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Neuroprotective effects of Retinal Ganglion cells by specific alpha 7 nicotine acetylcholine receptor agonists and an acetylcholineesterase inhibitor

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Acetylcholine receptors and their role in neuroprotection against loss of retinal ganglion cells in a glaucoma model

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ABSTRACT

In previous studies from this lab, an alpha7 nACh receptor was found to successfully modulate retinal ganglion cell neuroprotection against glutamate assault when bound to nicotinic acetylcholine receptors, or if an alpha7 nicotinic acetylcholine agonist, such as PNU-282987 was used in *in vitro* models (Wehrwein et al., 2004; Iwamoto et al., 2013). These *in vitro* studies support the hypothesis that activation of alpha7 nicotinic ACh receptors triggers neuroprotection against loss of retinal ganglion cells normally induced by excessive glutamate insult. The results from these *in vitro* studies instigated an *in vivo* study in our lab using a rat glaucoma model. However, further testing and manipulation of the specific alpha7 nAChR is required to confidently provide evidence concerning ACh receptor's modulatory capabilities. Therefore in our lab, a variety of acetylcholine agonists that bind to ACh receptors, like PNU-282987 and PHA 568487**,** will be used to analyze alpha7 nAChR neuroprotection in a rat model of glaucoma. In addition, an acetylcholinesterase inhibitor, Donepezil, will be administered to determine if inhibition of the acetylcholinesterase that normally degrades ACh, elicits a neuroprotective

response normally associated with activation of ACh receptors. Adult Long Evans rats will be used in *in vivo* glaucoma models, and the survival of retinal ganglion cells will be visualized using a Zeiss confocal microscope with Metamorph software. ANOVA statistical analysis will be performed on retinal ganglion cell survival in glaucoma-induced retinas and compared to control untreated retinas to provide evidence that activation of alpha7 nAChRs prevents loss of retinal ganglion cells in glaucoma. The long-term aim of this study is to find therapeutic preventative treatments against glaucoma and other neurodegenerative diseases.

INTRODUCTION

Anatomoy of the Eye

To understand the mechanisms behind glaucoma and the proposed therapeutic treatments in this thesis, it is necessary to have an understanding of the basic anatomy of the human eye (Figure 1). The human eye is composed of two separate chambers: the anterior chamber which is composed of a fluid called aqueous humour and the posterior chamber which is composed of a fluid called vitreous humour. In addition, the eye is segregated into three separate layers of tissue: the superficial layer is called the sclera, the middle layer is called the uvea, and the innermost layer is called the retina (Schwartz, 1999). The sclera is composed of a layer of tough, white fibrous tissue that works to provide protection and support for the eye. The anterior aspect of the sclera forms a transparent tissue called the cornea which is composed of transparent collagen fibers arranged in a lattice network that works to enable environmental light rays to enter the eye (Lens et al., 1999).

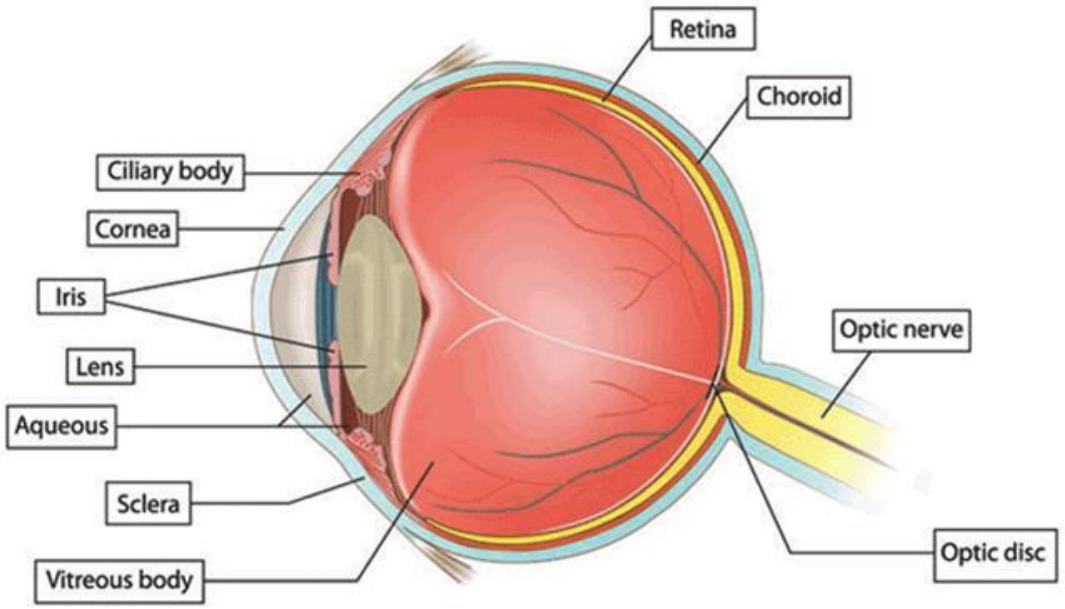

Figure 1

Anatomy of the human eye. (Image taken from the Glaucoma Research Foundation Website at http://www.glaucoma.org/glaucoma/anatomy)

The middle layer of tissue, the uvea, involves three distinct structures: the iris, the ciliary body, and the choroid. The iris is the colored part of the anterior portion of the eye. It is made up of two sets of smooth muscles under neural control that function to adjust the size of the opening at the center of the iris called the pupil. The ciliary body encircles the lens and is composed of both muscular and vascular structures. The muscular component of the ciliary body allows for the adjustment of the refracting power of the lens while the vascular component, also referred to as the ciliary processes, produces the fluid or aqueous humour that fills the anterior chamber and serves to nourish both the cornea and the lens. Lastly, the choroid is composed of a large capillary bed that functions as the main blood supple for the photoreceptors of the retina, the innermost layer of the eye (Purves et al., 2001).

The retina is the innermost tissue layer found within the posterior aspect of the eye. The retina is part of the central nervous system and it consists of a multitude of cells that function to translate visual information in the form of light stimuli into electrical signals that are then transported to the brain for interpretation purposes. This process is called phototransduction and the cells within the retina involved in this process include: photoreceptors (rods and cones), horizontal cells, bipolar cells, amacrine cells, and retinal ganglion cells (RGCs) (Figure 2). The primary focus of this thesis is concerned with RGCs and their role in visual perception.

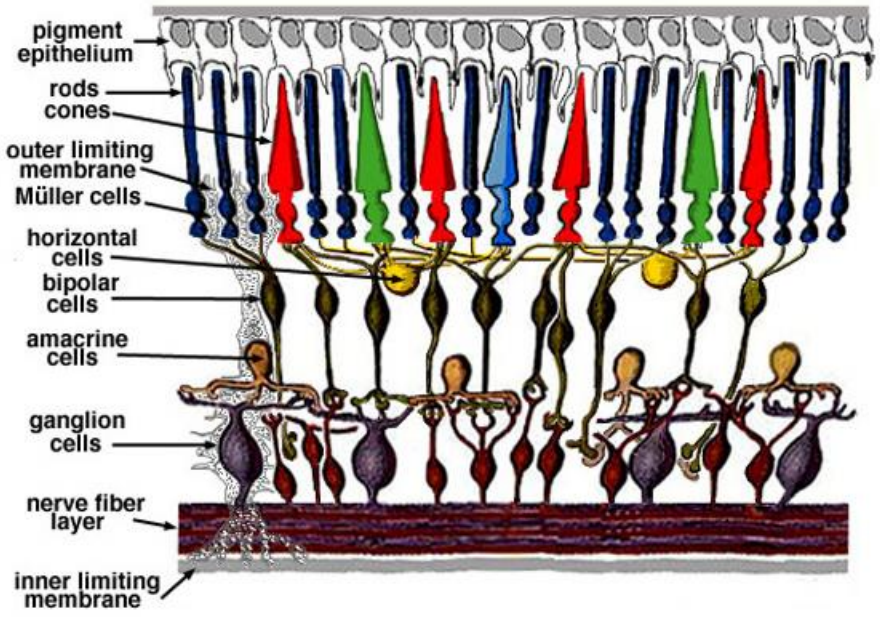

Figure 2.

Anatomy of the retina. A visual signal is converted into an electrical impulse at this layer of tissue. The cells involved in this conversion include: photoreceptors (rods and cones), bipolar cells, horizontal cells, amacrine cells, and retinal ganglion cells. (Image adapated from WebVision at http://webvision.med.utah.edu/imageswv/schem).

Light that enters the eye is directed to a location of the retina called the fovea centralis. It is at this particular spot that the maximal visual acuity can be realized. This is true because it is at this specific location that the greatest number of rods and cones, photoreceptors involved in the

conversion of light into electrical signals, are found. Rods are responsible for dark and dim vision, whereas, cones are responsible for color vision. Typically, rods are found throughout the retina and in the periphery while cones are concentrated more specifically at the fovea.

Once light enters the eye and stimulates the photoreceptors, the visual signal is then transferred to the retinal ganglion cell layer with the aid of the bipolar cells, horizontal cells, and starburst amacrine cells. The bipolar cells are interneurons that are connected vertically to both the photoreceptors and RGCs. The horizontal cells synapse on the photoreceptors and are responsible for regulation of the visual signal. The starburst amacrine cells are found displaced among RGCs and, along with the bipolar cells and horizontal cells, play a role in transmission of the visual signal. The axons of the RGCs form the optic nerve which transfers the electrical signal to the brain where visual processing occurs.

Starburst amacrine cells are of interest in this thesis not only because of their role in the regulation of the visual signal, but also because they serve as the only source of acetylcholine within the retina. This is significant because given that studies have shown acetylcholine to be an important endogenous neurotransmitter linked to the protection of cells within the central nervous system (Thompson et al., 2006). It is this protective mechanism that may in fact suggest another role of starburst amacrine cells, a role devoted to preventing neuron cell death.

Phototransduction

Phototransduction begins when a photon of light isomerizes rhodopsin, an integral membrane protein found within photoreceptors. This isomerization elicits a conformational change in rhodopsin, converting the 11-cis retinal structure to an 11-trans retinal structure. Once activated, rhodopsin stimulates transducin, a GTP binding protein,that subsequently activates

cGMP phosphodiesterase, an enzyme found in the cytoplasm of photoreceptor cell. The activation of cGMP phosphodiesterase results in a decrease in cytoplasmic cGMP which closes cGMP-gated cation channels and hyperpolarizes the photoreceptor membrane, which serves as the beginning of sight, by decreasing the amount of the photoreceptor neurotransmitter, glutamate, from being released (Houbin, 2003).

Photoreceptors are connected vertically to bipolar cells which synapse onto RGCs. There are two types of bipolar cells, OFF cells and ON cells. The difference between the two lies in the type of glutamate receptor expressed on their membrane. The OFF bipolar cells contain an ionotropic glutamate receptor, whereas, the ON bipolar cells express a metabotropic glutamate receptor. In a dark state, glutamate is continuously released and synapses with OFF bipolar cells, thus, depolarizing the bipolar cell membrane. However in a light state, when a photon elicits a response that acts to decrease glutamate release, the OFF bipolar cells become hyperpolarized. However, the ON bipolar cells' response to such a decrease in synaptic glutamate concentration is depolarization which serves to generate an electrical signal that can then propagate to RGCs (Massey and MIller, 1987).

The electrical signal is transferred to RGCs by synaptic glutamate release which elicits RGC depolarization when glutamate is bound to an excitatory kainate receptor on RGCs membrane. The axons of RGCs then converges at the optic nerve head, found at the posterior aspect of the eye, and axons travel to the lateral geniculate nucleus of the thalamus. From this location on the thalamus, the signal is then propagated to the visual cortex, the site of visual perception within the occipital lobe of the brain (Gao et al., 2013).

Glaucoma

Glaucoma is a retinal degenerative disease that results in optic neuropathy, cupping of the optic disk and retinal ganglion cell death, leading to visual field loss and blindness (Guo et al., 2005). High risk groups include African Americans, those over the age of 60, those whose family members were previously diagnosed, and diabetics. Glaucoma is characterized most frequently by a subsequent increase in intraocular pressure (Morrison et al., 1997; Chauhan et al., 2002; Levkovitch-Verbin et al., 2002). This increase in intraocular pressure is believed to be caused by a blockage or back-up within the eyes' filtration system, the trabecular meshwork. Such a block prevents the outflow of aqueous humor, fluid within the anterior chamber of the eye that provides nutrients and oxygen to the eye. Blockage of aqueous humor results in a build-up of pressure within the anterior part of the eye, leading to an overall increase in intraocular pressure. This build up of pressure, pushes on the suspensory ligaments of the lens and applies pressure to the retina, resulting in loss of retinal ganglion cells. However treatment to hinder the effects of glaucoma focus only on reducing intraocular pressure, and in some cases, this treatment proves to be insufficient to prevent the onset and progression of glaucoma, suggesting that other treatments could be developed to prevent the loss of retinal ganglion cells.

Excitotoxicity

Excitotoxicity, or neuronal cell death caused by excessive excitatory neurotransmitters, has been shown to have some correlation with visual field loss characteristic of retinal degenerative diseases such as glaucoma, as well as, retinal ischemia, and diabetic retinopathy (Quigley, 1998, Choi., 1988, Lafuente et al., 2001). In particular, glutamate induced excitotoxicity may be the missing component to understanding glaucoma and retinal ganglion cell death that is not associated with increased intraocular pressure. Glutamate is an excitatory

neurotransmitter released by bipolar cells and photoreceptors within the retina (Ehinger et al, 1988; Marc et al., 1990; Jujich and Pourcho, 1996). In previous research, an excessive amount of glutamate in the vitreous humor within the posterior chamber of the eye showed to propagate an influx of calcium into the retinal ganglion cells through glutamate receptor channels, initiating an apoptotic signaling cascade and leading to retinal ganglion cell death (Olney, 1977; Choi, 1987; Manev et al., 1989). Death of RGCs prevents the visual signal from being conveyed to the brain, thus resulting in visual field loss.

Neuroprotection

One proposed mechanism to protect against excitotoxcity is through neuroprotection of retinal ganglion cells. Neuroprotection is defined as the process of hindering excitotoxic events by inducing a protective mechanism to cease apoptotic cascades. Previous studies have demonstrated that acetylcholine and nicotine can provide neuroprotection against glutamate induced excitotoxicity (Wehrwein et al. 2004). Another study then determined that the specific nicotine acetylcholine receptor, an alpha7 nAChR, mediates neuroprotection (Brandt et al. 2011). However to deduce whether activation of this specific alpha7 nAChR provided neuroprotection of RGCs lost in association with glaucoma, a study was done that used an alpha7 nicotine agonist, PNU 282987, in an *in vivo* rat model experiments. An agonist works by mimicking the effects of nicotine and/or acetylcholine on the nAChR. This study demonstrated that the neuroprotective effects of PNU 282987 under glaucoma-like conditions prevented RGC loss within the retina, signifying that RGC loss is mediated through an alpha7 nAChR (Iwamoto et al. 2013).

The main objective of this Honors thesis project is to more thoroughly test and provide clearer evidence that the alpha7 nAChR is responsible for RGC neuroprotection by testing multiple alpha7 nAChR agonists such as, PNU-282987 and PHA-568487, and the acetylcholinesterase inhibitor, Donepezil. Acetylcholinesterase is an enzyme that acts to degrade acetylcholine from its receptor, thus ceasing acetylcholine's neuroprotective effects on retinal ganglion cells. However, if a specific acetylcholinesterase inhibitor was used to stop this enzyme's activity, by preventing acetylcholinesterase from degrading acetylcholine from the alpha7 nAChR, the hypothesis is that it will result in prolonged protection of the RGCs and prevent retinal degeneration and death.

PNU-282987

PNU-282987 (N-[(3R)-1-azabicyclo[2.2.2]oct-3-yl]-4-chlorobenzamide hydrochloride) is an alpha7 nAChR agonist that was originally designed by Pharmcia and Upjohn for the treatment of schizophrenia with the intent of providing neuroprotection to the neurons in the brain (Bodnar et al, 2005). However, its systemic use was found to cause detrimental effects in the heart by fostering inhibition of essential potassium channels (Walker et al., 2006). Although, systemic application was found to be unviable in treatment against schizophrenia, in glaucoma the use of PNU-282987 in localized application by way of eye drops had proved to be both attainable and effective without the aforementioned detrimental systemic effects.

In previous studies from our lab, bi-daily drops of 1 mM PNU-282987 applied for three days prior to inducing a glaucoma-like environment followed by one month post procedure application, have demonstrated pronounced RGC neuroprotection effects. In addition,when applied as eye drops, no trace of PNU-282987 was found in cardiac tissue, thus, proving

evidence that the previously hindering effects of systemic application is not applicable for consideration when using local eye drops (Mata, 2013).

PHA-56487

PHA-56487 (N-[(3R,5R)-1-azabicyclo[3.2.1]oct-3-yl]furo[2,3-c]pyridine-5-carboxamide) is a specific alpha7 nAChR agonist developed for the therapeutic treatment against cognitive deficits in people with schizophrenia and Alzheimer's disease. PHA-56487 has been shown to be active in both *in vitro* and *in vivo* studies (Acker et al., 2008). It is capable of rapidly penetrating the blood brain barrier and accomplishing effective auditory sensory gating and object recognition in an *in vivo* rat model (Wishka et al., 2006). Due to their specificity to alpha7 nAChRs, both PNU-282987 and PHA-56487 were used in this neuroprotective study.

Donepezil

Donepezil is a selective and reversible acetylcholinesterase inhibitor that is produced by both Pfizer and Eisai under the trade name Aricept. It is used for the therapeutic treatment of mild to moderate Alzheimer's disease. It binds to peripheral anionic sites and delays amyloid plaque deposition by maintaining acetylcholine levels in the brain (Colovic et al., 2013). As the use of Donepezil may enhance the neuroprotective effect of ACh released from the starburst amacrine cells onto RGCs, donepezil was used in this study as well.

Although these drug treatments seem promising, other cellular factors may come into play preventing the desired prolonged neuroprotection. Therefore, the goal of this thesis was to manipulate the amacrine cell to RGC synapse and analyze whether application of drugs such as Donepezil, PNU-282987, and PHA-568487 alone or in combination would result in preventing

retinal ganglion cell death and subsequent blindness in our glaucoma model. Results from these studies could lead to an alternate protective therapeutic treatment to prevent glaucoma.

Method

Animals

Male and female adult Long Evans rats between the ages of 3 to 6 months were used for this study. Animals were purchased from the Charles Rivers Lab in Portage, MI, and held at Western Michigan University's animal facility for research purposes. All animals were handled and cared for under the guidelines set forth by the Institutional Animal Care and Use Committee of Western Michigan University in accordance with IACUC protocol.

Inducing a Glaucoma-like Environment

The procedure to induce glaucoma in Long Evans rats used in this experiment has previously been published by Iwamoto et al. (2014). Rats were anesthetized via a 1ml/Kg interperitoneal injection of KAX, a cocktail consisting of 5 mL of ketamine (100mg/mL), 2.5 mL of xylazine (20mg/mL), 1 mL of acepromazine (10mg/mL), and 3 mL of sterilized water. The procedure was initiated once there was no indication of reflex activity. This was tested by pulling on the hind legs and/or pinching the skin.

To establish a glaucoma-like conditions, 50 µl of 2M NaCl was injected into the episcleral veins of each anesthetized experimental rat's right eye. This creates scar tissue in the trabecular meshwork that decreases the outflow of aqueous humor from the anterior chamber of the eye (Morrison et al., 1997). The effect is a slow build-up of intraocular pressure that mimics

the main risk factor associated with glaucoma. A glass needle, 40 micrometer in diameter, was pulled from a Narishige electrode puller to be used for the injection. The glass needle was attached to tapered polyethylene tubing (PE-50, Clay Adams, Parsippan, NJ) that was then placed into a 23-gauge needle with the tip filed off. The glass needle's tip was then beveled to allow for easy penetration into the episcleral vein upon injection of saline into the right eye of the animals (the left eye was left untreated and used as an internal control). The saline causes blanching within the episcleral vein and was used as an indicator of successful injection. Following the procedure to induce glaucoma, antibiotic cream was applied to the rat's right eye and the rat was returned to its cage where it was monitored until it was fully awake, prior to being placed back within the animal facility.

Drug Application

To determine the effects of PNU-282987, PHA-568487, and Donepezil, one drop of each drug was applied to the right eye of the specified rats (three rats for PNU-282987, 3 rats for PHA-568487, 3 rats for donepezil, and 3 rats for PNU-282987 and Donepezil in combination), twice a day for three days prior to surgery to induce glaucoma. This initiated the preventative neuroprotective mechanism of RGCs in the retinal ganglion layer. Following the procedure to induce glaucoma, PNU-282987 alone was applied as eye drops twice daily to the right eye of 3 rats for three weeks. Bi-daily drop application was deemed necessary based on previous studies that found detectable levels of PNU-282987 in the retina for up to twelve hours after eye drop application (Mata, 2013). The same procedure was conducted with Donepezil, PHA-568487, and Donepezil and PNU-282987 respectively. One exception was animals treated only with donepezil were only treated once each day. This was deemed sufficient based on previous studies

showing detectable levels twenty-four hours following application (Acker et al., 2008, Colovic et al., 2013, and Wishka et al., 2006).

All eye drop solutions were made up as 100 mM using DMSO and then diluted down to 1 mM using PBS for PNU and PHA compounds to 100 μM for Donepezil. Preliminary dose response studies demonstrated that these concentrations of each agent provided the maximal neuroprotective response.

Retina Isolation and RGC Quantification

One month following the procedure to induce glaucoma, animals were euthanized by carbon dioxide asphyxiation and both control and experimental eyes from each rat were surgically removed. A coronal slit was then made in the animals' eyes and surgical scissors were used to cut around the circumference of the eye in such a way that upon completion, anterior and posterior eyecups are made. The anterior eyecup, the lens, and the vitreous humour were discarded and the retina was carefully removed, in one piece, from the posterior eyecup after detaching the optic nerve. Four slits, 90 degrees apart, were then made at the periphery of all four quadrants of the retina in order to flat mount the retinas onto sylgard plates. Cactus needles were used to pin the retinas to the plates. The retinas were then fixed with 10% formalin overnight at 4 degrees C. Following fixation, the retinas were then rinsed with PBS and incubated with a specific antibody agonist Thy 1.1 in PBS (1:300 dilution) containing .02% saponin for one week at 4 degrees Celsius in a dehumidified chamber. Thy 1.1 is a mouse anti-rat IgG glycoprotein found exclusively on retinal ganglion cells in the retina (Barnstable and Drager, 1984).

Following the conclusion of one week, the retinas were then washed three time with PBS and incubated in a secondary goat antibody, Alexa Fluor 595 (1:300 dilution), for 5 days at 4

degrees C. for microscopic viewing purposes. Alexa Fluor 595 is a goat anti-mouse IgG (Invitrogen Molecular Probes). After 5 days, the retinas were then rinsed three times with PBS and transferred to glass slides with coverslips and mounted using a 50% glycerol and 50% PBS solution.

After fluorescently labeling RGCs, they were viewed using the 60 x objective lens of a Zeiss confocal microscope from each quadrant of the retina. Images were obtained in 1 micron increments transversely through the retinal ganglion cell layer at a location of 400 micrometers from the ONH, (optic nerve head) (Figure 3). Four images were taken from each quadrant of each retina and averaged to obtain the most accurate RGC survival data using Metamorph software. Thy1.1 labeled RGCs were counted using an 100 micrometer frame in Metamorph software. The total frame area was divided by the total cell area (axons were not included in this number) to obtain a percentage that was then applied to the visible cell count to determine the theoretical cell count. This method was used to ensure that the cells covered by axons and subsequently not visualized were accounted for.

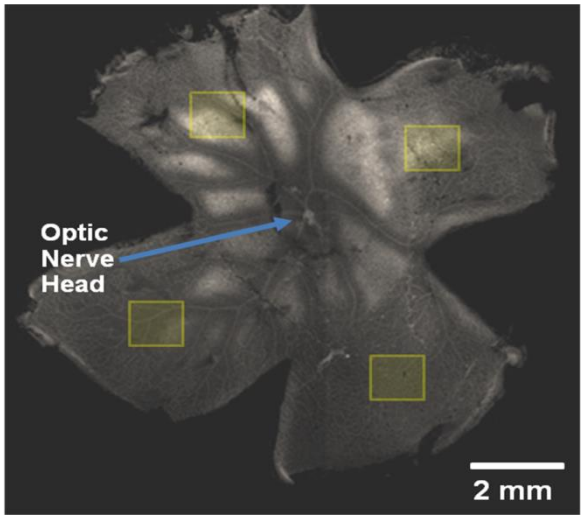

Figure 3 Image of a Flat Mount Retina.

The yellow boxes represent the areas 4 mm from the optic nerve head where RGC images were obtained.

Statistical Analysis

To analyze the effects of the drug treatments, RGC counts in experimental eye retinas were compared to their respective internal control retina using Student T-tests. All values were documented with the mean standard error of the sample-mean. A value of $p < 0.05$ was considered statistically significant.

Results

Effects of Glaucoma Induction on RGC

In order to determine the effects on RGCs in a glaucoma like environment, the episcleral vein of the right eyes of the tested rats were injected with 50 µl of 2mM NaCl. This fostered scar tissue formation in the trabecular meshwork, which served to increase intraocular pressure, a main risk factor associated with glaucoma. One month following saline injection, the retinas were removed and processed with antibodies against Thy1.1 glycoprotein, a protein found on the RGC plasma membrane. The RGCs were then counted and compared to their respective left internal controls. It was found that glaucoma induction surgery alone caused a 26% (± 1.5, N=3) decrease in RGCs (figure 5). This is a statistically significant difference from the control eye.

Effects of PNU-282987 on RGC

In order to determine the effects of PNU-282987 on RGC in an induced glaucoma state, 1mM of PNU-282987 was applied to right experimental rat eyes in a bi-daily drop-wise manner for three days preceding glaucoma induction surgery and for three weeks following surgery. One month after surgery, the retinas were removed and processed with antibodies against Thy1.1

glycoprotein, a protein located on the RGC plasma membrane. The RGCs were then counted and compared to their respective left internal control. It was found that after 1 mM of PNU-282987 drop-wise treatment, there was only a 7% (± 2.8, N=3) decrease in RGC total (Figure 4). This is a statistically significant difference from the control eye. The collected results show a pronounced improvement from the 26% decrease in RGC counts that normally follows glaucoma induction surgery (Figure 5) (Mata 2013).

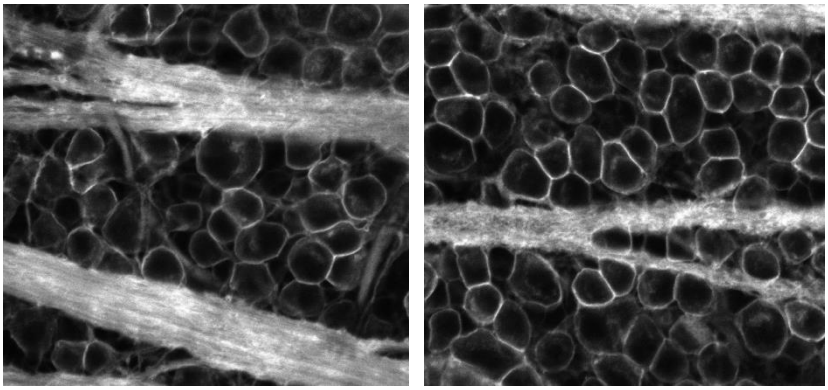

Figure 4. Right and Left Eye Images from a Glaucoma Induced Rat treated with PNU-282987

The right image is the experimental retina, the eye that had undergone glaucoma induction and PNU-282987 treatment. The left image is the left eye and the internal control. This eye did not undergo glaucoma induction or PNU-282987 treatment.

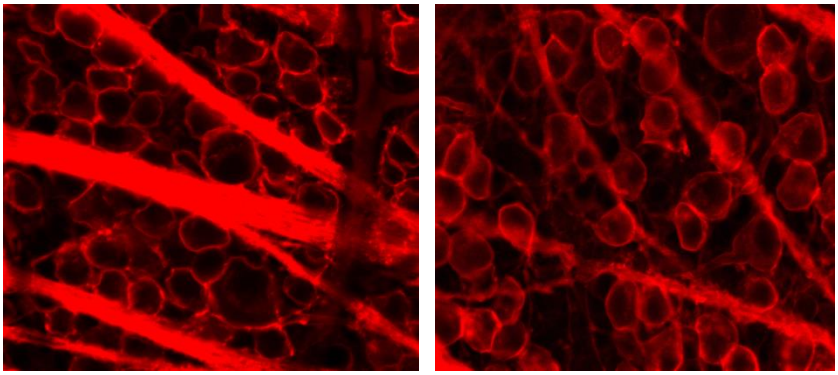

Figure 5. Right and Left Eye Images from a Glaucoma Induced Rat with No Treatment

The right image is the experimental retina, the eye that underwent glaucoma induced surgery without receiving treatment. The left image is the internal control retina, the eye that did not go through glaucoma surgery and did not receive any treatment.

Effects of PHA-568487 on RGC

In order to determine the effects of PHA-568487 on RGC in an induced glaucoma state, 1 mM of PHA-568487 was applied to the right experimental eyes of rats in a daily drop-wise manner for three days prior to surgery to induce glaucoma and for three weeks following surgery. One month after surgery, eyes were removed and retinas were processed with antibodies against a Thy1.1 glycoprotein on the RGC membrane. RGCs were counted and compared to internal controls. It was found that 1 mM of PHA-568487 had provided a 43% (± 21, N=3) increase in RGC. This is a statistically significant difference from the untreated control counts. The collected data shows that PHA-568487 alone would be sufficient in preventing RGC loss in a glaucoma-like environment. However, this data also suggests that PHA-568487 may be capable of fostering a proliferation-like effect on RGC given the substantial increase in cell counts.

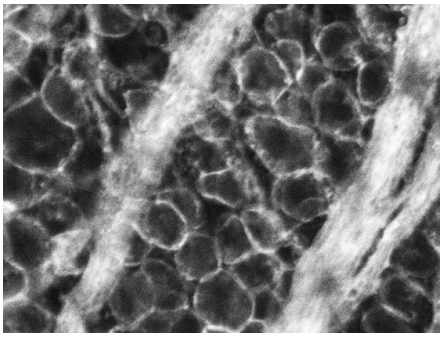

Figure 6. Right and Left Eye Images from a Glaucoma Induced Rat treated with PHA-568487

The right image is the experimental retina, the eye that underwent surgery to induce glaucoma and PHA-568487 treatment. The left image is the internal control retina, the eye that did not undergo glaucoma induction surgery and did not receive PHA-568487 treatment.

Effects of Donepezil on RGC

In order to determine the effects of Donepezil on RGC in a glaucoma-like environment, 100 micromoles of Donepezil was applied to the right experimental eyes of rats in a daily drop-wise manner for three days proceeding glaucoma inducing surgery and for three weeks following surgery. One month after surgery, eyes were removed and retinas were processed with antibodies against Thy1.1 glycoprotein found on the membrane of RGC. RGCs were counted and compared to internal controls. It was found that 100 micromoles of Donepezil produced a 36% (± 11, N=3) increase in RGC numbers (Figure 7). This is a statistically significant difference from untreated control counts. The collected data is evidence that Donepezil alone is sufficiently capable of protecting against RGC degradation in a glaucoma[illegible]dition to this protection, the data also suggests that Donepezil [illegible]iferation-like effect within the retina.

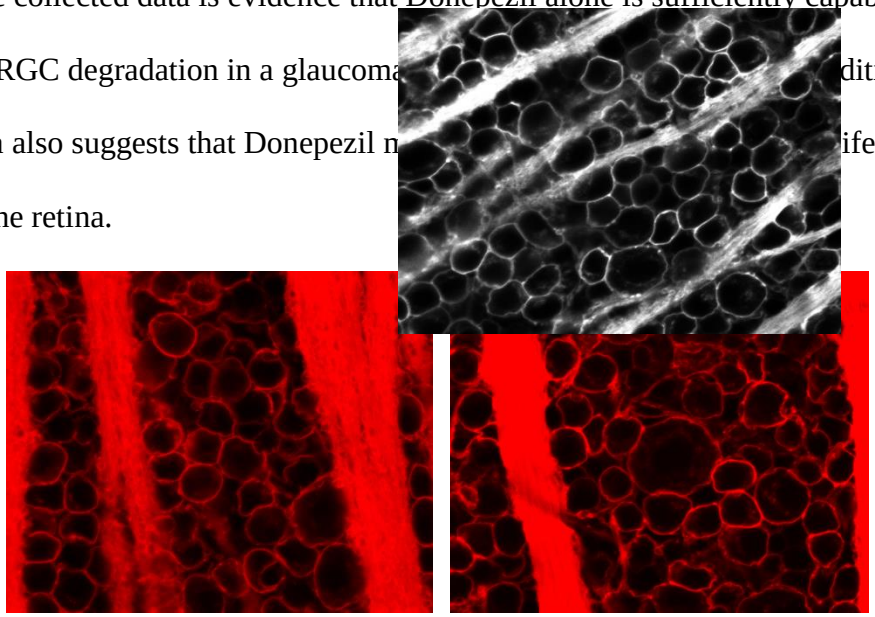

Figure 7. Right and Left Eye Images from a Glaucoma Induced Rat treated with Donepezil

The right image is the experimental retina, the eye that underwent glaucoma induction surgery and Donepezil treatment. The left image is the internal control retina that did not undergo surgery or treatment.

Effects of Donepezil and PNU-282987 on RGC

In order to assess the combined neuroprotection of an acetylcholinesterase inhibitor and an alpha7 nAChR agonist on RGC in a glaucoma induced environment, a cocktail of 100 micromoles of Donepezil and 1 mM of PNU-282987 was applied in a daily drop-wise manner to

the right experimental eyes of rats for three days prior to glaucoma induction surgery and for three weeks following surgery. One month after surgery to induce glaucoma, eyes were removed and retinas were processed with antibodies against Thy1.1 glycoprotein on the RGC membrane. RGCs were counted and compared to internal controls. The combined effects of Donepezil and PNU-282987 provided a 3.7% (± 6.5, N=3) increase in RGC counts (Figure 8). This is a statistically significant difference when compared to internal control counts. This data suggests that the combination of an alpha7 nAChR agonist and acetylcholinesterase inhibitor was effective at preventing RGC degradation in a glaucoma-like environment. The slight increase in RGC was not significantly different from control untreated counts.

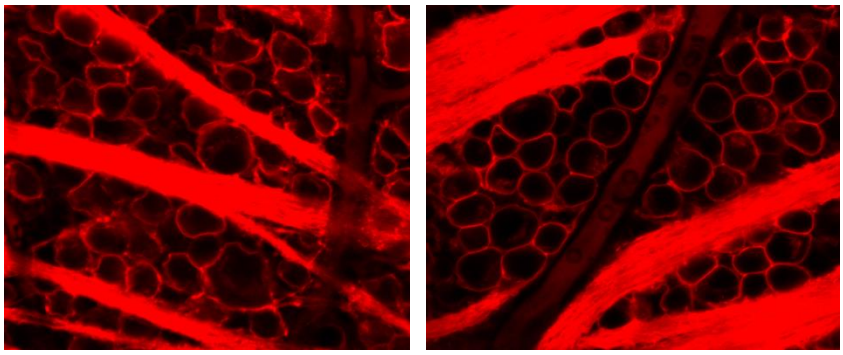

Figure 7. Right and Left Eye Images from a Glaucoma Induced Rat treated with Donepezil and PNU-282987.

The right image is the experimental retina, the eye that underwent glaucoma induction surgery and treatment with Donepezil and PNU-282987. The left image is the internal control retina, this eye did not undergo glaucoma induction surgery or treatment.

The aforementioned results of the pharmacological drop treatments used in this study are easily visualized and conveniently summarized in Figure 8, which shows the respective increase and decrease in RGC numbers when compared to internal controls (x axis).

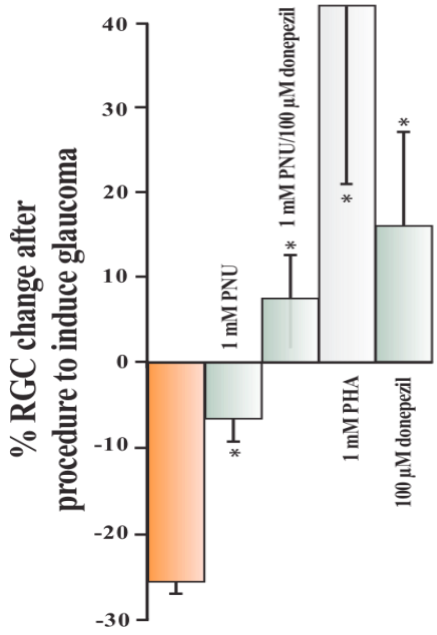

Figure 8. Percentage of RGC Damage After Procedure to Induce Glaucoma.

Rats that underwent the procedure to induce glaucoma had a 26% decrease of RGC. Rats that underwent the glaucoma-induction procedure and treatment of 1 mM PNU-282987 had only a 7% decrease of RGC. However, rats that underwent the glaucoma-induction procedure and treatment of 1 mM of PNU-282987 with 100 micromoles of Donepezil, 100 micromoles of Donepezil alone, and 1 mM of PHA-568487 all showed an increase in RGC of 3.7%, 36%, and 43% respectively. This increase appears to be suggestive of a proliferation-like effect on RGC subjected to the aforementioned treatment in that more cells are accounted for than what was visualized in their respective internal controls.

Discussion

All of the tested pharmacological drops provided some sort of protection against RGC loss that is typically of the glaucoma inducing treatment. However, some of the treatments went beyond protection and showed evidence of proliferation. Being that the cells under analysis are adult mammalian neurons which typically do not go through mitotic division, this concept of proliferation of retinal ganglion cells was unexpected. However after further research, it was discovered that the noted proliferation-like effect upon activation of an alpha7 nAChR is not an entirely novel finding. In fact, previous studies have reported similar observations with an alpha7

nAChR found associated with neurons in the granular cell layer of the hippocampus and olfactory bulb.

In one study, both Donepezil and PHA-568487 were used to analyze neuron proliferation. In this study, it was found that PHA-568487 was successful at promoting proliferation in the granular cell layer of the dentate gyrus (an area of the hippocampus). However, no such proliferation or survival effect was noted with the administration of Donepezil (Kita et al., 2014). Whereas the former finding on the proliferation potential of PHA-568487 is consistent with the findings in this thesis, the latter data is inconsistent with both this study's findings and a previous study's findings that analyzed both acute and chronic Donepezil treatment in a stress-induced rat brain model.

In the stress-induced rat brain model, rats were placed in close-fit cylinder restrainer for 6 hours every day for 4 weeks to mimic a stressful environment, Donepezil (both acute or short-lived treatment and chronic or long-term treatment) was administered, and neurons in the dendate gyrus and olfactory bulb areas of the brain were counted. It was found that chronic Donepezil treatment promotes the survival of newborn neurons in a stress-induced state but does not effect the proliferation of progenitor cells (pluripotent stem cells). On the other hand, acute Donepezil treatment showed to increase cell proliferation but had no long term effects on newborn neurons' survival (Kaneko et al., 2006).

It was hypothesized that these contrasting results may be in relation to treatment duration. Immediately following Donepezil treatment, the extracellular ACh concentration is greatly increased, thus fostering acute proliferation-like effects. However over time with chronic Donepezil treatment, these effects may become less significant and equilibrated by the down-regulation of other AChRs found on the neuron membrane (Kaneko et al., 2006). Although this

thesis's focus did not analyze varying treatment durations with Donepezil or other pharmaceutical drops, the results of the Donepezil study on neuron survival and proliferation potential are consistent with this thesis's results, which essentially shows that Donepezil and other drop treatments can protect against neuron loss in a stress-and/or glaucoma-induced state.

The mechanism behind the initiation of an additional mitotic division or proliferation is not currently known. However, three out of four of the specific drop treatments tested in this thesis had suggested some sort of proliferation-like effects. These findings were noted in treatment regimens that include: PHA-568487, Donepezil, and PHA-282987 in combination with Donepezil. In fact, the only treatment used that did not show evidence of proliferation was found in the rats who received 1 mM of PNU-282987 drop-wise solution.

When comparing the experimental retinas of Figure 4 and 5, it is evident that the RGC density is greater in the retinas that had received the PNU-282987 treatment (Figure 4) then in the retinas that had not. In addition, the RGCs of the eye that had not received treatment (Figure 5) appear less round and uneven in comparison to the treated eye (Figure 4). These observations suggest that PNU-282987 provides some level of neuroprotection against glaucoma. This scenario was validated based on the data that shows that PNU-282987 prevented the average loss of 17% of RGCs normally lost during the glaucoma inducing procedure. However, complete protection was not provided by PNU-282987 alone, thus suggesting that 1 mM of PNU-282987 is incapable of eliminating RGC loss in a glaucoma state. These findings are consistent with a previous study in the lab that showed that 1mM of PNU-282987 caused 6.9% RGC loss when compared to internal controls. However, this previous study also analyzed the effects on RGCs following 10 mM of PNU-282987 and it was found that with 10 mM of PNU-2282987, there

was a 13.5% increase in RGC density, thus suggesting that PNU-282987 is capable of fostering proliferation if a higher concentration is utilized (Mata 2013).

Contrary to these PNU-282987 results, PHA-568487, another alpha7 nAChR agonist, was analyzed for neuroprotective effects and showed an increase of 43% in RGC count. When compared to PNU-282987, which showed a moderate decrease in cell loss but no increase in cell numbers, PHA-568487 may prove to be an even better and more specific target for neuroprotection of retinal ganglion cells. These findings are consistent with the aforementioned study on brain tissue, which showed proliferation-like effects in cells treated with PHA-568487 (Kita et al., 2014).

In addition to assessing the effects of two different alpha7 nAChR agonists, Donepezil a acetylcholinesterase inhibitor, was also tested. Donepezil functions to prolong agonist binding to the alpha7 nAChR and it was suspected that by using Donepezil in conjunction with an alpha7 nAChR agonist, an even greater neuroprotective and possible proliferation effect might ensue. However before this study was done, it was necessary to assess the effects of Donepezil alone in order to establish a baseline marker that would allow for better stratification of its effects when used with an alpha7 nAChR agonist.

Donepezil by itself, had a larger effect of RGC survival than PNU-282987. After PNU-282987 treatment, there was an average RGC loss of 7% compared to Donepezil's 36% increase in RGCs. The combination treatment of Donepezil and PNU-282987 was not as effective as Donepezil alone, as the combined treatment only resulted in a 3.7% increase in RGCs. The subsequent next step would be to assess the combined effects of Donepezil and PHA-568487 and analyze varying concentrations of PNU-282987, alone and with Donepezil, in order to, determine if a better neuroprotective and proliferation-like effect is found. However due to both

time constraints and resources, the combination of PHA-568487 and Donepezil was not evaluated in this study.

Future Application

As previously mentioned, the next step would be to complete this study by analyzing the combined effects of Donepezil and varying concentrations of both PNU-282987 and PHA-568487 to develop a better idea as to what concentrations provide the most positive proliferative and neuroprotective effect on retinal ganglion cells. From there, a closer analysis of the specific mechanisms behind both the survival and proliferation effects is necessary to develop the best treatment against neurodegenerative diseases, such as, glaucoma.

In addition, further investigation into the identity and capabilities of what appears to be proliferated cells is warranted. If these cells are in fact retinal ganglion cells, then one is left to question as to whether these cells function in accordance with RGC. Are these additional cells capable of relaying the electrical impulse initiated by a visual stimuli to the brain as part of visual processing? At some point, a visual test would be necessary to determine whether the retinal ganglion cells protected by the pharmacological drops in this study are actually improving, or at the very least, sustaining one's visual potential.

Lastly, seeing that glaucoma is one type of neurodegenerative disease, future aims should be directed toward identifying the similarities and differences between the retina and the brain in hopes of discovering better treatment and/or a cure for neurodegeneration. Other neurodegenerative diseases that would be targeted include: Alzheimer's and Parkinson's.

The results of this study show that PNU-282987, PHA-568487, and Donepezil, separately and in combination, are capable of providing neuroprotection against the onset of glaucoma. However as previously mentioned, further dosage and behavioral studies are warranted (Mata 2013; Iwamoto et al., 2014). Nonetheless, the pharmacological drops in this study have the potential to serves as a novel drop-wise treatment option to prevent against, and possibly, with further research, one day reverse, the onset of glaucoma, thus, improving the vision and overall livelihood of the sector of our population that this life-altering disease affects.

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