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Neuroprotective Effects of a Nicotinic Acetylcholine Receptor Agonist and Modulator in the Rodent Retina

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NEUROPROTECTIVE EFFECTS OF A NICOTINIC ACETYLCHOLINE RECEPTOR AGONIST AND MODULATOR IN THE RODENT RETINA

by

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NEUROPROTECTIVE EFFECTS OF A NICOTINIC ACETYLCHOLINE RECEPTOR AGONIST AND MODULATOR IN THE RODENT RETINA

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The purpose of this dissertation is to assess the potential neuroprotective effect of an alpha7 nAChR agonist, PNU-282987, and modulator, PNU-120596, in an *in vitro* model of excitotoxicity and an *in vivo* model of acute glaucoma. In *in vitro* studies, retinas obtained from Long Evans rats were cultured using various concentrations of the PNU compounds to analyze neuroprotection against glutamate-induced excitotoxicity. After 3 days in culture, RGCs were identified using an antibody against Thy 1.1, visualized using a fluorescent dye and quantified. In culture, glutamate significantly decreased the number of RGCs. However, if either PNU compound was introduced before the glutamate insult, higher survival of cells was observed. In *in vivo* studies, PNU compounds were injected intravitreally to determine their potential neuroprotective effect in a rat glaucoma model. Following treatments with PNU compounds, surgery was performed to induce glaucoma-conditions by injecting 0.05ml of 2mM NaCl into the episcleral veins of right eyes in each rat. The left eye in each rat was left untreated. After one month, rats were euthanized, retinas were removed, flat mounted, fixed and nuclei were stained with cresyl violet or immunostained with an antibody against Thy 1.1. Stained nuclei in the RGC layer in glaucoma-induced retinas were counted and compared to cell counts from untreated retinas. The surgery to induce glaucoma-like conditions

caused a significant loss of cells in the RGC layer within 1 month after surgery. However, this effect was eliminated if either PNU compound was injected into the right eye an hour before surgery. The results from this thesis support the hypothesis that the alpha7 agonist, PNU-282987 and modulator, PNU-120596, have a neuroprotective effect in the rat retina. PNU-282987 and PNU-120596 may be viable candidates for future therapeutic pretreatment of glaucoma.

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SPECIFIC AIMS

Glaucoma is a degenerative retinal disease characterized by loss of vision due to the progressive loss of retinal ganglion cells (RGCs). In several *in vitro* models of glaucoma, death of RGCs is induced by excessive neurotransmitter that triggers excitotoxicity and apoptosis. Recently, a great deal of research has explored agents and mechanisms that provide neuroprotection against excitotoxicity. Neuroprotection is a process where neurons are protected from injury or degeneration. In previous studies from this lab, both acetylcholine (ACh) and nicotine have been found to be neuroprotective in the retina and act to prevent glutamate-induced excitotoxicity in an isolated cultured pig RGC preparation (Wehrwein et al., 2004). Other pharmacological studies using pig cultured RGCs have demonstrated that activation of specific α7 nicotinic acetylcholine receptors (nAChRs) are linked to this neuroprotection (Wehrwein et al., 2004; Thompson et al., 2006). However, it is unclear if these α7 nAChRs have any significant neuroprotective role against RGC loss associated with glaucoma. To address this issue, the α7 nicotinic agonist, PNU-282987, and the α7 nicotinic allosteric modulator, PNU-120596, will be analyzed in an *in vitro* model of excitotoxicity and

an *in vivo* rat model of glaucoma to test the hypothesis that introduction of α7 nicotinic agonists or modulators can reduce the loss of cells in the RGC layer that is normally associated with glaucoma. To test this hypothesis, 2 specific aims were designed.

1) **To determine if the α7 nicotinic agonist, *PNU-282987*, has a neuroprotective effect in the rat retina**

Recent pharmacological advances have developed PNU-282987, an agonist specific for the α7 nicotinic receptor. In this study, I will introduce this specific α7 nicotinic agonist to cultured rat cells and to an *in vivo* model of glaucoma in the rat to determine if PNU-282987 reduces the cell death normally associated with excitotoxicity (*in vitro* model) and to determine if PNU-282987 reduces the loss of cells in the RGC layer normally associated with the *in vivo* rat glaucoma model. In the *in vitro* model, rat retina will be dissociated and cultured in the presence or absence of PNU-282987 compound before inducing excitotoxicity with excessive glutamate. After 3 days, rat RGCs will be identified, counted and compared to untreated conditions using a fluorescently labeled antibody against Thy1.1 (a glycoprotein on RGCs in the retina). In the *in vivo* model, PNU-282987 will be

injected into the vitreous of a rat eye prior to inducing glaucoma-like conditions. Glaucoma-like conditions will be induced in the right eye of each rat used in the study by injecting hypertonic saline into the episcleral vein according to the method described by Morrison et al. (1997). This technique creates scar tissue and increases intraocular pressure, which is the primary risk factor associated with glaucoma. Using this method, significant loss of cells in RGC layer occurs within one month (Morrison et al., 1997). After a month, the retina of the control left eye and the glaucoma-induced right eye will be removed, cells in the retina will be stained with cresyl violet or an antibody against Thy 1.1 and the number of cells in the RGC layer will be counted and compared.

2) To determine if the α7 modulator, PNU-120596, enhances the neuroprotective effect of PNU-282987 on rat RGCs.

The compound PNU-120596 has been shown to be an allosteric modulator that binds to the α7 nicotinic receptor (Hurst et al., 2005). In previous *in vitro* studies using pig isolated RGCs, this modulator, in conjunction with PNU-282987, has been shown to enhance the neuroprotective effect of PNU-282987 alone (Linn C. et al., 2011). In this study, *in vitro* studies will be duplicated in the rat using a

combination of the α7 agonist and modulator to determine if the modulator enhances the neuroprotective effect described in pig studies. The combination of the α7 PNU agonist and modulator will also be used in the *in vivo* rat glaucoma model to determine if the modulator enhances the effect of the agonist and provides neuroprotection against loss of cells in the RGC layer normally associated with glaucoma. The results from these studies provides evidence that ACh-induced neuroprotection against glutamate-induced excitotoxicity in cultured rat RGCs is mediated through α7 nicotinic receptors and that loss of cells in the RGC layer due to glaucoma-like conditions can be prevented with α7 PNU compounds. These are the first studies that analyze the effects of these α7 agents in an *in vivo* model of glaucoma.

INTRODUCTION

The Retina

The sense of vision is one of the fundamental senses we use to perceive the world around us. The neuronal part of the eye, the retina, converts photons into electrochemical signals. For the light to reach the retina, it must pass through several parts of the eye before being detected in the retina. Figure 1 illustrates the anatomy of the vertebrate eye. Light must pass through the anterior parts of the eye before it reaches the photoreceptors in the retina where phototransduction occurs. Photons must first pass through the cornea, which is modified sclera. The cornea works to protect the anterior parts of the eye and to refract light into the eye. The iris, which is characterized as the colored pigmented part of the eye, is a group of muscles that define the size of the pupil to adjust the amount light that enters the eye. After light passes through the pupil and lens, majority of the signal it is focused onto the fovea. The fovea contains the highest concentration of cone receptors and produces the highest visual acuity. The lens is controlled by a group of muscles called the ciliary body. When the ciliary body contracts, the lens becomes convex; when it relaxes the lens becomes more flat. The changing shape of the lens allows us to focus on things at different distances, a process called accommodation. The

ciliary body also plays an important role in the eye by producing aqueous humor. Aqueous humor is a liquid that provides oxygen and nutrients to the anterior chamber of the eye. The pressure produced by aqueous humor plays an important role in our visual health.

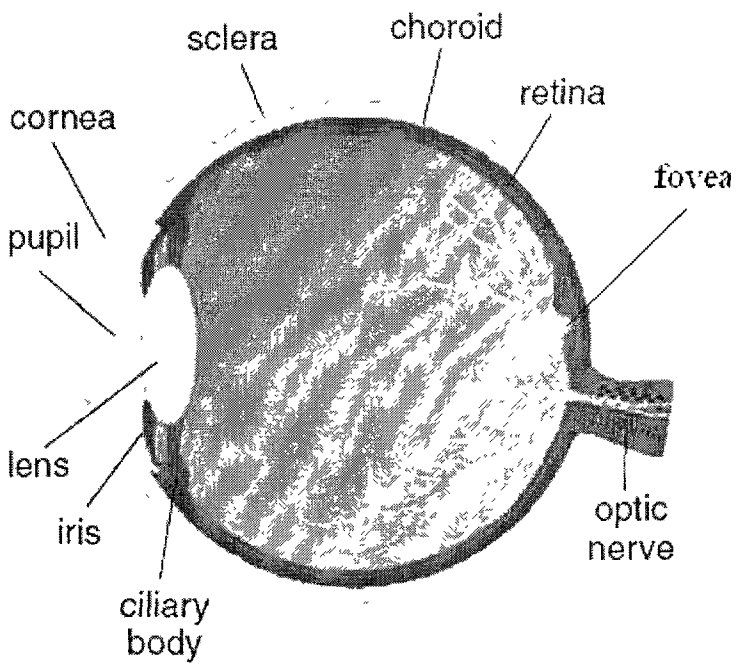

Figure 1: Anatomy of the eye

Light must pass through the cornea and lens before it is focused on the fovea of the retina. (Modified from Kolb, 2003)

The retina is part of the central nervous system and is located at the back of the eyeball. Its main function is to receive light information and to transduce it into electrochemical impulses and send that information into the brain. Light that enters the eye is converted into electrical signals in the photoreceptors. In the mammalian

retina, the photoreceptors can be divided into 2 different types, rods and cones. Rods have broad rod-shaped cell bodies and are used for dark and dim vision. The other type of photoreceptor is called the cone, which has a smaller conical shaped head and is used for color vision. In the human, the cones can be separated into 3 different types depending on the color of light that is perceived, blue, green, and red. Rods are located throughout the retina with more rods around the periphery, whereas cones are centralized and concentrated on the fovea of the eye. Rods are more numerous than cones in the human retina, containing approximately 120 million rod and 6 million cones.

Compared to the human trichromatic vision, rats have 2 types of cones, blue and green, but lack the red cones (Jacobs et al., 2001). This means rats are virtually blind to the color red and relatively longer wavelengths. However, rats can see into the ultraviolet range, which humans cannot see, as a result of blue cones that perceive shorter wavelengths (Jacobs et al., 1991).

Rats in general are considered to have relatively poor vision. Visual acuity is measured by the ability to distinguish between distinct small details of an object. The unit for this measurement is called cycles per degree (CPD), and in humans this number is around 30, compared in 1 CPD in Long-Evans rats (Prusky et al., 2000). This translates to rat vision being 20/600 compared to a normal human's 20/20

vision. However, even though their visual acuity is not as good as human eyes, the rat model remains an excellent model to analyze retinal diseases that affect RGCs, as their retinas have similar tissue structure and neurotransmitters as human retina.

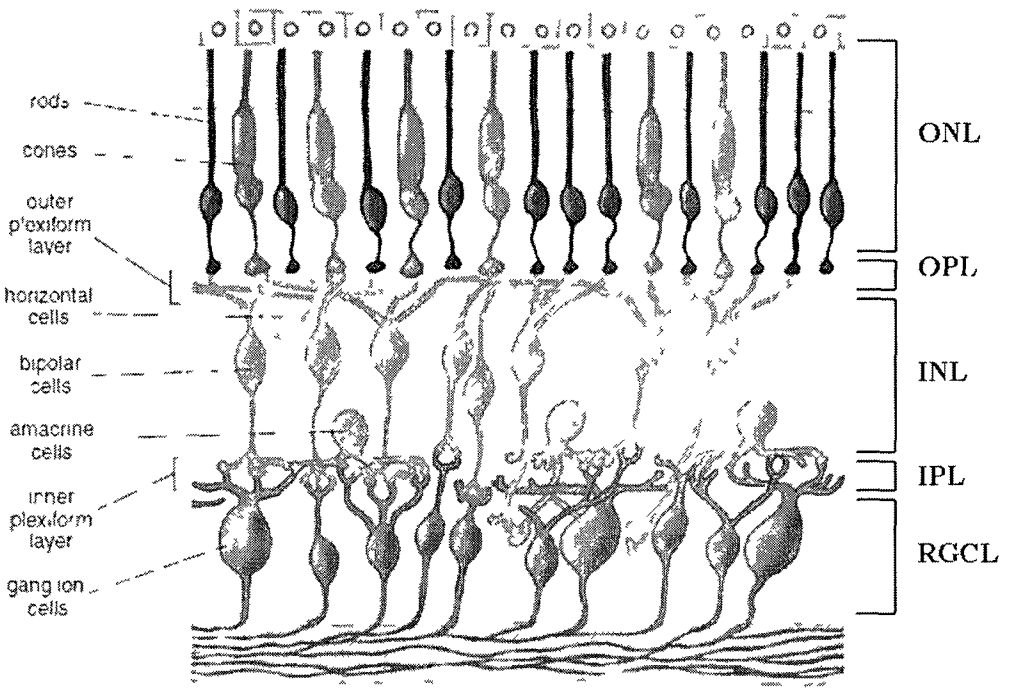

Figure 2: Schematic of mammalian retina

This figure illustrates the connection and different layers formed by neurons in the vertebrate retina. Each cell type works to pass the light signal from the photoreceptors through the layers of the retina to the brain. Some are directly connected vertically while others work to modulate the signal as it travels through the retina. (Modified from Kolb, 2003)

Within the retina, the photoreceptors are located in the deep layer of the retina, situated against the pigment epithelium. All vertebrate retinas can be divided into 3 nerve cell layers and 2 synaptic layers (fig. 2) The outer most cell layer,

called the outer nuclear layer (ONL), is where the photoreceptors are located. This layer transduces the light signal into electrochemical signals and transmits it through the retina. This process of the photoreceptors converting light signal into electrochemical signals is called phototransduction. From the outer most layers where the photoreceptors are located, the rest of the layers through the retina are defined as the outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL) and the retinal ganglion cell (RGC) layer. The RGC layer contains cell bodies of the RGCs and is the last layer to receive before sending visual information to the brain. The axons from the RGCs form the optic nerve and send the signals to the brain.

The plexiform layers are where synaptic connections occur. The first of these plexiform layers is the OPL where the synapse of photoreceptors and bipolar and horizontal cells occur. The bipolar cells are connected between the vertical processes, while horizontal cells are horizontally located within the OPL layer (Fig 2). The bipolar cells are interneurons that connect the photoreceptors to RGCs. Horizontal cells synapse with photoreceptors within the OPL to help regulate the signal and are responsible for surround inhibition in the outer retina.

Within the INL, cell bodies of horizontal, bipolar, and amacrine cells work together to process the visual signal. The IPL is where these inner neurons synapse

onto ganglion cells, the next order of neuronal cells. The last layer or organization in the retina is called the RGC layer and contains the ganglion cells and a small percentage of displaced amacrine cells (Perry et al., 1980; Keyser et al., 2000).

Many retinal diseases, including glaucoma and ischemia, affect the RGC layer and all retinal diseases that damage these cells result in loss of vision and can lead to blindness (Selles-Navarro, 1996). Since the RGC layer is the last layer to receive information before the information is sent to the brain, protecting the RGCs from apoptosis due to disease is a relevant area of research directed towards preventing blindness. This is also the focus of my research.

There are 2 major functional types of RGCs, characterized by their cell body size and their projections to the lateral geniculate nucleus (LGN), the primary processing center in the brain for visual information. The small RGCs have relatively small cell bodies and relatively short dendrites. These types of RGCs projects into the parvocellular layer of the LGN and responds mainly to color changes and not to contrast, which suggests their role in color vision. The larger type of RGC is called parasol cells and projects their axons mainly into the magnocellular layer of the LGN. Parasol cells are used for contrast detection and are not very sensitive to color changes.

From the LGN, the visual information is sent to the visual cortex. Each

hemisphere of the brain contains a region designated as the visual cortex, which is divided into the primary visual cortex (V1), and extrastriate visual cortical areas, V2-V5. From the V1, the visual information is split into 2 pathways, the dorsal and the ventral stream. The dorsal stream, which is also called the "where pathway", goes to the V2 and V5 pathway and is associated with visual location and motion. The ventral stream, which is also called the "what pathway", is associated with object recognition.

The eye is considered to be an extension of the central nervous system (CNS) as the tissue is derived from central neural tissue during development. Many CNS treatments used in the brain for Alzheimer's, Parkinson's and other neurodegenerative diseases are applicable to the retina because of this fact. In my experiments I will be focusing on neuroprotection in the RGC layer and use this layer of the retina to quantify cell survival or cell loss and will propose treatments that specifically affect cell survival in the RGC layer.

Phototransduction

The photoreceptors are responsible for phototransduction, which is the process where photons are converted into electrical signals. When stimulated with a photon of light, vertebrate photoreceptors respond with a hyperpolarization. The

key molecule responsible for phototransduction is called rhodopsin, which consists of opsin, a G protein-coupled receptor and an aldehyde of vitamin A, called 11-cis retinal (Hubbard and Wald, 1952).

When photons of light hit this pigment molecule, the retinal is converted from 11-*cis* to all-*trans* retinal (Mcbee et al., 2001). This conformational change starts a signaling cascade known as phototransduction. In this process, opsin that is bound to the retinal can no longer fit because of the conformational change and rhodopsin changes to metarhodopsin II. This in turn activates transducin, which is responsible for activating phosphodiesterase. Phosphodiesterase plays a role in controlling cGMP levels and when activated, hydrolyzes cGMP into GMP. This in turn decreases the cGMP concentration which in turn closes cGMP-gated channels. The closure in the non-specific cGMP-gated channels causes a hyperpolarization of the cell in the vertebrate retina, decreasing the influx of sodium and calcium through the channels (Baylor, 1996). The resulting hyperpolarization of the photoreceptors decreases the amount of the photoreceptor neurotransmitter, glutamate, to be released.

The photoreceptors are synaptically connected to bipolar cells, which make synaptic connections onto RGCs. In between these direct vertical connections are horizontal and amacrine cells that are involved in lateral inhibition. The bipolar

cells that bind to glutamate can be depolarized or hyperpolarized. The bipolar cells that depolarize to glutamate released from photoreceptors are considered off-bipolar cells whereas the ones that hyperpolarize in the presence of glutamate are called on bipolar cells. The 2 different types of bipolar cells contain different types of glutamate receptors. The off-bipolar cells contain AMPA/kainate receptors which are ionotropic glutamate receptors and the on-bipolar cells have metabotropic glutamate receptors that are linked to a type of G-protein coupled receptor (Dowling 1969).

At least 25 types of amacrine cells have been identified in mammals based on the variety of neurotransmitters located in the cells (Vaney et al., 1989; Mariani, 1990; Kolb et al., 1992). Some amacrine cells release neurotransmitters like GABA, dopamine and serotonin while others release peptides such as somatostatin (Vaney, 1986; Ishimoto et al., 1986). In this study, I am particularly interested in the starburst amacrine cells that release ACh, as we have previously demonstrated that ACh can induce neuroprotection in cultured pig studies (Wehrwein et al., 2005).

The photoreceptors, on- and off-bipolar cells, and lateral inhibition from the horizontal and amacrine cells, produce what it called a receptive field at the RGC level. The receptive field of the visual system is shaped in an annular manner where stimulation of the middle inner ring of the receptive field produces a response

opposite to responses recorded when the outer ring of the receptive field is stimulated. There are 2 types of receptive fields found in RGCs. The "on" center receptive field responds best when light is shined on the center of the ring but does not react to light that is shined onto the outer ring. The "off" center receptive field on the other hand responds maximally to light that is shined onto the outer ring (Fig 3). This receptive field structure produces size selectivity as well as a spatial selectivity. Each RGC is tuned to respond to a certain-sized light stimulus and has a preference to the direction of the light stimulus. This preference in direction of light is imperative to movement detection (Tadin et al., 2003). RGCs receive a wide variety of visual stimuli that is filtered and processed for contrast detection and movement detection before it is sent to the brain and perceived in the visual cortex.

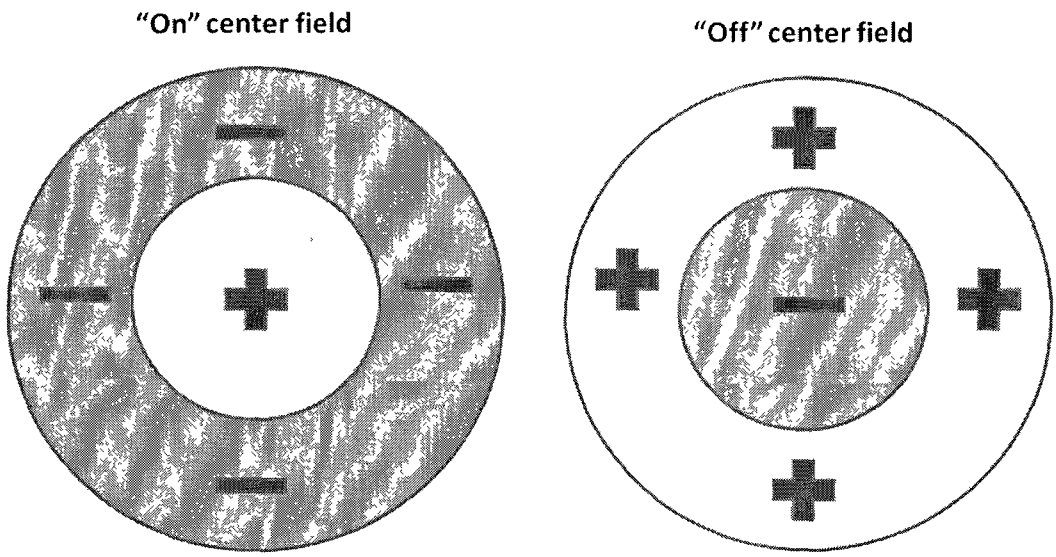

Figure 3: Receptive fields of RGCs

There are 2 main classes of receptive fields. The on-center receptive fields react best to light that is shone onto the center part of the ring, the off-center receptive field responds best to annular illumination that shines on the outer ring, but does not respond to light shone in the middle.

Neurotransmitters and receptors

The key-to-lock comparison is usually used to describe the neurotransmitter and receptor relationship. For example, a key is needed to unlock a door, and though there may be one key that unlocks a certain door, there can be various other keys that can open the same door as well. Many neurotransmitters in the retina work like a master key that can unlock many doors to transmit visual information.

The vertebrate retina uses several different neurotransmitters including, gamma aminobutyric acid (GABA), glycine, glutamate and ACh, to name a few

(Miller et al., 1981, Glickman et al., 1982). A substance that binds to a receptor and causes a response is called an agonist. There are many agonists in the mammalian system that are produced endogenously and others agonist drugs that can be applied exogenously to mimic the endogenous agents.

Within a receptor family, there are metabotropic and ionotropic receptors. Ionotropic receptors are receptors that are linked to channels that pass through ions such as Na^+ or Ca^{2+} when activated. These produce a depolarization or hyperpolarization in the cell, depending on what ion is passing through. Metabotropic receptors, when activated, trigger a G-protein and a second messenger system to start a cascade of events (Nicholls et al., 2001). Activation of metabotropic receptors results in phosphorylation of proteins. In general, the ionotropic receptors are very quick activating and the open or closure of the channels do not last long and only affect a very small region near the receptor. Activation of metabotropic receptors initiate a cascade of biochemical events that are triggered by activation of a G protein complex and ultimately cause activation of a protein kinase that phosphorylates proteins to create a physiological response. Activation of metabotropic receptors leads to longer duration responses, and can have a wide variety of effects within the cell.

Glutamate receptors (GluR) are a class of receptors that are found throughout

the mammalian body because of its wide use in the nervous system. Glutamate receptors are also important receptors within the retina, and glutamate functions as the main transmitter within the retina. GluRs can be found on a number of cells in the retina, including photoreceptors, bipolar cells and RGCs. The glutamate receptor family can be characterized into ionotropic and metabotropic subtypes. Ionotropic GluRs can be further divided into 3 major subtypes. These subtypes, N-methyl-D-aspartic acid (NMDA) receptor, kainic acid (KA) receptor and α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors, are named for the agonist that has the highest affinity for these subtypes (Seeburg, 1993). These subtypes of ionotropic GluRs are found in the retina. In the mammalian retina, both ionotropic and metabotropic types of GluRs can be found (Gilbertson et al., 1991; Duvoisin et al., 1995).

Another important receptor on the retinal cells is the AChR. Both ionotropic and metabotropic AChRs can be found in the retina. All of the AChRs in the retina are located on the amacrine cells and RGCs. The ionotropic AChRs are usually referred to as the nicotinic AChRs (nAChR), as the receptor has a high affinity for nicotine. There are also other ionotropic types that are not affected by nicotine. The metabotropic AChR are referred to as the muscarinic AChRs (mAChR) because of the high affinity to muscarine.

Vertebrate RGCs contain both the nicotinic (ionotropic) and muscarinic (metabotropic) classes of ACh receptors (Keyser el at. 1993). ACh is released onto RGCs from a population of cells in the retina called starburst amacrine cells (Tauchi, 1984). This population of amacrine cells that release ACh look like exploding fireworks (Famiglietti 1983, Masland 1998) and are located in the inner nuclear layer of the retina and as well as in the RGC layer. They receive strong inputs from the bipolar cells and synapse directly onto RGCs. It is unknown if ACh release from amacrine cells is compromised under glaucoma conditions.

Nicotinic AChRs have been particularly well studied because of their function in many tissues throughout the body. Functional nAChRs are composed of 5 nAChR subunits. Twelve subunits have been cloned in mammals. Nine different α (α2- α10) and three different β (β2- β4) subunits have been cloned. (Sargent, 1993; McGehee et al., 1995; Elliott et al., 1996). These subunits are further divided into different families and further into smaller categories depending on their protein structure. Functional AChRs can contain different combinations of nAChR subunits or can be composed of five α7 subunits (Hellström-Lindahl et al., 1999). Nicotinic receptors have been a target for potential neuroprotective mechanisms in schizophrenia, Alzheimer's, and even as an analgesic agent (Arneric et al., 2007). Earlier studies

done in our lab have implicated nAChRs on RGCs as a potential target for neuroprotection in the retina and RGCs (Wehrwein et al., 2004).

Glaucoma and excitotoxicity

Glaucoma is a progressive neurodegenerative disease that results in vision loss due to loss of RGCs. Although there are many types of glaucoma, the one common characteristic of all types is optic neuropathy. When axons in the optic nerve are lost or damaged, visual information is not conveyed to the brain and visual perception is lost. Glaucoma is one of the leading causes of blindness worldwide and affects over 2.2 million Americans a year. With the population rapidly aging, the number is suspected to increase by 70% in the relatively near future to over 3 million new cases a year (Congdon et al., 2004). Blindness caused by glaucoma is irreversible. The primary risk factor associated with glaucoma is the increase in intraocular pressure (IOP).

There are 2 types of glaucoma that do not result from genetic abnormalities or drug side effects. These two types of glaucoma are known as open-angle glaucoma and closed-angle glaucoma. To understand these 2 types of glaucoma, it's important to understand the drainage system in the front part of the eye. The anterior part of the eye contains a chamber in front of the lens that is filled with a

clear liquid called the aqueous humor. This fluid, secreted by the ciliary body, is responsible for providing oxygen and nutrients to the front part of the eye. This aqueous humor is continuously produced and filtered out via the trabecular meshwork. However, when the trabecular meshwork's filtering process is slowed for any reason, the aqueous humor builds up in the front of the eye and causes an increase of IOP. The most common type of glaucoma, open-angle glaucoma, occurs from the gradual increase in the IOP. This may be associated with a gradual blockage of the meshwork, or when the aqueous humor inflow does not equal the outflow. People with a family history of open-angle glaucoma are at a higher risk (Tielsch et al., 2004). African Americans and people from African decent also have a higher occurrence of this type of glaucoma which provides evidence for a hereditary aspect in glaucoma (The Eye Diseases Prevalence Research Group, 2004). The other type of glaucoma is called closed-angle glaucoma, which is an acute form of glaucoma resulting from blockage of the drainage system of the eye. This is usually associated with pain in the eye and, if not treated immediately, can result in blindness.

Currently the two main treatments for glaucoma are drugs or surgery. Either applied topically or orally, drugs can be administered to decrease the production of aqueous humor or can increase the drainage of fluid to decrease the IOP (Damji et

al., 2003). Beta-blockers are a popular class of drugs that are usually applied as eye drops and are used to decrease aqueous humor production (Brooks and Gillies, 1992). Another class of drugs, called prostaglandins, act by increasing aqueous outflow from the eye (Toris et al., 1992). Both popular classes of drugs, sometimes used in combination with one another, result in decreasing IOP. In other instances, a surgical tool is used to cut small holes in the eye to drain the aqueous humor, or a laser can be used to poke holes through the trabecular meshwork (Cairns, 1968; Savage et al., 1988). All of the current treatments of glaucoma are designed to decrease the IOP. However, in most cases, decreasing the IOP is insufficient to stop the disease and the progression of blindness does not cease. In some cases, people with normal IOP still develop vision loss, which indicates that something other than increased IOP may be causing the vision loss. Another condition, known as excitotoxicity, is also associated with glaucoma and may be the reason for the progression of the disease, even if IOP pressure is under control (Quigley, 1999).

Excitotoxicity

In recent years, glutamate is thought to be involved in excitotoxic conditions involved with glaucoma (Vickers et al. 1995). Glutamate is the main neurotransmitter released from photoreceptors and bipolar cells. Previous studies

done in this lab have determined that high concentrations of glutamate (500uM for 3 days) in cultured pig RGCs can result in upwards of 40% RGC loss (Wehrwein, 2004). Using specific antagonists, it was determined that the excitotoxic effect on these pig RGCs is mediated by both NMDA and non-NMDA ionotropic glutamate receptors.

Excitotoxicity is the process of apoptosis of neuronal cells caused by over stimulation of receptors. Excessive stimulation of these excitatory amino acids receptors is associated with many neuronal diseases including Parkinson's disease and Alzheimer's (Choi, 1988). This same concept can be applied in the retina as well, and is thought to be involved in glaucoma and ischemia. One of the theories involving excitotoxicity in glaucoma is that as pressure builds up in the anterior part of the eye, it can cause the RGCs in the retina to die. Cell death causes glutamate to be released in the retina and vitreous humor. With excess amounts of glutamate released from dying RGCs, there is overstimulation of glutamate receptors on the RGCs. This causes a positive feedback effect that causes non-specific cations channels to open and leads to apoptosis of the RGC by activating kinases and cell death pathways (Olney, 1977). In my proposed study, high concentrations of glutamate will be used to induce excitotoxic effects in the rat retinal cells in an *in vitro* model of excitotoxicity. In addition, to supplement the *in vitro* model, in this

study, hypertonic solution will be injected into the episcleral veins of rat eyes in an *in vivo* model to induce glaucoma. This *in vivo* glaucoma model will be used to determine if neuroprotection of cells in the RGC layer can occur under glaucoma-like conditions.

Apoptosis

One of the mechanisms involved in excitotoxic death of RGCs is apoptosis. Apoptosis is defined as programmed cell death, and is mediated by a family of proteins that send the signal to the cell to initiate cell suicide. One of the mechanisms for apoptosis is through molecules released from mitochondria that activate cleaving proteins called caspases (Fesik, 2001). When apoptotic signals, such as high intracellular calcium, are evident, cytochrome c is released from the mitochondria to activate the caspases (Martinez-Cballero, 2005). These apoptotic proteins are associated with creating pores on the outer membrane of the mitochondria or increasing the permeability of the membrane. The outer membrane permeability is also regulated by anti-apoptotic genes such as the bcl-2 family of proteins (Dejean et al., 2005). In previous studies from this lab, it has been shown that glutamate excitotoxicity in cultured pig RGCs involves phosphorylation of the p38 MAPK signaling pathway during apoptosis, while ACh triggers PI3K and

phosphorylation of Akt, which is involved in neuroprotection (Asomugha et al., 2010).

Neuroprotection

Neuroprotection is defined as prevention of excitotoxicity. Studies done in the brain have shown that in Alzheimer's disease, nicotine can prevent brain plaque formation, which is considered to be involved in pathogenesis of the disease (Wang et al., 2000). Nicotine has also been shown to improve cognitive and motor functions in Parkinson's disease by triggering dopaminergic neurons and protecting them from damage and loss. (Kelton et al., 2000) Smokers have a lesser incidence of Parkinson's than non-smokers (Morens et al., 1995). The possible neuroprotective effects of nicotine have just begun to be examined in the retina and may have promising results in neurotoxicity models.

Recently in this lab, ACh and nicotine have been shown to prevent glutamate-induced excitotoxicity through $\alpha 7$ and $\alpha 4\beta 2$ nicotinic acetylcholine receptors in isolated adult pig RGCs (Wehrwein et al., 2004; Thompson et al., 2006). There are several pathways that can ultimately lead to apoptosis or can protect cells from apoptosis. In the CNS, activation of nAChRs has been shown to protect against α and β amyloids, which are involved in Alzheimer's disease through several

neuroprotective pathways such as pathways that involve Janus Kinase2 (JAK2) or phosphoinositide 3-kinase/akt (PI3K-AKT) (Shaw et al., 2002; Buckingham et al., 2009). Studies from the Linn lab have demonstrated that activation of ACh receptors activate a second messenger system that increases cell survival via 2 pathways. ACh receptor activation leads to up-regulation of PI3 kinase-Akt-Bcl2 to enhance cell survival, and simultaneously decreases the p38 MAP kinase pathway that is involved in glutamate-induced apoptosis in this system (Asomugha et al., 2010). The link between ACh receptor activation and activation of cell survival pathways in the cultured pig RGC system was found to be calcium influx through the nAChR channels (Brandt et al., 2010). In this proposed study, I will perform experiments designed to determine if pretreatment of retinal tissue with an α7 agonist before inducing glaucoma will provide neuroprotection and prevent loss of cells from the RGC layer.

Previous studies

The initial glutamate excitotoxicity and ACh neuroprotection work in the retina came from the Wehrwein et al. (2004) study where isolated pig RGCs were used to determine what receptors were involved in glutamate excitotoxicity, as well as to determine if ACh provided neuroprotection against excitotoxicity. This study

also developed a method of isolating the RGCs to use as an *in vitro* model of excitotoxicity and glaucoma. In this study, a technique first proposed by Barres et al. (1988) was modified to purify the RGCs from other retinal tissue. This technique involved using anti-rabbit IgG antibody to remove non-specific binding and an anti-goat IgM antibody with an antibody against Thy1.1, a glycoprotein specific for RGCs in the retina, to isolate them from all other retinal tissue. This 2 step isolation method isolated pure RGCs, which were cultured and treated with various pharmacological agents.

In the study by Wehrwein et al., (2004), glutamate and other glutamate receptors agonists, such as NMDA and KA, were used to determine what receptors were involved in cell excitotoxicity. The experiments with glutamate using different culture time intervals suggested that a chronic exposure of 500μM glutamate over 3 days resulted in over 40% of RGC death. These studies also demonstrated that glutamate affected both large and small RGCs. Glutamate excitotoxicity was found to be mediated through both NMDA and non-NMDA receptors. These results were confirmed using a competitive NMDA antagonist, AP-7, and a noncompetitive NMDA antagonist, MK-801. When cells were pretreated with these glutamate antagonists, the excitotoxic effects were eliminated.

The other experiments performed in the study by Wehrwein et al. (2004) explored the potential neuroprotective effects of ACh against glutamate-induced excitotoxicity and determined what ACh receptors were responsible. When ACh was preapplied to the RGC culture 2 hours prior to glutamate application, the glutamate excitotoxic effects was eliminated and cell survival was near control levels. To determine if this effect was through nicotinic or muscarinic receptors, nicotine and muscarine was used in similar experiments. At low concentrations of nicotine, neuroprotection against glutamate-induced excitotoxicity occurred but there was no neuroprotection associated with activation of muscarinic ACh receptors, which indicated the neuroprotective effect was through nAChRs. When the nicotinic antagonist, α-Bgt, was applied before ACh or nicotine, the neuroprotective effect of ACh and nicotine was significantly reduced. This supports the hypothesis that neuroprotection was mediated by nAChRs. α-Bgt is an antagonist for the α7 receptor but can also bind to other subtypes of nAChRs at high concentrations and did not provide enough evidence that ACh-induced neuroprotection in pig RGCs was mediated through just α7nAChR. To explore this, MLA, an antagonist specific for the α7 receptor at 1-10nM, was used to determine if nicotine and ACh's neuroprotective effect was affected. When MLA was applied before ACh or nicotine,

a significant reduction in cell survival was observed. The use of MLA confirmed that ACh's neuroprotective effect was partially mediated through α7nAChRs.

This study raised further questions about neuroprotection in the retina. Though α7AChR were shown to play an important role, were other receptors within the nAChR family involved as well? The Thompson et al. (2006) study done in this lab further explored other possible neuroprotective targets using the pig retina.

In the CNS, besides the homomeric α7nAChR subunits, there are heteromeric types, which contain a combination of α and β subunits. For instance, functional AChR channels that are composed of $(\alpha3)_2(\beta4)_3$ and $(\alpha4)_2(\beta2)_3$ subunits are both functional types of nAChR channels that can be found in the brain (Le Novere et al., 1995). These subunits may exist in the retina, and could be involved in the neuroprotective effect, which could explain why only a partial effect was seen using an α7 antagonist.

To determine if different subunits could be found on pig RGCs, monoclonal antibodies against the α4, β2, α7 ACh subunits were used and labeled using fluorescent secondary goat anti-rat antibody. Double-label experiments were performed staining the as α4 subunits with Alexa-fluor 488 while the β2 subunits were stained with Texas red. α4 and β2 subunits typically form functional channels in the brain Thompson et al., 2006). The results showed that both α4 and β2

subunits exist on RGCs. However, results also demonstrated that these specific subunits can be found only on the small parvocellular RGCs. In the pig, the α7nAChR subunits, exist exclusively only on large magnocellular RGCs (Thompson et al., 2006).

Pharmacological studies were performed to determine if activation of the $(\alpha4)_2(\beta2)3$ nAChR subtypes had a neuroprotective effect against excitotoxicity. The methods used in the Wehrwein study were used with different agonists for specific ACh subtypes. Agonists specific for the α4 receptor were tested, including anatoxin, UB 165 fumarate, RJR 2403, lobeline hydrochloride, and epibatidine. Each of these α4 agonists provided partial neuroprotection against glutamate-induced excitotoxicity. This result was confirmed when the α4 nAChR antagonist, Dihydro-β-erythroidine hydrobromide (DHbE) was applied to cells before ACh and glutamate. In the presence of this antagonist, DHbE significantly reduced ACh's neuroprotective effect when applied to small RGCs before ACh and glutamate but had no effect on the large RGCs. Thus, both α7 and α4 nAChR activation was found to be involved in ACh induced neuroprotection in the pig retina.

These experiments identified what nAChRs are found on pig RGCs, as well as what receptors trigger neuroprotection. The next experiments done in the lab were

designed to examine the mechanism involved in ACh-induced neuroprotection in pig RGCs.

In the CNS, activation of metabotropic receptors has been clearly linked to triggering different kinase enzymes to produce a physiological response. However, neuroprotection in pig RGCs did not occur through activation of metabotropic receptors. Instead, previous pig studies from this lab have shown that neuroprotection was caused by calcium influx through nAChR channels on RGCs that initiate cell survival pathways and inhibit apoptotic pathways (Asomugha et al., 2010; Brandt et al., 2010).

The apoptotic and cell survival enzymes involved with glutamate-induced excitotoxicity and ACh-induced neuroprotection in pig RGCs were examined using ELISA techniques to quantify and measure protein content of the enzymes involved in neuroprotection and apoptosis (Asomugha et al., 2010). Two enzymatic pathways were identified from these studies. p38 mitogen-activated protein kinase (MAPK) levels were upregulated during glutamate-induced excitotoxicity and involved with apoptosis induced by neurotoxicity. ACh affected two different pathways to induce neuroprotection. In the presence of ACh, the phosphatidylinositol 3-kinase (PI3)-Akt pathway was triggered to enhance cell survival while p38-MAP kinase levels involved in the apoptotic pathways were reduced (Asomugha et al., 2010). Further

calcium imaging studies by Brandt et al. (2011), demonstrated that calcium permeation through nicotinic channels initiated the intracellular cascades.

PNU compounds

Two compounds used in this study, PNU-282987 and PNU-120596, are specific ligands that bind to the α7nAChR. PNU is an acronym for Pharmacia and Upjohn, the company where these drugs were developed and analyzed. These specific agents that bind to α7nACh receptors will be used in an *in vitro* model of toxicity and in an *in vivo* model of glaucoma in Long Evans rats.

PNU-282987 is a compound discovered by Pfizer using a high-throughput process which utilizes robotics and automated systems to test millions of drugs genetically and biochemically. Binding studies with rat chimera cells and electrophysiology studies in rats have demonstrated that PNU-282987 is a potent specific agonist for α7nAChRs (Bodnar et al., 2005). These binding studies have shown that PNU-282987 can displace MLA, an α7 antagonist. Using rat hippocampal neurons, application of the PNU compounds cause currents through α7nAChR channels in a dose dependant matter. This effect was blocked with MLA (Bodnar et al., 2005). As a result of these previous studies, this α7nAChR specific agonist will be used in this study to understand the role of the α7nACh receptor in rat eyes without

worrying about cross reactivity or cross binding with other nAChRs that may make the analysis and interpretation of data difficult.

PNU-120596 is another specific α7 compound that has been screened using the high-throughput process by Pfizer and tested in electrophysiological experiments. PNU-120596 acts as an allosteric modulator at the α7 receptor and binds to a different site than the agonist (Hurst et al., 2005). It has been suggested that nicotinic receptors have neuroprotective effects against retinal diseases including glaucoma (Wehrwein et al., 2004). However, with receptor desensitization, which is characteristic of nicotinic channels, prolonged exposure to a specific agonist may not be effective. PNU-120596 however, not only increases potency and maximal effects of the agonist alone, electrophysiology studies have shown that it prolongs the current as well (Hurst et al., 2005). This compound has not previously been tested in the retina, but has shown potentiation of responses from studies using brain slices (Livingstone et al. 2009) In this proposed study, I plan to use this compound in conjunction with the specific α7nAChR agonist to enhance and/or prolong the neuroprotective effect against excitotoxicity, and to determine the effects of these PNU agents in an *in vivo* glaucoma model.

With these α7nAChR specific compounds, I will be able to identify the neuroprotective effects of stimulating the α7nAChRs in the rat retina, as well as

analyze modulation of α7 nicotinic receptors in an *in vitro* and *in vivo* model of excitotoxicity and glaucoma respectively.

METHODS

Animals

Adult male and female Long Evans rats (breeding colony, WMU) were used for these experiments. Long Evans are docile by nature and have prominent eyeballs, thus making the surgical manipulations easier to perform than other rat strains. Each animal was kept in the animal colony prior to experiments and handled daily 1 week prior to surgery to minimize discomfort. All animals were treated and eventually euthanized according to IACUC protocol at WMU.

In vitro studies

Preparation

Before obtaining retinas from Long Evans rats, 8 welled glass chamber slides used in these experiments were precoated using poly-l-lysine and laminin. Briefly, 0.1 mg/ml of poly-l-lysine was added into each chamber and incubated at 37°C with 5% CO_2 overnight. The next day, the slides were washed with H_20, dried and treated with 10 μg/ml laminin overnight.

After slides were prepared, eyes were removed from Long Evans rats euthanized with CO_2. The remaining euthanized rat carcasses were placed in a freezer until disposed of through the animal care facility at WMU. The removed eyeballs were briefly dipped in 70% EtOH and the cornea and lens were carefully removed from the eye to create an eyecup. The retina was scraped off from the back of the eyecup using a flattened spatula after cutting the optic nerve. To begin the dissociation of the retina, the removed retina was incubated in a papain solution containing 2mg/ml papain (Sigma), 0.4mg/ml DL-cysteine (Sigma) and 0.4 mg/ml BSA (Sigma) in Neurobasal medium (Gibco/Invitrogen) for 25 minutes at 37°C, then washed 3 times with RGC culture medium. The RGC culture medium contained Neurobasal/B27 medium (Gibco) containing 100units/mL penicillin (Sigma), 100 μg/mL streptomycin (Sigma), 1mM pyruvate (Gibco/Invitrogen), 2mM glutamine (Gibco/Invitrogen), 5μg/mL insulin (Sigma), 100 μg/mL transferrin (Sigma), 100μg/mL bovine serum albumin (Sigma), 60ng/mL progesterone (Sigma), 16μg/mL putrescine (Sigma), 40ng/mL sodium selenite (Sigma), 40ng/mL thyroxine (Sigma), 40ng/mL tri-iodothyronine (Sigma), 50ng/mL BDNF (Biosource, Camarillo, CA), 10ng/mL CNTF (Biosource), 10ng/mL bFGF (Biosource), 5μM forskolin (Sigma), and 1% fetal calf serum (Atlas Biologicals, Fort Collins, CO) (Pang et al., 2007). After papain treatment, cells were mixed with 10mls of culture medium and the retina

was further dissociated by trituration with a glass pipette. The retinal tissue was then evenly divided into precoated 8 chamber welled slides at a density of 5 x 10^6 cells/ml, treated with various pharmacological conditions and incubated at 37^0C in 5% CO_2. During the plating procedure, great care was taken to ensure that the dissociated retina was evenly distributed between culture wells so that each culture well contained the same number of cells at the beginning of the culture procedure. To do this, all dissociated cells were pooled together and continuously mixed while 200µl of evenly dispersed cells were plated into each well. Once the cells were evenly distributed into precoated chamber-welled slides, they were treated with various pharmacological agents and cultured at 37°C in 5% CO_2 condition for 3 days. The α7 nicotinic agonist, PNU-282987, and modulator, PNU-120596, was added 1 hour prior to the glutamate treatment. The antagonist, MLA, was added 1/2 hour prior to PNU-282987. The application and duration time that produced maximal effect of each pharmacological agent was worked out from preliminary studies. Concentrations that produced the greatest effects were used in these studies.

After 3 days of incubation, the cells were fixed with 10% formalin for 30 minutes and then rinsed 3 times in PBS. After rinsing, fixed RGCs were incubated with a primary monoclonal antibody against Thy 1.1 (Chemicon) in 0.02% saponin and PBS at a final dilution of 1:500. Thy 1.1 is a glycoprotein found only on RGCs

and was used as a specific marker for RGCs (Barnstable and Drager, 1984). The slides were placed in a humidified chamber overnight in 4°C.

The following day, the slides were rinsed 3 times to remove the primary antibody. To visualize the cells, the cells on the slides were incubated with a fluorescently labeled secondary antibody Alexa-Fluor 594 goat anti-mouse IgG antibody at 1:300 dilution. (Invitrogen/Molecular Probes) for 30 mins and rinsed with water. The well walls of the chamber slides were removed and cells were mounted on the glass slide using a mixture of 50% glycerol and 50% PBS mounting medium.

In other *in vitro* studies, cholinergic amacrine cells were stained with an antibody against choline acetyltransferase (ChaT), which is an enzyme required for ACh synthesis. In the retina, only cholinergic amacrine cells stain with ChaT. The culture was fixed using 10% formalin for 30 minutes and then rinsed 3 times in PBS. The cells were then placed in the polyclonal primary goat anti-rat antibody for ChaT (Millipore) diluted to 1:100 in 0.4% Triton-X in PBS overnight in 4°C. After 3 subsequent rinses with PBS, the cells were secondarily labeled with Alexa-Fluor 388 donkey anti-goat IgG (Invitrogen) diluted to 1:500 in PBS (Bhagwandin et al., 2006). In separate experiments, to determine the specificity of the antibodies, cells were processed and incubated without primary antibody. When the primary antibody was

omitted, no staining was observed.

The method of immunostaining RGCs in rat retina was modified from the Wehrwein study (2004) where Thy 1.1 antibody was used in an isolated RGC culture. In my rat study, the RGCs were not isolated from other retinal tissue, but all retinal tissues were dissociated and incubated together. However, Thy 1.1 antibody was later used to specifically tag RGCs for identification. All rat retinal tissues were dissociated and cultured together to increase the viability of RGCs as the amount of retinal tissue obtained from each rat eye was much less than the amount of tissue obtained from each pig eye. We found that isolating rat RGC using the 2 step panning procedure before culturing did not produce a high enough density of cells for RGC survival in the rat unless large numbers of rat eyes were used in each experiment. Therefore, we modified the culture condition in the rat system so RGCs were cultured with other retinal tissue.

Microscopy was performed using the Nikon Diaphot epifluorescent research microscope illuminated by a 100-W mercury arc lamp with appropriate excitation wavelengths. Four pictures were taken from each cultured well (top, bottom, left and right section of each well) from the same locations. Images were captured using the Hamamatsu XC-77 CCD camera and Metamorph Imaging system (Universal Imaging). Based on size and intensity, the fluorescently labeled RGCs were divided

into large and small RGCs, counted and averaged. The number of cells that were treated pharmacologically was compared to the number of cells incubated under untreated control conditions obtained from each internal control. Data was always normalized to the internal controls from the same animal. In some studies, RGC counts were normalized based on percent of cells compared to control.

In vivo studies

Inducing glaucoma

Before surgery to induce glaucoma, adult Long Evans rats (aged 3-6 months; males and females) were anesthetized with 0.1ml/100g KAX via intraperitoneal injections until no reflexes were observed. KAX is a combination cocktail consisting of 5 ml ketamine (100 mg/ml), 2.5 ml xylazine (20 mg/ml), 1 ml acepromazine (10 mg /ml), and 0.5 ml sterile water.

The method to induce glaucoma is based on Morrison's method of injecting 2M hypertonic saline into the episcleral vein of the eye (1997). The 2M salt solution causes scarring in the trabecular meshwork and decreases the outflow of aqueous humor. This results in a gradual increase of intraocular pressure, mimicking glaucoma-like conditions. A topical anesthetic of 0.5% procain hydrochloride was applied to the eye before the surgery to induce glaucoma. To expose the targeted

episcleral veins and to restrict movement of the eye, a hemostat was used to pinch the bottom eyelid, which caused the eye and corresponding episcleral veins to bulge. The conjunctiva was incised with a fine scissors to further expose the vein, under a 25X to 40X magnification. To inject 50 µl of 2M hypertonic saline into the right episcleral veins, a 3mm long glass microneedle, 30 to 50 um in diameter, was glued onto a tapered polyethylene tubing (PE-50, Clay Adams, Parsippan, NJ). The microneedle was inserted into a 23 gauge needle with the tip filed off, and attached to a 1ml syringe. The glass needle was made using a Sutter horizontal microelectrode puller. When the salt was injected into the right episcleral veins, the anterior chamber of the eye turned white, which acted as verification that the salt made it into the circulatory system of the eye. Blanching of the anterior chamber was always associated with loss of cells from the RGC layer within a month. Additional doses of KAX were administered if any involuntary movements were observed during surgery. The rats were confirmed to be fully awake with no irregular effects before they were transported back to the animal colony. Injections of 2M hypertonic saline were always performed on the right eye in each rat. The left eye for each rat was left untreated and acted as an internal control.

Histology

One month following the glaucoma-inducing treatment, rats were anesthetized and euthanized by decapitation before the control and glaucoma-induced eyes were removed. The cornea and lens were subsequently cut away. The retinas at the back of the eyecups were carefully removed in one piece from each eye and 4 incisions at every 90 degrees were made in order to flat mount the retinas onto small sylgard plates using cactus needles. Pinned out retinas were then fixed with 4% paraformaldehyde overnight. Once fixed, the retina were transferred to a pig skin gelatin-coated glass slide and the standard Nissl (cresyl violet) procedure was used to stain cell nuclei. During this procedure, the retinas were washed with ascending concentrations of ethanol, stained with cresyl violet and further dried for mounting with DPX mounting medium.

Once mounted, the stained tissues were viewed throughout the ganglion cell layer using 1 micron increments using the capabilities of a confocal microscopy with a rhodamine filter. This process gives good contrast between RGCs and background, allowing for easy quantification. Cell bodies were opaque and could easily be counted. The stained cells were counted using Metamorph software. In some experiments cell counts were normalized to the internal control for each animal by

comparing the average counts from samples of both conditions.

Figure 4 depicts an image of the flat mounted retina. Using the optic nerve head as a visual landmark, 8 series of images were obtained using the 10x objective with the confocal microscope. Images were obtained from the top of the RGC layer through the entire RGC layer using 1 micron increments. The top layer that was in most focus within the series of photos was used. In each quadrant, one series of images were obtained from the periphery (red squares) of the retina at 400μms from the optic nerve head (ONH) and the 2nd series of images were obtained at 200μm from the ONH (yellow circles).

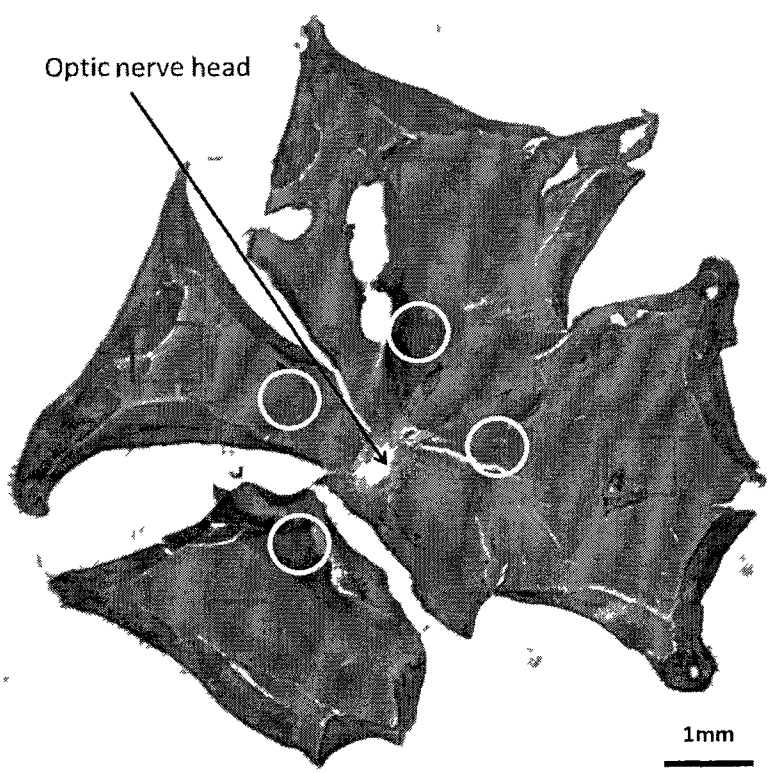

Figure 4: Flat mounted retina

The retina stained with cresyl violet and regions photographed using the Zeiss confocal microscope. The yellow circles indicate 200μm from the optic nerve head (ONH) red squares indicate 400μm from the ONH.

Figure 5 depicts low power images obtained from the periphery in a control untreated eye (fig. 5A) and a glaucomatous eye from the same animal (fig. 5B). Surgery to induce glaucoma was performed one month before the retinas were removed, stained with cresyl violet and visualized using the confocal microscope. The glaucomatous eye (fig. 5B) had significantly lower cell density then a control eye and fewer defined axon tracts.

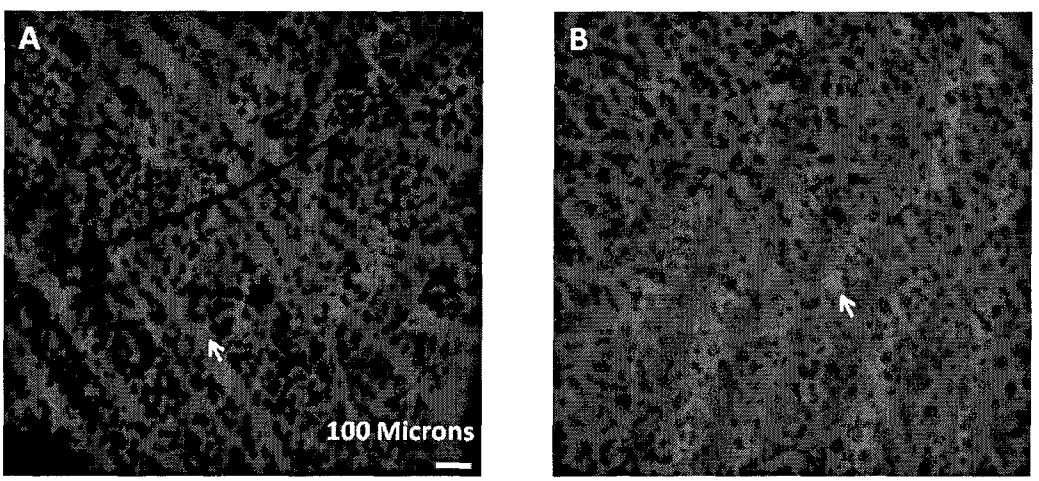

Figure 5: Visualization of cell loss from the in vivo glaucoma model

Stained retina was removed from the eye and flat mounted onto a sylgard plate. (A) is an image from a control eye and (B) is taken from a hypertonic saline injected eye. Both images were taken from a control untreated and an experimental eye from the same rat. Images obtained from both eyes were obtained from the same depth in the RGC layer and from the same region of the retina. Arrows point to defined axon tracts.

The number of stained cells in the RGC layer were counted at both distances and compared. The bar graphs in fig. 6 summarize the results of these experiments. Each bar graph represents the average number of stained cell bodies counted from the periphery and proximal RGC layer. The peripheral part of the retina had an average of 27.35% (+/-2.12) cell loss 1 month after injecting 2M NaCl hypertonic saline, compared to the proximal distance which had an average of 20.01% (+/-5.21) cell loss (Fig 6). As more cell loss in the periphery is typically associated with glaucoma, all other experiments counted cells 400 μm from the ONH.

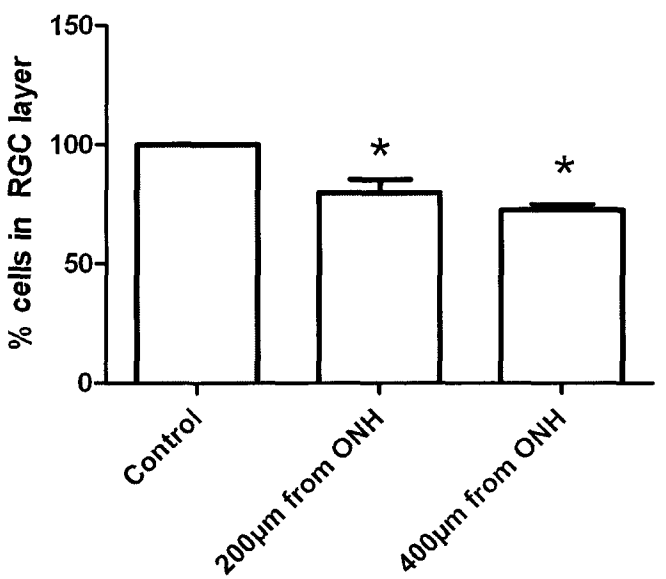

Figure 6: Percent cell loss associated with glaucoma-inducing procedure

This graph depicts the percent survival of cells in the RGC layer in both proximal and distal distances from the ONH 1 month after surgery to induce glaucoma. Each bar was produced using between 6 and 9 rat eyes. * represents significance from internal controls. Error bars represent SEM from 1 way-ANOVA.

To determine the timeline for the glaucoma model, animals were euthanized at 1 month, 2 months and 3 months following the hypertonic injections. At 1 month intervals, the retina were carefully removed and flat-mounted onto a sylgard plate with cactus needles, fixed and cells in the RGC layer were stained with cresyl violet. Cell counts were obtained from the periphery in experimental eyes and compared to cell counts obtained from the periphery in internal control eyes. As can be seen by the summarized results in fig. 7, significant loss of cells in the periphery of the RGC layer occurred 1 month following the surgery and there was no additional significant difference in the loss of cells at 2 and 3 months compared to 1 month results. Though longer studies were done at 4 and 6 months, (data not shown), at 1 month, hypertonic injections of saline into the episcleral vein always caused a significant amount of cell loss in the RGC layer if blanching occurred in the anterior chamber following the injection ($p<0.05$). Based on these results, all rats in subsequent glaucoma-inducing experiments were sacrificed 1 month following surgery to induce glaucoma and cell counts were obtained 400 μm from the ONH.

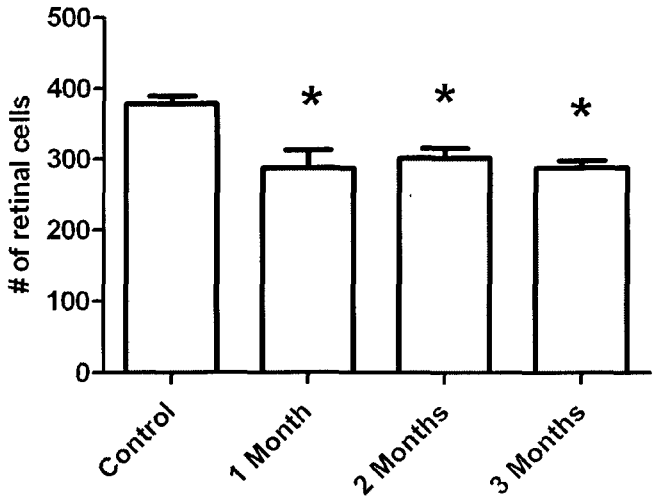

Figure 7: Cell loss associated with the glaucoma procedure at various time points

Animals were euthanized at different time points after surgery to induce glaucoma and the loss of cell in the RGC layer were quantified in a time-dependent manner. Each bar graph represents the average number of retinal cells that were counted in the RGC layer after inducing glaucoma with hypertonic saline injections. (* indicates significance from the untreated control). Each bar graph was generated from between 3 to 9 experiments. Each experiment used 3 rats and were repeated a minimum of 3 times. Error bars represent SEM.

Intravitreal injection of pharmacological agents

For all *in vivo* studies, drugs were injected intravitreally in volumes of 5 µl using a 5µl Hamilton syringe. The injections of various pharmacological agents included the α7 nicotinic agonist, *PNU-282987*, the α7 modulator, *PNU-120596*, and the α7 antagonist MLA. These agents were injected into the eye before surgery that normally induces glaucoma according to the timeline obtained from rat *in vitro*

studies.

Data analysis

All cell counts were compared to the internal control counts for each experiment. Student T-tests were used for single comparisons, and one-way ANOVA was used for multiple comparisons to statistically measure cellular loss with Tukey posthoc tests using Graphpad statistical software and minitab. For normalized data, statistical analysis was performed using Kruskall-Wallis non parametric analysis of variance with post hoc comparisons (Dunn's test). A p-value of < 0.05 (95% confidence) represents significance. Graphs were plotted with Prism GraphPad version 4.0 software (GraphPad Software, Inc., San Diego, CA)

RESULTS

In vitro studies

Glutamate excitotoxicity

To determine if the glutamate excitotoxic effects that were observed in pig studies are also demonstrated in rats, rat retinas were cultured for 3 days with 500μM glutamate, 100μM kainic acid (KA), 100μM *N*-Methyl-D-aspartate (NMDA), or with a combination of KA and NMDA. When glutamate alone was

applied (Fig. 8B), an average of 45.21% (SE ±2.10) cell reduction was observed compared to control untreated RGCs (Fig. 8A). However as seen in figure 8C, when the cultured cells were incubated with the KA receptor antagonist, CNQX (10μM) and the NMDA antagonist MK801 (100μM) for 30mins before applying 500μM glutamate, the toxic effect of glutamate was eliminated. This suggested that KA and NMDA glutamate receptors are involved in glutamate's excitotoxic effect.

Figure 8: Effects of glutamate on cultured RGCs

After 3 days in culture, RGCs were fixed and stained with an antibody against Thy 1.1. and labeled with Alexa-Flour 595 for visualization. Glutamate caused obvious RGC loss, and the RGC loss was blocked by CNQX and MK-801. Images were obtained after 3 days in culture under the various pharmacological conditions: (A) control untreated conditions (B) cultured for 3 days with 500μM glutamate, and (C) pretreated with both MK-801 and CNQX before application of 500μM glutamate. Arrows indicated stained RGCs.

The bar graphs shown in fig. 9 demonstrate that both KA and NMDA had negative effects on RGC survival when applied 30mins before 500μM glutamate. KA reduced cell survival by an average of 32.33% (SE ±2.19) while NMDA reduced RGC survival by an average of 21.28% (SE ±4.03) when applied before glutamate. However, when a combination of KA and NMDA was applied to the cells before

glutamate, the RGC loss observed were similar to glutamate alone, by decreasing cell survival by 46.23% (SE ±3.54). The RGC loss due to the pretreatment with a combination of NMDA and KA was eliminated if glutamate receptor antagonists, consisting of 10μM CNQX and 100μM MK-801, were applied before the glutamate agonist cocktail (Fig 9). These results provide evidence that glutamate-induced excitotoxicity of rat cultured RGCs is mediated through both KA and NMDA glutamate receptors similar to results obtained in pig retina.

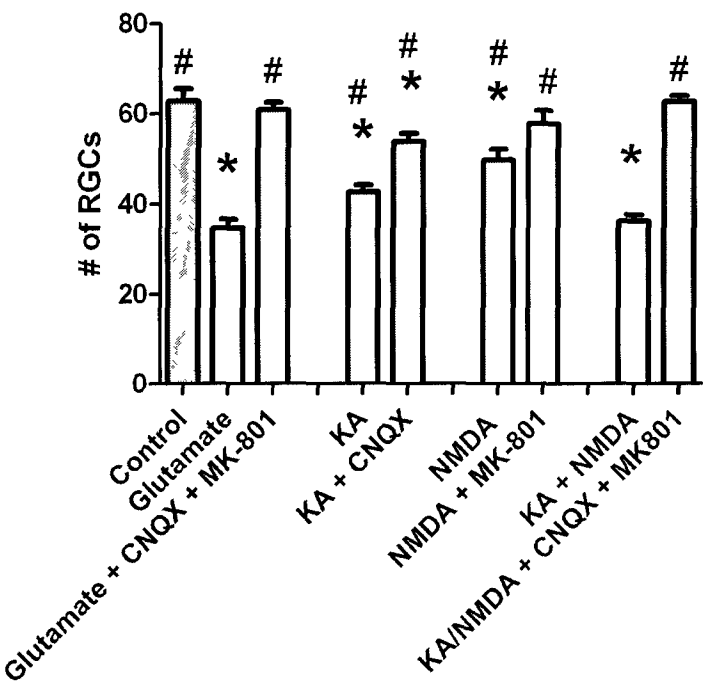

Figure 9: RGC loss due to glutamate agonists

Each bar graph represents the average numbers of RGCs that survive after 3 days of culture with various pharmacological treatments. A significant decrease in cells was observed when the cells were cultured with 500μM glutamate. Partial toxic effects

were observed when RGCs were incubated with KA or NMDA separately. However when both glutamate agonists KA and NMDA were applied to the culture together, the amount of cell death mimicked the effect of glutamate alone. A combination of KA and NMDA receptor antagonists, CNQX and MK-801 respectively, blocked this effect. Averages were obtained from between 6-9 animals. * represents significance from control. # represents significance from glutamate's effect. Error bars represent SEM.

Glutamate excitotoxicity was also tested at different time points. When cells were cultured in 500μM glutamate for 1, 3, 5 and 7 days, a significant decrease in cell survival was observed by day 3 (Fig. 10C). When separating glutamate effects by large and small RGCs, it was found that both types of RGCS were prone to glutamate damage. Glutamate significantly reduced the number of both types of RGCs after 3 days in culture (Fig. 10A, Fig. 10B). As a result, all future *in vitro* experiments cultured cells for 3 days before assaying for cell survival. One thing to note is that the in the control untreated conditions, the large RGC had loss of cells presumably due to cell death associated with the culturing process, where the small RGCs were more resistant to that cell loss after 7 days.

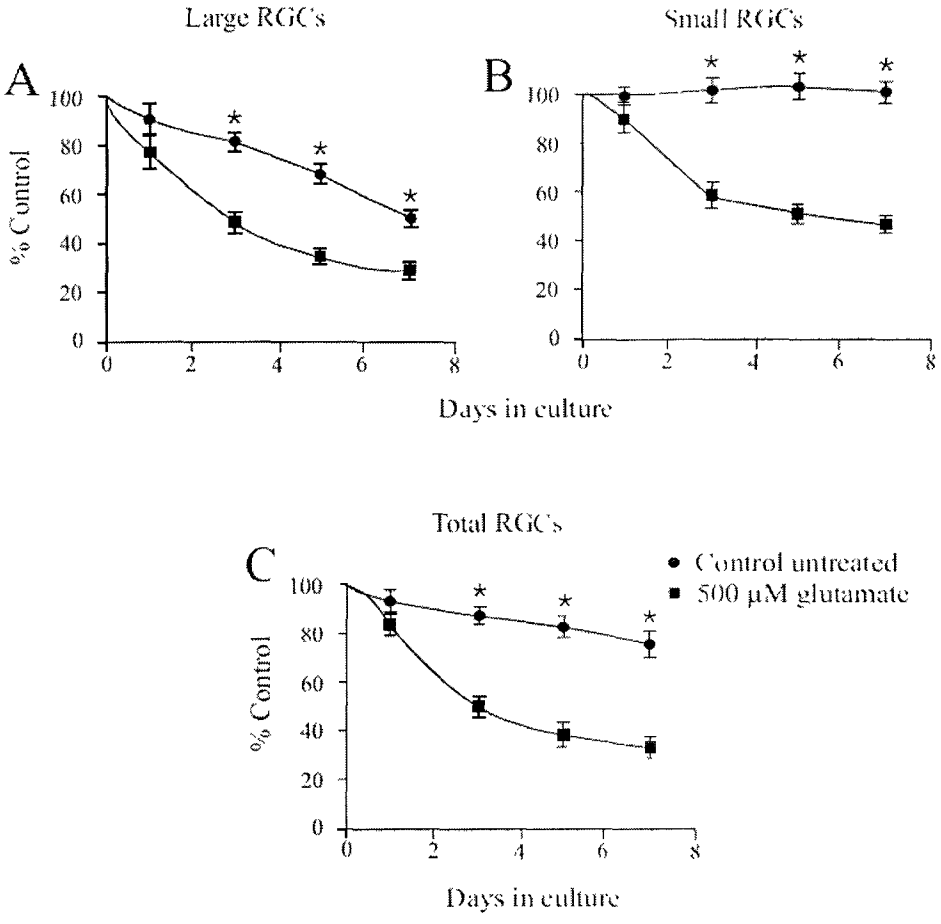

Figure 10: Glutamate excitotoxicity timeline

Glutamate causes a decrease in RGC survival in a time-dependent manner in both large and small RGCs. Solid circles represent the percent survival of RGCs after various amounts of time in culture under control untreated conditions. Solid squares represent percent survival of RGCs at various time points after being cultured with 500μM glutamate. (A) Percent cell survival for cultured large RGCs. (B) Percent cell survival for cultured small RGCs and (C) Percent cell survival for all RGCs pooled together. * Represents significance from glutamate. Normalized data points were obtained from a minimum of 3 experiments. Each experiment used tissue from 3 rats. Error bars represent SEM.

ACh neuroprotection

When rat RGCs were cultured with 10μM ACh or 100μM nicotine, both

compounds resulted in higher cell counts compared to glutamate conditions. The results of these experiments are summarized in Fig 11. As shown by the bar graphs in figure 11, when 100μM nicotine or 10 μM ACh was applied 1 hour before 500μM glutamate, glutamate excitotoxicity was eliminated. Both 100μM nicotine and 10 μM ACh produced the maximal cell against glutamate-induced excitotoxicity based on dose-response studies. 100μM nicotine increased cell survival by an average of 104.47% (SE ±7.062) compared to control conditions, while 10μM ACh increased cell survival by an average of 113.88% (±11.46) compared to the control untreated condition. However, when the α7 nicotinic antagonist MLA (10nM), was applied to cultures of rat retina before ACh and nicotine and the glutamate insult, neuroprotection was not observed. In the presence of MLA, ACh and nicotine had no significant neuroprotective effect against glutamate-induced excitotoxicity. These results were consistent with the results obtained from previous pig studies (Wehrwein et al. 2004) and support the hypothesis that neuroprotection against glutamate-induced excitotoxicity is mediated through α7nAChRs on rat RGCs. To further support this hypothesis, the next experiments used the specific α7nAChRagonist, PNU-282987.

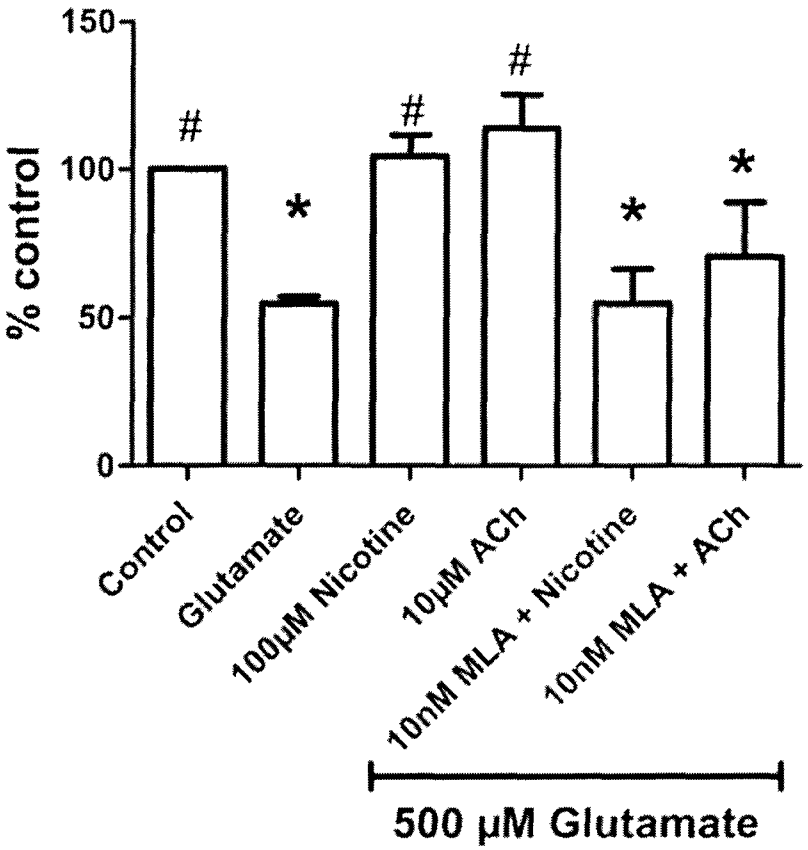

Figure 11: Effects of ACh and nicotine on rat RGCs (in vitro)

Each bar represents the average percent of RGCs that survive compare to control untreated conditions. Both nicotine and ACh provided significant neuroprotection against glutamate excitotoxicity. When the 10nM α7 nicotinic antagonist MLA was applied at 30mins before ACh and nicotine, the cell survival significantly decreased. # represents significance from glutamate's effect and * represents significance from control untreated conditions. Experiments were repeated between 3 and 9 times.

Effects of an α7 ACh agonist *in vitro*

To determine if the specific α7 ACh agonist, PNU-282987, has neuroprotective effects against glutamate-induced excitotoxicity in cultured rat retina, retinas were dissociated and cultured after pretreatment with various concentrations of PNU-282987 for 1 hour before addition of 500 μM glutamate. After 3 days, RGCS were fluorescently labeled with an antibody against Thy 1.1. Figure 12 represents sample images of RGCs fluorescently labeled with an antibody against Thy 1.1. Fig. 12A was obtained from retinal tissues cultured for 3 days under untreated control conditions. Fig. 12B represents an image obtained when the same density of retinal tissue was cultured for 3 days in the presence of 500μM glutamate. When PNU-282987 was applied before glutamate, higher cell survival was observed (Fig. 12C), but when the RGCs were pretreated with 10nM MLA before PNU-282987 and 500μM glutamate, the cell survival was significantly reduced (Fig 12D, Fig. 14).

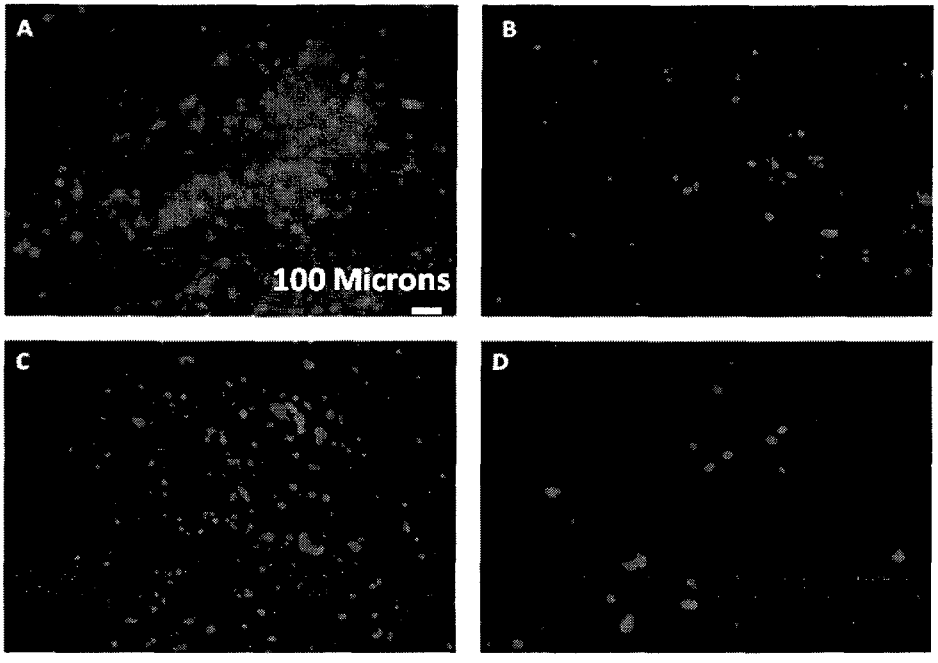

Figure 12: Effects of PNU-282987 in vitro

PNU-282987 provides neuroprotection against glutamate-induced excitotoxicity. Images of labeled RGCs were obtained after 3 days in culture under the following conditions: (A) under control untreated conditions. (B) in 500μM glutamate. (C) pretreated with PNU-282987 for 1 hour prior to 500μM glutamate and (D) when the specific α7 antagonist, MLA (10nM), was applied 30 minutes prior to PNU-282987 and 1 1/2 hours prior to 500 am glutamate.

Pretreatment of cells with 100nM of PNU-282987 before glutamate application produced the greatest survival of large RGCs suggesting that PNU-282987's effects are dose dependent. As shown in the summarized data shown in fig. 13, when 10nM, 1μM or 10μM PNU-282987 was applied before glutamate, there was no significant cell survival effects. At 10μM PNU, a significant decrease in cell survival occurred, compared to glutamate's effect, which was likely due to non-specific effects associated with this relatively high concentration used (Fig. 13).

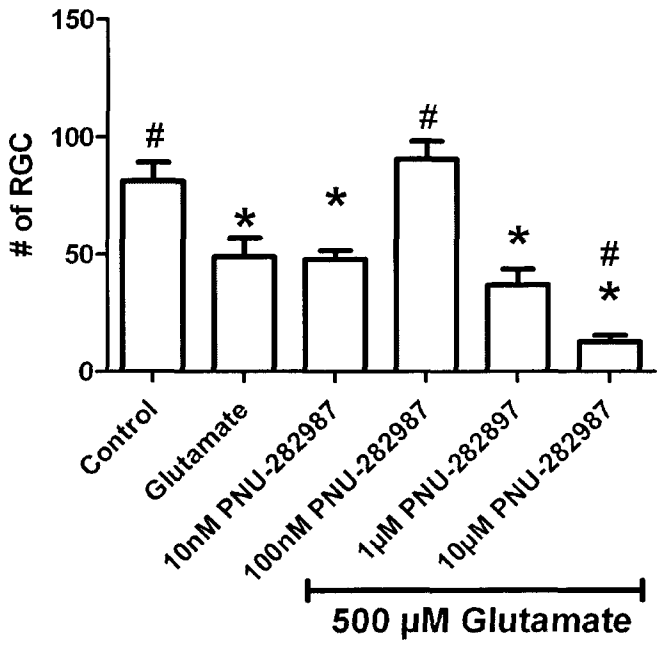

Figure 13: Dose dependent effects of PNU-282987 on cells in the RGC layer

Bar graphs represent the average number of labeled RGCs counted after culturing them for 3 days with different concentrations of PNU-282987 and 500μM glutamate. PNU-282987 was applied one hour before the glutamate insult. * indicates significance from control. # indicates significance from glutamate conditions ($P<0.05$). Averages were obtained from N's of 3-9.

In other experiments designed to provide evidence of α7nAChRs in neuroprotection, the cell culture was pretreated with MLA, an antagonist specific to the α7 receptors. In the presence of 10nM MLA, PNU-282987 had no effect on glutamate-induced excitotoxicity (Fig 14).

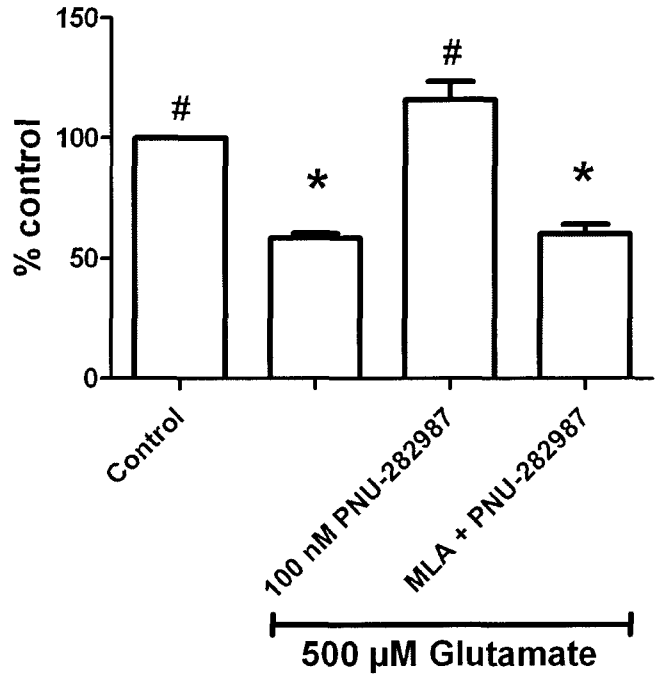

Figure 14: PNU agonist's neuroprotective effects

100nM of PNU-282987 provided neuroprotection against glutamate. When MLA was injected into eyes before the PNU compound, the PNU-induced neuroprotection was blocked. (* indicates significance from control. # indicates significance from glutamate conditions). Averages were obtained from between 6 and 9 experiments.

In pig studies, the α7nAChR were only found on large RGCs. To determine if this is also true in rats RGCs, the effect of the α7 agonist was also compared in large and small populations of the RGCs. Figure 15 represents the effects of glutamate and the PNU α7 agonist over a 3 day time period on large and small RGCs. In both the large and small RGCs, glutamate had an excitotoxic effect and significantly decreased RGC numbers by the 3rd day. The small RGC were slightly more resistant to the glutamate damage compared to large RGCs. However, while

the large RGCs demonstrated a gradual decrease in the control untreated conditions presumably due to the dissociation process, the percent of small RGC population did not significantly decrease by the 3rd day in control untreated conditions. Therefore, the small RGCs were more resistant to cell loss due to the dissociation and culture conditions.

Application of PNU-282987 protected the large RGCs and the cell survival was similar to the control levels (Fig 15A). PNU-282987 also had neuroprotective effects on the small RGC population (Fig 15B), although it did not provide complete neuroprotection against glutamate-induced excitoxicity in small RGCs like it did in large RGCs (Fig 16). However, these results suggest that unlike pig studies, α7nAChR may be found on both large and small RGCs in rats. Certainly, in rat the small RGCs show a response to these compounds presumed to interact specifically with α7nAChR.

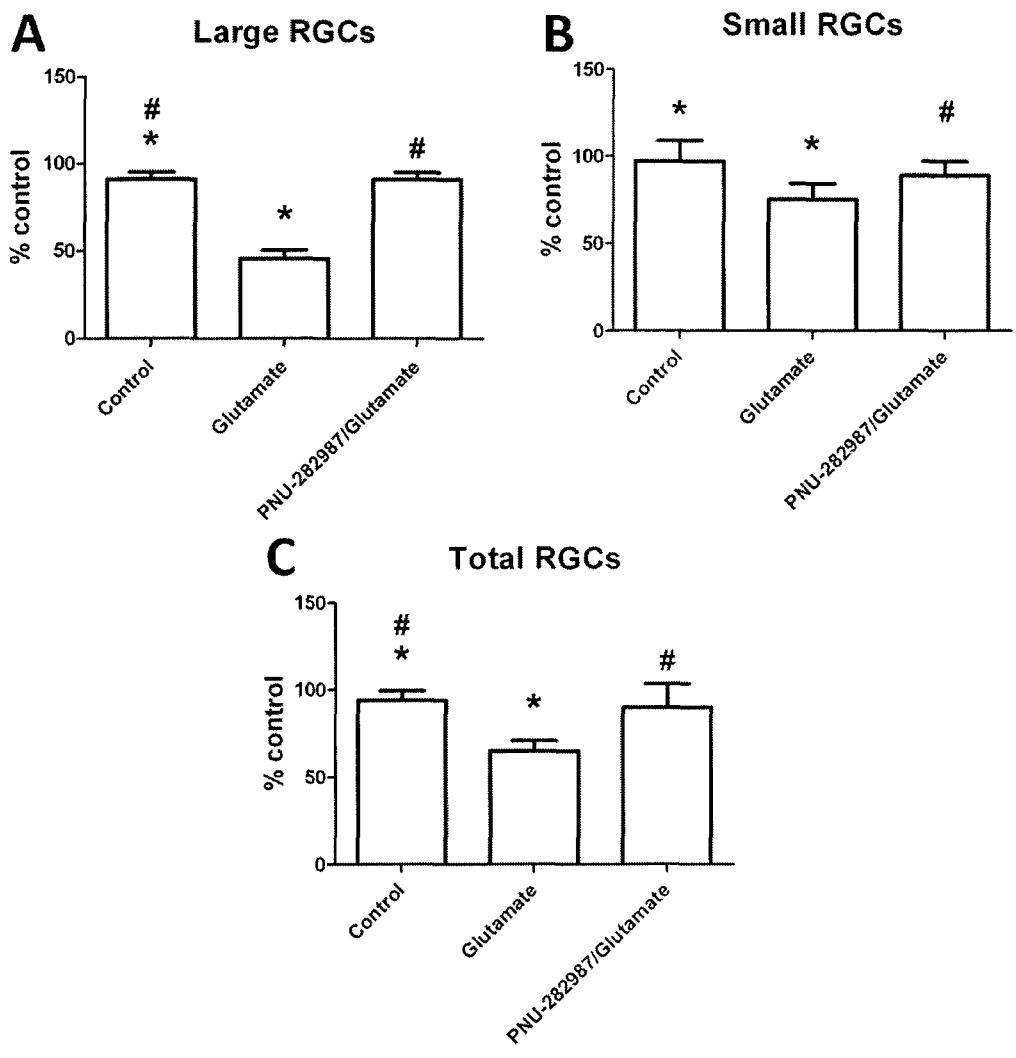

Figure 15: Effects of PNU-282987 on large and small RGCs

PNU-282987 caused an increase in the percent of RGC survival in both large and small RGCs if applied to cultures one hour before 500μM glutamate. Each normalized data point represents the percentage of cells that survived compared to control untreated conditions at Day 0. Each bar represents the percent survival of RGCs after three days in culture. A) Percent cell survival for cultured large RGCs. B) Percent cell survival for cultured small RGCs C) Percent cell survival for all RGCs pooled together # represents significance from glutamate, * represents significance from control. Normalized data points were obtained from between 6-9 rats. Error bars represent SE.

In vivo studies

Effects of PNU agonist *in vivo*

The objective of the next experiment was to determine if the neuroprotective effects seen using the *in vitro* excitotoxic model can be observed in an *in vivo* model of glaucoma. The vitreous in the right eye of each rat was injected with 5µl of 100µM PNU-282987 1 hour before the hypertonic saline injection. Figure 16 represents sample images obtained during these experiments. One month after hypertonic saline was injected to induce glaucoma-like conditions, a significant amount of cell loss in the RGC layer was observed (Fig. 16B) compared to control retinal images obtained from the internal control eye (Fig. 16A). In addition, the defined axon tract characteristics in control retinas (arrows; fig. 16A) are fragmented in the peripheral retina under experimental conditions (arrowheads; fig. 16B). However, when the α7 agonist, PNU-282987, was injected into the eye before the hypertonic injection to induce loss of cells in the RGC layer, the decrease in cell survival was eliminated (Fig. 16D) and cell counts were similar to control cell counts obtained from the internal control (Fig. 16C). In addition, if PNU-282987 was injected before surgery to induce glaucoma, the defined axon tract remain intact. However when 10nM MLA was injected before the PNU agonist, neuroprotection

against cell loss did not occur, as seen when comparing panels E and F. Panels G and H are images obtained from a sham study where 5µl of PBS was injected into the vitreous (Fig. 16H) before the glaucoma inducing surgery. G represents the internal control from the same animal. As demonstraed in panel H, the injection itself was not sufficient enough to prevent loss of cell in the RGC layer and is not sufficient to prevent loss of defined axon tracts. All of these experiments that produced images for fig. 16 were repeated between 3 and 9 times with similar results.

The summary of the in vivo experiments using PNU-282987 are illustrated in the bar graphs shown in figure 17. After injection of hypertonic saline into the episcleral veins, there was an average loss of 23.86% (SE±6.68, n=8) in the RGC layer. However, when retinas were pretreated with the α7 nAChR agonist PNU-282987, the cell death associated with the saline injection was eliminated and cell counts in the RGC layer in the periphery were statistically the same as in control conditions. When MLA was injected prior to the PNU agonist and surgery, the neuroprotective effect of PNU was eliminated and a significant loss of cells from the RGC layer occurred.

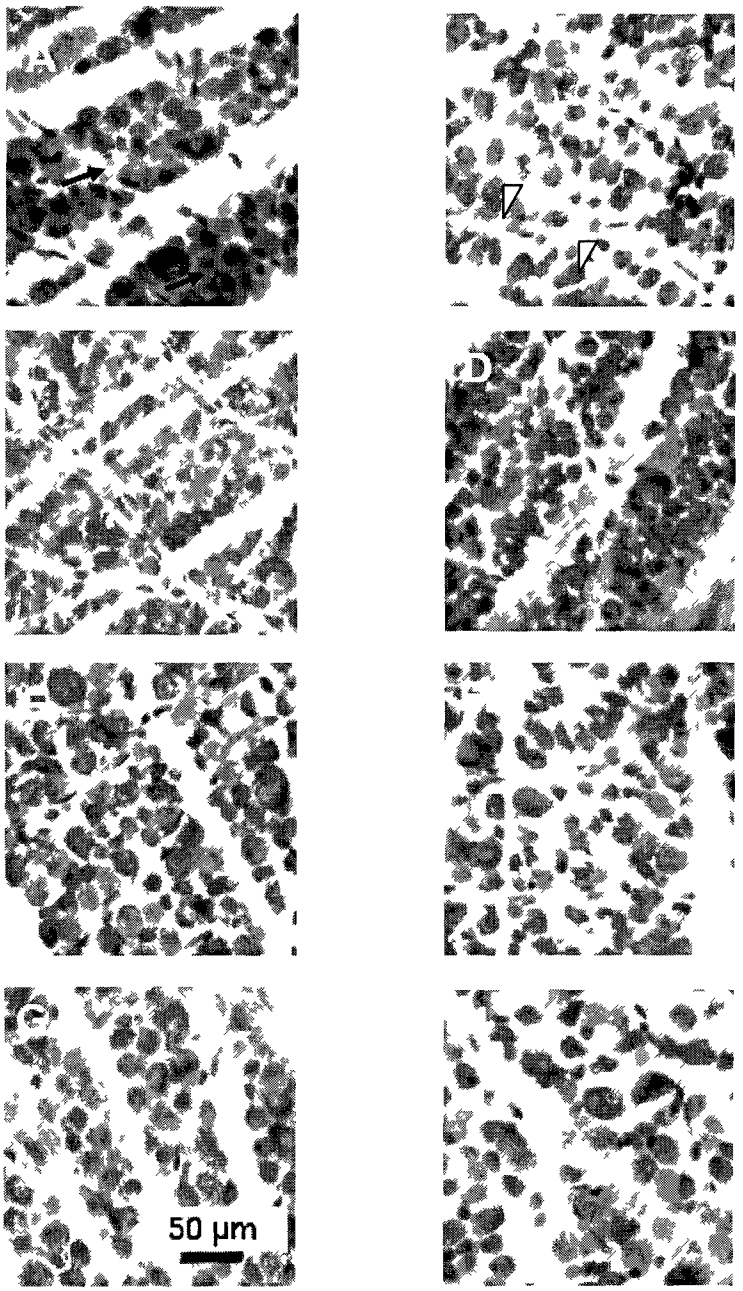

Figure 16: Sample of in vivo images with and without PNU agonist

The left columns represents the internal control images obtained from the same rat that the right column images in the same row were obtained from. B: image obtained after 2M hypertonic injection into eye. Arrow represent axon tracts and arrow heads represents cresyl violet stained retinal cells. D: 50 µl of 100 µM PNU-282987 injected into eye 1 hour before surgery to induce glaucoma. F: 10 nM MLA was injected into eye before PNU agonist and before surgery. H: PBS was injected

into eye before surgery to induce glaucoma. Scale bar is equal to 50μm.

In sham experiments, 5μl of PBS vehicle was injected into the eye before the glaucoma surgery to determine if the surgery alone was responsible for the increase of cell survival. The sham treatment had no significant effect on cell survival in the RGC layer. Even with an injection of 0.9% saline there was significant cell loss in the RGC layer 1 month after surgery.

Effects of PNU-120596, an α7 nAChR modulator: in vitro and in vivo

Binding studies have demonstrated that PNU-282987 acts as an agonist on α7nAChRs and that PNU-120596 acts to enhance α7nAChR receptor activity (Bodnar et al.,2005). To examine any modulatory effect of PNU-120596 in rat cultured retinal tissue, intitial *in vitro* experiments were performed to examine if PNU-120596 provided neuroprotection against glutamate-induced excitotoxicity. As shown in fig. 18, when cells were cultured with different concentrations of the α7 ACh modulator, PNU-120596, before the glutamate insult, 10nM of the modulator provided neuroprotection against RGC loss. Higher concentrations of the α7 modulator (100nM, 1μM 10μM) had little effect on cell survival or led to a significant decrease of cells compared to control conditions, likely due to toxic effects caused by relativly high concentrations of the agent (Fig. 18).

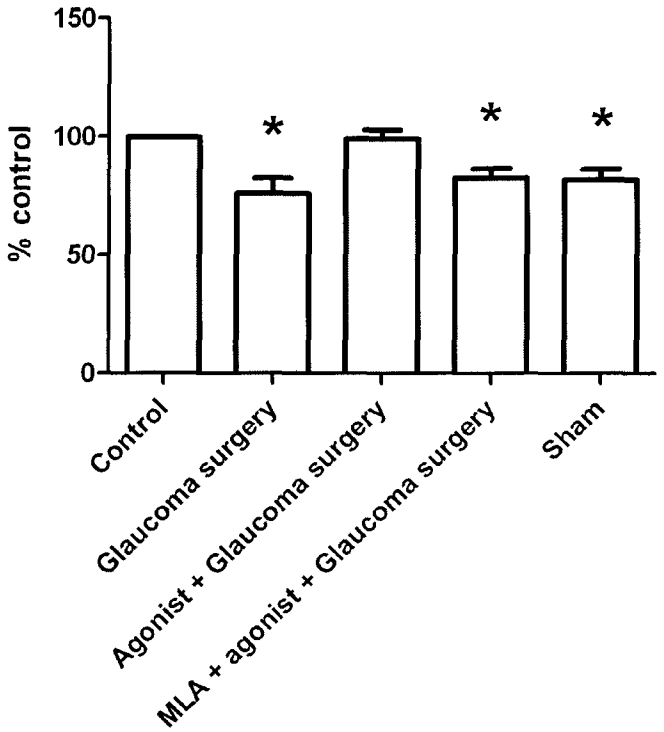

Figure 17: Effects of PNU-282987 on cell loss in the glaucoma model

Bar graphs summarize the results associated with using PNU-282987 (agonist) in the glaucoma model. To generate this figure, the number of cells in the RGC layer was counted and normalized to their corresponding internal control (left eye). Each bar represents the mean % of cells in the RGC layer compared to the internal control.* represents significance from control. Averages were obtained from between 3-9 experiments. Error bars represent SEM.

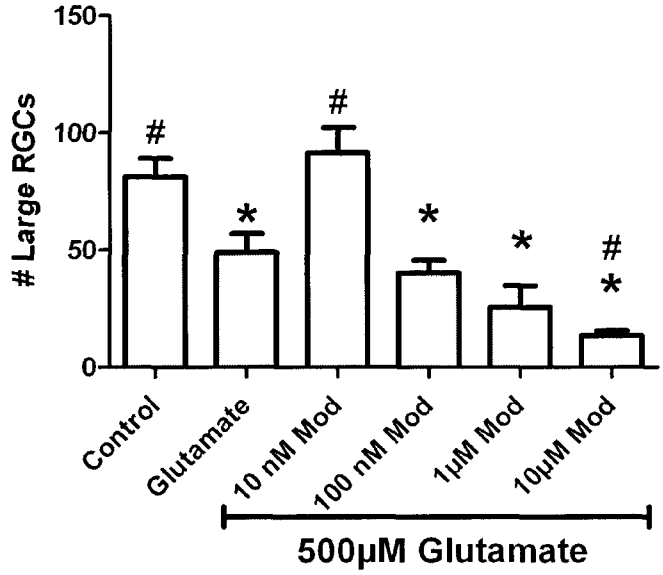

Figure 18: Dose dependent effects of PNU-120596 on cultured RGCs

Bar graphs summarize the effect of the PNU modulator, on cultured large RGCs. The RGCs were cultured with various concentrations of the PNU modulator and 500 μM glutamate. At 10nM, the α7 nAChR modulator by itself provided neuroprotection against glutamate-induced cell loss. * indicates significance from control. # indicates significance from glutamate conditions. Bar averages were obtained from cell counts obtained from between 3-9 rats.

These results suggests that the α7 modulator, by itself, had neuroprotective effects against glutamate-induced excitotoxicity at one concentration. However a modulator should only modulate the receptor if ACh or ACh agonists are bound to the receptors. Since the RGCs were not isolated during the culturing process, it was hypothesized that there may be starburst amacrine cells present in culture that would release ACh in the *in vitro* model. This would allow the PNU modulator to act on the

receptor to modulate levels of neuroprotection. To test this hypothesis, the cultures of retinal cells were stained with a antibody against choline acetyltransferase (ChaT) to verify the existence of cholinergic starburst amacrine cells in the rat RGC culture (Fig. 19). When stained with the anti ChaT antibody, cholinergic cells were visible throughout the retinal culture in relatively low numbers (Fig. 19). When cells were stained without the primary antibody in control studies, no cells were visible (data not shown). This result supports the hypothesis that cholinergic amacrine cells are present in the primary culture of retinal cells, and is a plausible reason that the PNU α7 modulator could provide neuroprotection in culture.

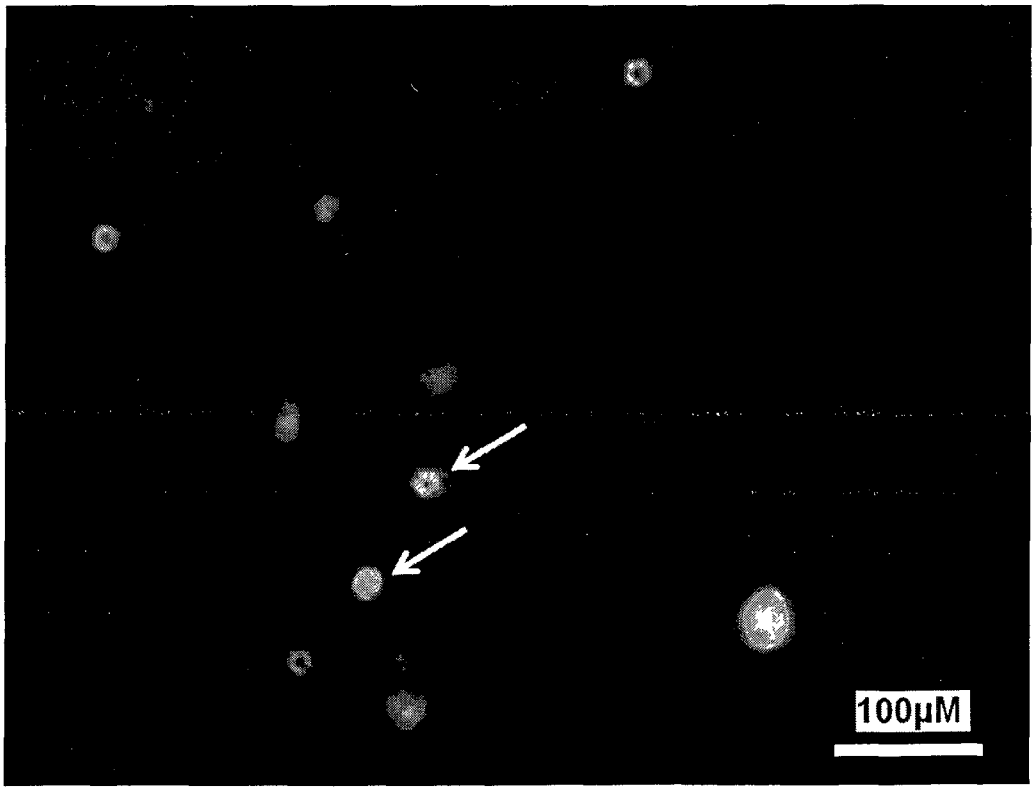

Figure 19: Amacrine cell staining using ChaT

Dissociated rat retina was cultured for 3 days, fixed and labeled with a primary antibody against Choline Acetyltransferase and secondarily labeled with Alexa Fluor 388 for visualization. Arrow represents amacrine cells stained with ChaT.

Combined effects of both PNU compounds

In culture, the PNU α7 agonist and modulator had considerable neuroprotective effects against glutamate-induced excitotoxicity when applied separately. I was interested in seeing what the effects of the α7 agonist and modulator would be if they were applied together before glutamate insult. Interestingly, when 100 nM PNU-282987 and 10nM PNU-120596 were combined together before applying the glutamate insult, cell survival was significantly higher than control levels at 154.38% (SE ±2.74) after 3 days in culture (Fig. 20) Thus, the combination of these agents appears to not only provided neuroprotection against glutamate-induced excitotoxicity, but also prevented loss of RGCs that normally occur during the cell culture process. This is likely due to the combination of both PNU compounds protecting against normal damage occuring during the 3 days the cells are cultured. These results are consistent with our hypothesis that introduction of α7 nicotinic agonists or modulators can significantly reduce the loss of cells in the RGC layer that is normally associated with glaucoma.

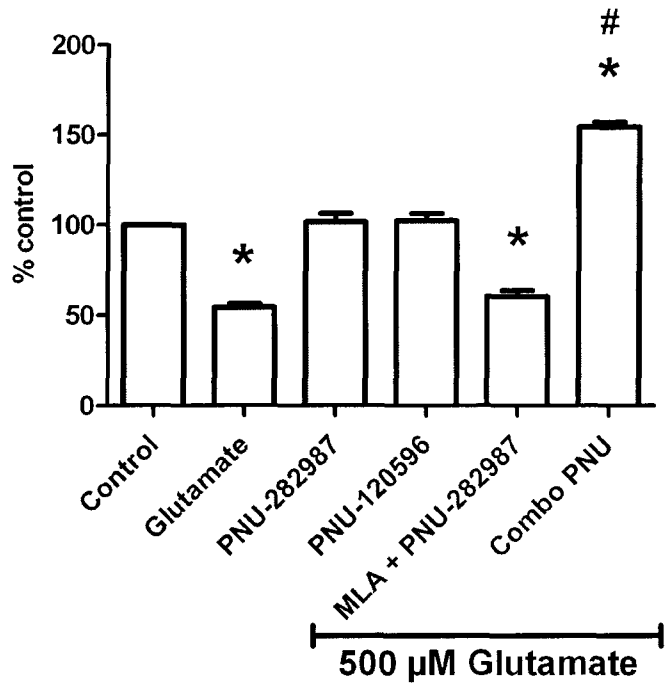

Figure 20: Summary data of RGCs in vitro

These bar graphs summarize the results of the effects of the PNU compounds in cell culture. To generate this figure, the # of RGCs cultured under the various conditions were counted and normalized o the corresponding control conditions for each experiment. * indicates significance from glutamate conditions. # indicates significance from control conditions. Bar averages were obtained from counts obtained from between 3-9 rats. Combo indicates that both PU-282987 and PNU-120596 were applied simultaneously.

Effects of PNU agents *in vivo*

Figure 21 represents sample images obtained from the rat's RGC layer 1 month after surgery to induce glaucoma in the rat's right eye. The left columns represent control untreated images obtained from the same area of the peripheral retina as shown in the corresponding right column. The images in each row were

obtained from the same animal. Panel A was obtained from peripheral retina under control untreated conditions. To obtain the image in fig. 21B, hypertonic saline was injected into the episcleral vein to induce cell loss in the RGC layer and fragmentation of distinct axon tracts. Panel C and D were obtained from another rat, where the right eye was injected with 5μl of 100μM PNU α7 agonist into the vitreous 1 hour before surgery to induce glaucoma. As shown in these 2 images, the α7nAChR agonist prevented the cell loss normally associated with hypertonic saline injection. This is also the effect seen when the α7nACh modulator PNU-120596 was injected before surgery. There is no significant difference between the number of cells in the treated eye compared to the internal control, obtained from the same regions of the retina from the same animal and there was no obvious change in the thickness or direction of the axon tracts. Figure 21F depicts an example image obtained from the right eye of a rat injected with α7 ACh modulator, PNU-120596, before surgery and the figure E represents an internal image from the left eye control of the same rat. When both PNU agonists were injected prior to the glaucoma surgery, there was no significant difference between the number of cells in the RGC layer comparing the left and right eye (figs. G and H). The results observed from *in vitro* studies that demonstrated a higher number of cells in the culture treated with both PNU compounds compared to control were not evident in the *in vivo* model.

These results support the hypothesis that the introduction of α7 nicotinic agonists or modulators can significantly reduce the loss of cells in RGC layer associated with our glaucoma model.

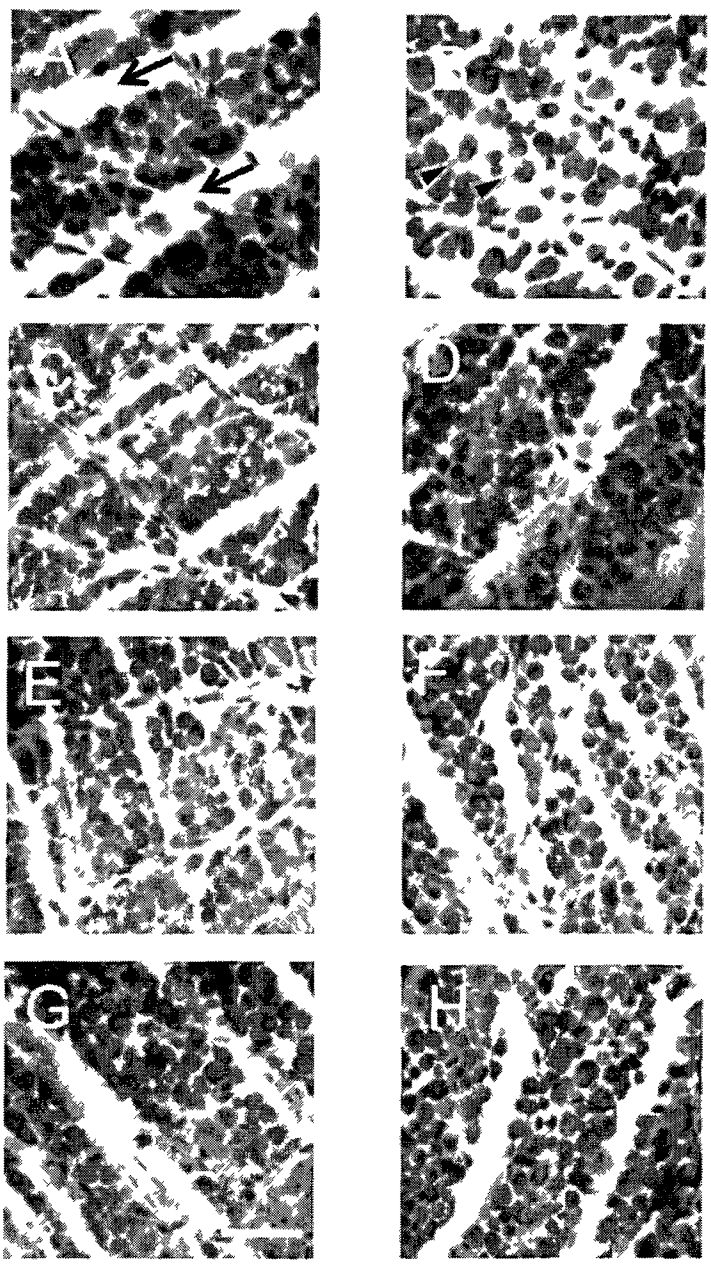

Figure 21: Effects of both PNU compounds in vivo

Each panel represents an image obtained from the peripheral of the rat retina under the following conditions. (A) control untreated eye, (B) glaucoma-induced eye from the same animal and from the same region of the retina as shown in A. (D) an image sample from a rat eye injected with the PNU α7 agonist before surgery to induce glaucoma, (F) an image obtained 1 month after the α7 modulator was injected prior to surgery, (H) is an image obtained in an eye co-injected with the PNU modulator and agonist together prior to surgery to induce glaucoma. The left column represents the internal control from the same animals that the experimental images were obtained from (right colum). All iamges were obtained 1 month following surgery to the right eye. Scale bar represents 50μm.

The bar graphs represented in Fig. 23 summarize the results of the PNU agonist and modulator experiments in the *in vivo* rat glaucoma model. Injection of hypertonic saline to induce loss of cells in the RGC layer resulted in an overall average loss of cells in the RGC layer by 23.86% (SE±6.68). Injecting the the α7 ACh agonist, PNU-282987 or the α7 ACh modulator, PNU-120596 before surgery to induce cell loss, eliminated the glaucoma effect and had a significant neuroprotective effect in increasing cell survival to 99.36% (SE±3.46) and 102.00% (SE±6.06) respectively. When a combination of PNU agonist and modulator was injected prior to the glaucoma surgery, the cell survival was comparable to the control eye (Fig. 22). These results support the hypothesis that introduction of α7 nicotinic agonists and/or modulators can significantly reduce the loss of cells in RGC layer associated with our glaucoma model.

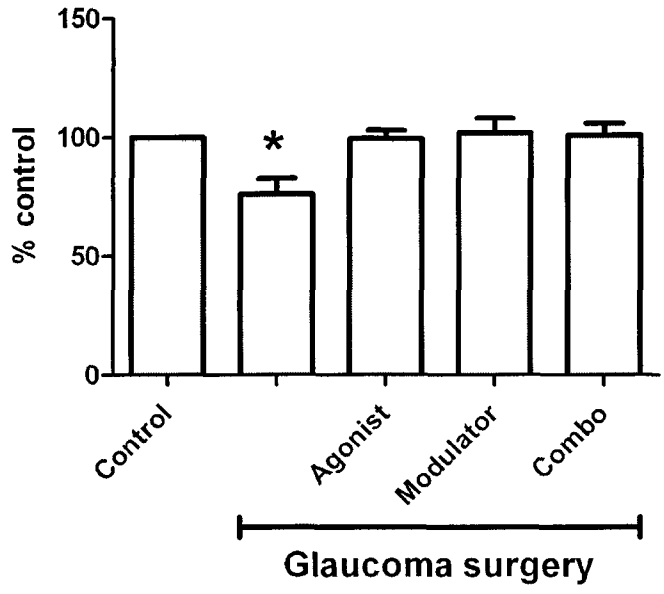

Figure 22: In vivo studies using PNU compounds

Cells in the RGC layer were counted and normalized to their internal control (left eye) 1 month after various treatments. Agonist indicated application of PNU-282987, modulator indicated application of PNU-120596 and combo indicates co-application of both PNU compounds before surgery to induce glaucoma. * indicates significance from control. Error bars represent SEM.

Labeling RGCs *in vivo*

Although careful consideration was given to count cells from specific preipheral regions in the RGC layer *in vivo,* cresyl violet stains all cell bodies that contain nissl substance, so it is uncertain if the cell counts fully represent the RGC population. Figure 23 represents images obtained from the peripheral retina after RGCs were stained with the Thy 1.1 antibody. Figure 23A is an image obtained from a untreated control retina. An average of 11.22% (SE+/-2.21) of cells in images

obtained from the periphery in the RGC layer did not label with the primary antibody (n=6). This suggests that the majority of cells in the peripheral rat retina in the RGC layer are RGCs.

Fig. 23B was obtained from the same animal's right eye 1 month after glaucoma surgery. There was an average loss of 28.12% (±3.2, n=6) of RGCs compared to the internal control. Though we have not identified the 11% of cells in the RGC layer that did not stain with antibody against Thy 1.1, previous studies have demonstrated that there is a population of displaced amacrine cells in the RGC layer in rats (Perry et al. 1980). However, the displaced amacrine cells are not prone to cell death induced by glaucoma (Kielczewski, 2005), therefore we can speculate that the loss of cells in the RGC layer from the surgical procedure to induce glaucoma, are primarily RGCs.

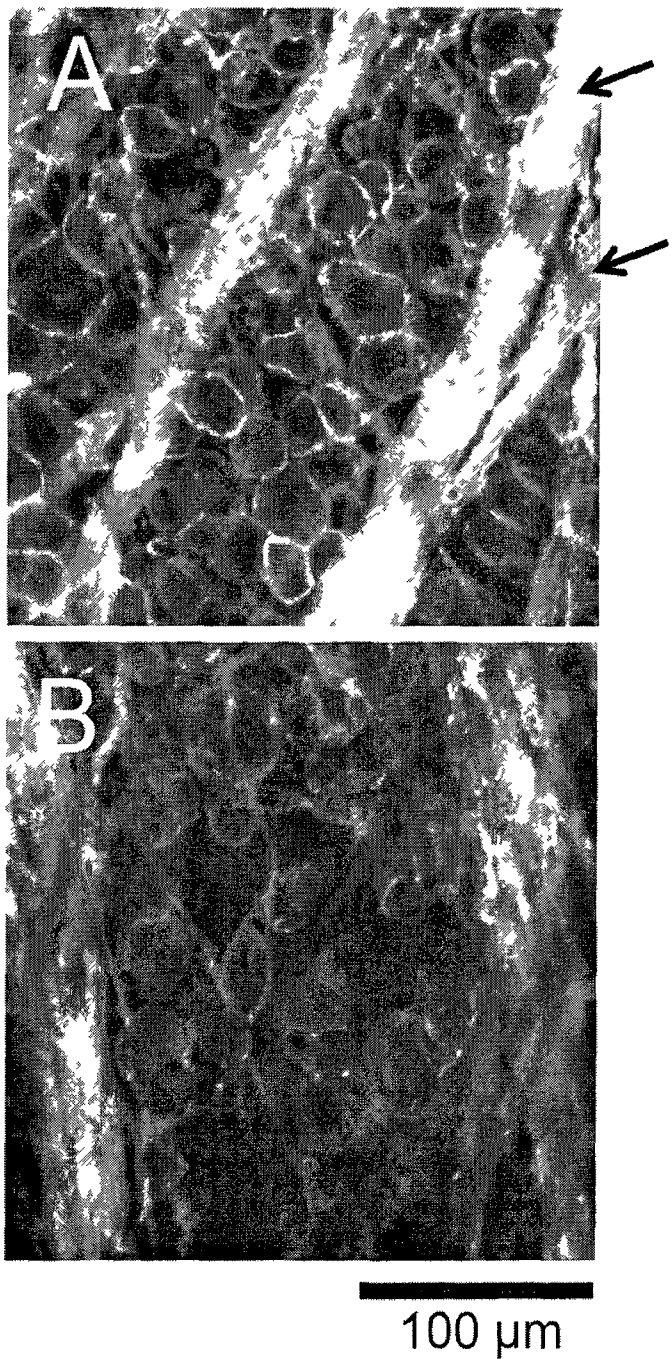

Figure 23: RGCs labeled with Thy 1.1

A) an image obtained from the untreated left eye of a rat. Arrows indicate axon tracts. B) an image obtained from the right eye from the same animal 1 month after surgery to induce glaucoma.

DISCUSSION

Excitoxicity

This study was designed to test the neuroprotective properties of a specific nAChR agonist and modulator against excitotoxicity in rat cell culture and against glaucoma-like conditions using an *in vivo* rat model. The excitotoxic effect of glutamate on cultured RGCs was previously examined using isolated pig RGCs (Luo et al., 2001; Wehrwein et al., 2005). We found that glutamate-induced excitotoxicity was also present in rat. *In vitro* studies demonstrated that 500μM glutamate caused an average of ~45% RGC loss after 3 days in dissociated rat retinal cell culture. When excitotoxicity was induced using KA, RGC survival decreased by ~34%. This cell loss was significant compared to control conditions, however it did not equal the cell loss with glutamate. The excitotoxic effect of KA was eliminated when CNQX, an antagonist specific to the KA receptor was applied. NMDA also induced excitotoxicity and caused an average loss of RGC of 23% compared to control. Again NMDA's effect represented a significant decrease of RGCs but it did not match the degree of loss associated with glutamate. The effect of NMDA was eliminated when MK-801, an antagonist specific to the NMDA GluR, was applied before NMDA. Thus it appeared that both KA and NMDA receptors

play a role in excitotoxicity in rat RGCs. To confirm this, when cells were cultured in a combination of NMDA and KA, cell survival decreased by an average of 43% which mimicked the excitotoxic effect of glutamate. If KA and NMDA were applied after the application of the antagonists, MK-801 and CNQX, their excitotoxic effect was eliminated. This suggests that glutamate-induced excitotoxicity was mediated though both NMDA and non-NMDA receptors which is consistent with data that indicates both types of GluRs can be found in the ganglion cell population (Muller et al., 1992, Hamassaki-Britto et al., 1993). These results are also consistent with pig studies done in this and other labs (Luo el al., 2001; Wehrwein et al., 2004).

Excitotoxicity and apoptosis has been associated with glaucoma in the eye and other neurodegenerative diseases in the brain. For instance, in Alzheimer's diseases, the loss of cognitive function is caused by apoptosis of neuronal cells (Niikura et al., 2006). Similar to results obtained in these glaucoma studies, neuroprotection against Alzheimer's disease has been linked to activity of α7nAChRs. Epidemiological studies showed that nicotine decreased the risk for AD (Fratiglioni and Wang, 2000). This negative association is in agreement with postmortem studies showing significant reduction of amyloid plaque deposits in former smokers with AD (Hellstrom-Lindahl el al., 2004a). Nicotine treatment

appears to interfere with the formation of the amyloid plaques *in vitro* and *in vivo* (Ono et al., 2002; Utsuki et al., 2002; Hellstrom-Lindahl el al., 2004a) and to reduce the accumulation of insoluble β-amyloid peptides (Nordberg et al., 2002) through a mechanism mediated by α7nAChRs as demonstrated in inhibition studies using neuroblastoma cells. In these inhibition studies, the α7nAChR antagonist, mecamylamine, was used to block nicotine's effect (Hellstrom-Lindahl el al., 2004b).

Neuroprotection

In this study, we examined the neuroprotective properties of 2 compounds specific to the α7nAChR. PNU-282987 is an agonist specific to the α7nAChR, and PNU-120596 has previously been shown to modulate this α7 receptor. Many laboratories have examined potential neuroprotective agents against glutamate excitotoxicity, by directly affecting the glutamate receptor with antagonists or by using other pharmacological agents that affect other specific receptors (Niikura et al., 2006). Other studies have looked at neurotrophic factors that affect the physiology to provide neuroprotection (Jiang, 2007). In this study I propose that neuroprotection can be triggered through ACh receptors found on RGCs.

In excitotoxic models, RGC death is thought to be caused by a large influx of

Ca^{2+} through glutamate receptor channels. However, we have shown that by adding ACh before a large glutamate insult, we are able to protect the RGCs. This suggests that ACh perhaps released from cholinergic starburst amacrine cells, may have a physiological neuroprotective effect under normal conditions. If so, this would represent a new role previously not understood associated with starburst amacrine cells. However when the retina is glaucomatous, these naturally occurring neuroprotective defense mechanisms could either be overwhelmed or ACh release from the starburst amacrine cells could be diminished. Since previous studies have shown that cholinergic amacrine cells are not lost in glaucoma-like conditions, other factors are likely responsible for the loss of neuroprotection (Kielczewski et al., 2005). For example, ACh release from the cells could be compromised. A decrease in ACh could be due to other extrinsic effects that compromise the starburst amacrine cell population directly, or indirectly affect the release of ACh from the amacrine cells. There is also the possibility that glutamate excitotoxicity or some aspect associated with the glaucoma condition could be affecting the ACh receptors at the RGC level by modifying the binding site or causing internalization of receptors (Shen et al., 2010).

Pig vs. rat

The *in vitro* studies using rat retina were done to determine if the excitotoxic and neuroprotective studies done in previous pig studies could be mimicked using rat tissue. In the rat study, the retina was dissociated but RGCs were not isolated. This procedure was done in the rat to increase cell density. Rat eyes are significantly smaller than pig eyes and many more rat eyes would be required in each experiment to maintain cell densities sufficient to sustain healthy culture systems. However in both methods, Thy 1.1 was used to identify the glycoprotein found exclusively on RGCs in the retina.

When comparing RGC death in pig and rat studies, 500μM of glutamate caused similar reductions of large RGCs in 3 days (rat 45% vs pig 42%). In both models the small RGCs were more resistant to damage than large RGCs. An average of 32% of small RGCs were eliminated with glutamate in pig studies while an average of 25% of small RGC were lost in rat studies. Surgery to induce glaucoma-like resulted in cell loss by more than 25%. Since starburst amacrine cell are not normally eliminated under glaucoma conditions (Kielczewski et al., 2005), it is likely that a significant number of cells that are lost in the RGC layer after hypertonic injections are RGCs.

In respect to neuroprotection from ACh and nicotine, both compounds protected against excitotoxicity in a dose dependent manner. For both the rat and pig, if the cells were pretreated with ACh, there was significant neuroprotection against 500μM glutamate. ACh applied at 10μM eliminated RGC death due to excitotoxicity completely. Nicotine had similar neuroprotective properties in both pig and rat studies. For the pig studies, maximum neuroprotection against excitotoxicity was observed when 10μM nicotine was used. From *in vitro* results using the rat, 100μM concentrations had maximal protection before cell survival began to decrease due to non-specific effects. Therefore nicotine and ACh provided neuroprotection against excitotoxicity in rat and pig studies, but to different degree and at different concentrations

Counting cells in the RGC layer

In original *in vivo* studies, cell counts were obtained from a flat mounted retina and cell bodies were stained with cresyl violet to visualize the cell bodies in the RGC layer. The cresyl violet stains cell bodies that contain nissl substance which is composed of rough endoplasmic reticulum. In the RGC layer, besides RGCs, there are Mueller cells, displaced amacrine cells and microglia. In some areas of the retina, the contribution of these other cells can be significant (Bernstein

and Guo, 2011). To address this issue, Thy 1.1, a glycoprotein found exclusively on RGCs in the retina, was used to verify that the loss of cells in the RGC layer that results from inducing glaucoma-like conditions are from the RGC population. The results of the specific staining of RGCs demonstrated that only an average of 11.22% (±2.21) of cells in the peripheral RGC layer were not RGCs, as they did not label with an antibody against Thy 1.1.

Implications and future studies

The main risk factor for glaucoma is an increase in IOP and all current therapies are designed to decrease IOP. However, other characteristics of the disease involve excitotoxicity and loss of RGCs and are more proximal to loss of function. In this study, I propose that pretreatment of the retina with an α7nAChR agonist might be used to prevent the excitotoxicity and loss of RGCs normally associated with glaucoma-like conditions. This would represent a different strategy for dealing with glaucoma and would be most beneficial for patients with a history of glaucoma in their family or could be beneficial to aged patients by preventing glaucoma from ever developing. Since the PNU compounds directly affect RGCs, this treatment could be used in any type of patient regardless of IOP. It is however best suited for patients as a preventative measure for people at high risk for glaucoma based on age,

genetics or race, since all our studies demonstrated that cells have to be preconditioned with α7 agonist. In fact, no neuroprotection occurs if the agents are given at the same time or after the glutamate insult (Wehrwein et al., 2004).

Though nicotine and other ACh agonists can produce similar neuroprotective effects, there are benefits of using specific agonists and modulator against the α7 nicotinic receptor on RGCs. Both PNU compounds used in this study are specific to the α7 nAChR. This specificity can be a great benefit physiologically. If used as an anti-glaucoma drug, the more specific an agent is to a certain receptor, the less side effects it will exert in the body. Broad general agonists can bind to multiple subtypes of receptors and can cause unwanted side effects. To avoid unwanted side effects, the method of drug delivery is also important. In this study, the PNU compounds were directly injected inside the eye via intravitreal injections. However, other non-invasive drug delivery methods to the eye may be beneficial. Delivery to the retina via topical eye drops has been shown to get PNU-282987 to the rabbit retina. When applied as eye drops, PNU-282987 reaches concentrations in the retina comparable to other glaucoma medications and remains in the retina at least 8 hours after application (Linn D. et al 2011). This could provide extended neuroprotection within the eye and is enough time to trigger cell survival pathways (Asomugha et al. 2010). More invasive methods such as systemic delivery though IP injections or

through transdermal patches can also produce prolonged effects and have been shown to provide neuroprotection in the rat glaucoma model (Linn C. et al., 2011), but these delivery methods will produce more side effects, as there are α7nAChR throughout the body and CNS.

One of the objectives of the present study was to compare previous excitotoxicity studies done with the pig with the current animal model using the rat. I also wanted to explore the effect of an α7 specific agonist and modulator against glutamate excitotoxicity and to test these agents against glaucoma-like conditions in an *in vivo* rat model. The results of this study suggest that neuroprotection against glutamate-induced excitotoxicity is not species specific as both pig and rat RGC can be protected against glutamate –induced excitotoxicity with pretreatment of ACh, nicotine and α7 specific PNU compounds.

The mechanism of cellular loss due to glutamate in rat RGCs is still unknown. However, previous studies done in pig studies have linked overstimulation of glutamate receptors to apoptosis (Asomugha et al., 2000). These previous studies found that excessive glutamate triggered p38 MAP kinase to initiate apoptotic cascade. This same mechanism could also occur in the rat, though further studies would be needed to support this.

The neuroprotective properties of both the PNU-282987 and PNU 120596

are promising but the mechanism responsible for neuroprotection in the *in vivo* system is still unknown and still need to be studied. Based on previous studies in pig, it can be postulated that glutamate and glutamate agonist, NMDA and KA, can trigger apoptotic pathways while ACh and ACh agonists decrease these apoptotic pathway and increase cell survival and proliferation through neuroprotective intracellular pathways (Asomugha et al., 2010). Other studies have shown that activation of PI3 kinase triggers a cell survival pathway in the retina. (Alvarez, 2009; Asomugha et al., 2010). Other compounds, such as brain-derived neurotrophic factor, can also protect RGCs through the PI3 kinase pathway (Nakazawa et al., 2002). Previous studies done in this lab have linked Ca^{2+} influx though nAChR channels to neuroprotection in pig RGCs (Brandt et al., 2011), but similar studies must be repeated in the rat using ACh and the PNU compounds to determine if similar links prevail.

In glaucoma conditions, it is still unknown if excitotoxic conditions overwhelm endogenous neuroprotective mechanisms through AChRs, or if neuroprotective mechanisms are blocked or decreased. Previous studies have demonstrated that the number of amacrine cells is not affected in experimental glaucoma models, and that most of the cell loss associated with glaucoma is from RGCs (Kielczewski et al., 2005). This was supported in my results. When Thy 1.1

was used as a specific label for RGCs in a flat mounted retina, approximately 90% of observable cells in the RGC layer were labeled, indicated that the vast majority of cells located in the RGC layer in the peripheral retina are RGCs. One month after injection of hypertonic saline, there was a significant loss of these labeled RGCs.

If glaucoma conditions target only the RGCs specifically and there is no decrease in number of amacrine cells, why doesn't endogenous ACh protect against the injection of 2M hypertonic saline that induces glaucoma-like condition? There may be other mechanisms that decrease neuroprotection. Though the amacrine cells may be intact, the release of ACh from these starburst amacrine cells could be compromised. There could also be a change in the acetylcholinesterase (AChE), an enzyme that breaks down ACh. In fact, a study done using Galantamine, an AChE inhibitor, has been shown to have neuroprotective effects in glaucoma models (Almasieh et al., 2010). Future studies need to be conducted that analyze any change in release of ACh from the amacrine cells. Alternatively, a change in the number or affinity of receptors on the RGC may occur to compromise the neuroprotective properties of ACh in our glaucoma model.

In this study, we reported that both large and small RGCs are affected by excitotoxicity. In previous studies done in lab using pig, it was shown that the α7 receptors are primarily found on the large RGC type, however, it is unknown if this

is the case for Long Evans rats. When analyzing PNU's effect on rat RGCs in culture, PNU agonist provided complete neuroprotection on large RGCs. In small RGCs, PNU-282987 provided partial neuroprotection against glutamate excitotoxicity. This suggests that α7 receptors are also present on small RGCs found in the retina in rat.

Although, PNU compounds prevent loss of cell from the RGC layer after the hypertonic injection, future studies must be done to address the function of these protected cells. Even though the number of RGCs do not change in the presence of PNU, it is important to verify that these cells function normally. To test this, electrophysiological studies must be done on the RGCs to determine if there are any changes in ion channel activity or to determine if there are any differences in response to protection.

When both PNU compounds were applied *in vitro*, there was a significant increase in the number of cells compared to control (Fig 20). What caused this increase in cell numbers beyond control levels in the *in vitro* model is unknown but a few speculations can be made. In the *in vitro* model, untreated control RGCs began to decrease in numbers by 3 days due to the dissociation and culture procedure (fig. 24). By day 7, approximately 50% of cells originally plated survived in control untreated conditions. PNU-282987 and PNU 120596 alone protected the

cells against glutamate excitotoxicity, however when used together, they protected RGCs from the excitotoxic effects of glutamate as well as from cell loss normally associated with the dissociation process (fig. 24). This is a plausible explanation as it is unlikely that there's an increase of RGCs in culture as adult neurons do not divide. It is, however, possible that there's an increase of RGCs due to the combination of PNU agonist and modulator, which acts together to lower the amount of normal cell death that normally accompanies the culture procedure. The increase in cell numbers beyond untreated conditions was not observed in the *in vivo* model of glaucoma.

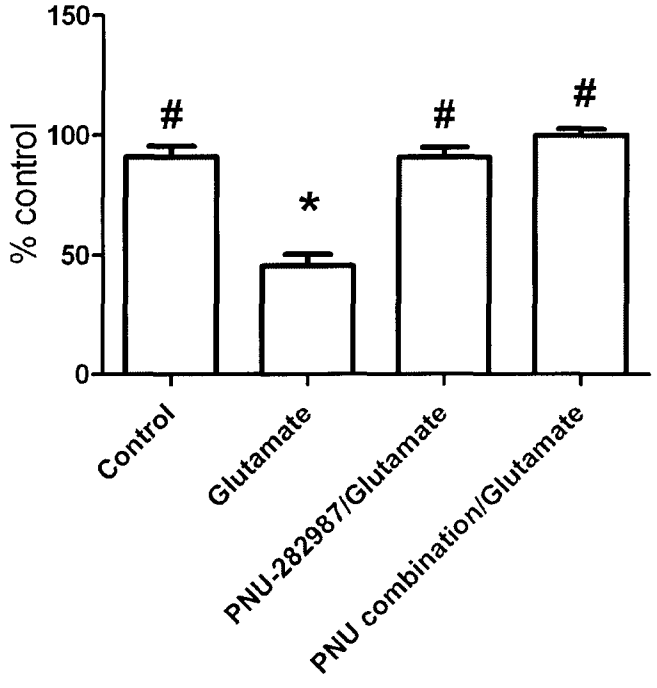

Figure 24: Both PNU compounds increase RGC counts

Each normalized data bar represents the percentage of cells that survived compared to control untreated conditions at Day 0. The PNU combination represents the percent survival of RGCs after three days in culture with 100nM PNU-282987 and 10nM PNU-120596 before 500μM glutamate. # represent significance from glutamate, and * represents significance from control untreated conditions. Normalized data were obtained from between 3-9 experiments. Error bars represent SE.

If using the PNU agonist and modulator together in culture can protect against normal cell damage and cell death associated with the dissociation process, these compounds could be included in standard culture media for RGCs to increase cell viability for researchers interested in culturing RGCs. Use of these agents may also have promising effects in an *in vivo* model, or in human clinical settings. Long term exposure to both compounds could be used to prevent normal cell death that occurs in disease states such as glaucoma, but also as a preventative measure for populations at high risk for RGC related ocular diseases.

In conclusion these data suggest that introduction of α7 nicotinic agonists and the PNU modulator can significantly reduce the loss of RGCs associated with glaucoma and glutamate excitotoxicity. It also strengthens the potential of the α7 nicotinic receptor as a potential therapeutic target for neuroprotection.

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APPENDIX

IACUC approval letter

WESTERN MICHIGAN UNIVERSITY

Institutional Animal Care and Use Committee

Centennial 1903-2003 Celebration

Date: May 13, 2009

To: Cindy Linn, Principal Investigator

From: Robert Eversole, Chair

Re: IACUC Protocol No. 09-03-02

Your protocol entitled "Investigation of Acetylcholine in an *in-vivo* Model of Glaucoma" has received approval from the Institutional Animal Care and Use Committee. The conditions and duration of this approval are specified in the Policies of Western Michigan University. You may now begin to implement the research as described in the application.

The Board wishes you success in the pursuit of your research goals

Approval Termination: May 13, 2010

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