloid-beta Plaques....5
Neurofibrillary Tangles....9
Neurodegeneration....12
Cellular Mechanisms....13
Axonal Outgrowth....13
Mitochondrial Dysfunction....16
Methylene Blue....18
Anti-Tau Aggregation Drug....19
Figure 1: Possible MB Treatments for AD....20
Figure 2: Potential Cellular Mechanisms of MB in relation to AD....21
Mitochondrial Energy Enhancer....23
Present Study....25
MATERIALS AND METHODS....27
Primary Neuronal Cultures....27
Stimulations of Neurons….....28
Table 1: Overview of Experiments....30
Immunocytochemistry....30
Immunofluorescence Microscopy....31
Quantification of Immunocytochemistry Images....32
MTS Cell Viability Assay....32
Figure 3: Sample Image of Distinctive Axonal Lengths....33
Figure 4: Sample Image Illustrating Axonal Tracing....34
CellTier-Glo® Luminescent Cell Viability Assay....35
Exclusion Criteria....35
Statistics....35
RESULTS....36
MB and Axonal Outgrowth....36
Figure 5: MB Was Not Toxic to Primary Neuronal Cultures....37
Figure 6: Sample Fluorescent Images of Tau....38
Figure 7: Exposure to MB Did Not Inhibit Axonal Outgrowth....40
Figure 8: Exposure to MB Enhanced Axonal Lengths....41
Figure 9: MB Did Not Inhibit Axonal Growth....43

Figure 10: Axonal Lengths were Enhanced with MB Stimulations.....44
Table 2: Assessment of Percentage of Long Axons by MB Dose.....45
MB and Generation of ATP.....45
Figure 11: Exposure to MB Increased ATP levels.....46
Pilot Study.....47
Figure 12: Little to No Attenuation of Glutamate Toxicity with MB.....48
DISCUSSION.....49
Overview of Research.....49
Model System Analysis.....49
Effect of MB.....50
Neurotoxicity Analysis.....*50*
Axonal Outgrowth.....*51*
Generation of ATP.....*56*
Pilot Study.....*59*
Future Experiments.....62
REFERENCES.....64
ACKNOWLEDGEMENTS.....77

ACRONYMS AND ABBREVIATIONS

AD: Alzheimer Disease

Aβ: β-amyloid peptide

APP: β-amyloid precursor protein

ATP: Adenosine Triphosphate

CSF: Cerebral spinal fluid

HA: healthy aging

MAP: microtubule-associated protein

MB: Methylene blue

MCI: Mild cognitive impairment

MT(s): Microtubule(s)

PBS: Phosphate buffered saline

ROS: reactive oxygen species

SirT1: NAD-dependent deacetylase sirtuin-1

SirT2: NAD-dependent deacetylase sirtuin-2

INTRODUCTION

Alzheimer Disease Overview

Alzheimer disease (AD), the sixth leading cause of death in the U.S. (Alzheimer's Association (AA) 2016), is a debilitating cognitive illness that impacts numerous individuals every day. AD is the most common cause of dementia in older populations (Saunders et al. 1993; Jacobsen et al. 2006; Takada-Takatori et al. 2006), accounting for nearly 60% to 80% of all dementia cases (AA 2016). Currently, an estimated 5.4 million Americans are living with this progressive illness (Alzheimer's Association (AA) 2016); approximately, 96% of them are 65 years of age and older, while 4% of them are under the age of 65 years (Herbert et al. 2013). By 2050, approximately 13.8 million individuals, ages 65 and older, may be living with AD (Herbert et al. 2013).

The less common form of AD is early-onset AD, which is seen in patients younger than 65 years. Early-onset consists of less than 5% of all AD cases and has been previously linked to mutations in the following three genes: amyloid-β precursor protein (*APP*), presenilins-1 (*PS-1*) and presenilins-2 (*PS-2*) (Duff et al. 1996; Schneuder et al. 1996). Since AD is underdiagnosed (AA 2016), many AD patients may be entirely unaware of this complex illness already running its course; definitive diagnosis is only confirmed postmortem (Markesbery 1997). Examining the largest risk factor for this disease may provide a better understanding as to why so many individuals currently have or will develop AD in the future.

Aging has been identified as the biggest single risk factor for developing late-onset AD (Knowles 2004; Gold and Budson 2008). Late-onset is the most common form

of AD that is characterized as occurring in individuals' ages 65 years and older. In addition, ε4 allele apolipoprotein E on chromosome 19 has been identified as the largest genetic risk factor for developing sporadic AD (Handley et al. 2006; Theendakara et al. 2013). With the probability of having AD doubling every five years after the age of 65 (Knowles 2004), as patients' age, they are placed at a greater risk of developing this disease. Aging comes with a number of physiological and cognitive manifestations. Some changes include synaptic losses (Terry et al. 1991), disruptions to synaptic plasticity (Crews and Masliah 2010), imbalances in energy metabolism (Bratic and Trifunovic 2010; Roberts and Rosenberg 2006) and neuronal losses (Gavidia-Bovadilla et al. 2017). Cellular changes at synapses may be conflicting with the normal cognitive functioning of neurons. One particular cognitive change occurs when recalling information from the short-term memory becomes a difficult task as individuals approach their 60s (Reddy and Beal 2011). For many years it has been widely debated whether or not AD is an exaggerated form of aging (Ohnishi et al. 2001; Spaan 2016). However, some suggest that a decline in cognitive abilities throughout the years indicates the pathogenesis of an illness (Linn et al. 1995). So while AD is not a normal part of aging, there may be factors associated with aging that increases one's risk of developing AD.

While scientists remain unaware of the cause of AD (Clinton et al. 1991), they are mindful of the physiological deficits that are associated with this decade-long progression. AD patients experience three hallmarks: amyloid-beta plaques, neurofibrillary tangles (Casley et al. 2002) and neurodegeneration (Crews and Masliah 2010). AD patients experience disruptions to synaptic plasticity (Crews and Masliah

2010) as well as a loss of synapses and communication between nerve cells, which eventually lead to damaged and destroyed neurons. In addition, mitochondrial dysfunction has been identified as a characteristic of AD (Chen et al. 2006, Kim et al. 2017). Evidence suggests that several neuropathological alterations occur years before the onset of cognitive impairments (Bennett et al. 2006). These physiological changes jeopardize the normal functioning of a person's cognition and memory.

AD patients experience a significant cognitive decline (Yeung et al. 2015; Haj 2016) with symptoms taking nearly ten years to appear. One of the first indicators of the clinical onset of AD is memory impairment (Yang et al. 2016). Individuals may also begin to find daily-living activities, such as eating and walking, quite difficult to perform (Staedtler and Nunez 2015). With language also jeopardized (Lee et al. 2017), communication between the patient and their families becomes difficult. Names of familiar people, places and items may gradually become problematic to recall (Choi and Twamley 2013); an increase in recall time has also been noted in previous research (Corbett et al. 2012). Other cognitive changes relate to thinking and reasoning, making judgments and decisions (Helmes and Østbye 2002) as well as to behaviors (Mega et al. 1996) and emotions (Drago et al. 2010). These cognitive impairments in AD patients suggest the importance of developing drugs that aim to alleviate cognitive changes. Prior to the discussion of one possible cognitive enhancing drug, the main pathologies of AD will be reviewed and some cellular mechanisms of plasticity, which depend upon energy, will be described.

Main Hallmarks of AD

Amyloid Beta Plaques

One of the main hallmarks of AD is the extracellular amyloid-beta plaque (Wilson et al. 1999; Leu et al. 1996). Plaques are made up of aggregated amyloid-beta (Aβ) protein (Friedrich et al. 2010), which can consist of 39 to 43 amino acids (Shoji et al. 1992). The β-amyloid precursor protein (APP) can be cleaved by various sequential proteases (O'Brien et al. and Wong 2011) such as α-secretase, β-secretase or γ-secretase (Haass et al. 1995; Strooper et al. 2010) to generate different sizes of Aβ peptides (Schneuder et al. 1996). Cleavage of APP by the proteases, β- and γ-secretase, may generate Aβ (Schneuder et al. 1996; Gu and Guo 2013), while cleavage of APP by α-secretase protease inhibits Aβ production (Strooper et al. 2010). Results from previous research have suggested that the cutting of APP by γ-secretase allows for a greater production of Aβ42, the more toxic form of Aβ *in vitro* (Wilson et al. 1999), as opposed to Aβ40. One finding by Kim and colleagues (2007) determined that decreased Aβ42/Aβ40 ratios reduced Aβ burden in transgenic mice; this finding supported a high degree of cellular toxicity from the fibril form of Aβ42. It has also been suggested that the oligomeric form of Aβ plays a role in the toxicity of the neurons (Dorostkar et al. 2014), which will be explored shortly. To date, the exact damaging effects of Aβ plaques on neurons are unknown.

Aβ deposition has been tightly linked to the initiation of AD pathology (Spires-Jones and Hyman 2014; Duff et al. 1996). Evidence suggests that genetic mutations cause alterations in the amount of Aβ being produced (Duff et al. 1996), which may generate

early-onset AD. This suggests that Aβ could initiate the disease pathway. In addition, imaging studies demonstrated that MCI, as well as asymptomatic individuals, already demonstrated Aβ staining (Zhao et al. 2017). This evidence indicated that deposition of Aβ might be an early event in AD, prior to the intervention of the rest of the characteristics of AD.

Aβ deposition has also been linked to the pathogenesis of AD (Seubert et al. 1992; Haass et al. 1995; Citron et al. 1997; Leung et al. 2011). In addition, these extracellular plaques have been correlated with disruptions in neurite morphologies (Spires-Jones et al. 2014), which may produce cognitive dysfunctions in AD patients following neuropathological alterations (Strooper et al. 2010). One particular *in vivo* study utilized Pittsburg Compound B (C-PIB) PET, a dye that binds to Aβ plaques (Pike et al. 2007), to investigate the linkage between Aβ plaques and memory performance in different stages of the disease. Healthy aging (HA) individuals as well as those found in the transitional stage between AD and healthy aging, mild cognitive impairment (MCI), were utilized in this study. Results indicated that 22% of HA patients, 61% of MCI cases and 97% of AD subjects had higher amounts of PIB binding, which indicated deposition of Aβ plaques. Additionally, higher PIB binding correlated with greater cognitive impairments of episodic memory performances among individuals (Pike et al. 2007). These results suggested a potential relationship between the presence of Aβ plaques and the likeliness of developing AD as well as the linkage between Aβ plaques and memory impairments.

One particular hypothesis suggests that Aβ plaques may be playing a role in

synapse dysfunction and loss (Dorostkar et al. 2014). A synapse is a region where two neurons can communicate with each other. Synaptic transmission is vital for learning, voluntary movement and perception while synaptic plasticity plays a central role in higher brain functions, such as in the formation of new memories and the disposal of old memories (Mayford et al. 2012). Without proper synaptic function, neurons can no longer respond to stimuli from another neuron, which can eventually lead to the loss of communication between these neurons. Previously, it has been reported that Aβ plaques may also contribute to inflammation (Tuppo and Arias 2005) and excitotoxicity (Mattson et al. 1992), which may be linked to synaptotoxicity. Thus, disruptions to synapses may contribute to cognitive decline in AD (Dorostkar et al. 2014; Yi et al. 2016). One study was interested in understanding how passive immunotherapy on an AD animal model (Tg2576) could alleviate synaptic loss from oligomeric Aβ toxicity. For a duration of eight weeks, twelve-month-old mice received 500 μg of oligomeric-specific (A-887755) or conformation-unspecific (6G1) antibodies by intraperitoneal injection, for a total of eight times. Results indicated that in hippocampal and cortical regions, oligomeric Aβ disrupted DLG4-positive post-synapses. Also, after they analyzed the pre- and post-synaptic structures closest to the Aβ plaques, they determined that both structures had been lost. However, the A-887755 treatment appeared to have protective effects in the mice (Dorostkar et al. 2014). Their results indicated that synaptic loss was facilitated by oligomeric Aβ structures and passive immunotherapy may aid in targeting this pathology of AD.

In addition to synaptic dysfunction and loss (Spires-Jones and Hyman 2014), Aβ

plaques have also appeared to work in collaboration with mitochondrial dysfunction. A hypothesis for the role of the toxic form of Aβ relates to an increase in reactive oxygen species (ROS). ROS may damage the membrane, placing great stress on the cells. Leung and colleagues utilized female rhesus monkeys' cortexes that were previously demonstrated to be sensitive to fibrillar Aβ (2011). When fAβ was introduced into the primates, neuronal losses occurred. In addition to these consequences, microglia, an immune cell in the brain (Lull and Block 2010), became activated which produced ROS as well as cytokines and chemokines (Leung et al. 2011). These researchers were interested in understanding how microglia affected nerve cells due to fAβ and whether neuronal losses was seen in this neurodegenerative disease model from this inflammatory response. Interestingly, they made the argument that inflammatory responses from fAβ may be playing a role in the pathogenesis of AD (Leung et al. 2011). This study utilized Tuftsin, a peptide that allows for stimulation of immunological responses, and a microglia/macrophage inhibitory factor, which aimed to protect these nerve cells against microglia activation as well as from damage done by inflammation. Results indicated that the volume of damage by fAβ was reduced when microglia activation was inhibited. In addition, in response to fAβ, microglia were activated, and they produced ROS. Microglia/macrophage inhibitory factor prevented this ROS production (Leung et al. 2011). Not only does this provide evidence for a potential immunological component to the irreversible damage seen in neurodegenerative diseases such as in AD, but this also provides some insight into why fAβ may be so harmful to the nerve cells.

Energy metabolism abnormalities have also been observed in previous AD

research (Pereira et al. 1998; Casley et al. 2002). Evidence has suggested a potential linkage between mitochondrial function impairment and the energy metabolism imbalances seen in AD (Pereira et al. 1998). Several enzyme complexes, including cytochrome oxidase, have been previously analyzed to be deficient in AD (Kish et al. 1992). No solid evidence exists on how Aβ deposition may be causing an impairment of energy metabolism in AD (Casley et al. 2002).

To explore the role of Aβ in energy deficiency, a separate study stimulated embryonic primary cell cultures with 50 μM $A\beta_{25-35}$ for 24 hours. $A\beta_{25-35}$ has been known to cause aggregation in a shorter period in comparison to $A\beta_{1-40/42}$, (Casley et al. 2002) and has showcased similar toxicity effects as $A\beta_{1-40/42}$ (Pike et al. 1993). By utilizing a spectrophotometer to measure mitochondrial enzyme activities of complexes I, II, III, IV, the following percentage of reductions in their activities were seen: 40%, 33%, 27%, 27%, respectively (Casley et al. 2002). These reductions were indicative of a loss of mitochondrial function induced by $A\beta_{25-35}$, suggesting that Aβ may be responsible for the impaired energy metabolism seen in AD. Later in this paper, the connection between improving energy metabolism and combating the cognitive decline in AD, will be raised.

Neurofibrillary Tau Tangles

The second hallmark of AD is the formation of intracellular neurofibrillary tau tangles (Busciglio et al. 1995; Goedert 1996; Leu et al. 1996), which mainly consists of paired helical filaments (PHF) (Serrano-Pozo et al. 2011). Tau tangles are associated with the progression of AD (Braak and Braak 1991; Arriagada et al. 1992; Fein et al. 2008; Congdon et al. 2012) and the severity of dementia (Arriagada et al. 1992, Brion 1998;

Giannakopoulos et al. 2003). While the complete mechanism of tangle formation is unknown, neurofibrillary tangles (NFT) develops from the accumulation of abnormally hyperphosphorylated, misfolded and self-aggregated tau proteins (Busciglio et al. 1995; Goedert 1996; Wang and Liu 2008). These aggregations of tau protein are usually insoluble (Bloom 2014), and may pose a significant threat to normal neuronal signaling.

Because the normal functioning of neurons requires the regulation of microtubule dynamics by tau, minimal tau phosphorylation exists in the brains of healthy individuals (Seubert et al. 1995). Phosphorylation of tau is a normal post-translation modification in neurons. One particular study generated a monoclonal antibody (12E8) that would bind Serine 262 (Ser^{262}), a phosphorylation site in tau (Seubert et al. 1995). Ser^{262} has been previously thought to play a role in the interaction between tau and microtubules. Phosphorylation at the site of Ser^{262} did not inhibit the binding between tau and microtubules. Furthermore, this study suggested that over accumulation of hyperphosphorylated tau caused by a lack of phosphatase activity might be driving the formation of tangles in the brains of AD patients (Seubert et al. 1995). Similarly, the deregulation of kinase and phosphatases activity may be contributing to the formation of tangles (Brion 1998).

In AD patients, previously stabilized tau with microtubules become detached from one another, disrupting their vital interaction, and allowing for the dissociation of microtubules and formation of tau aggregates to occur (Spillantini and Goedert 2013). This disruption may alter the normal conformation of tau (Hu et al. 2016) often leading to the gradual dissociation of the axon and neuronal death. With disturbances to the axon,

imbalances in neuronal activity, such as with membrane trafficking occurs (Wang and Liu 2008). This imbalance may facilitate in cognitive decline, which has been previously associated with tau tangles in AD patients (Arriagada et al. 1992; Giannakopoulos et al. 2003; Wang and Liu 2008). With this information, it becomes evident how tau tangles may be contributing to the progression of AD.

A more recent hypothesis of tangle formation focuses on the truncation of tau. One study provided evidence in support of the cleavage of tau by the protease, asparagine endopeptidase. The researchers proposed that the cleavage of tau by asparagine endopeptidase produces tau fragments which may facilitate hyperphosphorylation of tau and lead to disrupted microtubules and aggregations of tau, followed by synaptic losses and cognitive deficits (Zhang et al. 2014).

Alternatively, the most widely studied hypothesis suggests that tau tangle formation occurs from abnormal hyperphosphorylation of tau. Hyperphosphorylated tau can be found in normal adult human brains, but not to the extent that is seen in AD patients (Iqbal et al. 2010). Although it has been previously noted that hyperphosphorylation of tau may occur in a cell independent manner, a more recent hypothesis suggested that hyperphosphorylation may arise from a prion-like spreading mechanism from one neuron to another (Peeraer et al. 2015). It is possible that the spread of the disease to areas that were not previously affected may be attributed to misfolded proteins promoting further misfolding of normal proteins with no previous tau pathology (Braak and Braak 1991; Calignon et al. 2012). Currently, there is no direct evidence of this prion-like mechanism. However, if this hypothesis were true, one previous study was

interested in developing a way to model this spread of the disease *in vivo*, and in the future, explore ways to combat this potential prion-like mechanism (Peeraer et al. 2015). Their results demonstrated an increase in phosphorylated tau as well as in insoluble tau (Peeraer et al. 2015). This suggests that tau has fallen off from its interaction with microtubules, an important complex that will be discussed in a later section. Thus, there is a higher chance that tau may aggregate into neurofilaments. Interestingly, one study suggested that abnormal tau hyperphosphorylation might induce the aggregation and spread of tau tangles (Hu et al. 2016). Because of the possibility of tau aggregates being able to propagate as mechanisms that may contribute to the progressive cognitive decline in AD, drugs, which have the ability to prevent tau aggregation or to sequester tau, may demonstrate to be an effective treatment strategy.

Neurodegeneration

The third neuropathological AD hallmark is neurodegeneration (Yankner 1996), which is accompanied by synaptic damage and neuronal loss (Crews and Masliah 2010). When compared to other pathological features of the disease, the strongest linkage is present between synaptic losses and cognitive impairments (Crews and Masliah 2010; Tiwari et al. 2016). Some have hypothesized that neurodegeneration may be rooted from the effects of plaques (Pike et al. 1993; Butterfield 2002) and tangles (Zhang et al. 2014). Pike and colleagues demonstrated a short- and long-term relationship between neurotoxicity and the significant aggregation of Aβ in neuronal cultures (1993), while a separate study suggested that truncations of tau by the protease, asparagine endopeptidase, might facilitate neurodegeneration (Zhang et al. 2014). However, a human

study, which looked at 19 autopsied and confirmed AD brains, demonstrated that a decrease in cytochrome oxidase activity was also seen in the brains of deceased AD patients (Kish et al. 1992). This evidence supported the hypothesis that energy metabolism imbalances may contribute to neuronal cell death and thus, neurodegeneration as seen in AD (Kish et al. 1992). Apoptosis of nerve cells induced from stress by the endoplasmic reticulum have also been viewed as an alternative explanation (Hitomi et al. 2004). During the late stages of AD, approximately 50% of all preexisting neurons are lost. Cerebral atrophy, which is defined as the physical shrinking of the brain, occurs. This great loss in neurons severely impacts an individual's cognitive state. Thus, any drug treatment that could prevent neuronal loss under AD-like conditions should be considered as a potential treatment.

Cellular Mechanisms

Axonal Outgrowth

Neurons consist of the following basic parts: the cell body or the soma, dendrites, extensions of a neuron that receive incoming signals from pre-synaptic neurons and axons, long projections that form connections with other nerve cells, and are responsible for sending valuable information to other neurons (Kandel et al. 2012). Axons can grow to be over one meter long and one micrometer (μm) in width (Goldberg 2017). To date, we have limited knowledge on the regulation of the following axonal processes: the rate at which the axon elongates and the halting of this outgrowth, among others. In addition, the scientific community's comprehension of how axons grow is limited (Goldberg 2017). Both intrinsic and extracellular guidance cues appear to influence axonal

development; investigations of these cues are ongoing (Goldberg 2017). During axonal development, growth cones, which are extensions of highly motile actin, aid in axon growth, as well as in axonal guidance to their respective synaptic partners. Growth cones must travel long distances (Campbell and Holt 2001) and are located in front of the axon (Meiri et al. 1986), enabling the formation of appropriate neural connections.

Due to its massive growth, the axon has enormous cellular demands (Vaarmann et al. 2016). Some requirements for axonal development include the production, shipment, insertion and accurate timing of appropriate proportions of molecules to the growing axons (Goldberg 2017). The regulatory processes of these requirements are not well understood. In addition, cytoplasmic proteins were only thought be derived from the cell body. However, it is possible that the axon itself may be producing some of these required proteins to aid in its outgrowth. Certainly, with such a high demand in requirements, a large amount of energy formation is needed for the axon's proper development.

Since axons are a vital asset for communication among neurons, appropriate axonal elongation to other neurons is necessary (Goldberg 2017). In addition to actin, which is a dynamic cytoskeletal polymer necessary for proper growth cone movement (Kim et al. 2001), another important polymer of the axon structure is an array of microtubule filaments, which allows for both structural support and transportation of cellular material to their destination. Microtubules (MTs) are a vital part of the cytoskeleton and they provide tensile strength to the neuron and are important for the normal trafficking of cellular cargo along the axon. MTs work together with microtubule-

associated proteins (MAPs), with MTs' plus end pointing in the direction of post-synaptic neurons (Goldberg 2017). MAPs aid in stabilizing MT polymerization (Goldberg 2017). Tau protein is an example of a MAP (Yang et al. 2009), which is present in all axons. One of its main purposes is to drive the formation of axons by binding to MTs under normal physiological conditions and stabilizing them (Wang and Liu 2008). A lack of tau expression may render a decline in the growth cone's motility (Liu et al. 1999).

The two main players of neuronal MAPs are tau, which was previously described, and the microtubule-associated protein 1B (MAPIB) (Takei et al. 2000). To understand the relationship between tau and MAP1B, an *in vivo* study utilized a mutant mice model system (Takei et al. 2000) and compared them to single-knockout mice phenotypes as well as to primary hippocampal cultures. After they had analyzed mice with disrupted MAP1B and tau genes, disorganization of the neuronal layers became evident (Takei et al. 2000). Also, when the researchers investigated the effects of double mutants on primary hippocampal neurons, they discovered improper growth of axons from inhibition of tau and MAP1B (Takei et al. 2000). These results elucidated the importance of these proteins for axonal stabilization.

Moreover, not only is tau essential for MT stabilization (Wang et al. 2008) and neurite outgrowth (Takei et al. 2000) but also for the selective dynamic properties of MTs (Liu et al. 2012). Some level of tau phosphorylation by kinases on serine and threonine residues (Biernat et al. 2002) is responsible for regulation of tau's interaction with MTs (Fuster-Matanzo et al. 2012). When tau becomes phosphorylated, it temporarily falls off MTs, reducing the binding of tau to MTs and allowing for the remodeling of MTs

through retraction. When depolymerization of MTs occur, an overall decrease in the length of the axon takes place. When the phosphate is removed from tau, tau can once again bind to MTs, helping to stabilize them and helping with the elongation of the MTs. These processes contribute to the dynamic properties of MTs (Fuster-Matanzo et al. 2012), an important factor for synaptic plasticity in the brain. In AD, when tau is found in tangles, tau cannot reattach to MT and stabilize them, and there is also less available tau to bind MTs, leading to MT depolymerization (Spillantini and Goedert 2013). Thus, the healthy availability and function of tau are necessary for the axon. Because of this, any drug treatment that alters the function of tau by binding to it and altering its availability to bind to MTs might have serious consequences on axonal function. Therefore, neuronal signaling, which is vital for proper cognitive abilities, might also become compromised.

Mitochondrial Dysfunction

Mitochondria are dynamic organelles (Zhou et al. 2016) that can be transported to different areas of the neuron and supply energy for neuronal development and survival (Nicholls and Budd 2000). In AD, the function of mitochondria declines (Grimm and Friedland 2016). Whether changes in mitochondrial function are a cause or an effect of AD is unknown. Several aspects of mitochondrial function that may be compromised are decreases in energy formation, increases in reactive oxygen species (ROS), inabilities to detoxify ROS or changes in intracellular calcium, which is an important signaling molecule (Hroudová and Fišar 2011). More specifically, an increase in ROS or an inability to detoxify ROS may facilitate the aggregative properties of Aβ and tau, which may serve as a potential cause of the disease. Alternatively, it is possible that Aβ may

contribute to mitochondrial dysfunction in AD (Reddy et al. 2010) by potentially generating more ROS. Another possibility is the deregulation of transport of mitochondria to sites in need of energy. This deregulation may occur from MT destabilization in AD, thus preventing mitochondria from effectively reaching their targeted locations. In summary, with mitochondrial dysfunction, the neurons would be unable to perform their foundational activities appropriately and effectively. Therefore, a potential treatment that seeks to boost mitochondrial function may alleviate this abnormality.

One specific study utilized stem cell transplantation to aid with mitochondrial dysfunctions (Zhang et al. 2015). The aim of this experiment was to understand whether stem cells might interfere in the decline of mitochondrial function, which may allow cells to recover their normal number of mitochondria. Unlike several studies that utilized asymptomatic models, this study used a model that showcased AD-like pathology. One-year-old mice were expected to demonstrate similar deficits as disease pathologies (Zhang et al. 2015). After ten weeks of stem cell transplantation, deficits declined, and mice were able to learn tasks and recall them (Zhang et al. 2015). It is possible that this improvement occurred because cells may have incorporated the stem cells into a neuronal network, which promoted the recall process. This research supported the hypothesis that mitochondrial dysfunction is present in AD and that reversing that dysfunction could improve cognitive ability. Given this involvement of mitochondria and oxidative stress, it is not surprising that several AD researchers turned to chemical compounds that are

known antioxidants, to potentially improve energy in cells as a potential treatment strategy.

Methylene Blue

One possible drug candidate that may serve as a cognitive enhancing treatment for AD patients is methylene blue (MB). MB is a common and inexpensive dye, synthesized in 1866 (Lin et al. 2012) that has been widely used as a biological staining agent for over a decade. It is a type of phenothiazine drug that has been previously discussed as an antioxidant (Bruchey and Gonzalez-Lima 2010; Poteet et al. 2012) and an energy enhancer (Shen et al. 2013; Jiang et al. 2015). This redox agent (Wen et al. 2011) was previously used as a treatment strategy for several diseases including malaria (Poteet et al. 2012; Dormoi et al. 2013), dementia and depression (Wainwright and Crossley 2002), among others. This drug has almost no side effects *in vivo* (Naylor et al. 1986; Riha et al. 2005) and thus, has been safely utilized in humans.

In 2012, TauRX Pharmaceuticals utilized Rember, an altered form of MB, in a phase II clinical drug trial (Wisnick et al. 2009) that consisted of dosing and efficacy studies. For 50 weeks, 321 patients with mild to moderate AD were involved in this trial. Individuals who were administered this drug demonstrated an 81% slower cognitive decline in comparison to participants who were not given Rember (Hosokawa et al. 2012). The company proposed that MB might be behaving as an anti-tau aggregation drug in AD. After a successful Phase II trial with Rember, a phase III drug trial was initiated.

The Food and Drug Administration (FDA) approved a Phase III clinical drug trial

on patients with mild to moderate AD. This was the first an anti-tau aggregation drug underwent Phase III clinical trials. When this 15-month trial ended in 2016, results indicated that Leuco-methylthioninium-bis (hydromethanesulfonate) (LMTM), a newly modified MB drug (Gauthier et al. 2016a), failed in this final phase (Gauthier et al. 2016b). LMTM had no significant effects in slowing cognitive or functional decline in the population of AD patients that were tested. Disease progression was nearly identical for both placebo and experimental groups. It is possible that a vital component of MB was absent in LMTM or other pathological markers of AD were not being targeted by LMTM. In addition, if the mechanism of LMTM was better understood, reformulations of this drug may aid in accentuating its potential positive effects.

To date, it is possible that MB may serve as a treatment for AD patients (**Figure 1**). While the scientific community remains uncertain of the direct mechanism of MB in relation to AD, several hypotheses have been proposed (**Figure 2**). One hypothesis suggests that MB may be acting as an anti-tau aggregation drug (Hosokawa et al. 2012) while a separate hypothesis shines a light on MB acting as an energy-enhancing compound (Poteet et al. 2012).

Anti-tau Aggregation Drug

Hosokawa and colleagues (2012) investigated the effects of oral administration with MB in combatting abnormal tau accumulation in an *in vivo* model. The JNPL3 (P301L) tau transgenic mice experience several hallmarks of AD, including neuronal loss and tangles, but no plaques. Mice between the ages of 8-11 months were provided with 2 μg/mL or 6 μg/mL MB daily in their water. Following the collection of immunoblotting

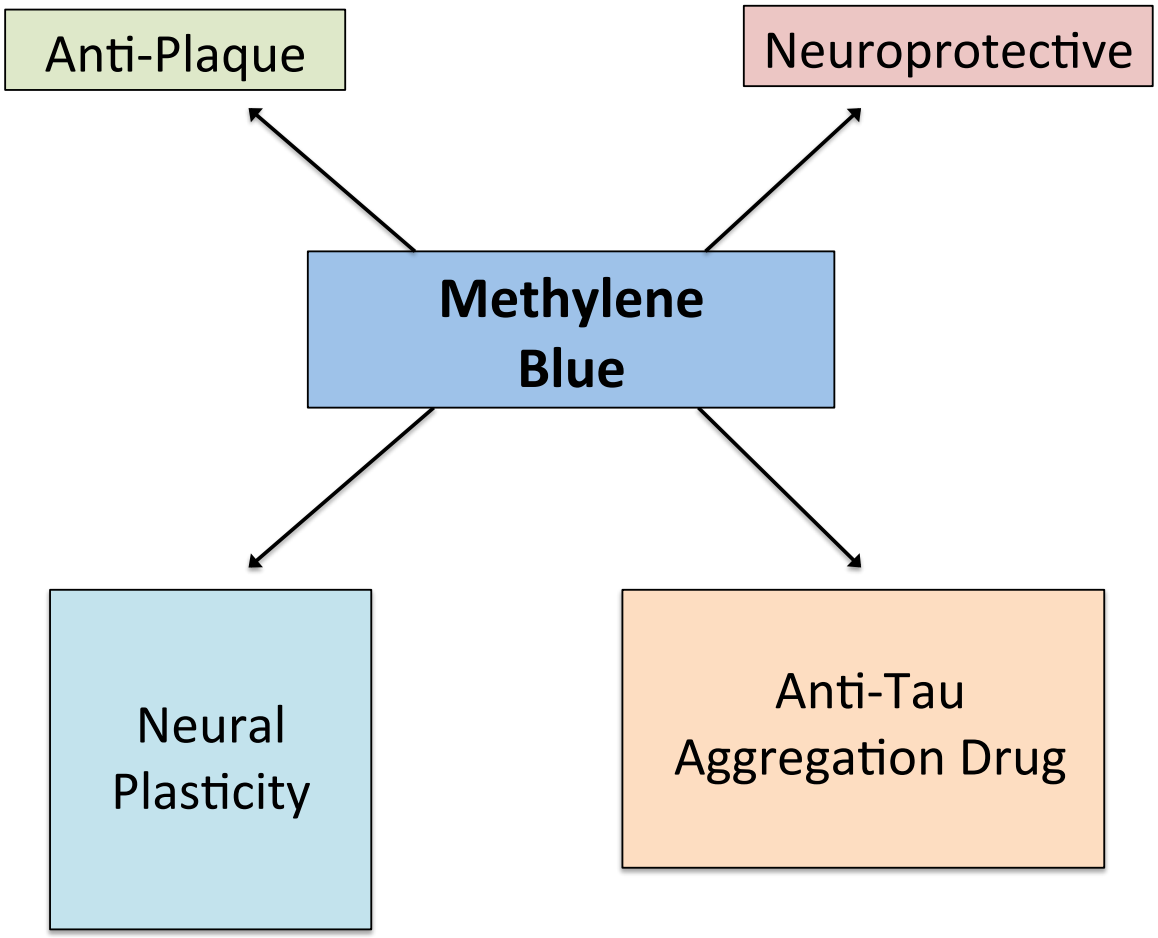

Figure 1: Possible MB Treatments for AD. MB may serve as treatment for AD in the follows ways: as an anti-tau aggregation drug, a neuroprotective drug, a neural plasticity enhancement drug or an anti-plaque drug (Guaman 2017).

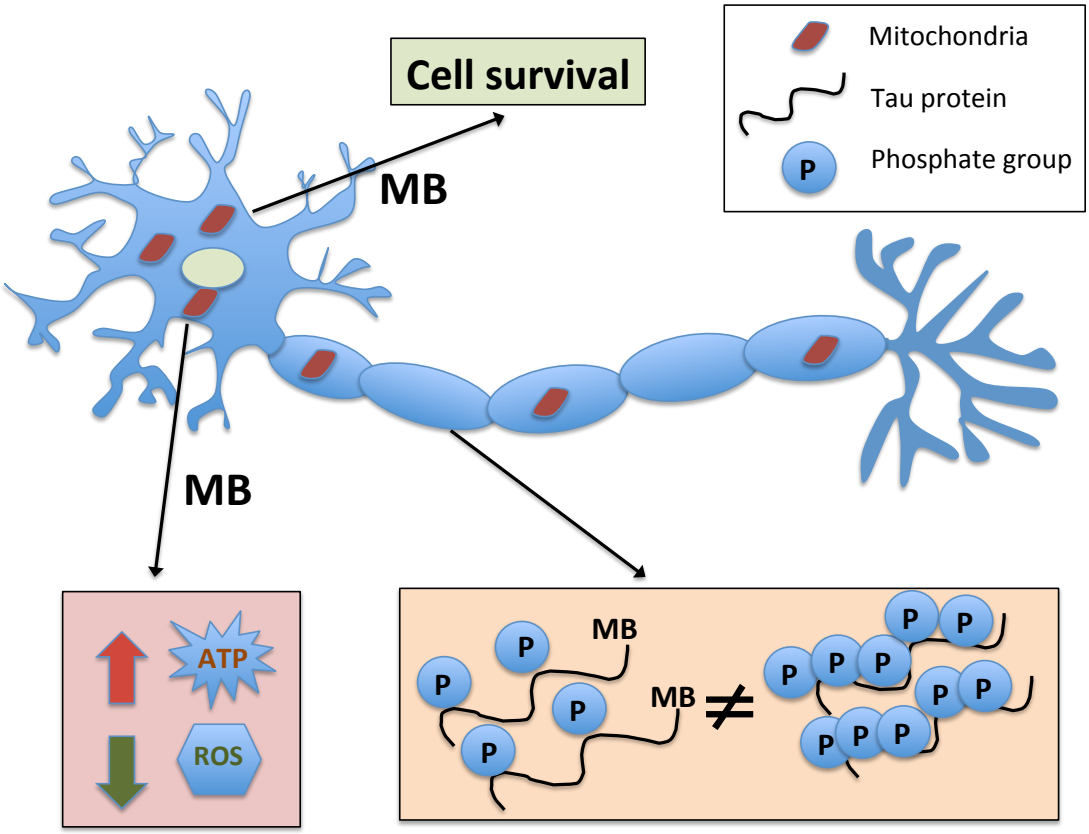

Figure 2: Potential Cellular Mechanisms of MB in relation to AD. MB may block aggregation of hyperphosphorylated tau, increase ATP levels, decrease ROS, and/or aid in cell survival through some unknown mechanism (Guaman 2017).

data, results indicated a reduction in phospho-tau with MB, in comparison to the control of water only (Hosokawa et al. 2012). This study was in agreement with Congdon and colleagues (2012), where a reduction in phospho-tau levels was also seen with MB treatment. Overall, this study suggested that MB targets the inhibition of tau aggregation in AD and thus, may be a potential treatment strategy for tauopathies, diseases stimulated by aggregations of tau, such as AD.

Congdon and colleagues (2012) examined the relationship between MB and tau levels *in vivo* and *in vitro*. They noted that previous studies had demonstrated a decrease in tau levels with MB. The scientists hypothesized that tau levels would be modulated by MB through autophagy, a process that delivers unnecessary substances to the lysosome. Tau transgenic pups (JNPL3; postnatal day 10) that overexpressed human tau were used in this study. When *ex vivo* slice cultures were introduced with MB (nM) or DMSO for five days, sarkosyl aggregated tau levels were significantly reduced to $87.2 \pm 0.94\%$ of the control. Sarkosyl is a detergent that aids in cellular lysing (Frankel et al. 1991). Also, they utilized male transgenic (JNPL3) mice to potentially find similar effects of MB on live mice, as they saw with the *ex vivo* slices. For a two-week duration period, mice were treated with no MB (water only) or with one of the three doses of MB (0.02 mg/kg, 2 mg/kg, 20 mg/kg). MB was administrated orally. Following an immunoblotting protocol, mice treated with 0.02 mg/kg and 2 mg/kg MB showed reduced total tau levels. Interestingly, phospho-tau levels also appeared to be reduced with MB stimulations (Congdon et al. 2012). Results also suggested induction of autophagy by MB, which facilitated the clearance of aggregated proteins, such as tau, and is important for cell

survival. These findings supported the potential role of MB as a therapeutic agent, through the promotion of autophagy and attenuation of tauopathy in neurodegenerative diseases, such as AD.

Mitochondrial Energy Enhancer

Some have suggested that MB behaves as an alternative electron carrier (Poteet et al. 2012). It has been documented that MB increases the rate of cytochrome C (mitochondrial complex IV) reduction (Poteet et al. 2012), which allows for an increase in ATP levels as MB stimulates electrons to enter the mitochondrial electron transport chain. This particular activity facilitates energy formation, cell survival and reduces ROS production. The reduction in ROS production may also be due to the ability of MB to bypass complex I-III activity (Shen et al. 2013; Jiang et al. 2015; Watts et al. 2016). In this manner, MB might be working to improve cognitive abilities by potentially delaying mitochondrial dysfunction as individuals' age.

In contrast to the findings by Congdon and colleagues (2012), a separate study did not see reductions in hippocampal phosphorylated tau levels of 6-month-old male 3xTg-AD and non-transgenic mice with MB; data were collected using an immunoblot following 16 weeks of MB treatment (Medina et al. 2011). The 3xTg-AD transgenic mice have been widely utilized in *in vivo* studies as they develop plaques, tangles and experience cognitive impairments and synaptic dysfunctions (Sterniczuk et al. 2010). For every 100 grams of food, the mice were administered 25 mg of MB. MB treatment improved memory and learning via the Morris Water Maze after a 16-week stimulation with MB, but not after eight weeks of treatment. In addition, they demonstrated a

significant reduction in soluble but not in insoluble $A\beta_{40}$ and $A\beta_{42}$ using an enzyme-linked immunosorbent assay. Because cytochrome C oxidase levels and ATP levels remained unchanged, the researchers suggested no changes in mitochondrial function with MB. However, these scientists recommended that the relationship between mitochondrial function and MB treatment should not be ignored. It is possible that other concentrations of MB may aid in mitochondrial function (Medina et al. 2011). They also emphasized the importance of future studies focusing on understanding the mechanism of action of MB in relation to AD.

A separate study suggested that MB can aid in complex learning. These researchers indicated that low doses of MB might help in memory retention by using spatial memory and object recognition activities (Wrubel et al. 2007). Male Long-Evans Hooded rats underwent several days of training, which consisted of the following phases: habituation, food search training (days 1-6) and discrimination training (days 7-12). Rewarded and non-rewarded trials were included in the hole-board maze food search task. MB (1 mg/kg) or saline was introduced to the subjects after the last trial. Results indicated that during days 10-12, rats treated with MB showed better overall mean performance. More specifically, MB rats were able to understand the difference between rewarded and non-rewarded trials. When looking at a potential mechanism, the authors of this study found an increase in cytochrome C oxidation in comparison to the control following three daily MB injections to Sprague-Dawley rats. This increase in cytochrome C oxidation provided evidence for MB's brain metabolic enhancer ability to increase ATP levels in neurons. Based on their findings, this behavioral study suggested that low

doses of MB might serve as memory enhancing when given during post-training periods (Wrubel et al. 2007).

In agreement with the experiments performed by Wrubel and colleagues (2007), an *in vitro* study investigated whether MB behaves as a cerebral metabolic enhancer (Lin et al. 2012). To explore this possibility, researchers isolated mitochondria from 3-month-old Sprague-Dawley rats. Using a mitochondrial complex activity assay, researchers determined that MB (10 μM) significantly increased complex I-III activity *in vitro* in comparison to the media control. In a separate experiment, immortalized mouse hippocampal cells (HT-22) were treated with MB and showed increased cellular oxygen (O_2) consumption. Even with the introduction of rotenone, a reagent that is highly toxic to cells (Satish and Kondapi 2015), cells with MB treatments had a higher cellular oxygen consumption rate than the control (Lin et al. 2012). This study demonstrated MB's property to serve as a cerebral metabolic enhancer. Overall, there are encouraging studies suggesting the possible involvement of MB to aid AD patients.

Present Study

This present study seeks to understand the overall effects of MB in an *in vitro* primary neuronal cell culture. We were first interested in characterizing the interaction of MB with normal and healthy neurons. Will MB disrupt any normal molecular process and potentially pose a threat to the dynamic functioning of neurons? The first hypothesis is that MB exposure at nanomolar concentrations would not be toxic to neurons. Since one line of research suggested that MB molecularly interacts with phosphorylated tau (Wischik et al. 2009), the second hypothesis is that MB exposure would not inhibit axon

growth, during a time when tau's interactions with MTs are crucial for axon elongation. In this manner, if MB only interacts with tau when tau is aggregating, then we predicted that MB would not jeopardize the stable interaction between tau/MTs and would not inhibit axon outgrowth. If MB interacts with mitochondria and produces ATP, we predicted that axonal elongation would be enhanced. To test these hypotheses, one-day-old cortical embryonic neurons were utilized. These neurons had relatively high levels of phosphorylated tau and dynamic microtubules (Fuster-Matanzo et al. 2012), making this a reasonable model system to examine whether MB would interfere with tau's normal function. Neurons were stimulated, and the longest tau-positive neurites were measured. To understand how axonal lengths were distributed following MB exposure, the data were plotted into histograms. We also sought out to determine whether there was a relationship between dose and axonal lengths for both exposure times.

In parallel experiments, ATP levels were measured at the same time points in which axon growth measurements were performed. We hypothesized that there would be an increase in ATP levels following stimulations with varying nanomolar concentrations of MB. These studies aimed to examine MB's effect on neurons at a developmental stage in which evidence could support one or both of these hypotheses.

A final aim of this thesis was to understand a potential role of MB as a protective agent in a pilot study. MB may attenuate the effects of glutamate, a compound that can induce neurotoxicity at elevated concentrations, or perhaps display no effects to counteract against losses of neurons. Given that AD is a neurodegenerative disease, any therapy that does not also slow down neuronal loss will, in the long term, not be

effective. We hypothesized that MB will aid with counteracting the neuronal loss following exposure to glutamate toxicity. To determine any potential attenuation of neurotoxicity by MB, 2-week-old neurons were stimulated with glutamate and varying concentrations of MB for 24 hours under conditions of high or low O_2. While we were mainly interested in understanding the trends seen in this pilot study, we suspected that generation of ROS would induce neurotoxicity under high O_2 conditions. Overall, these experiments may provide supporting evidence for distinctive beneficiary MB properties on young embryonic neurons.

MATERIALS AND METHODS

Primary Neuronal Cultures

In preparation for the Sprague-Dawley rat dissection, 24-well sterile cell culture plates (Greiner Bio-One, Cellstar), 96-well sterile cell culture plates (Greiner Bio-One, Cellstar), 96-well assay black plates with sterile clear bottoms (Corning Incorporated - Costar) or Corning™ 96-well clear bottom white polystyrene microplates with flat bottoms (Fisher Scientific) were utilized. Plates were coated with Poly-L-lycine Hydrobromide (Sigma) for 24 hours. Poly-l-lycine allowed adherent neurons to attach to the bottom of the well. Two washes with autoclaved water or Hank's Balanced Salt Solution (HBSS, Gibco by Life Technologies) were performed. Following these washes, plates were filled with plating media (NeurobasalQ Medium - Sigma, fetal bovine serum, Primocin (InvivoGen)) and placed in an incubator, at conditions of 37°C and 5% carbon dioxide (CO_2.) The pilot study was conducted utilizing two distinctive incubators at high or low O_2 levels. All other experiments, excluding this pilot study, were conducted under

conditions of high O_2 levels.

Following the sacrifice of a pregnant Sprague-Dawley mother rat using a CO_2 chamber, primary cortical neurons were cultured. Embryos at E17-19 were isolated and decapitated. After their brain was secluded, the skulls were punctured and peeled back to expose and isolate the two frontal cortexes. Cerebellum and midbrain regions were placed aside. Meninges, which are thin, red films of blood vessels, were removed from two frontal hemispheres. Cells were placed in pre-warmed trypsin, at conditions of 37°C for 5 minutes and twice in HBSS, at the same temperature, for 3 minutes. After dissociation of the neurons, they were counted with a hemacytometer. Cells were plated at $1*10^6$ cells/mL in plating media. After incubation of 1 hour, plating media was replaced with serum-free growth media (Fetal Bovine Serum, NeurobasalQ Medium - Sigma, Primocin (InvivoGen), B-27 Supplement (50X) (ThermoFisher Scientific)). Cells were fed every 2 – 3 days with serum-free growth media.

Stimulations of Neurons

In order to perform our experiments, we utilized cortical neurons that were one-day in culture (experiments #1-3) or two weeks in culture (experiment #4) (**Table 1**). One-day-old neurons represented a period in which the axon was dependent on the normal functioning of tau to stabilize the dynamic MT network (Fuster-Matanzo et al. 2012). Because phosphorylation of tau occurs in both healthy (Biernat et al. 2002) and disease states (Iqbal et al. 2010) and phosphorylation of tau is highest in fetal neurons (Brion et al. 1994), we used healthy embryonic neurons to test the effects of MB on them. In addition, axonal growth is the most prominent during these times, which is a property

that facilitated our axonal tracing experiments. Alternatively, we utilized two-week-old neurons for our pilot study. Cells at this stage resembled mature neurons that had been widely used in experiments (Dotti et al. 1988; Kim et al. 2004). The establishment of stabilized synapses and long dendritic processes also appear during this time (Kim et al. 2004).

Following an incubation period of days or weeks, neurons were treated with MB (0, 50, 100, 200 nM) alone, a combination of MB and 50 micromolar (μM) glutamate or 50 μM glutamate alone for a duration of 24 or 48 hours. All control samples consisted of growth media only. Each experiment called for specific stimulants and durations, which are described in **Table 1.** The nanomolar concentrations of MB were chosen based on previous literature (Rojas et al. 2012). Using a serial dilution technique, MB was diluted from its stock concentration (1 mM) to 10 μM, and then to 50, 100 or 200 nM. Glutamate (glutamic acid, growth media, NaOH) was a compound made in our lab. Usage of glutamate represented a cost-efficient method to induce neurotoxicity in our cultures (Choi et al. 1987, Lau et al. 2010). This compound was diluted from its original stock (50 mM) to a working concentration of 50 μM. The concentration of glutamate was chosen based on the previous testing in our lab, where we observed that 50 μM was the lowest concentration of glutamate that induced neurotoxicity (data not shown).

Table 1: Overview of Experiments

Experiment	*Question Asked*	*Age of Neurons Before Stimulations*	*Stimulants*	*Assay*	*Figure (F) or Table (T)*
#1	Is MB toxic to the cells?	1-day-old	0, 50, 100, 200 nM MB (24 or 48 hours)	MTS assay (# of survived neurons)	5 (F)
#2	Does MB influence axonal outgrowth?			Immunocytochemistry, Microscopy, Tracing of axons	6-10 (F), 2 (T)
#3	Can MB facilitate ATP formation?			ATP assay (# of ATP cells)	11 (F)
#4	Pilot Study: Can MB protect cells from toxicity?	2-week-old	0, 50, 100, 200 nM MB; with or without 50 µM Glutamate (24 hours)		12 (F)

Immunocytochemistry

Immunocytochemistry aided in visualizing our proteins of interest under a microscope. Following stimulations, neurons were fixed with 4% paraformaldehyde for 20 minutes at room temperature. Three subsequent washes with Phosphate Buffered Saline (PBS) (Sigma – Aldrich) were performed, each for three minutes. All wells were filled with PBS until further usage. A 30-minute incubation with the blocking reagent, 0.5% Bovine Serum Albumin (Sigma - Aldrich) dissolved in PBS was performed.

Following one brief wash with PBS, a 10-minute incubation with 0.5% Triton X-100, a detergent solution was also performed. After one additional wash with PBS, the primary antibody, Monoclonal Anti-Tau antibody produced in mouse 2mg/mL, clone Tau46, purified immunoglobulin, buffered aqueous solution (Sigma – Aldrich), was added and covered the complete bottom of all wells. The cells were placed overnight on a shaker, for approximately 24 hours, at room temperature. For long-term storage, parafilm was utilized to cover the sides of the plate. After the completion of a 24-hour incubation, the primary antibody was removed, and three further washes with PBS were performed. The secondary antibody, Anti-mouse IgG (whole molecule) F (ab'), fragment – Cy3 antibody produced in sheep (Sigma – Life Science) or Anti-Mouse IgG (Whole molecule) - FITC antibody produced in goat (Sigma – Life Science) was added to all wells for one hour at room temperature. After the removal of the secondary antibody, three additional washes with PBS were performed, and the neurons were placed in PBS and stored in the refrigerator.

Immunofluorescence Microscopy

Cells were imaged using the Zeiss LSM 510 Inverted Fluorescent Microscope (Hitech Instruments, Inc.). The program, AxioVision SE64 Rel. 4.9.1 (Carl Zeiss Microimaging, Inc.) was used to take these images. Typically, 25 – 45 images were taken for each condition in each experiment. Depending on which secondary antibody was utilized, each plate received exposure of Cy3 (Cyanine 3) or FITC (Fluorescein isothiocyanate) fluorescence. Prolonged exposure to fluorescence dampened the image's

brightness. Therefore, all plates received minimal fluorescence exposure prior to being imaged.

Quantification of Immunohistochemistry Images

The images were visualized and quantified using the NIS-Elements Advanced Research Microscope Imaging Software (Nikon). Following staining for tau, the wells were sequentially imaged across the slide. An example of distinctive sizes of axons is seen in **Figure 3.** The longest tau-positive neurite in every neuron, which we identified as the axon, was traced from its base to its end (**Figure 4**). Since the axonal lengths were collected in pixels, all values were converted to microns using the following equation: X (Pixels) * 0.44 = Y (microns). The width of every pixel was equivalent to 0.44 microns.

MTS Cell Viability Assay

In order to collect data through cell viability assays, a solution consisting of 2 mL of the tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium, inner salt (MTS) and 10 mL of growth media was prepared. The media in the wells was replaced with 100 µL of the pre-made solution; the 96-well plate was incubated at conditions of 37°C and 5% CO_2 for 2 hours. Data were collected following the insertion of the plate into the SpectraMax M3 M-Series Multi-Mode Microplate Plate Reader (Molecular Devices). Settings were set to read absorbance, at a wavelength of 490 nm, which correlated with the number of living cells. The program, SoftMax Pro Microplate Data Acquisition and Analysis Software Version 6.2 (Molecular Devices), was utilized to acquire the raw data. Excel for Mac 2011 Version 14.4.4 (Microsoft®) was used to graph the data.

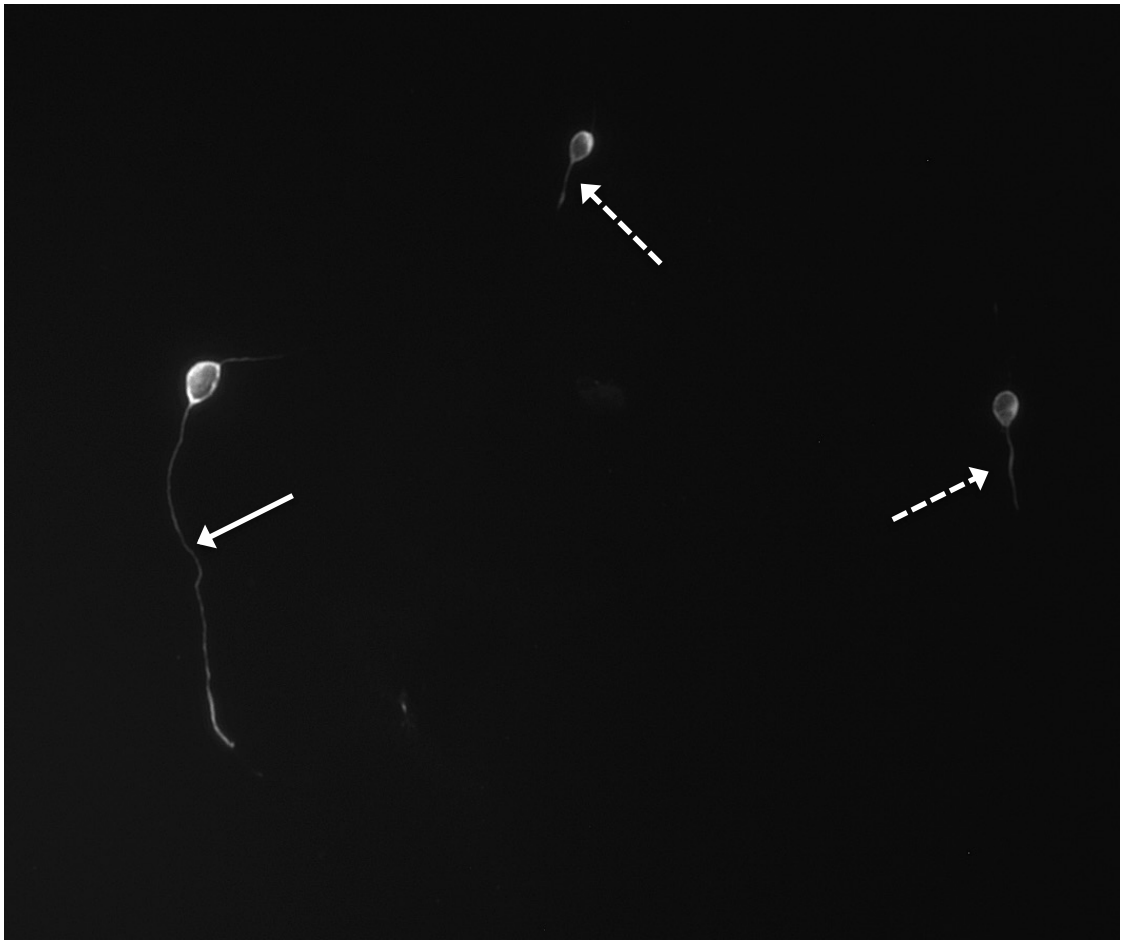

Figure 3: Sample Image of Distinctive Axonal Lengths. These are a few examples of the different sized axons in culture, following tau staining with immunocytochemistry.

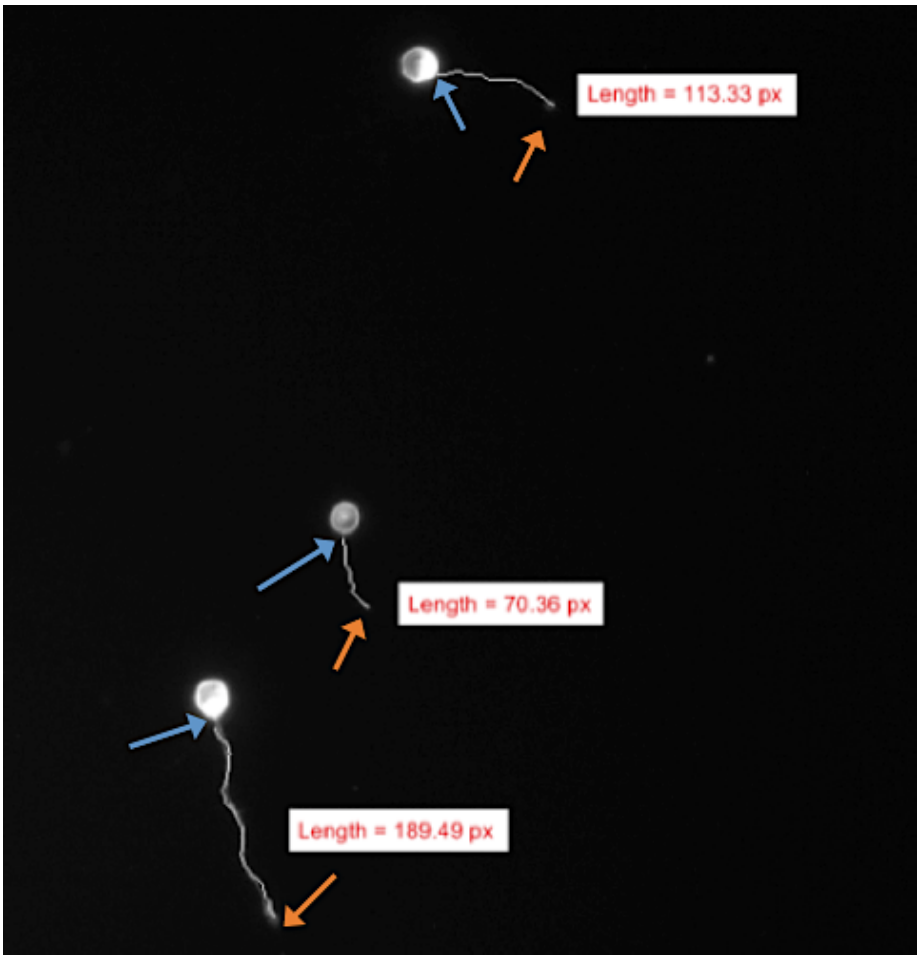

Figure 4: Sample Image Illustrating Axonal Tracing. Axons were traced from base (blue arrows) to end (orange arrows). All lengths were converted from pixels to microns.

CellTiter-Glo® Luminescent Cell Viability Assay

Ninety-six well plates with clear bottoms were placed at room temperature for 30 minutes. One hundred microliters of the CellTiter-Glo® 2.0 Reagent were added to the already present 100 µL of growth media with cells. Cells were placed on a plate shaker for two minutes, enabling the CellTiter-Glo® 2.0 Reagent to lyse the cells. Plates were left out at room temperature for 10 minutes to allow for stabilization of the luminescent signal. Plates were read on the SpectraMax M3 M-Series Multi-Mode Microplate Plate Reader (Molecular Devices) with settings set to read luminescence. Metabolically active neurons in culture correlated with the quantification value of ATP. Data were graphed using Excel for Mac 2011 Version 14.4.4 (Microsoft®).

Exclusion Criteria

For experiments that used MTS assays, control wells were excluded if they had an absorbance value of 0.2 or less. Anything below an absorbance of 0.2 represented insufficient neuronal survival. For all axonal length tracing experiments, if axons had no visible staining of tau under the microscope, they were not considered as part of our data. For the pilot study, negative controls that were greater than 80% of the growth media only control were not included. These negative controls did not demonstrate a high enough toxic effect from glutamate.

Statistics

Statistical analysis was performed using a one-way analysis of variance (ANOVA). If the p-value of this one-way ANOVA was less than 0.05, we determined that there was a main effect of concentration between the independent groups. If the main

effect was detected in the data, a post hoc Tukey honest significant difference (HSD) test was performed. For these post hoc tests, statistical significance was identified if $p < 0.01$. Two chi-squared tests of independence were conducted to investigate the relationship between doses and axonal lengths after 24 and 48 hours of MB exposure. Standard deviations were displayed as error bars in all applicable graphs.

RESULTS

MB and Axonal Outgrowth

Before testing potential effects on axon elongation, the general viability of neurons under various concentrations of MB was tested (**Figure 5**). One-day-old embryonic rat cortical cultures were exposed to 50 – 200 nM MB for a period of 24 or 48 hours. Cell survival numbers were determined using an MTS assay. Percentage cell viability averages for all treatment groups were normalized to the control value, and the control was set at a standard 100%. After a one-way ANOVA test was run ($p = 5.2 * 10^{-2}$), there was no main effect of MB concentration for both 24-hour and 48-hour exposure periods.

To examine the effects of nanomolar concentrations of MB on tau, neurons were stimulated with MB for a period of 24 hours. Following stimulations, cells were stained for tau using an immunocytochemistry protocol, and fluorescent images were collected. The longest tau-positive neurites were traced and analyzed. We observed many different cell types with great variance in axonal lengths (**Figure 6**).

In order to test the hypothesis that MB exposure will not interfere with axonal

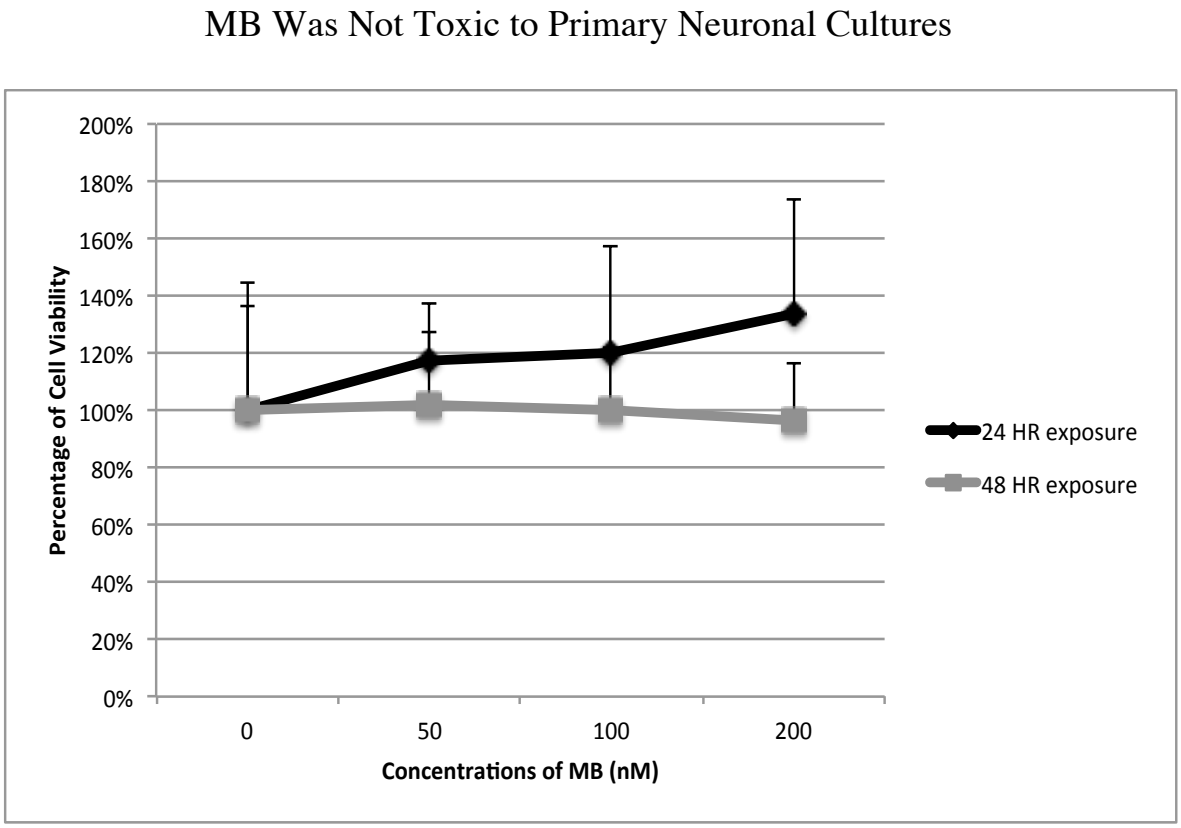

Figure 5: **Percent cell viability on cortical neurons with a 24-hour or a 48-hour exposure to MB.** Twenty-four hour exposure percentages were as follows: 100 ± 37% (0 nM MB), 117 ± 20% (50 nM MB), 120 ± 38% (100 nM MB), 134 ± 40% (200 nM MB). Forty-eight hour exposure percentages were as follows: 100 ± 44% (0 nM MB), 102 ± 25% (50 nM MB), 100 ± 22% (100 nM MB), 96 ± 21% (200 nM MB). There was no main effect of MB concentration in these data ($p > 0.05$). Each condition consisted of an n of 12 wells.

Sample Fluorescent Images of Tau

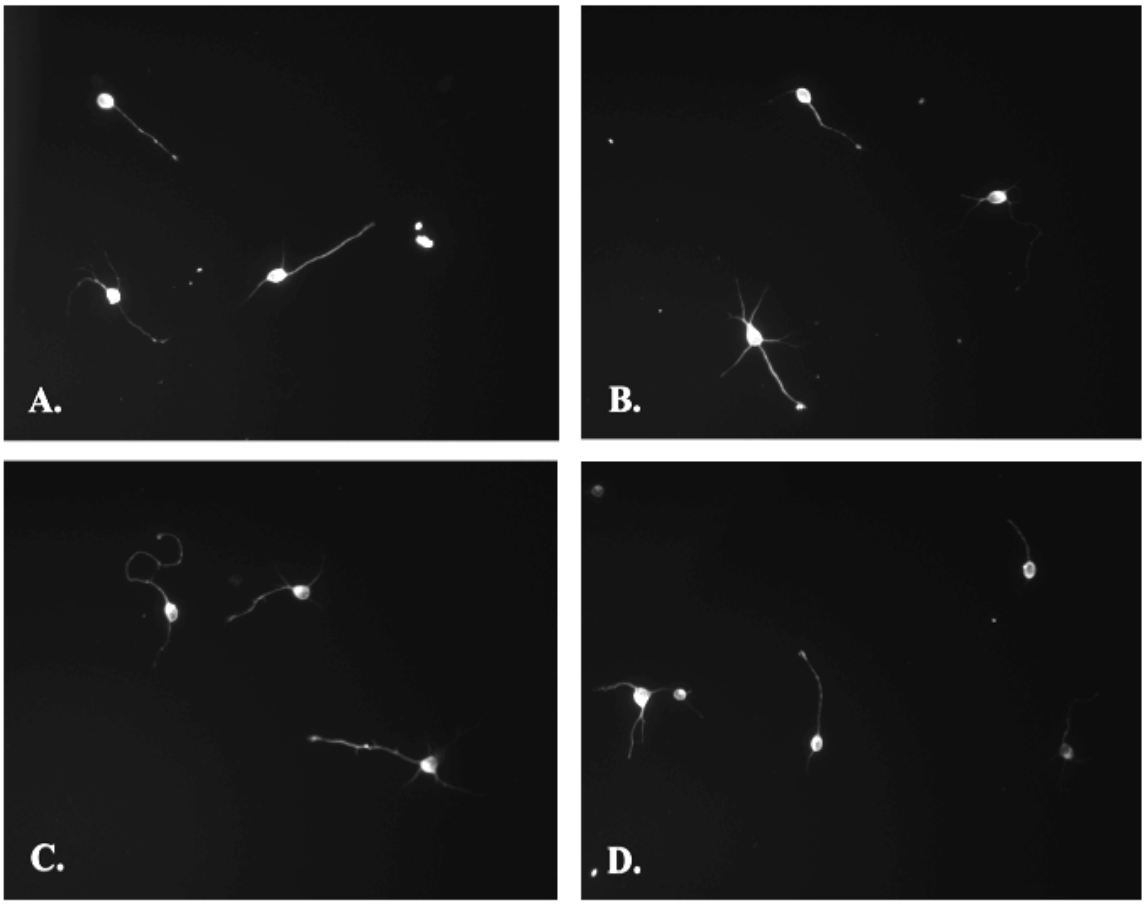

Figure 6: Representative fluorescent images depicting tau staining with a 24-hour MB stimulation period. A. Growth media only. B. 50 nM MB sample. C. 100 nM MB sample. D. 200 nM MB sample.

methodologies facilitated in the staining of tau and the visualization of this protein along the axon. One-day-old embryonic rat cortical cultures received 50 – 200 nM MB for 24 hours (**Figure 7**). After a one-way ANOVA was performed, a main effect of concentration was detected ($p = 2.55 * 10^{-6}$). Based on the Tukey HSD tests, the only statistical significance was seen with the 100 nM MB sample. The mean axonal lengths in this sample were significantly higher than the control ($p = 0.001$), the 50 nM MB sample ($p = 0.001$) and the 200 nM MB sample ($p = 0.001$). Axons in the 100 nM MB sample were approximately 26% longer than the control after a 1-day MB exposure.

Given the huge variation in the axonal lengths seen in the previous experiment (Figure 7), we created a histogram to explore the effect of MB on the frequencies of distinctive axonal lengths. Bins of 25 microns were used to categorize the different sized axons. There was one peak at the bin of 25 – 50 microns, which marked a transition in the distribution of the frequencies. Interestingly, it seemed as though the distribution curve was lower on both ends of the peak. The frequency distribution of the 50 nM MB and 200 nM MB appeared to be relatively similar to that of the control (**Figure 8A, B, D**). The one condition that did not have this appearance was 100 nM MB (**Figure 8C**). The 100 nM MB sample had the highest number of longer axons and the lowest number of smaller axons in comparison to the rest of the treatment groups and the control group.

We next tested to see whether a longer MB exposure would not inhibit axon outgrowth, as was previously seen with the 24-hour stimulation period of MB. In this experiment, 1-day-old neurons received 50 – 200 nM MB for 48 hours instead of the 24

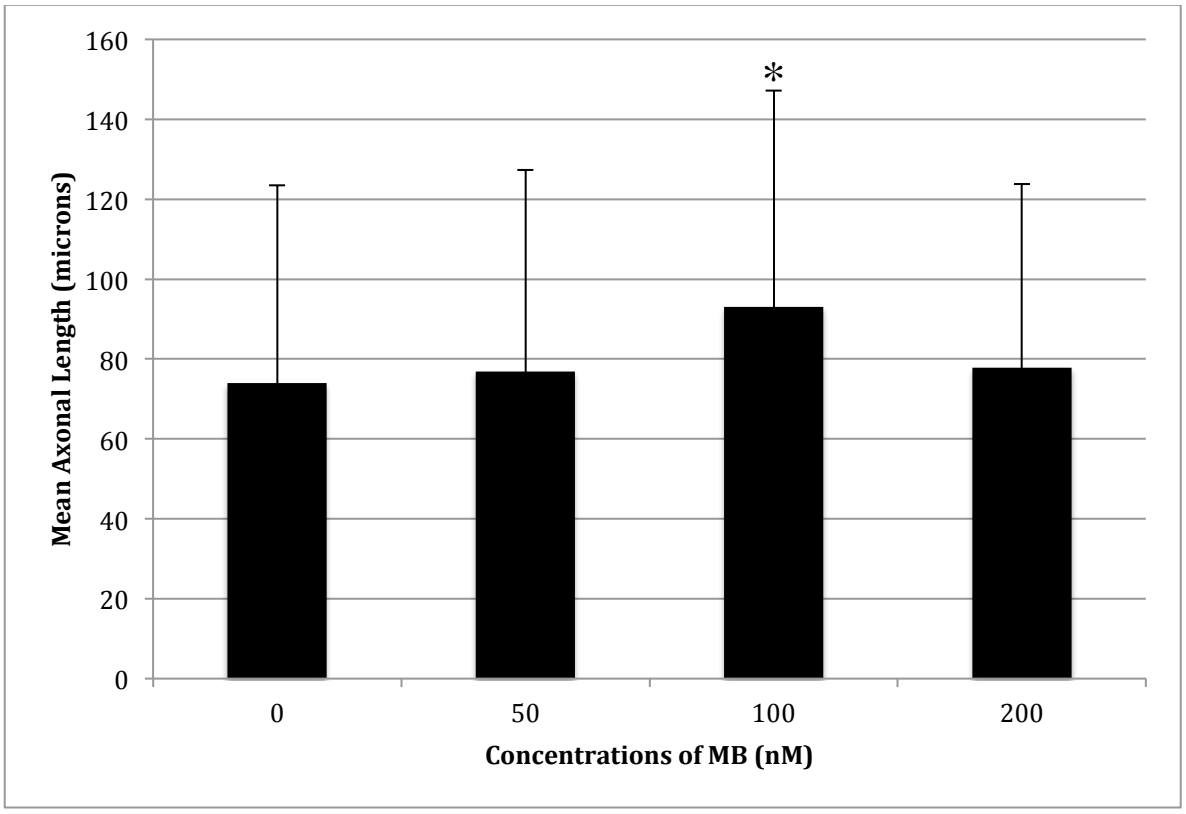

Figure 7: Mean axonal length measurements for a 24-hour exposure period with MB. Mean axonal lengths (in microns) were as follows: 73.99 ± 49.44 (0 nM MB), 76.92 ± 50.40 (50 nM MB), 93.12 ± 54.03 (100 nM MB) and 77.80 ± 45.99 (200 nM MB). There was a main effect of MB concentration in the data ($p < 0.05$). The asterisk (*) over the 100 nM MB indicates that this sample is significantly higher than 50 nM MB and 200 nM MB, as well as the control. (N = 1190 axons).

Exposure to MB Enhanced Axonal Lengths

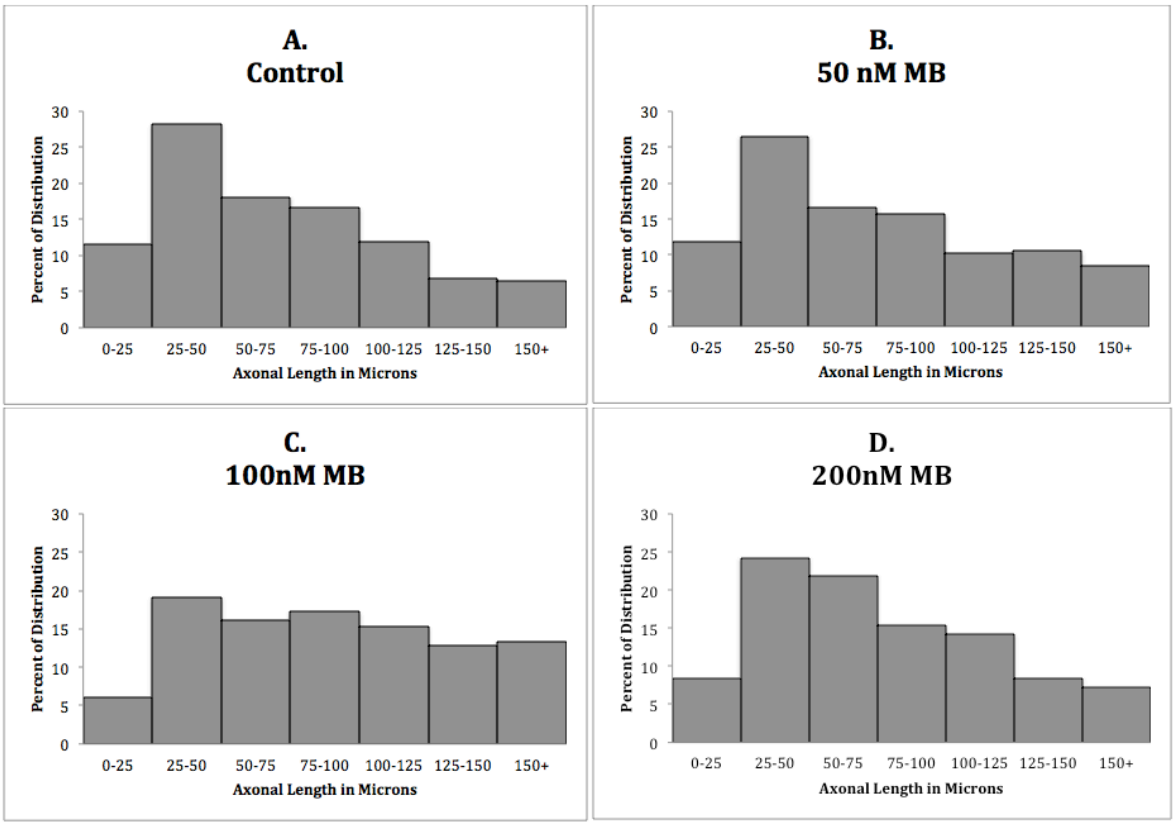

Figure 8: Assessment of axonal lengths by percent of distribution for a 24-hour exposure period with MB. Axonal lengths, in microns, were grouped into populations. The n for each condition was as follows: 276 (control), 294 (50 nM), 360 (100 nM) and 260 (200 nM). (N=1190 axons).

hours previously tested. A one-way ANOVA test revealed a main effect of concentration in this sample ($p = 0.01$). A Tukey HSD test indicated that none of the treatment groups were significantly different from the control (**Figure 9**). However, 100 nM MB was statistically higher than 50 nM MB ($p = 0.018$), and 200 nM MB ($p = 0.047$) conditions, but not in comparison to the control.

In order to understand the distribution of raw values from an experiment that stimulated the neuronal cultures for 48 hours (Figure 9), axonal length values were plotted into histograms (**Figure 10**). Bins of 25 were utilized to categorize the different sized axons. Interestingly, we observed two peaks with the control, which marked transitions in the frequency distribution data; the first peak was seen in the 50 – 75 microns bin while the second peak was seen in the 150+ microns bin. In a similar manner to the previous histogram (Figure 8), the 100 nM MB sample had the greatest number of longer axons and lowest number of smaller axons, in comparison to the rest of the groups, including the control (**Figure 10C**). However, this effect was not as pronounced as was previously seen with the 24-hour duration period (Figure 8C). Both 50 nM and 200 nM samples displayed a slight decrease in longer axons and an increase in shorter axons in comparison to the control **(Figure 10A, B, D).**

Data from the axonal lengths for 24-hour and 48-hour exposures (Figures 7, 9) were utilized to examine the relationship between dosages and axonal lengths. Axon lengths were divided into short (<75 microns) and long (>75 microns). There was a similar effect for both exposure times (**Table 2**). After a 24-hour exposure to MB, there was a significant difference in the percentage of long axons detected by dose, chi-square

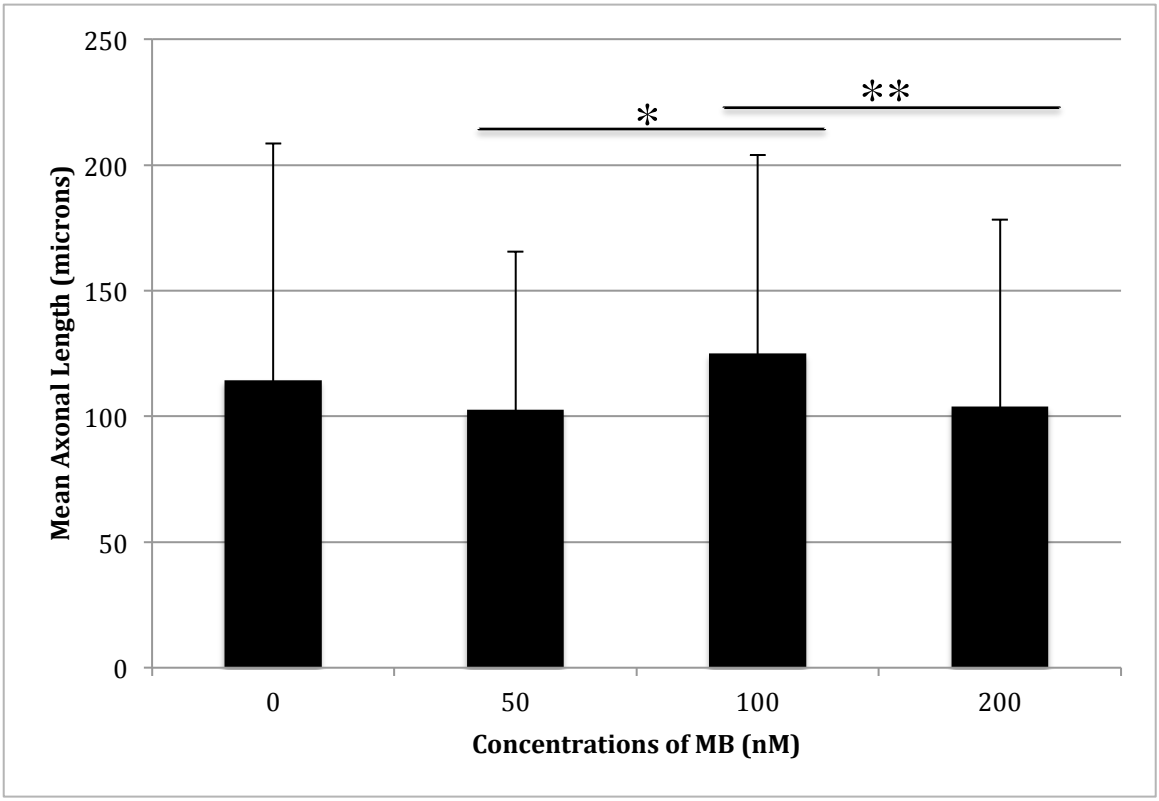

Figure 9: Mean axonal length measurements for a 48-hour exposure period with MB. Mean axonal lengths (in microns) were as follows: 114.42 ± 94.13 (0 nM MB), 102.51 ± 63.11 (50 nM MB), 124.94 ± 79.05 (100 nM MB) and 103.96 ± 74.25 (200 nM MB). There was a main effect of concentration ($p < 0.05$). The solid line with one asterisk (*) represented that 100 nM MB was significantly higher than 50 nM MB while the solid line with two asterisks (**) indicated that 100 nM MB was significantly higher than 200 nM MB. (N = 798 axons).

Axonal Lengths Were Enhanced With MB Stimulations

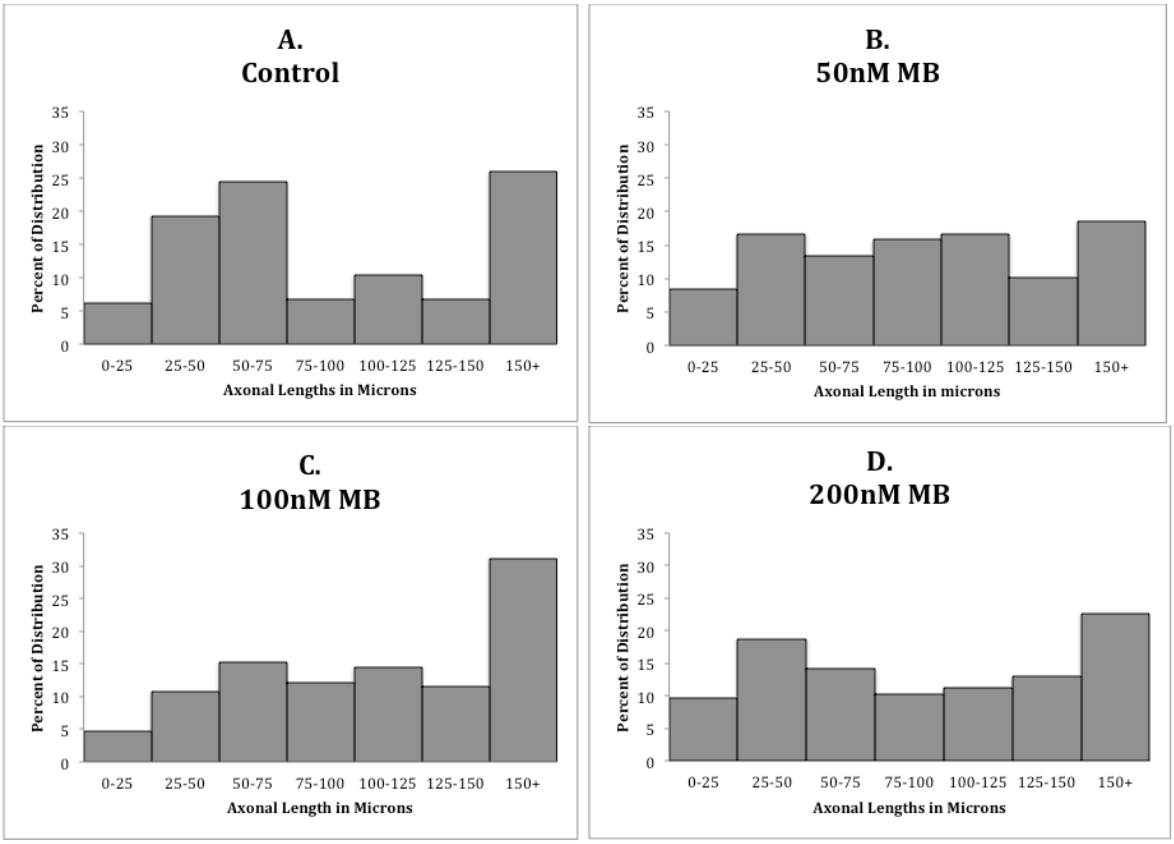

Figure 10: Assessment of axonal lengths by percent of distribution for a 48-hour exposure period with MB. Axonal lengths, in microns, were grouped into populations. The n for each condition was as follows: 192 (control), 215 (50 nM), 215 (100 nM) and 176 (200 nM). (N = 798 axons).

(N = 1190, *df* = 3) = 21.90, p < 0.0005. Similarly, with the 48-hour stimulation time with MB, there was a significant difference in the percentage of long axons detected by dose, chi-square (N = 798, *df* = 3) = 16.41, p = 0.001. In both the 24-hour and 48-hour exposures, a higher dose yielded an increased percentage of long axons until the 100 nM MB sample. Neurons stimulated with 200 nM for 24 hours had a decline in the percentage of long axons when compared to 100 nM yet the same percentage of long axons when compared to 50 nM. With a 48-hour stimulation period with MB, 200 nM had a lower percentage of long axons in comparison to both the 100 nM MB and 50 nM MB. The percentages of long axons for all conditions remained above the control value.

Table 2 Assessment of Percentage of Long Axons by MB Dose

	Dose (nM)				
		0	50	100	200
Exposure	24 hours	42	45	57	45
	48 hours	50	61	69	57

*Adapted from Dr. Sarah Abramowitz (Drew University).

MB and Generation of ATP

Because of the increase seen in longer axons with MB treatment, ATP levels were measured in parallel experiments. As before, embryonic rat cortical cultures were stimulated with 50 – 200 nM MB for 24 hours or 48 hours (**Figure 11**). All values were normalized to the control, which was set at a standard value of 100%. No statistical significant differences were seen with the 24-hour exposure to MB following a one-way ANOVA test (p = 0.196). When we ran a one-way ANOVA test on the experiment with the 48-hour stimulation period, it was evident that there was a main effect of MB

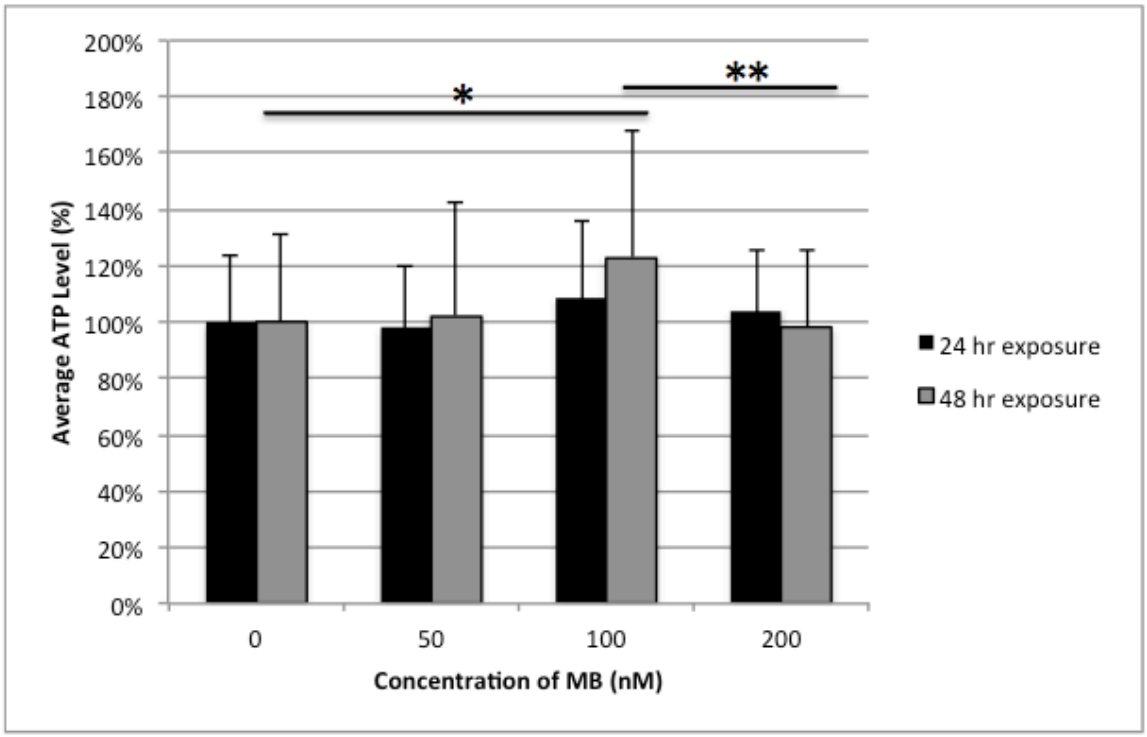

Figure 11: Assessment of ATP levels with a 24-hour or a 48-hour stimulation using MB. The 24-hour exposure ATP level values were as follows: 100 ± 24% (0 nM MB), 98 ± 22% (50 nM MB), 108 ± 27% (100 nM MB) and 104 ± 21% (200 nM MB). There was a main effect of concentration for 24 hours ($P > 0.05$). (N = 180 wells). The 48-hour exposure ATP level values were as follows: 100 ± 31% (0 nM MB), 102 ± 40% (50 nM MB), 123 ± 45% (100 nM MB) and 98 ± 28% (200 nM MB). There was a main effect of concentration for 48 hours ($P < 0.05$). The longest solid line with one asterisk (*) indicated that the average ATP levels for the 100 nM MB sample was significantly higher than the control while the shorter solid line with two asterisks (**) demonstrated that 100 nM MB was significantly higher than the 200 nM MB for the 48-hour MB exposure. (N = 180 wells).

concentration ($p = 0.008$). Tukey HSD tests revealed that 100 nM MB was significantly higher than the control ($p = 0.011$) and significantly higher than the 200 nM MB sample ($p = 0.016$).

Pilot Study

To examine any potential neuroprotective effects of MB, 2-week-old neurons were exposed to a 24-hour or 48-hour stimulation periods with MB and glutamate (50 μM) or MB alone. We assessed whether MB may attenuate the effects of glutamate neurotoxicity. Because we were interested in understanding what effect the changes in O_2 levels had on the treated cells, we tested these neurons under low O_2 and high O_2 conditions. Percentages for all treatment groups were normalized to the control value; control was set at a standard 100%. Our findings demonstrated little to no attenuation to glutamate neurotoxicity (**Figure 12**). The only sample in these two experiments that slightly overcame the neurotoxicity induced by glutamate was 200 nM MB at low O_2 conditions. This dose had a higher ATP level in comparison to our negative control consisting of 50 μM glutamate alone, although its value was less than the control. No statistical analysis was conducted for these experiments.

Little to No Attenuation of Glutamate Neurotoxicity with MB

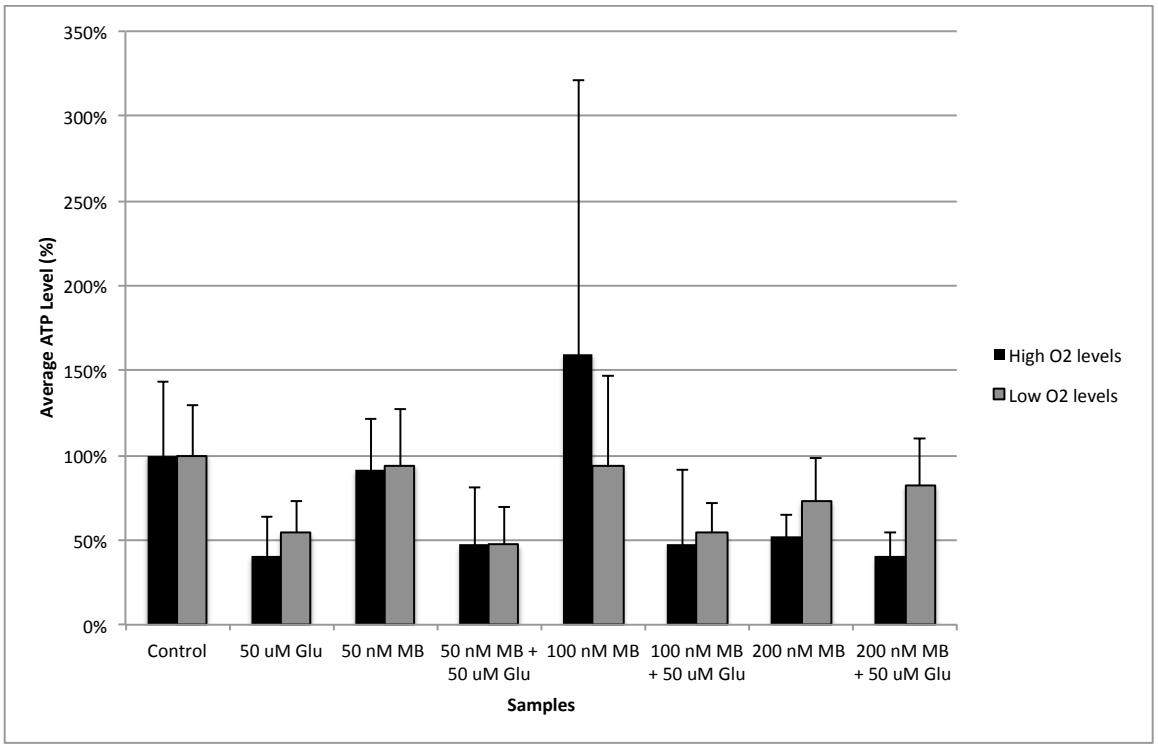

Figure 12: Pilot Study assessing neuroprotective effects of MB by measuring ATP levels on 2-week-old neurons with a 24-hour stimulation using MB and glutamate. Neuronal cultures were exposed to high or low O_2 conditions. There was an n = 6 for each condition.

DISCUSSION

Overview of Research

The main focus of these experiments was to investigate the direct effects of MB on early neuronal primary cultures. MB has been suggested to have antioxidant properties (Bruchey and Gonzalez-Lima 2010), attenuation effects of ROS (Poteet et al. 2012), and a possible role as neuroprotective (Lin et al. 2012) or therapeutic (Paban et al. 2014). In a Phase II clinical drug trial, individuals who were given Rember, an altered form of MB, demonstrated a lesser cognitive decline as opposed to those who were not given this drug (Wisnick et al. 2009). However, Phase III drug trials with LMTM were not successful (Gauthier et al. 2016b). While some studies have previously proposed MB as an anti-tau aggregation agent (Hosokawa et al. 2012), an energy enhancing drug (Shen et al. 2013; Jiang et al. 2015) or even a neuroprotectant agent (Lin et al. 2012), there have been no data on how MB has been directly influencing neurons in these drug trials. By conducting more basic research into MB's effects on neurons in a cell culture model, our research aims to better understand both the potential strengths and weaknesses of MB as a treatment for AD.

Model System Analysis

Early embryonic rat neurons were utilized as the model system for this study. The advantage of this model system is that these cultures are relatively hardy compared to neurons harvested from adult animals (Brewer 1997), and within a few days in culture, they go from small round cells to neurons that grow fairly long axons. Inhibition of tau in this crucial period prevents axonal growth (Caceres and Kosik 1990). Because highly

phosphorylated tau is present in growing axons (Brion et al. 1994) and in the diseased state of AD (Iqbal et al. 2010), usage of younger neurons facilitated in the testing of anti-tau aggregation properties of MB. Because growing axons require high levels of cellular respiration (Vaarmann et al. 2016), this model system also enabled the testing of possible mitochondrial properties of MB. A potential weakness of this model system is that it is a relatively poor representation of the specific population targeted by AD (Eide and McMurray 2005). In other words, AD targets individuals that are normally 65 years and older (Herbert et al. 2013) and our neurons may not be representative of the entire aging process that patients undergo all these years. In the future, developing a method to effectively culture primary neurons from aged rats may help better represent this aging process.

Effects of MB

Neurotoxicity Analysis

The first major aim of this thesis was to investigate whether the introduction of nanomolar concentrations of MB would be toxic to our primary neurons. We were interested in understanding what concentrations of MB would not be harmful to our cells, in order to utilize them in subsequent experiments. We expected to see no neurotoxicity with our nanomolar concentrations of MB because MB has been previously approved by the FDA (Wainwright and Crossley 2002). To assess the effects of MB on healthy neurons, cell viability assays were performed. Because no significant differences were detected in all cell survival percentages for 24-hour and 48-hour exposure lengths with MB (**Figure 5, 6**), doses up to 200 nM MB did not display toxic effects on our neurons.

In addition, previous studies have utilized concentrations at a similar nanomolar range (Rojas et al. 2012). Based on these results, we utilized these nanomolar concentrations to continue elucidating the properties of MB in the following experiments.

Axonal Outgrowth

While there are several hypotheses regarding the mechanism of action of MB in relation to AD, one potential mechanism of action of MB is as an anti-tau aggregator (Hosokawa et al. 2012). If MB were to serve as an anti-tau aggregation drug, our second aim identified whether MB could behave in a manner that would not disrupt the normal functioning of tau. Tau has several roles including MT stabilization and promotion of axon outgrowth, as well as facilitation in synaptic plasticity (Wang and Liu 2008). As memories form, evidence suggests that modifications of synapses, including the growth of new axon terminals, are vital (Kandel et al. 2012). It is possible that if MB binds to phosphorylated tau that is not bound to MTs, MB might act as an inhibitory agent during axonal growth. Thus, making sure that no disruptions occur to the normal function of tau is especially important because the selective dynamic properties of axons, as well as MT stabilization, contribute to synaptic plasticity in the brain (Wang and Liu 2008). If MB were to be given as treatment to AD patients, it is critical to make sure that MB would not interfere with this important process. We hypothesized that MB would interact with tau in such a way that would not harm the normal elongation of axons and thus, would not jeopardize the normal functioning of tau.

To begin the evaluation of nanomolar concentrations of MB on our primary neurons, we first observed how a 24-hour exposure with MB impacts the growth of the

axon by using immunocytochemistry and looking at tau staining of fluorescent images (**Figure 6**). All conditions showed great variability in the length of the axons. These results were expected because the primary neurons were at different stages of development when we introduced MB. In addition, this cell culture had a mixture of different types of cortical neurons with distinctive rates of maturation. Therefore, these noticeable differences were normal. Thus, in this experiment, there was a great variation in the manner in which axons developed normally in culture.

Following these observations, we collected axonal length averages to comprehend what MB was doing to our primary neurons. If axonal length means were lower than the control condition, this may have potentially signaled to a negative interaction between MB and tau, suggesting a potential detrimental side effect of MB. Following stimulations with various nanomolar concentrations of MB for 24 hours or 48 hours, the longest tau-positive neurite was measured for each neuron. One study suggested that tau is not phosphorylated until being 4 to 5 days in culture (Brion et al. 1994), which may have played a role in the results of our experiments. Like our observations from the fluorescent images (**Figure 6**), all conditions displayed great variability in the length of the axons, as is shown by the large standard error bars (**Figure 7 and 9**). Because we had a large sample size (1,190 axons – 24 hours; 798 axons – 48 hours), we attributed these large standard error bars to a great variation in the development of axons in culture. For the 24-hour exposure to MB, all means appeared to be relatively consistent with the control, with the exception of 100 nM MB, which had a higher value than the control (**Figure 7**). In essence, assessment of these average axonal lengths indicated that MB did not disrupt

tau's function to drive the formation of the axon; we also made an intriguing finding that 100 nM MB may have positive enhancement effects on neurons, following a 24-hour exposure time. However, this enhancement property of 100 nM MB was not observed with our 48-hour MB stimulation experiment (**Figure 9**), because 100 nM MB was not significantly higher than the control, although it was significantly higher than 50 nM MB and 200 nM MB. Thus, this previous effect (**Figure 7**) did not hold with a 48-hour exposure time, suggesting that the enhancement effects of MB on cells may only be held for a limited time. In order to test whether similar growth enhancements can also be seen in mature neurons, long-range experiments can be utilized to address the potential long-term effects of MB on these normally functioning neurons. While our current protocols introduced MB for a maximum of 48 hours during the initial growth development of axons, in AD, axons are more mature. Certainly, further experimentation is required to address the effects on neurons older than two or three days in culture. These findings may shed light on whether MB may have positive or negative effects *in vitro* for longer exposures to MB.

While we previously hypothesized that there would be no inhibition of axonal outgrowth by MB, we did not expect an enhancement of axonal lengths from the 100 nM MB sample at 24 hours (**Figure 7**). Based on this finding, we were intrigued to find out that this particular dose promoted axon elongation in these cultures. Given that AD is a disease in which there is a steady loss of synapses during the course of this illness (Scheff et al. 2006), any treatment that could promote the growth of axons may greatly benefit patients by stimulating their cognitive function. We examined the distribution of axons

(N = 1,988) that had grown under the various concentrations of MB (**Figure 8, 10**), and the data were highly suggestive that at a concentration of 100 nM, MB is capable of enhancing the growth a subset of axons. This was most pronounced during the 24-hour incubation period (**Figure 8**) as opposed to the 48-hour exposure period to MB (**Figure 10**), where we also saw an increase in longer axons and a decrease in shorter axons, in comparison to the control. This indicated that these particular dosages of MB might have participated in significantly enhancing the length of at least a subset of the axons growing in culture. Also, MB promoted the generation of separate distributions of axonal lengths, which were represented by peaks, a feature seen in the histograms (**Figure 8, 10**). Interestingly, one peak was evident with the 24-hour stimulation while two peaks were seen with the 48-hour stimulation period, which categorized the frequencies of axonal lengths into distinctive groups. The peak at 25 - 50 microns with the 24-hour stimulation was seen at a lower bin than the peak at 50 - 75 for the 48-hour stimulation period of MB; one explanation of these data is that neurons had not grown as long as with the 48-hour stimulation period. In both conditions, 100 nM had a decrease in shorter axons and an increase in longer axons in comparison to the control; this effect was more pronounced with the 24-hour stimulation period. More specifically, the percentage of neurons with axons less than 50 microns and greater than 150 microns for the control were 39.85% and 6.52%, respectively. On the other hand, the percentage of neurons with axons less than 50 microns and greater than 150 microns for the 100 nM MB sample were 25.27% and 13.33%, respectively. This supported the idea that 100 nM MB was enhancing the growth of the axon by doubling the number of axons that were greater than 150 microns. These

findings further contributed to our understanding that MB was positively affecting axonal outgrowth of the neuron. Because we previously saw a separation of axonal length frequencies to the right and left of 75 microns with a 48-hour stimulation, we utilized this mark to categorize axonal lengths and determine if there was a relationship between dose and axonal lengths using statistical analysis.

When we investigated the relationship between MB dose and axonal lengths, it became evident that MB had significantly increased the number of long axons (>75 microns) for both exposure times. There was an increasing impact of dose in the percent of long axons until 100 nM MB, in which there was a visible decrease with 200 nM MB (Table 2). All values, however, remained above the control value. A similar effect was seen for both exposure times. MB participated in significantly enhancing the length of at least a subset of the axons growing in culture. Because MB appears to have an ability to aid in enhancement of axonal lengths, it is possible that it may be playing a role in synaptic plasticity. Further experimentation should focus on looking into the role of MB in attenuating synaptic dysfunction. One possible study will be described in a later section.

From our observations, we did not see any evidence of MB interfering with axonal outgrowth. While it is possible that MB could have disrupted another function that we did not measure, MB did not do so in a way that disrupted tau's normal function. Because we did not directly test whether MB bound to phosphorylated tau, we cannot discount that MB did bind to phosphorylated tau. While this is one possibility, it is also possible that MB did not become bound to phosphorylated tau at all, or that MB may

have regulated a separate mechanism that aids in axonal growth. For example, MB may have assisted with membrane insertion by aiding in the transport of membrane vesicles, in local, intra-axonal production of proteins for the developing axon, or in the guidance of growth cones, which are crucial for axonal development (Goldberg 2017). Future research should focus on trying to distinguish which of these critical axonal elongation mechanisms MB can aid.

A separate suggestion for future studies is the growth of primary neurons at lower concentrations to understand how MB behaves with mature neurons. Because neurons grow very close to one another, plating them at lower concentrations would allow for a clearer distinction of individual neurons stained for tau, which was nearly impossible with our concentration of $1*10^6$ cells/mL. If we observe enhancement effects in these older neurons by MB, this could further support MB as being able to potentially aid in synaptic plasticity in these older neurons.

Given MB's positive effect on axonal outgrowth, it is reasonable to ask whether this growth is limited to axons or if dendrites could also be affected. Researchers have previously observed phosphorylated tau in both axons and dendrites (Brion et al. 1994), and while the role of tau in dendritic processes is not well known, there may be an effect of a tau binding drug in those domains as well. Furthermore, if MB's primary effect on axon growth is mediated by generation of ATP, there is reason to suspect that dendrites would also benefit from increased energy to generate longer and more branched processes.

Generation of ATP

Based upon the observation that axonal growth could be enhanced by MB exposure, it seemed reasonable to look for a potential mechanism of action. One hypothesis suggested that MB might be acting through mitochondria by increasing ATP levels (Weinstein et al. 1964; Wen et al. 2011). In AD, imbalances in cellular energy production may prevent cells from maintaining a homeostatic environment, which may cause cells to die. We hypothesized that MB may be interacting with mitochondria to increase ATP levels, which may provide supporting evidence for MB assisting in energy formation. We ran two separate experiments, which paralleled the axon growth experiments, where one-day-old neurons were treated for 24 hours (**Figure 9**) or 48 hours (**Figure 11**) with 50 – 200 nM MB. We expected that with a 24-hour stimulation period of MB, we would see increases in ATP levels, and in particular, with 100 nM MB, which would correlate with the significant increase of axonal lengths in comparison to the control. Although 100nM MB displayed the highest value of ATP means among all groups, there was no statistical significance with the 24-hour stimulation period. On the other hand, with a 48-hour stimulation period, there were significantly higher ATP levels between 100 nM MB and the control, as well as between 100 nM MB and the 200 nM MB. Because we previously observed no neurotoxicity being induced by nanomolar concentrations of MB and thus no changes in neuronal survival with these concentrations (**Figure 5**), we suggested that changes in ATP levels were not attributed to a lowering neuronal count but an increase of ATP levels per neuron. One possible explanation for why the 24-hour exposure to MB did not enhance ATP levels could is that during the time of early and high growth, neurons may be hydrolyzing ATP at faster rates.

Therefore, there might not be significantly high ATP levels that could be detected with the assay. Based on these findings, MB may aid in the facilitation of energy formation when incubated with neurons for 48 hours, under our conditions.

My results agreed with the findings of one particular study (Gonzalez-Lima and Bruckey 2004), where low-dose (4 mg/kg) MB was administered intraperitoneally to eight rats on a daily basis. The purpose of this experiment was to take notice of improvement in the retention of extinction memory following MB administration. Results indicated that there was an improvement in memory in rats as well as an increase in overall brain oxidative metabolism. This suggested that MB was acting favorably for cognitive activity in the brain. Since my results indicated that MB might be aiding through the facilitation of energy formation, perhaps this particular mechanism of action explains why these researchers observed an improvement in memory retention.

One interesting manipulation to our methods that might prove beneficial is an increase in the number of doses of MB that are delivered to the neurons. From our results, it became clear that when the neurons were exposed to 48-hours with MB, the effects on the axonal lengths of neurons were not as drastic as they were with a 24-hour stimulation period. This suggests that there may be a period between 24 and 48 hours in which MB reaches its peak effect on the primary neurons and after this period it is possible that the effect becomes less dramatic. Providing the cells with multiple rounds of MB may enhance the effects that we originally saw with a 24-hour stimulation. In the future, introducing MB on axons for several consecutive days may provide us with an understanding on whether or not these MB “boosters” may aid in preventing a loss of

effect from MB. It is possible that MB may serve as a booster drug to maintain healthy mental functioning by allowing axons to form new connections, thereby rescuing synapses and aiding with neural plasticity. Future experimentations are required to test out this hypothesis.

Moreover, studies have indicated that growth mechanisms can occur from the soma and the axon. With this in mind, future research can look at measuring concentrations of mitochondria in different areas of the neuron. Primary neurons can be stimulated with nanomolar concentrations of MB for 24 or 48 hours. By staining the neurons with a mitochondrial dye, we can identify any location-based effects and better understand what part of the neuron MB may be having the greatest impact.

Pilot Study

Since MB was previously suggested to serve as a therapeutic agent (Paban et al. 2014), we conducted a pilot study to understand whether MB may aid with neuronal loss. We investigated whether 2-week-old neurons stimulated with 0 – 200 nM MB would be protected from 50 μM glutamate, a neurotoxic agent that can induce neurotoxicity (Lau et al. 2010). These neurons were incubated in conditions of high or low O_2 levels. We suspected that higher levels of O_2 would create greater toxicity through a generation of ROS. While we believed that MB might demonstrate neuroprotective effects, we did not have a clear idea of what exactly would happen. We were mainly interested in observing trends in the data. Our data from ATP assays indicated that almost no attenuation occurred with MB since the neurons were not being kept alive **(Figure 12).** The only dose that appeared to demonstrate some level of attenuation was the 200 nM MB with 50

μM glutamate under conditions of low O_2 levels. In the future, utilizing doses higher than 200 nM MB may highlight MB's potential neuroprotective abilities. Overall, little to no attenuation took place with nanomolar concentrations of MB.

In this pilot study, we also tested whether changes in O_2 levels would affect ATP levels. Based on our findings, the cells appeared to be more sensitive to glutamate when they had were placed under higher concentrations of oxygen **(Figure 12)**. This may be due to oxidative damage occurring in these neuronal cultures even in the absence of glutamate. One interesting data point to support this hypothesis is that the 100 nM MB sample, grown under high O_2 levels and without glutamate, was higher than the controls. This indicated that MB may have protected the neurons from oxidative stress, but once neurons were pushed to a stressed state with glutamate exposure, MB could not rescue them. The usage of other stressors, such as Aβ or rotenone (Poteet et al. 2012), may potentially highlight any neuroprotective abilities that may not have been demonstrated with glutamate neurotoxicity. Our data did not support previous data in which low nanomolar concentrations of MB were neuroprotective *in vitro* (Wen et al. 2011, Poteet et al. 2012).

When we compare the unsuccessful results from the phase III clinical drug trial of MB with our results, it is evident that there is consistency among our findings. No neuroprotective effects were seen with MB in our study, and no slowing of cognitive decline was seen in the phase III trial. One explanation for these similarities may lie in the timing of both experiments. Because MB drugs may have relatively short-term effects on preventing cognitive decline in AD, it is possible that with the shorter duration of the

phase II trial, scientists were not able to collect data that would alert them of this limitation. Perhaps, the shorter Phase II trial was successful because of the timing. In addition, it is possible that with a longer duration of the phase III clinical drug trial, AD pathologies promoted a great loss of neurons and MB lost its effectiveness to behave as a cognitive enhancer.

Understanding why this drug did not showcase positive effects during the phase III clinical drug trials may require the re-evaluation of the following three AD hallmarks: neurofibrillary tangles (Goedert 1996; Leu et al. 1996), Aβ plaques (Seubert et al. 1992; Haass et al. 1995; Citron et al. 1997) and neurodegeneration (Busciglio et al. 1995). With this in mind, having a drug that only targets one of these three hallmarks may initially show a positive response on neurons. For example, if we were to only focus on utilizing MB to aid in the impairment of energy metabolism, it may briefly provide assistance to these neurons. This effect, however, may not be long lasting as the other two hallmarks may progressively cause adverse effects on the neurons. This indicates that in order to tackle this disease, it may be vital to not only focus on one of part of the illness but the other two hallmarks as well. With this in mind, a method that targets more than one hallmark may provide longer lasting effects on AD patients.

Interestingly, a drug cocktail may be designed to have multiple targets. For example, we may use MB, a drug that may have cognitive-enhancing properties, in collaboration with drugs such as RD82. RD82 is an allosteric modulator of mGlu4 G-protein-coupled glutamate receptors; glutamate is a neurotransmitter that is heavily involved in memory, learning and synaptic plasticity (Pearce 2017). In a thesis study

performed by Lindsay Pearce (2017), RD82 displayed neuroprotective abilities in an embryonic neuronal culture, after neurons had been exposed to N-methyl-D-aspartate (NMDA) neurotoxicity. NMDA receptors have been identified as playing a role in neurodegenerative mechanisms (Pearce 2017). If MB and RD82 were combined into one solution, the cognitive-enhancing properties of MB and the neuroprotective abilities of RD82 may enhance cognitive function and display prolonged neuroprotection in our primary neuronal cultures. We suspect that this drug combination may attack pathologies and at the same time, protect the cells from toxicity seen in neurodegenerative illnesses such as in AD. Assessments of these effects need to be tested on an *in vitro* and *in vivo* model systems. Because these compounds target several detrimental properties of AD, this potential drug therapy may be a better representation of an optimal drug treatment for AD.

Future Experiments

Because MB may function as a cognitive-enhancing drug, using an *in vivo* model may help elucidate any attenuation of cognitive dysfunction with MB. These studies could utilize six-month-old transgenic female mice expressing P301S, the mutant human tau (Takeuchi et al. 2011), to model tauopathy. Since memory consists of distinctive time-dependent phases, it would be interesting to inject low doses of MB (1 – 3 mg/kg) 1 – 48 hours after each learning session, in order to highlight some beneficial effects of MB in enhancing cognitive functioning. This study could use a hole-board spatial memory task to observe the effects of MB. If these transgenic mice can learn and remember under

these conditions, this may be an indicator that MB enhances cognitive functioning. These findings may further support MB behaving as a cognitive-enhancing drug.

It would also be interesting to assess if MB may aid in synapse dysfunction by helping keep synapses intact. One future study could look at the specific synapses being formed following exposure to nanomolar concentrations of MB while placing careful attention to 100 nM MB, which may potentially showcase an increased number of synapses. Neurons that have been at least seven days in culture begin to display synapses (Cullen et al. 2010) and therefore, should be used in this experiment. One potential measurement of this study may be the number of axon terminals; a greater number of axon terminals present may indicate a higher number of synaptic vesicles. In addition, it may be useful to visualize these synaptic vesicles under a fluorescent microscope after staining for synaptophysin protein, a major synaptic vesicle protein. These findings may suggest that MB can aid in synaptic dysfunction.

Ultimately, my thesis aimed to highlight the beneficiary effects of MB on young, normally functioning primary neurons. The central focus of our study was making sure that MB does not interfere with tau, one of the major proteins needed in axonal elongation. Our fluorescent images that were stained for tau following exposure to MB demonstrated great variation in growth rates of neurons. Axonal length mean data indicated no inhibition of axonal growth with MB in addition to an intriguing enhancement effect of axonal lengths with 100 nM MB, signaling at MB's potential ability to serve as an axonal enhancement drug. This property may allow MB to potentially aid in synaptic plasticity, which may also have cognitive benefits for AD

patients. It is possible that MB enhanced axonal outgrowth through the facilitation of energy formation, which was supported by our study. Given the number of diseases that affect the elderly, the use of this drug to help improve energy might be useful under a number of conditions. Preliminary results from our pilot study indicated that nanomolar concentrations of MB were not able to rescue neuronal losses from exposure to glutamate excitotoxicity. Because we remain unsure of the direct involvement of MB in the neuron, future research could aim at trying to distinguish which axonal length mechanism is working closely with MB. Although our data did not provide evidence that suggests MB targets pathologies of AD, our data did support MB as enhancing axon outgrowth and energy levels. In essence, these finding may suggest a role of MB in helping with cognitive deficits in AD. Our preliminary results suggest a promising future for MB as a potential cognitive-enhancing drug.

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