12-2014

Neuroprotective Effect of an A-7 Nicotinic Acetylcholine Receptor Agonist and a Positive Allosteric Modulator in an In Vitro Model of Glaucoma

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NEUROPROTECTIVE EFFECT OF AN α-7 NICOTINIC ACETYLCHOLINE RECEPTOR AGONIST AND A POSITIVE ALLOSTERIC MODULATOR IN AN *IN VITRO* MODEL OF GLAUCOMA

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A Thesis Submitted to the Graduate Faculty of

GRAND VALLEY STATE UNIVERSITY

In

Partial Fulfillment of the Requirements

For the Degree of

Master of Physician Assistant Studies

Department of Physician Assistant Studies

December 2014

ACKNOWLEDGEMENTS

I would first like to acknowledge Dr. Cindy Linn, Professor in the Department of Biological Sciences at Western Michigan University. Allowing me to work in her laboratory as an undergraduate student built my foundation in research and sparked my interest in this field of study. With her guidance and support I was able to pursue a Master's degree in Physician Assistant Studies while continuing to work on research in the basic sciences. I appreciate her role in developing me into the student and professional I am today.

Next I would like to thank Dr. Martina Reinhold, Professor in the Department of Physician Assistant Studies at Grand Valley State University. Dr. Reinhold also served as my research advisor throughout the completion of this project and has been nothing but supportive throughout. She was eager to help as I decided to "stray from the norm" in the Physician Assistant Studies department and complete a solo project in the basic sciences. Her expertise in this field of research and her constant guidance through email and long office conversations was immensely helpful. I'm truly grateful for her help in completing this project. I would also like to acknowledge Dr. Sango Otieno, a professor in the Statistics Department at Grand Valley State University. He and his team of graduate students in the Statistical Consulting Center helped to sort through and process the mountain of data I had collected over the previous year. I appreciate their help and willingness to share in their expertise as it relates to statistical analysis.

I would also like to extend my gratitude to Dr. David Geenen, Professor in the Department of Physician Assistant Studies at Grand Valley State University. Dr. Geenen was

willing to jump in and join my research committee and his vast knowledge and expertise was much appreciated as I began to analyze and interpret my data.

Finally, I would like to thank Dr. David Linn, professor in the Biomedical Sciences Department at Grand Valley State University. Dr. Linn graciously allowed me to join his team as I entered Grand Valley as a graduate student in 2012. Dr. Linn's former student assistant, Jenna Fredrickson, was immensely helpful in sharing her knowledge when I arrived and I wish her the best of luck as she begins her Physician Assistant Studies program this fall. Working in Dr. Linn's lab has been both challenging and rewarding. It has helped me to develop the skills to think as a scientist, to critically solve problems, to work independently in the laboratory setting, and to address a problem that had yet to be answered. I have learned and grown an immeasurable amount throughout this experience and am thankful to Dr. Linn for granting me that opportunity. I truly appreciate the time he took to teach and share his expertise with me throughout my time at GVSU; it was a pleasure learning from him and I know that I will be a better health care provider because of this experience.

PREFACE

Definition of Terms

ABT 199: molecule designed to selectively bind and inhibit Bcl-2, a critical regulator of apoptosis. Bcl-2 is hypothesized to play a vital role as the final protein in the neuroprotective pathway utilized by PNU-282987.

ACh: acetylcholine, a neurotransmitter utilized in the human body at all autonomic ganglia, at many autonomically innervated organs, at the neuromuscular junction, and at many synapses in the CNS. In the mammalian retina, acetylcholine is released from starburst amacrine cells and acts on both nicotinic and muscarinic receptors on retinal ganglion cells.

Allosteric modulation: the modification of an enzyme or protein via the process of ligand binding at an allosteric site (a site physically distinct from the protein's active site) which enhances or diminishes the action of substrate at the active binding site.

α7 nAChR: alpha-7 nicotinic acetylcholine receptor, located on retinal ganglion cells within the mammalian retina and hypothesized to play a role in neuroprotection.

Excitotoxicity: neuronal cell death caused by overstimulation of receptors, specifically by the amino acid neurotransmitter, glutamate. In the retina, diseases associated with excitotoxicity include glaucoma, retinal ischemia, and diabetic retinopathy.

IOP: intraocular pressure, or pressure within the eye which is determined by the coupling of production of aqueous humor from ciliary epithelium and drainage of aqueous humor through the trabecular meshwork. Often this pressure is increased in a glaucomatous state.

LY 294002: a potent inhibitor of class I phosphoinositide 3-kinases, which signal through Akt to hypothetically enhance the action of Bcl-2, an anti-apoptotic protein.

Neuroprotection: any therapeutic strategy aimed at the prevention or delay of neurodegenerative processes.

PAM: positive allosteric modulator, a ligand that binds an allosteric site of a protein or enzyme to structurally modify and enhance the activity of an orthosteric (primary) agonist at the active site.

PNU-120596: a novel positive allosteric modulator of the α7 nAChR which not only increases the potency and maximal efficacy of agonists but also is the first to demonstrate modification of neuronal activity *in vivo*.

PNU-282987: a potent, specific agonist of the α7 nAChR which is found on retinal ganglion cells within the retina.

RGC: retinal ganglion cell, a type of neuron located on the inner surface of the retina which receives visual information from photoreceptors. Retinal ganglion cells transmit vision-forming information to the brain via their axons, which collectively form the optic nerve. Death of these neurons is the hallmark pathologic feature of glaucoma.

SB 203580: an inhibitor of MAP kinase signaling, specifically of p38 MAP kinase which is implicated in the excitotoxic pathway of retinal ganglion cells.

ABSTRACT

Glaucoma is one of several neurodegenerative diseases of the central nervous system for which a pharmacologic cure is yet to be discovered. In previous studies acetylcholine (ACh) has provided neuroprotection for retinal ganglion cells (RGC) in the mammalian retina under glaucomatous conditions (Wehrwein et al., 2004). More specifically, an α7 nicotinic ACh receptor (nAChR) agonist has demonstrated neuroprotection for RGCs in both *in vitro* and *in vivo* models of glaucoma (Iwamoto et al., 2014). In this study, this α7 nAChR agonist was combined with a positive allosteric modulator (PAM) in dissociated adult porcine retinas to evaluate its effect on isolated RGCs and identify a potential molecular signaling pathway of neuroprotection.

Porcine retinas were dissociated using a two-step panning technique to isolate RGCs. Once isolated, RGCs were cultured under various pharmacologic conditions and incubated for 3 days. Pharmacologic conditions utilized throughout the course of this research included: agonist alone, agonist with low, medium or high dose PAM, and PAM without agonist. In subsequent experiments, enzyme inhibitors were applied thirty minutes prior to pharmacologic intervention to evaluate effects of the drugs in the absence of specific proteins utilized in their hypothesized cellular signaling pathway.

Retinal ganglion cells treated with the α7 nAChR agonist alone demonstrated a 28.0% (± 12.8%) increase in cell survival over untreated control. This agonist in combination with medium or high dose PAM resulted in increased cell survival at 43.0% (± 11.6%) and 52.0% (± 20.9%),

respectfully. However when the PAM was used as monotherapy, cell survival increased by only 3.2% (± 10.4%) over untreated control, supporting its hypothesized allosteric mechanism of action. Enzyme inhibition results suggest that the α7 nAChR agonist utilizes the PI3 to Bcl-2 signaling pathway to produce this neuroprotective effect, and that the PAM works in an allosteric manner through PI3 kinase to produce an enhanced effect. These studies provide support for future research in analyzing the effects of an α7 nAChR agonist and PAM in *in vivo* models of RGC death. Further understanding of these pharmacologic agents could provide important information in the development of new therapeutic options for glaucoma and other neurodegenerative diseases.

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CHAPTER 1

INTRODUCTION

Background

Glaucoma is a pathologic state in which a combination of increased intraocular pressure, damage to the optic nerve, and death of retinal ganglion cells result in loss of vision and ultimately, irreversible blindness. According to the World Health Organization's 2012 statistics, there are an estimated 285 million people in the world who are visually impaired, 39 million of who are legally blind (WHO Media Centre, 2012). Of those, approximately 44.7 million people are blind or visually impaired as a result of open-angle glaucoma, and this number is expected to increase to 58.6 million people by 2020 (Jacobs, Trobe & Park, 2014). Consequently, glaucoma is the second-leading cause of blindness in the world. In addition to this staggering data, the American Academy of Ophthalmology estimates only 50% of the 3 million Americans who suffer from open-angle glaucoma are aware that they have the condition (The Ophthalmic News & Education Network, 2010). The insidious, chronic nature of open-angle glaucoma in combination with the lack of curative treatment renders glaucoma a devastating medical problem that needs to be addressed.

Treatment for glaucoma is limited. The American Academy of Ophthalmology lists pharmacologic treatment, laser therapy, or incisional glaucoma surgery as treatment options, with pharmacological eye drops being the most common (The Ophthalmic News & Education Network, 2010). The problem with these options is that they all aim to treat the initial

pathology of increased intraocular pressure (IOP), but none addresses the underlying cause of blindness- optic nerve damage and retinal ganglion cell (RGC) death. An increase in IOP results when the outflow channels between the trabecular meshwork and the uveoscleral blood vessels resist aqueous drainage from within the eye (American Glaucoma Society, 2013) or when production of aqueous humor exceeds the capacity of the drainage system. When the aqueous humor is unable to drain, either completely or at a rate sufficient for production, the pressure within the eye rises. Elevated IOP is the number one risk factor for glaucoma, but it is not widely accepted as the direct cause of blindness and is not present in all cases of glaucoma. Increased IOP and RGC death have a cause and effect relationship. Most often, an increase in IOP results in activation of pathways that are involved in apoptotic RGC death. However not all cases of glaucoma present with IOP, and studies have shown lowering IOP does not ensure prevention of RGC death (Sena et al., 2010). A specific class of glaucoma called normotensive glaucoma presents with the same pathological findings as open-angle glaucoma in the absence of elevated IOP (Munemasa & Kitaoka, 2013). All currently accepted therapies are targeted at decreasing IOP since this is the only modifiable risk factor (American Glaucoma Society, 2013). However, not all cases of glaucoma exhibit this risk factor and therapies focused on direct protection of the optic nerve remain to be utilized.

There are numerous theories regarding the molecular mechanism behind RGC degeneration associated with glaucoma. In addition to IOP elevation, the processes of oxidative stress, autoimmune regulation, glial cell activation, endoplasmic reticulum stress, endothelin receptor activation, neurotrophic factor deprivation, and excitotoxicity have all been implicated in the degeneration of RGCs (Munemasa & Kitaoka, 2013; Kuehn et al., 2005). The process of

excitotoxicity, specifically excitotoxicity caused by the neurotransmitter glutamate, has been a focus of extensive background study in this lab and others for decades. Glutamate is an excitatory neurotransmitter that is released by presynaptic neurons in the mammalian nervous system. Glutamate excitotoxicity is a pathological process involved in numerous degenerative conditions throughout the central nervous system and within the retina (Munemasa & Kitaoka, 2013). Toxicity in the retina was first described in 1957 by Lucas and Newhouse who injected glutamate subcutaneously in mice and observed severe destruction of the retinal ganglion cell layer (Lucas & Newhouse, 1957). The term "excitotoxicity" was coined in 1969 to describe this type of neuronal damage when similar effects were observed in neonatal mice (Olney, 1969).

In previous studies in both *in vivo* and *ex vivo* models, glutamate excitotoxicity via increased concentrations of glutamate in the eye has shown to lead to a prolonged influx of cations into RGCs, resulting in activation of apoptosis (Brandt et al., 2011; Asomugha et al., 2010; Thompson et al., 2006). In the pig retina, glutamate has been demonstrated to bind to both NMDA and non-NMDA glutamate receptors, activating a calcium-induced cascade that ultimately results in RGC death through an apoptotic mechanism (Thompson et al., 2006). Although the specific role of glutamate excitotoxicity in the overall pathology of glaucoma remains unclear, drugs that target the prevention of this pathway have shown promise in preventing RGC apoptosis (Danesh-Meyer, 2011).

With regards to glaucoma, neuroprotection is defined as an intervention administered to prevent any one of the previously proposed mechanisms from causing optic nerve damage or cell death (Danesh-Meyer, 2011). In recent years there have been numerous studies

conducted to explore the development of novel neuroprotective strategies. According to a review done by Prof. Helen Danesh-Meyer of the University of Auckland in 2011, 37 different neuroprotective therapeutic agents were tested on the glaucoma model from 2010-2011. Of these, a wide spectrum of RGC survival was attained through prevention of many of the aforementioned target pathways. Previous studies from this lab and others have demonstrated success in preventing RGC loss and axon degeneration through stimulation of nicotinic acetylcholine receptors (nAChR) within the mammalian retina (Iwamoto et al., 2013; Brandt et al., 2011; Thompson et al., 2006; Wehrwein et al., 2004).

Previous studies have demonstrated that neuroprotection results from the activation of nicotinic acetylcholine receptors (nAChRs) with ACh or the receptor agonist, nicotine (Brandt et al., 2011; Asomugha et al., 2010; Bader and Linn, 2007; Thompson et al., 2006; Wehrwein et al., 2004). Further studies were then performed to investigate the intracellular mechanism of ACh neuroprotection. Results of these studies suggest that ACh provides RGC protection by directly stimulating an enzyme survival pathway, the PI3 kinase signaling pathway, and indirectly inhibiting an excitotoxic pathway, the p38 MAP kinase pathway (Asomugha et al., 2010). It is also hypothesized that this excitotoxic pathway which ACh inhibits is the same pathway that is up-regulated in the presence of glutamate, a known cause of excitotoxic processes throughout the CNS (Asomugha et al., 2010).

To further address the effectiveness of ACh neuroprotection, an *in vivo* study was designed to test the role of ACh under physiologic conditions (Iwamoto et al., 2014). Specifically, the α7 nAChR agonist PNU-282987 (Tocris Bioscience, 2006) was administered via

eye drops to the intact eye of Long Evans rats. This compound was originally developed by Pfizer but sold when learned to have toxic effects systemically. Use of this substance in the retina remains a viable option as drugs administered in eye drop form have minimal systemic absorption. This was specifically tested by Linn et al. in 2011 in an *in vivo* model; PNU-282987 in eye drop form penetrates retinal tissue while trace amounts could be detected in blood plasma systemically (Linn DM et al., 2011; Farmen et al., 2011). Further results of *in vivo* use of PNU-282987 in eye drop form in the intact retina demonstrated prevention of RGC loss in a dose-dependent manner. This *in vivo* study utilized rats with surgically modified elevations in IOP to mimic glaucoma conditions. When glaucomatous rats were treated with low-dose agonist in eye drop form for 30 days post-surgery there was no significant reduction in RGC loss. However, with a medium dose of agonist, significant neuroprotection resulted with total cell numbers approaching that of the control eye. With a high dose, the percentage of RGCs increased in the experimental eye in comparison to the untreated control eye (Iwamoto et al., 2014).

Under normal conditions, neurons are not actively dividing cells. In humans the majority of the neocortex is formed from stem cells before birth and neurogenesis ceases in adulthood. However, the application of PNU-282987, a nAChR agonist, at a high concentration dosage resulted in increased numbers of RGCs in the experimental eye as compared to the control eye (Iwamoto et al., 2014). The medium dose resulted in similar numbers of RGCs under high IOP conditions versus the control eye, suggesting successful use as a neuroprotective agent. Further research is needed to investigate the cause of increased RGC counts at a higher dose of this agonist. It is not clear if neurons are being induced to divide in a physiologic or pathologic manner. It is possible that oncogenes are being activated resulting in neoplastic growth masked

as increased numbers of RGCs. It is equally possible that a normal growth factor pathway is being activated resulting in controlled differentiation (Linn CL et al., 2011).

Expounding on these questions will be an important target of future research. If the nAChR agonist is indeed stimulating an enzyme pathway utilized in RGC survival and inhibiting the enzyme pathway utilized in excitotoxicity, these neuroprotective effects could be enhanced with the addition of a positive allosteric modulator (PAM). A PAM is a compound that binds to a site distinct from the agonist-binding site on the target receptor complex. This site on the receptor complex is called the allosteric site, and its binding serves to indirectly amplify the effect of an agonist at another discrete target (orthosteric) site on the same receptor complex. Generally this is performed through induction of a conformational change in the orthosteric binding site. In contrast to an orthosteric agonist, the allosteric modulator does not bind directly to the active receptor site and thus does not produce a direct agonist effect of its own (Schwartz & Holst, 2007). Instead, they enhance the sensitivity and/or efficacy of the receptor during agonist activation (Hurst et al., 2005).

The prototypic example of a positive allosteric modulator is Diazepam (Valium). Diazepam is a drug of the benzodiazepine class, which work by binding stereospecific benzodiazepine receptors on the postsynaptic GABA neurons, located at several specific sites throughout the CNS (Brunton, Chabner & Knollmann, 2011). Binding of Diazepam to a GABA neuron enhances membrane permeability to chloride ions and thus induces hyperpolarization and cell stabilization, but only in the presence of GABA. In the absence of natural GABA binding Diazepam exerts no effect. It cannot activate receptors that are not being activated and

therefore has a ceiling effect, allowing for a greater safety profile that other GABA agonists like barbiturates. In this study the α7 nAChR PAM, PNU-120596, is hypothesized to work in this same manner by enhancing the action of the α7 nAChR agonist, PNU-282987, at its target receptor.

PNU-120596 is a novel positive allosteric modulator of the α7 nAChR which not only increases the potency and maximal efficacy of agonist but also is the first to demonstrate modification of neuronal activity *in vivo* (Hurst et al., 2005). Several PAMs of α7 nAChRs have been previously described; these agents function to minimize the rapid desensitization of α7 nAChRs and maximize the peak evoked response that is characteristically induced by orthosteric agonists (Gill et al., 2013). The PAM used in this study comprises an entirely new class of PAM of the α7 nAChR as it the first drug of its kind to substantially modify the duration of agonist evoked response (Hurst et al., 2005). If the agonist in question, PNU-282987, provides neuroprotection to RGCs via binding to α7 nAChRs and up-regulating an enzymatic survival pathway, an α7 nAChR PAM should amplify and prolong these effects to provide enhanced protection from cell death. This study aims to address this hypothesis with the α7 nAChR positive allosteric modulator, PNU-120596 (Tocris Bioscience). Further research in this field of study may have great implications in the search for a neuroprotective agent that can prevent, or even possibly reverse, the loss of RGCs associated with glaucoma.

Problem Statement

Glaucoma is one of the leading causes of blindness in the world. There has been a significant amount of research dedicated to finding a cure, however at this point none has been obtained. The drugs currently on the market do not target the true cause of vision loss, but rather aim to slow the progression of severe neuron damage. With current IOP-lowering medications, RGC death can be slowed but not fully stopped. A compound that acts directly on the RGCs and their axons could be of benefit alone and in combination with current medications. An analysis of the cellular effect of an α7 nAChR agonist and PAM could reveal their effectiveness as a pharmacologic therapy to prevent the RGC loss associated with glaucoma at a cellular level.

Aims of Study

The main objective of this study is to determine if the use of a positive allosteric modulator can amplify the survival of retinal ganglion cells treated with the nAChR agonist PNU-282987 under glaucomatous conditions. If amplified survival is achieved, further investigation into the role of known signaling cascades utilized by various combinations of agonist and PAM will be conducted.

Significance of Study

Evaluation of the result of isolated RGC treatment with PNU-282987 and a PAM may reveal an increase in cell survival over the level which PNU-282987 can achieve as mono-therapy. An amplification of cell survival would increase the clinical efficacy of this drug and

promote advancing research in this field of study. The researchers in this study aim to promote advancement in this field to develop a drug that can effectively prevent the death of RGCs associated with vision loss in glaucoma.

Research Questions

- Previously, *in vitro* glaucoma studies were conducted on RGCs that had their axons severed (axotomy) during removal of the eye globe in the course of dissection. Thus, one could hypothesize that the observed drop of RGCs in culture to a plateau level (Wehrwein et al. 2004) reflects this damage. The addition of high levels of glutamate in previous studies leads to an additional drop observed with this system. Without the additional insult of glutamate, will an α7 nAChR agonist and PAM increase RGC survival under axotomy conditions?
    - Does the neuroprotection occur in a dose dependent manner?
    - Is the neuroprotection seen with the combination of drugs significantly higher than neuroprotection provided by agonist alone?
    - At which concentration of α7 nAChR agonist and PAM does optimal neuroprotection occur?
- Previously, the α7 nAChR agonist has been proven to provide neuroprotection through stimulation of the PI3 kinase pathway resulting in phosphorylation of Akt and upregulation of Bcl-2 (Asomugha, Linn, & Linn, 2010). If the combination of α7 nAChR agonist and PAM also provide neuroprotection, is it a result of activation of this same pathway?

- If so, is the effect of the α7 nAChR agonist enhanced by the presence of the PAM in an allosteric manner? Or, is an additional pathway activated?

CHAPTER 2

LITERATURE REVIEW

Introduction

Glaucoma is a devastating, worldwide disease. In just the United States there are over 2 million estimated cases with 120,000 of those responsible for blindness. Unfortunately, only half of those 2 million people are estimated to be aware of their condition (Glaucoma Research Foundation, 2012). Angle-closure glaucoma is an acute condition associated with rapid onset of severe pain, erythema and visual loss, and thus emergent surgery is employed as a therapeutic intervention. In contrast, open angle glaucoma, the most common subtype of glaucoma, is an insidious process that results in slow, progressive, and irreversible loss of the visual field (Munemasa & Kitaoka, 2013). Early stages of open angle glaucoma may be asymptomatic, allowing the disease to go unnoticed and progress to optic nerve damage. At this point no form of treatment can restore lost vision (Sena et al., 2010) and thus screening for at-risk individuals is vital in diagnosis. Still others will initiate treatment before this point of progression and still experience optic nerve damage as a result of inadequate treatment options. Unlike most other eye diseases, glaucoma is a lifelong neurodegenerative disorder that cannot be cured, only treated (American Glaucoma Society, 2013).

The inadequacy of pharmacological therapy for glaucoma has landed it at the forefront of numerous research studies in the recent past. According to a review done at the University of Auckland in 2011, 37 different therapeutic agents were tested on the glaucoma model from 2010-2011 (Danesh-Meyer, 2011). The current medical strategy for treatment of glaucoma is

medication or surgery to decrease intraocular pressure (IOP) - a strategy proven to slow progression of the disease (The Ophthalmic News and Education Network, 2010). However, axon deterioration and visual field loss have been observed in eyes with normal or decreased IOP, suggesting that the disease process is at least partially independent of this variable (Munemasa & Kitaoka, 2013). Despite these findings, reducing pressure in the eye remains the only proven pharmacological therapy to slow the progression of glaucoma (American Glaucoma Society, 2013). A true pharmacological cure for glaucoma is dependent upon the prevention of RGC death as these are the cells responsible for transmitting visual impulses along their axons to the visual processing center of the brain (Munemasa & Kitaoka, 2013).

Numerous strategies have been employed to prevent RGC death. These neuroprotective strategies and their corresponding pharmacological agents have been tested on pre-clinical animal models with varying success rates. One of the many challenges faced in development of a successful pharmacotherapy is lack of knowledge of the underlying mechanism of glaucomatous optic neuropathy (Danesh-Meyer, 2011). At this point in time considerable research is still aimed at discovery of the true cause of optic neuropathy, if a single cause does exist. Numerous methods of injury have been implicated including: oxidative stress, autoimmune regulation, glial cell activation, endoplasmic reticulum stress, endothelin receptor activation, neurotrophic factor deprivation, hypoperfusion of the optic nerve, and excitotoxicity (Kuehn et al., 2005; Danesh-Meyer, 2011; Munemasa & Kitaoka, 2013). Drugs have been successfully developed to target many of these injury processes with no specific therapy showing significant success over the others. With widespread promise demonstrated in cellular

and pre-clinical studies it is important to continue further research into narrowing the field of study and translating it to a clinical setting for therapeutic use (Danesh-Meyer, 2011).

In 2010 a review was published by the Cochrane Collaboration detailing the state of clinical advancement in neuroprotection as treatment for glaucoma in adults. The review was performed to systematically examine the effectiveness of topical and oral neuroprotective agents in prevention of RGC death in adults aged 30 years and older with open angle glaucoma. Selection criteria was limited to randomized controlled trials with a minimum five year follow up period in order to fully assess progression of visual field loss. Initial literature review returned 1716 articles and 29 were chosen for primary inclusion. After further evaluation none met the criteria for review (Sena et al., 2010). The results of this review reiterate the pressing need for further clinical research in the field of glaucomatous neuroprotection. Although a sufficient amount of cellular and animal research has been completed to date, very little has been carried out to the clinical phase with results to provide evidence of clinical importance. Of the 1716 articles initially returned, 15 potentially relevant trials were found with follow-up periods ranging from two hours to two and a half years (Sena et al., 2010). This is a step in the right direction although it is clear that further research needs to be directed into long-term visual field preservation as glaucoma is a progressive and lifelong disease (Sena et al., 2010).

Comparative Literature

As stated previously, there are currently many theories and strategies in regards to RGC and optic nerve injury and their appropriate treatment. One of those treatments investigated in the Department of Ophthalmology at Harvard Medical School is Etanercept, marketed as

Enbrel, a widely used tumor necrosis factor-α (TNF-α) antagonist (Roh et al., 2012). TNF-α is a pro-inflammatory cytokine that is secreted in response to infection and trauma and plays a role in apoptosis of susceptible cells throughout the body (Roh et al., 2012). Prior to this study by Roh et al., TNF-α was hypothesized to play a connecting role between increased IOP and RGC death although its exact role was unclear. To investigate the role of TNF-α, Roh et al. used an *in vivo* rat model with episcleral vein cauterization (EVC). This technique is used to elevate IOP through cauterization and generation of scar tissue in the drainage system of the eye with subsequent obstruction of outflow channels of aqueous humor resulting in rapid-onset elevations in IOP. EVC was performed on the right eye to simulate the glaucoma-like condition of elevated IOP, leaving the left eye untreated as a control. This is a widely-accepted and commonly used mechanism for glaucoma model experimentation in animals (Danesh-Meyer, 2011). The TNF-α inhibitor was injected intraperitoneally and several parameters were measured post-sacrifice (Roh et al., 2012).

Results from this study were suggestive of further use of Etanercept as a neuroprotective agent for glaucoma. Eyes in rats subject to EVC demonstrated increased IOP with a subsequent dramatic increase in retinal TNF-α levels, axonal degeneration, and a 38% loss of RGCs (Roh et al., 2012). Eyes subject to EVC with Etanercept treatment also demonstrated reduced TNF-α levels and axon degeneration as well as near-control levels of RGCs. This study also determined that microglia cells activated by increased IOP are the source of TNF-α in the retina and therefore provide the foundation for activation of RGC apoptosis. Confirming both an increased level of TNF-α and a source for its production clarifies its role in RGC loss (Roh et al., 2012). The results of this study are significant because they identified both

a cause and treatment of RGC loss in a glaucomatous state, charting a path for future research into TNF-α antagonists as a treatment strategy for glaucoma.

Another theory in RGC degeneration is apoptosis as a result of hydrostatic pressure-induced oxidative stress on the retina. To further evaluate this theory, researchers at Shandong University of Traditional Chinese Medicine studied the effects of alpha-lipoic acid (ALA), an agent proven to provide protection against neurodegenerative disorders in humans and experimental animals (Liu et al., 2012). The aim of study was to determine if ALA also produced the same neuroprotective effects in the retina under glaucoma-like conditions. The study was performed on an *in-vitro* model of cultured RGCs to determine if ALA provides the same neuroprotective effect for RGCs as it has proven to provide for neurons in other neurologic disorders related to oxidative stress such as Alzheimer's disease, diabetic peripheral neuropathy, subarachnoid hemorrhage, and traumatic brain and spinal cord injury (Liu et al., 2012). The *in-vitro* model is useful for glaucoma because it facilitates assessment of molecular changes and enzyme regulation of RGCs independent of environmental factors (Liu et al., 2012).

Liu et al. first isolated RGCs, followed by pretreatment with varying concentrations of ALA (50 μM-200 μM). Pretreated and non-pretreated experimental cells were exposed to six hours of hydrostatic pressure (50 mmHg) while pretreated and non-pretreated control cells remained in a normal pressure environment. Results revealed cells that were subject to high pressure without ALA pre-treatment experienced increases in apoptosis and intracellular reactive oxygen species (ROS) production in comparison to non-pretreated control cells in a normal pressure environment. In contrast, cells pretreated with ALA experienced significant

reduction in ROS production and prevention of apoptosis through the mRNA expression of manganese superoxide dismutase (MnSOD), an antioxidant enzyme (Liu et al., 2012). MnSOD functions to counteract the injury inflicted by hydrostatic pressure through deactivation of ROS. Researchers concluded that pre-treatment with ALA significantly increased the expression of MnSOD leading to reduced production of ROS, resulting in inhibition of hydrostatic pressure-induced RGC degeneration in a dose-dependent manner. Through its antioxidant activity, ALA supplementation has potential as a neuroprotective agent in glaucoma therapy. In addition, ALA has already been used for years in the treatment of diabetic peripheral neuropathy and has an established safety profile (Liu et al., 2012). Therefore, these results dictate further studies directed towards the therapeutic value of ALA for glaucoma in a clinical setting.

Another recent theory suggests a correlation between glaucoma and Alzheimer's disease, as both involve neuronal loss through an apoptotic process. To further assess this correlation, a team of Canadian researchers from universities in Quebec and Nova Scotia worked together to study the effectiveness of the Alzheimer's drug Galantamine as a neuroprotective agent for RGCs (Almasieh et al., 2010). Galantamine is an acetylcholinesterase (AChE) inhibitor and allosteric ligand of nAChRs that is used for symptomatic treatment of Alzheimer's. Through inhibition of AChE, the drug prevents breakdown of ACh and allows ACh levels in the retina to increase and supposedly counteract the functional loss of cholinergic cells due to disease process. Increased levels of ACh have proven to provide neuroprotection through increased stimulation of AChRs *in vitro*. The mechanisms of neuroprotection *in vivo* remain poorly defined, but results have proven that Galantamine increases survival of hippocampal neurons and dopaminergic neurons in the treatment of Alzheimer's. To determine

if its neuroprotective effects translate to the treatment of glaucoma, an *in vivo* rat model was used. Rats were subjected to elevated IOP through limbal vein obliteration via hypertonic saline injection to mimic the effects of glaucoma. Treatment with Galantamine produced significant neuroprotection for RGCs under these conditions (Almasieh et al., 2010).

Researchers next evaluated the functional effect of Galantamine on RGCs through measurement of visual evoked potentials (VEP) after flash stimulation (Almasieh et al., 2010). VEPs are evaluated by a recording electrode placed on the visual cortex which receives predominantly RGC input, and therefore serve as a functional assay to determine if RGCs that demonstrate structural protection also demonstrate functional protection. Initial results revealed that both phosphate buffered saline (PBS)-treated and Galantamine-treated eyes showed complete obliteration of VEPs five weeks post-EVC, despite a structural protection rate of 70% of RGCs with Galantamine treatment. Researchers hypothesized that visual impairment was mediated by sustained elevations in IOP and tested their hypothesis with a combination therapy of Galantamine and Timolol, a topical β-adrenergic receptor blocker commonly used in glaucoma therapy to decrease IOP. This combination therapy restored 47% of the VEP response in Galantamine/Timolol treated eyes compared to a 0% response in PBS/Timolol treated eyes. Lastly, researchers determined the mechanism of action of Galantamine through selective pharmacological blockade of the two hypothesized pathways, nAChRs or muscarinic AChRs (mAChRs). Galantamine provides neuroprotection in Alzheimer's treatment through activation of nAChRs, however results proved neuroprotection of RGCs was mediated through mAChRs (Almasieh et al., 2010).

This study produced important findings in the search for an effective pharmacological therapy for glaucoma. First, Galantamine proved to be an effective pharmacological therapy in the protection of RGCs in an *in vivo* model of glaucoma. Second, functional deficits seen in glaucoma were markedly improved by a combination therapy of Galantamine and a β-adrenergic receptor blocker. This supports further research into the use of novel neuroprotective drugs in combination with currently used IOP-lowering drugs for long term functional RGC protection and subsequent visual field preservation. Finally, Galantamine's effect was modulated by mAChRs, suggesting further research into their use as a therapeutic target for prevention of RGC death and visual loss associated with glaucoma (Almasieh et al., 2010).

Summary and Implications for Study

For the past decade, research has been conducted in this lab with the purpose of determining the cause and prevention of RGC degeneration associated with glaucoma. Studies have been directed at the prevention of neuronal degeneration as a result of glutamate-induced excitotoxicity, one of the major theories behind glaucomatous optic neuropathy, as well as other implicated degeneration models. Previous studies have identified an excess of glutamate in the vitreous humor of eyes affected by glaucoma, and that excess glutamate has been proven to lead to a prolonged influx of nonspecific cations that activate pathways of apoptosis within RGCs (Dryer et al., 1996). In a previous study, glutamate excitotoxicity was tested on an *in vitro* model of isolated pig RGCs exposed to 500µM L-glutamate for various time periods. By day 3, cells exposed to chronic glutamate had a mean 58% survival rate as

compared to untreated isolated cells, suggesting glutamate had an excitotoxic effect on 42% of isolated RGCs after a 72 hour period (Wehrwein et al., 2004). Further research determined this excitotoxic effect was mediated through activation of both NMDA and non-NMDA glutamate receptors (Wehrwein et al., 2004).

Next, a neuroprotective strategy was developed to prevent neuron degeneration associated with glutamate excitotoxicity. As stated in the previous study, nAChRs are considered to play a role in neuroprotection of hippocampal and cortical neurons in Alzheimer's therapy, although the exact mechanism of neuroprotection is unclear. Agonists of nAChRs, acetylcholine (ACh) and nicotine, were tested for neuroprotective effectiveness on glutamate-induced excitotoxicity in the retina. Results showed increased RGC survival as a result of pretreatment with ACh or nicotine before chronic glutamate exposure. Further experimentation in combination with previous evidence suggested the neuroprotective effect of the nAChR agonist was mediated through α7 nAChRs (Wehrwein et al., 2004). These findings have since been supported with further research and expanded to include a potential additional role of α4 nAChRs (Thompson et al., 2006).

Subsequent research was aimed at determining the mechanism of correlation between nAChRs and neuroprotection. Two signaling pathways were hypothesized to be involved: p38 MAP kinase and PI3 kinase (Asomugha et al., 2010). The p38 MAP kinase pathway is associated with inflammation and apoptosis. On the other hand, PI3 kinase is involved in a pathway that induces activation of two other proteins that are actively involved in cell survival processes. ELISA studies were performed on cultured pig RGCs and results revealed that glutamate

excitotoxicity is mediated through the p38 MAP kinase signaling pathway while ACh neuroprotection is mediated through stimulation of the PI3 kinase pathway and inhibition of the p38 MAP kinase pathway (Asomugha et al., 2010). Further research revealed that the PI3 kinase survival pathway is activated by calcium permeation through nAChR channels activated by an ACh agonist. Therefore, calcium is the trigger linking activation of nAChRs to activation of neuroprotection (Brandt et al., 2012).

The next step was to evaluate an *in vivo* model to determine if ACh had a neuroprotective effect under physiological conditions. A rat model was used and rats were subject to limbal vein obliteration via hypertonic saline injection (Iwamoto et al., 2014) as was used by Almasieh et al. in the galantamine study described previously. Injection of a hypertonic solution leads to scarring of vessel lumen, decreasing diameter and increasing resistance to out-flow. This technique is similar to the EVC technique described in previous studies as the outcome of both procedures is resistance to aqueous humor drainage and ensuing elevation of IOP mimicking glaucoma. EVC is rapid process with cauterization resulting in complete obliteration; this in contrast to limbal vein occlusion which is a much more gradual process taking up to a month for scarring to obliterate the venous lumen. Using the limbal vein occlusion procedure, an α7 nAChR agonist, PNU-282987, was applied in eye drop form for 30 days post-procedure to 3 different dosing groups. Results revealed that rats treated with low-dose agonist for 30 days post-surgery experienced no significant reduction in RGC loss. However, with a medium dose of agonist significant neuroprotection resulted, and with a high dose the percentage of RGCs increased in comparison to the untreated control eye (Iwamoto et

al., 2014). These results support the use of a nAChR agonist for RGC neuroprotection and raise the ensuing question of the mechanism of increased RGC counts in the high dose agonist group.

This study aims to further elucidate the mechanism of nAChR agonist neuroprotection in the absence of additional elevated glutamate insult, as well as to examine the effectiveness of the nAChR agonist in combination with a positive allosteric modulator (PAM). A PAM is a ligand that binds an allosteric site distinct from the agonist-recognition site on its target receptor. It has no agonistic activity when applied alone, but enhances the activity of agonist-evoked responses when co-applied with an orthosteric agonist (Gill et al., 2013). In the setting of this study, the application of a specific PAM of the α7 nAChR, PNU-120596, in combination with the α7 nAChR agonist PNU-282987 should theoretically potentiate an increased neuroprotective response. To test this hypothesis an *in vitro* model of isolated porcine RGCs will be used. The same enzyme pathways implicated in previous study, p38 MAP kinase and PI3 kinase, will be analyzed in the setting of both a nAChR agonist and PAM.

As previously stated, the p38 MAP kinase pathway is associated with inflammation and apoptosis while the PI3 kinase pathway is involved in cell survival processes (Asomugha et al., 2011). ELISA studies provided evidence to support glutamate's role in excitotoxic cell death through the p38 MAP kinase pathway that ultimately decreases levels of Bcl-2, an anti-apoptotic regulator protein. Studies also provide evidence of the PI3 kinase pathway in phosphorylation of Akt, which ultimately leads to increased expression of Bcl-2 (Asomugha et al., 2011). If a nAChR agonist and PAM indeed provide neuroprotection by these enzyme pathways, the neuroprotection would be eliminated in the presence of a Bcl-2 inhibitor. This

concept will be the one of the main focuses of this study. Further elucidation of the role of nAChR agonists, specifically in the presence of a PAM, will be beneficial in the search for novel pharmacotherapeutic strategies for glaucoma.

Throughout the course of this study several hypotheses are tested, and these can be summarized into five major parameters. First, in the absence of an elevated glutamate insult the presence of an α7 nAChR agonist will increase RGC survival over control levels after three days in culture. Second, RGC survival will be enhanced in the presence of an α7 nAChR PAM in combination with the aforementioned agonist. Third, the increased RGC survival mediated by PI3 kinase will be completely or nearly completely inhibited by a specific PI3 kinase inhibitor, LY 294002, in the presence of agonist alone or in combination with a PAM. Forth, the increased cell survival expected via blockade of the cell death pathway, p38 MAP kinase, will be more than observed for PI3 kinase inhibition. Fifth, Bcl-2 serves as the final target protein for the increased survival mediated by PI3 kinase, thus inhibition of this protein will result in inhibition of the cell survival mediated by PI3 kinase. Determination of the mechanism of cell survival through evaluation of these pathways is crucial in understanding the pathway to and facilitating the development of a clinically effective neuroprotective treatment for glaucoma.

CHAPTER 3

METHODOLOGY

RGC Isolation Procedure

The study was performed using an *in vitro* preparation of isolated RGCs from adult porcine eyes. The eyes were obtained from a local slaughterhouse (DeVries Meats, Coopersville, MI) and kept on ice during transfer to the Cook-DeVos Center for Health Sciences at Grand Valley State University. Severance of the optic nerve occurs upon removal of the eye from its anatomic location in the orbit, therefore this mechanism of injury was employed even before attaining eyes for dissection. Upon arrival the retina was dissected away from the choroid and sclera posteriorly. Four retinas were then prepared for further dissociation using a two-step panning process previously described by Wehrwein et al. (2004), Thompson et al. (2006) and Asomugha et al. (2011). The panning process is schematically displayed in Figure 1. To begin, isolated retinas were placed into a solution of CO_2-independent media (Gibco) enriched with 4 mM glutamine, 5% fetal bovine serum (FBS), 5% antibiotic/antimycotic (Gibco) and 4 mM HEPES. The retinas were each cut into eight pieces and enzymatically dissociated with papain (27 u/mg) activated with cysteine for 20 minutes at 37°C. After 20 minutes the enzymatic dissociation was inactivated with the addition of fresh CO_2-independent media and 1 mg/ml DNase to break up free DNA released from damaged cells. Physical dissociation by titration and centrifugation for 2 minutes at 2000 rpm completed the retinal dissociation process.

The enzymatically and physically dissociated retinas were then transferred onto Petri dishes pretreated with goat anti-rabbit IgG antibody (Jackson ImmunoResearch; 0.5 mg in 48 ml of 20 mM Tris buffer). Petri dishes were prepared 24 hours prior by application of IgG antibody evenly dispersed over the surface of four large (150 x 15 mm) Petri dishes, which were then kept at 4°C overnight. On the day of experimentation, IgG-treated Petri dishes were washed with three rinses of PBS and one rinse of PBS with 0.2% BSA. After 60 minutes of incubation with PBS with 0.2% BSA this rinse was removed and dissociated retinas were applied. Dissociated retinas were incubated on IgG-coated plates for 1 hour at 37° C to bind nonspecific cellular content such as vascular tissue within the dissociated retina. This completed the first step in the panning procedure.

After incubation for one hour, media containing unbound RGCs was removed from IgG-coated plates and transferred to Petri dishes pretreated with goat anti-mouse IgM (Jackson ImmunoResearch; 0.3 mg in 48 ml of 20 mM Tris buffer) and mouse anti-rat Thy 1.1 antibody (BD Biosciences; 10 µg in 10 ml PBS containing zero calcium and zero magnesium). These plates were prepared in a similar manner as described previously with application of IgM antibody to twelve small (60 x 15 mm) Petri dishes 24 hours prior to experimentation. On the day of experimentation Ig-M coated plates were further prepared by removing excess IgM and applying Thy 1.1 antibody (0.165 µg in 48 ml of 20 mM Tris buffer). Thy 1.1 antibody is used because Thy 1.1 antigen is a RGC-specific marker in the retina (Asomugha et al., 2011). After incubation for 60 minutes at 37° C, excess Thy 1.1 was removed and dishes were washed with three rinses of PBS and one rinse of PBS with 0.2% BSA. After an additional 60 minutes of incubation at 37° C the final rinse was removed and dishes were ready for tissue. The dishes

were incubated with remaining retina contents for 1 hour at 37°C; after this period RGCs were bound to Thy 1.1 on the dishes and the supernatant containing unbound cells was then removed and discarded. This completed the second and final step in the panning procedure as the RGCs were bound to plates treated with Thy 1.1 antibody and therefore isolated from all other retinal tissue. Fresh CO_2-independent media containing glutamate, FBS, antibiotic/antimycotic, and HEPES was further enriched with nerve growth factor (0.3 μg/ml), transferrin (1 μg/ml), and insulin (6 μg/ml) and added to the dishes to nourish isolated RGCs.

This isolation process has been thoroughly established and studied by Wehrwein et al. who demonstrated that RGCs isolated in this nature, subject only to injury via optic nerve severance, die off in a predicable manner over time. In enriched CO_2-independent media, survival of RGCs with severed axons gradually decreases from 100% to a plateau at 61% survival over the first 7 days in culture (Wehrwein et al., 2004). This model allows for evaluation of RGC survival in the absence of the additional variable of added insult, much in the manner that an *in vivo* study would employ.

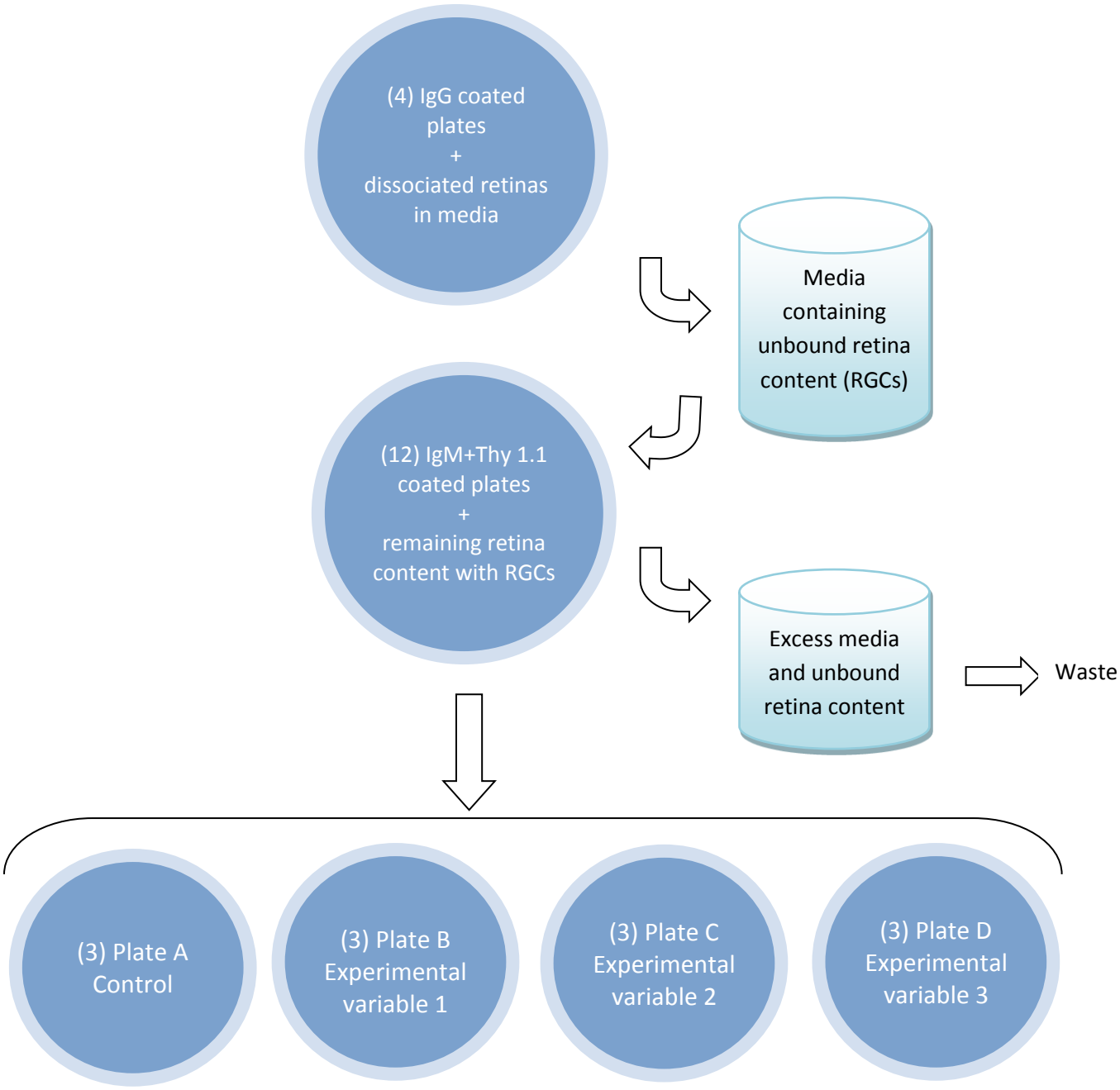

Figure 1. Schematic of panning and RGC isolation protocol. IgM plates contain Thy 1.1 antibody which binds Thy 1.1 antigen that is specific for retinal ganglion cells. RGCs remain bound to the twelve IgM plates while all other retinal content is removed and discarded. Fresh media is applied to each of the twelve plates in preparation for pharmacologic treatment. Plates A are left untreated as control while Plates B, C & D receive pharmacologic treatment.

Experimental Design

Before final incubation the isolated RGCs were treated with an α7 nAChR agonist, PNU-282987 (Tocris, 10 µM in PBS), and varying doses of an α7 nAChR PAM, PNU-120596 (Tocris, 10 µM in PBS) to evaluate their effectiveness as neuroprotective agents for RGCs. Dosage of agonist sufficient for neuroprotection was a subject of previous study by Bader and Linn, 2007, therefore an effective dose of 100 nM agonist was utilized throughout this experiment. Five experimental conditions were performed throughout the course of this study. Conditions 1 & 2 involved agonist and differing doses of PAM. Condition 1 tested the effect of increasing doses of PAM and agonist while Condition 2 tested extreme doses of PAM to further evaluate its role in neuroprotection. Because a PAM is an allosteric ligand it is proposed to have no effect when working alone; this hypothesis is tested in treatment group D of Condition 2.

Conditions 3, 4 & 5 involved pre-treatment with enzyme pathway inhibitors prior to application of agonist and PAM. The enzyme pathway inhibitors served to evaluate the involvement of potential cell signaling cascades implicated in the aforementioned excitotoxic and neuroprotective pathways. By pre-treating with one inhibitor per experiment, a single enzyme or protein's effect can be evaluated. Two enzymes and one protein were evaluated throughout the course of this study. PI3 kinase, p38 MAP kinase, and Bcl-2 were inhibited in Conditions 3, 4 & 5, respectively. A schematic of proposed enzyme pathway involvement in RGC neuroprotection is provided in Figure 2. All five experimental conditions were completed with a control variable (A) and three experimental variables (B, C & D); three dishes were created for

each variable totaling 12 dishes in each experiment. The five experimental conditions are further described in Table 1.

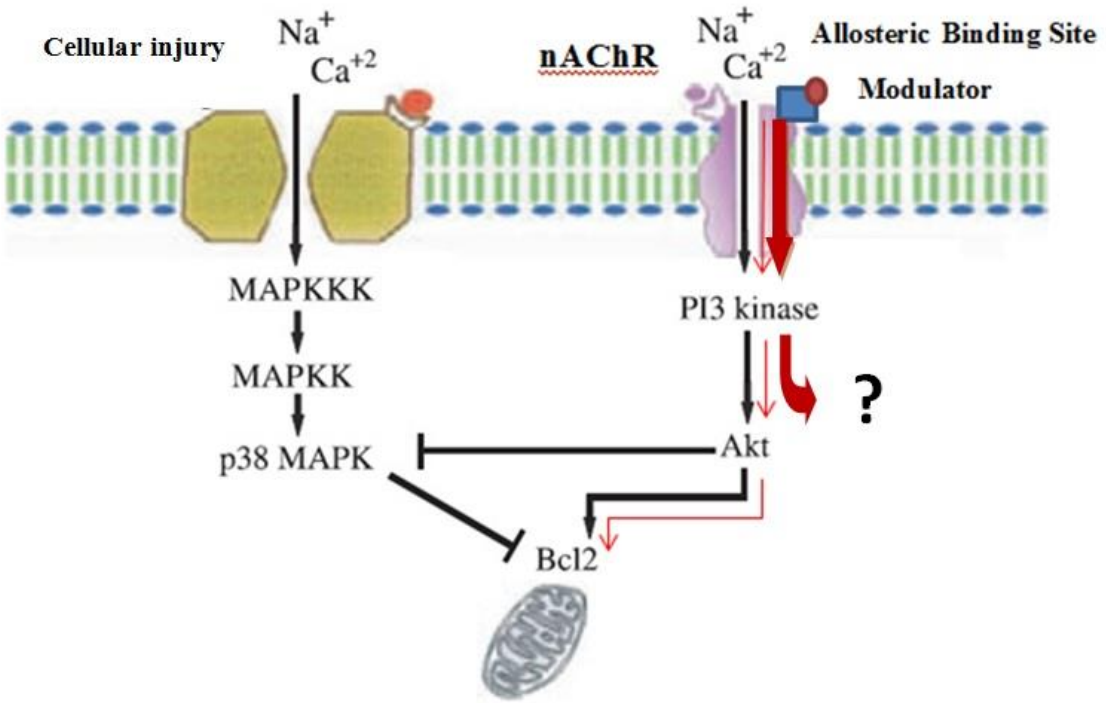

Figure 2. The proposed mechanism of neuroprotection of RGCs is illustrated on the right side of diagram. PNU-282987 has been shown to utilize a binding site on nAChR to initiate an influx of Na^+ and Ca^{2+} and activate PI3 kinase. Ultimately, PI3 kinase activation promotes Bcl-2, an anti-apoptotic protein. The PAM binds to an allosteric transmembrane site on the nAChR and is proposed to act in an allosteric manner by increasing the influx of Na^+ and Ca^{2+} in the presence of PNU-282987 binding. If this is the true mechanism of action the PAM would have no activity in the absence of PNU agonist. It is also possible that the PAM works through a different unidentified pathway; this hypothesis is tested in Condition 2. The left side of the diagram illustrates a proposed mechanism of excitotoxicity. Through activation of p38 MAP kinase, the excitotoxic pathway inhibits Bcl-2 and thus promotes apoptosis. Activation of the PI3 kinase pathway has inhibitory effects on this action of p38 MAP kinase.

Pharmacological Treatment

Doses used in all five conditions can be found in table format in Table 1. Doses of PNU-282987 agonist sufficient for use as a neuroprotective agent were previously studied by Bader and Linn, 2007, and employed in this study. In Condition 1 (n=9) the following pharmacologic doses were used: A) cells were left untreated as a control, B) cells were treated with 100 nM agonist, C) cells were treated with 100 nM agonist and 50 nM PAM, and D) cells were treated with 100 nM agonist and 100 nM PAM. In Condition 2 (n=6) the following pharmacologic doses were used: A) cells were left untreated as a control, B) cells were treated with 100 nM agonist, C) cells were treated with 100 nM agonist and 20 nM PAM, and D) cells were treated with 100 nM PAM alone. In Condition 3 plates B, C & D were pre-treated with 10 nM LY 294002 to inhibit PI3 kinase. After 30 minutes the following pharmacologic doses were used: A) cells were left untreated as a control, B) cells were treated with 100 nM agonist, C) cells were treated with 100 nM agonist and 50 nM PAM, and D) cells were treated with 100 nM agonist and 100 nM PAM. Conditions 4 & 5 were performed in an identical manner to Condition 3 but a different enzyme inhibitor was used for pre-treatment in each. Condition 4 utilized treatment with 10 nM SB 203580 to inhibit p38 MAP kinase. Condition 5 utilized treatment with 10 nM ABT 199 to inhibit Bcl-2. After application of inhibitor and/or drugs, cells were incubated at 37°C for three days. An illustration of the enzyme inhibitor experiments, Conditions 3-5, is found in Figure 3.

Experimental Condition	A	B	C	D
Condition 1	Control	100 nM agonist	100 nM agonist 50 nM PAM	100 nM agonist 100 nM PAM
Condition 2	Control	100 nM agonist	100 nM agonist 20 nM PAM	 100 nM PAM
Condition 3	Control	10 nM LY 294002 100 nM agonist	10 nM LY 294002 100 nM agonist 50 nM PAM	10 nM LY 294002 100 nM agonist 100 nM PAM
Condition 4	Control	10 nM SB 203580 100 nM agonist	10 nM SB 203580 100 nM agonist 50 nM PAM	10 nM SB 203580 100 nM agonist 100 nM PAM
Condition 5	Control	10 nM ABT 199 100 nM agonist	10 nM ABT 199 100 nM agonist 50 nM PAM	10 nM ABT 199 100 nM agonist 100 nM PAM

Table 1. Pharmacologic treatment as classified by experimental condition.

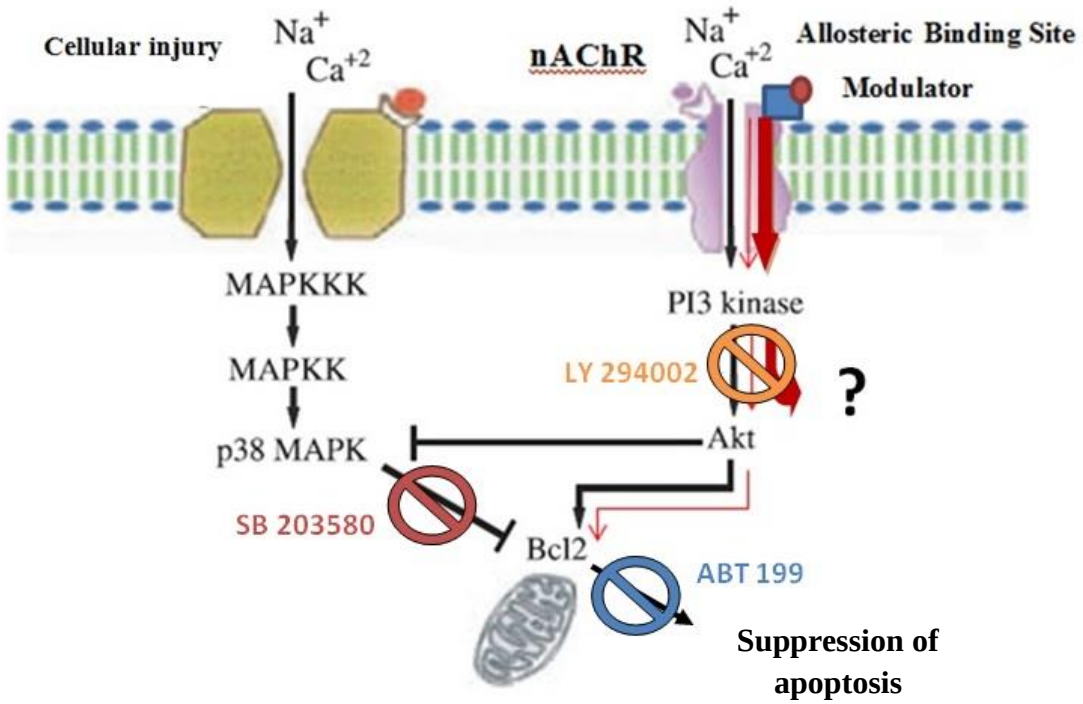

Figure 3. Demonstration of enzyme inhibitor experiment mechanism of action. In Condition 3, LY 294002 is used to inhibit PI3 kinase, which is proposed to up-regulate the anti-apoptotic protein, Bcl-2. In Condition 4, SB 203580 is used to inhibit p38 MAP kinase, which is proposed to inhibit Bcl-2. In Condition 5, ABT 199 is used to inhibit Bcl-2 itself.

Equipment and Instruments

After a three day incubation period, pharmacologically treated and untreated RGCs were labeled with 2 μM of membrane-permeable calcein, a fluorescent dye, for visualization with fluorescence microscopy. Cells treated with calcein were incubated at 37°C for 30 minutes to fluorescently label viable cells (Asomugha et al., 2011). Microscopy was performed with a Nikon Diaphot epifluorescent research microscope illuminated by a 100-W mercury arc lamp with an excitation filter FITC 460-490, dichroic mirror DM 580 and barrier filter BA 590. Images were captured using a Hamamatsu XC-77 CCD camera and QCapture Pro 7 imaging system from Image Analysis, Inc. To quantify cell density, four pictures of fluorescent cells were captured at a consistent magnification from a predetermined location (12, 3, 6 and 9 o'clock positions) on each dish. The number of cells in each of these four pictures was counted and averaged to obtain a total representative of cell survival on each dish.

Data Analysis

The number of viable cells in each image was counted using Image-Pro software from Media Cybernetics, Inc. An average was calculated for the four images captured for each dish, and this average value was obtained for each of the 12 dishes in each experiment. Although all RGCs were originally isolated under the same conditions, there is some variation in cell death between experiments due to aspects of the dissociation process such as titration, variation in time between eye removals and culture, etc. (Asomugha et al., 2011). Comparison of experimental cell survival to untreated control survival within the same experiment eliminated this variation between experiments. Data was organized such that randomized complete design

with subsampling could be performed (Hinklemann & Kempthorne, 1994). Statistical analysis was implemented on non-normalized data using two-way analysis of variance (ANOVA) with interaction between plate and treatment. A P-value of less than 0.05 was considered statistically significant for all tests.

Results of this study are limited to an evaluation of RGCs in isolation and extrapolation to an *in vivo* system should be used with caution. However, this allows for true evaluation of the effectiveness of pharmacological therapy and involvement of targeted enzyme pathways in the absence of confounding variables. There are also numerous steps throughout the dissociation process that leave room for human error in procedure. Nonetheless, consistent results utilizing this technique have been published in several peer-reviewed journals. In addition, only specific enzyme pathways were targeted for study. Involvement of other enzyme signaling pathways were neither detected nor analyzed.

CHAPTER 4

RESULTS

Cell Survival in Response to Pharmacotherapy

Experimental Conditions 1 & 2 investigated the effects of varying doses of α7 nAChR agonist and PAM in an *in vitro* model of glaucoma using isolated RGCs. A membrane-permeable fluorescent dye, calcein, was taken up by viable cells and results viewed under fluorescence microcopy. An example of the results obtained following this procedure is shown in Figure 4. Statistical analysis was used to evaluate significant differences in cell survival between variables A, B, C & D within each experiment. Condition 1 (n=9) compared viable cells in control plates to those in plates with agonist alone (B), agonist and low-dose PAM (C), and agonist and high-dose PAM (D). Control plates (A) contained a significantly lower amount of viable cells than did all other pharmacologically-treated plates in Condition 1 ($p<0.0001$). The difference among plates (1, 2 & 3) within each treatment group (A, B, C & D) was not statistically significant ($p=0.8069$) and there was no interaction between plate and treatment ($p=0.1562$). This is a testament to the precision of experimental results and is true for all five experimental conditions as none produced a p-value for interaction less than the accepted value of 0.05.

Figure 4 demonstrates images taken from treatment groups A, B, C & D of a Condition 1 experiment. Pictures demonstrate viable RGCs stained with calcein three days after isolation. There is a noticeable and direct increase in viable cells sequentially from A to D that corresponds with increasing doses of pharmacotherapy. The least squares means for viable

cells of Condition 1 are as follows: treatment A = 34.73 (± 2.29), treatment B = 44.29 (± 3.39), treatment C = 48.96 (± 3.75), treatment D = 50.12 (± 5.13). Control group A was statistically distinct from experimental variables B ($p<0.01$), C ($p<0.01$) and D ($p<0.01$), however B, C & D were not statistically significant from each other. Addition of a low or high-dose PAM in these experiments did produce a noticeable increase in the survival of RGCs, and although not statistically significant, resulted in a 14.9-23.3% increase in mean cell survival over agonist alone.

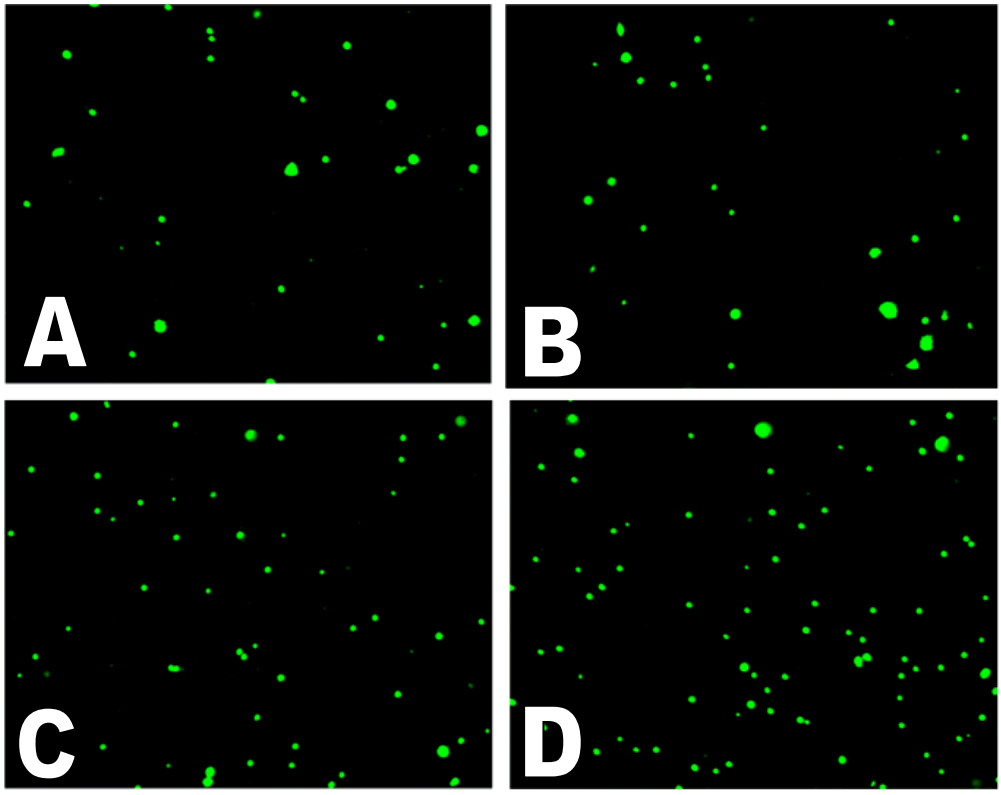

Figure 4. Images obtained from similar locations on Plates A, B, C & D of a single experiment. Viable cells take up calcein dye and fluoresce under fluorescence microscopy with FITC excitation filter (460-490). Images were analyzed with QCapture Pro Imaging system and cell counts were obtained with Image-Pro software. A) cells were left untreated as a control, B) cells were treated with 100 nM agonist, C) cells were treated with 100 nM agonist and 50 nM PAM, and D) cells were treated with 100 nM agonist and 100 nM PAM.

Condition 2 differed from Condition 1 in the dosages of agonist and PAM used. In Condition 2, treatment C contained very low dose concentration of PAM with agonist and treatment D contained PAM without any agonist. The least squares means for viable cells of Condition 2 are as follows: treatment A = 35.10 (± 3.93), treatment B = 44.35 (± 5.07), treatment C = 62.96 (± 15.44), treatment D = 36.01 (± 5.73). Control group A was statistically distinct from experimental variables B ($p<0.01$) and C ($p<0.01$), but not from D ($p=0.38$). In treatment group D with PAM without any agonist, cell survival was nearly equal to that of control. A comparison of the results from Condition 1 and Condition 2 can be found in Figure 5.

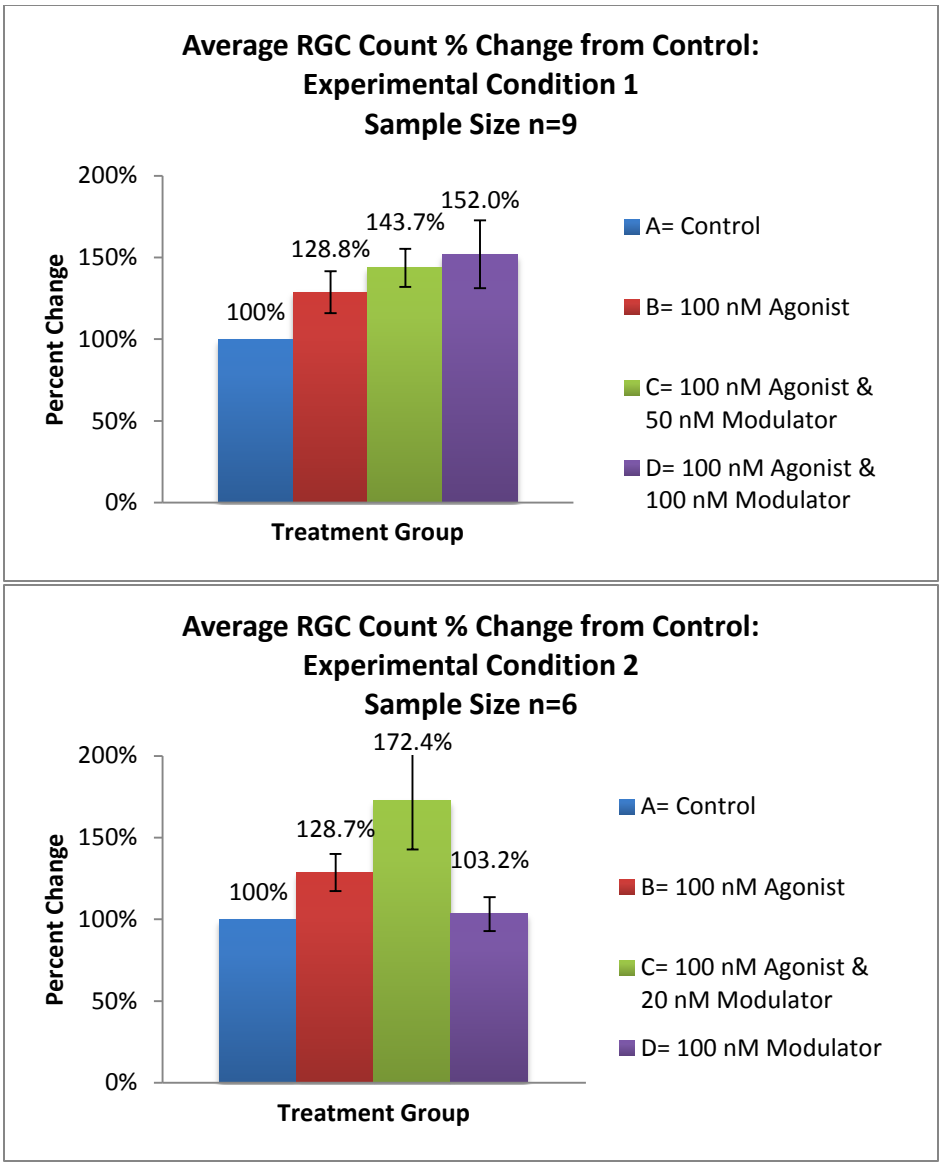

Figure 5. Summary of results obtained in Conditions 1 & 2. Cell counts for each treatment group were compared to internal controls to obtain a percent change. Percent change values were averaged to produce results displayed above. For Condition 1, treatment groups B, C, and D produced a 28.8%, 43.7%, and 52.0% increase in cell survival, respectfully. For Condition 2, treatment groups B, C, and D produced a 28.7%, 72.4%, and 3.2% increase in cell survival, respectfully. Error bars represent standard error. Refer to Table 1 for a breakdown of pharmacologic treatment groups.

Enzyme Inhibitors Produce a Change in Cell Survival Response

Conditions 3, 4 & 5 were conducted to analyze and evaluate proposed mechanisms of enzyme pathway involvement in RGC neuroprotection. In Condition 3 experiments, treatment groups B, C & D received pre-treatment with LY 294002 to inhibit PI3 kinase. PI3 kinase is proposed to play a role in the ACh neuroprotection pathway; therefore, the hypothesized finding for this condition would be a decrease in neuroprotection and subsequently no survival difference between control plates and pharmacotherapy plates. Statistical analysis of Condition 3 (n=4) revealed no significant difference in cell survival between control and treatment groups B, C & D ($p= 0.052$, $p=0.32$, $p=0.31$, respectively). Least squares means for the treatment groups were as follows: treatment A = 19.75 (± 5.26), treatment B = 24.56 (± 7.60), treatment C = 18.58 (± 5.62), treatment D = 20.90 (± 5.12). This result is graphically displayed in Figure 6.

Condition 4 was conducted under similar methods but utilized the compound SB 203580 to inhibit p38 MAP kinase. This enzyme plays a role in the glutamate pathway that is proposed to participate in excitotoxicity and subsequent RGC death in a glaucomatous state. The hypothesized finding through inhibition of p38 MAP kinase is a decrease in toxic insult and subsequent increase in cell survival. Statistical analysis of Condition 4 (n=4) revealed control group A was statistically distinct from experimental variables B ($p<0.01$), C ($p<0.01$) and D ($p<0.01$), however B, C & D were not statistically significant from each other. Least squares means for the treatment groups were as follows: treatment A = 23.19 (± 3.38), treatment B = 33.65 (± 9.15), treatment C = 29.29 (± 4.99), treatment D = 32.00 (± 6.08). The result is graphically displayed in Figure 6.

Finally, Condition 5 mirrored Conditions 3 & 4 but utilized the compound ABT 199 to inhibit Bcl-2. Bcl-2 is a regulator protein involved in the suppression of apoptosis; it is proposed to play a role in the ACh neuroprotection pathway targeted by PNU-282987. As such, inhibition of Bcl-2 would result in suppression of neuroprotection on pharmacologically-treated plates and similar cell survival between control and experimental variables. Statistical analysis of Condition 5 (n=4) revealed no significant difference in cell survival between control and treatment groups B, C & D ($p= 0.27$, $p=0.39$, $p=0.37$, respectively). Least squares means for the treatment groups were as follows: treatment A = 50.00 (± 16.8), treatment B = 56 (± 17.75), treatment C = 53.04 (± 16.97), treatment D = 53.63 (± 13.10). The result is graphically displayed in Figure 6 alongside the results of Conditions 3 & 4.

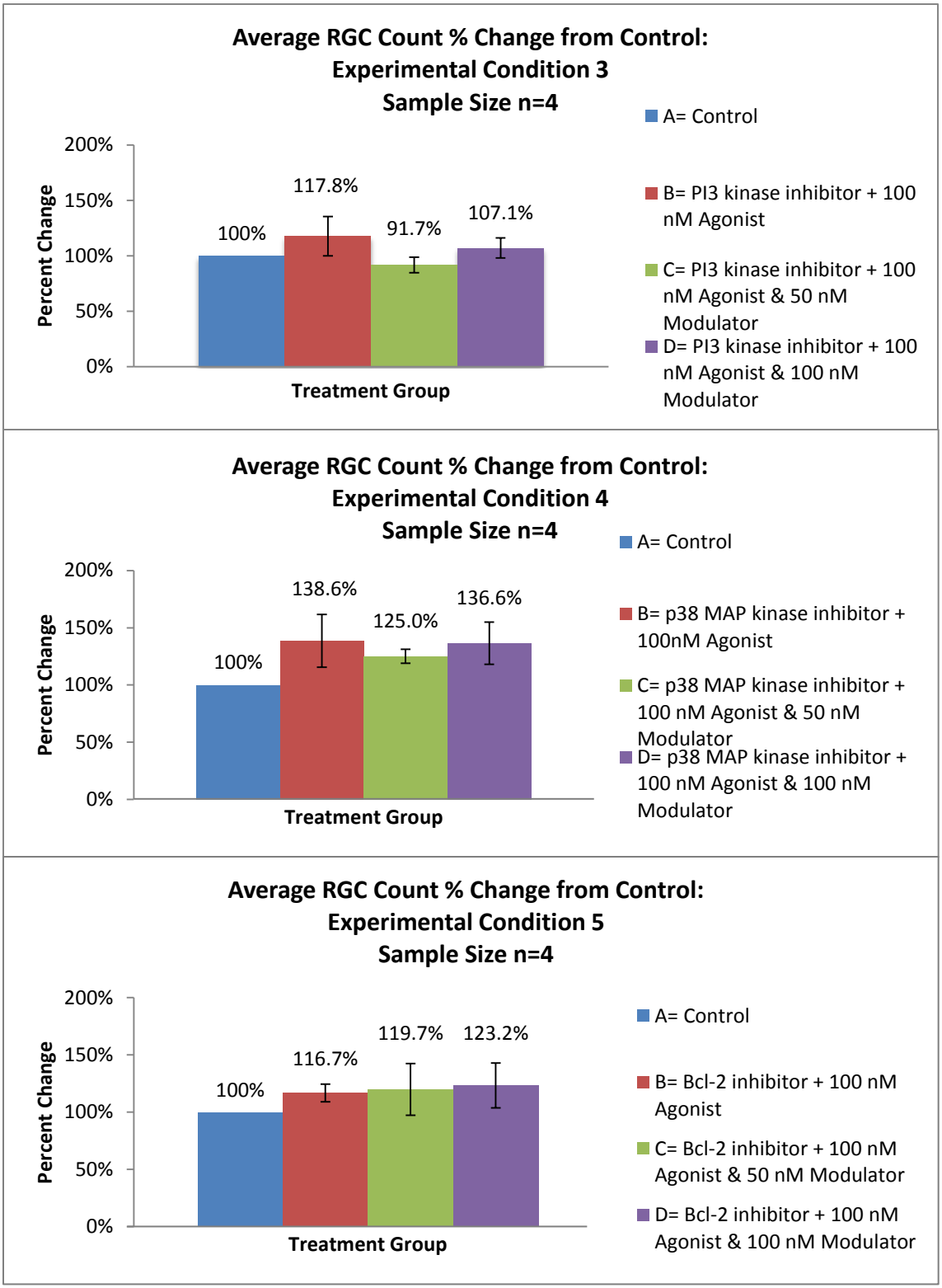

Figure 6. Comparison of RGC counts in enzyme inhibitor experiments. There was no statistically significant difference among the treatment groups in Conditions 3 or 5. In Condition 4 the experimental variables B, C, and D were all statistically significant from control. Error bars represent MSE.

CHAPTER 5

CONCLUSION

Neuroprotection

This study was designed to test the neuroprotective properties of a specific nAChR agonist, PNU-282987, in combination with a positive allosteric modulator, PNU-120596, in a cell culture model of glaucoma. In previous studies using cultured porcine RGCs and *in vivo* rat RGCs, ACh and nicotine have demonstrated neuroprotection through activation of the α7 nAChR (Wehrwein et al., 2004, Thompson et al. 2006, Iwamoto et al., 2013). The goal of this study was to replicate these results and evaluate the additive effect of an allosteric modulator that is specific for the α7 receptor. Treatment of cells with the α7 nAChR agonist prior to incubation resulted in an increase in cell survival, as seen in previous experiments. This increase was statistically significant. Addition of the PAM produced a dose-dependent increase in cell survival over that of agonist alone, also statistically significant compared to control. The combination of agonist and PAM was not statistically significant over agonist alone; however, the trend of increased neuroprotection with dose-dependent PAM is evident in the data. PAM in absence of agonist produced no change in cell survival, suggesting that the PAM works allosterically as hypothesized. Much in the same way a benzodiazepine like Diazepam (Valium) works to modulate the GABA receptor, PNU-120596 likely works to increase the effect of the agonist rather than producing an additive neuroprotective effect of its own.

As mentioned previously, there are many theories pertaining to the mechanism of cell death associated with glaucoma. Previous studies in this lab have focused on a model of excitotoxicity related to overstimulation of cellular receptors by the neurotransmitter, glutamate. In this study no additional insult (i.e. excess glutamate) was employed. Prior to retinal dissociation and culture, the axons of all retinal RGCs were severed via cut of the optic nerve. RGCs die off in a gradual, predictable pattern in this model as described by Wehrwien et al. in 2004. In this study, agonist and PAM provided neuroprotection for RGCs in the absence of a specific insult, suggesting their use is not limited to an excitotoxic model. While release of excess glutamate from injured cells may still be the insult producing cell death, it is possible that another proposed mechanism is responsible for RGC death (i.e. oxidative stress, autoimmune regulation, glial cell activation, endoplasmic reticulum stress, endothelin receptor activation, neurotrophic factor deprivation, or hypoperfusion of the optic nerve). Treatment with α7 nAChR agonist and PAM provide neuroprotection for RGCs in a model of nonspecific cellular injury which suggests their usefulness as a neuroprotective agent may extend to injured neurons regardless of mechanism of injury.

Mechanism of Action

A previous study by Asomugha et al. utilized ELISA techniques to evaluate the enzymatic pathways utilized by the α7 nAChR. This previous study revealed that ACh neuroprotection is mediated through stimulation of the PI3 kinase ⟶ Akt ⟶ Bcl-2 pathway as well as inhibition of the p38 MAP kinase pathway. Conversely, glutamate excitotoxicity is mediated through stimulation of the p38 MAP kinase signaling pathway. To evaluate the additive effect of

a PAM on this model, specific enzyme inhibitors for PI3 kinase, p38 MAP kinase, and the final protein target of both pathways, Bcl-2, were added to cell culture prior to incubation. When PI3 kinase was blocked in this study, the cell survival of RGCs in agonist and agonist + PAM groups was similar to that of control. The neuroprotective effect of agonist and PAM was completely eliminated in the absence of PI3 kinase. This finding suggests that neuroprotection by the combination of agonist and PAM is mediated through PI3 kinase, as is agonist alone.

Although glutamate was not specifically added to culture to evaluate its role in cell death through excitotoxicity, an inhibitor for the hypothesized cell death pathway was tested. P38 MAP kinase plays a role in cell death through down-regulation of Bcl-2, or antagonistic effect of the PI3 kinase pathway (Asomugha et al. 2010). When p38 MAP kinase was blocked, RGC cell survival in agonist and agonist + PAM groups was significantly increased as compared to control, however not to the level of survival seen in its presence. This result raises questions about the interaction between the p38 MAP kinase death pathway and the PI3 kinase survival pathway. Does the PI3 kinase pathway require some input from p38 MAP kinase to substantiate its neuroprotective properties? Or, is the action of the p38 MAP kinase pathway different in the absence of an added glutamate insult? It is also possible that blocking cell death is different from increasing cell survival, and thus a significant increase in cell survival is not to be expected. That is, the p38 MAP kinase cell death pathway may play a direct role in decreased numbers of RGCs in vitro, but does not have a direct effect on the increased number of RGCs in the presence of agonist and PAM.

Finally, an inhibitor for Bcl-2 was used to analyze its role in cell survival. Bcl-2 is an anti-apoptotic protein that is up-regulated by PI3 kinase and down-regulated by p38 MAP kinase. It is hypothesized to be the final target of the ACh neuroprotective pathway as well as the p38 MAP kinase death pathway. In its absence one would expect a total loss of neuroprotection and RGC survival with agonist + PAM to be equal to that of control. Interestingly, RGC survival in absence of Bcl-2 was increased with agonist + PAM as compared to control. The amplitude of this increase was less than that in the presence of Bcl-2 from previous experiments. This result would need to be strengthened for statistical significance with a greater experimental value than achieved in this study (n=4). However, the data trend in this study suggests that while some of the increase in RGC survival is mediated through the PI3 kinase $\longrightarrow$ Akt $\longrightarrow$ Bcl-2 pathway, another pathway may be contributing to cell survival. That is, if the Bcl-2 pathway was really the essential end target then blocking it should drop the increase in survival to zero, as seen in PI3 kinase inhibition. The persistent increased survival is suggestive of an additional mechanism for survival. This additional pathway must be activated downstream of PI3 kinase as its absence resulted in total blockade of neuroprotection, and upstream of Bcl-2 as its absence resulted in cell survival at a lesser incidence.

Each of the aforementioned enzyme inhibitor experiments utilized a known inhibitory compound for targeted enzymes in the hypothesized signaling cascade of PNU-282987 and PNU-120596. Each of the inhibitory reagents utilized in this study are widely produced and used with proven efficacy for their target function. The inhibitor reagents were applied at a consistent concentration of 10 nM thirty minutes prior to drug application on experimental plates B, C & D. Cell survival on these plates was then compared to control plate A which

contained no inhibitor or pharmacotherapy. It is warranted to repeat these methods with an additional variable of inhibitor alone. Inhibitor in the absence of pharmacotherapy is a justified variable because it would allow for observation of cell survival in the absence of enzymes of interest but without the influence of pharmacotherapy. This would help to further clarify the role of PNU-282987 and PNU-120596 in the activation of Bcl-2 as a final target protein. It would also further clarify what, if any, role p38 MAP kinase plays in activation of apoptotic RGC death to determine if this pathway has a direct or indirect role in the neuroprotection provided by the α7 nAChR agonist and PAM. Continuation of this enzyme inhibitor experimentation will be conducted in this laboratory in the near future to augment the data obtained in this study.

Implications

The results of this study suggest that a PAM can be used in combination with an α7 nAChR agonist, PNU-282987, to enhance its effect as a neuroprotective agent. The PAM has no neuroprotective effect on its own, but rather works allosterically to enhance the effect of the agonist. This neuroprotective effect was assessed on an *in vitro* model of porcine RGCs that were subject to axotomy. The absence of a specific cellular injury model suggests that the protection provided by these agents may be applicable in a number of different models, as well as an *in vivo* model of nonspecific neurodegeneration. It has been proposed that increased IOP presses against RGC axons as they make a 90° turn at the optic nerve head to exit the eye, damaging RGCs and contributing to neurodegeneration. This is referred to as the "Achilles heel" of the anatomy of the retina (Dreyer et al., 1996). If this natural mechanism of injury leads to

RGC injury in absence of a specific insult, pharmacotherapy with an α7 nAChR agonist and PAM would be effective form of neuroprotection.

The role of glutamate excitotoxicity remains yet controversial, and further experiments with measured levels of glutamate would be needed to determine if glutamate plays a role in this nonspecific model of injury. Enzyme inhibitor experiments propose that the combination of agonist and PAM work via the PI3 kinase ⟶ Akt ⟶ Bcl-2 pathway to up-regulate Bcl-2, which has known anti-apoptotic properties. This cell survival pathway via PI3 kinase has been linked to calcium influx through activated α7 nAChRs (Brandt et al. 2010). Results of this study also suggest that an additional pathway may be contributing to enhanced cell survival, and this pathway occurs somewhere between PI3 kinase and Bcl-2. Further research is needed to identify this additional pathway. Once identified, inhibition of this pathway in combination with inhibition of Bcl-2 should produce a complete loss of neuroprotection under these culture conditions to confirm its validity.

Limitations and Potential Applications

This study was limited to findings of an *in vitro* culture model of glaucoma utilizing isolated porcine RGCs. The question remains, does a combination of PNU-282987 and PAM provide neuroprotection for RGCs in an *in vivo* model of glaucoma? Would the PAM continue to work allosterically to enhance the effect mediated by nAChRs under physiologic conditions *in vivo*? Previous studies in this laboratory have demonstrated that PNU-282987 inhibits the loss of RGCs in glaucoma-like conditions in a dose-dependent manner in both *in vitro* and *in vivo* studies through activation of α7 nAChRs (Linn et al. 2011, Iwamoto et al. 2013). Acetylcholine

and nicotine provide neuroprotection through this same mechanism within the retina (Wehrwein et al. 2004, Thompson et al. 2006, Asomugha et al. 2010). In this study the PAM was tested only with the specific agonist PNU-282987, but it is possible that it would also enhance the effect of naturally-occurring ACh. In the retina, ACh is released from starburst amacrine cells, which synapse onto RGCs (Masland, 1988). The effect of a PAM in combination with naturally-occurring ACh was not able to be evaluated in this study because RGCs were isolated and cultured in the absence of starburst amacrine cells. If the PAM used in this study could allosterically enhance the effect of ACh as it does for PNU-282987, it could potentially be used to substantiate the body's natural protective response against RGC stress. If not in combination with ACh, the potential still remains for PNU-282987 and PAM to be used as a pharmacotherapeutic strategy for patients with neurodegenerative diseases of the retina, such as glaucoma.

There is also a potential for application of these agents for neuroprotection against neurodegenerative diseases elsewhere in the nervous system, such as Alzheimer's disease. Alzheimer's disease is characterized by neurodegeneration resulting from apoptosis of neurons and formation of neurotoxic amyloid plaques in the brain (Nikura et al., 2006). Previous epidemiologic studies identified a decreased incidence of Alzheimer's disease in nicotine users, and thus a neuroprotective effect of nicotine with regards to formation of amyloid plaques (Fratiglioni and Wang, 2000). Nicotine affords this effect through a mechanism mediated by α7 nAChRs, demonstrated by Hellstrom-Lindahl et al. (2004) through inhibition studies with an α7 nAChR antagonist, Mecamylamine, to block nicotine's effect in transgenic mice containing Alzheimer's disease. In the absence of the α7 nAChR, nicotine did not have a neuroprotective

effect. Therefore, the α7 nAChR is involved in neuroprotection of diseases in the retina and elsewhere in the nervous system, providing opportunity for future application of an α7 nAChR agonist and α7 specific PAM in other neurodegenerative disease states.

Conclusion

The findings of this study are suggestive of future application of an α7 nAChR agonist in combination with a PAM for protection of RGCs in the setting of glaucoma, and possibly in other neurodegenerative diseases as well. Previous studies have demonstrated success with PNU-282987 as a neuroprotective strategy for RGCs in an *in vivo* rat model of glaucoma (Iwamoto et al. 2014). This study's *in vitro* finding suggests that a PAM could enhance the neuroprotection provided by PNU-282987. An *in vivo* approach to the repetition of this study would further elucidate the role of a PAM in the protection of RGCs both in combination with a specific α7 nAChR agonist and in combination with naturally-occurring ACh in a physiologic setting. This study has implicated the PI3 kinase ⟶ Bcl-2 pathway in the action of α7 nAChR neuroprotection, and also suggested the utilization of an additional unidentified pathway downstream from PI3 kinase. Further insight into any additional pathway utilized by these agents is also warranted in future research. Use of these agents could ultimately lead to therapeutic prevention of glaucoma and potentially other neurodegenerative diseases.

As members of the healthcare team it is important to not only provide the highest level of care, but also to continue research to advance the highest level of care. Glaucoma, a chronic and debilitating disease that is responsible for current visual impairment in over 6 million people worldwide, remains without a cure. To date, all treatment strategies for glaucoma are

designed to decrease IOP within the anterior chamber of the eye (The Ophthalmic News & Education Network, 2010). Elevated IOP remains the only risk factor currently recognized by the FDA despite the fact that not all cases of glaucoma present with IOP, and studies have shown lowering IOP does not ensure prevention of RGC death (Sena et al., 2010). Previous studies in this lab have shown that treatment with the α7 nAChR agonist, PNU-282987, in eye drop form after the onset of glaucomatous conditions inhibits the loss of RGCs *in vivo* (Iwamoto et al., 2014). Results from this study demonstrate an enhanced level of PNU-282987 neuroprotection with the addition of a PAM, and possible application of a PAM to enhance the physiologic response to neurodegenerative insult. Clinically, these results prompt further research into a novel therapeutic approach to glaucoma that is not targeted at altering IOP. It also warrants further research into the clinical effectiveness of a PAM on the physiologic neuroprotective response of ACh and the α7 nAChR. The results from this study prompt further research that could dramatically change the treatment approach for patients with glaucoma by targeting the disease at the level of the retina where irreversible damage and vision loss ultimately occurs.

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