

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 μ M-200 μ M). 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 500 μ M 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 $\alpha 7$ nAChRs (Wehrwein et al., 2004). These findings have since been supported with further research and expanded to include a potential additional role of $\alpha 4$ 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 outflow. 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 $\alpha 7$ 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 $\alpha 7$ nAChR, PNU-120596, in combination with the $\alpha 7$ 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 $\alpha 7$ 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 $\alpha 7$ 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.