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Mechanisms of Neuroprotection Against Retinal Ganglion Cell Loss Using an *in vivo* Glaucoma Model

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ABSTRACT

Glaucoma is an incurable ocular disease characterized by apoptotic cell death and degeneration of retinal ganglion cells (RGCs), and is one of the leading causes of blindness worldwide (Quigley and Broman, 2006; Quigley et al., 1995). While current treatments are effective at slowing vision loss, glaucoma’s multifactorial etiology has made it difficult for researchers to identify a target for treatment that will permanently halt RGC degeneration. Previous studies have proposed a glutamate-induced excitotoxic mechanism of RGC death in glaucoma, and neuroprotective agents have been investigated to prevent this effect. One agent, acetylcholine, has been shown to initiate pro-survival signaling cascades by binding to $\alpha_7$ nicotinic acetylcholine receptors (nAChRs) on RGCs, and prevents glutamate excitotoxicity when applied prior to glutamate insult.

PNU-282987, a drug developed by Pharmacia and Upjohn, is a specific $\alpha_7$ nAChR agonist that has been shown to prevent RGC death in an in vivo rat model of glaucoma in a dose-dependent manner. In this study, immunofluorescent staining techniques were utilized to investigate the effects of 2mM PNU-282987 on RGC survival when applied as an eye drop for varying amounts of time before performing a procedure to elicit glaucoma-like conditions in rats. In addition, experiments investigating the involvement of caspase-3 in the apoptotic mechanism of the in vivo glaucoma model were conducted. It was determined that 2mM PNU-282987 provided a neuroprotective effect against RGC death when applied to eyes for short periods of time prior to the onset of glaucoma-like conditions, but also resulted in apoptotic and proliferative effects when applied for extended periods of time. Additionally, it was found that caspase-3 was involved in the apoptotic mechanism associated with the in vivo model of
glaucoma. The results from these studies suggest that PNU-282987 may be a valuable preventative treatment option for individuals susceptible to glaucoma development.

INTRODUCTION

Eye Anatomy and Physiology

In order to understand the mechanism of retinal damage associated with glaucoma, a basic understanding of human eye anatomy is required (Figure 1). The whole eye is enclosed by three separate layers of tissue, and the interior of the eye is divided into an anterior chamber filled with fluid called aqueous humour, and a posterior chamber filled with fluid called vitreous humour. The sclera is the most superficial layer of tissue that composes the “white” of the eye, and consists of a dense collagen fiber network to provide protection and support for the eye’s internal components (Schwartz, 1999). On the anterior surface of the eye rests a transparent tissue continuous with the sclera called the cornea. The cornea’s transparency is a result of its lattice-like organization of collagen fibers and allows photons of light from the environment to enter the eye (Lens et al., 1999).
Figure 1

Anatomy of the human eye. (Image adapted from Virtual Medical Centre at http://www.virtualmedicalcentre.com)

Beneath the sclera is the uvea composed of the choroid, ciliary body, and iris. The choroid consists of a large vascular network which nourishes the retina, as well as dark melanin pigmentation to prevent the scattering of photons that are not absorbed by the retina (Durairaj et al., 2012; Lens et al., 1999). The anterior portion of the choroid forms a specialized structure called the ciliary body. The ciliary body contains smooth muscle and is attached to the crystalline lens of the eye through suspensory ligaments. Contraction of this muscle allows the shape of the lens to be adjusted and focus light entering the eye (Schwartz, 1999). In addition, the ciliary body continuously produces aqueous humour in the anterior chamber of the eye, which nourishes the cornea and lens (Records, 1979). The most anterior portion of the uvea is the iris which surrounds a central opening called the pupil. The iris also contains smooth muscle, and its contraction allows the size of the pupil to be altered in order to mediate the amount of light entering the eye (Schwartz, 1999).
The innermost tissue layer, located at the back of the eye, is the retina. The retina is a complex ocular tissue consisting of multiple cell layers including photoreceptors (rods and cones), horizontal cells, bipolar cells, amacrine cells, and retinal ganglion cells (RGCs) (Figure 2). While RGCs are the primary focus of this thesis, all the cells of the retina play a key role in visual perception. The retina is ultimately responsible for converting photons of light from visual stimuli into recognizable electrical signals that are sent to the brain through a process called phototransduction.

**Figure 2**

Anatomy of the human retina. Photons of light pass through five cellular layers upon coming in contact with the retina. From anterior to posterior these layers are: retinal ganglion cell layer, amacrine cell layer, bipolar cell layer, horizontal cell layer, and photoreceptor cell layer consisting of rods and cones. (Image adapted from WebVision at http://webvision.med.utah.edu/imageswv/schem)
The first step of phototransduction occurs as photons of light pass through the retinal layers and activates the pigment rhodopsin found in photoreceptor cells. Photoactivated rhodopsin stimulates the GTP-binding protein transducin to activate cGMP phosphodiesterase enzymes that hydrolyze cGMP found in the cytoplasm of photoreceptor cells (Schwartz, 1999; Shastry, 1997). This hydrolysis results in a reduction of cGMP concentration, closure of cGMP-dependent cation channels, and hyperpolarization of the photoreceptor cell.

During dark conditions with no photoactivated rhodopsin present, photoreceptors are in a slightly depolarized state and continuously release the neurotransmitter glutamate at their synapses with bipolar cells (Records, 1979; Schwartz 1999). Upon hyperpolarization, however, the amount of glutamate they release is reduced leading to a variety of effects on postsynaptic bipolar cells depending on which receptors they express. OFF-bipolar cells contain ionotropic glutamate receptors and experience hyperpolarization in response to decreased glutamate exposure (Hack et al., 1999). Conversely, ON-bipolar cells express metabotropic glutamate receptors and depolarize when synaptic glutamate levels fall. This depolarization leads to the generation of an electrical signal that is then transmitted to RGCs (Baylor and Fettiplace, 1976; Hack et al., 1999; Massey and Miller, 1987).

Bipolar cells transduce their electrical signals to RGCs by releasing glutamate at their synapses in response to depolarization (Baylor and Fettiplace, 1976; Massey and Miller, 1987). This glutamate binds primarily to excitatory kainate receptors on RGCs leading to their depolarization and furthering the transmission of the electrical signal (Massey and Miller, 1987). RGC axons converge and form the optic nerve which exits through the posterior region of the eye known as the optic nerve head (Lens et al., 1999). This optic nerve eventually converges in the brain at the lateral geniculate nucleus (LGN) of the thalamus. The LGN conveys signals from
the optic nerve to the visual cortex of the occipital lobe where visual processing occurs and results in the conscious perception of sight (Zeman, 1998).

In addition to bipolar cells, specialized cholinergic starburst amacrine cells also synapse with RGCs in the retina. These cells release acetylcholine onto RGCs and are thought to play a role in the directional selectivity of their signal transmission in response to visual stimuli movement (Chalupa and Werner, 2004). In this thesis, the neuroprotective effects of an acetylcholine agonist have been demonstrated in glaucoma-like conditions suggesting another role for starburst amacrine cells. Further investigation into the pathogenesis of glaucoma may reveal that interference with the functionality of starburst amacrine cells under glaucomatous conditions could compromise the neuroprotective effect of naturally occurring acetylcholine in the retina.

**Glaucoma and Excitotoxicity**

Individuals with glaucoma slowly lose vision as RGCs degenerate and die. While the direct cause of RGC loss is not known, elevated intraocular pressure (IOP) is often exhibited in glaucoma patients. IOP is thought to cause RGC death by introducing excitotoxicity and mechanical stress, as well as interfering with anterograde and retrograde axoplasmic flow necessary for survival (Anderson and Hendrickson, 2013; Quigley, 2011). Currently, all medications available to treat glaucoma aim to reduce IOP levels. However, while reduction of IOP can slow the rate of RGC degeneration, it is insufficient to completely halt RGC death in glaucoma once it has begun. In addition, some patients with glaucoma never exhibit elevated IOP levels to begin with. As a result, researchers have investigated other possible mechanisms of damage that could be responsible for RGC death.
It has been found that individuals suffering from glaucoma exhibit increased levels of the excitatory neurotransmitter glutamate in their vitreous humour leading researchers to investigate a possible excitotoxic mechanism of RGC death. (Lam et al., 1999; Quigley, 2011). Excitotoxicity refers to the apoptotic death of neurons that results from overexposure to an excitatory neurotransmitter leading to their prolonged stimulation. Previous studies have found that exposure of cultured porcine retinas to 500 µM of glutamate for three days lead to a 42 ± 5% decrease in RGCs (Wehrwein et al., 2004). In addition, it was found that this excitotoxic effect occurs through the binding of specific NMDA and non-NMDA glutamate receptors present on RGCs. Under normal conditions, glutamate binds to both types of receptors on the ganglion cell surface and opens ionotropic channels that allow cations necessary for proper functioning to enter the cell. Under excitotoxic conditions, however, chronic and excessive exposure to glutamate leads to the prolonged influx of cations and results in apoptosis. The mechanism leading to apoptosis in this system involves an excessive influx of calcium that has been shown to activate a mitogen-activated protein kinase (MAPK) mediated apoptotic signaling cascade (Asomugha et al., 2010).

MAPK signaling cascades are highly conserved in eukaryotes and regulate a variety of cellular functions including differentiation, movement, mitotic division, and apoptosis (Schaeffer and Weber, 1999). Typically, these cascades occur via sequential phosphorylation and activation of enzymes specific to different cellular pathways starting with the activation of a MAPK kinase kinase in response to some stimuli (Schaeffer and Weber, 1999; Zarubin and Han, 2005). MAPK kinase kinase phosphorylates a MAPK kinase enzyme, which then proceeds to activate one of three classes of MAPKs. Extracellular signal-regulated MAPKs are generally involved in cell differentiation and growth, while c-Jun N-terminal (JNK) and p38 MAPKs play a role in
inflammatory responses and apoptosis (Schaeffer and Weber, 1999; Zarubin and Han, 2005). In both human and rodent glaucomatous retinas, an increase in p38 MAPK signaling has been observed (Dapper et al., 2013; Levkovitch-Verbin et al., 2007; Tezel et al., 2003). Furthermore, previous studies investigating glutamate toxicity in cultured porcine retinas found that phosphorylated p38 MAPK levels increased over twofold after glutamate insult lending evidence that glutamate-induced RGC apoptosis is mediated by p38 MAPK (Asomugha et al., 2010).

Initiation of p38 MAPK-mediated apoptotic signaling cascades generally leads to the activation of tumor protein 53 (p53). p53 up-regulates the expression of Bcl-2-associated X protein (BAX), which then proceeds to associate with the mitochondrial outer membrane and form a mitochondrial apoptosis-induced channel. This channel allows cytochrome c to be released from the mitochondrial inner membrane and enter the cytoplasm of the cell (Buytaert et al., 2006). Once in the cytoplasm, cytochrome c activates caspase-9 enzymes, which in turn activate caspase-3 enzymes and ultimately leads to apoptosis. Interestingly, while previous studies have shown increased p38 MAPK levels after glutamate insult to cultured porcine RGCs, phosphorylated p53 levels did not significantly increase compared to controls (Asomugha et al., 2010). However, levels of phosphorylated Bcl-2, an anti-apoptotic protein that acts to block cytochrome c release from the mitochondria, were significantly less than in control retinas. This suggests that p38 MAPK’s apoptotic effect may act primarily through the down-regulation of Bcl-2 rather than the direct activation of p53 (Asomugha et al., 2010; De Chaiara et al., 2006).

In this study, caspase-3 activity was analyzed in the in vivo rat model to provide evidence that apoptosis is associated with the procedure used to induce glaucoma-like conditions.
Acetylcholine as a Neuroprotective Agent

In response to the proposed excitotoxic mechanism of damage in glaucoma, researchers have tried to identify neuroprotective agents that could prevent glutamate-induced RGC death from occurring. In other diseases of the CNS, such as Alzheimer’s disease and Parkinson’s disease, nicotine has been shown to provide neuroprotection by binding to nicotinic acetylcholine receptors (nAChR) on neurons, and triggering a cell survival signal transduction pathway (Bordia et al., 2007; Hejmadi et al., 2003; Shaw et al., 2002). Since nicotine binds to nAChR’s, previous studies have tested the neuroprotective effects of both nicotine and ACh in cultured porcine RGCs exposed to glutamate. It was found that when pretreated with 5µM nicotine or 5µM ACh two hours before glutamate insult, there was no significant loss of RGCs when compared to controls (Wehrwein et al., 2004). In addition, it was found that this neuroprotective effect was mediated by the binding of both α4 and α7nAChR subunits found on porcine RGCs (Thompson et al., 2006, Wehrwein et al., 2004). These findings suggest that ACh provides neuroprotection against glutamate excitotoxicity in the retina.

The binding of ACh to nAChRs on RGCs opens ligand-gated ion channels allowing calcium to enter the cell (Burnashev, 1998). Instead of inducing an excitotoxic effect, the calcium influx through nAChR channels activates a survival pathway. This raised the critical question of how calcium influx was able to cause two different effects when entering the same cell. While excessive influx of calcium through the activation of glutamate channels triggers apoptosis, calcium permeation through nAChR channels triggers cell survival. To address this, a study conducted by Brandt et al. (2011) measured levels of calcium entering cultured porcine RGCs when exposed to varying concentrations of ACh, nicotine, and glutamate. It was found that even at high concentrations, exposure to ACh and nicotine only allowed relatively low levels
of calcium into the cell compared to the influx of calcium resulting from the activation of glutamate channels. This is likely due to the desensitization of nAChRs that occurs following prolonged binding of agonists (Giniatullin et al., 2005; Yamodo et al., 2009). In fact, even when applied at a concentration of 500µM, nicotine still provided neuroprotection instead of toxicity. These results suggest that initiation of calcium-induced survival and apoptotic pathways are dependent upon the amount of calcium entering the cell. A small influx of calcium prior to a large influx initiates a survival pathway to precondition the cell against a later insult.

In other systems exhibiting ACh-induced neuroprotection, calcium influx resulting from the binding of α7nAChRs has been shown to activate a phosphatidylinositol 3-kinase (PI3K) → protein kinase B (AKT) → Bcl-2 survival pathway (Dasari et al., 2008; Kihara et al., 2001; Jonge and Ulloa, 2007). Previous studies investigating this survival pathway in cultured porcine RGCs measured phosphorylated AKT and Bcl-2 levels after exposure to acetylcholine. It was found that when ACh was applied alone or prior to glutamate insult, both AKT and Bcl-2 levels increased significantly compared to control levels (Asomugha et al., 2010; Brandt et al., 2011). It was also found that when inhibitors of PI3K enzymes were applied before exposure to ACh, AKT and Bcl-2 levels were significantly lower than ACh-induced levels (Asomugha et al., 2010). In addition, p38 MAPK levels significantly decreased in RGCs treated with ACh alone or prior to glutamate insult when compared to RGCs that were only exposed to high concentrations of glutamate. These findings suggest that the binding of α7nAChRs on RGCs may provide neuroprotection in two ways: by activating the PI3K→AKT→Bcl-2 survival pathway, as well as down-regulating the expression of p38 MAPK that is critical for glutamate-induced excitotoxicity.
PNU-282987

PNU-282987 (N-[(3R)-1-azabicyclo[2.2.2]oct-3-yl]-4-chlorobenzamide hydrochloride) is an α7nAChR agonist developed by Pharmcia and Upjohn that was initially intended for use as a neuroprotective treatment in individuals with schizophrenia (Bodnar et al., 2005). However, it was found to be an unviable treatment option due to its inhibition of vital potassium channels in the heart (Walker et al., 2006). In glaucoma the use of PNU-282987 as an eye drop may be able to safely provide neuroprotection as long as it stays local to the retina and never reaches the heart. To investigate this, previous studies in this lab topically applied 100µM, 1mM, and 10mM concentrations of PNU-282987 to the eyes of Long Evans rats (Mata, 2013). At different time points following each application the animals were sacrificed, and retinal and cardiac tissues were isolated and underwent LC/MSMS processing to detect levels of PNU-282987. It was found that PNU-282987 reached the retina, and that detectable levels were found up to 12 hours following topical application. In addition, no detectable levels of PNU-282987 were found in cardiac tissues, thereby eliminating the concern of potassium channel inhibition in the heart.

Because it has been demonstrated that α7nAChRs are present on the surface of rat RGCs, studies were conducted to investigate the neuroprotective potential of PNU-282987 as an α7 nicotinic agonist using an in vivo rat model of glaucoma (Iwamoto et al., 2013; Mata, 2013). As in vitro studies demonstrated that ACh and nicotine could provide neuroprotection of RGCs when applied before glutamate insult, in the in vivo model bi-daily eye drops of PNU-282987 were applied for three days before a procedure was performed to induce glaucoma-like conditions (Mata, 2013). After the procedure, the bi-daily application of PNU-282987 continued for one month before sacrificing the animals and quantifying RGCs. PNU-282987-induced neuroprotection was observed in a dose dependent manner. While 100µM PNU-282987 did not
prevent significant RGC loss, both 500µM and 1mM concentrations provided significant neuroprotection when compared to untreated controls (Mata, 2013). Strangely, it was also found that when a concentration of 2mM or 10mM was applied, there was a 13.5% increase in RGCs. Overall, these findings suggest that PNU-282987 has a neuroprotective effect against RGC loss under glaucoma-like conditions induced in rats.

The objectives of this thesis were designed to follow-up on the findings of previous studies using PNU-282987. Because neuroprotection of RGCs was observed when 1mM PNU-282987 was used, and RGC proliferation occurred when 2mM or 10mM PNU-282987 was applied, multiple studies were conducted to analyze the neuroprotective effects of 2mM PNU-282987 when applied as eye drops for varying amounts of time using an in vivo glaucoma model in Long Evans rats. The goal of these studies was to determine the extent of neuroprotection conferred following each period of PNU-282987 application, and to analyze when 2mM PNU-282987 induces RGC proliferation. Determining the timeline of PNU-282987’s neuroprotective effects will allow for a better understanding of its impact on RGCs. In addition, since evidence for a p38 MAPK-mediated apoptotic mechanism of glaucoma was found in previous in vitro studies using cultured porcine RGCs, studies to analyze changes in retinal caspase-3 levels in vivo were conducted. Caspase-3 is a downstream enzyme of p38 MAPK that is involved with apoptosis, and evaluating its activity following the procedure to induce glaucoma-like conditions will yield a better understanding of the apoptotic mechanism associated with the in vivo model.
MATERIALS AND METHODS

Animals

Adult male and female Long Evans Rats between 3 and 6 months of age were used as an in vivo model for glaucoma. Animals were obtained from breeding colonies at Charles Rivers Labs in Portage, MI, and kept at Western Michigan University’s animal facility until needed. All animals were cared for following the guidelines of the Institutional Animal Care and Use Committee of Western Michigan University.

Inducing Glaucoma-Like Conditions

These studies utilized the same procedure to induce glaucoma-like conditions as previous studies that investigated the neuroprotective capabilities of PNU-282987 in an in vivo rodent model (Mata, 2013; Morrison et al., 1997). Rats were anesthetized via a 1ml/Kg intraperitoneal injection of KAX cocktail consisting of 5mL of ketamine (100mg/mL), 2.5mL of xylazine (20mg/mL), 1mL of acepromazine (10mg/mL), and 0.5mL of sterile water. To ensure proper anesthetization, the procedure to induce glaucoma-like conditions was not performed until animals lacked reflex activity caused by a toe or tail pinch.

The procedure to induce glaucoma-like conditions consisted of injecting the episcleral veins surrounding the iris with a 2M hypertonic saline solution. As the saline drains from the veins, NaCl salt crystals precipitate out of solution and cause scarring at the trabecular meshwork of the eye. This scarring results in inadequate aqueous humour drainage from the eye’s anterior chamber and an increase in IOP (McKinley et al., 2012; Schwartz, 1999). Previous studies have confirmed that the elevation of IOP following this procedure is sufficient to cause significant RGC loss (an average of 26% decrease) (Mata, 2013). A glass needle 40µm in diameter was pulled from a Narishige electrode puller and used to inject the episcleral veins. The glass needle
was attached to tapered polyethylene tubing (PE-50, Clay Adams, Parsippan, NJ) that was then inserted into a 23-gauge needle with the tip filed off. The tip of the glass needle was beveled prior to injection to ensure easy penetration into the episcleral veins. 50µL of sterile 2M saline solution was then injected into the episcleral veins of the right eye causing them to blanch. The blanching effect ensured a proper injection. The left eye was left untouched to serve as an internal control. Antibiotic cream was applied to the right eye following the procedure, and the rat was placed back into its cage and monitored until it had completely recovered from anesthesia before being returned to the animal facility.

**Application of PNU-282987**

To investigate the effects of 2mM PNU-282987 on retinal ganglion cells, it was applied as eye drops 2 times each day for different periods of time prior to performing the procedure to induce glaucoma-like conditions. In some studies, PNU-282987 was applied twice a day via eye drop for 3 days, 1 day and 1 hour prior to NaCl injection into the episcleral veins. In other studies, 2mM PNU-282987 was applied for three days prior to injection, and then added bi-daily for 1 week, 2 weeks, and 4 weeks following the procedure. All animals were sacrificed 4 weeks after the NaCl injection as previous studies have demonstrated that significant loss of RGCs normally occurred 1 month following the hypertonic injection into the episcleral veins (Iwamoto et al., 2013). Drops were administered bi-daily because previous LC/MSMS studies have found detectable levels of PNU-282987 in the retina for up to twelve hours following ocular application (Mata, 2013).

The eye drops were prepared by dissolving PNU-282987 in dimethyl sulfoxide to make a stock solution that was then diluted to 2mM using phosphate buffered saline (PBS) to allow it to diffuse into the retina after reaching the back of the eye. Eye drops were applied to rats using
plastic pipettes while they remained in their cages. Following application, the rats received Cheerios® as positive reinforcement.

**Isolation of Retinas and Quantification of RGCs**

After animals received PNU-282987 treatment for the designated lengths of time they were euthanized in a carbon dioxide chamber for at least 3 minutes to ensure death. After 3 minutes, animals were examined for the absence of breathing and reflexes to confirm they were deceased. The left and right eyes were surgically removed and cut circumferentially behind the iris to separate the eye’s anterior and posterior halves. After removal of the lens and vitreous humour, the retinas were carefully extracted from the posterior half of the eyecup. Care was taken to keep the retinas in one piece. Four cuts towards the optic nerve head were made in the retinas, which allowed them to be flat mounted and pinned to a sylgard dish using cactus needles with the RGC layer facing upward (Figure 3). Pinned retinas were then fixed in 10% formalin overnight at 4°C. After fixation, retinas were rinsed three times with PBS. To block nonspecific binding, the retinas were incubated in 2% bovine serum albumin (BSA) in PBS containing 0.02% saponin for 30 minutes at 25°C before the application of the primary antibody. The monoclonal primary antibody used for studies investigating the effects of PNU-282987 was Thy1.1 (mouse anti-rat IgG; BD Biosciences), which is specific against Thy-1 glycoproteins only found on RGCs in the retina (Barnstable and Drager, 1984). The retinas were incubated in a 1:300 dilution of Thy1.1 in PBS containing 0.02% saponin for 1 week at 4°C in a humidified chamber. After 1 week, the retinas were rinsed three times with PBS and incubated in a 1:300 dilution of fluorescent secondary antibody Alexa Fluor 595 (goat anti-mouse IgG; Invitrogen/Molecular Probes) for another week at 4°C to allow for visualization of labeled RGCs.
under a confocal microscope. After incubation was complete, the tissues were rinsed three times with PBS and mounted onto glass slides with a 50% PBS and 50% glycerol solution.

Retinal images were obtained using the 60x objective lens of a Zeiss confocal microscope. Previous glaucoma studies found that the most damage to RGCs occur in the peripheries of the retina, so pictures of RGCs were taken 4mm from the optic nerve head (Mata, 2013). Four pictures were taken per retina in each of four quadrants 4mm from the optic nerve head to ensure adequate sampling of RGC density (Figure 3). Pictures were taken throughout the RGC layer in 1μm increments. Thy1.1 labeled RGCs were counted within an 80μm² frame in each of the four quadrants for each retina and averaged. RGC counts from experimental retinas were directly compared to the internal untreated control RGC counts to determine if any significant change occurred in the number of RGCs. The 80μm² frame was placed so as to minimize inclusion of labeled RGC axon tracts which may have obscured RGC cell bodies.
Figure 3
Image of a flat mounted retina taken with a light microscope. Green boxes represent areas 4mm from the optic nerve head where cell counts were performed. (Image taken with modified labeling from Mata, 2013)

Caspase-3 Labeling and Assessment

In other studies, caspase-3 activity was assessed to determine whether there was a change in caspase-3 activity in RGCs under induced glaucoma-like conditions. The right eyes of four adult Long Evans rats were subjected to NaCl injection as described previously while leaving the left eyes untouched to act as an internal control. The animals were euthanized at 1 day, 1 week, 2 weeks, and 4 weeks following the injection procedure, and their retinas were isolated, flat mounted, and pinned to a sylgard dish. The retinas were fixed in 10% formalin overnight at 4°C followed by three rinses with PBS. To block nonspecific binding the retinas were incubated in
2% bovine serum albumin (BSA) in PBS containing 0.02% saponin for 30 minutes at 25°C before the application of the primary antibody. The primary monoclonal antibody used for staining was specific against caspase-3 enzymes (rabbit anti-rat IgG; Millipore). The retinas were incubated in a 1:300 dilution of the primary antibody in PBS containing 0.02% saponin for 1 week at 4°C in a humidified chamber. After one week the retinas were rinsed three times with PBS followed by incubation in a 1:300 dilution of fluorescent secondary antibody Alexa Fluor 594 (donkey anti-rabbit IgG; Life Technologies) for 1 week to allow for visualization of caspase-3 enzymes under the confocal microscope. After incubation was complete, the tissues were rinsed three times with PBS and mounted onto glass slides with a 50% PBS and 50% glycerol solution.

Retinal images were obtained with a Zeiss confocal microscope using a 10x objective lens. Pictures of the RGC layer were taken in 4µm increments 4mm from the optic nerve head, and in the region surrounding the optic nerve head itself. While the greatest damage to RGCs has been shown to occur in the peripheries of the retina, pictures around the optic nerve head were also taken because of this region’s sensitivity to IOP changes (Mata, 2013; Selbach et al., 1999; Novack et al., 1990). Experimental and control retinal pictures were analyzed for fluorescence that would indicate a change in caspase-3 activity.

**Statistical Analysis**

For studies analyzing the effects of PNU-282987, RGC counts in experimental retinas were compared to RGC counts in internal controls using Student’s T-tests. P-values < 0.05 were considered statistically significant. All data values are reported with the mean standard error of the sample-mean (SEM).
In studies analyzing changes in caspase-3 activity following the procedure to induce glaucoma, experimental retinas and internal controls were examined and compared for fluorescence to determine the presence of activated caspase-3 in the RGC layer. 950µm × 950µm representative images of both retinal peripheries and regions surrounding the optic nerve head were analyzed. Since it was impossible to determine whether labeled caspase-3 was present within apoptotic vesicles, cellular debris, or RGC bodies, isolated points of fluorescence in each image were quantified as caspase-positive units (CPUs) to verify changes in caspase-3 activity. Because the aim of this study was to qualitatively determine whether caspase-3 was involved in the apoptotic mechanism associated with the *in vivo* model, statistical analysis of CPU counts was not performed.

**RESULTS**

**Effects of PNU-282987 on RGC Levels**

To investigate the effects of PNU-282987 on RGC survival, 2mM PNU-282987 was applied as eye drops for various amounts of time before and after the procedure to induce glaucoma-like conditions. One month following NaCl injection, the retinas were removed and processed with antibodies against the glycoprotein Thy-1. It was found that exposure to PNU-282987 treatment for different periods of time had different effects on RGC survival. Figures 4A and 4B are images of retinas taken by Mata (2013) that show the effects of NaCl injection on RGC survival without any treatment with PNU-282987. Figure 4A demonstrates the left untreated eye that served as an internal control. Single arrows indicate RGC bodies, and double arrows indicate RGC axon tracts that run through the retina. Figure 4B demonstrates the effect on RGCs 1 month after 2M NaCl injection into the episcleral veins. The region displayed in Fig. 4B was obtained from the same rat that produced Fig. 4A and from the same region of the retina.
The RGC densities in the experimental retinas (4B) appeared dramatically reduced when compared to untreated internal controls (4A). In addition, the shapes of RGCs in experimental retinas had a blebbed and uneven appearance that differed from the smooth rounded control RGCs. Figures 4C and 4D illustrate the effects on RGCs from another rat when a single drop of 2mM PNU-282987 was applied one hour prior to the procedure to induce glaucoma-like conditions. The RGCs in the experimental retinas (4D) were similar in shape and density to those in the internal control retinas (4C). The region displayed in Fig. 4D was obtained from the same rat that produced Fig. 4C and from the same region of the retina. It was evident that 1 drop of 2mM PNU-282987 prior to NaCl injection provided some level of protection against RGC loss.

Similar results occurred if eye drops were applied 1 and 3 days before NaCl injection into the episcleral veins. Figures 4E and 4F illustrate the effect of 2mM PNU-282987 on RGCs when applied twice a day for 1 day prior to injection. RGC shape and density in experimental retinas (4F) appeared similar to RGCs of the internal control retinas (4E). Likewise, RGC shapes and densities in experimental retinas that were treated with 2mM PNU-282987 for 3 days prior to injection (4H) were similar to RGC densities observed in the untreated control retinas (4G) indicating protection against loss of RGCs that is typically associated with the NaCl injections.

Different effects on RGC survival occurred when 2 mM PNU-282987 was applied for longer periods of time after NaCl injection into the episcleral veins. The images shown in figures 4I and 4J illustrate the effects that 2mM PNU-282987 had on RGC survival when it was applied for three days prior to NaCl injection and one week post-injection. While the shape of RGCs in the experimental retinas (4J) was similar to those in control retinas (4I), RGC density was reduced in experimental retinas. Protection against RGC loss did not appear to occur under these conditions.
The images shown in figures 4K and 4L illustrate the effects that 2mM PNU-282987 had on RGC survival when it was applied for three days prior to NaCl injection and two weeks post-injection. The shapes of RGCs in experimental retinas (4L) appeared similar to RGCs in control retinas (4K); however, RGC density was now greater in experimental retinas. Likewise, the shapes of RGCs in experimental retinas treated with 2mM PNU-282987 for 3 days prior to NaCl injection and 4 weeks post-injection (4N) appeared similar to control RGCs (4M), but their density had also increased. Protection against RGC loss and potential RGC proliferation was evident under both of these conditions.
Figure 4

Grayscale images of RGCs stained with a fluorescent antibody against Thy 1.1. Images shown in the left columns represent control untreated retinas obtained from the same animal and from the same location as the right experimental images, 4mm from the ONH. The right experimental images were obtained after 2mM PNU-282987 was applied for different periods of time before and after NaCl injection to induce glaucoma-like conditions. All images were taken 1 month following NaCl injection to the right eye. Frame B depicts RGCs labeled after NaCl injection with no PNU-282987 treatment taken from previous studies conducted by Mata (2013). Frame D depicts RGCs labeled after treatment with 1 drop of PNU-282987 before NaCl injection. Frame
F depicts RGCs labeled after 1 day of treatment with PNU-282987 before NaCl injection. Frame H depicts RGCs labeled after 3 days of treatment with PNU-282987 before NaCl injection. Frame J depicts RGCs labeled after 3 days of treatment with PNU-282987 before NaCl injection followed by 1 week of treatment post-injection. Frame L depicts RGCs labeled after 3 days of treatment with PNU-282987 before NaCl injection followed by 2 weeks of treatment post-injection. Frame N depicts RGCs labeled after 3 days of treatment with PNU-282987 before NaCl injection followed by 4 weeks of treatment post-injection. Single arrows indicate labeled RGC bodies, and double arrows indicate RGC axon tracts running through the retina.

Quantification of the change in RGC survival after different periods of PNU-282987 application is summarized in Figure 5. Retinas analyzed in studies by Mata (2013) four weeks after the procedure to induce glaucoma-like conditions without any PNU-282987 treatment had an average of 26% (± 5.2, N=6) RGC loss, and is included in Figure 5 for comparison. Retinas that were exposed to 1 drop of PNU-282987 prior to NaCl injection showed statistically significant protection against RGC loss as indicated by an average RGC decrease of 4.2% (± 4.1, N=3). Likewise, retinas exposed to PNU-282987 1 day prior to NaCl injection, and for 3 days prior to NaCl injection also exhibited statistically significant protection. 1 day of treatment only averaged a loss of 6.7% (± 3.2, N=3) RGCs, and 3 days of treatment averaged a loss of 6.3% (± 3.1, N=3) RGCs. However, this neuroprotective effect changed when 2mM PNU-282987 was applied for longer periods of time. Retinas exposed to 3 days of PNU-282987 treatment prior to the NaCl injection and for an additional week post-injection demonstrated significant RGC loss of 18.3% (±.05, N=3). If PNU-282987 was applied longer than 1 week after the NaCl injection, proliferation of cells occurred. Significant proliferation of RGCs was observed in retinas that were treated with PNU-282987 for 3 days prior to the NaCl injection followed by 2 weeks and 4 weeks of subsequent treatment. Retinas treated for 2 weeks following injection averaged a RGC gain of 28.9% (±10.2, N=3), and retinas treated for 4 weeks following injection had an average RGC gain of 24.2% (± 2.3, N=3). Overall, statistically significant protection against RGC loss was only observed when short periods of exposure to 2mM PNU-282987 occurred before the
procedure to induce glaucoma-like conditions was performed. Prolonged post-procedural treatment with PNU-282987 appeared to result in the initial death of RGCs, which was then followed by a period of proliferation.

Figure 5

Summary of the results following bi-daily eye drop application of 2mM PNU-282987 for varying amounts of time. Each bar represents the average percent change in the number of RGCs when compared to untreated internal control conditions 1 month following the NaCl injection procedure to induce glaucoma-like conditions. Data for 1 drop (N=3), 1 day (N=3), and 3 days (N=3) of PNU-282987 application prior to injection is included, as well as data from 3 days of PNU-282987 application prior to injection followed by 1 week (N=3), 2 weeks (N=3), and 4 weeks (N=3) of subsequent application following injection. Error bars represent SEM. Because the SEM for 1 week of post-injection PNU-282987 treatment was only ±0.05, the error bar does not expand beyond the data bar’s boundary. *represents data taken from Mata (2013) when eyes were subjected to the procedure to induce glaucoma-like conditions without any PNU-282987 treatment (N=6) and is included for comparison.
Changes in RGC Caspase-3 Activity Following NaCl Injection

To investigate the involvement of caspase-3 in the apoptotic mechanism associated with an in vivo rat model of glaucoma, retinas of left control eyes and right eyes subjected to NaCl injection were isolated at 1 day, 1 week, 2 weeks, and 4 weeks following the procedure to induce glaucoma-like conditions. Retinas were subsequently stained, and the peripheral and optic nerve head regions were analyzed for fluorescence. Because consistent levels of fluorescence were observed throughout these regions in each retina, a 950µm × 950µm representative image was taken of each periphery and optic nerve head, and isolated points of fluorescence were quantified as caspase-positive units (CPUs) to verify changes in caspase-3 activity. The most dramatic change in caspase-3 activity occurred 1 day following NaCl injection (Figure 6). In the peripheral region of the control retina there were 111 CPUs (6A), while the experimental retina exhibited 492 CPUs (6B). Additionally, only 11 CPUs were observed in the region surrounding the optic nerve head of the control retina (6C), while 175 CPUs were counted in the experimental retina. This indicates a large increase in caspase-3 activity throughout the retina 1 day following the procedure to elicit glaucoma-like conditions.
FIGURE 6

Images of retinas labeled with a fluorescent antibody against caspase-3 taken 1 day after NaCl injection was performed to induce glaucoma-like conditions. All images were taken from the same animal. Images in the left column came from the left eye that served as an internal control. Images in the right column came from the right eye that was subjected to NaCl injection. Frames A and B depict images taken of the peripheral region of the retina 4mm from the optic nerve head. Frames C and D depict images taken of the area directly surrounding the optic nerve head. Single arrows indicate caspase positive units.

Significant changes in caspase-3 activity in retinal peripheries were absent in retinas analyzed 1 week, 2 weeks, and 4 weeks following the NaCl injection procedure to induce glaucoma-like conditions. Figure 7 portrays pictures of these retina peripheries, and also includes the pictures of retinas analyzed 1 day post-injection already depicted in Figure 6 for comparison (7A,7B). In peripheral images taken of retinas isolated 1 week following the injection procedure
there were 102 CPUs present in the control tissue (7C) and 114 CPUs present in the experimental tissue (7D), which did not indicate a significant change in caspase-3 levels under these conditions. Likewise, CPU counts in the peripheral regions of retinas isolated 2 and 4 weeks following injection did not differ significantly from controls. In retinas isolated 2 weeks following NaCl injection the control tissue exhibited 5 CPUs (7E) and the experimental tissue exhibited 6 CPUs (7F). In retinas isolated 4 weeks after NaCl injection the control tissue only exhibited 4 CPUs (7G) and the experimental tissue exhibited 3 CPUs (7H). In summary, no significant change in caspase-3 activity appeared to occur in the retinal peripheries at 1 week, 2 weeks, or 4 weeks following NaCl injection.
Figure 7

Images of retinas labeled with a fluorescent antibody against caspase-3 taken in the peripheral regions 4mm from the optic nerve head at different times after NaCl injection to induce glaucoma-like conditions. Images in rows were taken from the eyes of the same animal. Images in the left column came from left eyes that served as internal controls. Images in the right column
came from right eyes that were subjected to the injection procedure to induce glaucoma-like conditions. Frames A and B depict the images already presented in Figures 6A and 6B of retinas labeled 1 day after NaCl injection. Frames C and D depict images of retinas labeled 1 week after NaCl injection. Frames E and F depict images of retinas labeled 2 weeks after NaCl injection. Frames G and H depict images of retinas labeled 4 weeks after NaCl injection.

Figure 8 depicts the regions surrounding the optic nerve head in both experimental and control retinas, and also includes pictures of the retinas analyzed 1 day post-NaCl injection that were in Figure 6 for comparison (8A,8B). The experimental retina isolated 1 week following injection exhibited 142 CPUs (8D) while the control retina only exhibited 7 CPUs (8C). This indicates that a significant increase in caspase-3 activity is present at the optic nerve head for at least a week following the procedure to induce glaucoma-like conditions. However, experimental retinas isolated 2 weeks and 4 weeks following NaCl injection did not exhibit a significant increase in CPUs when compared to internal controls. In retinas isolated two weeks following NaCl injection experimental tissue only exhibited 4 CPUs (8F), and the control tissue exhibited 3 CPUs (8E). In retinas isolated four weeks after NaCl injection the experimental tissue exhibited 4 CPUs (8H), and the control tissue also exhibited 4 CPUs (8G). While caspase-3 activity appeared dramatically increased at the optic nerve head 1 week post-NaCl injection, no significant change in caspase-3 activity occurred at 2 weeks or 4 weeks following injection.
Figure 8

Images of retinas labeled with a fluorescent antibody against caspase-3 taken at the region directly surrounding the optic nerve head at different times after NaCl injection to induce glaucoma-like conditions. Images in rows were taken from the eyes of the same animal. Images in the left column came from left eyes that served as internal controls. Images in the right column
came from right eyes that were subjected to the injection procedure. Frames A and B depict the images already presented in Figures 6C and 6D of retinas labeled 1 day after NaCl injection. Frames C and D depict images of retinas labeled 1 week after NaCl injection. Frames E and F depict images of retinas labeled 2 weeks after NaCl injection. Frames G and H depict images of retinas labeled 4 weeks after NaCl injection.

**DISCUSSION**

**Effects of PNU-282987**

In this study the neuroprotective effects of 2mM PNU-282987 against RGC death were investigated when it was applied bi-daily as an eye drop for varying amounts of time using an *in vivo* rat model of glaucoma. There was not statistically significant RGC loss when eyes were pretreated with PNU-282987 for 3 days and 1 day before performing the NaCl injection to induce glaucoma-like conditions. Likewise, a single drop of PNU-282987 applied one hour before injection was sufficient to prevent significant RGC death. Significant RGC loss did occur when it was applied for 3 days prior to the injection procedure and for 1 week following injection. Under these conditions there was an average RGC loss of 18.3%. Oddly, when PNU-282987 was applied for 2 weeks and 4 weeks following NaCl injection dramatic RGC proliferation was observed. 2 weeks of application led to a 28.9% increase in RGCs, and 4 weeks of application resulted in a 24.2% increase. While short exposure of PNU-282987’s provided neuroprotection against the loss of RGCs associated with the NaCl injection, prolonged use appeared to have a severe effect on cell survivorship.

Studies have shown that the binding of nicotinic agonists to nAChRs allows calcium to enter the cell, and that prolonged binding desensitizes the receptors and doesn’t allow the influx of calcium to reach toxic levels (Brandt et al., 2011; Giniatullin et al., 2005; Yamodo et al., 2009). However, the binding of PNU-282987 to nAChRs for a week appears to be causing cell death. This apoptosis could be occurring due to greater levels of calcium entering RGCs when
PNU-282987 binds to nAChRs than when other nicotinic agonists bind resulting in cell death. Alternatively, RGC death may be due to other mechanisms such as changes in calcium tolerance that may result from a relatively high concentration of PNU-282987 exposure.

The proliferative effect of PNU-282987 observed after 2 weeks and 4 weeks of application is strange in that it appears to follow a period of cell death. This effect was unexpected as adult mammalian retinal neurons do not normally undergo mitotic division. While the mechanism surrounding this switch from apoptotic to proliferative cell signaling is unknown, prolonged binding of nAChRs on various non-neuronal cells such as pulmonary endothelium, keratinocytes, and esophageal epithelium has been shown to induce mitogenic division and lead to cancer (Egleton et al., 2008; Grando et al., 1996; Jensen et al., 2012). The nicotinic receptor mediated initiation of cell replication has been shown to occur in a variety of ways, one of which is the activation of a class of MAPKs known as external signal-regulated kinases (ERKs) (Egleton et al., 2008; Schaeffer and Weber, 1999). ERKs are involved in a variety of functions related to cell survival, differentiation, and division. Activation of ERKs occurs in response to prolonged calcium influx, and initiates a signaling cascade that leads to the transcription of genes and expression of proteins necessary for cells to enter S phase and prepare for mitosis (Harding et al., 2003; Schaeffer and Weber, 1999). Normally, RGCs are arrested in G₀ phase and have ERKs that mediate survival functions in the cell such as preventing hypoxia-induced apoptosis and aiding in axonal regeneration (Park et al., 2004; Zhou et al., 2005; Zhou et al., 2007). However, perhaps the continuous influx of subtoxic levels of calcium that results from prolonged exposure to PNU-282987 is sufficient to activate ERK-mediated cell replication pathways that can push RGCs into S phase and lead to mitotic division.
**Caspase-3 Activity**

In addition to investigating the neuroprotective effects of PNU-282987, this study also analyzed the involvement of caspase-3 in the apoptotic mechanism associated with an *in vivo* rat model of glaucoma. Fluorescent immunocytochemical techniques were utilized to visualize caspase-3 enzymes present in RGCs at different time points following the procedure to induce glaucoma-like conditions. A large increase in caspase-3 activity was observed in the peripheries and in the regions surrounding the optic nerve head at 1 day following NaCl injection. At 1 week post-injection, caspase-3 activity did not appear to differ significantly from controls in retinal peripheries, but there was still an increase in activity around the optic nerve head. By 2 weeks and 4 weeks after NaCl injection, however, caspase-3 activity did not significantly differ from controls in the retinal peripheries or around the optic nerve head. These findings support that caspase-3 is involved in the mechanism of RGC death observed in the *in vivo* glaucoma model. In addition, because caspase-3 is a downstream target of the proposed p38 MAPK-mediated mechanism of apoptosis observed in *in vitro* studies analyzing glutamate excitotoxicity, these findings provide evidence that this same apoptotic mechanism may be involved in the *in vivo* model (Asomugha et al., 2010).

The most prominent increase in caspase-3 activity observed at just 1 day following NaCl injection is most likely due to the nature of the injection procedure itself. When NaCl crystals from the injected saline solution precipitate and drain from the eye, they pass through the trabecular meshwork causing substantial damage and scarring (Morrison et al., 1997; Mata, 2013). This immediately impedes the outflow of aqueous humour from the anterior chamber, and IOP levels quickly begin to rise. While this increase in IOP simulates glaucomatous conditions, its rapid onset does not reflect the process of slow IOP elevation that occurs in patients with
The significant increase in caspase-3 activity observed 1 day after NaCl injections may be due to a sudden sharp elevation of IOP, which immediately introduces profound cellular stress and triggers apoptotic signaling.

**Future Studies and Implications**

Because application of 2mM PNU-282987 had a variety of effects on RGCs depending on how long eyes were exposed to it, future studies will need to analyze what specific signaling pathways PNU-282987 is initiating. Since neuroprotection was observed when it was only applied prior to NaCl injection into the episcleral veins, analyses of PI3/AKT levels following 2mM PNU-282987 application will need to be performed to determine if the PI3-AKT-Bcl-2 survival pathway proposed by previous studies is a plausible mechanism of protection in the *in vivo* rat model of glaucoma (Asomugha et al., 2010; Brandt et al., 2011). In addition, studies investigating how long the neuroprotective effect of PNU-282987 lasts when applied prior to NaCl injection need to be conducted. While protection was observed at four weeks following the procedure to induce glaucoma-like conditions, analysis of RGC survival at 5 weeks, 6 weeks, etc. post-procedure should be performed. This will provide a time frame for PNU-282987’s efficacy, and may be able to prevent some of the adverse effects on RGC survival observed when exposed to the drug for extended periods of time.

To address the apoptotic and proliferative effects that prolonged exposure to 2mM PNU-282987 had on RGCs following NaCl injection, this study should be repeated and the levels of pro-apoptotic and mitogenic enzymes should be analyzed at each time point. Investigating the fluctuating levels of enzymes like p38 MAPK and ERK in RGCs subjected to different periods of PNU-282987 exposure will allow for a better understanding of this drug’s action when bound to nAChR’s. Specifically, the enzyme levels associated with exposure to 2mM PNU-282987 for
7-14 days should be investigated to determine how RGCs are switching from apoptotic activity to proliferation.

Because adult mammalian RGCs are not supposed to undergo mitotic division, the RGC proliferation induced by prolonged exposure to high concentrations of PNU-282987 is puzzling. The physiology of the RGCs involved needs to be investigated. The key question to address is whether newly formed RGCs are able to successfully participate in the transmission of electrical signals generated during phototransduction. If so, could the induction of RGC proliferation under glaucomatous conditions lead to the restoration of sight in individuals? Studies need to be conducted to determine whether newly developed RGCs are successfully synapsing with bipolar and starburst amacrine cells in the retina since these play key roles in electrical signal transmission. In addition, studies looking at the axonal development of new RGCs need to be performed to determine whether they are successfully extending to the lateral geniculate nucleus of the brain. Even if new RGCs are developing correctly, behavioral studies will have to be performed to determine whether a noticeable improvement in sight is actually occurring as a result of their induced proliferation. If the controlled induction of functional RGC proliferation under glaucomatous conditions is perfected, it could have huge implications in current glaucoma research and treatments. Rather than simply controlling RGC loss, this discovery could reverse glaucomatous damage and potentially lead to the restoration of sight in patients.

In addition to further characterizing the effects of PNU-282987, this study confirmed that caspase-3 is involved in the apoptotic mechanism associated with an in vivo rat glaucoma model. However, further studies need to be conducted to determine what upstream components are responsible for the activation of caspase-3. Since glutamate is highly suspected to be involved in RGC death, levels of apoptotic enzymes associated with glutamate excitotoxicity as determined
by past studies such as p38 MAPK and BAX should be investigated. This will provide a better understanding of how RGC apoptosis is occurring in the in vivo model, as well as provide more insight into the neuroprotective mechanism of PNU-282987. Additionally, double labeling experiments should be conducted to label both caspase-3 enzymes and RGC membranes in order to determine the specific location of activated caspase-3 following the procedure to induce glaucoma-like conditions. Distinguishing whether caspase-3 is more prominent within RGCs, apoptotic vesicles, or cellular debris will provide a more thorough understanding of the process and rate of apoptosis occurring in vivo.

The results of this study provide evidence for the effectiveness of PNU-282987 as a potential neuroprotective agent against RGC death in glaucoma. However, because apoptotic and proliferative effects were also observed at 2mM concentrations, it needs to be further investigated. It is important to note that previous studies have found that the application of lower concentrations of PNU-282987 between 500μM-1mM provided only neuroprotection when applied prior to the onset of glaucomatous conditions without any noticeable adverse effects to RGCs (Mata, 2013; Iwamoto et al., 2013). Perhaps these lower concentrations could be developed as a drug that, when used in conjunction with current treatments to lower IOP levels, may serve as a preventative treatment option for those at risk for developing glaucoma due to age or genetic predisposition. In addition, if the proliferative effect observed when RGCs are exposed to high concentrations of PNU-282987 is further characterized, it has the potential to become a groundbreaking treatment option that could reverse glaucomatous damage.
References


Thorofare, NJ: SLACK Incorporated


