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Use of a Specific Alpha7 Nicotinic Acetylcholine Receptor Agonist Can Prevent Loss of Retinal Ganglion Cells in an In Vivo Rat Glaucoma Model

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USE OF A SPECIFIC ALPHA7 NICOTINIC ACETYLCHOLINE RECEPTOR
AGONIST CAN PREVENT LOSS OF RETINAL GANGLION CELLS
IN AN *IN VIVO* RAT GLAUCOMA MODEL

by

David Mata

A thesis submitted to the Graduate College
in partial fulfillment of the requirements
for the degree of Master of Science
Biological Sciences
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David Mata, M.S.

Western Michigan University, 2013

Acetylcholine (ACh) has been shown to have a neuroprotective effect against glutamate-induced excitotoxicity in cultured retinal ganglion cells (RGCs) through activation of $\alpha 7$ nicotinic ACh receptors (nAChRs). In this study, the neuroprotective effects of the $\alpha 7$ nAChR agonist, PNU-282987, were investigated using an *in vivo* model of glaucoma in adult Long Evans rats. Hypertonic saline injections were used to induce a glaucoma model. After one month, retinas were removed, flat mounted, fixed and labeled with an antibody against Thy 1.1 to label RGCs and fluorescently tagged for visualization. Eye drop application of PNU-282987 resulted in neuroprotection against RGC loss in a dose-dependent manner compared to untreated controls when applied 3 days before hypertonic injections and for 30 days following the procedure. Liquid chromatography with mass spectroscopy with quad capabilities (LC/MSMS) demonstrated that PNU-282987 can be detected in the retina with small amounts in blood plasma and no detectable levels in heart samples. These results support the hypothesis that eye drop application of the $\alpha 7$ nAChR agonist, PNU-282987, can prevent loss of RGCs associated under glaucoma-like conditions.

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SPECIFIC AIMS

Glaucoma is characterized as a neuropathic disease caused by an increase in intraocular pressure (IOP) that causes damage to the optic nerve and progressive degeneration of retinal ganglion cells (RGCs), resulting in a loss of vision (Guo et al., 2005). In previous studies from Iwamoto (2011), the loss of RGCs was prevented in an *in-vivo* rat model of glaucoma by using intravitreal injections of a specific $\alpha 7$ nicotinic acetylcholine receptor agonist ($\alpha 7$ nAChR), PNU-282987, before hypertonic injections to induce glaucoma-like conditions. In this model, glaucoma-like conditions were induced in the right eye of anesthetized Long Evans rats using hypertonic injections of 2M saline into the episcleral veins of the eyes, which create scar tissue and cause an increase in IOP to mimic glaucoma-like conditions. Previous studies observed that significant loss of RGCs in the retina occurs one month following hypertonic injections and the IOP increases gradually by an average of 12 mmHg. Retinal ganglion cell death resulted after injection of hypertonic saline unless intravitreal injections of PNU-282987 were given prior to the hypertonic injections.

However, the use of intravitreal injections to eliminate the effects of glaucoma is an invasive procedure and reduces the appeal of developing PNU-282987 as a possible glaucoma treatment. This issue might be addressed by development of eye drop applications. The hypothesis of this thesis is that eye drop applications of a specific $\alpha 7$ nicotinic acetylcholine receptor agonist can prevent loss of retinal ganglion cells in an *in-vivo* rat glaucoma model.

INTRODUCTION

The eye is a highly developed sensory organ that allows the body to react to the environment and to communicate with our surroundings. Like many other sensory organs, the eye works in profound ways to transduce energy into a recognizable message that allows us to react to specific stimuli from the environment. Signal transduction, which occurs in the retina, is the first step leading to visual perception and is required for the visual process. The retina is composed of many different cell types that work together to create and send electrical messages to the brain, which ultimately leads to visual perception. Because of the physiological importance of the retina for quality of life, there is a high priority in promoting ocular health and research in retinal function. As this thesis involves an understanding of the vertebrate retina, a brief description of the anatomy of the eye is included.

The Eye

The eye contains many anatomical features that are important for its function. The cornea is the first lens of the optic system and functions to refract rays of light from the environment to the retina (Jacob, 1823; Maycock, 1932). Light passes through the cornea, which refracts rays of light and enters through the pupil in the anterior chamber of the eye. The iris is the smooth muscle in the eye that controls how much light is allowed in by controlling pupil diameter (Jacob, 1823; Maycock, 1932). After the light passes through the pupil, it is further focused by the lens onto the retina lying in the back of the posterior chamber of the eye (Jacob, 1823; Maycock, 1932).

The Retina

The vertebrate retina contains five main types of sensory neurons that work together to detect light stimuli from the environment. The five main cell types in the retina include; photoreceptors, horizontal cells, bipolar cells, amacrine and retinal ganglion cells (Dowling, 1967; Werbin and Dowling, 1969). As light is focused onto the retina from the lens, photoreceptors transduce incoming light into electric potentials in the nervous system. Photoreceptors are responsible for carrying out phototransduction, a process where photons of light are converted to electrical energy so the brain can interpret visual stimuli (Tomita, 1970; Baylor and Fettiplace, 1977). When photoreceptors are stimulated by light, pigments in the outer discs of these neurons are stimulated to initiate a series of events that result in photoreceptor hyperpolarization (Werbin and Dowling, 1969; Tomita, 1970; Kaneko, 1970). In the dark, photoreceptors remain depolarized. This constant depolarization is called the “dark current” (Hagins et al., 1970; Baylor et al., 1979). However, photoreceptors do not play an important role in this study and will not be mentioned further.

The next step in the visual pathway is communication from primary receptors to post-synaptic sensory neurons in the rest of the retinal circuitry. Photoreceptors form synapses with bipolar cells (Dowling, 1967; Werbin and Dowling, 1969; Baylor and Fettiplace, 1977). The synapse onto bipolar cells continues the direct electrical pathway to retinal ganglion cells (RGCs) (Hartline, 1938; Kaneko, 1970; Baylor and Fettiplace, 1977). The RGCs then relay visual information through the optic nerve to the occipital lobe where the first stages of visual perception takes place (Hartline,

1938; Baylor and Fettiplace, 1977). Between the photoreceptors and RGCs, horizontal and amacrine cells use lateral inhibition to enhance contrast differences before information is sent to the brain (Kaneko, 1970; Cervetto and Piccolino, 1974; Walong and Pak, 1975; Kaneko and Shimazaki, 1975). As bipolar and horizontal cells are not the focal point of this study, their physiology will not be described in detail.

Amacrine Cells

One type of sensory neuron in the retina that will be discussed in this study is amacrine cells. Amacrine cells were initially described by Cajal (1892), Famiglietti (1983) and Masland (1988). These cells are an extremely diverse class of interneurons in the retina and more than two dozen morphologically distinct subtypes have been described (Cajal, 1892; Kidd, 1962; Dowling and Boycott, 1966; Grimes, 2011). The particular subtype of amacrine cell that is pertinent to this study is known as starburst amacrine cells. Starburst amacrine cells communicate with RGCs using acetylcholine (ACh) (Famiglietti, 1983; Massland, 1988; Grimes, 2011). The possible roles of starburst amacrine cells in this study will be elaborated upon when discussing neurotransmitter function in the retina.

Retinal Ganglion Cells

RGCs are the main focus of this study. Hartline (1938) made significant contributions to what is currently known about the physiology of the RGCs. RGCs are

essential for visual characterizations such as contrast detection and spatial sensitivity (Hartland, 1938; Kuffler, 1953; Rodieck, 1965; Sharpley and Victor, 1986). RGCs, by virtue of their response properties to photoreceptors, are also specialized to respond preferentially to certain intensities of light and interpret rates of change in light intensity (Kuffler, 1953; Barlow and Hill, 1963). This characteristic is important for motion detection (Barlow and Hill, 1963).

RGCs are the final sensory neurons in the retina (Dowling, 1967; Werbin and Dowling, 1969). The axons of the RGCs (along with glial components) make up the optic nerve that leads to the occipital lobe where visual perception is initiated. RGCs can be susceptible to various ocular diseases, such as glaucoma, that can affect visual processing. This is a primary focus of this study.

There is much to understand about RGCs and RGC neuropathies. To understand the RGCs neuropathies that can develop, it is important to understand the RGC channels and neurotransmitters in the retina that activate specific receptors. These neurotransmitters and receptors play a significant role in many neurodegenerative diseases, including glaucoma, that cause degeneration of RGCs. A brief overview of neurotransmitters and receptors are mentioned below.

Neurotransmitters and Receptors in the Retina

There are many different types of neurotransmitters and receptors that are released by neurons in the vertebrate retina (Yazulla, 1986; Ehinger et al., 1988; Stryer, 1988; Marc et al., 1990). In the dark, photoreceptors release glutamate (Werbin and Dowling, 1969; Marc et al., 1990; Jujich and Pourcho, 1996). This release of

glutamate from photoreceptors activates receptors located on bipolar cells. Bipolar cells in turn release glutamate onto the RGCs and the electrical signals continue to the brain (Ehinger et al, 1988; Marc et al., 1990; Jujich and Pourcho, 1996). Signals throughout the retinal circuitry are modified by a process called lateral inhibition that increases contrast detection (Kuffler, 1953; Barlow and Hill, 1963). This process is achieved by horizontal and amacrine cells by the release of gamma-aminobutyric acid (GABA) and glycine (Yazulla, 1986, Stryer, 1988, Marc et al., 1995) to inhibit neighboring signals. In this thesis study, the release of glutamate and ACh in the retina will be examined.

Glutamate

In the retina, photoreceptors, bipolar cells and RGCs release glutamate as their primary small molecule neurotransmitter (Ehinger et al, 1988; Marc et al., 1990; Gilbertson et al. 1991; Jujich and Pourcho, 1996). Glutamate can cause a depolarization or hyperpolarization in bipolar cells depending on their type (Werbin and Dowling, 1969; Baylor and Fettiplace, 1977). Glutamate receptors are classified as ionotropic or metabotropic receptors (Mayer and Westbrook, 1987). There are three main ionotropic receptors for glutamate; AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), kainate, and NMDA (*N*-Methyl-D-aspartate) receptors (Mayer and Westbrook, 1987). AMPA and kainate glutamate receptors are collectively called non-NMDA receptors (Mayer and Westbrook, 1987). OFF bipolar cells contain AMPA and NMDA receptors, which are responsible for the depolarizing response from glutamate (Gilbertson et al., 1991). The ON bipolar cells contain metabotropic

glutamate receptors and are responsible for the hyperpolarizing response (Pin and Duvoisin, 1995). In this study, glutamate is key neurotransmitter proposed to be involved in RGC cell death by over-stimulation of receptors.

Acetylcholine

ACh is found in starburst amacrine cells of the retina and was originally characterized by Cajal (1892), Famiglietti (1983) and Massland (1988). Starburst amacrine cells are the only cells in the retina known to synthesize and release ACh onto RGCs. Both muscarinic and nicotinic ACh receptors (nAChRs) have been demonstrated on RGCs in the mammalian retina (Keyser et al. 1988, Hughes, 1991). Multiple subunits of nAChRs have been cloned (Sargent, 1993; McGehee et al., 1995; Elliott et al., 1996). Under physiological condition, these subunits form heteromeric and homomeric combinations (Sargent, 1993; McGehee et al., 1995). In the central nervous system (CNS), the most common combination of nAChR subunits consist of heteromeric $\alpha 4$ - $\beta 2$ subunits and homomeric $\alpha 7$ subunits (Elliot et al., 1996). Previous studies done by Thompson et al. (2006) observed that both of these functional nAChRs are found on pig RGCs. Furthermore, Iwamoto et al. (2013) demonstrated that the homomeric $\alpha 7$ nAChR is found on rat RGCs.

During early development, starburst amacrine cells are necessary for generation of retinal waves due to the excitatory effect of ACh release (Zhang et al., 2004; Massland, 2011). The results from this thesis suggest that there may be an additional function for ACh in the adult retina.

Glaucoma

In this study, glaucoma-like conditions were modeled in Long Evans rats to provide an insight into the manifestation and possible prevention of the disease. Currently, all treatments for glaucoma aim to reduce an increase of intraocular pressure (IOP), which is the primary risk factor associated with glaucoma. However, these treatments alone are insufficient to halt the progression of blindness associated with glaucoma. Cell death correlated with over-stimulation of receptors has been known to be associated with glaucoma, as well as other neurodegenerative diseases in the CNS, and may provide a mechanism associated with the manifestation of the disease (Choi, 1988; Tielsch et al., 1991; Gupta and Weinreb, 1997).

Currently, there are approximately 60 million people worldwide with glaucoma. An estimated 8.4 million people are blinded by glaucoma. These numbers are expected to increase to 80 million by 2020 (Cook and Foster, 2012). Glaucoma is characterized as a progressive neurodegenerative disease that targets retinal components (Foster et al., 1992). Neuropathy in glaucoma specifically targets the axons of RGCs that make up the optic nerve (Foster et al., 1992). The axons and somata from RGCs slowly degenerate under glaucoma-like conditions. This, in turn, causes progressive loss of vision that lead to blindness in the individual. Glaucoma patients tend to initially develop loss of visual function in the periphery and slowly develop tunnel vision that becomes worse over time due to the cupping effect of the disease (Madeiros et al., 2009; Hendry et al., 2012). This progressive tunnel vision ultimately leads to blindness in individuals, if the disease is not treated. Blindness caused by glaucoma is irreversible. Once adult neurons in the mammalian retina are

lost, there is no known method or treatment to regenerate them *in-situ*.

Causes for glaucoma are unclear. Because of this, the manifestations of glaucoma are not well understood. Although there is a small genetic influence for glaucoma cases (Fingert, 2011), there are two variations of glaucoma that are not due to these genetic conditions, specifically open-angle and closed-angle glaucoma. Open-angle glaucoma, the most common type of the disease, is likely associated with the gradual congestion of the trabecular meshwork, disturbing the inflow and outflow of aqueous humor in the anterior portion of the eye (Glaucoma Research Foundation, 2012). The other major type of glaucoma, closed-angle glaucoma, arises very quickly and damage is usually very noticeable. Onset of closed-angle glaucoma can cause symptoms of pain and if not treated immediately, can quickly result in blindness (Glaucoma Research Foundation, 2012).

Several studies have demonstrated an association between loss of RGCs and an increase in IOP under glaucoma-like conditions (Morrison et al., 1997; Chauhan et al., 2002; Levkovitch-Verbin et al., 2002). The eye contains two chambers, the anterior and posterior chamber, which is filled with aqueous and vitreous humor, respectively. The aqueous humor is continually produced by the ciliary body and is filtered through the trabecular meshwork as it exits the eye (Jocson and Sears, 1971; Bill and Phillips 1971; Gelatt et al., 1977). In glaucoma, it has been observed that the outflow system becomes congested, decreasing aqueous drainage (Jocson and Sears, 1971; Bill and Phillips 1971; Gelatt et al. 1977). Since the input of aqueous humor to the anterior chamber continues, decreased drainage leads to increased pressure in the anterior chamber (Gelatt et al., 1977). The retina can be the target of this increase in pressure. As the pressure increases in the anterior chamber it continues to be conveyed

to the posterior chamber where vitreous humor is pushed through the optic nerve head (ONH) causing cupping of the ONH. There is concomitant death of RGCs at this point. The mechanism(s) behind this particular manifestation of glaucoma remains debatable and is the topic of much discussion.

Today, there are two main treatments for glaucoma; medication or optic surgery. These treatments ultimately focus on controlling the increase of IOP that accompanies glaucoma (Damji et al., 2003). Drugs can be administered to decrease the production of aqueous humor or can be administered to increase the drainage of fluid in the trabecular meshwork. Both methods act to reduce the IOP (Damji et al., 2003). These drugs can be administered topically or orally. Surgical procedures are also used to control aqueous flow through the trabecular meshwork when medication is not sufficient to lower IOP. Surgical procedures to lower IOP include cutting small holes in the eye to drain the aqueous humor. Alternatively, lasers can be used to produce holes in the trabecular meshwork to increase outflow of aqueous humor (Cairns, 1968).

Glaucoma in its early stages is typically asymptomatic. Symptoms do not typically begin until retinal damage causes peripheral vision loss. Often, by the time patients receive medical care, the disease has progressed, some already partially blind. These advanced stages of the disease decrease the chance of slowing the progression of the disease (Cronenmberger et al, 2009). This is a particular problem in developing countries where medical care is not easily accessible or affordable. Numerous studies have reported that many patients know nothing about ocular diseases at the time they receive medical care (Costa, 1995; Mello, 2003). For this reason, early detection of this disease is essential if there is to be any success in slowing down the rapid

progression of optic nerve damage. The rapid degeneration of RGCs and optic nerve are believed to be multi-factorial. One of these factors involves cell death caused by over-stimulation of receptors.

Cell Death Caused by Over-stimulation of Receptors

One way that cell death can occur is by over-stimulation of receptors due to excessive abundance of ligands in the extracellular space (Olney et al., 1977; Michaelis, 1998). This over activation of receptors allows excessive influx of ions, such as calcium, into cells which can lead to activation of apoptotic pathways. Over-stimulation of these receptors has been correlated to many neuronal diseases including Parkinson's, Alzheimer's and glaucoma (Quigley, 1998, Choi., 1998, Lafuente et al., 2001).

Glutamate is the main neurotransmitter that is released from photoreceptors and bipolar cells in the retina (Ehinger et al, 1988; Marc et al., 1990; Jujich and Pourcho, 1996). Excessive glutamate release in the retina has been hypothesized to trigger RGC loss associated with glaucoma (Onley, 1969; Neal et al., 1994; Vickers et al., 1995; Kaushik, 2003). It has been observed that when pressure increases in the anterior chamber of the eye, cells in the retina begin to release an excess of neurotransmitter as cells in the retina begin to degenerate. As more glutamate is released from dying cells, it leads to further over-stimulating of receptors on RGCs, (Onley, 1969, Choi, 1987, Neal et al., 1994, Zhang et al., 2004 and Levkovitch-Verbin et al., 2002). When non-specific cation NMDA channels allow an excess of calcium influx into cells, intracellular calcium levels can rise to a point where apoptosis is

triggered through activation of protein kinases involved in apoptotic and cell death pathways (Olney, 1977; Choi, 1987; Manev et al., 1989). The over-stimulation of glutamate receptors has also been shown to regulate enzymes that break down cellular structures associated with apoptotic events (Zhang et al., 2004). Previous studies from our laboratory have documented the toxic effects of glutamate in cultured pig RGCs that is mediated through NMDA receptors (Wehrwein et al., 2004). Additionally, Brandt et al. (2011) observed increases of intracellular calcium during glutamate-induced cell death in cultured pig RGCs and linked this increase of calcium influx to activation of apoptotic pathways.

Apoptosis

Apoptosis in glaucoma affects a wide range of neural cell types, particularly RGCs, whose axons make up the optic nerve. Apoptosis occurs when a group of enzymes are activated that cause the cell to degenerate (Kerr et al., 1972). This is thought to be a built-in defense mechanism that allows cells to self-destruct when they are introduced to abnormal environmental cues or to abnormal stimuli (Kerr et al., 1972). Under chronic conditions, this may provide an adaptive advantage. Yet, in acute or transient events, protection from these adverse environments may provide a new approach to prevent cell death caused by over-stimulation of receptors in neurodegenerative diseases, such as glaucoma. During apoptosis an increase of calcium in the cell activates caspases from the mitochondria (Earnshaw et al., 1999; Fesik, 2001). Cytochrome-c is then released from the mitochondrial matrix and activates caspases to cleave intracellular proteins (Martinez-Caballero, 2005). Many

studies have demonstrated that loss of RGCs, in both human and animal models of glaucoma, take place by apoptosis rather than by a non-specific trauma from mechanical deformation resulting in IOP (Garcia-Valenzuela et al., 1995; Quigley, 1995; Pease et al., 2000; Brandt et al., 2011). Particularly, apoptosis has been shown to occur after an elevated IOP (WoldeMussie et al., 2001; Levkovitch-Verbin et al., 2002).

Previous studies from this laboratory investigated the possible mechanisms during glutamate-induced cell death in cultured RGCs (Asomugha et al., 2010). Results from these studies showed that there was an up-regulation of P38 mitogen-activated protein kinase (MAPK) that occurred in response to glutamate-induced cell death. P38 MAPK was found to be associated with glutamate-induced apoptosis in isolated pig RGCs (Asomugha et al., 2010).

Neuroprotection

Neuroprotection is a therapeutic strategy aimed at preventing or slowing the neurodegenerative process. A variety of processes can lead to cell degeneration in neurons including; increased levels of oxidative stress, mitochondrial dysfunction, inflammatory changes, iron accumulation, protein aggregation, and as already outlined, cell death caused by over-stimulation of receptors (Dunnet, 1999; Page et al, 1999; Agarwal et al, 2009; Seidl and Potashkin, 2011). Common neuroprotective agents that have been used to prevent cell death caused by over-stimulation of receptors have included; glutamate antagonists, neurotrophic factors, agonists and antioxidants. These agents aim to limit cell death caused by over-stimulation of

receptors and oxidative stress, mediated through activation of respective receptors (Agawal et al., 2009).

Previous studies from this laboratory have demonstrated the neuroprotective effects of various nAChR agonists (Wehrwein et al., 2004; Thompson et al., 2006; Asomugha et al., 2010; Brandt et al., 2011; Birkholz, 2011; Iwamoto 2011). Asomugha et al. (2010) investigated the possible neuroprotective mechanisms in glaucoma-like conditions using ACh as a neuroprotective agent in cultured pig RGCs. Results from this study proposed two major pathways that could prevent loss of RGCs associated with glutamate-induced cell death. One of these pathways, triggered by pre-treatment of cultured RGCs with ACh, was the cell-survival phosphatidylinositol 3-kinase (PI3)-Akt pathway. Activation of this pathway enhanced cell survival against glutamate-induced cell death. In addition, ACh pre-treatment of cultured RGCs also resulted in inhibiting the apoptotic pathway by decreasing phosphorylated P38 MAPK level (Asomugha et al., 2010). Additionally, calcium imaging studies conducted by Brandt et al. (2011), suggested that calcium permeation through nAChRs was responsible for activation the intracellular cascades engaged with neuroprotection. The studies in this thesis focused on using an $\alpha 7$ nAChR agonist, PNU-282987, to determine its neuroprotective effects *in-vivo*.

PNU-282987

PNU-282987 (N-[(3R)-1-azabicyclo[2.2.2]oct-3-yl]-4-chlorobenzamide hydrochloride) is a specific $\alpha 7$ nAChR agonist (Bodnar et al., 2005; Hajos et al., 2005). This high specificity of PNU-282987 makes it a beneficial agonist for

addressing neurodegenerative diseases such as glaucoma. PNU is an acronym for Pharmacia and Upjohn, the company where this pharmaceutical was produced and developed. Initially, this agonist was used as a treatment of schizophrenia, but proved to be unsuitable for human use because of its excessive inhibition of hERG potassium channels located in the heart (Walker et al., 2006). However, this agent may prove to be a promising treatment for glaucoma if it is applied as eye drops, but only if eye drop application does not reach the heart and affect cardiac physiology. In this study, PNU-272987 was applied as eye drops in Long Evans rats that underwent hypertonic saline injections to induce glaucoma to determine if PNU-282987 prevents degeneration of RGCs. Liquid chromatography: mass spectroscopy with triple quad capabilities (LC/MSMS) was also used to detect PNU-282987 in the retina, blood plasma and heart tissue after eye drop application.

Previous Studies

Results from Iwamoto et al. (2013) demonstrated that the homomeric $\alpha 7$ nAChRs are present on rat RGCs. These findings encouraged us to determine if activation of these receptors could provide neuroprotection against loss of RGCs associated with glaucoma-like conditions in an *in-vivo* model of glaucoma in Long Evans rats. Iwamoto (2011) first demonstrated the neuroprotective effects of the specific $\alpha 7$ nAChR agonist, PNU-282987, in glaucoma-like conditions *in-vivo*. It was observed that when PNU-282987 was administered as intravitreal injections, cell death of RGCs in the rat retina was minimized in glaucoma-like conditions. An acute model of glaucoma was generated in Long Evans rats using hypertonic saline

injections as described in Morrison et al. (1997). The results from Iwamoto (2011) and Iwamoto et al. (2013) support the hypothesis that the $\alpha 7$ nAChR agonist, PNU-282987, has a neuroprotective effect in the rat retina under glaucoma-like conditions when delivered as intravitreal injections *in-vivo*. We were encouraged about the possible development of this compound to be used for therapeutic treatment of glaucoma, although intravitreal injection is not a likely preferred route of administration.

Previous studies in our laboratory have labeled cell bodies in the RGC layer using cresyl violet (Birkholz, 2011; Iwamoto, 2011) before and after PNU-282987 treatment and the procedure to induce glaucoma-like conditions. However, labeling cell bodies in the RGC layer using this method leads to inconclusive results concerning the identity of the labeled cells as cells other than RGCs can be found in the RGC layer. Iwamoto (2011) therefore, used an antibody against Thy 1.1 glycoprotein to exclusively label RGCs and Thy 1.1 glycoprotein is only found on RGCs in the retina. This method revealed retinal changes associated with the glaucoma-like condition that had not been previously characterized or examined.

To follow up on these studies, the neuroprotective effect of PNU-282987 against the loss of RGC under glaucoma-like conditions was repeated using a less invasive delivery method. This thesis focused on the delivery of PNU-282987 as eye drops to determine if neuroprotection against loss of RGCs under glaucoma-like conditions could be achieved. In addition, this study was designed to detect levels of PNU-282987 in the retina after eye drop application using LC/MSMS to determine if PNU-282987 reaches the retina and remains in the eye after eye drop application. LC/MSMS analysis was also conducted in the blood plasma and heart tissue after eye

drop application to determine if PNU-282987 could be detected in these tissues. Finally, general retinal morphometrics were analyzed in this study before and after inducing glaucoma-like conditions. These retinal morphometric issues have not been previously examined in our laboratory and provide an insight into how experimentally-induced glaucoma manifests itself.

The neuroprotective characteristic of PNU-282987 in the retina opens up the possibility for a new treatment for glaucoma that does not solely deal with reducing IOP. Using these new neuroprotective methods in conjunction with already existing treatments could potentially lead to preventative care in glaucoma. The results from these experiments strengthen the hypothesis that activation of $\alpha 7$ nAChRs on rat RGCs can prevent the loss of RGCs that occurs under glaucoma-like conditions.

METHODS

Animals

Adult Long Evans rats (males and females between 3 and 6 months) were used for all *in-vivo* studies. Rats were kept at Western Michigan University's animal facility until they were needed. Outbred Long Evans rats were used as a model strain because visual deficits are not prominent in this strain (Jeffery et al., 1997), retinal dissections are easily done against a pigmented background and their docile nature allows them to be easily handled. A breeding colony of Long-Evan rats (Charles River Labs, Portage, MI) was established. All animals were cared for in accordance with the approved guidelines of the Institutional Animal Care and Use Committee (IACUC) of Western Michigan University.

Inducing Glaucoma-Like Conditions

The procedure to induce glaucoma-like conditions in rats was modified from the procedures initially described by Morrison et al., (1997). Figure 1 schematically displays the organization of the hypertonic injection procedure. Long Evans rats were anesthetized with 0.1ml/100ml KAX by intraperitoneal injections until no rat reflexes were observed. KAX is a combination of 5 ml of ketamine (100 mg/ml), 2.5 ml xylazine (20 mg/ml), 1 ml acepromazine (10 mg/ml), and 0.5 ml sterile water. A topical anesthetic of 0.5% procaine hydrochloride was applied to the eye before the hypertonic saline injections to induce glaucoma-like conditions. Once the animal had

no signs of reflexes, a hemostat was used to pinch the eyelid, so the eye bulged out of the eye socket, revealing the episcleral vein for injection of the hypertonic saline solution (Figure 3C). The right eye was used for surgical manipulation while the left eye served as a control for each experiment unless noted otherwise. The needle used for injection was a long glass micro needle, 40 μm in diameter that was pulled from a Narishige electrode puller (Figure 3B). The glass electrode was glued onto a tapered polyethylene tubing (PE-50, Clay Adams, Parsippan, NJ) and inserted into a 23 gauge needle with the tip filed off (Figure 3B). The glass needle was subsequently beveled for easy penetration into the episcleral veins. An injection of 50 μl sterile 2 M hypertonic saline was injected into the vein with sufficient pressure to cause blanching of the episcleral veins. This causes scarring due to sclerosis of the hypertonic saline in the trabecular meshwork and decreases aqueous outflow which, in turn, slowly increases IOP (Johnson and Tomarev, 2011, Nissirios et al, 2009, Morrison et al., 1997). Following the injection, the hemostat was released, antibiotic cream was added to the region of the eyelid that was pinched and the rat was put back in its cage until it fully recovered. The entire procedure was completed before the animal woke and animals were closely watched to ensure full recovery before they were returned to the animal facility.

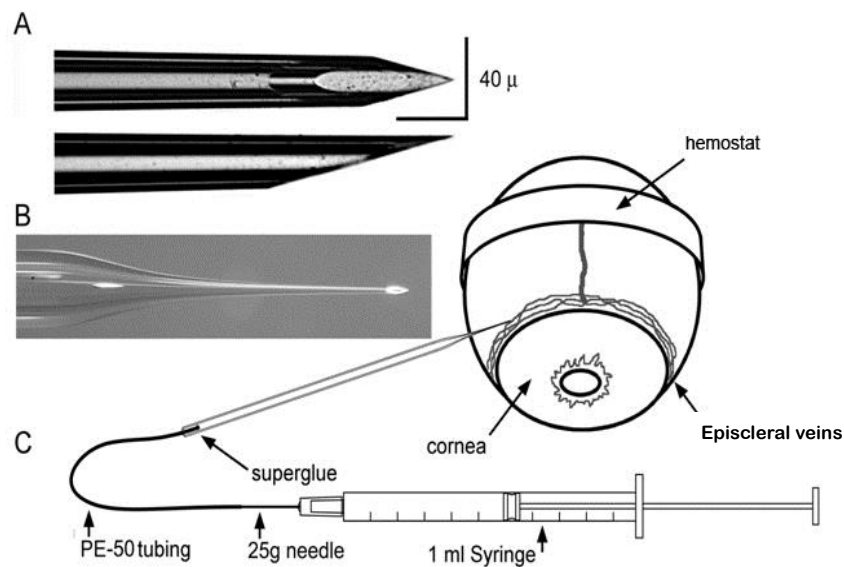


Figure 1

Schematic of hypertonic saline injection. The needle used for injection is a 3 mm long glass micro needle, 30 μm in diameter (B), which is glued onto a tapered polyethylene tubing, inserted into a 23 gauge needle with the tip filed off (A). The glass needle was subsequently beveled for easy penetration into the episcleral veins. An injection of 50 μl sterile 2 M hypertonic saline was injected into the vein with sufficient pressure to cause blanching of the episcleral veins (C). (Modified labeling from Linn^b et al., 2011)

Eye Drop Application

In-vitro studies previously demonstrated that ACh has a neuroprotective role against glutamate-induced cell death in RGCs (Wehrwein et al., 2004; Thompson et al., 2006) and that neuroprotection is mediated through $\alpha 7$ nAChR subunits (Thompson et al., 2006). Therefore, to determine if the $\alpha 7$ nAChR agonist, PNU-282987, has a neuroprotective effect under physiological conditions, the $\alpha 7$ nAChR specific agonist was introduced by eye drops to Long Evans rats prior to the procedure that induced glaucoma-like conditions. Four different concentrations of

PNU-282987 were used to determine which concentration of PNU was the most effective at preventing loss of RGCs. The four different concentrations chosen were at 10 times the concentration used in studies where PNU-282987 was injected intravitreally (Iwamoto, 2011). The four different concentrations of PNU eye drops used in this study were 100 μ M, 500 μ M, 1 mM, and 10 mM PNU-282987. The PNU compound was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution and then diluted in PBS to allow transportation of PNU-282987 through the sclera to the retina. The eye drops were applied for three days before the injection of hypertonic solution designed generate glaucoma-like conditions. Following hypertonic saline injections, eye drops were applied twice a day for one month. Eye drops were delivered twice a day since LC/MSMS studies demonstrated that there was PNU-282987 detected in the retina up to 12 hours after application (Linn^a et al., 2011). Animals were sacrificed after one month since previous studies have demonstrated that significant loss of RGCs occurs one month following hypertonic saline injections in the periphery (Iwamoto, 2011). Application for longer periods could cause retinal detachment from the effects of the hypertonic injection procedure (Morrison et al., 1997). Rats remained in their cages as the eye drops were applied. After each eye drop, the rats were given cheerios as a positive reinforcement. Eye drops were delivered using separate plastic syringes for each dose and were aimed at the bulbar conjunctiva, since that was the easiest access for the eye drops to seep into the interior of the eye socket while also retaining consistency in the administration of PNU-282987.

Quantification of RGC Loss

After rats were exposed to PNU-282987 for a month, they were sacrificed and retinas were removed from the eyes. Rats were euthanized in a carbon dioxide chamber for 5 minutes to ensure the animal was deceased. After 5 minutes, the animal was checked to ensure that there was no respiratory or reflex activity. The animals had both left and right retinas surgically removed from the eyes after removal of the cornea, lens and vitreous humor. Care was taken to peel the retina off the back of the remaining eyecup in one piece to maintain anatomical landmarks. Whole retinas were then flat mounted, pinned out in a sylgard dish with the RGC layer facing upward using cactus needles, and fixed in 10% formalin overnight at 4° C. Figure 2 illustrates an example of a retinal flat mount. After the samples were fixed, the tissue was rinsed with PBS three times. To block nonspecific binding, the tissue was incubated in 2% BSA in PBS containing 0.02% saponin for 30 minutes at room temperature before applying the primary antibody. Thy 1.1 (BD Biosciences), is a monoclonal antibody against glycoproteins only found in RGCs in the retina (mouse anti-rat) (Barnstable and Drager, 1984). Preliminary serial dilution studies determined that optimal results were obtained when the primary antibody was diluted 1:300 in 0.02% saponin in PBS with 2% BSA for 1 week at 4° C in a humidified chamber. After a week, the primary antibody was rinsed 3 times using PBS and incubated in fluorescent secondary antibody (goat anti-mouse IgG), Alexa Fluor 595 (Invitrogen/Molecular Probes), for visualization. After one week, the incubated tissue was rinsed and mounted on glass slides using 50% PBS and 50% glycerol.

In control studies, experiments were conducted to determine specificity of

the antibodies used. Negative control experiments were performed to examine antibody specificity. In some experiments designed to determine antibody specificity, large RGCs were processed with the primary antibody omitted, while other experiments substituted non-immune mouse immunoglobulin (dilution: 0.1 – 1.0 $\mu\text{g/ml}$) for the monoclonal antibody. In other experiments designed to determine antibody specificity, preabsorption controls were performed where the primary antibody and Thy 1.1 antigen were added together before applying to tissue. No significant epifluorescence was observed under any of these conditions.

Glass slides with retinal tissue were visualized using a Zeiss confocal microscope. Using the Z stack function of the confocal microscope, images were obtained from the periphery of the retina 400 microns from the optic nerve head (Figure 2). This measurement was based on previous studies showing that the greatest amount of damage occurs in the periphery (Iwamoto, 2011).

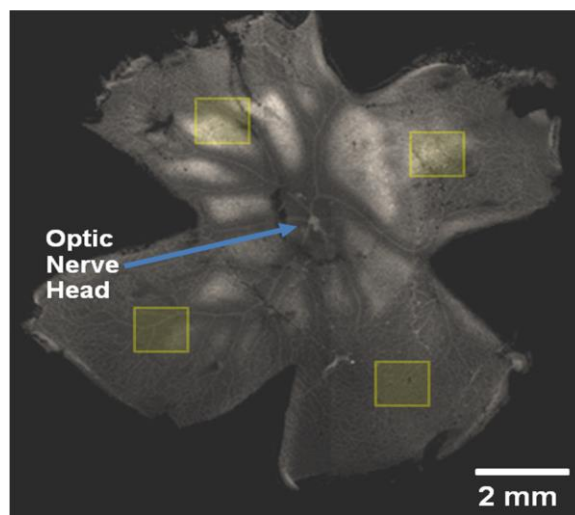


Figure 2

Rat retina flat mount. Unlabeled rat retina flat mount visualized under compound light on Zeiss confocal microscope with added frames 400 μm from ONH. The yellow squares indicate regions where cell counts were performed.

Images were obtained throughout the entire RGC layer in 1 μm increments, to properly account for RGC density. As the distribution of RGCs can be uneven in different regions of the rat retina (Dreher et al., 1985), images were obtained from four 80 μm^2 spaces that surround the optic nerve head at 400 μm away from the center of the optic nerve head (Figure 2). The total number of Thy 1.1 labeled RGCs in each square 400 μm from the optic nerve head were counted in experimental and control retinas. Counts were taken from similar anatomical landmarks in each eye using Metamorph software. RGC and axon fascicle counts were analyzed and graphed using GraphPad software.

Quantification of RGCs was formulated using an 80 μm^2 frame placed on every retinal image that was taken. The position of this square was placed to avoid axon fascicle interference 400 μm from the ONH. Preliminary studies used the 80 μm square frame at multiple identified locations 400 μm from the ONH to take images, count and average the number of RGCs. There were significant differences in RGC counts if 2 or 3 squares were averaged 400 μm from the ONH. However, if 4, 6 or 8 squares were evenly distributed around the ONH 400 μm from the ONH, the average RGC counts were significantly similar. As a result, 4 images were taken from each retina and RGC counts were averaged in all studies.

LC/MSMS Analysis of Rat Retina, Blood Plasma and Heart Tissue

LC/MSMS (liquid chromatography: mass spectroscopy with triple quad capabilities) was performed according to standard procedures on retina, plasma and heart samples removed from sacrificed Long Evans rats at various time points

following eye drop applications. Retinas, blood plasma and heart samples (atria and ventricle) were removed from asphyxiated rats after different concentrations of PNU-282987 was applied to rat eyes as eye drops for various amounts of time. Collected samples were removed from experimental animals, weighed and placed on ice immediately after removal and sent to the Michigan Innovation Center of Kalamazoo, MI to perform LC/MSMS analysis for quantification of PNU-282987 in each sample. Five samples were collected for each time and dose response experiment. Three different concentrations of PNU-282987 was applied to rat eyes (100 μ M, 1 mM and 10 mM) for five different amounts of time before the animals were sacrificed and tissue was collected and put on ice. Animals were sacrificed 1, 2, 4, 8 and 12 hours after eye drop application of PNU-282987. At the Innovation center, each sample collected were labeled and placed in -80°C until processed for LC/MSMS analysis. Preliminary studies using LC/MSMS detected PNU-282987 in rabbit retina when the agent was applied as eye drops (Linn^b et al, 2011). These studies were repeated using the glaucoma rat model and performed on plasma and heart tissue in rat to examine the idea that PNU-282987 can be detected in the retina when applied as eye drops, but may not be detected in blood plasma or heart tissue.

LC/MSMS Sample Preparation

At the Michigan Innovation Center, the tissue was transferred to homogenation tubes and appropriate volumes of saline were added to each tube to make a tissue concentration of 50 mg/ml. Each retinal tissue was homogenized for approximately 3 minutes using a pestle and homogenation tube (Kimble-Chase). A

volume of homogenized rat tissue or plasma was combined with an equal amount of blank acetonitrile and an equal amount of acetonitrile containing internal standard for total volume of 1 ml. Standards were prepared by combining an aliquot of control retinal tissue homogenate with calibration spiking solution in acetonitrile and internal standard in acetonitrile. All samples were placed in a vortex to precipitate proteins and then centrifuged for 10 minutes at 14,000 rpm at 4°C. Supernatants were transferred to autosampler vials and dried in a SpeedVac and reconstituted in 0.1% formic acid/10% acetonitrile for LC/MSMS.

LC/MSMS Analysis

LC/MSMS was performed on a Waters Quatro Micro triple quadrupole mass spectrometer using positive ion electrospray ionization. A Waters CapLC capillary HPLC was configured for on-line SPE. Additional experimental details can be found in Linn^b et al., (2011).

Statistical Analysis

All cell counts were compared to the internal control counts for each trial. Student T-tests were used for single comparisons. Statistical analysis was performed on all normalized data using Kruskal-Wallis non-parametric analysis of variance (ANOVA) with post hoc multiple comparisons (Dunn's test). Two-way ANOVA with correction for multiple comparisons was conducted on data that was not normalized. $P < 0.05$ was considered statistically significant for all tests.

RESULTS

Inducing Glaucoma-Like Conditions Causes Loss of RGCs

The left eye in each experimental animal was left untreated to act as an internal control and right eyes were manipulated with hypertonic injections to induce glaucoma-like conditions. One month following injection of the hypertonic saline into the episcleral vein of the right eye, animals were sacrificed, the retinas were removed in one piece, flat-mounted onto sylgard dishes and the retinas were process for visualization using an antibody against Thy 1.1 to label RGCs. An example of the results obtained following this procedure is shown in Figure 3. Figure 3A illustrates an image of labeled RGCs and labeled RGC axon fascicles that was obtained from the control untreated eye of a Long Evans rat 400 μm from the ONH. Figure 3B illustrates an image from a similar retinal location, from the same rat, but in the right eye, which underwent a procedure to generate a glaucoma model. Rats injected with hypertonic saline experienced a noticeable RGC count loss compared to the internal control.

It was observed from RGC labeling experiments that hypertonic saline injections had different effects in the retina. Panels shown in Figure 3A and 3B, show the results when the left eye was not manipulated and the right eye underwent hypertonic saline injections. Panel 3A shows RGCs (arrow heads) and main axon fascicles (double-headed arrows). Defasciculation off the main axon fascicles was also observed (single arrows). Panel 3B also shows labeled RGCs (arrow heads) and

main axon fascicles (double-headed arrows). Defasciculation off the main axon fascicles was also observed (single arrows), and was more prominent than compared to the internal control. It was also observed that the main axon fascicle diameter was altered when compared to the internal control.

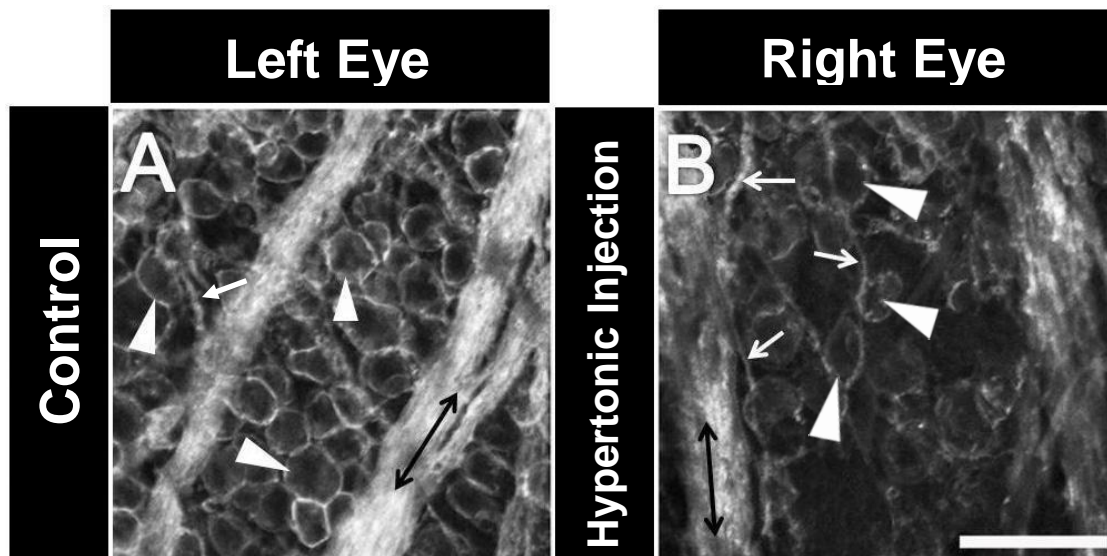


Figure 3

Injection of hypertonic saline. (A) Left eye: untreated internal control with labeled RGCs using antibody against Thy1.1 in grayscale. (B) Right eye: glaucomatous retina one month following injection of 50 μ l hypertonic saline. Scale represents 50 μ m. The images were obtained from the same animal. Arrow heads indicate labeled RGCs. The double arrow indicates axon fascicles.

The bar graph in Figure 4 summarizes the effects of hypertonic injections on RGC loss compared to control untreated conditions. Rats eyes injected with hypertonic saline experienced a significant decrease in cell counts of 26% (± 2.19 ; N=9) when normalized to internal controls. Studies to analyze morphological differences in the retina are discussed later in this thesis

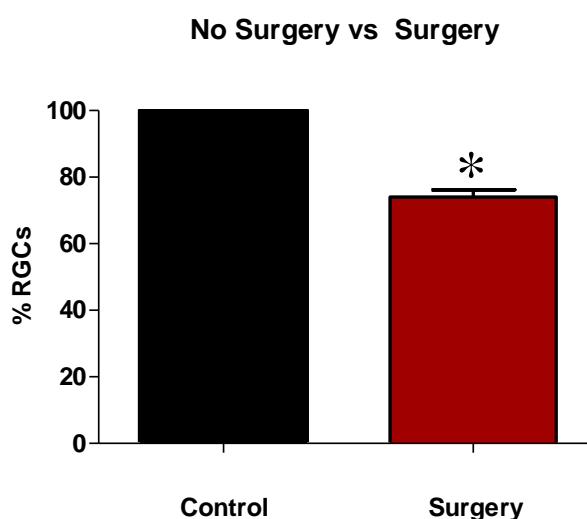


Figure 4

RGC counts compared in control and hypertonic saline injected retinas. Bar graph represents the normalized average RGCs in the right eye (red bar) compared to the left internal control (black bar) (N=9). Hypertonic saline injection to the episcleral vein significantly decreased RGC counts when compared to internal control. Error bars represent SEM. *represents significance to internal control.

Dose-dependent Response of PNU-282987

The images shown in Figure 5A and Figure 5B illustrate the effect of hypertonic saline injections to induce glaucoma-like conditions in a Long Evans rat that was already shown in Figure 3. To determine if PNU-282987 had any neuroprotective effects when applied as eye drops, left control and right experimental retinas were removed from animals after treatment of using PNU-282987 as described in Materials and Methods. It was observed from RGC labeling experiments that each dose of PNU-282987 had different effects in glaucoma-like conditions. Panels shown in Figure 5C and 5D, show the results when the right eye of another animal was treated with 100 μ M PNU-282987 under glaucoma-like conditions (Figure 5D) and

the left eye was left untreated (Figure 5C). These images were obtained from the same animal. Panel 5C shows RGCs (arrow heads) and main axon fascicles (double-headed arrows). Defasciculation off the main axon fascicles was also observed (single arrows). Panel 5D also illustrates labeled RGCs (arrow heads) and main axon fascicles (double-headed arrows) under glaucoma-like conditions, with the treatment of 100 μ M PNU-282987. Defasciculation off the main axon fascicles was also observed (single arrows), and was more prominent than compared to the internal control. It was also observed that the main axon fascicle diameter was reduced when compared to the internal control, similar to results shown in Panel 5B. There was also apparent RGC loss in the right eye even when treated with 100 μ M PNU-282987 when compared to the left internal control. RGC morphology was also altered in the right eye having a blebbed membrane appearance that was not observed in the left internal control. In summary, 100 μ M PNU-282987 did not act to protect against changes associated with a procedure to generate glaucoma-like conditions.

Panels 5E and 5F, illustrate the result when the right eye was treated with 500 μ M PNU-282987 under glaucoma-like conditions. Both images in the panels were obtained from the same animal. Panel 5E illustrates RGCs and main axon fascicles with defasciculation. Panel 5F illustrates RGCs and main axon fascicles under glaucoma-like conditions with the treatment of 500 μ M PNU-282987. Although there was still some defasciculation off the main axon fascicles, reduced diameter in the main axon fascicles and decreased RGC counts compared to the internal control, 500 μ M PNU-282987 had measurable neuroprotective effects. The neuroprotective effect of PNU-282987 increased in the presence of 1 mM PNU-282987. When the retina was treated with 1 mM PNU 282987, there was less defasciculation, the diameter of

the main axon fascicles appeared similar to the diameters in the control retina and RGC densities in 1 mM PNU treated retinas were similar to RGC densities in the untreated control retina (Figure 5G and 5H).

A substantial degree of neuroprotection was evident when eyes were treated with 10 mM PNU-282987 (Figure 5I and 5J). Little to no defasciculation off the main axon fascicles was observed in the experimental eye (Figure 5J). When treated with 10 mM PNU-282987, the diameters of the main axon fascicles were similar in comparison to the control panels (Figure 5I). Finally, when treated with 10 mM PNU 282987, the RGC profiles were smooth and RGC density was similar to internal control counts.

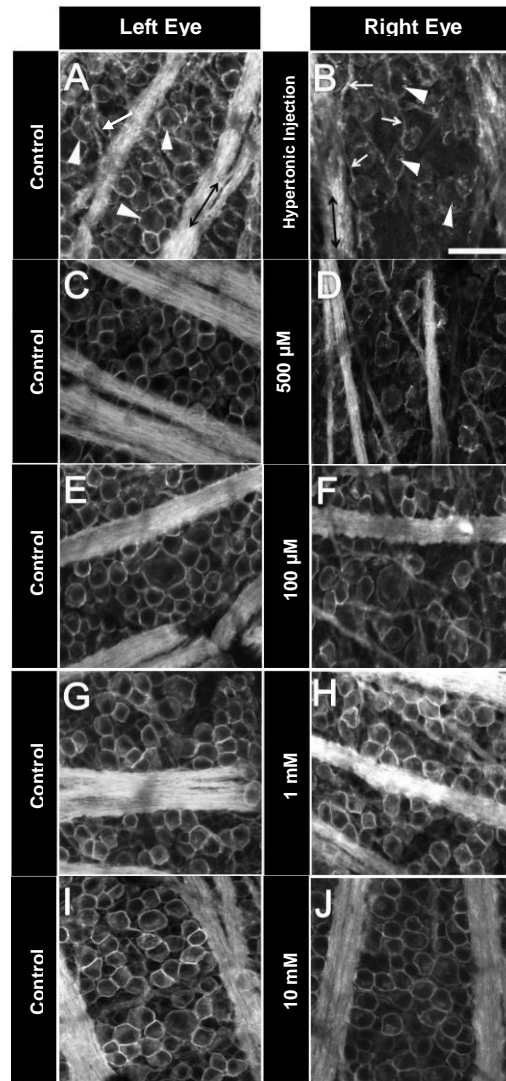


Figure 5

Treatment of RGCs using different concentrations of PNU-282987. Samples of images obtained from RGCs in rat retina labeled with fluorescently tagged antibody against Thy 1.1 in grayscale. Images in the left column represent control untreated images for the experimental images shown in the right column. Images were obtained 1 month following procedure to induce glaucoma-like conditions from the same animals in each row. (B) Labeled RGCs after eyes were injected with hypertonic saline injections. (D) Labeled RGCs after treatment of 100 μ M PNU-282987 before and after procedure to induce glaucoma-like conditions. (F) Labeled RGCs after treatment of 500 μ M PNU-282987 before and after procedure to induce glaucoma-like conditions.. (H) Labeled RGCs after treatment of 1 mM PNU-282987 before and after procedure to induce glaucoma-like conditions. (J) Labeled RGCs after treatment of 10 mM PNU-282987 before and after procedure to induce glaucoma-like conditions. The images in each row were obtained from the same animal and from the anatomical location. Arrow heads indicate labeled RGCs. The double arrow indicates axon fascicles. Single arrows indicate defasciculation. Scale bar is 50 μ m.

From the fluorescent imaging shown above, it was apparent that RGC loss was minimized when eyes were treated with the higher doses of PNU-282987. The summarized results of these dose-response studies are shown in Figure 6. One month following induction of glaucoma-like conditions, there was an average of 26% (\pm 2.19, N=9) loss of RGCs. Eye drop application of PNU-282987 before and after hypertonic injection resulted in prevention of RGC loss in a dose-dependent manner. Application of 100 μ M PNU-282987 had no significant effect on RGC loss associated with the procedure to generate a glaucoma model. This was indicated with a 22.3% (\pm 5.81, N=5) RGC loss in the right eye treated with 100 μ M PNU-282987 when compared to the left internal control. However, when 500 μ M PNU-282987 was applied, 8.4% (\pm 3.8, N=3) of RGCs were lost compared to the internal untreated controls. This result was found to be significantly different from internal control conditions. When 1 mM PNU-282987 was applied, only 6.9% (\pm 3.84, N=5) of RGCs were lost compared to untreated controls and represents statistically significant neuroprotection from eyes treated with hypertonic injections. Protection of RGCs also occurred when 10 mM PNU-282987 eye drops were applied to manipulated eyes. When treated with 10 mM PNU-282987, RGC percentage increased by an average of 13.5% (\pm 4.19, N=5) compared to the untreated controls.

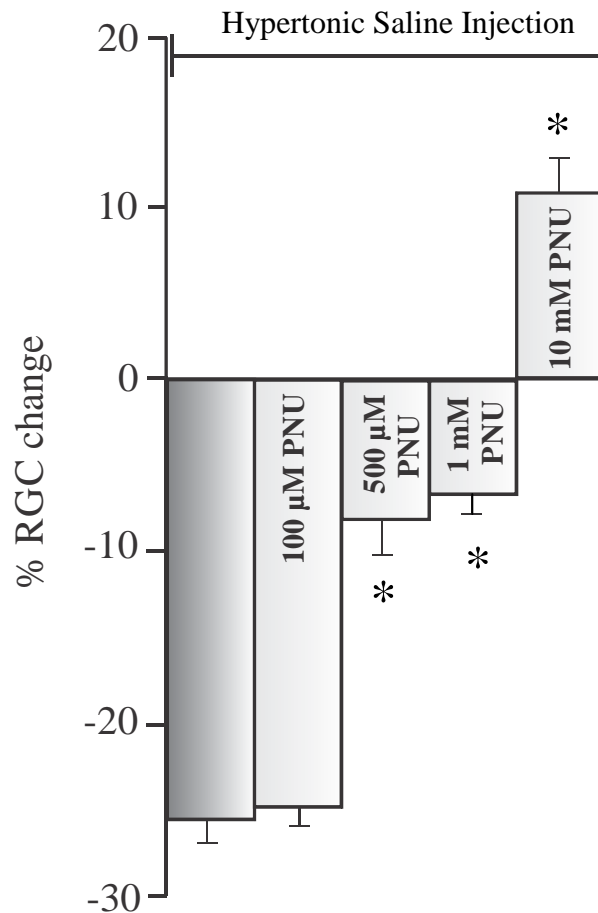


Figure 6

Summary of results following eye drop treatment of PNU-282987 before and after hypertonic injections. Each bar graph represents the normalized average change of RGCs compared to control untreated conditions that were measured 1 month following procedure to induce glaucoma. Treatment of 100 µM PNU-282987 (N=5), 500 µM PNU-282987 (N=3), 1 mM PNU-282987 (N=5), and 10 mM PNU-282987 (N=5) are shown. Eye drop application of PNU-282987 before and after hypertonic injections resulted in prevention of RGC loss in a dose dependent manner. Error bars represent SEM. *represents significant different from the glaucoma-induced results (dark grey bar).

Morphological Differences

Morphological changes occurred in the rat retina as a result of injecting hypertonic solution into the episcleral vein of the right eye, as demonstrated in earlier

experiments. These morphological changes were not previously described in our laboratory. By using a new marker to label RGCs, retinal changes could be analyzed that were previously obscure. These morphological changes in the retina decreased or diminished as higher doses of PNU-282987 were administered. Hypertonic injections with no treatment of PNU-282987 had the most visible changes on retinal organization. In comparison, using 10 mM PNU-282987 on retinal tissue before the procedure to induce glaucoma-like conditions had little or no changes on retinal arrangement, demonstrating a similar appearance to the internal control. The method of labeling RGCs using an antibody against Thy 1.1 opened up the opportunity to examine the anatomical changes in the retina during glaucoma-like conditions. In this thesis, 3 different morphological changes in the RGC layer were analyzed with and without PNU-282987 treatment to the eye. These morphological changes are discussed in the following sections.

Figure 5 (A, C, E, G, and I) displays images illustrating typical retinal arrangement and organization. These images were obtained from untreated eyes where RGCs and axons were labeled with the antibody against Thy 1.1. In control conditions, there are thick axon fascicles (double arrows), and little to no defasciculation off the main axon fascicles (tissue strands less than 2 μm). The morphology of the membrane in the RGCs typically has a smooth round appearance (arrow heads), and the vast majority (90%) of the image is covered with labeled RGCs. However, after the procedure to induce glaucoma-like conditions, significant differences in retinal arrangement and morphology occurred. The first morphological differences being discussed are changes in main axon fascicle diameter.

Main Axon Fascicle Diameter

When the retina was stained with antibody against Thy 1.1, the retina was layered in thick axon fascicles leading to the ONH as shown by the double arrows illustrated in Figure 5 (A, C, E, G, and I). If RGCs were lost after hypertonic injection, it was expected that the diameter of the axon fascicles carrying the axons of those RGCs would also change. To determine if the diameters of main axon fascicles changed with different manipulations, the thickest section of each fascicle in each image was measured, an averaged diameter was computed and the average was compared to the average diameter of main axon fascicles obtained under control untreated conditions 400 μm from the ONH. Measurements were taken from the widest part of all main axon fascicles from each image and analyzed. If the main axon fascicle branched into different axon fascicles, the axons were excluded from measurements and only the main axon fascicle was measured. Manipulated right eye measurements were compared to the left internal controls. There were two scenarios that compared differences in main axon fascicle diameter between left untreated controls and right experimental eyes. The first scenario included experiments where the right eye was only given injections of hypertonic saline. The second scenario included experiments where the right eye was given injections with hypertonic saline along with treatment with 10 mM PNU-282987.

Figure 7 illustrates the results obtained when the left internal control eyes were compared to the right eyes that only had hypertonic injections to induce glaucoma-like conditions. When manipulations were performed to generate a glaucoma model, the average axon fascicle diameter measured from the left untreated control retinas

was 15.7 μm (± 0.57 , N=6). The average main axon fascicle diameter from right experimental eyes was 12.1 μm (± 0.86 , N=6). The injections to induce glaucoma significantly decreased the main axon fascicle diameters when compared to the internal control. Thus, there was an average decrease in axon fascicle diameter of 25% (± 0.25 , N=3) compared to the left internal control.

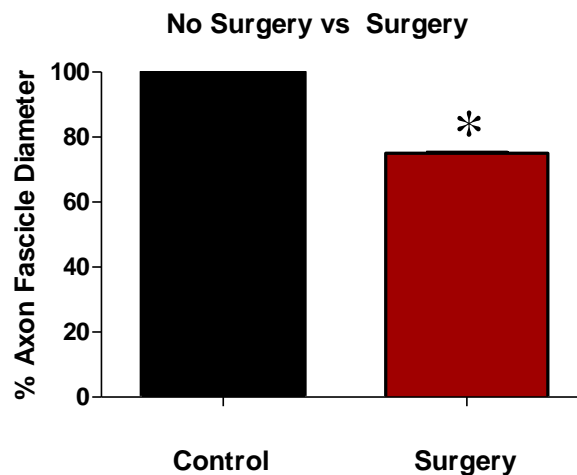


Figure 7

Axon fascicle diameter comparison between control and hypertonic saline injected retinas. The graph represents the normalized average axon fascicle diameter 1 month following procedure to induce glaucoma-like conditions in the right eye. Left internal control eyes were not manipulated in any way (black bar) and the right eyes were subjected to injection of hypertonic solution (red bar) to induce glaucoma-like conditions with no treatment of PNU-282987. Measurements were taken from the thickest diameter of the axon fascicles 400 μm from the ONH (N=6). Error bars represent SEM. *represents significance to internal control.

If retinas were treated with 10 mM PNU-282987 before and after hypertonic injections and for a month following hypertonic saline injections, the main axon fascicle diameter in the left internal controls averaged 20.1 μm (± 1.16 , N=5). When the right eyes were treated with 10 mM PNU-282987 before and after hypertonic injections resulted in an average diameter of 19.2 μm (± 1.03 , N=5). Results of these

experiments are summarized in Figure 8. When the experimental axon fascicle diameter was compared to the left internal control fascicle diameter, measurements only decreased by an average of 4.8% (± 9.06 , N=5) and were not significantly different from each other.

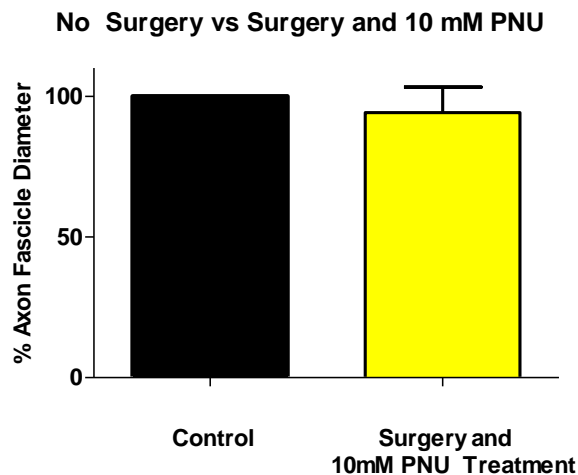


Figure 8

Axon fascicle diameter comparison between control and retinas with hypertonic injections and 10 mM PNU-282987 treatment. The graph represents the normalized average axon fascicle diameter 1 month following procedure to induce glaucoma in the right eye. Left internal control eyes were not manipulated in any way (black bar) and the right eyes were subject to hypertonic injections to induce glaucoma-like conditions and treatment with 10 mM PNU-282987 (N=5) (yellow bar). Error bars represent SEM.

The following control study was performed to determine if main axon fascicle diameter changed from the internal control using nothing but 10 mM PNU-282987 treatment. This control experiment was conducted to determine if PNU-282987 treatment would cause any unprecedented effects. When right eyes were treated with 10 mM PNU-282987 without an injection of hypertonic solution, there were no visible morphological differences. Specifically there were no changes in axon diameters and there was no noticeable change in the smoothness or shape of the RGCs

between control and PNU-282987 treated retinas. An example of this is shown in Figure 9A and 9B.

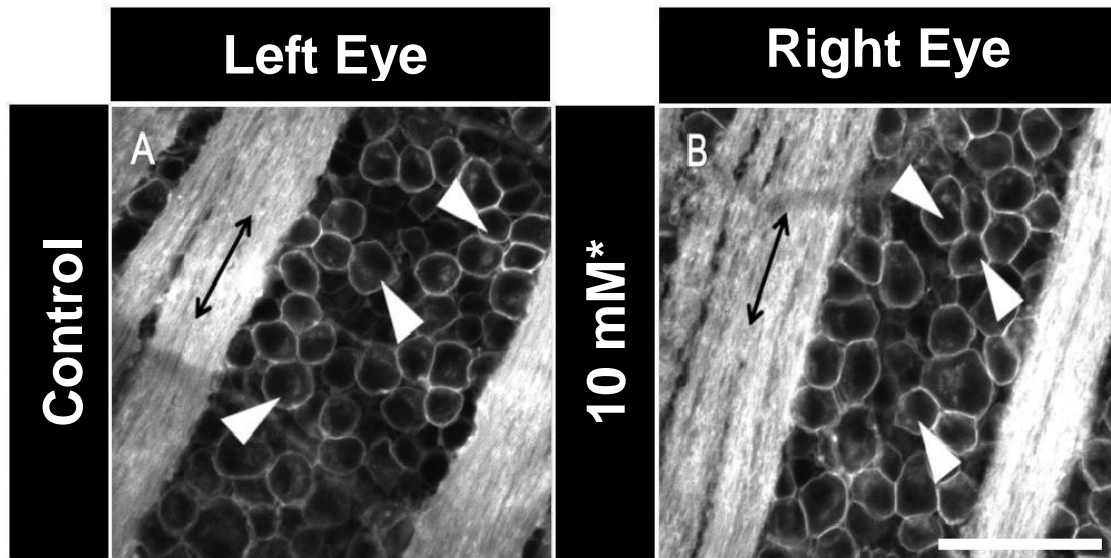


Figure 9

Treatment with 10 mM PNU-282987 without a hypertonic injection. (A) Untreated internal control with labeled RGCs using antibody against Thy1.1 in grayscale. (B) (*) Retina following treatment of PNU-282987 for one month. The images were obtained from the same animal, in similar anatomical locations 400 μm from the ONH. Arrow heads indicate labeled RGCs and double arrow indicates main axon fascicles. Scale bar indicates 50 μm .

Under these experimental conditions, it was observed that there was an average axon fascicle diameter of 21 μm (± 1.31 , N=3) in control untreated retinas. The right experimental eye with treatment of 10 mM PNU-282987 was observed to have an average axon fascicle diameter of 20 μm (± 1.06 , N=3). The summary of these control experiments is represented in Figure 10. The bar graphs in Figure 10 demonstrate that there was an average difference in axon fascicle diameter of 5.3% (± 7.86 , N=3) between the two conditions. No significant differences in axon fascicle diameter were observed.

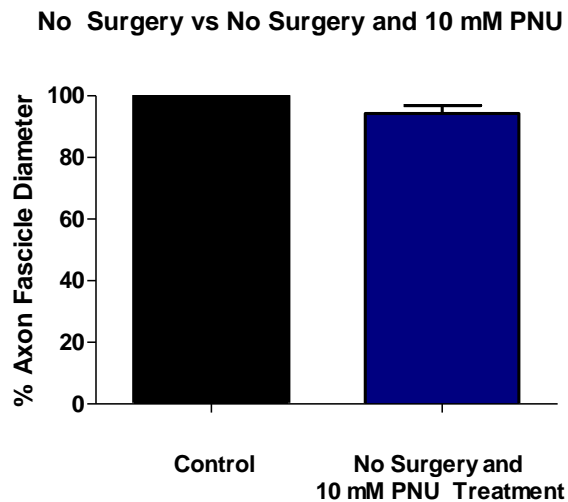


Figure 10

Axon fascicle diameter comparison between control untreated retinas and retinas treated with 10 mM PNU-282987 without hypertonic injections. The bar graph represents the normalized average axon fascicle diameter in conditions that were measured after the right eye was treated with 10 mM PNU-282987 for 1 month. The control eyes were not manipulated in any way (black bar) and the right eye was subject to only treatment of 10 mM PNU-282987 with no injection of hypertonic solution (blue bar). This shows that 10 mM PNU-282987 by itself has no significant effect on axon fascicle diameter size when compared to internal control (N=3). Error bars represent SEM.

A summary of axon fascicle diameter results from all three experiments is shown in Figure 11. Hypertonic saline injections designed to generate glaucoma-like conditions significantly decreased axon fascicle diameter size when compared to internal controls. However, hypertonic injections in conjunction with 10 mM PNU-282987 failed to produce a significant effect on axon fascicle diameter. Finally, 10 mM PNU-282987 treatment alone did not affect axon fascicle diameter.

Summary Results on Axon Fascicle Diameter

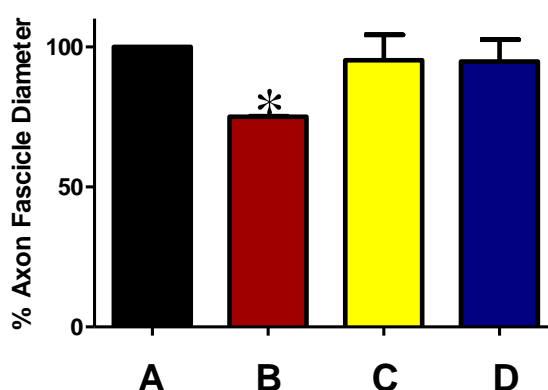


Figure 11

Axon fascicle diameter comparison between four different conditions. Each bar graph represents the normalized average axon fascicle diameter under various conditions. (A) Average axon fascicle diameter size from internal controls of all trials (N=14). (B) Right eye injected with hypertonic solution with no PNU-282987 treatment (N=6). (C) Right eye injected with hypertonic solution and treated with 10 mM PNU-282987 (N=5). (D) Right eye with no hypertonic solution and treated with 10 mM PNU-282987 (N=3). Error bars represent SEM. *represents significance to internal control.

Defasciculation Off Main Axon Fascicles

Another morphological difference that was examined in this thesis was in regard to the defasciculation that occurred from the main axon fascicles. If main axon fascicle diameter decreased after hypertonic injection, it could be solely the result of RGC death and axon loss. Alternatively, one might expect the fascicle to defasciculate into less tightly woven bundles as it dissociates. This would suggest additional, unforeseen events in the progressive changes seen with cell death in the retina. This experiment was to determine if there is any difference in defasciculation behavior under different experimental conditions. The total number of defasciculated axons from main axon fascicles was analyzed to determine if defasciculation of axons off

the main axon fascicles increased after procedures to induce glaucoma-like conditions. Defasciculated fascicles were defined as fibers extending from the main axon fascicles in an $80\ \mu\text{m}^2$ image obtained $400\ \mu\text{m}$ from the ONH with a diameter no larger than $2\ \mu\text{m}$. A continuous filament was counted as 1 count. If a fascicle branched into more filaments, each defasciculated branch was counted individually if each branch measured less than $2\ \mu\text{m}$ in diameter. Three different scenarios were analyzed for defasciculation. In the first scenario, the number of fascicles from left internal control images was compared to the fascicles counted from the right eye where hypertonic injections were delivered to induce glaucoma-like conditions. In the second scenario, defasciculation from left internal control images were compared to the amount of defasciculation from right eyes that were subject to hypertonic injections and 10 mM PNU-282987 treatment. Finally, the third comparison was made between defasciculation occurring in left internal control images and right eye images obtained after 10 mM PNU-282987 application. Images showing defasciculated axons off the main fascicles are displayed in Figure 12B.

Panels A, C and E in Figure 12 shows left internal control images obtained from each animal in each of the experimental scenarios. Left internal controls showed minimal or no defasciculation occurring from the main axon fascicles. Figure 12B illustrates the right eye treated with only hypertonic saline injections with no treatment of 10 mM PNU-282987. The main axon fascicle diameter was decreased and defasciculation off the main axon fascicle was higher compared to its internal control. Figure 12D shows a right eye with glaucoma-like conditions by hypertonic injections and treatment with 10 mM PNU-282987. Main axon fascicle diameter appeared unaffected compared to the internal control and there was no defasciculation

occurring from the main axon fascicles. Finally, Figure 12F illustrates an eye treated with 10 mM PNU-282987 with no hypertonic saline injections. Similar to results in Figure 12D, there were little or no changes in axon fascicle diameter and no prevalent defasciculation when compared to its internal control.

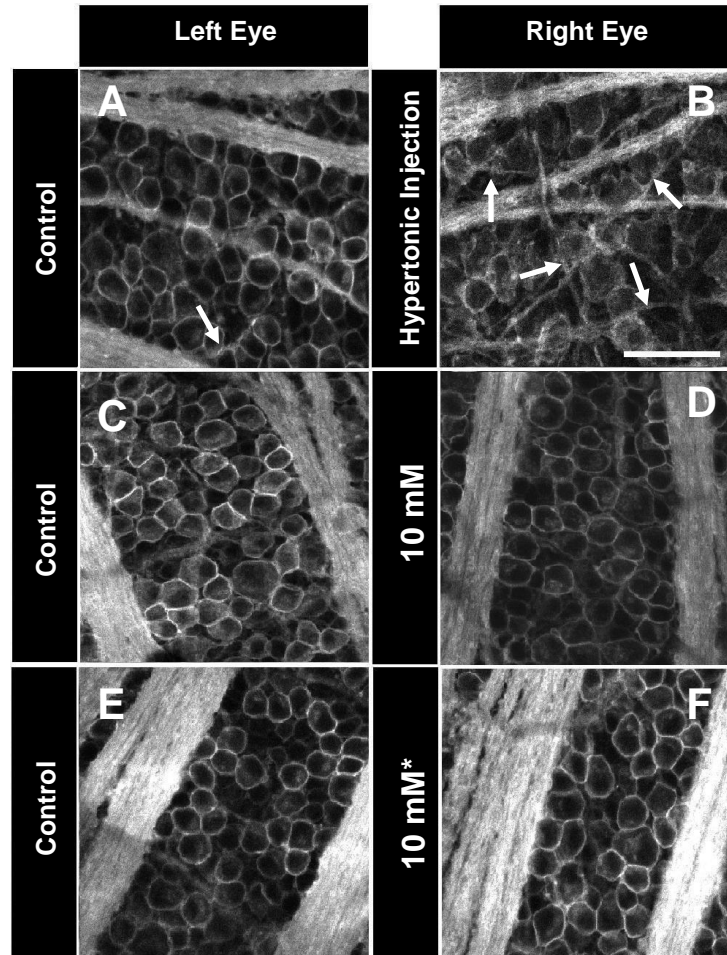


Figure 12

Defasciculation off main axon fascicles in different conditions. (A, C, and E) Left eye images obtained from each animal that acted as untreated internal controls illustrating labeled RGCs using antibody against Thy1.1 in grayscale. The images obtained in the left columns correspond to the right images in each row from the same animals (B) Image shows a right eye image obtained when the eye was injected with hypertonic saline with no PNU-282987 treatment. (D) Right eye image obtained after hypertonic injections and treatment with 10 mM PNU-282987. (F) (*) Right eye image obtained from an eye only treated with 10 mM PNU-282987 with no hypertonic saline injections. All images were taken after one month after hypertonic injections or treatment to the right eyes 400 μ m away from the ONH. Scale bar indicates 50 μ m.

The number of defasciculated axons counted under each condition is summarized in Figure 13. For the first manipulation, the left control eye averaged 2.43 (\pm 0.81, N=6) fascicles, while the manipulated right eye that averaged 5.23 (\pm 0.4, N=6) fascicles (Figure 13). This was found to be statistically significant compared to the internal control. Figure 13 summarizes the results obtained when the number of fascicles from untreated controls was compared to manipulated right eyes that had undergone 10 mM PNU-282987 treatment. Under these conditions, the left control eye had an average of 3.42 (\pm 1.36, N=5) fascicles and the right eye averaged 2.82 (\pm 1.06, N=5) fascicles (Figure 13). This does not represent a significant difference. Finally, Figure 13 summarizes the results obtained when comparing the left control eye to right eyes that were treated with 10 mM PNU-282987. Under these conditions, the left eye images obtained from 3 different animals averaged 3.0 (\pm 0.3, N=3) fascicles off the main axon fascicles while the right experimental eyes averaged 3.77 (\pm 1.37, N=3) fascicles. Defasciculation observed in this study was shown to increase from control untreated conditions after the procedure to induce glaucoma-like conditions, but not if 10 mM PNU-282987 was applied.

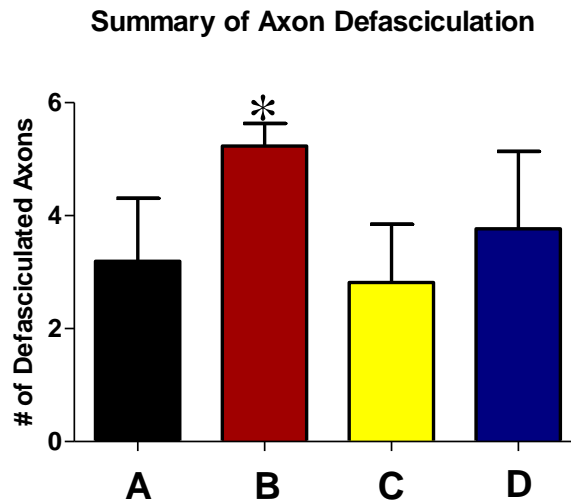


Figure 13

Axon defasciculation off main axon fascicles in comparison between three different conditions. Each bar graph represents the average number of defasciculated axons from main axon fascicles in conditions that were measured 1 month following procedure to induce glaucoma-like conditions. Control eyes were not manipulated in any way and the right eyes were subject to manipulations. (A) Average defasciculation in internal control for all three manipulations (N=14). (B) Right eye injected with hypertonic solution with no 10 mM PNU-282987 treatment (N=6). (C) Right eye injected with hypertonic solution and treated with 10 mM PNU-282987 (N=5). (D) Right eye with no hypertonic solution and treated with 10 mM PNU-282987 (N=3). Error bars represent SEM. *represents significant different from internal control.

Retinal Ganglion Cell Morphology

Other than differences in axon fascicle diameter and defasciculation, noticeable differences in RGC morphology were also observed under different conditions. These morphological differences were observed when; hypertonic saline was injected into the episcleral vein (Figure 5B); 100 μ M PNU-282987 was administered before and after glaucoma-like conditions; when PBS eye drops that were substituted instead of PNU-282987 during glaucoma-like conditions (Figure 18B) and injection hypertonic saline to the episcleral vein of the left eye (Figure 16A) when compared to their respective internal controls. It was observed in fluorescent

labeling experiments that under these conditions RGCs had a distinct morphology when compared to control and 10 mM PNU-282987 treated conditions such as those illustrated in Figure 5. In control and PNU-282987 treated conditions the RGC somata membrane was uniform with a smooth outline. Morphology of RGCs during glaucoma-like conditions was blebbed and not circularly uniform. Using Image J imaging and analysis software, quantification of this morphology was conducted. This program uses an index that determines a value for circularity in cells. A circularity value of 1.0 indicates a perfect circle. As the value approaches 0.0, it indicates polygonal characteristics maximally deviated from perfect circular geometry. Using Image J, the circumference from 3 different animals in both eyes was examined and obtained a circularity index for RGCs. This was repeated in different scenarios and compared to the left internal control. Circularity of RGCs was quantified in three different scenarios leaving left eyes as untreated internal controls. The first scenario had hypertonic saline injected to the right eyes to induce glaucoma-like conditions. The second had hypertonic saline injections with 10 mM PNU-282987 treatment. Finally, the third scenario had no hypertonic saline injections, only 10 mM PNU-282987 treatment.

Quantification of circularity in RGCs demonstrated that RGCs in eyes changed their circularity index under different conditions. In eyes that had hypertonic injections the circularity index for RGCs was $0.63 (\pm 0.02, N=3)$. This circularity index was significant when compared to the circularity index of $0.86 (\pm 0.01, N=3)$ in internal controls. The circularity index for eyes that had 10 mM PNU-282987 treatment with hypertonic injections resulted in a circularity index of $0.89 (\pm 0.01, N=3)$ and was not significant when compared to its internal control index of $0.89 (\pm$

0.01, N=3). The circularity index was also not significant in eyes that had 10 mM PNU-282987 treatment without hypertonic injections. Right eyes had a circularity index of 0.90 (\pm 0.06, N=3) when compared to the internal control index of 0.90 (\pm 0.01, N=3). Results summarizing these results are shown in Figure 14.

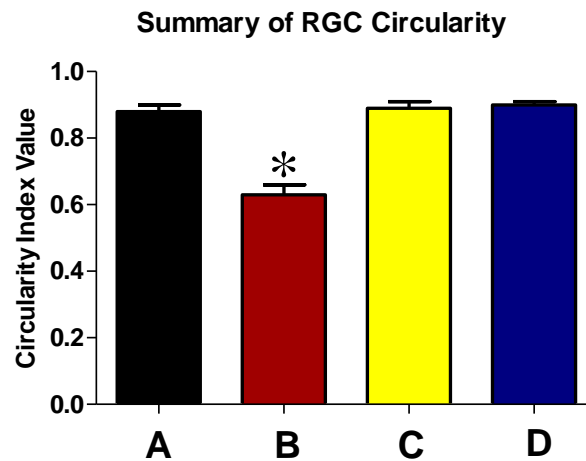


Figure 14

RGC circularity index in comparison between three different conditions. Each bar graph represents the average RGC circularity index in conditions that were measured 1 month following procedure to induce glaucoma-like conditions. Control eyes were not manipulated in any way and the right eye was subject to manipulations. (A) Average RGC circularity index in internal control for all three manipulations (N=9). (B) Right eye injected with hypertonic solution with no 10 mM PNU-282987 treatment (N=3). (C) Right eye injected with hypertonic solution and treated with 10 mM PNU-282987 (N=3). (D) Right eye with no hypertonic solution and treated with 10 mM PNU-282987 (N=3). Error bars represent SEM. *represents significant different from internal control.

In addition to quantification of RGC membrane morphology, RGC somata size was also examined. This analysis was conducted to determine if this change in morphology altered RGC some size. I wanted to investigate the effects that the hypertonic saline injection and treatment of 10 mM PNU-282987 had on the diameter of individual RGC somata. The observation being tested in this study was that the

injection of hypertonic saline and application of 10 mM PNU-282987 could affect the size of RGC somata that survive after hypertonic saline injections and PNU-282987 treatment. Three different sets of experiments were analyzed in these studies. In the first scenario, the diameter of RGC somata were measured in left control conditions and compared to the diameter of RGC somata from the right eye manipulated conditions. In the next condition, the diameter of RGC somata in left control eyes were compared to the diameter of RGC somata that had the procedure to induce glaucoma-like conditions and were treated with 10 mM PNU-282987. Lastly, the diameter from the left internal control RGC somata were compared to the diameters of right experimental RGC somata that were treated only with 10 mM PNU-282987. Measurements were obtained by taking a vertical and a horizontal measurement from each cell, then adding the two distances together and computing the average. Incomplete cells that did not show the complete circumference of the cell were excluded from quantification. RGCs were considered small when their diameter was less than 15 μm and large RGCs were characterized as RGCs with diameter greater than 15 μm .

Results illustrated in Figure 15 show that when the right eye was treated with hypertonic injections to induce glaucoma-like conditions, an average diameter of 10 μm (± 0.01 , N=4) was measured for small RGCs compared to an average diameter of 9.02 μm (± 0.41 , N=4) in the control untreated eye in small RGCs. There was also no significant difference in the diameter of small RGCs when eyes were treated with 10 mM PNU-282987 treatment before the procedure to induce glaucoma-like conditions. An average diameter of 10 μm (± 0.026 , N=4) was measured in small RGCs under these conditions. Finally, when the retina was treated with just 10 mM PNU 282987,

the average diameter of the small RGCs equaled $9.25\ \mu\text{m}$ (± 0.25 , $N=4$). None of these averages were significantly different from any other diameter measurement obtained from small RGCs and suggest that the RGC somata size does not significantly change after surgical or pharmacological treatment with PNU-282987.

Similar results were obtained when the somata diameter of large RGCs were measured. When large RGCs were analyzed, the average somata diameter measured $16.7\ \mu\text{m}$ (± 0.29 , $N=4$) under control untreated conditions and $17.5\ \mu\text{m}$ (± 0.36 , $N=4$) for eyes that underwent the procedure to induce glaucoma-like conditions. When eyes were surgically manipulated to induce glaucoma-like conditions but were treated with 10 mM PNU-282987, the average somata size for large RGCs equaled $18.75\ \mu\text{m}$ (± 0.48 , $N=4$). Finally, when retinas were treated with 10 mM PNU-282987 alone, the average RGC somata diameter equaled $17.75\ \mu\text{m}$ (± 1.11 , $N=4$). There was no significant difference detected in somata size that was associated with any treatments.

Small RGC Somata Diameter Frequency

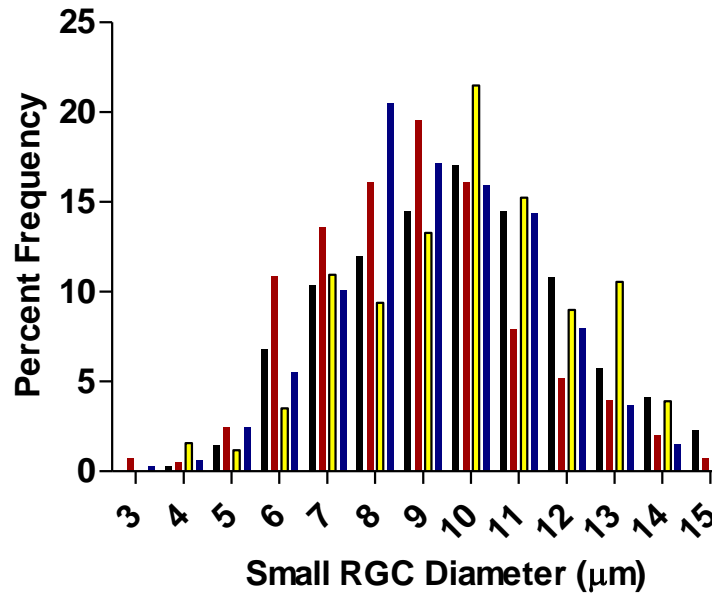


Figure 15

Individual histograms of small RGC diameter. Histogram represents the distribution in RGC somata size for small RGCs under 3 experimental conditions that were measured 1 month following procedure to induce glaucoma. Left control eyes (black bars) were not manipulated in any way and the right eye was subject to manipulations. (A) Left internal controls distributed with right experimental conditions with hypertonic injections and no treatment of 10 mM PNU-282987 (N=4) (red bars). (B) Left eye subject to hypertonic injections with no PNU-282987 treatment and right eye injected with hypertonic solution with 10 mM PNU-282987 treatment (N=4)(yellow bars). (C) Left internal control compared to the right eye with no hypertonic injection and only treated with 10 mM PNU-282987 (N=4) (blue bars).

Hypertonic Injections in both Eyes and PNU-282987 Treatment

In control studies, both eyes were subject to hypertonic injections to induce glaucoma-like conditions, while the right eye was also treated with 10 mM PNU-282987 eye drops. Results from fluorescent labeling experiments demonstrate that

there was a significant difference in RGC survival when retinas were treated with 10 mM PNU-282987. The left eye had a disorganized appearance, apparent decrease in RGC density and increased axon defasciculation when compared to the right eye treated with 10 mM PNU-282987 (Figure 16A and 16B).

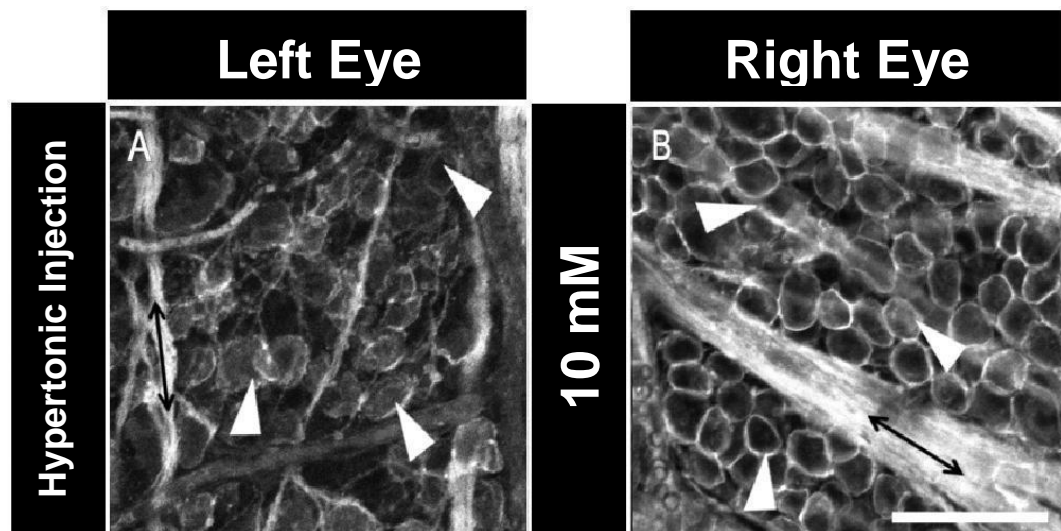


Figure 16

Hypertonic injections in both eyes with 10 mM PNU-282987 treatment on the right eye. (A) Left eye subject to hypertonic injections labeled with antibody against Thy1.1 in grayscale. (B) Retina following treatment of 10 mM PNU-282987 for one month following hypertonic injection. Arrow heads point to RGCs. Double arrow indicates the axon fascicles. The images were obtained from the same animal 1 month after hypertonic saline injections, from the same region of the retina 400 μ m from the ONH. Scale bar indicates 50 μ m.

Results from this experiment illustrated a 20% (± 4.1 , N=3) average increase in RGC density in the right eye treated with 10 mM PNU-282987 when compared to the left, glaucomatous, untreated eye. This result was shown to be significant when compared to the left eye condition. Figure 17 summarizes the differences in cell density between the right and left eye.

Surgery vs Surgery and 10 mM PNU Treatment

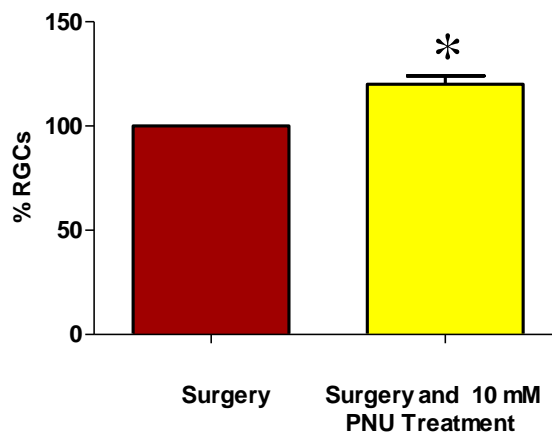


Figure 17

Hypertonic injections in both eyes with 10 mM PNU-282987 treatment in the right eye. The bar graph displays the normalized RGC change from the internal control. Internal control subject to hypertonic injections (red bar) (N=3). Retina following treatment of 10 mM PNU-282987 for one month with hypertonic injection (yellow bar) (N=3). Error bars represent SEM. *represents significance to internal control

Hypertonic Injections with PBS Treatment

To verify that the neuroprotective effects seen in this study were due to PNU-282987 and not the vehicle that the PNU-828987 was dissolved in; experiments were conducted using PBS drops instead of PNU-282987. The left eye of several rats was left as an untreated control and the right eye was subject to hypertonic injection to generate glaucoma-like conditions. PBS treatment was administered 3 days before hypertonic injections and for one month following hypertonic injections. Results from these experiments illustrated a significant difference between the two images due to the effects of the hypertonic injection (Figure 18A and 18B). Cell density was noticeably reduced in experimental eye with PBS drops and hypertonic saline

injections. There were similar morphological effects as those shown previously with only hypertonic injections. The axon fascicles were reduced in diameter and RGC density was also reduced. Defasciculation was also observed in eyes treated with PBS under glaucoma-like conditions. RGCs also had a prominent blebbed appearance in the plasma membrane. There was no clear neuroprotective effect of PBS when combined with hypertonic injections.

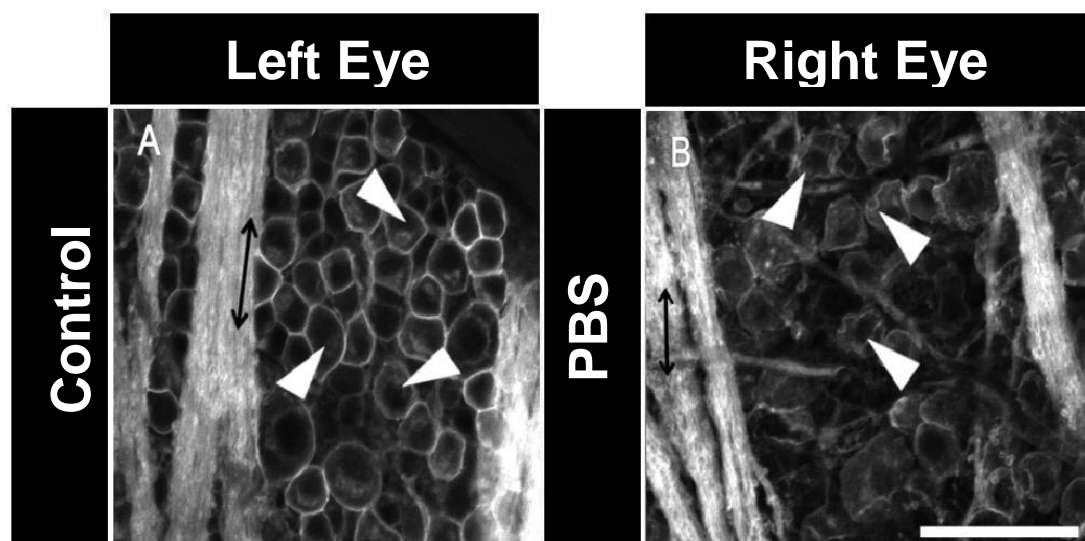


Figure 18

Hypertonic injections with PBS treatment in the right eye. (A) Untreated internal control with labeled RGCs using antibody against Thy1.1 in grayscale. (B) Retina following treatment of PBS drops for one month with hypertonic injection. Arrow heads point to RGCs. Double arrow indicates the axon fascicles. The images were obtained from the same animal. Scale bar represents 50 μm

The summary of these results is shown in Figure 19. There was an overall average decrease of RGCs by 16% (± 7.35 , $N=6$) in the right experimental eye compared to the internal control (Figure 19). This result was significant when compared to the left internal control.

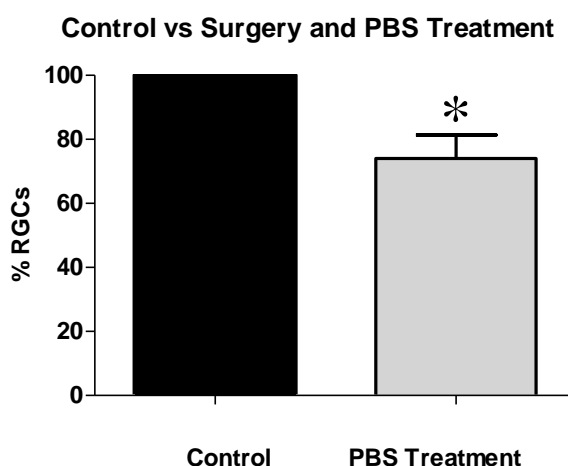


Figure 19

Hypertonic injections with PBS treatment. The bar graph displays the normalized RGC change from the internal control. Untreated internal control (black bar) (N=6). Retina following treatment of PBS drops for one month after hypertonic injection (grey bar) (N=6). The images were obtained from the same animal. Error bars represent SEM. *represents significance to the left eye.

Treatment of PNU-282987 with no Hypertonic Injections

To verify that PNU-282987 has no effect on the RGC counts by itself, 10 mM PNU-282987 was applied to the right eye with no hypertonic injections. Results were compared to the left untreated internal control and differences were recorded after one month of treatment. Results from fluorescent labeling experiments display similar appearance in both eyes. There are no apparent morphological differences in both eyes. Axon fascicles are organized and thick while RGCs are smooth in appearance. Cell densities were also similar in both control and 10 mM PNU-282987 treated retinas from the same animal. Figure 9 earlier in this study shows an example of the fluorescent labeling results.

These results are summarized in Figure 20. When the right eye was only

treated with 10 mM PNU-282987 drops, RGC counts were not significantly different, main axon fascicle diameter were not visibly different and there was no increase of axon defasciculation. The RGC counts between the right and left eyes under these conditions demonstrated an average difference of 5.3% (\pm 6.9, N=4) (Figure 20), which does not represent a significant difference in RGC counts between the two eyes.

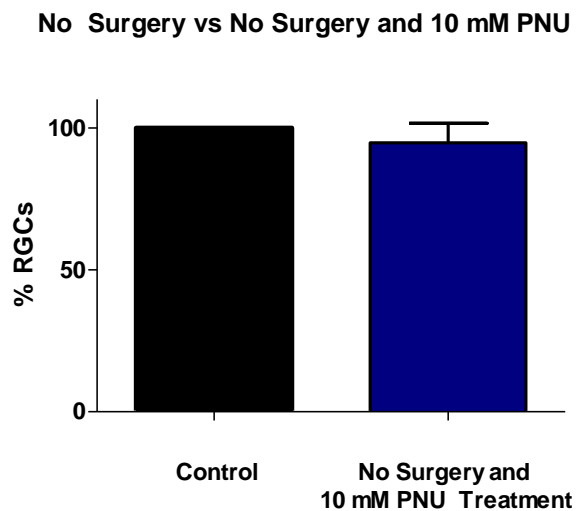


Figure 20

Comparison with no hypertonic injections with 10 mM PNU-282987 treatment in the right eye. Untreated internal control with labeled RGCs using antibody against Thy1.1 in grayscale (black bar) (N=4). Retina following treatment of 10 mM PNU-282987 drops for one month without hypertonic injection (blue bar) (N=4). Error bars represent SEM.

Hypertonic Injection with no Primary Antibody

To verify that our primary antibody Thy 1.1, is labeling the glycoprotein in RGCs, fluorescent labeling experiments omitting the primary antibody were conducted in a subset of rats. In Figure 21A, the left control eye was processed

normally for RGC staining. The right experimental eye underwent the procedure to induce glaucoma-like conditions while being treated with 10 mM PNU-282987 for 1 month. After one month, retinas were processed for RGC staining with (Figure 21A) and without (Figure 21B) using the monoclonal Thy 1.1 primary antibody. PBS was substituted for the primary antibody in these instances. Without the primary antibody, no fluorescence is observed (Figure 21B).

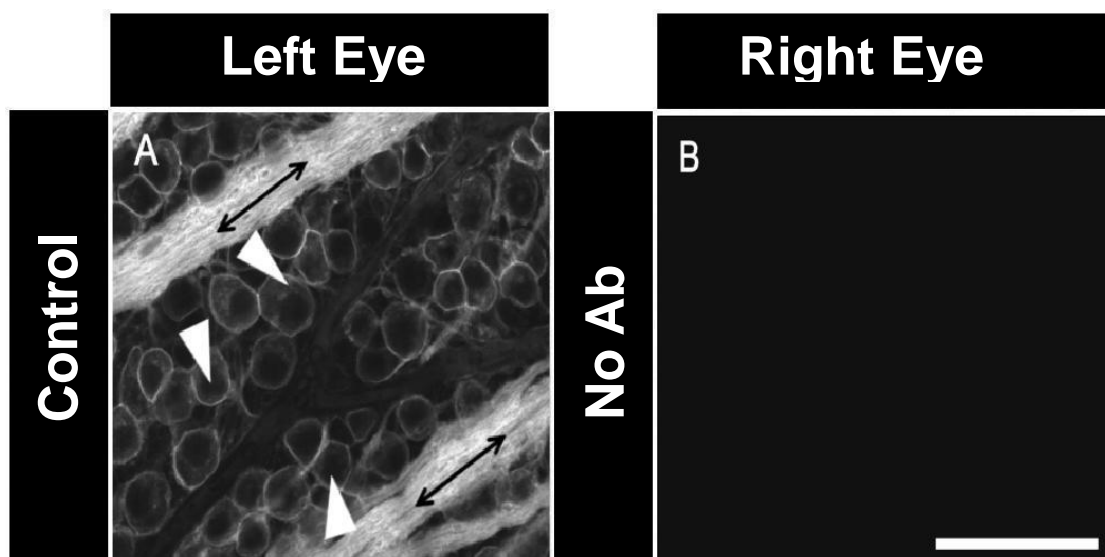


Figure 21

RGCs labeled with Thy 1.1 primary antibody Left internal control fluorescently labeled with thy 1.1 and right eye omitting thy 1.1 primary antibody in grayscale. (A) Untreated internal control with labeled RGCs using the Thy 1.1 primary antibody. (B) Retina following treatment of PNU-282987 after one month with hypertonic injection with no primary antibody. The images were obtained from the same animal. Scale bar indicates 50 μ m.

LC/MSMS

The next experiments were designed to verify that PNU-282987 reached the

retina when it was applied as eye drops, and to demonstrate that PNU-282987 could not be detected in heart tissue after eye drop application. LC/MSMS was performed on retina, plasma and heart samples removed from sacrificed Long Evans rats at various time points following eye drop applications using three different concentrations of PNU-282987. Specifically, retinas were removed from euthanized Long Evans rats 1, 2, 4, 8 and 12 hours after applying 100 μ M, 1 mM or 10 mM PNU-282987 directly to the rat eyes. Removed tissue was sent to the Michigan Innovation Center of Kalamazoo for LC/MSMS detection of PNU-282987. These time intervals corresponded to those that were done in the rabbit retina with the same compound (Linn^b et al., 2011).

Results from LC/MSMS studies demonstrated that all three doses of PNU-282987 reached the retina. Figure 22 demonstrates the results from LC/MSMS studies. The control bar measured from untreated retinal tissue indicates a low level of structures similar to PNU-282987, averaging 0.6 ngPNU/gm (\pm 0.15). PNU-282987 does not exist physiologically in the body, but the assay detected similar compounds that have a very similar chemical structure. The highest amount of PNU-282987 detected in the retina for any time interval was recorded at 2 hours. Two hours after application, LC/MSMS detected 2.1 ngPNU/gm (\pm 0.25) in the retina after 100 μ M eye drops were used, 3.35 ngPNU/gm (\pm 0.41) after 1 mM eye drops were applied and 3.40 ngPNU/gm (\pm 0.38) after 10 mM PNU eye drops were applied. Each of these values 2 hour calculations were significantly different from all other time intervals for each particular concentration. PNU-282987 detection is the lowest at 12 hrs.

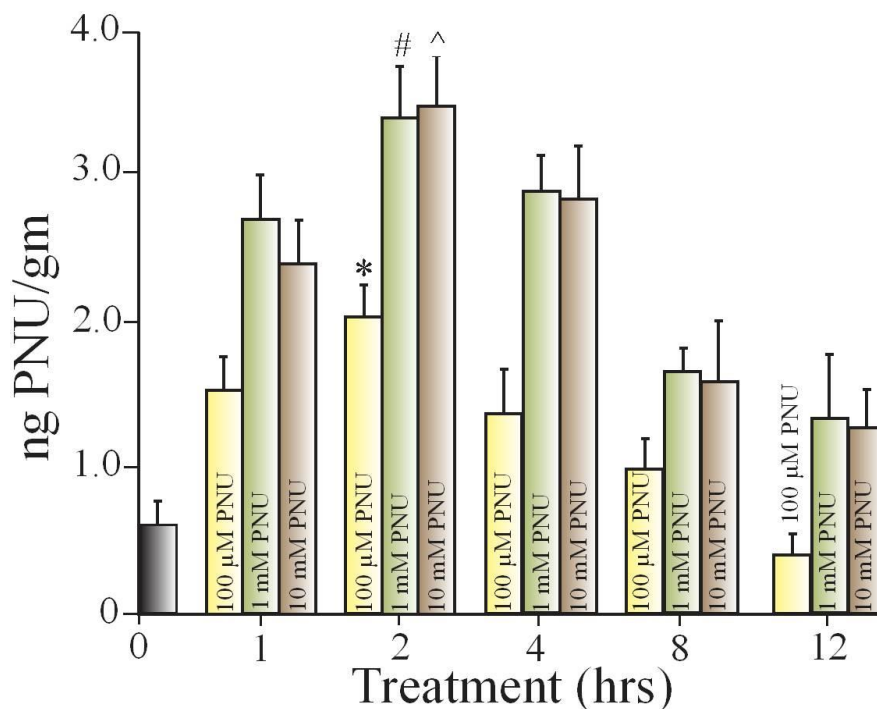


Figure 22

LC/MSMS results on the retina applying different concentrations of PNU-282987 for different amounts of time. The bar graphs demonstrate LC/MSMS results obtained from the Michigan Innovation Center of Kalamazoo on retinal tissue using different concentrations of PNU-282987. * represents significant differences of PNU-282987 detection when eyes were treated with 100 µM PNU-282987 for 2 hours compared to all other time points gathered using 100 µM PNU-282987. # represents significant differences of PNU-282987 detection when eyes were treated with 1 mM PNU-282987 for 2 hours compared to all other time points gathered using 1 mM PNU-282987. ^ represents significant differences of PNU-282987 detection when eyes were treated with 10 mM PNU-282987 for 2 hours compared to all other time points gathered using 10 mM PNU-282987. Each bar graph was generated from Ns of 3-6. Error bars represent SEM.

LC/MSMS experiments were also conducted on the vascular components of the rat to determine if traces of PNU-282987 were found in the plasma and heart tissues after eye drop application. Samples of retina, blood plasma, and heart tissue, (separated into atria and ventricle) were collected in lab and sent to the Kalamazoo Innovation Center for analysis after 10 mM PNU-282987 was applied as eye drops for various amounts of time. The results of these studies are shown in Figure 23. The

highest levels of PNU-282987 were measured in the retina when 10 mM PNU-282987 was applied for 2 hours and was followed by a steady decrease in detection up to 12 hours. Cooresponding to results shown in Figure 22. Significantly lower levels of PNU-282987 were detected in blood plasma, but levels in blood plasma peaked at 2 hours, similar to the peak measured in the retina. Two hours after eye drop application of 10 mM PNU-282987, only 0.35 ngPNU/gm (± 0.15) was detected in the blood plasma. No trace amounts of PNU-282987 were found in the heart tissue at any time points.

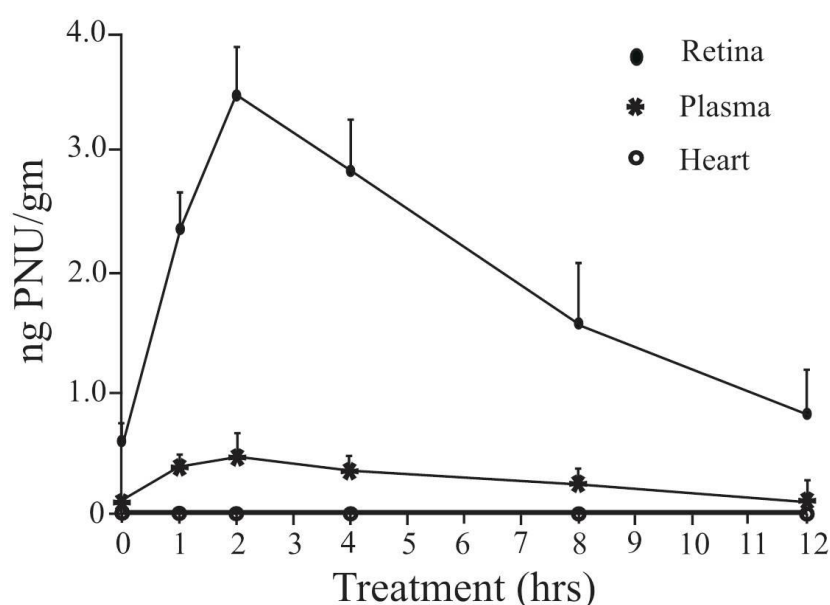


Figure 23

LC/MSMS results on the retina, blood plasma, and heart tissue using 10 mM PNU-282987 at different time points. The line graphs demonstrate LC/MS MS results obtained from the Kalamazoo Innovation Center on different tissues using PNU-282987. Each data point represents the average obtained under each condition. Each bar graph was generated from Ns of 3-6. Error bars represent SEM

DISCUSSION

In this thesis, I report on investigations of the possible neuroprotective effects of PNU-282987 administered as eye drops to prevent the loss of RGCs mediated through $\alpha 7$ nAChRs using an *in-vivo* rat glaucoma-like model. In this study, it was observed that an increase in IOP caused by injection of 2M NaCl into the episcleral veins significantly reduced RGC density in the periphery of the RGC layer after one month (26% RGC loss), and when PNU-282987 was applied as eye drops before and after the hypertonic saline injection, it reached the retina and prevented loss of RGCs due to hypertonic injections in a dose-dependent manner. For instance, 100 μ M eye drop concentration of PNU-282987 did not provide significant neuroprotection against loss of RGCs normally associated with the procedure to induce glaucoma, while 500 μ M eye drop concentration of PNU-282987 provided significant neuroprotection of RGCs in the retina (8.4% RGC loss), and 1 mM eye drop concentration of PNU-282987 provided near complete neuroprotection of RGCs in the retina (6.9% RGC loss). Surprisingly, it was also determined that 10 mM PNU-282987 eye drop application significantly increased the percentage of RGCs when compared to the control untreated eye (13.5% RGC gain). Overall, these results strongly support the hypothesis that eye drop application of the $\alpha 7$ nAChR agonist, PNU-282987, can prevent loss of RGCs associated with an increase in IOP in a dose-dependent manner.

It was also determined that the remaining RGC somata that survived hypertonic saline injections to induce glaucoma remained the same size even though many of the cells displayed a blebbed membrane appearance. The diameter of RGC

somata also did not change when treated with PNU-282987 before and after glaucoma-like conditions caused by injection of hypertonic saline. The diameter of main axon fascicles decreased under hypertonic saline injection conditions with no PNU-282987 treatment but remained the same diameter if treated with PNU-282987 before and after hypertonic injection to induce glaucoma-like conditions. In addition, analysis of defasciculation in the retina revealed that axons defasciculated off the main fascicles when eyes underwent hypertonic saline injections that generated a glaucoma model. However, this defasciculation did not occur if eyes were treated with PNU-282987 before and after the glaucoma-inducing procedures. Morphological analysis in control and glaucoma-like conditions in the retina are important to understand the possible manifestations of experimentally-induced glaucoma.

LC/MSMS studies demonstrated that when PNU-282987 was applied as eye drops; it can be detected and measured in the retina. This observation strongly supports the hypothesis that eye drop application of PNU-282987 can be used to prevent loss of RGCs associated with glaucoma-like conditions. LC/MSMS studies on the heart (atria and ventricles) and blood plasma indicated no detectable levels of PNU-289287 in the heart after eye drop application and relatively small amounts of were detected in the blood plasma.

Cell Death Caused by Over-stimulation of Receptors

It is important to note that there are many factors that may contribute to the manifestation of experimentally-induced glaucoma. A number of studies have argued

that cell death caused by over-stimulation of receptors play a role on the onset of glaucoma (Onley, 1969; Neal et al., 1994; Vickers et al., 1995; Kaushik, 2003), but different contributing mechanisms are also likely to play a role in the progression of the disease such as oxidative stress by reactive species, vascular dysregulation, cytoskeletal dysfunction, changes in growth factor levels, genetic contributions, and many other pathological pathways (Kuehn et al., 2005; Agarwal et al., 2009). Manifestation of glaucoma and other neurodegenerative diseases is unclear, but it is agreed that the primary risk factor associated with glaucoma is an increase in IOP (Gelatt et al., 1977; Morrison et al., 1997; Chauhan et al., 2002; Levkovitch-Verbin et al., 2002), leading to the degeneration of the optic nerve. The mechanisms behind this process seem to be pathologically multifactorial and synergize with each other. Although there are many contributing factors, the mechanisms for cell death caused by over-stimulation of receptors remain to be explored and understood during pathological manifestations.

This study draws many parallels with previous work in our laboratory. Previous *in-vitro* experiments conducted in the rat retina have demonstrated the effects of glutamate-induced cell death on isolated RGCs. Iwamoto (2011) demonstrated that when glutamate was applied to cultured rat RGCs resulted in significant cell loss. Loss of RGCs was also observed from glaucoma-like conditions generated by hypertonic saline injections and lends support to the hypothesis that cell death may be mediated by over-stimulation of receptors may also be involved in glaucoma-like conditions *in-vivo*.

Results from this study also are in agreement with the effectiveness of the hypertonic saline injection to produce glaucoma-like conditions that result in a

decrease of RGCs in the rat retina (Birkholz, 2011; Iwamoto, 2011) as well is in other laboratories (Morrison et al., 1997; Bouhenni et al., 2012). This consistency demonstrates that the methods initiated by Morrison et al. (1997) are appropriate to analyze RGC loss in an experimentally-induced model of glaucoma.

The mechanism of cell death due to glutamate on rat RGCs is still under investigation. Previous studies have shown that excessive amounts of glutamate in the eye leads to prolonged influx of cations, particularly calcium, in RGCs and causes cellular cascades that lead to apoptosis and loss of visual function (Quigley, 1998; Lam et al., 1999; Asomugha et al., 2010; Brandt et al., 2011). Previous studies from this lab have linked overstimulation of glutamate receptors in the pig retina to activation of the P38 MAPK pathway leading to apoptosis. Activation of this kinase was proposed to begin apoptotic mechanisms by blocking BCl₂, thereby inhibiting the anti-apoptotic role of BCl₂ (Asomugha et al., 2010). Similar investigations must be conducted in rat RGCs *in-vitro* to investigate the apoptotic pathway that may be activated during similar conditions as these mechanisms can differ between different models.

Many neurodegenerative diseases, including glaucoma, have been linked to cell death mediated by over-stimulation of receptors. A mechanistic understanding of cell death caused by over-stimulation of receptors in these diseases may lead to preventative and therapeutic measures to prevent neuropathy. There are many neurotransmitters found in the CNS that may contribute to homeostasis. When concentrations of these agents are disturbed, it can prolong or diminish the signaling from ligand-gated ionotropic receptors causing cell death mediated by apoptotic pathways (Dunnet, 1999; Doble, 1999; Page et al, 1999; Agarwal et al, 2009; Seidl

and Potashkin, 2011). Parkinson's disease, among many other neurodegenerative diseases, may be associated with cell death caused by over-stimulation of receptors as part of their pathological manifestation. Parkinson's disease is characterized by a selective loss of dopaminergic neurons which lead to inhibitory output to the thalamus caused by degeneration of the substantia nigra, resulting in motor dysfunction (Prezedborski, 2005). Substantia nigra cells contain NMDA receptors and receive input from the subthalamic nucleus in the form of glutamate. As substantia nigra neurons degenerate, the stimulation from the subthalamic nucleus is not restrained and further induces cell death in nigral cells, resulting in progressive neuronal degeneration (Simon et al., 2002). In Alzheimer's disease, cell death caused by over-stimulation of receptors may be manifested by an over abundance of β -amyloid peptides that follow a cellular cascade which ultimately results in stimulation of NMDA receptors, causing a high influx of calcium in the cell and leading to cell death (Miguel-Hidalgo et al., 2002).

The mechanisms involved with cell death caused by over-stimulation of receptors in other neurodegenerative diseases seem to be linked to glutamate-induced apoptosis mediated through ligand-gated NMDA receptors, parallel to glaucoma-like models (Manev et al., 1989; Aarts and Tymianski, 2004; Wang et al., 2010). In Alzheimer's disease some weak nonselective NMDA receptor antagonists have been shown to increase functionality (Gortelmeyer and Erbler, 1992). Other studies in Parkinson's disease have used similar antagonists on NMDA receptors such as procyclidine, amantadine, budipine, and memantine. These compounds have been shown to also increase functionality in individuals (Onley et al., 1987; Rabey et al., 1992; Jackisch et al., 1994), though no treatment can prevent cell loss entirely.

Future *in-vivo* studies in a rat glaucoma model need to include focus on mechanisms of cell death and neuroprotection associated with glaucoma-like conditions. A potential way to check that cell death mediated by over-stimulation of receptors is to use specific glutamate antagonists such as MK-810 that would target NMDA receptors. This may support that glutamate-induced cell death may be mediated by excessive abundance of glutamate through NMDA receptors. If RGC degeneration is inhibited, it would support previous studies that were done *in-vitro*. This could be repeated *in-vivo* to retain consistency and further support that glutamate-induced cell death is involved in glaucoma-like conditions. Another way to support previous *in-vitro* studies is to use ELISA techniques *in-vivo*. This could demonstrate the up or down regulation of apoptotic markers, analogous to the work done by Asomugha et al. (2010) that analyzed the apoptotic and cell survival pathways *in-vitro*. These results may draw parallels with previous work if performed *in-vivo* and give insights to possible mechanisms involved during cell death and neuroprotection during glaucoma-like conditions.

Neuroprotection

As previously mentioned, many neurodegenerative diseases have been associated with over-stimulation of receptors resulting in cell death. Neuroprotection against the over-stimulation of receptors has been used as a therapeutic approach in many neurodegenerative diseases, including glaucoma. In this study I analyzed the neuroprotective effect of PNU-282987, a $\alpha 7$ nAChR-specific agonist, to determine if it can prevent loss of RGCs caused by a glaucoma-inducing procedure. Results using

this particular agonist showed a dose-dependent response to prevent death of RGCs during glaucoma-like conditions. Increasing the concentration of PNU-282987 showed significant improvement in glaucoma-like conditions with every increasing dose. Previous *in-vitro* work in the pig and rat retina demonstrated that when nAChR agonists were applied, such as nicotine and ACh during glaucoma-like conditions, provided a protective effect to inhibit the degeneration of cultured RGCs (Wehrwein et al., 2004; Asomugha et al., 2010; Birkholz, 2011; Iwamoto, 2011). Other studies using a $\alpha 7$ nAChR antagonist, MLA, demonstrated that the neuroprotective effect was mediated through $\alpha 7$ nAChRs. This is consistent with previous *in-vivo* experiments using PNU-2827987. When PNU-282987 was injected intravitreally, it provided neuroprotective effects to prevent loss of RGCs during glaucoma-like conditions, similar to those in this study using eye drop application (Iwamoto, 2011).

Previous *in-vitro* studies from this laboratory have suggested mechanisms for neuroprotection against glutamate-induced RGC death in the pig retina. ELISA studies demonstrated that ACh activation of $\alpha 7$ nAChRs triggered phosphatidylinositol-3-kinase (P13). This activation of P13 phosphorylated Akt to activate BCl₂ and provided neuroprotective effects against glutamate-induced RGC death (Asomugha et al., 2010). The activation of this neuroprotective pathway was found to be initiated by calcium influx through $\alpha 7$ nAChR channels (Brandt et al., 2011). However, calcium influx through glutamate-activated NMDA receptors is also thought to be the main cause for RGC death in glaucoma models by over-stimulation of receptors. As discussed in Brandt et al. (2011), it was proposed that a preconditioning dose of calcium entering through the nAChRs triggered the neuroprotective pathways and preconditioned the cells against a subsequent larger

calcium influx. This preconditioning dose of calcium appears to be the main correlate of neuroprotective effects in these models.

The $\alpha 7$ nAChR is one of the main receptors that play a role in neuroprotection against neurodegenerative diseases in the brain (Conejero-Goldberg et al., 2008; Liu et al., 2012). Here, results are presented that support the hypothesis that $\alpha 7$ nAChRs are also involved in neuroprotection in a generated glaucoma model. However, does neuroprotection against RGC loss occur under physiological conditions in the retina?

Starburst amacrine cells are a type of amacrine cells that release ACh under normal conditions onto RGCs (Famiglietti, 1983; Massland, 1988; Grimes, 2011). It is possible that ACh release may be compromised under a glaucoma-like environment. This reduction in ACh may contribute to the loss of RGCs normally associated with glaucoma-like conditions. The justification for this proposition is that ACh has been found to have a neuroprotective effect in cultured cells (Wehrwein et al., 2004). If activation of AChRs has a neuroprotective role under physiological conditions, it should help to prevent cell death under glaucoma-like conditions that are mediated by over-stimulation of receptors. If the starburst amacrine cells are also lost under glaucoma-like conditions or if the release of ACh from them is compromised, it would unveil a previously unknown function of starburst amacrine cells in the retina. I propose that ACh release is reduced in glaucoma conditions. This remains to be determined. If true, neuroprotective role of ACh would be compromised in such a way that it induces loss of RGCs since there is an insufficient amount of ACh. In this study, activation of $\alpha 7$ nAChRs prevents loss of RGCs associated with glaucoma-like conditions in a rat glaucoma model, supporting the hypothesis that ACh levels may be compromised under glaucoma-like conditions. If future studies find that ACh has a

neuroprotective effect in the retina under normal conditions, it could greatly enhance our understanding of the retina and our appreciation of different ways that the retina naturally protects the RGC layer. Future work to investigate this possible function includes labeling of ACh with radiolabelled choline to assess the amount of the neurotransmitter in the retina during normal physiological conditions and glaucoma-like conditions. Once ACh is labeled, perfusates could be collected from control eyecups in response to light and from glaucoma-like eyecups in response to light. The amount of ACh released under each condition could be compared. If ACh content is reduced during glaucoma like conditions, it may be that starburst amacrine cells have compromised ACh release, since they are one of the few neurons in the retina known to release ACh. This would support that RGC cells may be receiving neuroprotective input from the starburst amacrine cells under normal physiological conditions.

Increased RGC Density Using PNU-282987

This particular study had unprecedented results using 10 mM PNU-282987 on a glaucoma-like model. This model demonstrated dose-dependent results using PNU-282987. The lowest dose of 100 μ M PNU-282987 had no significant effects, while 500 μ M PNU-282987 and 1mM PNU-383987 had visible neuroprotective effects leading to enhanced RGC survival when compared to the internal control. However, when 10 mM PNU-282987 was applied as eye drops, RGC counts increased by an average of 13.5% (\pm 4.19; N=5) when compared to the internal control. This was a surprising result, as adult retinal neurons do not typically divide.

This increase in cell density had been observed in previous work using PNU-

282987 and nicotine administration. In these *in-vivo* rat studies, cells in the RGC layer were stained using cresyl violet staining which allowed visualization of all cell bodies in the RGC layer (Birkholz, 2011 and Iwamoto 2011). However, as cresyl violet stains the nuclei of all cells in the RGC layer, it was uncertain if the increase in cell counts were due to an increase of RGCs. However, in this thesis, only RGCs were labeled by using the specific Thy 1.1 antibody. This suggests that the cell increase observed in the cresyl violet stained retinas may be due to an increase of differentiated RGCs.

The reason behind this apparent paradox remains unknown. There are many possible explanations for this outcome including, but not limited to; an increased expression of glycoprotein Thy 1.1 in other neurons due to unknown molecular factors, relatively high doses of PNU-282987 could cause mitotic dysfunction resulting in cancerous effects, or the dose of PNU-282987 used could cause stimulation of neuronal growth in the retina. This phenomenon was an unexpected and drawing any detailed conclusions would be premature. Studies are now investigating this perplexing, but exciting, observation. The work begun to assess this increase in RGCs includes double labeling experiments to ensure that the Thy 1.1 antibody is in sync with a different RGC marker. Other potential work to investigate increased RGC density includes using cell death markers, such as caspases-3 antibodies or TUNEL assays that label apoptotic cells. By labeling cells with cell death markers, we can gain an insight into what is happening in the glaucoma model used in our laboratory. A key future study is to use fluorescent staining against cell proliferating markers. PCNA (proliferating cell nuclear antigen) and BrdU (Bromodeoxyuridine) have begun to be used to examine the cellular proliferation that could be occurring.

Neural Morphology

Axon fascicle characterizations in the rat retina during glaucoma-like conditions have yet to be investigated and characterized using antibody against Thy 1.1. Previous work from our laboratory has not investigated the retinal organization using this particular method to label RGCs. By analyzing this morphology during normal and glaucoma-like conditions we may provide insight into the manifestation of experimentally-induced glaucoma. Results from this study have shown that RGC axon fascicle diameter changes under different conditions during a glaucoma-like environment. This study has shown the dose-dependent response of PNU-282987 under glaucoma-like conditions induced by injections of hypertonic saline. This study shows that RGC axon fascicle diameter decreases when injected with hypertonic saline. This decrease in diameter supports the observation that RGC density decreases during glaucoma-like conditions. If RGCs are degenerating during glaucoma-like conditions, their corresponding axons filaments would also subsequently degenerate, decreasing the diameter of the fascicle, as expected. Similar results have been shown to display a marked correlation between RGC death and axon degeneration (Trip et al., 2005; Soto et al., 2011; Kalesnykas et al., 2012). However, when a dose of 10 mM PNU-282987 was used, the degree of degeneration in axon fascicle diameter decreased. In glaucoma-like conditions, RGC axon fascicle diameter decreased 25% when compared to the diameter of axon fascicles in the internal control of the same animals. When 10 mM PNU-282987 was used, axon fascicle diameter did not demonstrate significant differences when compared to the internal controls of the

same animals. This parallel between axon fascicle diameter and RGC counts support the effects of the glaucoma-inducing procedure and the neuroprotective effects of PNU-282987. Characterization of RGC axon fascicle diameter has never been characterized in the rat retina during glaucoma-like conditions using Thy 1.1. The diameter of axon fascicles 400 μm from the ONH ranged between 16-22 μm under normal physiological conditions. Axon fascicle diameter changes 1 month following hypertonic injections to induce glaucoma because RGC degeneration is the highest at this point before retinal detachment is observed (Morrison et al., 1997). Loss of RGC components include the loss of RGC axons, which make up the main axon fascicles that make up the optic nerve. Variations in this diameter can fluctuate significantly as measurements can increase towards the ONH and decrease away from the ONH. However, measurements were only made 400 μm from the ONH. Future studies need to be conducted to get an accurate representation of the RGC axon fascicle diameter in the rat retina at different distances from the ONH.

The change in axon fascicle diameter is consistent with other studies that characterize the composition of the optic nerve during experimental glaucoma. Various manipulations and techniques to induce glaucoma-like conditions have shown that optic nerve damage is visibly seen under experimental glaucoma models (Quigley et al., 1987; Fetchner and Weinreb, 1994; Mabuchi et al., 2004). Although this study did not include optic nerve histology, as it only focused on activity in the retina, the degeneration of the RGCs and the loss of corresponding axons are parallel to damages that are seen in the optic nerve showing a marked decrease in optic nerve filaments and axonal death (Quigley et al., 1987; Fetchner and Weinreb, 1994; Mabuchi et al., 2004).

In the retina, defasciculation from the large axon fascicle bundles was also observed after hypertonic saline injections to induce glaucoma-like conditions. Defasciculation during glaucoma like conditions using PNU-282987 resulted in less defasciculation from main axon fascicles. The increase of defasciculation during glaucoma-like conditions and prevention of this process using PNU-282987 treatment corresponds to the decrease in axon fascicle diameter and then recovery of the axon fascicle diameter. This relationship has not been previously characterized and suggests that when RGCs are lost, or in the process of degenerating, there is a more complex cascade of damage that extends to the optic nerve than previously expected. When RGCs are lost, the main axon fascicle diameter decreases. This could be due to both the axonal defasciculation and death and subsequent loss of axons that is occurring at the RGC soma level. Different studies have observed defasciculation in the optic nerve during glaucoma-like conditions that also occurs with RGC loss and degeneration of optic nerve myelin (Fu and Sretavan, 2010; Soto et al., 2011). This observation may tie to other mechanisms that may be involved with the onset of experimentally-induced glaucoma. Onset of axon degeneration may be an early event during the manifestation of glaucoma and could be associated with degeneration of supporting proteins that bundle axon fascicles (Fu and Sretevan, 2010). Additionally, this correlation may be associated with myelin-degenerating diseases such as multiple sclerosis (Fu and Sretevan, 2010). Defasciculation of RGC axons and optic nerve may play a very important role in the manifestation of glaucoma that is currently not well investigated.

Among the fascicular changes in the retina during glaucoma-like conditions, morphological differences were also observed in the RGC somata. Blebbing of the

RGC membrane after hypertonic saline injections was also observed in this study. The blebbing of the RGC membrane was diminished as increasing doses of PNU-282987 was administered before and after hypertonic injections. Blebbing is defined as protrusions of the cell membrane (Charras, 2008) and can manifest in a number of instances including apoptosis (Mills et al., 1999 and Coleman et al., 2001). Blebbing of RGCs may demonstrate the execution phase of apoptosis during glaucoma-like conditions generated by injection of hypertonic saline and may support that RGC loss may be mediated by apoptosis associated in experimentally-induced glaucoma (WoldeMussie et al., 2001; Levkovitch-Verbin et al., 2002). This would support current proposed mechanisms that associate the manifestation of experimentally-induced glaucoma with apoptosis (Kerrigan et al., 1997; Quigley, 1999).

It is proposed that soma size decreases under apoptotic events (Kerr et al., 1972). With this knowledge in mind, it has been reported that although RGCs are the main cell type susceptible in glaucoma-like conditions, larger RGCs with larger axon diameters seem to degenerate before any other RGC type in human, primate, feline, and other animal glaucoma models (Kalensnykas et al., 2012). RGC characterizations during glaucoma-like manipulations have not been well investigated or understood. From this study, it was shown that the diameter of the remaining RGC somata remained consistent throughout different manipulations. However, there was a blebbed morphology observed in the plasma membrane of RGCs one month following hypertonic saline injections to induce glaucoma. Although RGC diameters remained consistent in various manipulations it is important to note that RGCs have already gone through apoptosis and can't be counted as apoptotic remnants would have been phagocytized. The blebbing in the remaining cells might indicate the

beginning of the onset of apoptosis in remaining cells.

Initially, I expected to find a decrease in RGC diameter during glaucoma-like conditions due to the glaucoma-like conditions existing in the eye. However, the results of this study 1 month after hypertonic injections demonstrate similar RGC soma diameters when compared to the internal controls. Similar results characterizing RGC soma size under glaucoma-like conditions has also been shown in other studies demonstrating no change in RGC soma size (Pavlidis et al., 2003). Although similar results have been achieved, different studies have also shown that there seems to be a discrepancy in the characterization of RGC soma size under experimental glaucoma models. Studies have shown an increase in RGC soma size (Ahmed et al., 2003) and decrease in RGC soma size (Jacobs et al., 2005; Kalesnykas et al., 2012) in glaucoma models. It is likely that the different results obtained from different researchers correspond to time tissue is sacrificed and processed. If tissue is processed early in the apoptotic process, more cells may appear smaller if they haven't been phagocytized. On the other hand, cells may appear to be similar in size if all cells that are destined to go through apoptosis have done so already before processing the tissue. Under this scenario, the remaining cells would likely not be affected.

Applications

In this study, the neuroprotective effect of the $\alpha 7$ nAChR agonist, PNU-282987, has been tested to show that it inhibits the loss of RGCs in glaucoma-like conditions in a dose-dependent manner. It was observed that in order to prevent the degeneration of RGCs, a pretreatment of PNU-282987 was necessary to provide

neuroprotective effects against a glaucomatous environment. No neuroprotection occurs if an agent was given at the same time or after the glutamate insult to prevent loss of RGCs (Wehrwein et al, 2004; Iwamoto et al., 2011). Clinically, this introduces a preventative approach to avoid the onset of glaucoma in patients that might be susceptible to the disease based on genetic probability, race and/or family history. To date, all glaucoma treatments are designed to decrease IOP measurements (Cairns, 1968; Damji et al., 2003). The results from this study could change the way that glaucoma may be treated as PNU-282987 treats the disease at the retina level instead of in the anterior chamber of the eye. Previous work by Iwamoto et al., (2011) used intravitreal injections of PNU-282987 to deliver the compound directly into the eye. This was an invasive method and was unappealing for therapeutic development. The application of PNU-282987 by eye drops used in this thesis provides greater appeal for preventative treatment and potential development for glaucoma patients.

A primary concern for these new neuroprotective compounds in the nervous system is their potentially harmful effects to other tissue. Introduction of these compounds may have hazardous effects on other systems in the body as there are $\alpha 7$ nAChRs in many organs throughout the body. PNU-282987 was initially developed by Pharmacia and Upjohn to treat Schizophrenia. However, its use was discontinued to treat Schizophrenia as systemic application reached the vascular system and the heart. In the heart, PNU-282987 was found to inhibit a potassium channel thus diminishing its development to treat the disease.

To circumvent this issue, PNU-282987 was applied as eye drops in this study and was not applied systemically. As a result, it seemed likely that PNU-282987 would not reach the heart and initiate damage. To confirm this, LC/MSMS results

demonstrated that PNU-282987 levels were detected and measureable in the rat retina when the agent was applied as eye drops and remained detectable for at least 12 hrs. Delivery of PNU-282987 to the retina by topical eye drops has also been detected in the rabbit retina and shown to stay in the eye for at least 8 hrs (Linn et al., 2011). This is more than enough time to trigger cell survival pathways (Asomugha et al., 2010). In addition, relatively low levels of PNU-282987 were detected in blood plasma and no PNU-282987 was detectable in the atria or ventricles of the heart. As a result, application of PNU-282987 may be developed as a pretreatment for diseases where it can be applied topically.

Implications and Future Studies

The main purpose of this study was to test the hypothesis that the $\alpha 7$ nAChR agonist, PNU-282987, had a neuroprotective effect against RGC loss in an experimentally-induced glaucoma rat model. Results demonstrated that RGCs had a significant survival rate under glaucoma-like conditions if rats were treated with PNU-282987 for 3 days before the hypertonic injections and after one month following hypertonic injections to induce glaucoma-like conditions. The mechanism behind this neuroprotective effect remains unknown, but one hypothesis based on previous *in-vitro* studies is that overstimulation of glutamate receptors results in apoptosis under glaucoma-like conditions. The same cellular response could be occurring in the rat model in response to over stimulation of glutamate receptors, but future studies are needed to confirm this. Similarly, neuroprotective pathways using ACh and respective agonists could trigger P-I3 kinase to begin an intracellular

cascade to promote cell survival (Alvarez et al., 2009 and Asomugha 2010). Other peptides, such as brain-derived neurotrophic factor, have also been shown to also activate the P13 kinase pathway to prevent death of RGCs (Nakazawa et al., 2002). This cell survival pathway by P13 kinase has been linked to calcium influx that is mediated through nAChRs to protect pig RGCs *in-vitro* (Brandt et al., 2011). I propose that preconditioning of a relatively low amount of calcium, by activation of the $\alpha 7$ nAChR agonist, PNU-282987; prior to hypertonic saline injection can provide neuroprotective effects by activating an intracellular survival pathway. This cell survival pathway may be similar to the P13 kinase pathway that is shown in the porcine model. I propose that similar pathways leading to apoptotic events could be similar as the pathways identified in the porcine model under conditions using hypertonic saline injections to mimic glaucoma-like conditions. These inferences are untested at present. However in the *in-vivo* rat model and future studies must be shown to determine the molecular mechanisms in the rat model that are responsible for RGC death and survival under glaucoma-like conditions.

An array of analyses was conducted to characterize the retinal components in a glaucoma-like rat model. Analysis looking at RGC axon fascicle diameter matched up with predictions. Diameter from main axon fascicles 400 μm from the ONH decreased under glaucoma-like conditions. This decrease in diameter was significantly reduced if eyes were treated with 10 mM PNU-282987 before and after hypertonic saline injections to generate a glaucoma model. Results from this study also indicated defasciculation from the main axon fascicles under glaucoma-like conditions increased from internal control conditions and was directly correlated with a decrease in axon fascicle diameter, but this decrease was less prevalent when treated

with PNU-282987. RGC soma size was expected to decrease under glaucoma-like conditions and remain a normal size when treated with PNU-282987 compared to their internal controls. Instead, we found that RGC soma size was virtually the same before and after the procedure to induce glaucoma. This result could be due to apoptotic cells already having been phagocytized. Additionally, it was observed that RGCs had a blebbed appearance during glaucoma-like conditions. Blebbing is common during apoptotic processes and gives an insight to what could be mechanistically occurring during glaucoma-like conditions in the retina.

From previous studies it has been shown that ACh provides neuroprotective effects in isolated pig RGCs. The hypothesis first introduced by Iwamoto (2011) remains at question if ACh release from amacrine cells is compromised during glaucoma-like conditions. Studies have demonstrated that RGCs are the main target in cell loss in glaucoma. However, a subset of cholinergic starburst amacrine cells are displaced amacrine cells that are also found in the RGC layer and may be affected by glaucomatous conditions. If ACh does in fact provide neuroprotective effects in normal physiological states, it may be that ACh release is compromised in glaucoma. Almasieh et al. (2010) has demonstrated the effects of acetyl cholinesterase (AChE) using galantamine, an AChE inhibitor, to provide neuroprotective effects in glaucoma models. This further supports the hypothesis that ACh levels may decrease in glaucoma. It could also be noted that the introduction of hypertonic saline to produce glaucoma-like conditions may be altering the amount of affinity of the receptors that are present on RGCs that may compromise the neuroprotective role of ACh. There are other propositions on why normal neuroprotective effects that occur physiologically are compromised. One of these propositions include that this compromise of ACh

release may be due to other outside factors that are indirectly effecting the normal release of ACh from amacrine cells. Another possibility includes the internalization of cholinergic receptors may be occurring due to the conditions that exist in glaucoma. This internalization of receptors may ultimately be affecting the effects of ACh on RGCs during glaucoma-like conditions (Shen et al., 2010). Future studies must be conducted to investigate the roll of ACh in glaucoma and determine its effect.

PNU-282987 has been shown to have a neuroprotective effect on RGCs when subject to hypertonic injection of saline, but the functionality of the surviving RGCs remains unknown. Although these cells remain visible under the microscope, it is important to verify that these neurons are still capable of producing electrical currents using electrophysiology techniques to assure that there are no changes in ion channel activity in response to the administration of PNU-282987. Following these electrophysiology studies, behavioral studies may be conducted to confirm if there are any changes in visual acuity, contrast detection or directional selectivity in the rat model. These behavioral studies will further the appeal the use of $\alpha 7$ nAChR agonists for therapeutic uses in the mammalian retina.

Using 10 mM PNU-282987 caused a significant increase in RGC counts when compared to the other doses that were administered. This unprecedented phenomenon was also observed by Birkholz (2011) and Iwamoto (2011) from this laboratory. Many studies must be done to fully understand these observations.

PNU-282987 was initially used to treat schizophrenia and deemed to be unsafe due to traces of the compound effecting ion channels in the heart. The introduction of this compound in the visual system has been successful and demonstrated that it can prevent loss of RGCs during experimental glaucoma. LC/MSMS studies indicate that

PNU-282987 reaches the retina when applied as eye drops, similar to results in the rabbit retina (Linn et al., 2011). Traces of PNU-282987 were not found in the heart and only relatively small traces were found in the blood plasma. Longer timelines must be developed to ensure that PNU-282987 administration will not have similar results as the trials conducted in schizophrenia models.

The results of PNU-282987 in culture studies and *in-vivo* have shown to have promising effects in preventing nerve damage associated with experimentally-induced glaucoma models. The use of PNU-282987 may be applicable in medical settings to prevent RGC loss typically associated with glaucoma. Long term use of this agent may have potential to be used as a preventative treatment for individuals that may be susceptible to RGC degeneration in ocular neuropathies.

In conclusion, the data presented in this study demonstrate the potential of $\alpha 7$ nAChR agonists, including PNU-282987, to significantly prevent the loss of RGCs associated with experimental glaucoma and possibly with glutamate-induced cell death. Results from this study also support the potential of nAChRs to be used as a target in other neurodegenerative diseases, including glaucoma, to decrease the probability of neuronal dysfunction.

APPENDIX – IACUC APPROVAL FORM

WESTERN MICHIGAN UNIVERSITY

Institutional Animal Care and Use Committee

ANNUAL REVIEW OF VERTEBRATE ANIMAL USE

PROJECT OR COURSE TITLE: Investigation Of Acetylcholine In An In-Vivo Model Of Glaucoma.

IACUC Protocol Number: 12-03-03

Date of Review Request: 03/01/13

Date of Last Approval: 03/01/2012

Purpose of project (select one): ☐ Teaching ☒ Research ☐ Other (specify):

PRINCIPAL INVESTIGATOR OR ADVISOR

Name: Cindy Linn

Title: Professor

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CO-PRINCIPAL OR STUDENT INVESTIGATOR

Name: Cynthia Gossman

Title: Student

Department: BIO

Electronic Mail Address: cynthia.gossman@wmich.edu

1. The research, as approved by the IACUC, is completed:

☐ Yes (Continue with items 4-5 below.)

☒ No (Continue with items 2-5 below.)

If the answer to any of the following questions (items 2-4) is "Yes," please provide a detailed explanation on an attached sheet of paper. Include details of any modifications made to the protocol based on new findings or publications, adverse events or mortalities.

2. Have there been any changes in Principal or Co-Principal Investigators? ☐ Yes ☒ No

3. Have there been any new findings or publications relative to this research? ☒ Yes ☐ No

Describe the sources used to determine the availability of new findings or publications:

☐ No search conducted (Please provide a justification on an attached sheet.)

☐ Animal Welfare Information Center (AWIC)

☒ Search of literature databases (select all applicable)

☐ AGRICOLA

☐ Current Research Information Service (CRIS)

☒ Biological Abstracts

☒ Medline

☒ Other (please specify): Annual conventions

Date of search: 02/13/13

Years covered by the search: 30

Key words: Neuroprotection, glaucoma, excitotoxicity, retinal ganglion cells

☐ Additional search strategy narrative:

4. Are there any adverse events, in terms of animal well being, or mortalities to report as a result of this research? ☐ Yes ☒ No

Cumulative number of mortalities: 0

5. Animal usage:

Number of animals used during this quarter (3 months): 50

Cumulative number of animals used to date: 150

1. Cindy Linn

Principal Investigator/Faculty Advisor Signature

2/18/13
Date

1. Cynthia Gossman

Co-Principal or Student Investigator Signature

2/20/13
Date

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