FKBP51 AND METHYLENE BLUE AS NEUROPROTECTIVE TARGETS

DISSERTATION

Presented to the Graduate Council of University of North Texas

Health Science Center at Fort Worth

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By
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Fort Worth, Texas
May 1, 2011

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my mentor, Dr. Thomas Yorio, for his exceptional guidance and leadership over the last five years. Words can not describe the pride that I have for all of the sacrifices Dr. Thomas Yorio as my mentor. His expertise, personality, and friendship have contributed to my current accomplishments and inspired me to pursue as similar level of success both professionally and personally that he achieved. I will always view our interactions fondly. Furthermore, I hope to one day encourage future scientists in the same way that Dr. Thomas Yorio inspired me.

I would also like to express my gratefulness for Dr. Abbott Clark, whose selflessness and sacrifices were instrumental to my research project. Dr. Abbott Clark all always found a way to make time to help me with our many research endeavors. Our lengthy interactions and conversations directly improved my dissertation and my education.

I am thankful for my committee members, including Dr. Glenn Dillon, Dr. Meharvan Singh, and Dr. Robert Wordinger, for the time that they freely gave and they way they helped shape my research and doctoral experience. Furthermore, I asked these individuals to be members of my graduate committee because of the pride and respect that I have toward them.

I would also like to express warmest thoughts to my laboratory. Both Dr. Raghu Krishnamoorthy and Dr. Adnan Dibas gave their time freely and always taught me several lesion about research and life. In the short time that Brett Mueller has been with our laboratory, he has greatly impacted my research and I am glad to have him as a peer. Hai-Ying Ma, Ming Jiang,

Yong Park, and Bridget Moty all made contribution to my research and graduate experience. Thank you all for the positive experiences.

I must, of course, thank my mother, Sharon Striefel, and my father, Andrew Striefel, for being the two most influential individuals in my life. Their selflessness has established the values of compassion, determination, and perseverance within me.

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CHAPTER 1

INTRODUCTION

Neurodegenerative diseases

The central nervous system is composed of a variety of specific neurons that are important for every physiological process. The health of the neurons is essential to maintaining control of these physiological processes. Neurodegeneration is an umbrella term that covers any stress that causes the premature loss of structure and function of neuronal cells. The loss of these neuronal cells can lead to the irreversible loss of vital physiological processes. Neurodegenerative diseases such as Alzheimer's, Parkinson's, and glaucoma afflict the lives of millions of aged Americans and are increasing in prevalence due to an increase in lifespan. Over 50 million Americans suffer from a neurodegenerative disorder, according to the National Institute of Neurological Disorders and Stroke. There are over 600 neurological disorders. It is estimated that over 5.4 million Americans suffer from Alzheimer's disease, 2.8 million suffer from Parkinson's disease, and 2.22 million suffer from glaucoma (Thies and Bleiler 2011; EDP Research Group 2004). Alzheimer's disease accounts for more than a 100 billion dollar burden on the United States a year (Brown et al., 2005). Typical treatments for the 600 neurological disorders specifically target treating the symptoms of each individual disease and not actually

curing the disease itself. However, it is known that apoptosis accounts for neuronal cell death in almost every neurological disorder. This similarity provides optimism that a neuroprotectant intervention could be uniformly beneficial to a number of neurodegenerative disorders; however, efficacious candidate neuroprotectants are lacking (Waldmeier and Tatton 2004).

Neuroprotection

Due to the increasing numbers of neurodegenerative diseases afflicting millions of individuals, an emerging field of scientific research is in the field of neuroprotection. A neuroprotective agent protects neurons within the nervous system from premature apoptosis or degeneration that occurs during a neurodegenerative disorder or neurotrauma. Even though several potential neuroprotective compounds exist, very few drugs have been developed and/or approved to treat neurodegenerative diseases and neurotraumas due poor efficacy or patient tolerance (Lipton 2007). An example of a compound that is currently going through clinical trails to treat wet Age-related Macular Degeneration (AMD) is Ranibizumab. Ranibizumab, produced by Genetech, is a monoclonal antibody fragment (Fab) that targets VEGF-A, which increases macular edema. Thus, Ranibizumab decreases macular edema throughout the entire retina. Ranibizumab significantly improves or stabilizes vision loss in AMD, as shown following a phase III clinical trial. However, a single intravitreal injection of Ranibizumab costs 2,000 dollars U.S., which limits access (Brown et al., 2009; Raftery et al., 2007). Two potential efficacious neuroprotective agents are FK506 Binding Protein 51 and methylene blue. Thes studies examined FK506 Binding Protein 51 (FKBP51) and the compound methylene blue (MB) as potential neuroprotective interventions.

FK506:

FK506, also known as Tacrolimus, is an immunosuppressant drug used to reduce the activity of a patient's immune system either locally to treat dermatitis (eczema), or systemically to treat allogeneic organ transplants. FK506 achieves its immunosuppressant properties through inhibiting calcineurin, also known as calmodulin-activated protein phosphatase 2B. Inhibition of calcineurin within T lymphocytes prevents dephosphorylation and the activation of Nuclear Factor of Activated T-cells (NFAT). Inhibition of NFAT prevents transcription of the cytokines interleukin-2 (IL-2) and interferon-γ (INF-γ). Therefore, FK506 causes immunosuppression and blocks inflammation (Yano, 2008).

The Food and Drug Administration (FDA) approved FK506 (Tacrolimus) in 1994 for liver transplantation. In contrast to other immunophilins such as cyclosporine, FK506 crosses the blood-brain barrier and is more potent (Yano, 2008). Within the central nervous system, FK506 exhibits profound neuroprotection and neuroregeneration. Neuroprotection from FK506 has been shown during several forms of trauma, including optic nerve crush, traumatic brain injury, brain ischemia, sciatic nerve injury, and focal and global ischemia (Freeman and Grosskreutz, 2000; Giordani et al, 2003; Kaminska et al, 2004). Intravitreal injections of FK506 increase gene transcription of molecules within the retina that are associated with neuroprotection, including the estrogen receptor, the erythropoietin receptor, protein kinase C, the gamma-aminobutyric acid receptor, fibroblast growth factor, the glial cell line-derived neurotrophic factor receptor and the neuropeptide Y receptor (Oh-i et al., 2007). FK506

increases the rate of neuronal recovery and functional recovery after nerve crush in rats (Yeh et al., 2007). *In vivo* and *in vitro* experiments indicate that FK506 increases transcription of GAP-43. These effects are blocked by rapamycin, an immunosuppressant that also inhibits FKBP51 (Stan et al., 1994). Within retinal ganglion cells, FK506 displays neuroprotection and neuroregeneration *in vivo,* following optic nerve crush (Freeman and Grosskreutz, 2000; Huang et al., 2005). FK506 caused greater than 33% retention of retinal ganglion cells following crush (Freeman and Grosskreutz, 2000). The characteristics of FK506 provide intriguing potential treatments for retinal ganglion cell loss observed during glaucoma.

Even though FK506 displays several beneficial properties, the neuroprotective downstream signaling molecules have not been completely defined. FK506 interacts through several binding proteins, leading to a number of neuroprotective and neuroregenerative traits independent of calcineurin inhibition (Herdegen and Klettner 2003). Characterization of these signaling pathways would be advantageous in treating neurodegenerative diseases without systemic immunosuppression.

FK506 Binding Proteins:

Immunophilins are peptidyl-prolyl cis/trans isomerases (PPIase) that interact with immunosuppressive drugs such as FK506, cyclosporine, or rapamycin. Proteins characterized for binding directly to FK506 are referred to as FK506 binding proteins (FKBP). FKBPs are present in all eukaryotes from yeast to humans and have a broad range of neuroprotective functions (Klettner and Herdengen, 2003). FKBPs are defined based on their molecular size. FKBP 12, 13, 22, 23, 25, 36, 38, 51, 52, 60, 63, 65 have been identified. FKBP12 is the most

studied and defined immunophilin, based on its inhibition of calcineurin and role in immunosuppression. FK506 binds to FKBP12, which inhibits calcineurin (Yano, 2008). This complex blocks the translocation of NFAT into the nucleus, preventing IL-2 expression and T cell activation, leading to neuroprotection (Kochel and Strzadala 2004). *In vitro* gene silencing of FKBP12 in T cells through short interfering RNA (siRNA) blocks immunosuppressive effects from the addition of FK506, indicating FKBP12 as the sole FKBP responsible for immunosuppression and inhibition of calcineurin (Xu et al., 2002). However an additional immunophilin may be responsible for some of FK506's neuroprotection because FK506 still protects neurons in FKBP12 knockout mice (Guo et al., 2001). *In vitro* studies resulted in FK506 being equipotent for neuroprotection in cell lines with or without FKBP12. Furthermore, FK506 drug analogues (GPI-1046), which function independently of FKBP12, have been shown to be neuroprotective (Burnett et al., 2003).

FK 506 Binding Protein 51

FKBP51 is a potential immunophilin responsible for FK506 induced neuroprotection. FKBP51 is expressed in numerous tissues, including the central nervous system. In most tissues, it is expressed in molar excess over FKBP12 (Weiwad et al., 2006); however, FKBP51 has not been extensively studied for its neuroprotective properties. The FK506-FKBP51 complex inhibition of calcineurin is controversial; however, the FK520-FKBP51 complex has been shown to directly inhibit calcineurin. Nonetheless, the FK520-FKBP51 complex is 400 times weaker than FK520-FKBP12 in regards to inhibiting calcineurin (Baughman et al., 1997; Xu et al., 2002). FKBP51 is a large molecular weight immunophilin, suggesting a greater capability of

diverse interactions and function than smaller molecular weight immunophilins, such as FKBP12, because it has two FK domains and three tetrati-copeptide repeats (TPR) domains (Li et al., 2011). Currently, FKBP51 is mostly characterized for its chaperone properties with native steroid receptors. This may result from the presence of one region containing TPR, which are responsible for protein-protein interactions (Li et al., 2011). FKBP51's TPR domain has high affinity for Heat Shock Protein 90 (hsp90) in steroid receptor complexes. FKBP51-hsp90 complex can bind with the glucocorticoid receptor, the progesterone receptor, the androgen receptor, and to a lesser degree with the estrogen receptor (Chen et al., 1998; Zhang et al., 2008; Nair et al., 1997). FK506 potentiates the transport and stability of the FKBP51-hsp90-hormone receptor complex (Zhang et al., 2008). Hsp90 is an important cofactor of cell survival promoting molecules, such as V-Raf-1 Murine Leukemia Viral Oncogene Homolog 1 (Raf-1) and Akt, suggesting a novel mechanism – FK506 increasing cell survival and inhibiting apoptosis through the FKBP51-hsp90 complex (Zhang et al., 2005; Neckers 2002; Sinars et al., 2003).

FKBP51 also plays a significant role in the activation of NFκB, an important cell survival protein. The activation of NFκB is initiated through the degradation of the inhibitory molecule, IκB. This leads to the activation and translocation of NFκB into the nucleus to initiate transcription of several pro-survival proteins, growth factors, and anti-apoptotic proteins. IκB is ubiquitinated through the serine/thereonine kinase, IKK, leading to the degradation of IκB through the proteosome. FKBP51 has been shown through a proteomic approach to be an important cofactor of the catalytic subunit (IKKα) of IKK (Giraudier et al., 2002). The over expression of FKBP51 upregulates NFκB expression in cancerous cell lines, which is believed to increase their resistance to apoptosis-inducing chemotherapeutical agents. This suggests a new

potential mechanism, resulting in FKBP51 being neuroprotective (Komura et al., 2005; Krappmann and Scheidereit 2004). Furthermore, NFκB regulates transcription of several anti-apoptotic proteins, including BCL-2, which has been shown to be neuroprotective and neuroregenerative (Fahy et al., 2003).

FKBP51 overexpression is observed in several cancers, including idiopathic myelofibrosis, prostate cancer, and haematopoiesis causing apoptosis resistance to several chemotherapy agents (Bock et al., 2004; Febbo et al., 2005). During idiopathic myelofibrosis, FKBP51 is overexpressed up to eight times greater than its basal levels. FKBP51 overexpression leads to apoptosis resistance during cytokine deprivation in cell lines (Giraudier et al., 2002). Furthermore, the JAK/STAT pathway is over activated during idiopathic myelofibrosis and is shown to be mediated through FKBP51 overexpression. Specifically, STAT3 and STAT5 over activation and translocation are observed. These STATs are commonly associated with tumorigenesis. Furthermore, sustained activation of the JAK/STAT pathway leads to the transcription of several anti-apoptotic proteins, including Bcl-xl (Komura et al., 2003; Giraudier et al., 2002; Yoshida et l., 2002). Interestingly, inhibition of the JAK/STAT pathway through the antagonist AG490 during acute elevated IOP had a detrimental effect on RGC survival (Yao et al., 2007). This provides evidence of another possible mechanism FKBP51 acts through to achieve neuroprotection and neuroregeneration. FKBP51's proposed mechanism of action is: NF-κB activation, transcription factor transport, and STAT 3/5 activation (**figure 1**).

Figure 1: Proposed mechanism of FKBP51

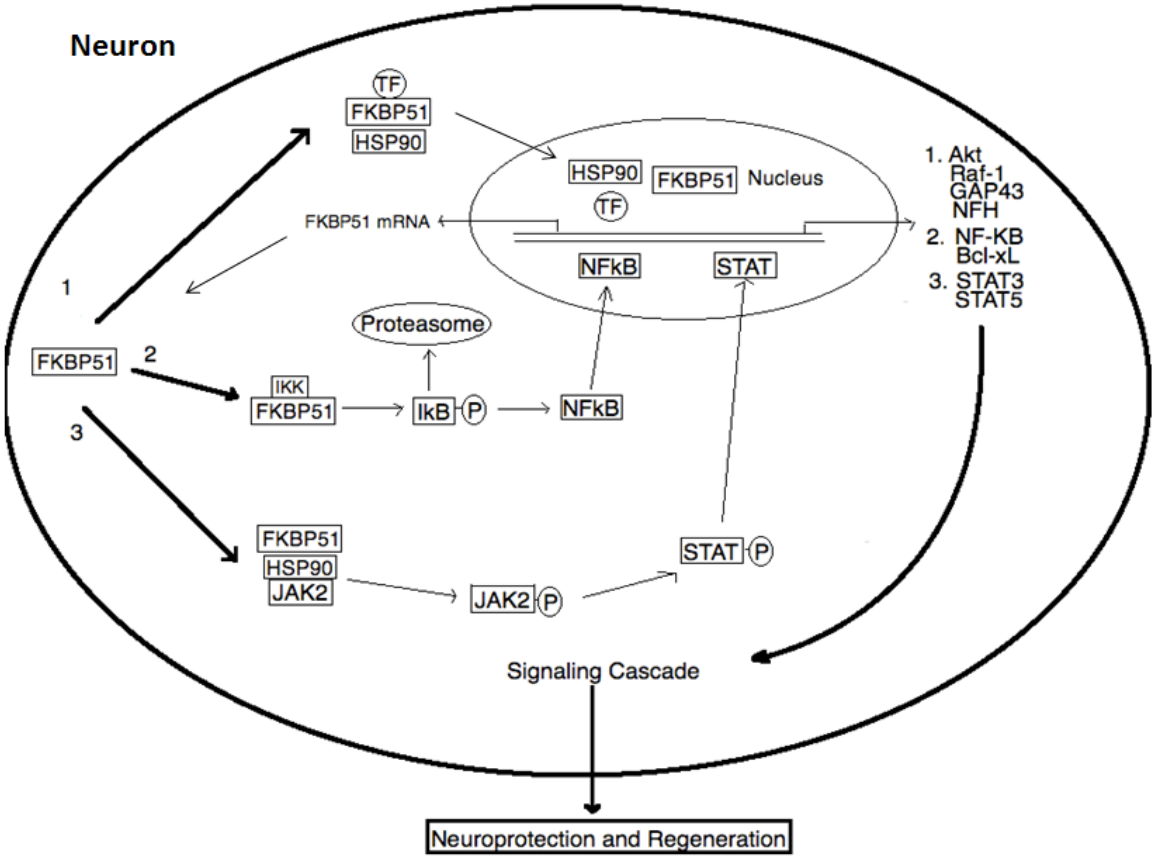

Figure 1: A schematic representation of FK Binding Protein 51's (FKBP51) hypothesized neuroprotective and neuroregenertative functions. **1:** FKBP51's Neurotrophic properties are achieved through heterodimerization with Heat shock protein 90 (HSP90) in order to aid transport of transcription factors (TF). In addition, Hsp90 can increase stability and function of survival promoting signaling molecules such as Raf-1 and Akt. **2:** FKBP51 sustains cell survival through association with IKKα, allowing disassociation with IκB-kinase complex. Degradation of IκB prevents inhibition on NF-κB allowing NF-κB to sustain cell survival and promote transcription of Bcl-2. **3:** FKBP51 anti-apoptototic properties are attained through NF-κB (previously mentioned) and activation of JAK-STAT pathway, which increases activation and translocation of STAT-3, STAT-5 and Bcl-xl, which promote cell survival pathways.

Actions of FKBP51 provide a compelling case for further investigating its role in neuroprotection and neuroregeneration. We therefore hypothesized that increases in FKBP51 protein levels lead to neuroprotection, while decreases in FKBP51 expression lead to an increase in neuronal cell death when exposed to a challenge inducing cell death. This investigation further evaluated changes to prosurvival NF-κBs and phosphorylated NF-κB proteins following changes in FKBP51 protein levels (**Figure 2**). FKBP51's ability to prevent neurodegeneration during neurotoxic incidences could potentially provide new insights for medical treatments to target during neurological disorders that are clinically important in treating neurodegenerative diseases.

Figure 2: FKBP51's Role in Neuroprotection

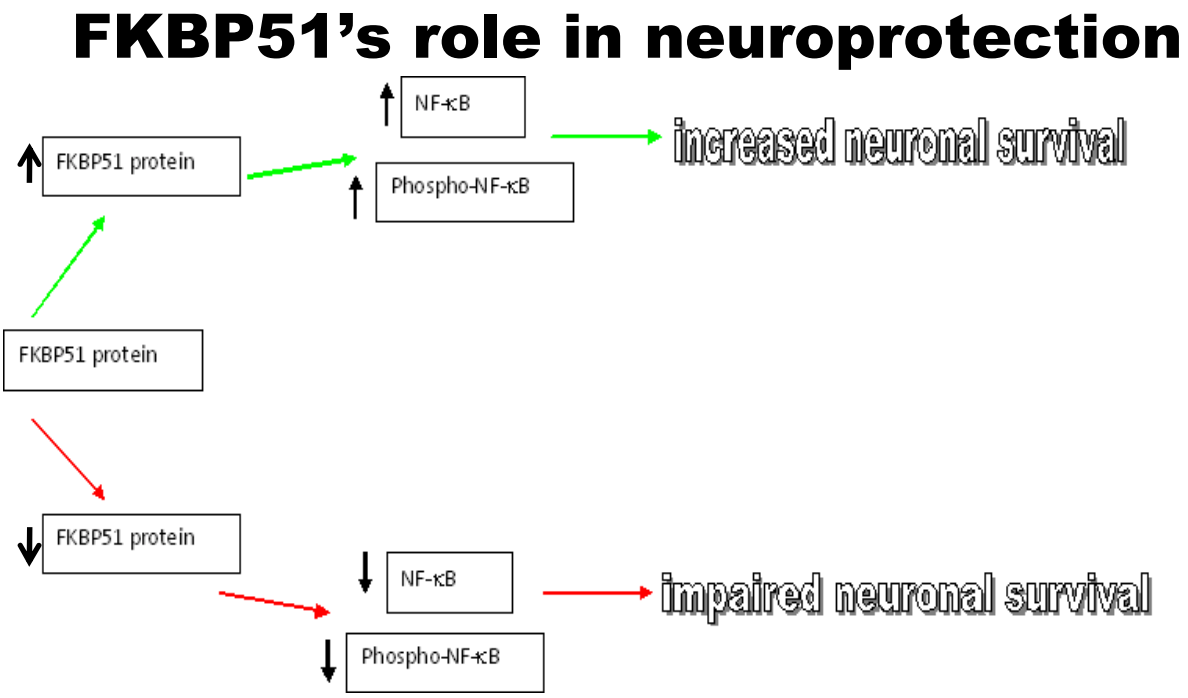

Figure 2: A schematic overview of FKBP51's mechanism of action in neuronal survival. An increase (green arrows) in FKBP51 protein levels leads to an increase in neuronal survival following a challenge promoting cell death by increasing prosurvival molecule NF-κB and phospho-NF-κB. Furthermore, a decrease (red arrows) or knockout of FKBP51 protein levels leads to impaired neuronal survival following a challenge promoting cell death by a decrease in NF-κB and Phospho-NF-κB.

Mitochondrial dysfunction during neurodegenerative diseases

Mitochondria are highly dynamic organelles typically referred to as the cell's powerhouse because they generate adenosine triphosphate (ATP) through aerobic respiration and oxidative phosphorylation (Detmer and Chan, 2007). Furthermore, the mitochondrion can also influence intermediary metabolism, calcium homeostasis, cell proliferation, development and apoptosis (Oakes and Korsmeyer, 2004; shaw and Nunnari, 2002; Chen et al., 2003). Due to the high energy demand of neurons, a growing body of evidence has linked mitochondrial dysfunction to age-related neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), and glaucoma (Reddy, 2009). For example, analysis of mitochondria structure within AD brains showed significant changes to the morphology of the mitochondria (Baloyannis, 2006). Furthermore, these neurons had increased oxidative damage and significant levels of cytochrome c oxidase within the cytosol (Hirai et al., 2001; Castellani et al., 2002). Decreased cytochrome c oxidase activity as well as a decrease in energy metabolism has been observed during Alzheimer's disease as well as a decrease in energy metabolism (Nunomura et al., 2001; Cardoso et al., 2004). During glaucoma, mitochondrial dysfunction is believed to be a secondary pathology. The increase in intraocular pressure leads to transient ischemia and thus mitochondrial dysfunction (Kong et al., 2009). This suggests that an efficacious mitochondrial metabolic enhancing compound could uniformly benefit neuronal survival during neurodegenerative disease, specifically protecting against mitochondrial dysfunction.

Methylene Blue

Methylene blue, also known as methylthioninium (C(16)H(18)N(3)SCl . 3H(2)O), is a remarkable compound that is already Food and Drug Administration approved, cheap and causes minimal side effects. MB is an autoxidizable phenothiazine with potent antioxidant and metabolic enhancing properties (Bruchey and Gonzalez-Lima 2008) (**Figure 3**). MB was the first phenothiazine compound developed, and its unique properties have been under investigation for over 120 years. MB was successfully transformed into several derivatives including; antimalarial agents quinacrine and chloroquine, the phenothiazine antihistamine promethazine, and the first antipsychotic drug chlorpromazine (Bruchey and Gonzalez-Lima 2008). MB is a metabolic poison antidote to induce methemoglobinemia (Clifton and Leikin, 2003). Chronic treatment with MB is safe and can protect against mental disorders and prevents encephalopathy in humans undergoing chemotherapy (Naylor et al., 1988; Kupfer et al., 1994; Kupfer et al., 1996; Pelgrims et al., 2000).

Methylene blue can protect against several forms of *in vivo and in vitro* neurotrauma. For example, methylene blue protects the retinal and retinal ganglion cell layer against rotenone toxicity (Rojas et al., 2009). Rojas showed the intravitreal injections of rotenone in rats significantly impaired the structure of the retina and visual acuity of the rats. Co-administration of methylene blue significantly improved the structure of the retina and the visual acuity of these rats (Rojas et al., 2009; Zhang et al., 2006). Methylene blue also significantly improved learning and memory of rats during amnestic mild cognitive impairment (Riha et al., 2011). Furthermore, methylene blue enhanced discrimination learning, extinction of fear, brain oxidative metabolism and memory in rats (Callaway et al., 2004; Riha et al., 2011; Wrubel et al., 2007; Wrubel et al., 2006).

Figure 3: Methylene Blue Structure:

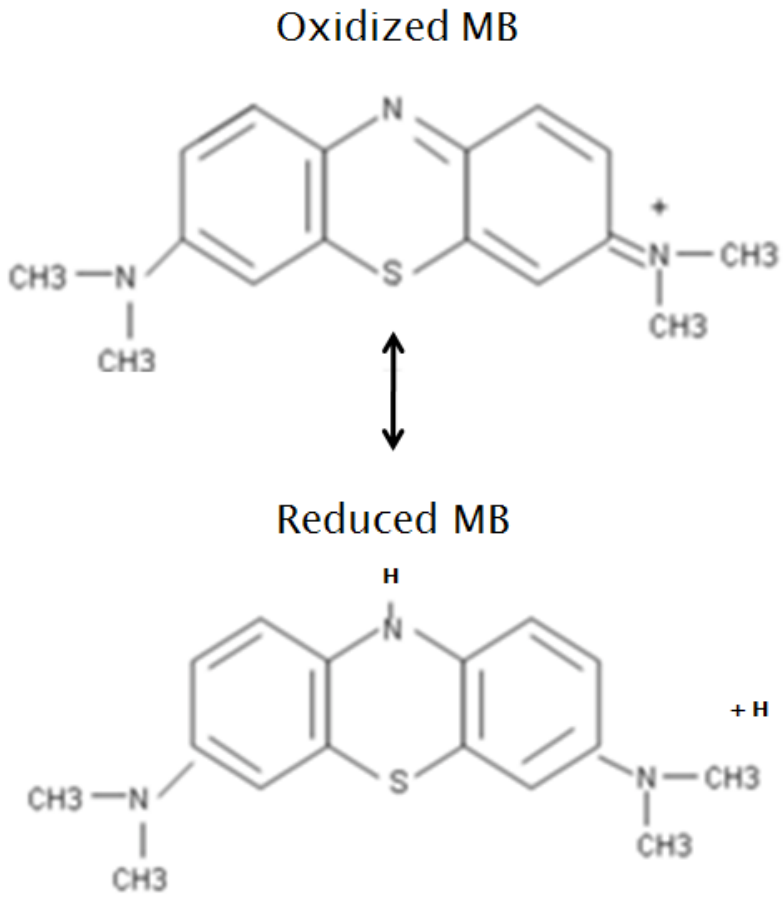

Figure 3: Methylene Blue Structure

Methylene blue is a compound capable of reversible reduction-oxidation. Methylene blue's oxidized state can accept electrons. Methylene blue's reduced form, leukomethylene blue, can transfer electrons to different molecules such as cytochrome c oxidase or oxygen to form water.

Methylene blue Protection in Central Nervous System

Methylene blue has a high bioavailability in the brain and readily crosses the blood brain barrier (Peter et al., 2000; Wainwright and Crossley, 2002). In pigs, MB protects against hypoxia-induced neuronal injury and systemic oxidative stress (Misclescu et al., 2006). Furthermore, MB protects the brain and retina from rotenone induced mitochondrial toxicity (Rojas et al., 2009; Zhang et al., 2006). MB also protects against learning and memory impairments induced by sodium azide (Callaway et al., 2002). Additionally, clinical trials are being performed with chronic MB treatment to prevent the progression of Alzheimer's disease (Wischik et al., 2008).

Methylene blue mechanism of action

MB is an autoxidizable phenothiazine with potent antioxidant and metabolic enhancing properties (Bruchey and Gonzalez-Lima 2008) that facilitates memory and promotes neuroprotection (Callaway et al., 2004; Riha et al., 2005; Gonzalez-Lima and Bruchey 2004; Zhang et al., 2006). MB is a compound capable of reduction-oxidation and can support electron cycling. This property allows MB to transfer electrons to oxygen, which is specifically important for the electron transport chain of the mitochondria (Calabrese et al., 2007) (**Figure 4**). MB cannot enter a neuron until it is reduced (MBH2) at the cell surface. Once MBH2 enters the cell it can be re-oxidized back into MB, maintaining it within the cell (Bongard et al., 1995). MB may be re-oxidized by a heme-protein such as cytochrome c or cytochrome c oxidase (Atamna et al., 2007). MB can accept electrons from electron donors such as nicotinamide

adenine dinucleotide (NADH) or flavin adenine dinucleotide (FADH2) and then donate electrons to coenzyme Q (CoQ) or cytochrome c. Within the cell, MB and MBH2 are maintained in equilibrium making a reversible reduction-oxidation system (May et al., 2004). The numerous neurodegenerative diseases that are potentiated by mitochondrial dysfunction could be uniformly protected with methylene blue treatment (Baltmr et al., 2010). Investigating methylene blue's protective properties during a model of retinal ganglion cell death could lead to a novel, inexpensive and efficacious treatment of glaucoma.

Figure 4: Methylene Blue's Mechanism of Action:

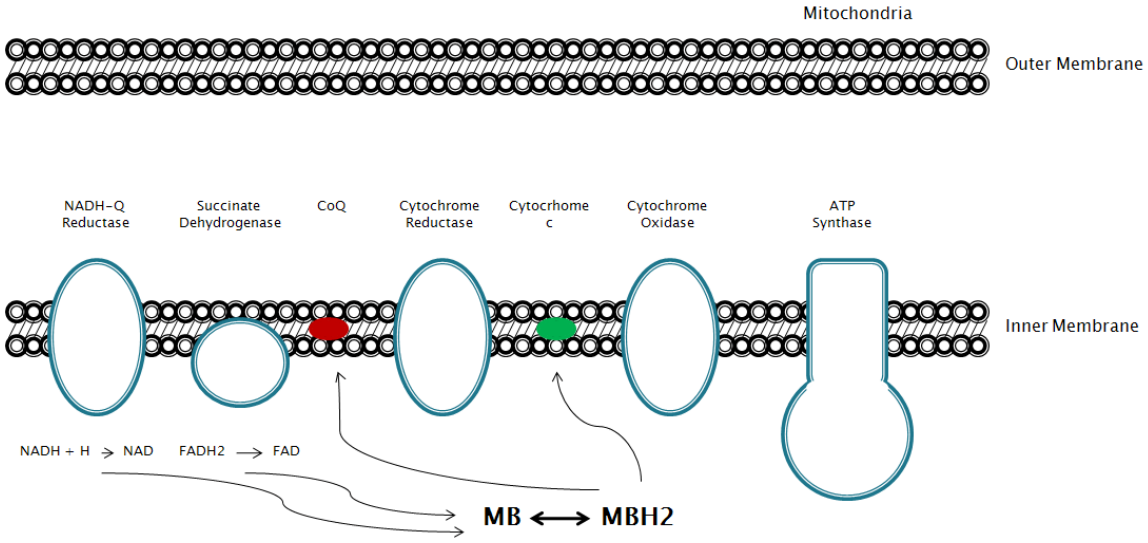

Figure 4: Methylene Blue's Mechanism of Action:

The mitochondrial electron transport chain couples electron transfer from electron donors (NADH and FADH2) and electron acceptors (O2) in order to create a proton gradient. The proton gradient between the outer and inner membranes is the driving force to create chemical energy in the form of adenosine triphosphate (ATP). During mitochondrial dysfunction, several of the electron transport chain complexes become impaired and can no longer transfer electrons from one molecule to another. Methylene blue can preserve the propagation of electrons by acting as an electron acceptor from NADH and FADH2 and then donate the electrons further down the electron transport chain at coenzyme Q (CoQ), cytochrome c and oxygen. This preserves the gradient of protons thus allowing ATP synthase to generate ATP.

Specific Aims

Neurodegeneration is a wide-ranging problem involving the progressive loss of structure and function of neurons. Neurodegeneration includes several types of neurotraumas and diseases such as glaucoma, Alzheimer's disease, and Parkinson's. As research advances to sub-cellular levels, these neurotraumas and diseases show an increasing amount of similarities. These similarities provide insight that a neuroprotectant intervention could be uniformly beneficial to a number of neurodegenerative disorders; however, efficacious candidate neuroprotectants are lacking. FK506, a widely used immunosuppressant drug, has profound neuroprotective and neuroregenerative properties throughout the central nervous system. FK506 treatment binds to the FK Binding Protein 51 (FKBP51), suggesting FKBP51's possible importance in neuroprotection and neuroregeneration. However, the specific mechanism related to FKBP51 actions still needs further investigation. Additionally, mitochondrial dysfunction is a common symptom of neuronal death during neurodegenerative diseases and neurotraumas (Nunomura et al., 2001; Cardoso et al., 2004). The FDA approved compound methylene Blue provides preliminary evidence that it could be useful as a potential therapeutic intervention. **The hypotheses tested was (1) increased FKBP51 protein levels promote neuroprotection while decreases in FKBP51 protein levels impair cell survival and that (2) methylene blue will specifically protect retinal ganglion cells from neuronal stress.** The specific aims tested were:

1. Determine FK506's effect on FKBP51 in retinal cell culture.

1.1: Measured if FK506 treatments increased protein levels of FKBP51 and related signaling molecules, NFkB and phospho-NFkB, in retinal cell cultures.

2. Determined if over-expressing FKBP51 through stable transfection with an over-expression vector affects survival of neuronal cells in culture following challenges promoting cell death in retinal cell cultures.

2.1: Measured changes in prosurvival proteins NFkB and phospho-NFkB following changes in FKBP51 protein levels.

2.2: Determine whether FKBP51 expression protects cells from stuarosporine-induced neurotoxicity by monitoring cell death using calcein AM/propidium iodide and caspase 3 detection.

3. Determined if methylene blue can protect retinal ganglion cells from neurotoxic insults.

3.1 Use calcein AM/ethidium homodimer-1 to determine whether methylene blue treatments protected primary rat retinal ganglion cells from rotenone, staurosporine and hypoxia induced cytotoxicity.

The experimental design developed determined the neuroprotective properties of two therapeutic agents, FKBP51 and methylene blue. The experimental design determined if FKBP51 overexpression or knockout affected the viability of neuronal cell cultures and retinal ganglion cells following toxic insults. Furthermore, the experimental design determined if methylene blue was a potential treatment to protect retinal ganglion cells and explored methylene blue's mechanism of action. Results from this investigation not only yielded insight into the actions of these neuroprotective agents but also provided insight into new potential drug targets.

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CHAPTER II

FK506 BINDING PROTEIN 51 (FKBP51) PROTECTS 661W CELL CULTURE FROM STAUROSPORINE-INDUCED APOPTOSIS

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Molecular Vision (2011)

Abstract

Purpose: Neurodegenerative diseases and neurotraumas typically result in apoptosis of specific neurons leading to the pathology observed during the disease state. Existing treatments target the symptoms instead of preventing the death of these neurons. Although neuroprotective drugs should be useful as a treatment to prevent further loss of neurons, efficacious molecules are lacking. FK506 (tacrolimus), a widely used immunosuppressant drug, has significant neuroprotective and neuroregenerative properties throughout the central nervous system, including the eye. FK506 achieves these properties through interaction with FK506 binding proteins (FKBP), including FK506 binding protein 51 (FKBP51). In this study, we examine the effects of FKBP51 as a neuroprotective agent on a neuronal cell line.

Methods: We cultured 661w cell cultures with or without FK506, or stably transfected them with an FKBP51 expression vector. These cells were then exposed to the apoptosis inducing agent staurosporine. Cell viability was determined using a calcein AM/propidium iodide assay. Protein levels and activation of nuclear factor kappa-light chain-enhancer of activated B cells (NF-κB) were determined by western immunoblot analysis.

Results: FKBP51 overexpression significantly protected 661w cell cultures from staurosporine-induced apoptosis. FKBP51 overexpression also significantly increased NF-κB p65 protein

levels and activated NF-κB p65. FK506 treatment significantly protected 661w neuronal cultures from staurosporine-induced apoptosis. FK506 increased FKBP51, NF-κB p65, and levels of activated NF-κB p65 protein.

Conclusions: These results suggest that FKBP51 protects 661w cell cultures from apoptosis induced by staurosporine. Additionally, FK506 protected 661w cell cultures from apoptosis and displayed a mechanism similar to that of FKBP51 overexpression. Both FK506 and FKBP51 appear to act through activation of NF-κB p65 protein, suggesting a common pathway for neuroprotection. These findings suggest that FKBP51 is a compound important to neuronal cell culture survival. FKBP51 may be a potential therapeutic drug target for preventing the neurodegeneration and neurotrauma that occur during neurodegenerative diseases.

Key Words: FKBP51, Neuroprotection, 661w, apoptosis, staurosporine

INTRODUCTION

Neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and glaucoma affect the lives of millions and are increasing in prevalence due to the progressive increase in human lifespan [1]. Each year, over 3 million people worldwide die from neurologic disorders such as Alzheimer's and Parkinson's disease [2]. Typical treatments for neurologic disorders specifically target treating the symptoms of each individual disease and are not directed to intervening in the disease process. However, it is known that apoptosis accounts for most neuronal cell death during neurologic disorders [3]. This similarity provides hope that neuroprotectant intervention could be uniformly beneficial to several neurodegenerative disorders; however, efficacious neuroprotectants are currently unavailable [4].

FK506 (tacrolimus) exhibits significant neuroprotective and neuroregenerative properties in several forms of neurotrauma, including optic nerve crush, traumatic brain injury, brain ischemia, sciatic nerve injury, and focal and global ischemia [5-8]. This protection is not limited to neurons; it extends to glia cells within the brain and several other organs [9]. These characteristics of FK506 make it potentially useful for neuroprotection; however, FK506 produces calcineurin-induced immunosuppression by binding FK506 Binding Protein 12 (FKBP12), which can increase the incidence of cancer [10,11]. FK506 was found to be equipotent in protecting cells lacking FKBP12 (U251 human glioma), compared to cells expressing FKBP12 (SH-SY5Y human neuroblastoma) [12]. Furthermore, FK506 also was

found to protect neurons in FKBP12 knockout mice [13]. FK506 drug analogs, such as GPI-1046, which function independently of FKBP12, were shown to be neuroprotective [12,14]. However, not all FK506 downstream signaling pathways have been defined. FK506 interacts through several binding proteins, leading to several neuroprotective and neuroregenerative traits devoid of calcineurin inhibition [15]. Characterization of these signaling pathways would be advantageous to treating neurodegenerative diseases without systemic immunosuppression.

FKBP51, an immunophilin that interacts with FK506, is a potential neuroprotective agent for preventing apoptosis during neurodegenerative disease and neurotrauma. FKBP51 plays a significant role in the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), an important cell-survival protein. The activation of NF-κB is initiated through the degradation of the the inhibitory molecule, IKappaB (IκB). This leads to the activation and translocation of NF-κB into the nucleus to initiate transcription of several prosurvival proteins, growth factors, and anti-apoptotic proteins. IκB is ubiquitinated through the serine/thereonine kinase, IKappaB Kinase (IKK), leading to the degradation of IκB through a proteosome. FKBP51 is an important cofactor of the catalytic subunit (IKKα) of IKK [16]. Overexpression of FKBP51 has been shown to upregulate NF-κB protein levels in hematopoietic cells [17]. This suggests a new potential neuroprotective and regenerative mechanism of FKBP51 [18]. Furthermore, NF-κB regulates the transcription of several anti-apoptotic proteins, including BCL-2 [19]. In a melanoma cell line, siRNA-mediated reduction of FKBP51 protein levels decreased expression of NF-κB and increased IκBα and IκBβ protein levels [20]. In UT7 cells, FKBP51 overexpression increased the protein levels of NF-κB p65 and NF-κB p50, and

decreased the protein levels of IκBα [17]. Sustained activation of NF-κB was neuroprotective against glutamate-induced excitotoxicity in primary cortical neurons [21].

FKBP51 is a potential neuroprotective target; however, it is unclear if FKBP51 plays a neuroprotective role. Currently, we are testing the hypothesis that increases in FKBP51 protein levels decrease 661w neuronal cell culture death in reaction to the apoptosis inducing agent staurosporine [22-24].

MATERIALS AND METHODS

661w cell culture

The 661w cells were derived from a murine retinal tumor (these have been shown to have the same relevant cellular and biochemical characteristics of cone photoreceptor neurons [25]). The 661w cells were grown in Dulbecco's modified Eagle's medium (DMEM, cat no. 23700–040; Invitrogen-Gibco, Grand Island, NY), supplemented with 10% heat inactivated fetal bovine serum (cat no. 26140–079; Invitrogen-Gibco), 100 U/ml penicillin, and 100 mg/ml streptomycin (Fisher Scientific, Pittsburgh, PA). The 661w cells were cultured at 37 °C in 5% CO2 and air for all experiments.

Stable transfection

The pCL-neo-FKBP51 overexpression vector and parental control pCl-neo vector were a kind gift from Dr. Marc B. Cox, University of Texas El Paso, El Paso, TX. Vectors were reconstituted in sterile Tris/EDTA buffer and transformed into DH5α E. coli. Competent bacteria were selected using ampicillin LB plates. Colonies that produced the highest levels of vectors were selected through miniprep. Maxipreps were performed using a CsCl gradient. Vectors were rehydrated in sterile TE buffer and maintained at −20 °C.

The 661w neuronal cell cultures (passage 6) were seeded in 100 mm dishes and grown with complete DMEM. After 24 h, the cell cultures were transfected through a lipophilic method (Metafectene Pro; Biontex, Toulouse, France), as instructed by the manufacturer. After an additional 24 h, 2 mg/ml of G418 were added to kill cells that did not incorporate the vectors. Cells were maintained on 0.2 mg/ml G418 and grown to a maximum 15 passages.

Western blot analysis

Cultured cells, from cells grown at 60%-80% confluence, were isolated and lysed as previously described [26]. Protease inhibitors (1 mM Dithiothreitol [DTT] and 500 μM Phenylmethanesulfonyl fluoride [PMSF]) were added. Cells were harvested at 60%–80% confluence. Protein concentrations were determined using Bio-Rad Bradford Protein Assay ((500-0006; Bio-RaD, Bio-Rad Laboratories, Hercules, CA). Samples of protein (25 μg) were run on a sodium dodecyl sulfate 7.5% polyacrylamide gel and immunoblotted according to previous published methods [27-29]. Briefly, the separated protein was transferred to 0.45 μm-

supported nitrocellulose membranes (162–0094; BioRad) and blocked with 7.5% nonfat dry milk in Tris-buffered saline with Tween. The following primary antibodies were used: mouse anti-FKBP51 (610582, 1:500; BD Transduction Laboratories, Lexington, KY); mouse anti-NF-κB (SC-8008, 1:500; Santa Cruz, Santa Cruz, CA); mouse anti-GAPDH (P04406, 1:1000; Millipore, Billerica, MA); rabbit anti-phospho-NF-κB (3033, 1:500; Cell Signaling, Cell Signaling Technology, Inc, Danvers, MA); and mouse anti-β-tubulin (T0198, 1:1,000; Sigma, Sigma-Aldrich, St. Louis, MO). Primary antibodies were incubated and rotated overnight at 4 °C. Blots were washed for 30 min at room temperature. Prior to the addition of secondary antibodies ECL-rabbit IgG, HRP-linked, or ECL-mouse IgG, HRP-linked (NA9340 and NA9310, 1:10,000; GE, Piscataway, NJ) for 30 min. Luminescence was detected using SuperSignal West Dura (34075; Thermo Scientific, Waltham, MA) in the BioRad Molecular Imager. Densitometric analysis was performed using the Bio-Rad Image Lab. GAPDH and β-tubulin were used as loading controls.

Calcein-acetomethoxy/propidium iodide cell-survival assay

Cell viability was determined using a calcein/propidium iodide (cat. no. C3099; Invitrogen-Molecular Probes, Carlsbad, CA) dual-staining assay. The cell cultures were treated with or without 100 nM–10 μM staurosporine, an apoptosis-inducing agent [22- 24], for 24 h (ALX-380–014-M001; Enzo Life Sciences, Plymouth Meeting, PA). After treatment, the culture medium was removed, and the coverslips were rinsed with 1´ phosphate buffer saline (PBS). Then, 1 μM calcein and 2 μg/ml propidium iodide in 2 ml 1´ PBS were added to each culture well. The culture dishes with the cells were incubated at 37 °C for 60 min, and fluorescence was measured (Microphot FXA digital fluorescent microscope; Nikon, Melville, NY).

Caspase-3 detection assay

Caspase-3 activity was determined using the SR-DEVD-FMK Caspase-3 detection kit, Cell Technology Inc (Mountain View, CA) following the manufacturer's protocol. Coverslips were coated with 10 μg/ml Poly-D-Lysine for 60 min, washed, and then placed into wells with 500 μl of DMEM. Either 10,000 empty vector or FKBP51-overexpressing 661w cells were added to each well and incubated for 24 h. Staurosporine or a vehicle was added to each well to reach a final concentration of 10 nM, 100 nM, or 1 μM for 6 h. Cells were washed three times with PBS. Prepared caspase-3 detection reagent (300 μl) was added to each well and incubated for 60 min. Cells were washed three times with PBS and then inverted onto a slide with Fluorosave. Images were taken at the same exposure times on a fluorescence microscope (Microphot FXA digital fluorescent microscope; Nikon). Images were measured for intensity using Image J software.

Statistical analysis

SigmaPlot 11.0 (Systat Software Inc., San Jose, CA) was used to perform all statistical analyses. Results were expressed as mean ± standard error. Paired comparisons were analyzed using a Mann–Whitney U test. Multiple comparisons were performed using a one-way analysis of variance (ANOVA) followed by a Mann–Whitney U test. Significance was defined as a p value of ≤ 0.05.

RESULTS

FKBP51 expressed in rat retina and brain. An adult Sprague Dawley rat was sacrificed and the brain, retina, heart, lung, liver, and kidney were collected in HEPES buffer containing protease inhibitors (1 mM DTT an 500 μM PMSF). Tissue and cells were lysed using sonication. Fifty micrograms of protein were added to each well. An anti-FKBP51 antibody (Cat: 610582; BD Transduction Laboratories) was used to detect FKBP51 protein. FKBP51 was detected in all tissues that were analyzed (Figure 1).

FKBP51 overexpression increased protein levels of NF-κB and activated NF-κB and decreases protein levels of IκBα. FKPB51 is expressed in many organs, including the retina (Figure 1). We used western blot analysis to determine the expression of FKBP51 and the potential downstream signaling molecules NF-κB p65 and phosphorylated NF-κB (n=6). FKBP51 was overexpressed 5 fold in FKBP51 transfected cells, compared to cells transfected with the empty vector controls. The overexpression of FKBP51 also increased protein levels of phosphorylated NF-κB p65 by 3.2±0.5 fold and NF-κB p65 by 18±14 fold. β-tubulin (IκBα and NF-κB p65) or GAPDH (FKBP51 and phosphor NF-κB p65) was used for normalization (Figure 2).

FKBP51 overexpression protected 661w cell culture from staurosporine. We wanted to determine whether an increase in FKBP51 protein would protect neuronal cell cultures from

staurosporine, the apoptosis-inducing agent. Cell viability was determined using calcein AM/propidium iodide double-staining. In the absence of staurosporine, 99%–100% of the FKBP51 and empty-vector transfected cells remained viable. The addition of 10 nM staurosporine for 24 h did not induce a significant level of apoptosis in either cell culture. FKBP51 cells had 98±0.02% viability, and 99±0.01% of empty vector cells survived. In contrast, the addition of 100 nM staurosporine induced cell death in both FKBP51-overexpressing and empty-vector cell cultures. However, FKBP51 overexpression significantly protected the 661w neuronal cells from 100 nM staurosporine-induced apoptosis (83±0.02% viability in FKBP51 cells versus 71±0.04% viability in control cells, p=0.013). Furthermore, FKBP51 overexpression significantly protected the 661w neuronal cell culture from the 24 h of 1 μM staurosporine-induced apoptosis. The FKBP51 overexpression significantly protected the neuronal cells (62±0.03% viability in FKBP51 cells versus 12±0.02% viability in control cells, p≤0.001; Figure 3).

FK506 treatment increased FKBP51 and NF-κB protein levels. Western blot analysis was used to determine the protein expression of FKBP51 and NF-κB p65 after 24 h of FK506 treatment (n=3). FK506 dose-dependently increased FKBP51 and NF-κB protein levels. FKBP51 was significantly elevated with 10 μM FK506 (p=0.023) and NF-κB at 100 nM (p=0.004). All three concentrations of FK506 (0.1 μM, 1 μM, and 10 μM) increased FKBP51 protein expression by 1.36±0.3 fold, 2.7±0.9 fold, and 2.6±0.4 fold, respectively. Furthermore, the same FK506 concentrations increased NF-κB p65 protein levels by 1.58±0.1 fold, 2.42±0.21 fold, and 5.6±0.4 fold, respectively. GAPDH was used as an equal loading control (Figure 4).

Additionally, there were no changes to phosphorylated NF-κB p65 protein levels when analyzed during the 24 h treatment (unpublished data).

One micromole FK506 treatment increased activation of NF-κB. NF-κB is a nuclear transcription factor with diverse activities, including the regulation of cell survival [30]. The majority, but not all, of the available research indicates that NF-κB increases anti-apoptotic actions and prevents cell death in various cells [31-33]. It would be helpful to determine if FK506, like FKBP51, activates NF-κB, because it may be a common downstream molecule activated by both FK506 and FKBP51 to achieve neuroprotection. Western immunoblot analysis determined that the FK506 (1 μM) phosphorylates NF-κB p65 after 30 min, 1 h, 2 h, and and 4 h (n=6 at each timepoint). FK506 significantly increased the phosphorylation of NF-κB p65 2.2±0.5 fold (p=0.004) after 30 min. It was also determined that phosphorylated NF-κB p65 returned to baseline after 1 h, 2 h, and 4 h. β-tubulin was used as the equal-loading control. Therefore, the protein levels of NF-κB p65 increased after 24 h of treatment, while the phosphorylated form of NF-κB p65 increased sooner (Figure 5).

FK506 protected 661w cell cultures from staurosporine-induced cell death. FK506 is neuroprotective against several forms of toxicity as well as in several in vivo [5,6] and in vitro models [7,8]. Although FK506 is significantly neuroprotective outside of the eye, we want to determine whether FK506 protects ocular neuronal cell cultures from staurosporine-induced-apoptosis. The 661w neuronal cell cultures were processed for determination of apoptosis using calcein AM/propidium iodide double-staining [34] following 1 μM staurosporine and 1 μM

FK506 treatments. Virtually all 661w neuronal cell cultures were alive when untreated or treated with 1 μM FK506. In contrast, when the 661w neuronal cell cultures were exposed to 1 μM staurosporine for 24 h, only 28±0.05% of the cell cultures survived. The addition of 1 μM FK506 significantly protected the 661w neuronal cell cultures ($p \leq 0.001$) from 1 μM staurosporine-induced apoptosis over the 24 h treatment, by increasing the survival rate to 95±0.01 (p=012; Figure 6).

FKBP51 overexpression protected 661w cell culture from staurosporine induced caspase-3 activation. Apoptosis accounts for most of the neuronal cell death observed during neurologic disorders [3]. It would be advantageous to prevent apoptosis in order to uniformly treat a wide range of neurologic disorders. Staurosporine was used to induce apoptosis [22-24]. We investigated whether an increase of FKBP51 protein protected against caspase-3 activation, a common marker of apoptosis. Six hours of staurosporine treatment was used to induce a significant amount of caspase-3 activation. FKBP51 overexpression significantly decreased the amount of caspase-3 activation at all three concentrations of staurosporine: 10 nM (p=0.006), 100 nM (p=0.001), and 1 μM ($p<0.001$; Figure 7).

DISCUSSION

FKBP51 has diverse physiologic functions. FKBP51 is a chaperone protein that aids in the transport of several hormones and hormone receptors. It is increased in several forms of cancer. It may also be increased in prostate cancer patients. This increase of FKBP51 is a suggested cause for positive feedback from androgen and the androgen receptors. This promotes cell survival and growth in these non-neuronal cells [35-37]. An increase in FKBP51 protein levels has been shown to cause resistance to chemotherapeutic agents that induce apoptosis in cancerous tumors [35,38]. Even though this is a disease state, in which homeostasis is out of balance, it would be advantageous to more thoroughly understand whether increases in FKBP51 activity can promote cell survival and growth. In neurons, FKBP51 promotes microtubule stability and elongation. FKBP51 works with Hsp90 to bind phosphorylated tau [39]. FKBP51 catalyzes the cis-trans isomerization of the peptidyl–prolyl bonds (the PPIase reaction), allowing tau to be recycled. With a mutant or defective FKBP51 molecule, the PPIase reaction will not occur, causing an accumulation of phosphorylated tau proteins, potentially leading to an Alzheimer's disease-like state [39]. Furthermore, FKBP51 is involved in several cell-signaling pathways that promote cell survival and neuroregeneration [16,40]. FKBP51's diverse physiologic functions, including its prosurvival properties, make it an important molecule to continue researching.

In this study, we have shown that FKBP51 neuroprotects 661w neuronal cell lines from the apoptosis-inducing agent, staurosporine. Even though FKBP51 has been well researched for its

protective properties outside of the central nervous system, its protective properties within neurons need more research [16,17]. FKBP51's neuroprotective efficacy and function in other in vitro and in vivo models of ocular neurodegeneration need to be tested to determine its potential for treating neurodegenerative diseases and neurotrauma. The downstream molecules that FKBP51 interacts with appear to be similar to those that interact with FK506. Furthermore, a drug that targets FKBP51 without inhibiting calcineurin through FK506 and causing systemic immunosuppression would be beneficial. A drug that is a potential candidate is GPI1046. GPI1046 is an FK506 analog, a non-immunosuppressive immunophilin ligand that appears to have the same neuroprotective properties [12]. GPI1046 displays neurosurvival and regenerative activities in vivo and in vitro [41,42]. Further investigation of GPI1046 is needed to determine its value not only as a neuroprotective molecule, but also as an aid to characterizing immunophilins such as FKBP51.

FKBP51 is an important coactivator of the NF-κB signaling pathway [34]. NF-κB has both detrimental and prosurvival effects in neurons. NF-κB activity still remains an important signaling molecule to investigate. Many argue that NF-κB has a large effect on prosurvival genes, supported by the finding that NF-κB knockouts are lethal during development [30,43,44]. This present study demonstrates that an increase of FKBP51 can increase protein levels and the activation of NF-κB's major subunit, NF-κB p65. Interestingly, FK506's effect on NF-κB in neurons is still controversial [45,46]. Nevertheless, 1 μM of FK506 caused a phosphorylation of NF-κB p65 in 661w neuronal cell cultures after 30 min. The mechanism of this activation is still

not understood; however, it may occur through FKBP51. Additional testing is needed to determine whether FKBP51 is essential to FK506 phosphorylation of NF-κB p65.

In summary, we have shown that an increase of FKBP51 protein protects 661w neuronal cell cultures from the apoptosis-inducing agent staurosporine. Both FK506 and FKBP51 share similar downstream signaling molecules, suggesting that utilization of FKBP51 by FK506. FKBP51 has has diverse physiologic functions in promoting several prosurvival pathways. A potential therapeutic intervention is to increase the function of FKBP51, which could increase the stability and duration of several FKBP51 downstream molecules to maintain or even increase cell survival.

ACKNOWLEDGEMENTS

Supported in part by NIH Grant T32AG020494 and NIH Grant 1R01EYZ16242. We would also like to thank Yong Park for his technical help, Dr. Xinyu Zhang for help with tissue localization of FKBP51 and Dr. Abbot Clark for his helpful discussions.

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Figure 1. FK506 (tacrolimus) binding protein 51 is expressed in rat retina and brain. Tissues were extracted from an adult Sprague Dawley rat and the protein was isolated. FK506 binding protein 51 (50 μg) was added to each lane. Western blot analysis detected the protein in the brain, heart, lung, liver, kidney, and retina.

FIGURE 1

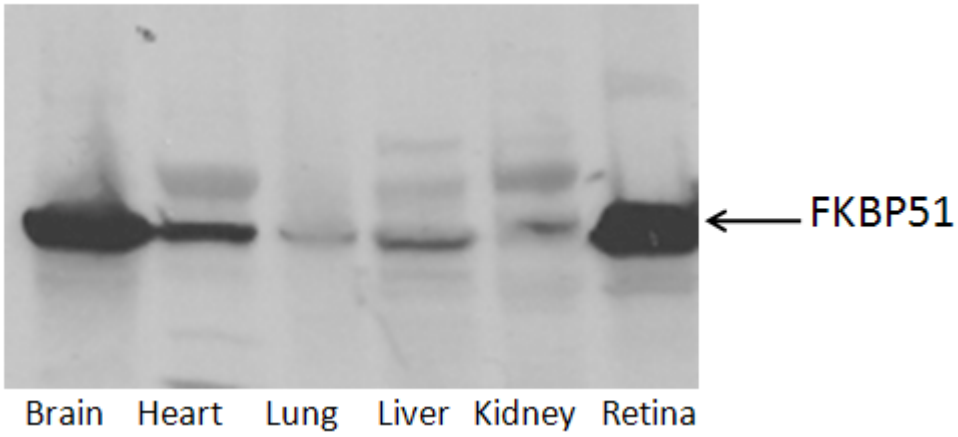


Figure 2. FK506 (tacrolimus) binding protein 51 overexpression increases protein levels of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), activated NF-κB, and decreased protein levels of inhibitory molecule, IKappaB (IκB). A: FK506 Binding Protein 51 (FKBP51) overexpression significantly increased NF-κB p65 and phospho NF-κB p65 protein levels while significantly decreasing IκBα protein levels. The 661w neuronal cell culture—stably transfected with an FKBP51 expression vector— increased FKBP51 protein levels fivefold, compared to the 661w neuronal cell culture stably transfected with the parental empty vector. (-) represents stably transfected empty vector control cells and (+) represents stably transfected FKBP51 overexpression cells. **B**, **C**: The increase in FKBP51 expression caused a significant increase in protein levels of NF-κB p65 and activated NF-κB p65 (NF-κB p65: n=6, *p=0.002; phospho NF-κB p65: n=6, *p=0.002). **D**: The increase in FKBP51 expression caused a significant decrease in protein levels of IκBα (IκBα: n=3, *p=0.044). The loading controls were β-tubulin for IκBα and NF-κB p65, while GAPDH was used for FKBP51 and for activated NF-κB p65. Significance was obtained through one-way analysis of variance (ANOVA) and the Mann–Whitney test. Error bars represent SEM.

FIGURE 2:

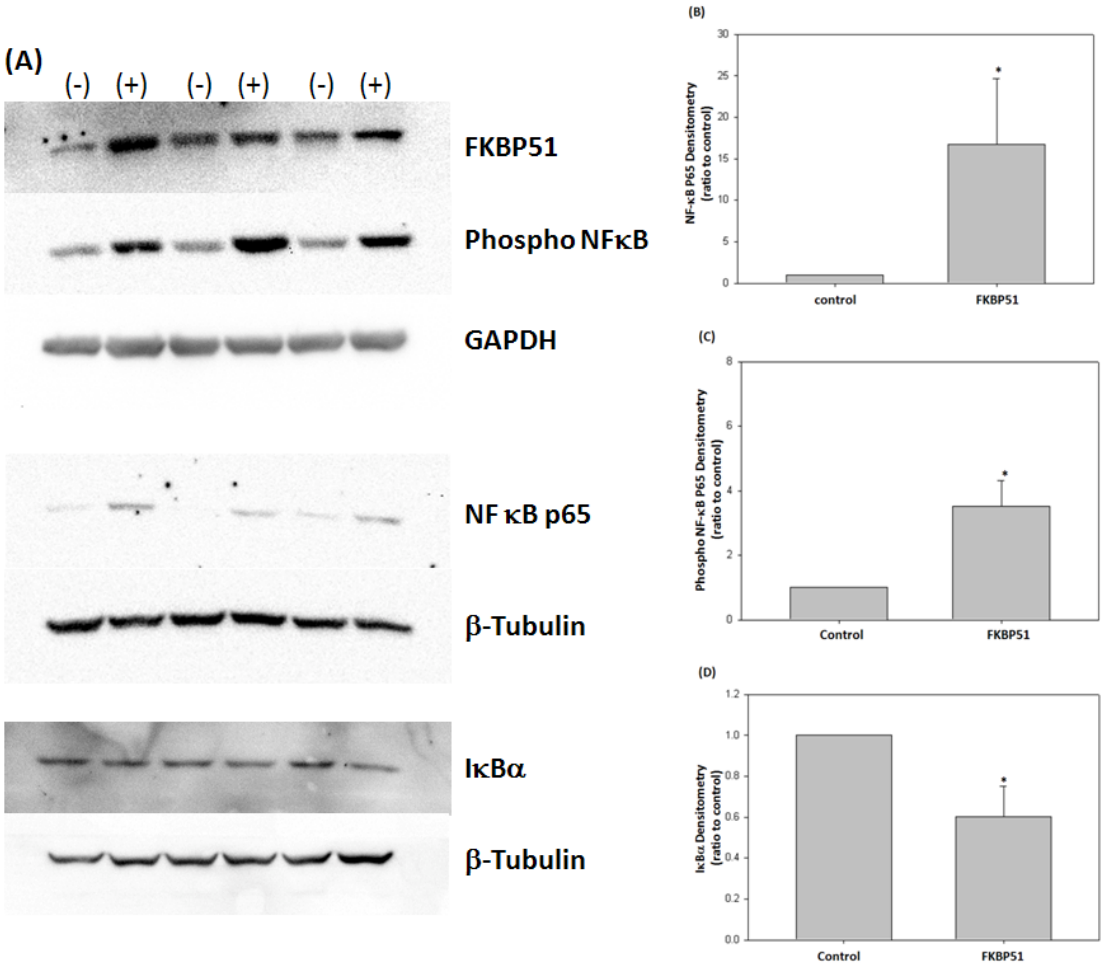

(A)
(-) (+) (-) (+) (-) (+)
FKBP51
Phospho NFκB
GAPDH
NF κB p65
β-Tubulin
IκBα
β-Tubulin
(B)
NF-κB P65 Densitometry (ratio to control)
control
FKBP51
(C)
Phospho NF-κB P65 Densitometry (ratio to control)
Control
FKBP51
(D)
IκBα Densitometry (ratio to control)
Control
FKBP51

Figure 3. FK506 (tacrolimus) binding protein 51 overexpression protects 661w cell culture from staurosporine. **A**: The FK506 Binding Protein 51 (FKBP51) protein significantly protected the 661w neuronal cell culture from staurosporine-induced apoptosis. Cell survival was monitored with a calcein-AM/propidium iodide cell-survival assay. The 661w neuronal cell cultures were stably transfected with either an FKBP51 expression vector or parental empty vector. Cells were treated with or without varying concentrations of staurosporine (10 nM, 100 nM, and 1 μM) for 24 h (scale bar=100 μm). **B**: FKBP51 overexpression significantly protected the 661w neuronal cell culture from staurosporine-induced apoptosis at 100 nM ($p=0.013$) and 1 μM ($p \square 0.001$) concentrations (n=3). Error bars represent SEM.

FIGURE 3:

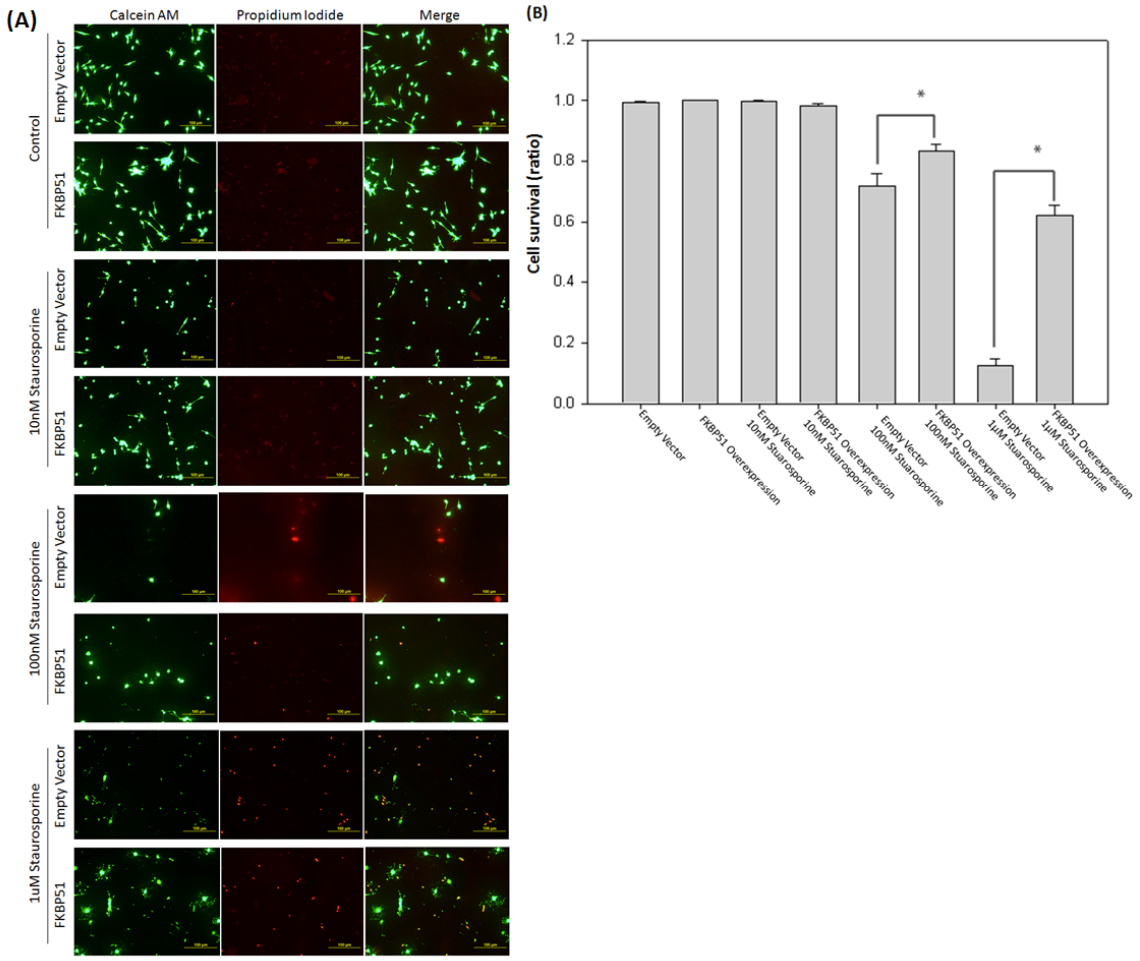

(A)
Calcein AM
Propidium Iodide
Merge
Control
Empty Vector
FKBP51
10nM Staurosporine
Empty Vector
FKBP51
100nM Staurosporine
Empty Vector
FKBP51
1uM Staurosporine
Empty Vector
FKBP51
(B)
Cell survival (ratio)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Empty Vector
FKBP51 Overexpression
Empty Vector 10nM Staurosporine
FKBP51 Overexpression 10nM Staurosporine
Empty Vector 100nM Staurosporine
FKBP51 Overexpression 100nM Staurosporine
Empty Vector 1μM Staurosporine
FKBP51 Overexpression 1μM Staurosporine

Figure 4. FK506 (tacrolimus) treatment increases FK506 binding protein 51 (FKBP51) and nuclear factor kappa-light-chain-enhancer of activated B (NF-kB) cell protein levels. A: Twenty-four hour FK506 treatments significantly increased protein levels of FKBP51 and NF-κB in the 661w neuronal cell cultures. The 661w neuronal cell cultures were treated for 24 h with a vehicle—100 nM FK506, 1 μM FK506, or 10 μM FK506. Cell lysate proteins (25 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), transferred to membranes, and immunoblotted for FKBP51, NF-κB, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The quantification of band intensity is represented as a percentage of FKBP51 or NF-κB of its corresponding GAPDH control band on the same membrane. **B**: FK506 significantly increased FKBP51 protein levels at a concentration of 10 μM ($p=0.03$), while 0.1 μM and 1 μM caused an increased in FKBP51 protein levels; however, significance was not obtained ($n=3$). **C**: Additionally, FK506 significantly increased NF-κB protein levels at a concentration of 0.1 μM ($p=0.004$), while 1 μM and 10 μM caused larger increases in protein levels; however, significance was not obtained ($n=3$ for each dose indicated).

FIGURE 4

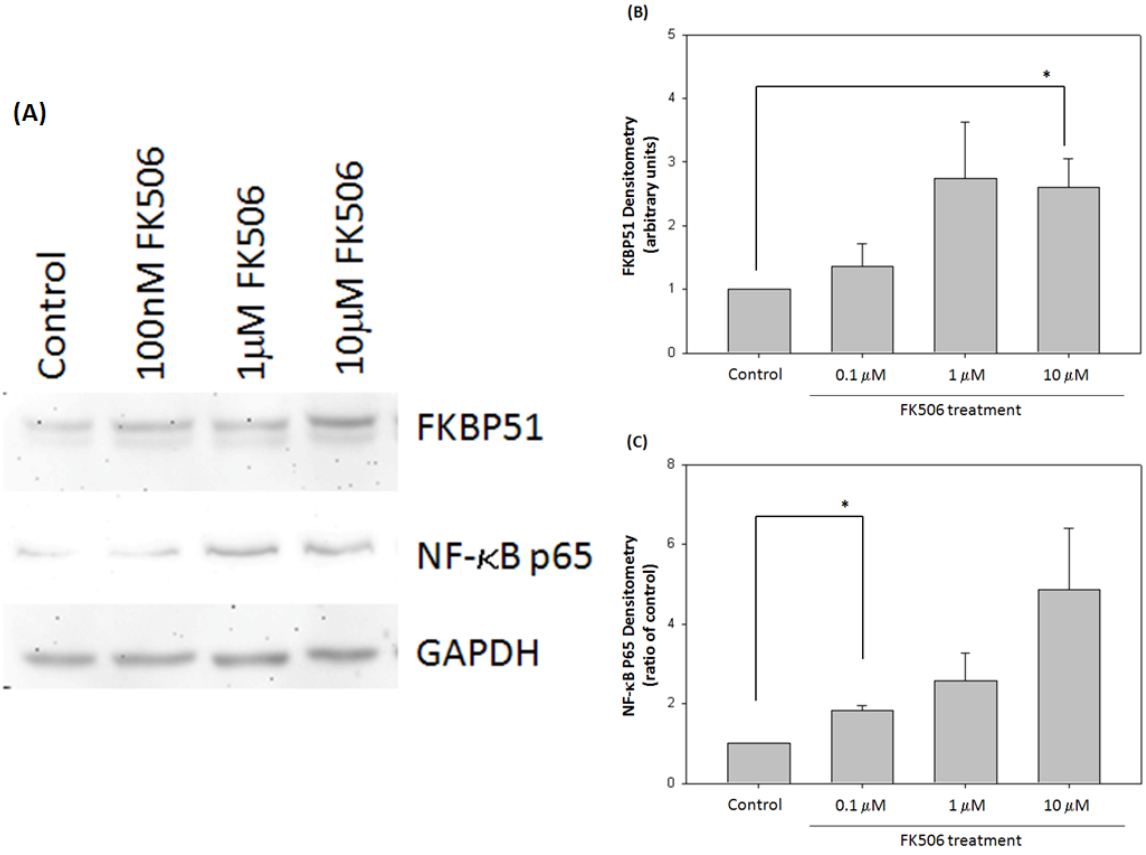

(A)
Control
100nM FK506
1μM FK506
10μM FK506
FKBP51
NF-κB p65
GAPDH
(B)
FKBP51 Densitometry (arbitrary units)
Control
0.1 μM
1 μM
10 μM
FK506 treatment
(C)
NF-κB P65 Densitometry (ratio of control)
Control
0.1 μM
1 μM
10 μM
FK506 treatment

FIGURE 5: **1 μM FK506 treatment increases the activation of NF-κB (A)** 1 μM FK506 treatment for 30 minutes significantly activated NF-κB protein in 661w neuronal cell culture. 661w neuronal cell cultures were treated with 1 μM FK506 or control (DMSO) for 30 minutes, 1 hour, 2 hours, or 4 hours. Cell lysate proteins (25 ug) were separated by western immunoblot analysis for phosphor-NF-κB and β-tubulin. The quantification of band intensity are represented as a percentage of phospho NF-κB to its corresponding control (β-tubulin) band on the same membrane. **(B)** 1 μM FK506 significantly increased Phospho NF-κB protein levels (P=0.004) at 30 minutes while phospho NF-κB protein levels returned near basal levels within 2 hours of FK506 treatment (n=6).

Figure 5

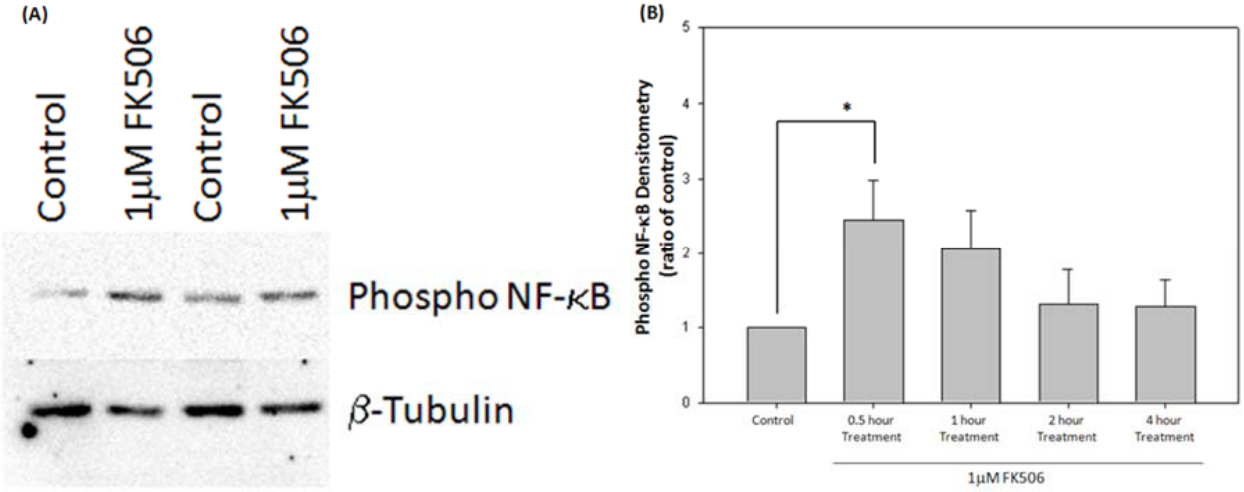

(A)
Control
1μM FK506
Control
1μM FK506
Phospho NF-κB
β-Tubulin
(B)
Phospho NF-κB Densitometry (ratio of control)
0
1
2
3
4
5
*
Control
0.5 hour Treatment
1 hour Treatment
2 hour Treatment
4 hour Treatment
1μM FK506

Figure 6. FK506 (tacrolimus) protects 661w cell cultures from staurosporine-induced cell death. A: FK506 (1 μM) significantly protected 661w neuronal cell cultures from staurosporine-induced apoptosis. Cell survival was monitored with the calcein- AM/propidium iodide cell-survival assay (scale bar=100 μm). The 661w neuronal cell cultures were treated with 1 μM FK506, with 1 μM staurosporine, with both 1 μM FK506 and 1 μM staurosporine, or with vehicle for 24 h. **B**: The 1 μM FK506 treatments significantly protected 661w neuronal cell cultures from 1 μM staurosporine-induced apoptosis ($p<0.001$, $n=3$). Error bars represent SEM.

FIGURE 6:

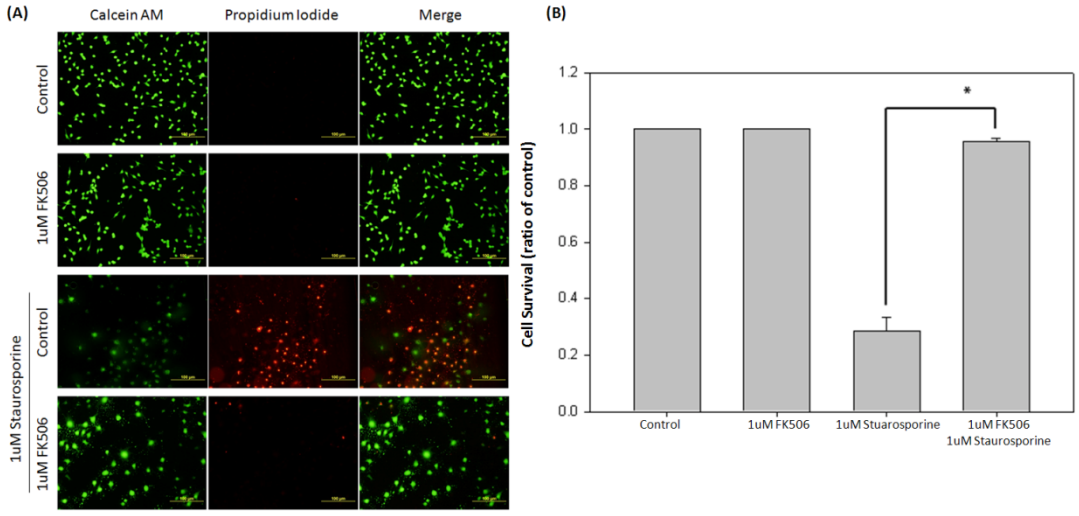
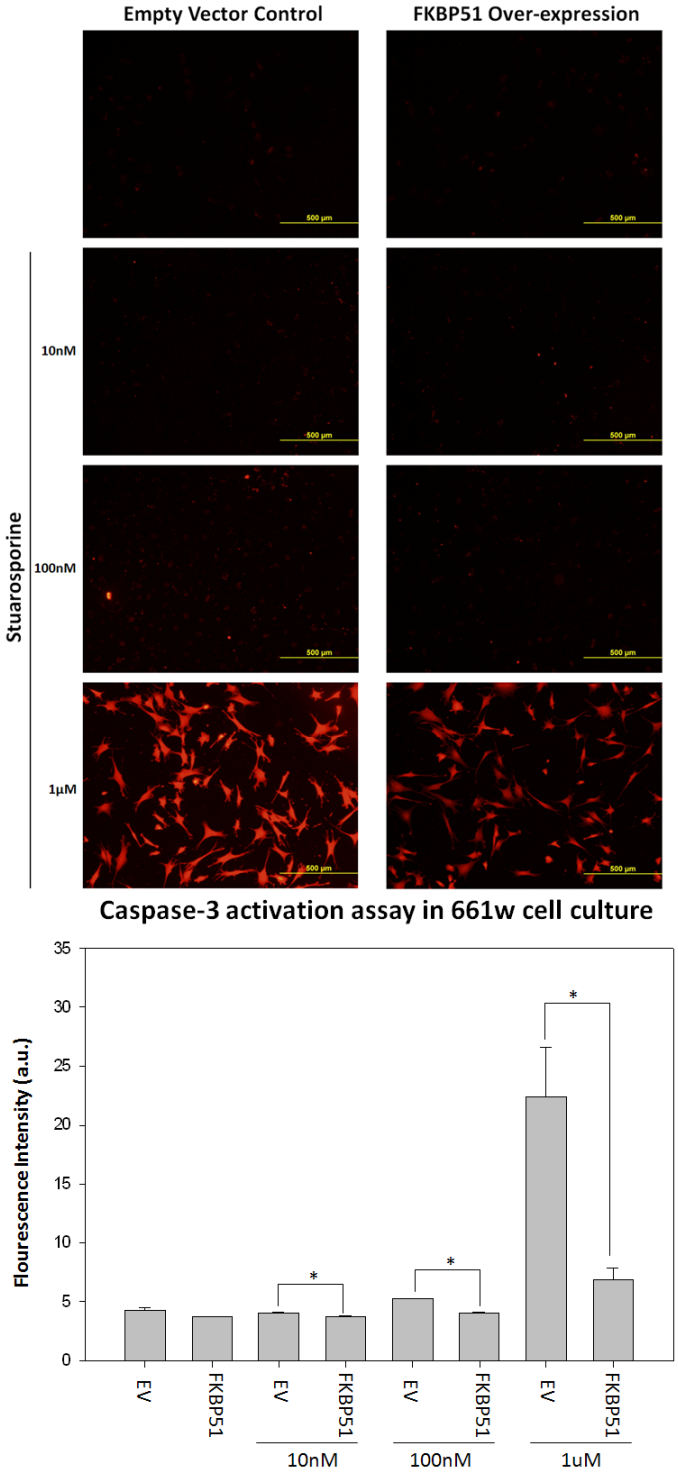

(A)
Calcein AM
Propidium Iodide
Merge
Control
1uM FK506
1uM Staurosporine
Control
1uM FK506
(B)
Cell Survival (ratio of control)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
1uM FK506
1uM Stuarosporine
1uM FK506
1uM Staurosporine

Figure 7. FK506 binding protein 51 overexpression protects 661w cell culture from staurosporine-induced caspase 3 activation. Staurosporine treatments for 6 h significantly increased caspase 3 activation, a marker of apoptosis, in 661w cell culture. FKBP51 overexpression in 661w cell cultures significantly decreased caspase 3 activation during staurosporine treatments. Caspase 3 activity was assayed using a colorimetric caspase 3 detection kit. Intensity was measured using Image J. FKBP51 significantly protected against caspase 3 activation during staurosporine treatment at 10 nM ($p=0.006$, $n=3$), 100 nM ($p=0.001$, $n=3$), and 1 μM ($p<0.001$). Significance was obtained through analysis of variance (ANOVA) and the Mann-Whitney test. Error bars represent SEM.

FIGURE 7:


Empty Vector Control
FKBP51 Over-expression
Stuarosporine
10nM
100nM
1µM
Caspase-3 activation assay in 661w cell culture
Flourescence Intensity (a.u.)
35
30
25
20
15
10
5
0
*
*
*
EV
FKBP51
EV
FKBP51
EV
FKBP51
EV
FKBP51
10nM
100nM
1uM

CHAPTER III

Methylene Blue Protects Primary Rat Retinal Ganglion Cells from Cellular Senescence

Donald R. Daudt III, Brett Mueller, Yong H. Park, Yi Wen and Thomas Yorio

(to be submitted)

ABSTRACT

Purpose: Glaucoma is a progressive optic neuropathy characterized by loss of retinal ganglion cells (RGC) and optic nerve degradation. Existing treatments focus on lowering intraocular pressure (IOP); however, vision loss may still progress. Neuroprotective drugs may be useful as an adjunct approach to prevent further loss of RGCs; though, efficacious drugs are lacking. One agent, methylene blue has been shown to protect neurons in several neurodegenerative models. Methylene blue potentiates the electron transport chain by shuttling elections from NADH and FADH2 to coenzyme Q (CoQ) and cytochrome c. The purpose of this study was to determine if methylene blue can protect RGCs from noxious stimuli. **Methods:** Primary rat RGCs were isolated and cultured following a sequential immunopanning technique using P3-P7 Sprague-Dawley rats. Approximately 25,000 RGCs were seeded per coverslip and cultured for 3 days before testing. The RGCs were treated for 24 hours with rotenone or staurosporine or for 72 hours of hypoxia. Methylene blue was then assessed for protection of RGCs during each of these insults. Cell viability was measured using calcein Am and ethidium homodimer-1. Cytochrome c oxidase activity was measured using a cytochrome c oxidase assay kit to monitor the health of mitochondria. **Results**: Methylene blue (1μM and 10μM) significantly protected RGCs against 24 hours of 1μM rotenone. Methylene blue (1μM and 10μM) significantly protected RGCs against 24 hours of 1μM staurosporine. Methylene blue significantly protected RGCs against 72 hours of hypoxia. Methylene blue increased activity of complex IV, cytochrome c oxidase, of the mitochondria in the prescence of hydrogen peroxide. **Conclusion**: Methylene blue is a neuroprotective compound that can protect retinal ganglion cells. Methylene

blue's ability to protect RGCs during rotenone, hypoxia, and staurosporine supports its suggested mechanism of action of preserving the electron transport chain. Further testing is needed to determine if methylene blue would be an efficacious treatment for the protection of neurodegeneration that occurs during optic neuropathy.

INTRODUCTION

Glaucoma is an optic neuropathy characterized by apoptosis of retinal ganglion cells (RGCs), cupping of the optic disc and progressive deterioration of optic nerve axons resulting in impaired structure and function. Glaucoma is the second leading cause of blindness worldwide. Glaucoma is defined as the impairment of structure and function of the optic nerve, which results in death of the retinal ganglion cells. During glaucoma, intraocular pressure (IOP) may be elevated which is believed to cause some of the pathology observed during this neurodegenerative disease. Due to increased lifespan, the prevalence of glaucoma is projected to increase to 3.6 million by 2020 (Friedman et al., 2004). Currently, treatments aim to increase aqueous humor outflow or decrease aqueous humor formation through surgical or drug intervention. However, vision loss may still progress in these patients. The exact mechanisms causing loss of retinal ganglion cells are unknown; however, the axons forming the optic nerve become strained between lamina cribrosa cells, resulting in neurotrophin deprivation. The lack of neurotrophin support leads to apoptosis, also known as programmed cell death (Stuart et al., 2008; Varma et al., 2008; Levin and Peeples 2008; Tezel and Wax 2007).

Methylene blue (MB) is a potential neuroprotective intervention for the treatment of retinal ganglion cell (RGC) death during glaucoma. MB is already approved by the Food and drug Administration to treat several ailments, including methemoglobinemia (Clifton and Leikin, 2003). Chronic treatment with MB is safe and can protect against mental disorders and prevents encephalopathy in humans undergoing chemotherapy (Naylor et al., 1988; Kupfer et al., 1994; Kupfer et al., 1996; Pelgrims et al., 2000). MB is an autoxidizable phenothiazine with potent antioxidant activity with metabolic enhancing properties (Bruchey and Gonzalez-Lima, 2008).

Methylene blue has a high bioavailability in the brain and readily crosses the blood brain barrier (Peter et a., 2000; Wainwright and Crossley, 2002). Additionally, clinical trails are being performed with chronic MB treatment to prevent the progression of Alzheimer's disease (Wischik et al., 2009).

Methylene blue protects against several models of neurodegenerations *in vivo*; including amnestic mild cognition impairment, neurotoxin-induced impairment, and optic neuropathy (Riha et al., 2011; Rojas et al., 2009; Zhang et al., 2006; Rojas 2009). In rats, methylene blue improves discrimination learning, facilitates the extinction of fear, improves brain oxidative metabolism and memory retention (Callaway et al., 2004; Wrubel et al., 2007; Bruchey and Gonzalez-Lima 2008; Zhang et al., 2006). In mice, optic neuropathy was induced using rotenone, which causes mitochondrial dysfunction. The experimental eye that received intravitreal injections of MB maintained visual acuity when compared to the control contralateral eye. MB also maintained structure and function of the retina during rotenone treatments (Rojas et al., 2009). Furthermore, MB protected the integrity of the retinal ganglion cell layer (Zhang et al., 2006).

MB is a potent metabolic enhancing and antioxidant agent that facilitate memory and promotes neuroprotection (Callaway et al., 2004; Riha et al., 2005; Gonzalez-Lima and Bruchey 2004; Zhang et al., 2006). MB is capable of both reduction and oxidation and such supports electron cycling. This property allows MB to transfer electrons to oxygen, which is an essential process that exists in the electron transport chain of the mitochondria (Calabrese et al., 2007). MB cannot enter a neuron until it is reduced (MBH2) at the cell surface. Once MBH2 enters the cell it can be re-oxidized back into MB, maintaining it within the cell (Bongard et al., 1995).

MB may be re-oxidized by a heme-protein such as cytochrome c or cytochrome c oxidase (Atamna et al., 2007). Within the cell MB and MBH2 are maintained at equilibrium making a reversible reduction-oxidation system (May et al., 2004). A number of neurodegenerative diseases are potentiated by mitochondrial dysfunction (Lin and Beal, 2006); so, MB's electron coupling ability could protect to neuronal cells during neuronal disease states. Investigating methylene blue's protective properties during a model of retinal ganglion cell death could lead to a novel, inexpensive and efficacious treatment of glaucoma.

MATERIALS AND METHODS

All animal procedures were performed in accordance and with the approval of the University of North Texas Health Science Center Institutional Animal Care and the ARVO Animal Use Committee guidelines. Female time-pregnant Sprague-Dawley rats were obtained from Charles River (Wilmington, Massachusetts, United States). Primary rat RGCs were isolated and cultured following a sequential immunopanning technique using P3-P7 Sprague-Dawley rats (Barres et al., 1988). Retinal ganglion cells were positively selected using a T11D7 (anti-thy1) antibody while macrophages were isolated using an antimacrophage antibody (CLAD51240 Cedarlane Laboratories). Primary RGCs were seeded 25,000 per coverslip, which were pretreated with poly-D-lysine (P6407 Sigma-Aldrich) and mouse laminin (3400-010-01 Trevigen-Celtrex). Cells were incubated at 37°C in 10% CO2 and air for all experiments, unless otherwise stated.

Immunocytochemistry

Dr. Ben Barres trained our laboratory to perform his method of primary retinal ganglion cell isolation, which yields 99.7% ± 0.3 pure cultures of RGCs (Barres et al., 1988). Primary RGCs were isolated and cultured for 7 days. Coverslips, containing RGCs, were washed 3X with PBS followed by incubation with cold 100% methanol for 20 minutes. Cells were washed 3X with PBS and then blocked using 5% Donkey serum in antibody buffer for 1 hour. Blocking solution was removed and cells were incubated with primary antibodies 1:10 anti-Thy 1.2 (550543 BD Pharmingen) and 1:100 anti-GFAP (SC-6170 Santa Cruz Biotechnology). Cells were incubated overnight at 4°C. Cells were washed 3X and then incubated with donkey or goat secondary antibodies for 2 hours at room temperature. Cells were washed 3X using PBS and then mounted with 15μL of prolong gold antifade reagent and DAPI. Images were taken on a Confocal Laser Scanning Microscope 510 Meta (Carl Zeiss, Maple Grove, Minnesota).

Calcein-acetomethoxy/ethidium homodimer-1 cell-survival assay:

Primary rat retinal ganglion cells were cultured for 72 hours post isolation before subjection to any treatment. Upon completion of treatment, cell viability was determined using LIVE/DEAD Viability/Cytotoxicity Kit, ethidium homodimer-1 and calcein AM (Invitrogen L3224, Carlsbad, California, United States). Cultured primary RGCs were washed 3X with phosphate buffered saline (PBS). Cells were incubated in 2μM calcein AM and 1μM ethidium bromide homodimer-1 in PBS at 37°C for 20 minutes. Cells were washed 3X in PBS and then mounted on slides containing fluorosave (345789 EMD Biosciences, Gibbston, NJ).

Fluorescence was measured on a Microphot FXA digital fluorescent microscope (Nikon, Melville, NY). All cell survival assays were counted by an individual that was masked to the experimental design.

Cytochrome c Oxidase Assay Kit:

Thc Cytochrome c Oxidase Assay Kit (CYTOCOX1 Simga, Saint Louis, Missouri) was used to measure cytochrome c activity. The assay kit works by converting ferrocytochrome c to ferricytochrome c by cytochrome c oxidase. Ferrocytochrome c is measureable at 550 nm while ferricytochrome c is not. This means, the greater amount of functional cytochrome c oxidase the greater the conversion of Ferrocytochrome c to ferricytochrome c and thus a decrease in absorbance at 550nm. RGCs were seeded 450,000 per well of a six well dish. Primary RGCs were cultured for 7 days at 37°C at 10% C02 and air. Then the RGCs were treated with a vehicle, 500 μM hydrogen peroxide (216763 Sigma, Saint Louis, Missouri), methylene blue (NDC 17478-504-10 Akorn), or both for 2 hours. Cells were removed using trypsin (T-9201 Sigma-Aldrich) and spun down at 1,000 RPM for 5 minutes. Liquid was removed from pellet. Before each assay, photospectometer was blanked using 900 μL of assay buffer and 50 μL of suspended pellet. Then 50μL of ferrocytochrome c substrate solution was added and mixed through inversion. Assay measurements at 550 nm were taken every 10 seconds for 120 seconds. The change in ferrocytochrome c was expressed as initial absorbance / final absorbance relative to the control primary RGCs.

Statistical Analysis

SigmaPlot 11.0 (Systat Software Inc., San Jose, CA) was used to perform all statistical analyses. Results were expressed as mean ± standard error. Paired comparisons were analyzed using a Mann-Whitney U test. Multiple comparisons will be performed using a one-way analysis of variance (ANOVA) followed by a Mann-Whitney U test. Significance was defined as a p value of ≤0.05.

RESULTS:

Primary Rat Retinal Ganglion Cell Culture

Retinal tissue was collected from P3 to P7 Sprague Dawley rat pups. Macrophages were selected through an anti-macrophage antibody and removed. Retinal ganglion cells were collected using the T11D7 (anti thy1) antibody. After 7 days in culture, the primary retinal ganglion cell cultures were incubated with DAPI, a thy1.2 antibody (red), and a GFAP antibody (green). Virtually all cells were expressed the thy1.2 antigen, while none of the cells were GFAP positive.

Rotenone Toxicity Concentration-Response

Rotenone induces mitochondrial dysfunction by inhibiting complex I of the electron transport chain (Zhu et al., 2011). Cell viability was determined using calcein AM/ethidium homodimmer-1 double staining. The vehicle treated primary RGCs were set at 100% survival and used to standardize each rotenone treatment group. The addition of 100 nM rotenone for 24

hours left 59% of the RGCs viable (P<0.001). The addition of 1 μM rotenone for 24 hours left 39% of RGCs viable (P<0.001). The addition of 10 μM rotenone for 24 hours left 7% of RGCs viable (P<0.001). The concentration 1 μM was used for all sequential rotenone experiments (Figure 2).

Methylene Blue Protects Primary Retinal Ganglion Cells Against Rotenone Toxicity

Rotenone induces mitochondrial dysfunction by inhibiting complex I of the electron transport chain (Zhu et al., 2011). Cell viability was determined using calcein AM/ethidium homodimmer-1 double staining. Untreated cells were used as a control to quantitate the average amount of viable cells following primary RGC isolation. Results are expressed as a ratio (survival % / Untreated survival %). The addition of 1 μM rotenone for 24 hours left 41% of RGCs viable viable. The addition of 100 nM methylene blue did not induce a significant level of protection from 1 μM rotenone (43.0% RGC survival). However, 1 μM and 10 μM methylene blue significantly protected against 1 μM rotenone toxicity (67% survival, P<0.001, and 68% survival, P<0.001) (Figure 3).

Staurosporine Toxicity Concentration-Response

Staurosporine is an apoptosis inducing agent and was used to determine if methylene blue can protect of primary retinal ganglion cells against this cytotoxic insult. Cell viability was determined in the presence and absence of staurospoine. The addition of 10 nM staurosporine for 24 hours left 43% of RGCs viable (P<0.001). The addition of 100 nM staurosporine for 24 hours left 41% of RGCs viable (P<0.001). The addition of 1 μM staurosporine for 24 hours left

35% of RGCs viable (P<0.001). The addition of 10 μM staurosporine for 24 hours left 0% of RGCs viable (P<0.001). The 100 nM staurosporine concentration was used for all subsequent staurosporine/cell viability experiments (Figure 4).

Methylene Blue Protects Primary Retinal Ganglion Cells Against Staurosporine

The addition of 100 nM staurosporine for 24 hours left 17% of cells viable. The addition of 100 nM staurosporine in the prescence of 100 nM methylene blue did not induce a significant level of protection whereas, 1 μM and 10 μM methylene blue significantly protected against 1 μM staurosporine toxicity (41% survival, P<0.001, 43% survival, P<0.001) (Figure 5).

Hypoxia Time-Response

Tissue hypoxia in the optic never head and retina is thought to develop as secondary response following elevated intraocular pressure and is associated with the pathology underlying optic nerve degeneration (Tezel et al., 2004). Cells were incubated at 37°C in 0.5% O2, 10% CO2 and air for 24 to 72 hours. There was not a significant amount of RGC cell death at 24 hours following hypoxia (93% survival), whereas hypoxia significantly killed the primary RGCs at 48 hours, leaving 71% of the cells viable (P<0.001). Hypoxia significantly killed the primary RGCs at 72 hours, leaving 62% of the cells viable (P<0.001). (Figure 6).

Methylene Blue Protects Primary Retinal Ganglion Cells Against Hypoxia

Cells were incubated at 37°C in 0.5% O2, 10% CO2 and air for 72 hours with or without methylene blue. Hypoxia for 72 hours left 25% of the cells viable. There was not a significant amount of protection after 72 hours with 100nM methylene blue treatment (26% survival). However, methylene blue, at concentrations of 1 μM and 10 μM, significantly protected primary retinal ganglion cells from 72 hours of hypoxia (38% survival, $P=0.001$, 42% survival, $P<0.001$) (Figure 7).

Methylene blue increases cytochrome c oxidase activity during hydrogen peroxide toxicity

In order to determine if methylene blue preserves activity of the electron transport chain in retinal ganglion cells during a toxic challenge, we directly measure cytochrome c oxidase (Complex IV). Approximately 400,000 RGCs were seeded in each well of a six well plate. The cells were incubated for 7 days before being challenged with 500 μM hydrogen peroxide for 2 hours. Cytochrome c oxidase activity is expressed as a percentage to the control group (figure 9). The control RGCs cytochrome c oxidase activity was set to the arbitrary unit of 1, while hydrogen peroxide decreased the cytochrome c oxidase activity to 50% of control. The addition of 1 μM methylene blue protected cytochrome c oxidase activity against hydrogen peroxide treatment and was 82% of control (Figure 8).

DISCUSSION

Glaucoma is classified as a neurodegenerative disease characterized by the progressive loss of retinal ganglion cells (RGCs) leading to the loss of visual field and eventually blindness. Glaucoma is the second leading cause of blindness worldwide and the most common form of glaucoma, primary open angle glaucoma (POAG), which accounts for 90% of the cases (Varma et al., 2008). Although changes in the outflow pathway cause increased intraocular pressure (IOP), the actual mechanism responsible for optic nerve damage is still unclear. Mitochondrial dysfunction is believed to contribute to the pathogenesis observed during glaucoma (Kong et al., 2009).

During the disease state of glaucoma, mitochondria are densely concentrated at the optic nerve head, which indicates the high recruitment of Adenosine triphosphate (ATP) at the primary site of glaucomatous axonal injury (Barron et al., 2004). When cultured retinal ganglion cells are exposed to elevated hydrostatic pressure, the pressure induces mitochondrial fission and disruption ATP production and predisposing the cells for apoptosis (Ju et al., 2007; Sappington et al., 2006). A mitochondrial enhancing compound, such as methylene blue, could be used to protect against the mitochondrial impairment. Methylene blue is especially intriguing due to its low toxicity because it could be prescribed to groups of people who have a higher genetic prevalence to the disease (Ramdas et al., 2011; Mackey and Craig, 2003).

Our results indicated that methylene blue preserved mitochondrial activity against oxidative stress caused by hydrogen peroxide induced mitochondrial dysfunction, specifically involving cytochrome c oxidase (Zhang et al., 2010). This suggests that the neuroprotective

agent, methylene blue, may prevent mitochondrial dysfunction during glaucoma, thus leading to increased retinal ganglion cell survival and preserved vision; however, further testing is needed.

Methylene blue significantly protected retinal ganglion cells during three challenges that we examined. This includes protection against mitochondrial dysfunction induced by rotenone, which selectively impairs complex I of the electron transport chain (Zhu et al., 2011). Methylene blue is known to significantly protect against rotenone and other hermetic decreasing agents (Wen et al., 2011; Wright et al., 1999; Zhang et al., 2006). Our results indicate that methylene blue's protection against rotenone is preserved within the retinal ganglion cells of rats.

Apoptosis is a common form of neuronal death during several neuronal diseases including glaucoma (Tatton et al., 2001; Yuan and Yankner, 2000). Even though the exact mechanism if unknown, staurosporine activates caspases and induces apoptosis (Nguyen et al., 2010; Chae et al., 2000). Our results indicate that methylene blue protects against staurosporine cytotoxicity. Even though the exact mechanism of this protection has not been thoroughly examined or is not completely understood, it suggests that methylene blue may protect against apoptosis.

Hypoxia and ischemia occur during age-related neurodegeneratives, including glaucoma, and neurotraumas (Schmidt et al., 2004; Simonian and Coyle, 1996). Hypoxia, oxygen deprivation, in neurons leads to a localized increased in excitatory amino acids and proteins that can result in the premature death of neurons and brain tissue (Banasiak et al., 2000). Methylene blue increases neuronal survival during *in vivo* ischemia (Miclescu et al., 2010; Bardakci et al., 2006). Methylene blue's mechanism of action suggests that it will increase the propagation of electrons, even during oxygen deprivation. Furthermore, methylene blue is a free radical

scavenger and can convert superoxide into water (Bruchey and Gonzalez-Lima, 2008), which can increase the viability of neurons during oxygen deprivation. Furthermore, our preliminary data suggest that methylene blue's mechanism of action is preserved in retinal ganglion cells by increasing the activity of cytochrome c oxidase (complex IV of the electron transport chain) (Atamna and Kumar, 2010); however, additional test are needed.

Dr. Gonzalez-Lima's laboratory showed that methylene blue can protect retinal tissue during rotenone toxicity (Rojas et al., 2009; Zhang et al., 2006); however, in order to develop methylene blue as a treatment for the neurodegeneration that occurs during glaucoma, it would be advantageous to use a more sentient animal with more relevant models of glaucoma. For example, examine if methylene blue can protect monkeys from elevated intraocular pressure. Additionally, photopic and scotopic electroretinograms (ERG) activity could be analyzed to determine the activity of retinal ganglion cells during this glaucomatous model.

Methylene blue could be used as a treatment once a neurodegenerative disease is detected; however, optimal treatment would be a prolonged treatment before the disease has manifested. This would require clinicians to recommend the treatment to individuals who are genetically predisposed to a higher prevalence of glaucoma; however, greater testing is needed.

ACKNOWLEDGEMENTS:

I would like to thank Dr. Ben Barres for his ample time and help teach us the primary retinal ganglion cell isolation techniques. I would also like to thank Dr. Abe Clark for his insight and helpful discussions.

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Figure 1: **Primary Rat Retinal Ganglion Cell Culture**

Characterization of the isolated primary retinal ganglion cells following Dr. Ben Barres' protocol (Barres et al., 1988). Cells were characterized by immunocytochemistry for normally expressed RGC marker Thy-1. Thy-1 was detectable in virtually all of these cells. Scale bar, 20 µm.

Figure 1: Primary Retinal Ganglion Cell Cultures

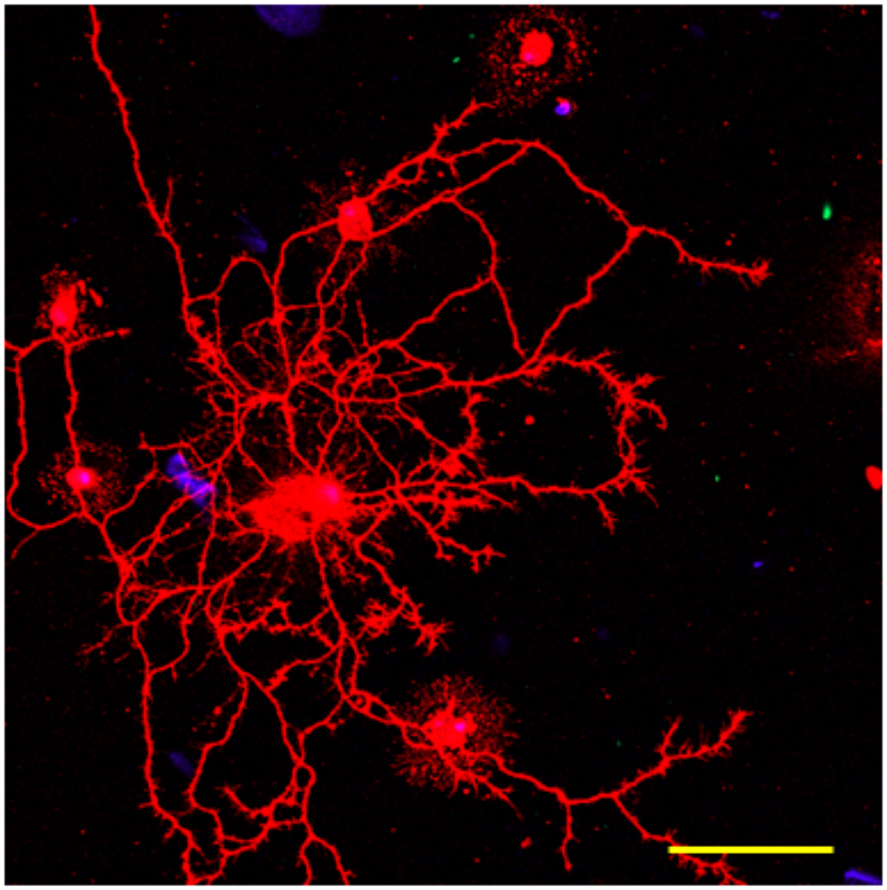
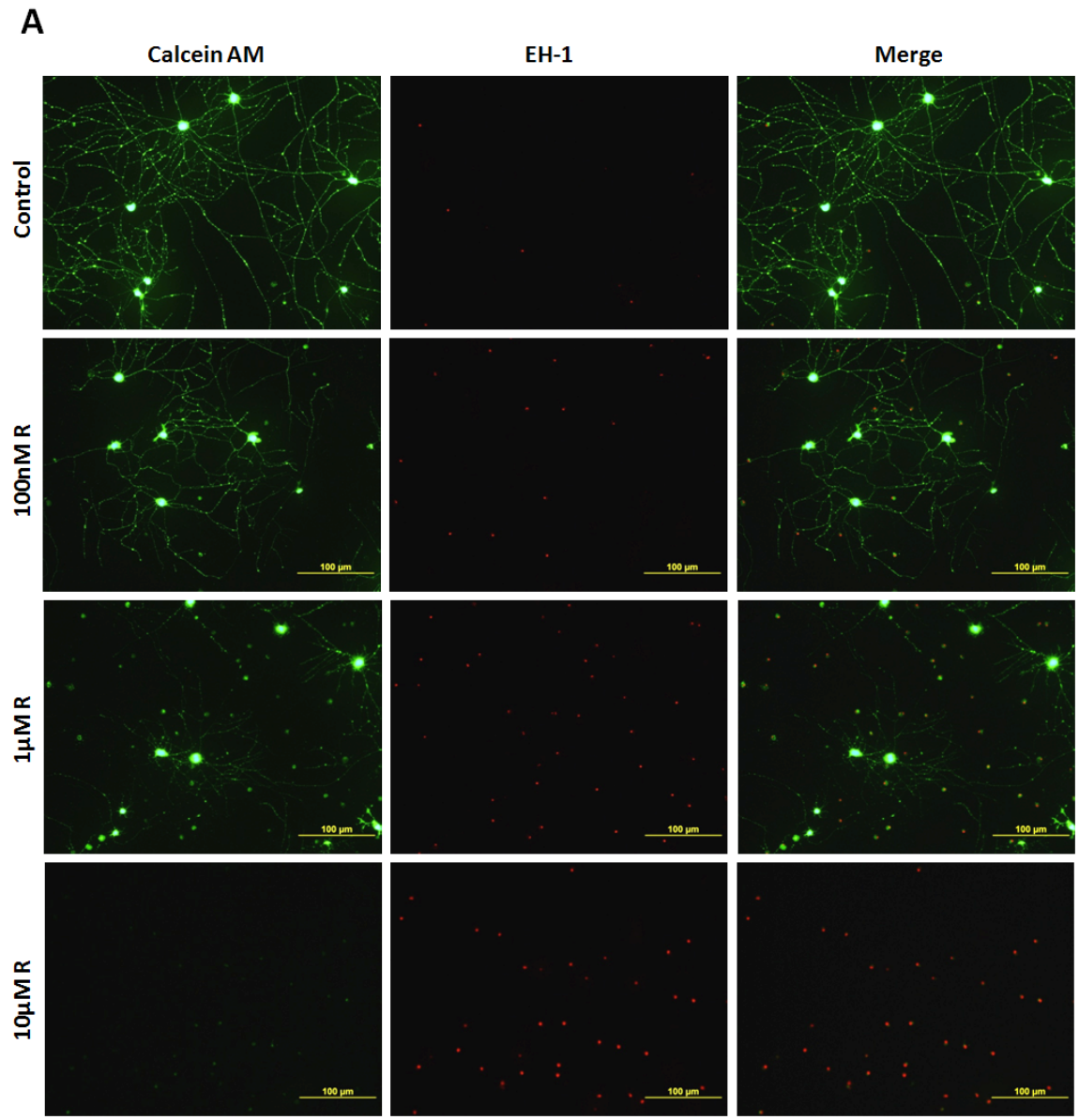


Figure 2: **Rotenone Toxicity Concentration-Response**

Rotenone significantly kills primary rat retinal ganglion cells. **(A)** Cell survival was measured by simultaneously staining with green-fluorescent calcein-AM to indicate intracellular esterase activity and red-fluorescent ethidium homodimer-1 to indicate loss of plasma membrane integrity. Primary rat retinal ganglion cells were seeded, 25,000 per well. Cells were cultured for 72 hours before beginning rotenone treatment. Cells were treated with or without varying concentrations of rotenone (100 nM, 1 µM, or 10 µM) for 24 hours (scale bar = 100 µM). **(B)** Rotenone significantly killed primary RGCs at 100 nM (59% survival $P < 0.001$), 1 µM (39% survival $P < 0.001$), and 10 µM (7% surival $P < 0.001$) ($n = 3$).

Figure 2:



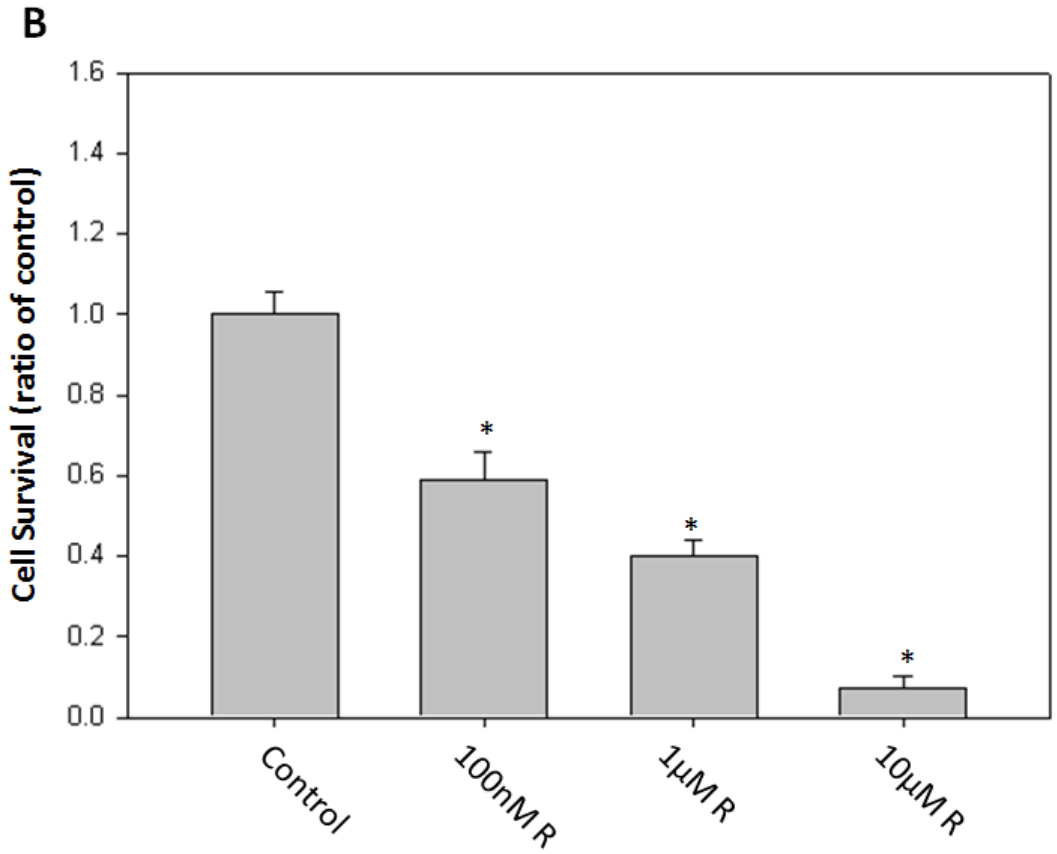
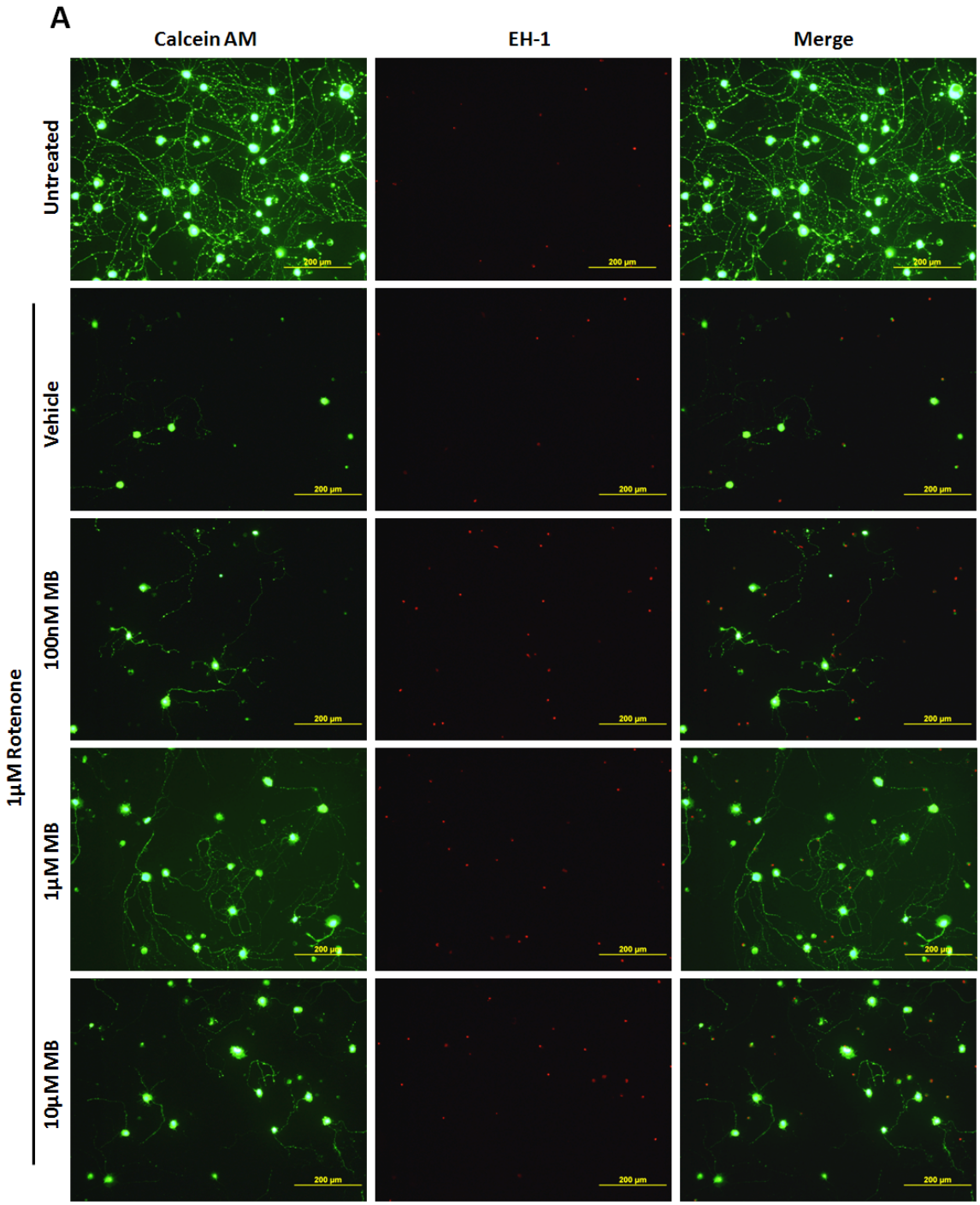

B
Cell Survival (ratio of control)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
Control
100nM R
1μM R
10μM R

Figure 3: **Methylene Blue Protects Primary Retinal Ganglion Cells from Rotenone Toxicity**

Methylene blue (MB) significantly protects primary RGCs from rotenone toxicity. **(A)** Cell survival was measured by simultaneously staining with green-fluorescent calcein-AM to indicate intracellular esterase activity and red-fluorescent ethidium homodimer-1 to indicate loss of plasma membrane integrity. Primary rat retinal ganglion cells were seeded, 25,000 per well. Cells were cultured for 72 hours before beginning rotenone treatment. Cells were treated with 1 µM rotenone and with or without varying concentrations of methylene blue (100 nM, 1 µM, or 10 µM) for 24 hours (scale bar = 100 µM). **(B)** 1 µM rotenone significantly (41% survival $P < 0.001$) killed primary RGCs. Methylene blue significantly protected primary RGCs from rotenone toxicity at 1 µM (67% survival $P < 0.001$), and 10 µM (68% survival $P < 0.001$) ($n = 6$).

Figure 3: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Calcein AM
EH-1
Merge
Untreated
1µM Rotenone
Vehicle
100nM MB
1µM MB
10µM MB
200 µm

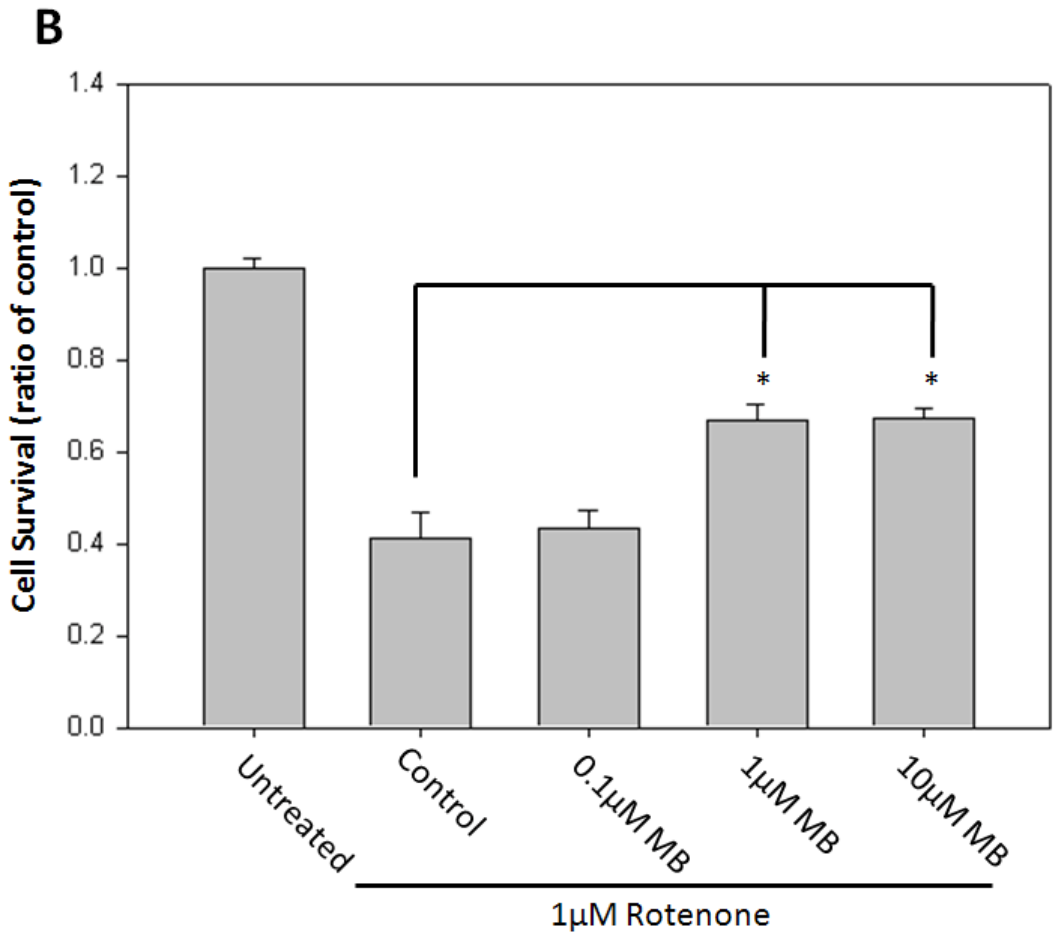
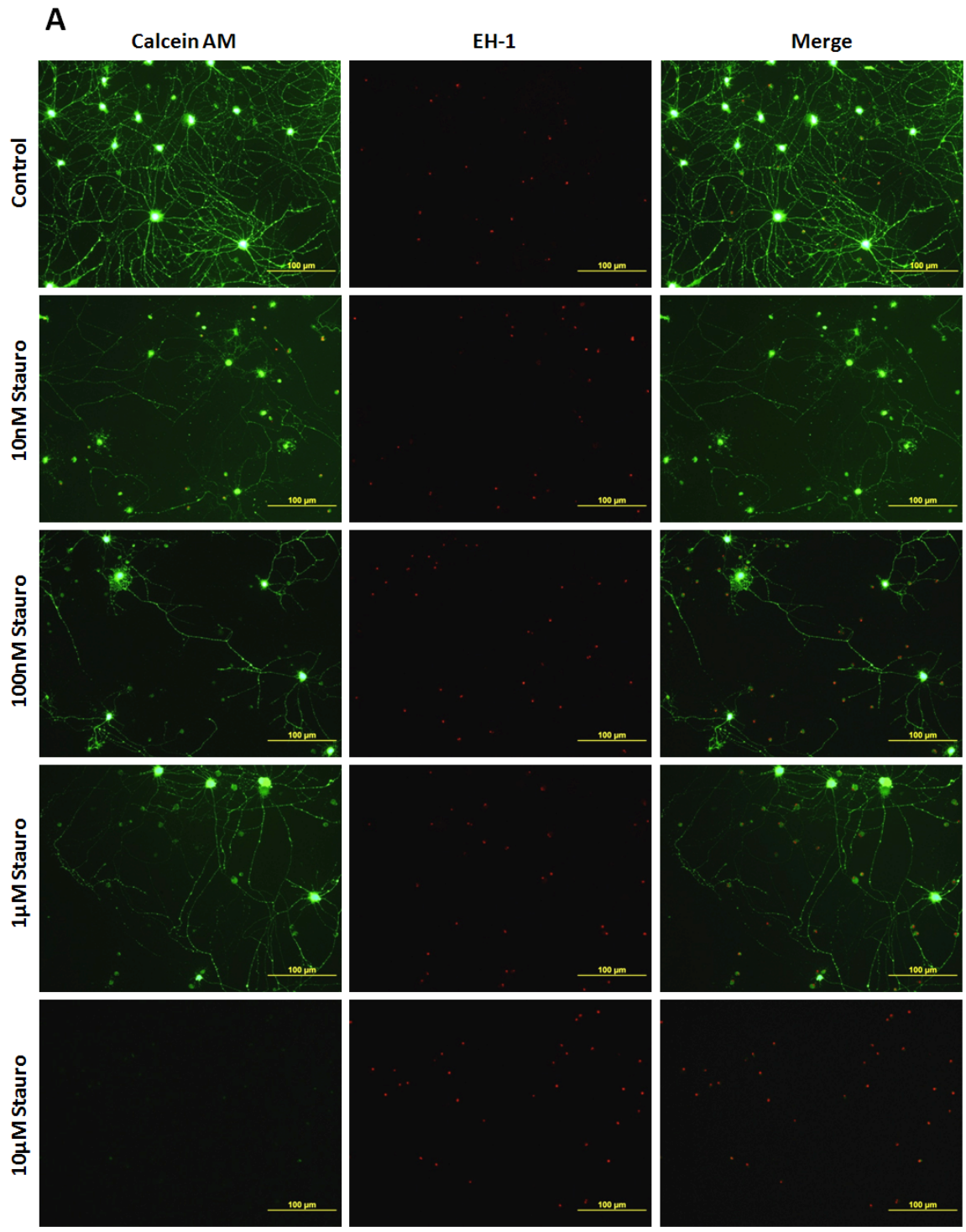

B
Cell Survival (ratio of control)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Untreated
Control
0.1μM MB
1μM MB
10μM MB
1μM Rotenone

Figure 4: Staurosporine Toxicity Concentration-Response

Staurosporine significantly kills primary rat retinal ganglion cells. **(A)** Cell survival was measured by simultaneously staining with green-fluorescent calcein-AM to indicate intracellular esterase activity and red-fluorescent ethidium homodimer-1 to indicate loss of plasma membrane integrity. Primary rat retinal ganglion cells were seeded, 25,000 per well. Cells were cultured for 72 hours before beginning staurosporine treatment. Cells were treated with or without varying concentrations of staurosporine (10 nM, 100nM, 1 µM, or 10 µM) for 24 hours (scale bar = 100 µM). **(B)** Staurosporine significantly killed primary RGCs at 10 nM (43% surival $P < 0.001$), 100 nM (41% survial $P < 0.001$), 1 µM (35% survival $P < 0.001$), and 10 µM (0% surival $P < 0.001$) ($n = 3$).

Figure 4:


A
Calcein AM
EH-1
Merge
Control
10nM Stauro
100nM Stauro
1µM Stauro
10µM Stauro
100 µm

B

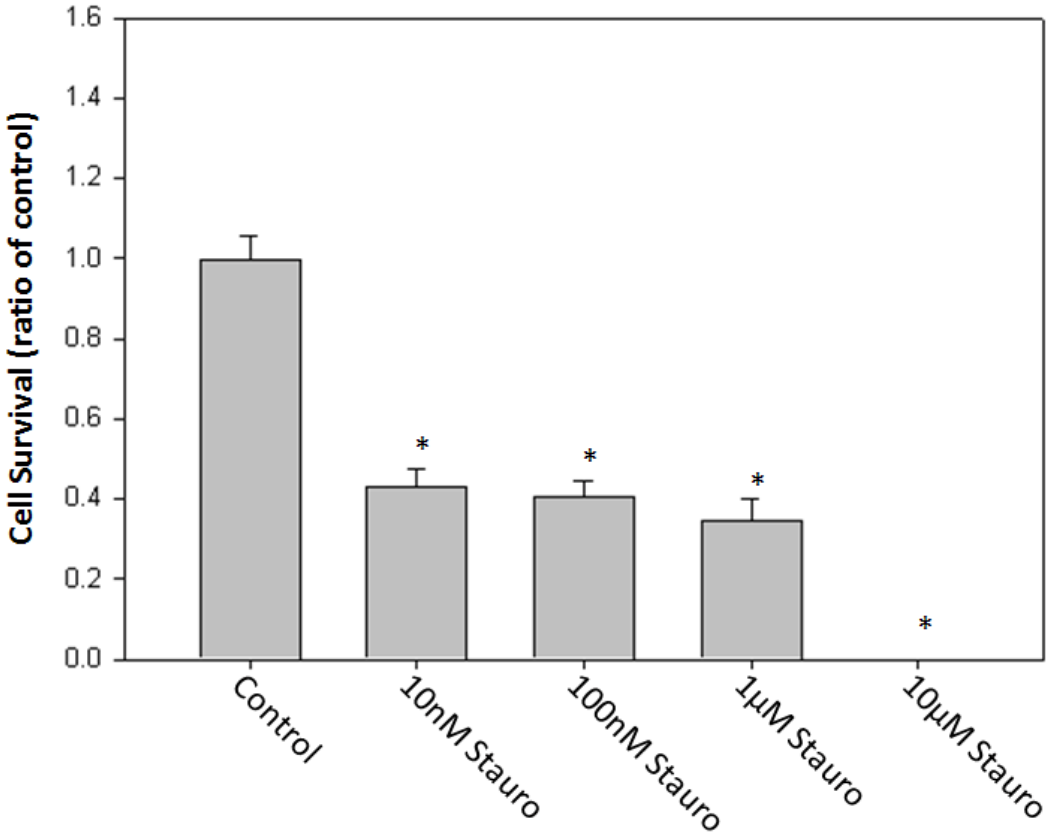
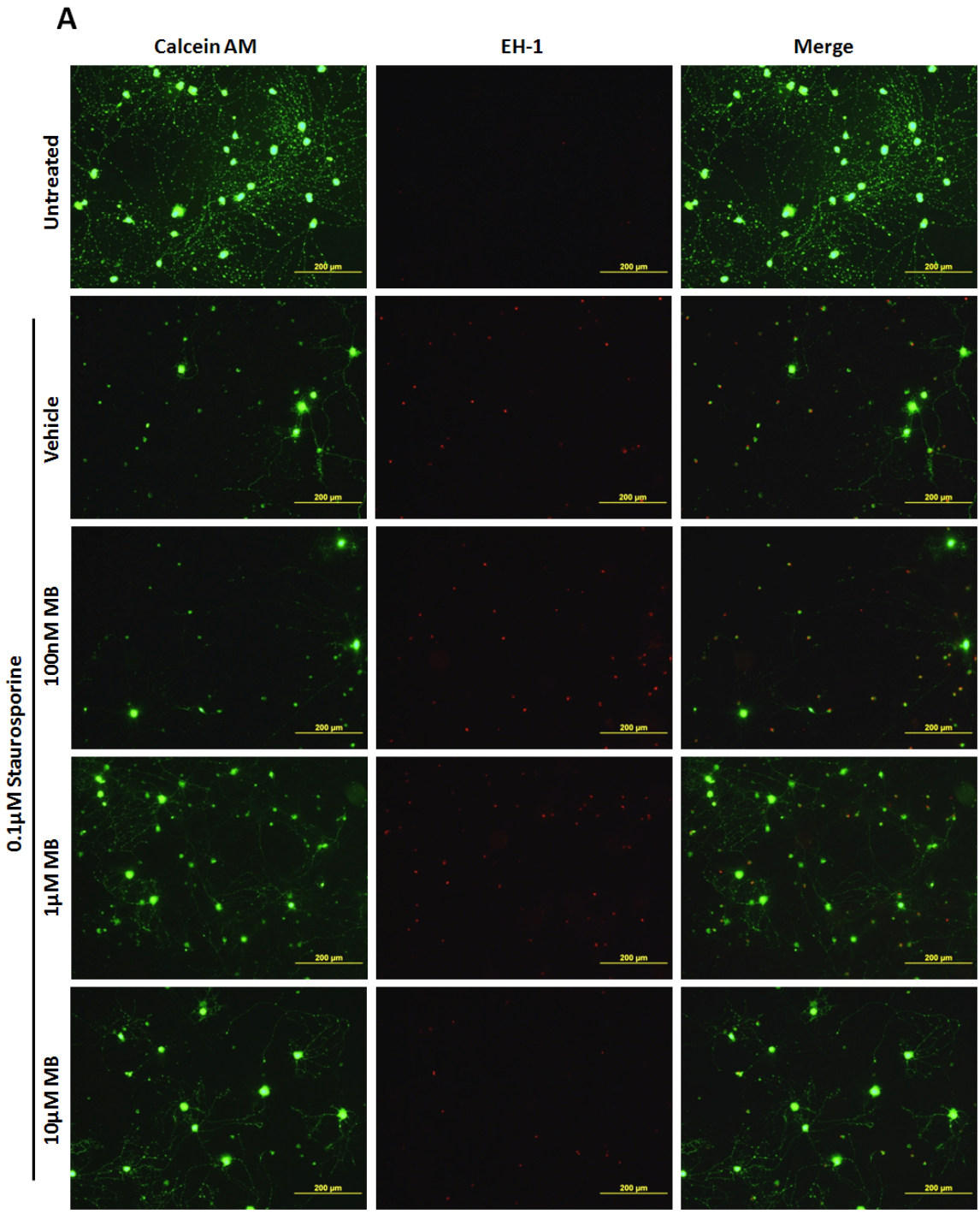

Cell Survival (ratio of control)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
*
Control
10nM Stauro
100nM Stauro
1μM Stauro
10μM Stauro

Figure 5

Methylene Blue Protects Primary Retinal Ganglion Cells Against Staurosporine

Methylene blue (MB) may protect primary RGCs from staurosporine. **(A)** Cell survival was measured by simultaneously staining with green-fluorescent calcein-AM to indicate intracellular esterase activity and red-fluorescent ethidium homodimer-1 to indicate loss of plasma membrane integrity. Primary rat retinal ganglion cells were seeded, 25,000 per well. Cells were cultured for 72 hours before beginning staurosporine treatment. Cells were treated with 100 nM staurosporine and with or without varying concentrations of methylene blue (100 nM, 1 μM, or 10 μM) for 24 hours (scale bar = 100 μM). Untreated cells were used as a control to quantitate average amount of cell viability following primary RGC isolation. All other experimental groups will be expressed as a ratio of untreated cell survival. **(B)** The addition of 100 nM staurosporine for 24 hours induced left 17% cells viable. The addition of 100 nM staurosporine with 100 nM methylene blue did not induce a significant level of protection. The addition of 1 μM and 10 μM methylene blue significantly protected against 1 μM staurosporine toxicity (41% survival, $P<0.001$, 43% survival, $P<0.001$) (n=6).

Figure 5


A
Calcein AM
EH-1
Merge
Untreated
Vehicle
100nM MB
1µM MB
10µM MB
0.1µM Staurosporine
200 µm

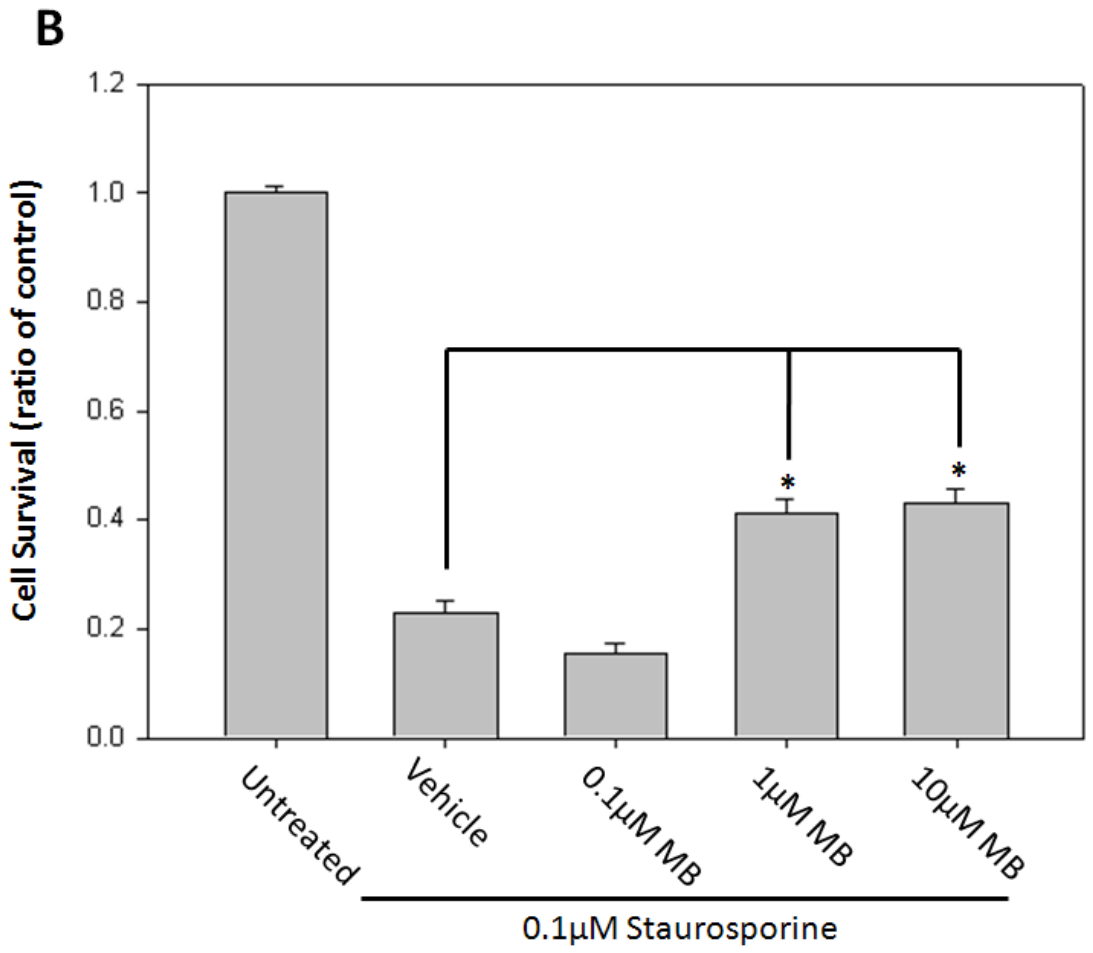
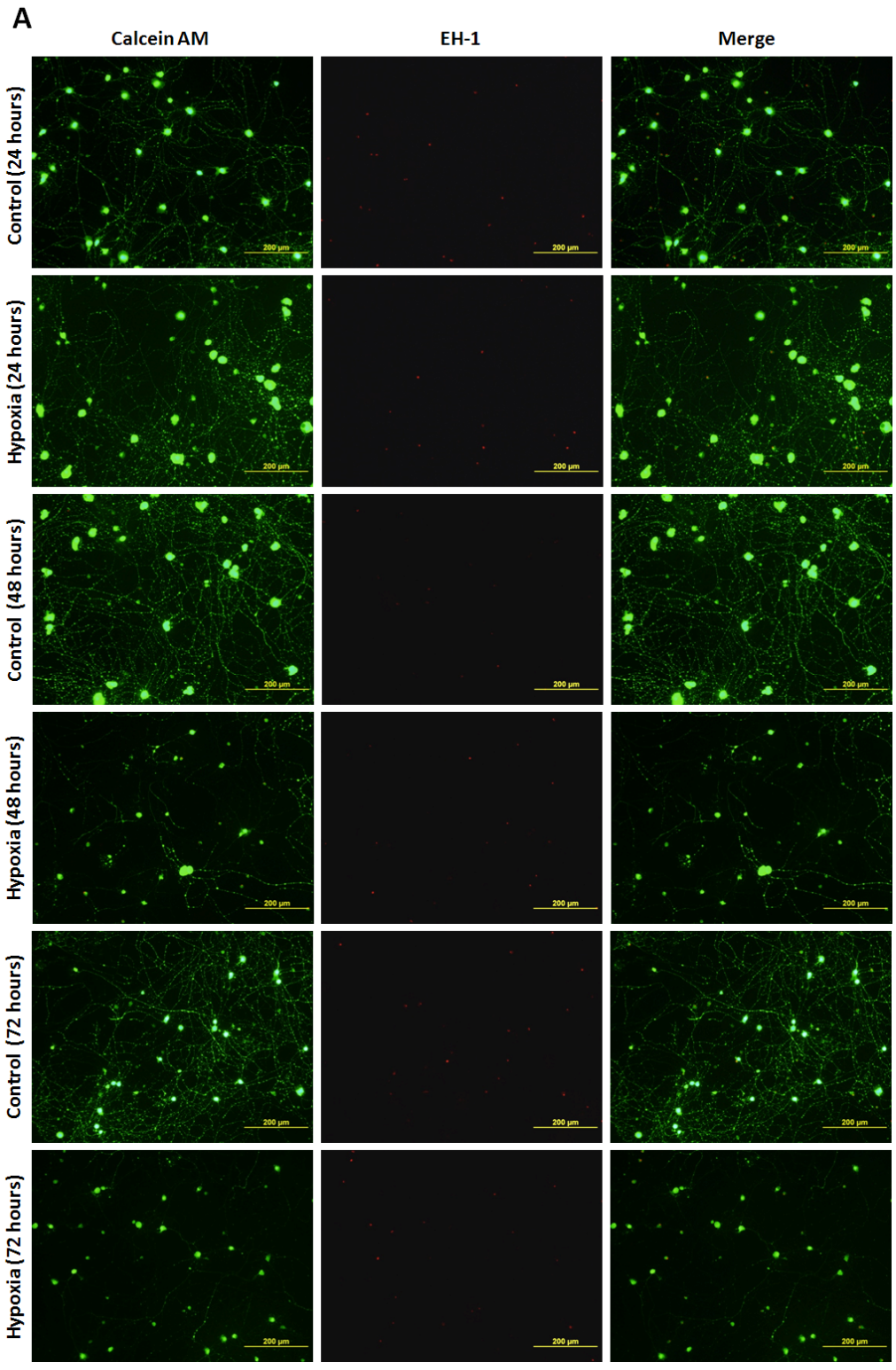

B
Cell Survival (ratio of control)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Untreated
Vehicle
0.1μM MB
1μM MB
10μM MB
0.1μM Staurosporine

Figure 6:

Hypoxia Time-Response

Hypoxia significantly kills primary rat retinal ganglion cells. **(A)** Cell survival was measured by simultaneously staining with green-fluorescent calcein-AM to indicate intracellular esterase activity and red-fluorescent ethidium homodimer-1 to indicate loss of plasma membrane integrity. Primary rat retinal ganglion cells were seeded, 25,000 per well. Cells were cultured for 72 hours before beginning hypoxia (0.5% oxygen). Cells were exposed to either hypoxic or normoxic conditions from 24 hours or 48 hours (scale bar = 200 µm). **(B)** There was not a significant amount of death at 24 hours of hypoxia (93% survival). Hypoxia significantly killed the primary RGCs at 48 hours, leaving 71% of the cells viable ($P<0.001$). Hypoxia significantly killed the primary RGCs at 72 hours, leaving 62% of the cells viable ($P<0.001$). Primary RGCs will be exposed to 72 hours of hypoxia for subsequential experiments (n=6).

Figure 6:


A
Calcein AM
EH-1
Merge
Control (24 hours)
Hypoxia (24 hours)
Control (48 hours)
Hypoxia (48 hours)
Control (72 hours)
Hypoxia (72 hours)
200 µm

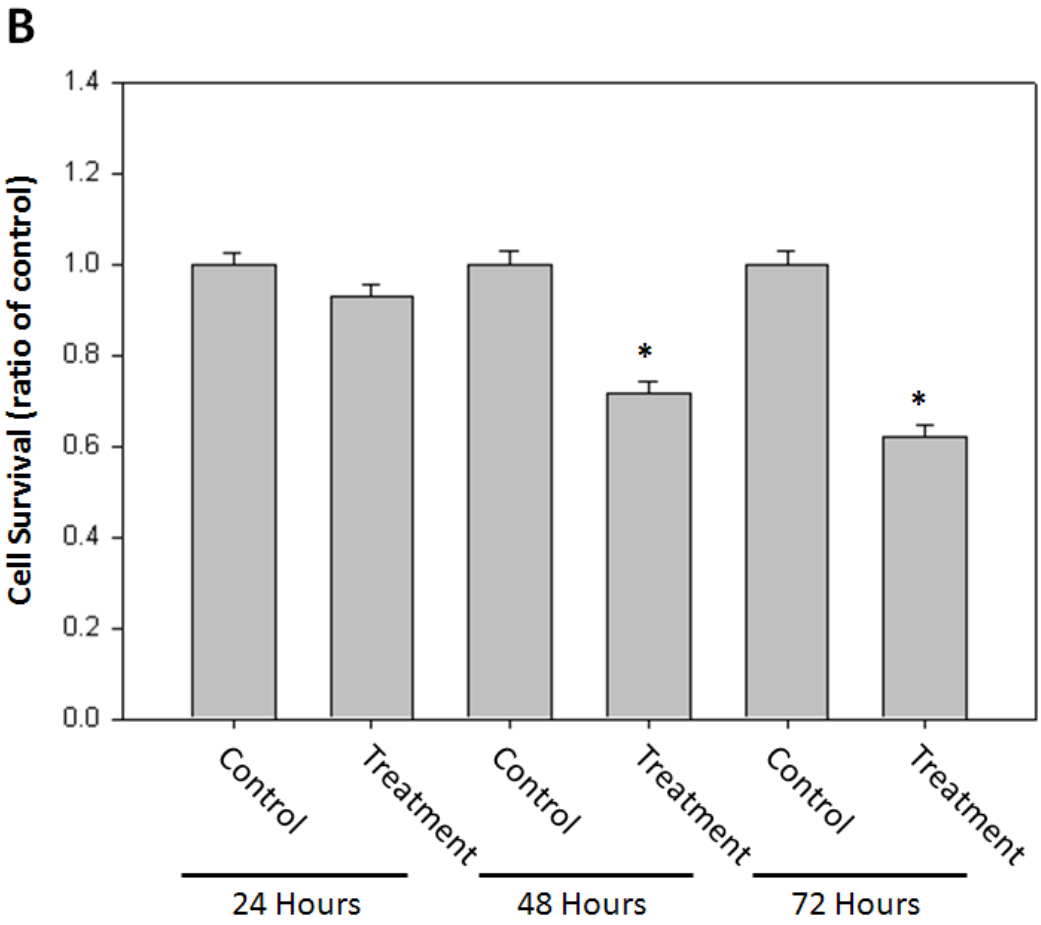
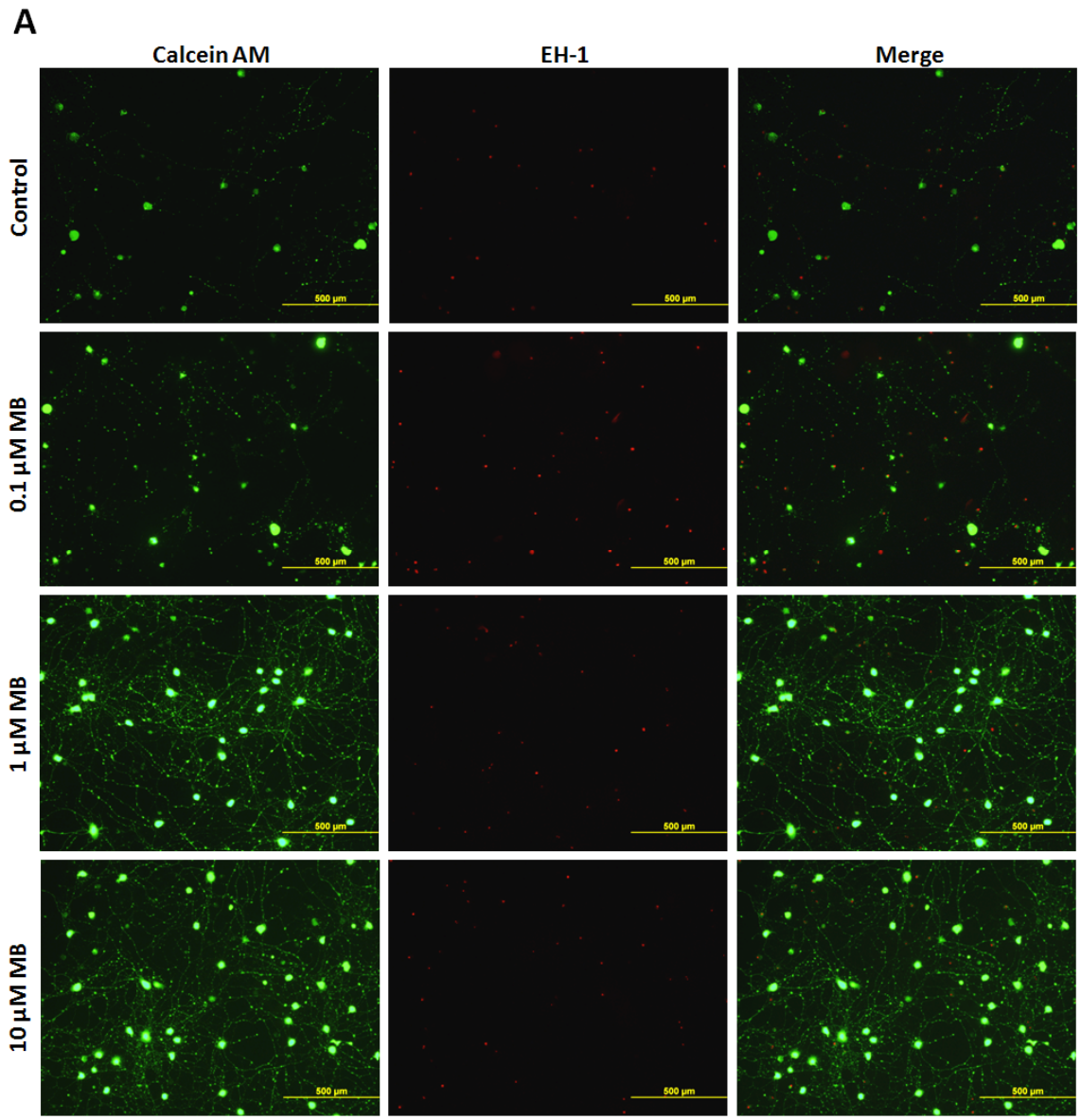

B
Cell Survival (ratio of control)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Control
Treatment
Control
Treatment
Control
Treatment
24 Hours
48 Hours
72 Hours

Figure 7:

Methylene Blue Protects Primary Retinal Ganglion Cells Against Hypoxia

Hypoxia significantly kills primary rat retinal ganglion cells. **(A)** Cell survival was measured by simultaneously staining with green-fluorescent calcein-AM to indicate intracellular esterase activity and red-fluorescent ethidium homodimer-1 to indicate loss of plasma membrane integrity. Primary rat retinal ganglion cells were seeded, 25,000 per well. Cells were cultured for 72 hours before beginning hypoxia (0.5% oxygen). Cells were exposed to either hypoxic or normoxic conditions from 24 hours or 48 hours (scale bar = 200 μM). **(B)** 72 hours of hypoxic conditions induced left 25% of the cells viable. There was not a significant amount of protection after 72 hours of 100nM methylene blue treatment (26% survival). Methylene blue (MB), at concentrations 1 μM and 10 μM, significantly protected primary retinal ganglion cells from 72 hours of hypoxia (38% survival, $P=0.001$, 42% survival, $P<0.001$) (n=6).

Figure 7



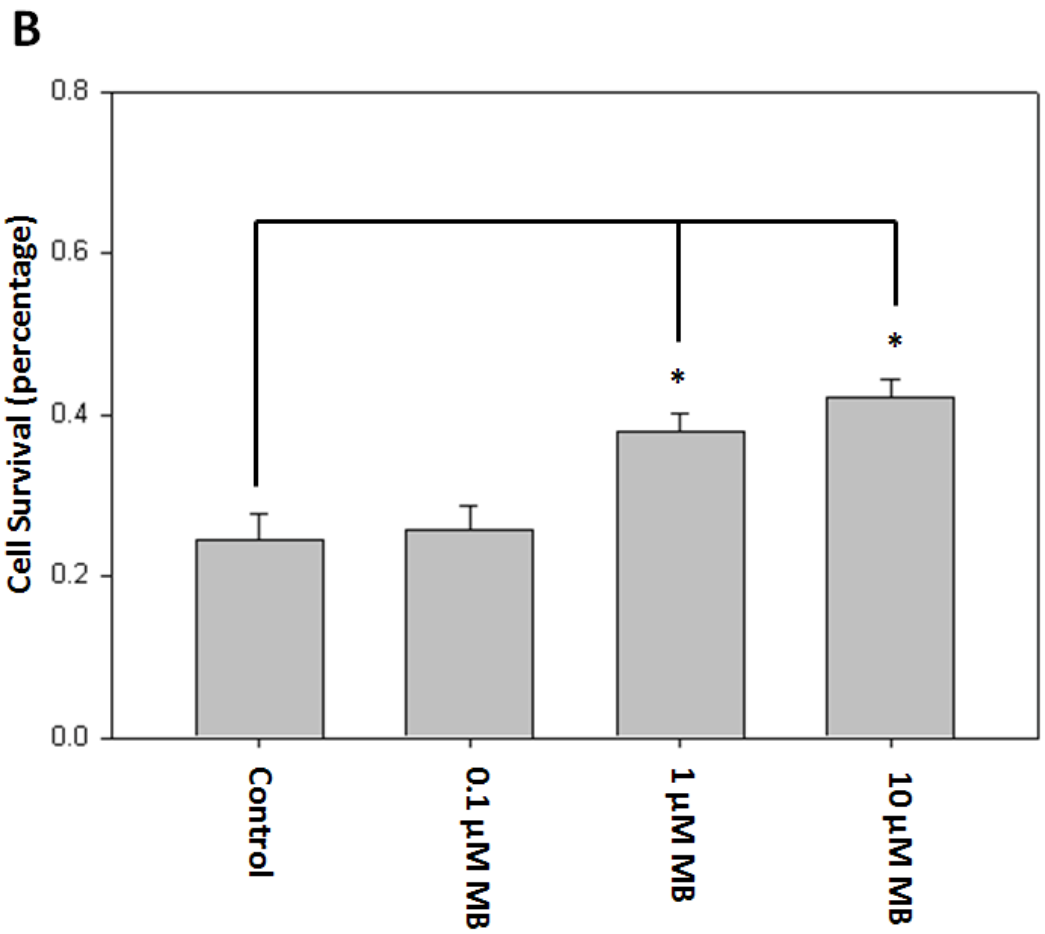
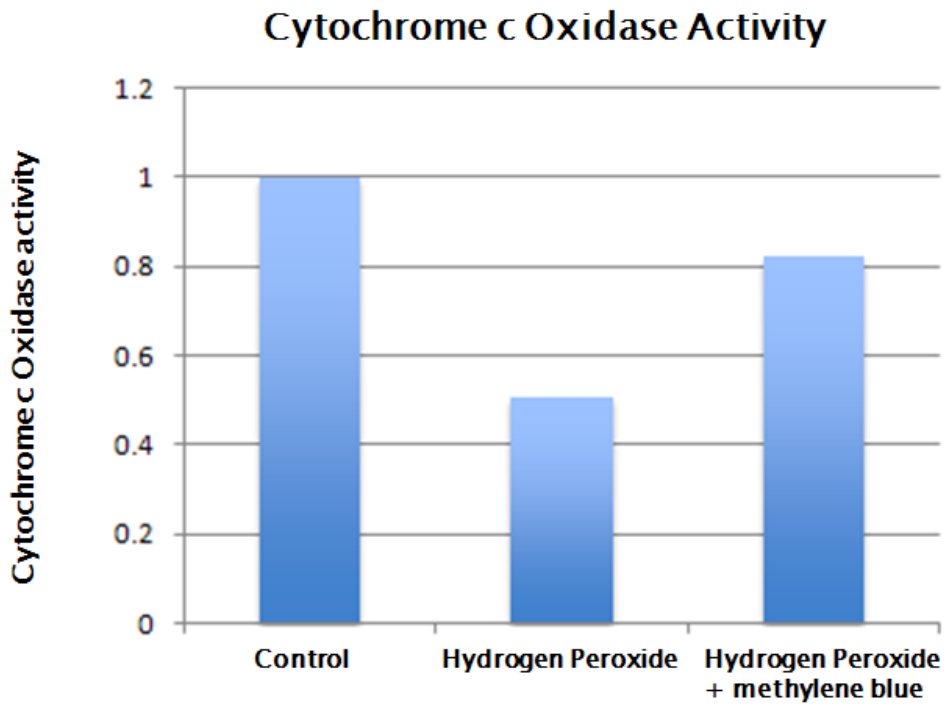

B
Cell Survival (percentage)
0.8
0.6
0.4
0.2
0.0
*
*
Control
0.1 µM MB
1 µM MB
10 µM MB

Figure 8:

Methylene blue increases cytochrome c oxidase activity during hydrogen peroxide toxicity

Methylene blue protects cytochrome c activity in RGCs exposed to hydrogen peroxide. 400,000 primary rat RGCs were cultured for 7 days before being subjected to treatment. Primary RGCs were treated for 120 minutes with a vehicle or 500 µM hydrogen peroxide with or without 1 µM methylene blue. Cytochrome c oxidase activity was measured by a cytochrome c oxidase assay kit (CYTOCOX1 Sigma). This assay utilizes cytochrome c oxidase activity to convert ferrocytochrome c, which has an absorance at 550nm, to ferricytochrome c, which does not have an absorbance at 550nm. A spectrophotometer was used to measure ferrocytochrome at 550nm. Results indicate that primary RGCs had the highest functioning cytrochrome c oxidase while hydrogen peroxide decreased it. The addition of methylene blue increased the amount of functioning cytocrhome c oxidase.

Figure 8:


Cytochrome c Oxidase Activity
Cytochrome c Oxidase activity
1.2
1
0.8
0.6
0.4
0.2
0
Control
Hydrogen Peroxide
Hydrogen Peroxide + methylene blue

CHAPTER IV

CONCLUSIONS

The purpose of this dissertation was to investigate new approaches to protect neurons during neurodegenerative states and neurotramas. This study design was necessary to ascertain not only if FK 506 Binding Protein 51 (FKBP51) or methylene blue is neuroprotective but to also define key mechanisms associated with these properties. The studies involving methylene blue protection in primary retinal ganglion cells was to advance research about this known neuroprotective compound to become an ocular treatment for glaucoma and other neurodegenerative diseases (Rojas et al., 2009). Specifically observing methylene blue's protection in retinal ganglion cells advances the compound's potential as a neuroprotective agent against ocular neurodegenerative disease such as glaucoma. Each insult that was applied to the primary retinal ganglion cells was selected for their specific pathology. Rotenone is a well-documented agent that induces mitochondrial dysfunction; specifically at complex I of the electron transport chain (Bruchey and Gonzalez-Lima 2008). A common pathology observed during glaucoma is mitochondrial dysfunction of the retinal ganglion cells, which leads to the selective apoptosis of these cells (Bruchey and Gonzalez-Lima 2008). Furthermore, rotenone was selected due to methylene blue's mechanism of action as an electron acceptor at the electron transport chain (Bruchey and Gonzalez-Lima 2008). Secondly, staurosporine was selected as an

insult because it induces apoptosis (Chae et al., 2000; Daudt et al, 2011). Glaucoma induces apoptosis of retinal ganglion cells (McKinnon 1997). Finally, hypoxia is a relevant model for glaucoma. Hypoxia is a secondary pathology to elevated intraocular pressure. As pressure increases within the eye, transient ischemic insults and oxygen deprivation occurs (Tezel and Wax 2004). We demonstrated that methylene blue increases viability of retinal ganglion cells following exposure to these three relevant models of neuronal death. Our results are what were expected based on extensive research of the literature. Our results provide evidence that methylene blue can protect retinal ganglion cell and thus potentially be given as a treatment for glaucoma; however, more research is needed.

Methylene blue is an autoxidizable phenothiazine with potent antioxidant and metabolic enhancing properties with a reduction-oxidation capacity for electron cycling. Methylene blue is reduced by accepting electrons from reduced electron transport donors and methylene blue transfers electrons to oxygen to form water, thus maintaining activity of the electron transport chain (Bruchey and Gonzalez-Lima 2008). We demonstrated that hydrogen peroxide decreased activity of the mitochondria, specifically at cytochrome c oxidase. Methylene blue increased cytochrome c oxidase activity following this insult. This suggests that methylene blue's mechanism of action includes sustaining cytochrome c oxidase activity preserving retinal ganglion cells.

Methylene blue is a neuroprotective compound that could potentially be used to treat neuronal loss during a several neurodegenerative disease. Methylene blue has several characteristics that make its use clinically more intriguing. For example, methylene blue has been used clinically for over 100 years and it is well documented to have low toxicity

(Wainwright and Crossley, 2002). Methylene blue could be administered orally by the patient because of MB's high bioavailability and ability to readily cross the blood-brain barrier (Peter et al., 2000; Wainwright and Crossley, 2002). This means a compound like methylene blue would have a great patient compliance than a neuroprotective compound that needs to be administered through intravitreal injections. Additionally, methylene blue could be administered to a patient once a neurodegenerative disease is detected; however, it would be more advantageous to treat a patient long before the disease manifests because methylene blue lacks neuroregeneration. Every individual could be thoroughly examined in terms of genetics and history in order to know if they are susceptible to a specific neurodegenerative disease, such as glaucoma, and then receive methylene blue treatment for a long duration before and after the disease manifest itself. This would help each patient preserve the greatest number of neurons and potentially decrease the progression of the disease. Because of these reasons, methylene blue is already undergoing clinical trails to treat the progression of Alzheimer's disease (Wischik et al., 2008)

Furthermore, we investigated the neuroprotective actions of FK506 (Tacrolimus). While FK506 is significantly neuroprotective within the central nervous system, it also induces systemic immunosuppression by binding to one of its downstream binding proteins, FK 506 Binding Protein 12 (FKBP12). Interestingly, FK506 is still significantly neuroprotective in cell lines lacking FKBP12 (Guo et al., 2001) and FK506 analogues that are devoid of calcineurin inhibition are still significantly neuroprotective (Burnett et al., 2003). This suggests that there may be additional FK506 binding proteins that mediate FK506's neuroprotective actions.

This lead to an investigation that suggests FKBP51, an FK506 binding protein, has neuroprotective properties and appears to be devoid of immunosuppression (Xu et al., 2002).

We designed our studies to determine if FKBP51 is expressed within the brain and retina. We also designed our experiments top determine viability of a neuronal origin cell culture. We selected the 661w neuronal cell line to perform our studies as these cells are of retina neuronal origin and are readily available. Furthermore, we performed a western blot analysis that displayed FKBP51 expression in the 661W neuronal cell cultures.

In order to confirm that FK506 was protective in these cell cultures, we designed our experiments to test if FK506 could protect against toxic insults. Staurosporine was selected because it specifically induced apoptosis. Staurosporine induced a significant amount of apoptosis in the 661w neuronal cell cultures (Nguyen et al., 2010; Chae et al., 2000). The addition of FK506 significantly protected the 661w neuronal cell cultures. We also stably transfected 661w cell cultures with an FKBP51 over-expression vector or an empty vector as a control. Staurosporine induced a significant amount of apoptosis in the empty vector control cells. This was observed through a caspase 3 detection assay and calcein AM/propidium iodide assay. The over expression of FKBP51 significantly protected the 661W neuronal cell cultures from cell death and specifically apoptosis. This result indicated that FKBP51 was neuroprotective and prevented apoptosis in this model.

We also examined FKBP51's downstream signaling molecules. FKBP51 was shown to interact with the NF-κB signaling pathway (Giraudier et al., 2002). The over-expression of FKBP51 caused an increase in the pro-survival molecules NFκB and activated NFκB while decreasing the inhibitory subunit IκB protein levels. Furthermore, we were interested if FK506 activates the same signaling proteins as FKBP51. The addition of FK506 to 661w cell cultures significantly increased the protein levels of NFκB while decreasing the protein levels of IκB. At

30 minutes of FK506 treatment, NFκB was significantly activated. This suggests that FK506 and FKBP51 act through a similar mechanism of action. This pathway may be the signaling pathway responsible for both of their neuroprotection properties.

FKBP51's neuroprotective actions make it an intriguing protein to treat neuronal loss and even potentially regenerate neurons. Because FK506 activates both FKBP51 and FKBP12 it can not be given to protect against neurodegenerative diseases because it will increase the incidence of cancer. While there are several molecules that are analogues of FK506 they need to be thoroughly examined for toxicity and which molecules they interact with before they could be given to patient. Ideally, creating a compound that specifically activates FKBP51 would be advantageous to treat neuronal loss during a neurodegenerative disease and neurotrauma; however, extensive research would need to be performed to make sure that the compound does not increase the incidence of cancer due to FKBP51 prevalence in several cancers. Ideally, a localized delivery system of the theoretical FKBP51 activating compound could protect the rest of the body from side effects.

Furthermore, several FK506 analogues are being examined to heal skin wounds. This means that FKBP51 could be investigated as a topical target to increase the rate of healing.

In summary, these studies identified two important pathways that are potential targets for neuroprotection: maintaining mitochondrial function (as shown with methylene blue) and increasing cell prosurvival pathways, such as NFκB signaling (as shown with FKBP51). Methylene blue treatments could be taken orally by a patient for several years before a disease manifest itself because of its low side effects and minimal toxicity. This may prevent the progression of a neurodegenerative disease before the disease even manifest itself. FKBP51 on

the other hand is neuroprotective and potentially neuroregenerative. This means that a compound, which increases FKBP51 activity, could be administered after a disease or trauma manifests itself. However, further studies are needed to advance each protective pathway and compound.

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CHAPTER V

PROPOSAL OF FURTHER RESEARCH

Although the research presented in this dissertation provided several striking new findings regarding novel therapeutic targets and potential drug interventions, many questions remain unanswered. For example, does the absence of FKBP51 decrease the neuroprotective properties of FK506? Does the absence of FKBP51 decrease the neuroprotective properties of FK506 drug analogues, such as GPI-1046? Can methylene blue increase neurosurvival during elevated intraocular pressure? Listed below are several future investigations:

Determine if knockout of FKBP51 is detrimental to RGC survival during ocular hypertension in primary retinal ganglion cells and if FKBP51 deficient mice are more susceptible to apoptosis following toxic challenges like hypoxia.

1: Quantitate through Calcein AM/propidium iodide and caspase 3 detection if primary retinal ganglion cell cultures lacking FKBP51 are more susceptible to apoptosis toxic insults compared to cells expressing FKBP51.

2: Determine if overexpressing FKBP51 using an overexpression vector in cells from FKBP51 knockout mice can re-establish neuroprotection when cells lacking the FKBP5 (FKBP51) gene are exposed to toxic stimuli.

3: Determine if the absence of FKBP51 causes impairment to visual acuity or decreases retinal ganglion cell survival in FKBP51 deficient mice through virtual optomotor training and quantitation of RGCs.

4. Determine if metheylene blue, when given orally, can protect against elevated intraocular pressure in rats. Rats could undergo the Morrison model of elevated IOP and then determine if methylene blue treatment before and during increases survival of retinal ganglion cells and visual acuity.

Hypothesis: Apoptosis in primary retinal ganglion cells lacking FKBP51 protein will increase when challenged through a stress inducing apoptosis. In FKBP51 deficient primary retinal ganglion cells, re-introducing FKBP51 through an overexpression vector will re-establish neuroprotection. Finally, the absence of FKBP51 will cause increased cell death of retinal ganglion cells during development in FKBP51 deficient mice, causing a significant change in the quantity of RGCs and visual acuity. Methylene blue could protect retinal ganglion cells against elevated intraocular pressure if given before and during the insult.