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# Inhibiting effects of Methyllycaconitine (MLA) on PNU-282987 induced neurogenesis in blast-exposed mouse retinas

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**Synopsis**

Irreversible vision loss can be caused by retinal damage, disease, or aging. In recent years, the prevalence of ocular damage due to blast trauma has been increasing due to the modernization of warfare. Adult mammalian retinal neurons were previously thought to lack the capacity for regeneration to reverse vision loss, which deemed the effects of many ocular damages permanent. However, recent studies from this lab have shown that PNU-282987, an agonist to the alpha 7 subtype of neural nicotinic acetylcholine receptors, will result in neural regeneration in adult mammalian cells. For this research project, I will be using Methyllycaconitine (MLA), an antagonist to the alpha 7 subtype of neural nicotinic acetylcholine receptors to inhibit the effect of PNU-282987 in blast-exposed mouse retinas. The objective of this experiment is to verify that PNU-282987's regenerative effect in the adult mammalian retina is mediated through the alpha 7 subtype of neural nicotinic acetylcholine receptors. We expect that methyllycaconitine (MLA) will inhibit the effect of PNU-282987 in blast-exposed mouse retinas. The hypothesis of this experiment is that PNU-282987 will act on the alpha 7 subtype of neural nicotinic acetylcholine receptors to induce neurogenesis. For this study, a blast exposure was introduced to adult mouse eyes to mimic ocular damage that typically occurs in soldiers after landmine blasts during combat. The blasts were introduced using a modified paintball gun at 35 psi, which caused a significant loss of cells in the INL, ONL, and GCL. This was followed by PNU treatment or MLA treatment before the introduction of PNU. After treatment, the animals were euthanized, and the retinas were then removed, fixed, and then immunostained with primary antibodies RFP to label the Müller glial cells in tdTomato transgenic mice, as well as with antibodies against recoverin to label photoreceptors. Secondary antibodies including Alexa Fluor 594 (red) and 488 (green) were used for visualization using retinal sections with a Nikon confocal microscope. Photoreceptor cell counts increased in PNU-282987 treated blast-exposed retina,

while retinas in the presence of MLA and PNU-282987 showed cell loss due to blast. Meanwhile, tdTomato-positive photoreceptors were observed in blast-exposed retinas after 3 weeks of PNU treatments. However, if eyes were treated with MLA before PNU-282987 treatment, no tdTomato-positive photoreceptors were seen. These results support the hypothesis that PNU-282987 works through alpha7 nACh receptors. If vision loss can be regained via PNU-282987, it can improve the quality of life for many people that have experienced damage to their eyesight due to blast trauma as well as in neurodegenerative diseases.

## Introduction

### *1.1 Blast Injury*

In recent years, ocular injuries under combat conditions have been on the rise. Urbanization of warfare such as increasing the explosive power of weapons and insufficient eye protection during combat results in increased ocular blast injuries. A study by Robert Scott shows that eye injuries in combat conditions went from <2% during World War 2 to 13% in Operation Desert Storm in 1991 (Scott, 2011). Another study by McMaster and Gerry showed that out of 13,700 patients with ocular injuries, 78.1% of these injuries were reported to result from explosive weapons (McMaster & Clare, 2021). There are 4 different categories of blast injuries, which are primary, secondary, tertiary, and quaternary. Primary blast injuries are caused by explosive shockwaves that travel through the body which can result in tissue damage. Secondary blast injuries result from the impact of shrapnel sourced from explosive devices, or surrounding debris propelled by the explosion. Tertiary blast injuries are a result of individuals being thrown by the blast, and quaternary injuries are all other explosion-related injuries not caused by the first 3 blast categories. Ocular blast injuries are commonly a combination of primary and secondary injuries, such as globe ruptures, or eye penetration (*"Explosions and Blast Injuries,*" n.d.). As most of these injuries are non-fatal, consequences of vision loss or impairment include a reduction in quality of life, independence, mobility, and mental health (National Academies Press (US), 2016). Vision loss also causes a strain on the national healthcare system and for individuals and their families, whereby a 2013 national report found that the expenditures as a result of vision loss cost a whopping 139 billion dollars. (National Academies Press (US), 2016b).

*1.2 Previous Studies*

The retinal neurons of adult mammals were previously thought to lack the capacity for regeneration to reverse vision loss. However, organisms such as adult zebrafish can regenerate retinal and brain neurons throughout their lifetime. This is made possible when the Müller glial cells in fishes act as stem cells, dividing mitotically in the inner nuclear layer, migrating along the Müller glia (MG) to the outer nuclear layer, and eventually differentiating into rod photoreceptors throughout their growth period. When the retinal neurons are damaged or destroyed, the Müller glia dedifferentiates and undergoes a series of processes to generate a retinal progenitor that replaces the damaged retinal neurons (Lenkowski & Raymond, 2014). A similar mechanism is observed during the retinal neuroregeneration of chicks (Fischer, 2005).

Previous studies from this lab have shown that PNU-282987, an agonist to the alpha 7 subtype of neural nicotinic acetylcholine receptors had a neuroprotective effect in rat retinas in a dose-dependent manner when the agent was injected intravitreally. PNU-282987 was able to prevent the loss of retinal ganglion cells (RGC) when glaucoma-like conditions were induced (Iwamoto et al., 2014). However, when PNU-282987 was applied as eye drops, an increased number of RGCs were observed in adult rats compared to the internal control retina (Webster et al., 2017) The increased number of RGCs labelled with antibodies against Thy 1.1 suggested the proliferation of new retinal ganglion cell bodies and RGC axon fascicles (Webster et al., 2017). The neurogenic effect effects of PNU-282987 were further analyzed using 5-Bromo-2'-deoxyuridine (BrdU). BrdU was used to label mitotically active cells (S phase entry) and cells undergoing unscheduled DNA synthesis. BrdU-positive cells that were Thy 1.1 positive appeared numerous in PNU-282987 treated eyes in adult rodents.

To test out the hypothesis that BrdU-positive cells originated from the Müller glia, retinal sections were analyzed for the presence of BrdU-positive cells after eye drop

treatments containing PNU-282987 and BrdU between 1-28 days. It was observed that BrdU-positive cells appeared first in the INL, followed by the ONL and the GCL, suggesting that the BrdU-positive cells likely originated from the INL before migrating to the other retinal layers via interkinetic nuclear migration.

The cell bodies of Müller glia are located in the INL and project processes toward the outer and inner limiting membranes of the retina. To determine the role of Müller glia, retinal sections were immunostained with antibodies against BrdU, proliferating cell nuclear antigens (PCNA) (labels cells undergoing mitosis), vimentin (labels Müller glia), nestin (labels neuronal precursor progenitor cells in dividing cells), and 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) (labels cell nuclei blue). The results showed positive BrdU staining in the ONL, INL, and some Müller glia. The INL also had nestin and PCNA-positive cells. These results support the hypothesis that PNU-282987 acts as an agonist on the alpha 7 neural nicotinic acetylcholine receptors, causing Müller glia-derived progenitor cells to proliferate and differentiate into retinal neurons.

The mechanism of Müller glial cells and its ability to artificially induce neurogenesis begins when PNU-282987 is applied in the form of eye drops. When PNU-282987 reaches the retinal pigment epithelium (RPE), it binds to the alpha-7 nACh receptors found on RPE, resulting in changes in the gene expression of the RPE (Webster et al, 2022). Upregulation of genes involved in immune system activation, molecular signaling and transportation, cell differentiation, and gene translation occurs, while downregulated genes include those involved in gliosis and cytokine activity. The hypothesis is that changes in gene expression will result in the release of signaling molecules from the RPE to the endfeet of Müller glia resulting in cell proliferation. This process induces dedifferentiation and production of progenitor cells, which re-enters the cell cycle and migrates to the retinal layers for the production of retinal neurons in adult mammals.

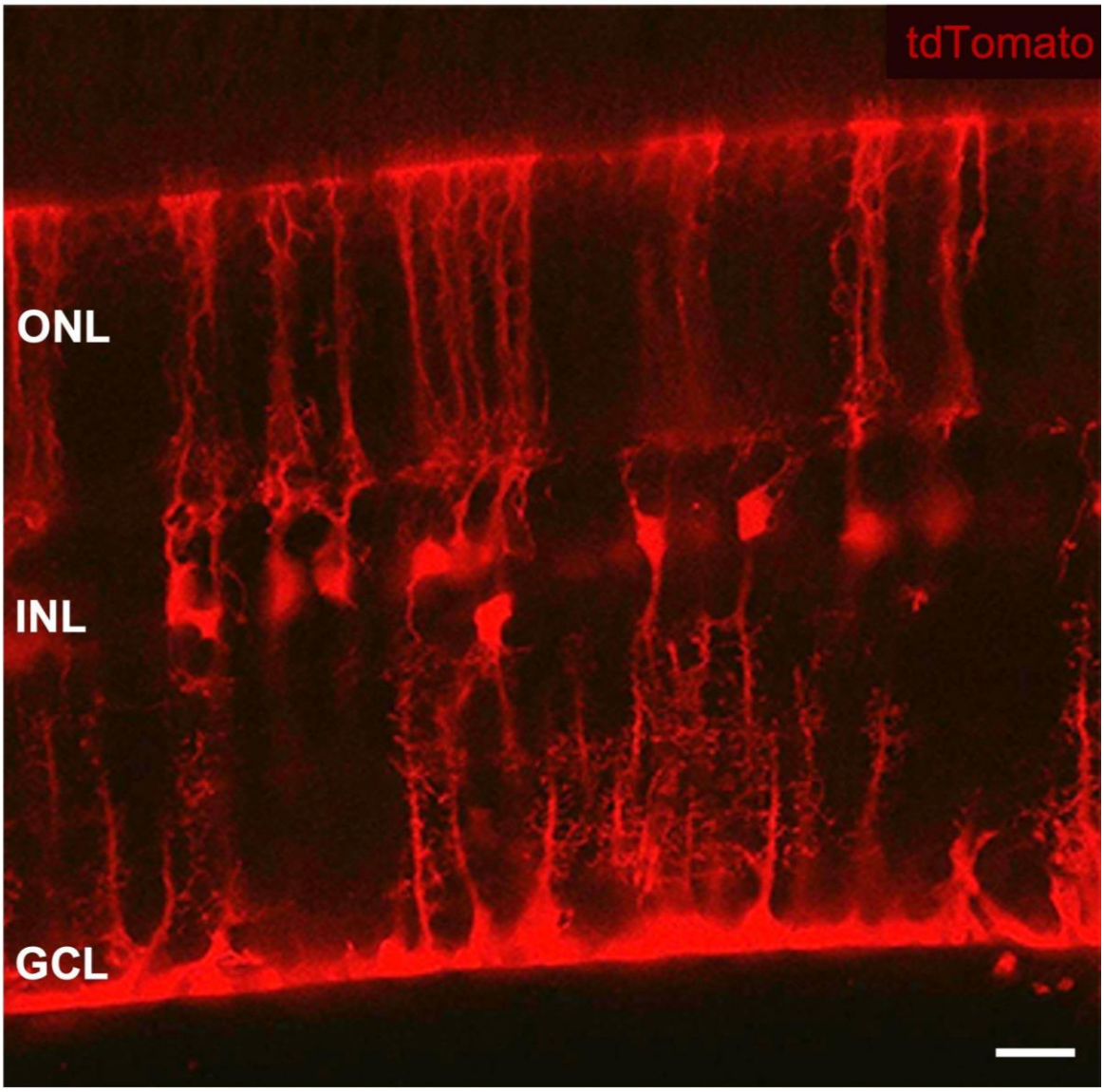


*Figure 1: Image of Müller glia from mice of the 129Svj strain that carries the RlbpCre-ER and Rosa-tdTomato transgenes.*

*1.3 Anatomy and Physiology of The Eye*

The eye can be distinguished into 3 layers, the outer region, the middle layer, and the inner layer (*Human Eye | Definition, Anatomy, Diagram, Function, & Facts*, 2023). The outer region consists of the cornea and sclera. The opaque sclera (white outer layer) is a connective tissue coat that maintains the shape of the eye and acts as a protective barrier against external and internal forces. A conjunctiva is a thin mucous membrane that covers the visible section of the sclera. On the other hand, the transparent cornea provides eye protection against infections or structural damage and refracts and transmits light to the lens and the retina. Together, the cornea and sclera are connected at the limbus.

The middle layer of the eye consists of the iris, choroid, and ciliary body. The iris regulates the amount of light that reaches the retina by controlling the size of the pupil, which is an opening at the center of the iris. The choroid lies between the sclera and the retina (*NCI Dictionary of Cancer Terms*, n.d.), and has blood vessels that provide oxygen and nutrients to the outer retinal layers. The ciliary body is a circular structure that is an extension of the iris. Besides producing aqueous humor, it also has ciliary muscles that change the shape of the lens (*Ciliary Body*, n.d.).

The inner layer of the eye contains the retina, which is responsible for converting photons of light into electrical signals. An optic nerve is formed by a bundle of ganglion cell axons, which runs to the brain via the optic disk (Purves, 2001) to transmit electrical impulses from the eye to the brain (*What Is Your Optic Nerve?*, n.d.). The retina also consists of blood vessels, which open into the retina to vascularize the neurons and retinal layers of the eye.

The eye consists of 3 chambers, the anterior chamber, the posterior chamber, and the vitreous chamber (*Eye Anatomy and Function*, n.d.). The anterior chamber is between the

cornea and the iris and the posterior chamber is between the iris and the lens (The Healthline Editorial Team, 2018). They both contain aqueous humor, a watery fluid that provides structural support, nutrients as well as the removal of waste products. The vitreous chamber is between the lens and the back of the eye and contains vitreous humor, consisting of 99% water and 1% collagen + hyaluronic acid which gives it a gelatinous consistency. The vitreous humor provides structural support, keeps the retina attached to the back of the eye, and absorbs shocks. Figure 2 illustrates the complete anatomy of the eye described.

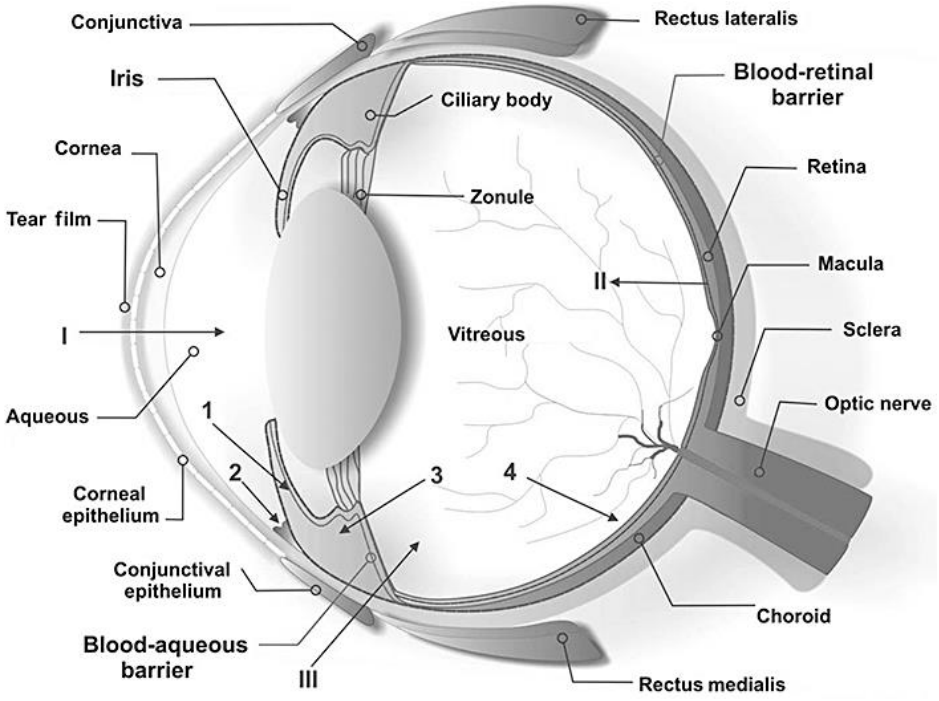


*Figure 2: Anatomy of the vertebrate eye. (Ocular Drug Delivery; Impact of in Vitro Cell Culture Models, 2009b)*

*1.4 Retina*

The retina lines the back of the eye and has a thickness of around 0.5mm (Mahabadi, 2022). It consists of several cell layers, including retinal ganglion cells, amacrine cells, bipolar cells, horizontal cells, Müller glial cells, and photoreceptors (rods and cones). The

retina plays the role of capturing and converting photons into chemical and electrical signals that are sent to the brain through phototransduction. It results in visual perception.

When viewed in a radial section, the retina contains 5 different categories of neurons which are interconnected via synapses. These neurons consist of photoreceptors, horizontal cells, bipolar cells, amacrine cells, and ganglion cells. Besides the neurons, the retina contains Muller glia cells, retinal pigment epithelium, and limiting membranes (Mahabadi, 2022), all of which are organized into 10 distinct layers with distinct roles. Starting from the layer closest to the pupil, we have the **inner limiting membrane**, which acts as a barrier between the vitreous body and the retina. It houses the end feet of Müller glial cells, which maintain homeostasis of the retina by supporting other cells and upholding retinal lamination. The **Retinal Nerve Fibre layer** contains axons of the retinal ganglion cells, astrocytes, and Müller cell processes. The next layer is the **ganglion cell layer**, which consists of ganglion cell bodies that form the optic nerve through the projection of their axons. Ganglion cells receive chemical signals from the retinal interneurons bipolar and amacrine cells (*Ganglion Cell Physiology by Ralph Nelson – Webvision*, n.d.). **The inner plexiform layer** is the location of synapse between the axons of bipolar cells, dendrites of amacrine cells, and ganglion cells. The amacrine cells modulate the electrical conduction between bipolar cells and ganglion cells to prevent lateral potentiation, enhancing contrast, light adaptation, and color discrimination in visual perception (Kramer & Davenport, 2015). Followed by that is the **inner nuclear layer**, which is composed of the cell bodies of horizontal cells, amacrine cells, bipolar cells, and Muller glia (Mahabadi, 2022). Horizontal cells modulate feedback onto rod and cone cells, and bipolar cells act as channels that transmit and encode synaptic inputs from photoreceptor cells to ganglion cells.

The **Outer Plexiform Layer** is where photoreceptor cell projections synapse with dendrites of cells in the inner nuclear layer, and the **Outer Nuclear Layer** contains cell

bodies of rod and cone photoreceptors. The **External Limiting Membrane** contains gap junctions between the Müller cells and the photoreceptor cells and acts as a separator between the inner and outer segments of rod and cone cell bodies. The **Photoreceptor Layer** is made up of the outer segments of rod and cone photoreceptors. The outer segments of the photoreceptors contain membrane-bound disks with photopigments like rhodopsin for phototransduction to occur. The inner segments are rich in mitochondria to meet the high metabolic demands of the photoreceptors. Lastly, the **Retinal Pigment Epithelium** is the outermost retinal layer that is one cell thick, and adjacent to the choroid layer. It plays vital roles such as regulating the blood-retinal barrier (BRB), responsible for ion and water transport, and secretes cytokines and growth factors. Because of its proximity to the outer segments of rods and cones, recycling the all-trans-retinal back into 11-cis-retinal is made possible, and phagocytosis of old worn-out rhodopsin pigments occurs. This explains the importance of the retinal pigment epithelium in the support and maintenance of photoreceptors and the capillary endothelium. Figure 3 shows the complete anatomy of the retina as described above.

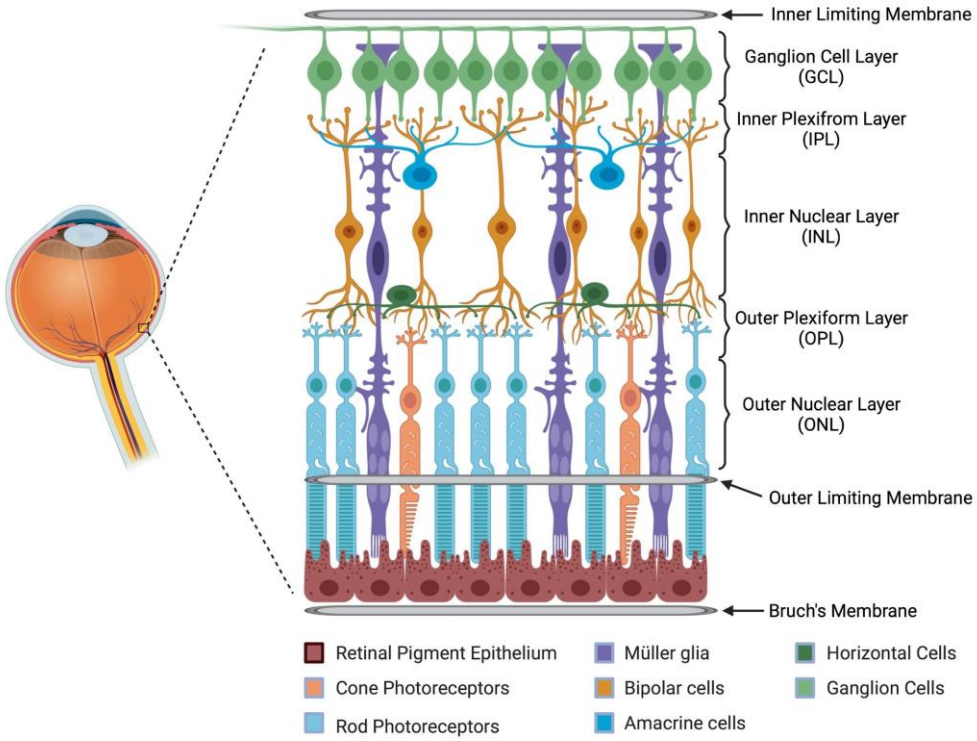


*Figure 3: Anatomy of the Vertebrate Retina.*

*1.5 Phototransduction*

The process of transduction is important for us to understand how the structures of the retina come together. The direct transmission of visual information from the eye to the brain involves the photoreceptor cell, bipolar cell, and ganglion cell (Lumen Learning, n.d.). This process begins from the rods and cones (photoreceptors) that contain photopigments. Rhodopsin is the main photopigment in vertebrates and exists in 2 parts. The first is the membrane protein opsin, and the second is the light-absorbing molecule retinal. When light strikes the rhodopsin, the G protein transducin which is composed of the alpha, beta, and gamma subunits will be activated. The GDP on the alpha subunit is replaced by GTP, resulting in its dissociation and binding to the phosphodiesterase. The phosphodiesterase will then convert cGMP to GMP, which will cause the closing of the sodium ion channels. Because sodium ions are unable to enter the cell, coupled with potassium ions leaving the cell

continuously, the membrane becomes hyperpolarized and driven further from the threshold. This will cause a decrease in glutamate release from the photoreceptor's axon terminal in response to light.

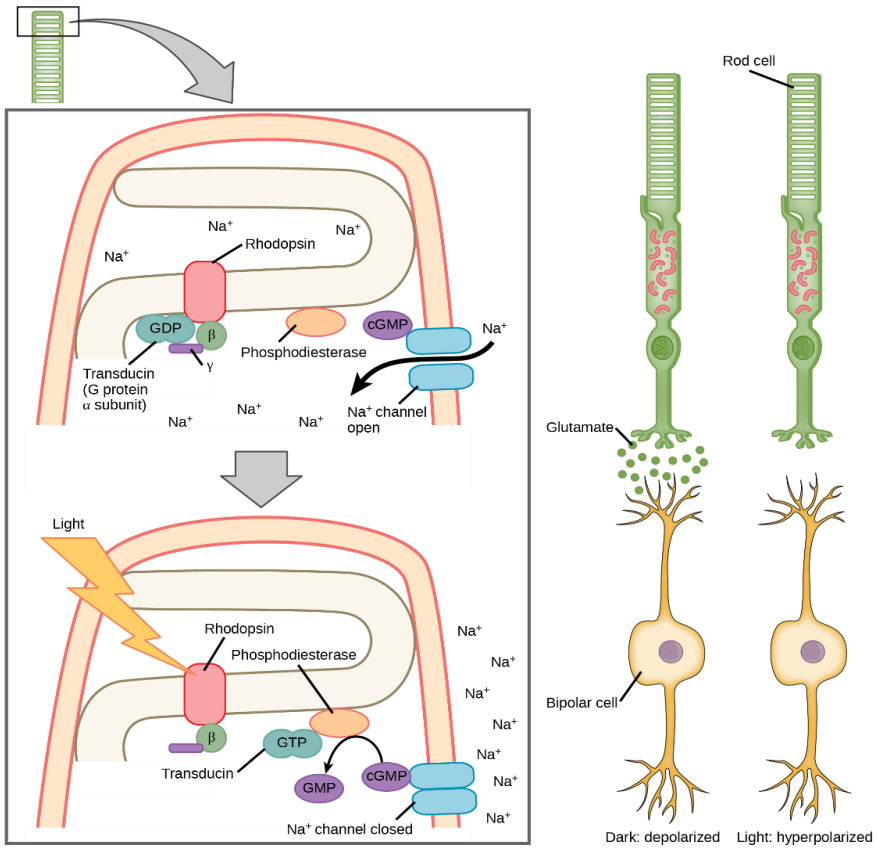


*Figure 4: Reactions in Rhodopsin. (Lumen Learning, n.d.-a)*

An estimated 125 million photoreceptor cells synapse with 10 million bipolar cells, and bipolar cells respond to glutamate release through graded potentials (Dragoi, 2019). There are at least two main types of bipolar cells, which are off and on bipolar cells. The off-bipolar cells are depolarized by glutamate when the photoreceptors are in the dark, due to the opening of an ionotropic glutamate receptor channel and hyperpolarizing in the light. The on-bipolar cells hyperpolarize in the dark due to the activation of a metabotropic glutamate receptor and depolarize in the light. Traveling down the bipolar cells, their axon terminals synapse with dendrites of ganglion cells. When bipolar cells are depolarized, the neurotransmitter, glutamate, is released. Ganglion cell depolarization can result in the

generation of action potentials that will carry visual information through the optic nerve to the occipital lobe in the cortex of the brain where final visual perception occurs. The horizontal cells modulate the synaptic activity of receptor cells and, thereby, indirectly affect the transmission of visual information by bipolar cells. Similarly, the amacrine cells modulate the synaptic activity of the retinal bipolar and ganglion cells, thereby affecting the transmission of visual information by the ganglion cells.

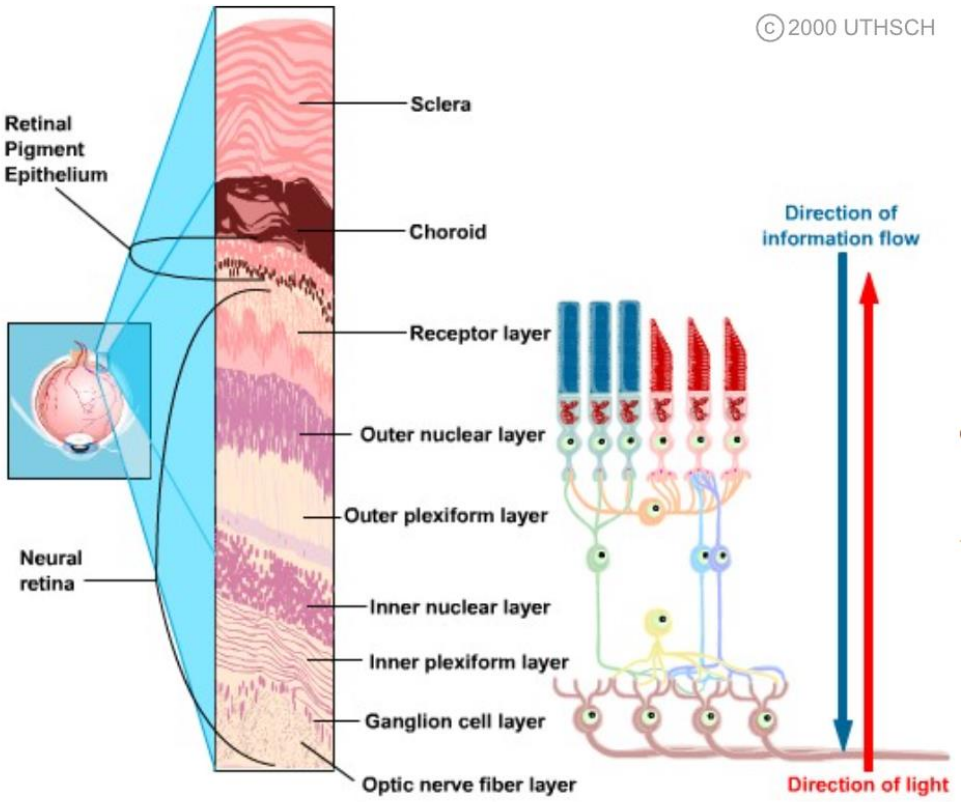


*Figure 5: Phototransduction across the retina. (Dragoi, 2019)*

*1.6 PNU-282987 and Alpha 7 Subtype of Neural Nicotinic Acetylcholine Receptors*

Alpha 7 neural nicotinic acetylcholine receptors (nAChRs) are found in the central and peripheral nervous systems and the neuromuscular junction (Walker et al., 2006). These receptors are pentameric ligand-gated ion channels formed from the combinations of the alpha and beta subunits, or as homopentamers. The alpha 7 nACh receptor channels are

formed from 5 alpha subunits and are essential in modulating the GABAergic synaptic transmission, whereby a deficit of its function is linked to diseases like Alzheimer's or mental illnesses like Schizophrenia (Noviello et al., 2021). In the past, α7 nAChR agonists correlated with improved cognitive performance in animal models and schizophrenic patients (Bodnar et al., 2005). Thus, more research was carried out on α7 nAChR agonists.

PNU-282987 (N-[(3'R)-1'-azabicyclo[2.2.2]oct-3'-yl]-4-chlorobenzamide hydrochloride) is a selective α7 nAChR agonist developed by Pharmacia and Upjohn and was seen to be useful in treating cognitive and attentional deficits of schizophrenia. However, further research showed that PNU-282987 inhibited the human Ether-a-go-go (hERG) potassium channel when it was applied systemically, which was essential for regulating electrical activity in the heart, making it unviable for human application (Sanguinetti & Tristani-Firouzi, 2006).

However, if PNU-282987 is applied as eye drops, it doesn't reach systemic levels that affect heart activity, and positive results from the use of PNU-282987 have been surfacing. In a study by Mata et. al. (2013), LC/MS/MS (liquid chromatography: mass spectroscopy with triple quad capabilities) was carried out that demonstrated PNU-282987 was detected in the retina when applied as eye drops, but not detectable in heart samples (Mata, 2013).

*1.7 Functional recovery of retinal function with PNU-282987 in a blast model*

Recently, a study by Spitsbergen et. al demonstrated the loss of cells in the ONL, INL, and GCL layers after a blast exposure of 35 PSI that was delivered using a modified paintball gun (Spitsbergen et al., 2023). After 4 weeks of PNU-282987 treatment in the blast-exposed retinas, an increase in cell count was observed across all three retinal layers. Functional changes in the adult mouse retina were also monitored across this period. Increased amplitudes in dark-adapted ERG recordings indicated improved electrophysiological function, and increased amplitudes in the dark-adapted flicker frequency series recordings indicated improvement of the ON- and OFF- cone-dependent pathways of the retina. PhNR recordings also showed significant recovery in amplitudes after PNU-282987 treatment, which translated to increased RGC functions.

*1.7 Transgenic Mouse Model*

In this study, we used mice of the 129Svj strain that carries the *Rlbp Cre-ER* and *Rosa-tdTomato* transgenes. The *Rlbp Cre-ER* mice carry a Cre-transgene under the *Rlbp* promoter which is tamoxifen-inducible and results in Cre recombinase expression in the Müller glia. The *Rosa-tdTomato* Cre-reporter is expressed after the Cre-excision of the stop codon in the transgene (Webster et al., 2019). This transgenic line allows visualization of retinal progenitor cells and Müller glia differentiation in the adult retina (Vázquez-Chona et al., 2009). These transgenic mice are generated by the crossing of a Cre+/tdTomato+ male and a Cre-/tdTomato + female mouse (Webster et al., 2019). The male mouse is heterozygous for *Rlbp Cre-ER*, and homozygous or heterozygous for the tdTomato reporter. The female animal lacked the *RlbpCre-ER* transgene and was homozygous for the tdTomato reporter. Through tail biopsies, the pups were genotyped through polymerase chain reaction (PCR) analysis.

These mice were used to trace the lineage of cells derived from the Müller glia, specifically photoreceptors located in the outer nuclear layer (ONL) of the retina after retinal blast exposure. These tdTomato-positive / Müller glia-derived photoreceptors should appear red in the photoreceptor cell bodies under the confocal microscope. When also labelled with antibodies against recoverin and secondarily labelled with Alexa Fluor 488, newly generated photoreceptors that originated from Muller glia should be in the ONL with a red cell body and green fluorescence surrounding them.

**MLA**

Methyllycaconitine (MLA) is a selective antagonist to the alpha 7 neural nicotinic acetylcholine receptor. In the study by Webster et al. (2019), transgenic mice were given MLA in the form of intravitreal injections before eye drop treatments of PNU-282987 + BrdU (Webster et al., 2019). There were significantly lower BrdU+ cell counts when compared to cells without MLA treatment, while chronic exposure to MLA inhibited the incorporation of BrdU mediated by PNU-282987.

Because of the absence of α7 nACh receptors on MG, further tests were carried out on α7 nACh receptors on the retinal pigment epithelium (RPE). RPE cells were treated with PNU-282987 or MLA with PNU-282987. The cells were cultured for different times (0-72 hours), washed, and their modified culture media was injected into the vitreous chamber of adult mice that were treated with BrdU eye drops for 2 weeks. Mice injected with PNU-282987 treated culture media (72 hours) exhibited BrdU+ cells in the INL and ONL, while the presence of MLA significantly reduced the number of BrdU+ cells. The results support the hypothesis that the proliferation of MG is mediated by the α7 nACh receptors located in the RPE when PNU-282987 is applied. For this research project, I will be using Methyllycaconitine (MLA), an antagonist to the alpha 7 subtype of neural nicotinic

acetylcholine receptors to inhibit the effect of PNU-282987 in blast-exposed mouse retinas. 1 drop of 1mM methyllycaconitine (MLA) in DMSO will be applied to each eye prior to PNU-282987 treatment. The objective of this study is to determine if Methyllycaconitine (MLA) will inhibit the effect of PNU-282987 in blast-exposed mouse retinas to test the hypothesis that PNU-282987's effect is mediated through α7 nACh receptors on RPE.

## Experimental Methods

### *2.1 Blast Procedure*

Three adult transgenic and wild-type 129 SVJ male mice were used in this experiment, which were aged between 3-6 months and weighed 20-25 grams. These mice were bred and kept in Western Michigan University's animal facility, with free access to food and water in a 12-hour light/dark cycled environment. They were cared for in accordance with the standards set by the Institutional Animal Care and Use Committee (IACUC) at Western Michigan University. All experiments involving animal subjects were also carried out according to the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised in 1996.

With permission from Dr. Edward Levine of Vanderbilt University, the transgenic 129/SvJ mice were generated with the RlbpCre-ER (Cre+) and Rosa-tdTomato (tdTomato+) transgenes. At six weeks, the mice genotyped to contain Cre+/WT; tdTomato+ were injected intraperitoneally with 10 mg/mL tamoxifen in corn oil for 3 consecutive days (300 µL total volume). This step results in the expression of the tdTomato fluorescent protein in the Müller glial cells. (Webster et al., 2019; Webster et al., 2021). To mimic the damage initiated from blast exposures in combat conditions, a commercially available paintball gun (Invert Mini,

Empire Paintball, NJ, USA) was modified according to Hines-Beard et al., (2012). Modifications include a shortened barrel with a modified front to increase the pressure of air and allow a more focused output on the mice's eyes. A pressure regulator is built into the paintball gun to allow control of the output pressure. The pressure was calibrated with a force transducer, which is connected to the PowerLab and LabChart software (AD instruments).

Before any blast experiments, the animals were anesthetized with ketamine/acepromazine/xylazine (KAX) through an intraperitoneal (IP) injection at a concentration of 66/1.3/6.6 mg/kg body weight. Approximately 10 minutes after the KAX injection, they were given a toe pinch, and a lack of response would confirm that the animals were completely anesthetized. Next, the mice were placed in a heavily cushioned open mouse holder to keep their head and eye positions well aligned. The head of each mouse was gently restrained to avoid whiplash from the blast pressure. The barrel of the gun was positioned directly perpendicular to the anatomical left eye with a distance of 1-2mm before a single blast of pressurized air at 35 PSI was delivered. Figure 6 illustrates the blast process as stated above. All three mice were given 35 mg/mL acetaminophen in their drinking water 3 days before and seven days after the blasting procedure.

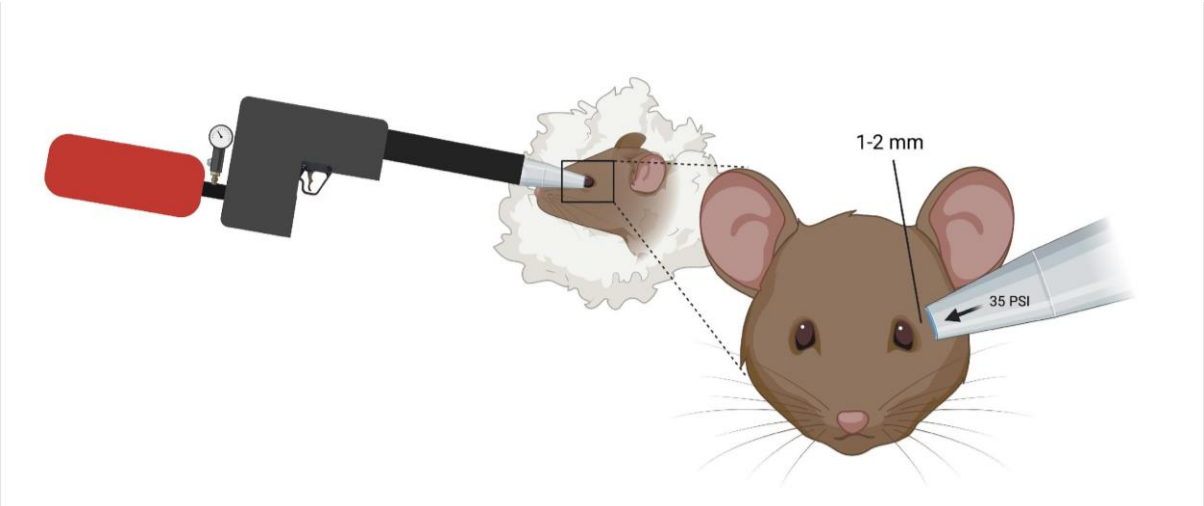


*Figure 6: Blast Procedure for Transgenic Mice. (Spitsbergen et al., 2023)*

*2.2 Eye Drop Treatment*

All three mice were treated with eye drops once a day on the left and right eyes, for a total period of 21 days. For the control mouse #687, 1 drop of 1mM PBS (Phosphate Buffered Saline) was dropped in each eye. Experimental mouse #685 received 1 drop of 1mM PNU-282987 on each eye. Experimental mouse #718 received 1 drop of 1mM methyllycaconitine (MLA) in DMSO on each eye and 1 drop of 1mM PNU-282987 on each eye after 1 hour.

*2.3 Retinal Isolation and Preparation*

After 3 weeks, all three mice were euthanized through CO2 asphyxiation. They were examined for the absence of reflexes and breathing to confirm death. The thoracic cavity was then punctured with a scalpel to ensure euthanasia according to IACUC protocols. Both their left and right eyes were then removed using curved forceps, and the excess tissue around the optic nerve was removed with Noyes scissors. A cut was made in a circumference behind the iris to separate the anterior and posterior sections of the eyes surgically. After the removal of the lens and the anterior chamber, a cut was made down the sclera, and the retina was gently nudged away from the interior surface of the sclera to ensure it remains intact, before cutting it away from the optic nerve. The intact retinas were then transferred into individually labelled Sylgard dishes. Next, four equidistant cuts were made on the edges of the retina towards the optic nerve head to allow the retina to be flat-mounted. They were then pinned down and flat mounted on the Sylgard dishes using cactus needles with the retinal ganglion cell layer facing upwards. The entire process was completed within 10-15 minutes. The pinned retinas were then fixed in 4% paraformaldehyde (PFA) in PBS at 4°C overnight.

The next day, PFA from each Sylgard dish was disposed of as hazardous waste using a micropipette, and the retinas were rinsed three times with PBS solution. After that, the retinas

were unpinned and moved into individually labelled microfuge tubes containing PBS. The PBS was removed and replaced with 100% Methanol (MeOH) and stored at -20°C until processed immunocytochemically.

*2.4 Immunohistochemistry*

**Day 1**

To initiate the rehydration step, the 100% Methanol was carefully removed from the microfuge tubes using a serological pipette. It was then replaced with 300 microliters of 75% MeOH/25% PBS solution and left for 5 minutes at room temperature. The entire process was repeated by replacing the 300 microliters of 75% MeOH/25% PBS solution with 50% MeOH/50% PBS solution, 25% MeOH/75% PBS solution, and then finally 100% PBS solution.

Next, the retinas were permeabilized using 1% PBSTr with 1% Donkey and 1% Goat serum for 1 hour and 45 minutes on a rocker at room temperature. The PBSTr was made using PBS and 1% of Triton-X-100. After this step, the retinas were rinsed with PBS. This was followed by refixing of the retinas in cold 4% paraformaldehyde (PFA) for 1 hour and 30 minutes at room temperature on a rocker. The PFA was discarded as hazardous waste, and the retinas were washed with PBS again. Lastly, 500 microliters of primary antibodies (Anti RFP (to label tdTomato positive Muller glia, and Anti Recoverin (to label photoreceptors) were diluted in 1% donkey serum and PBS and incubated overnight at room temperature on the rocker (Table 1).

**Day 2**

The primary antibodies were removed, and the retinas were rinsed with 0.1% PBSTr at room temperature on the rocker, followed by PBS. Next, the retinas were incubated in 500 microliters of secondary antibodies Donkey anti-goat AF 594 (red) and Donkey anti-rat AF 488 (green) diluted in PBS for visualization of the primary antibodies. The tubes were covered in foil and left overnight at room temperature on the rocker (Table 1).

**Day 3**

The secondary antibodies were removed from the tubes, and the retinas were washed in PBS. The retinas were stored in 50% PBS and 50% glycerol and were left for several days to clear before it was mounted. After antibody staining, retinas were counterstained with DAPI (0.1 microgram/ml) to stain all cell nuclei.

*Table 1: List of Primary and Secondary Antibodies Used.*

| **Primary Antibodies** | **Dilution** | **Label** |
|---|---|---|
| Anti RFP (anti-goat) | 1:200 | Transgenic Müller glia |
| Anti Recoverin (anti-rabbit) | 1:100 | Photoreceptors |
| **Secondary Antibodies** | **Dilution** | **Label** |
| Donkey anti-goat AF 594 (red) | 1:300 | Transgenic Müller glia |
| Donkey anti-rabbit AF 488 (green) | 1:300 | Photoreceptors |

*2.5 Mounting*

The retinas were sectioned into 40-micron strips and positioned all 3 nuclear retinal cell layers facing upwards. Sections were suspended in 50% PBS and 50% Glycerol on a coverslip on a glass slide.

*2.6 Microscopy and Cell Counts*

To visualize the processed retinas, a Nikon C2+ scanning laser confocal microscope (Tokyo, Japan) with a 60x water immersion objective lens was used. An average of 5 images were captured from retinas from the following retinal conditions: blast with PNU, blast with PBS, and blast with MLA and PNU. The 200 $\mu m^2$ images were obtained from different quadrants located 4mm from the optic nerve head (Iwamoto et al., 2014; Mata et al., 2015; Webster et al., 2021). Using ImageJ software, the total cell count of the outer nuclear layer (ONL) was obtained, and the change in cell percentage to the control data was calculated for each retinal condition. The total number of double-labelled photoreceptors (indicated by co-labelling of TdTomato (RFP) positive neurons and recoverin in photoreceptors) was obtained, and the percent of double-labelled photoreceptors against the total number of cells in the ONL was calculated and recorded.

*2.7 Statistical Analysis*

Statistical analysis was carried out using the data collected. A T-test was used to compare the means between the control group and the following experimental groups: blast, PNU, blast with PNU, and blast with MLA and PNU. A bar chart was created for data visualization. The result is statistically significant when the p-value is less than or equal to 0.05.

## Results

### *3.1 Effect of Blast Exposure on Photoreceptors In The ONL*

All 3 male mice underwent the blast procedures with the modified paintball gun with a blast pressure of 35 PSI. Previous studies by Spitsbergen et al. have shown that a blast of PSI was sufficient to cause significant loss of cells in the INL, ONL, and GCL retinal layers, with no occurrences of death in animals due to the damage and no changes in the thickness of the ONL (Spitsbergen et al., 2023). The confocal images in Figure 7 demonstrate the effect of a 35 psi blast on retinal cell loss in the photoreceptor layer. When quantified and compared to the control group (Fig. 7A), an average decrease of 14.58% in cell count was observed in the ONL with blast exposure, from an average cell count of 232.6 cells (control) to 198.7 cells (blast-exposed) (Fig. 7B). The T-test value of 0.05 indicates that the average cell count after blast exposure is significantly different from the control group. These cell counts were obtained from an average of 200 $\mu m^2$ (60x) images for both the control condition and the blast conditions.

### *3.2 The Effect of PNU-282987 After Blast Injury In The ONL*

To determine the neuro-regenerative properties of PNU-282987, both blast-exposed mice and mice in unblasted conditions were given PNU-282987 treatment in the form of eye drops for 3 weeks. After treatments, the retinas were viewed under the confocal microscope, and cell counts were obtained from 200 $\mu m^2$ (60x) images for both the unblasted retina with PNU-282987 and the blast-exposed retina with PNU-282987. The image obtained for the blast-exposed retina with PNU-282987 treatment showed a close resemblance in cell density to the ONL seen under control unblasted conditions. When compared to the control group (Fig. 7A), a 9.29% increase in the average cell count was observed in the ONL with blast exposure and PNU-282987 treatment, from an average cell count of 232.6 cells (control) to 254.16

cells (blast-exposed with PNU-282987) (Fig. 7C). The T-test value of 0.32 indicates that the average cell count of the blasted retina with PNU-282987 treatment is not significantly different from the control group. For the unblasted retina with PNU-282987 treatment, an 11.65% increase was observed when compared to the control group. The average cell count increased from 232.6 cells (control) to 259.7 cells (unblasted with PNU-282987). The T-test value of 0.13 indicates that the average cell count of the unblasted retina with PNU-282987 treatment is not statistically significant from the control group. No significant changes in retinal thickness were observed in the ONL.

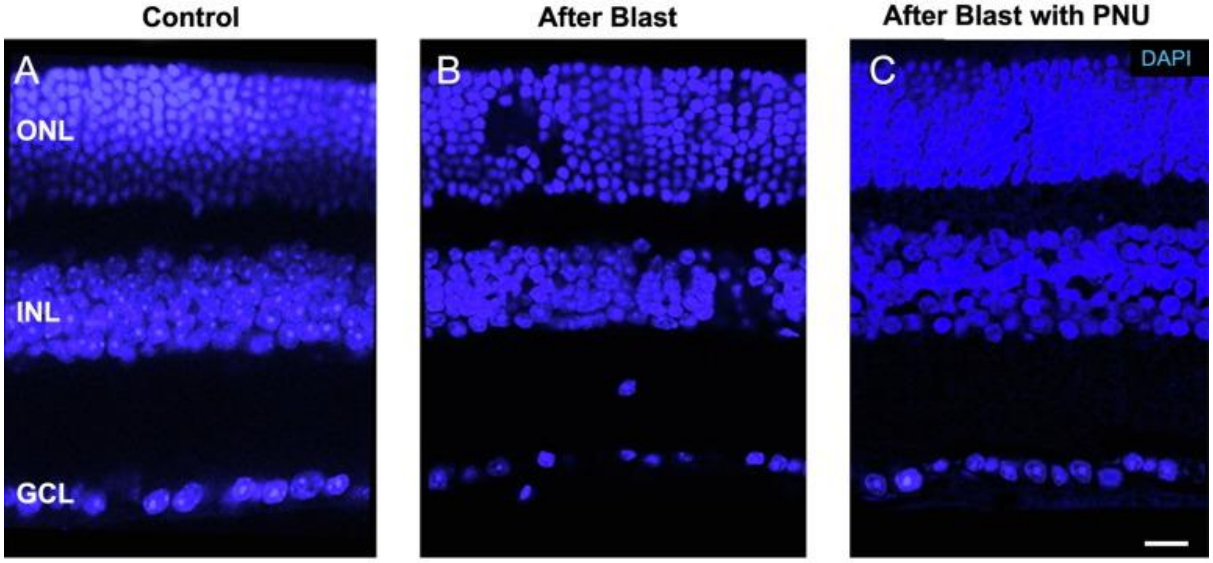


*Figure 7: Confocal Image of Retinal Cell Layers in (A) Control, (B) Blast, and (C) Blast with PNU conditions. (Spitsbergen et al., 2023)*

*3.3 Effect of MLA with PNU-282987 Treatment on Retinal Neural Regeneration in Blast-Exposed Retinas*

To determine the specificity of the agonist PNU-282987, to the alpha 7 subtype of neural nicotinic acetylcholine receptors in the retinal pigment epithelium, Methyllycaconitine (MLA) treatment was given in the form of eye drops 1 hour before daily PNU-282987 treatment for 3 weeks. The retinas were viewed under the confocal microscope, and cell

counts were obtained from an average of 200 $\mu m^2$ (60x) images for the blast-exposed retina with MLA and PNU-282987 treatment. After blast exposure, a significant loss of photoreceptors in the outer nuclear layer (ONL) of the retina was observed (Fig. 8B) when compared to the control group (Fig. 8A). It resembled the confocal image of blast-exposed retinas with no PNU-282987 treatment. Table 2 quantifies these findings. When compared to the control group (no blast exposure and no PNU-282987 treatment), a 16.15% decrease in the average cell count was observed in the ONL, from an average cell count of 232.6 cells (control) to 195 cells (blast-exposed with MLA and PNU-282987). The T-test value of 0.02 indicates that the average cell count of the blasted retina with MLA and PNU-282987 treatment is statistically different from the control group providing evidence that MLA blocked the regenerative effect of PNU-282987.

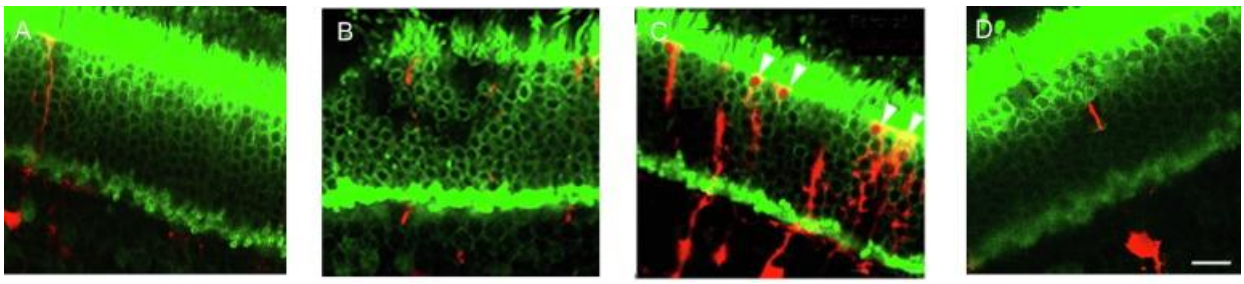


*Figure 8: Confocal Image of Retinal Cell Layers in (A) Control, (B) Blast, (C) Blast with PNU, (D) Blast with MLA and PNU (Spitsbergen et al., 2023)*

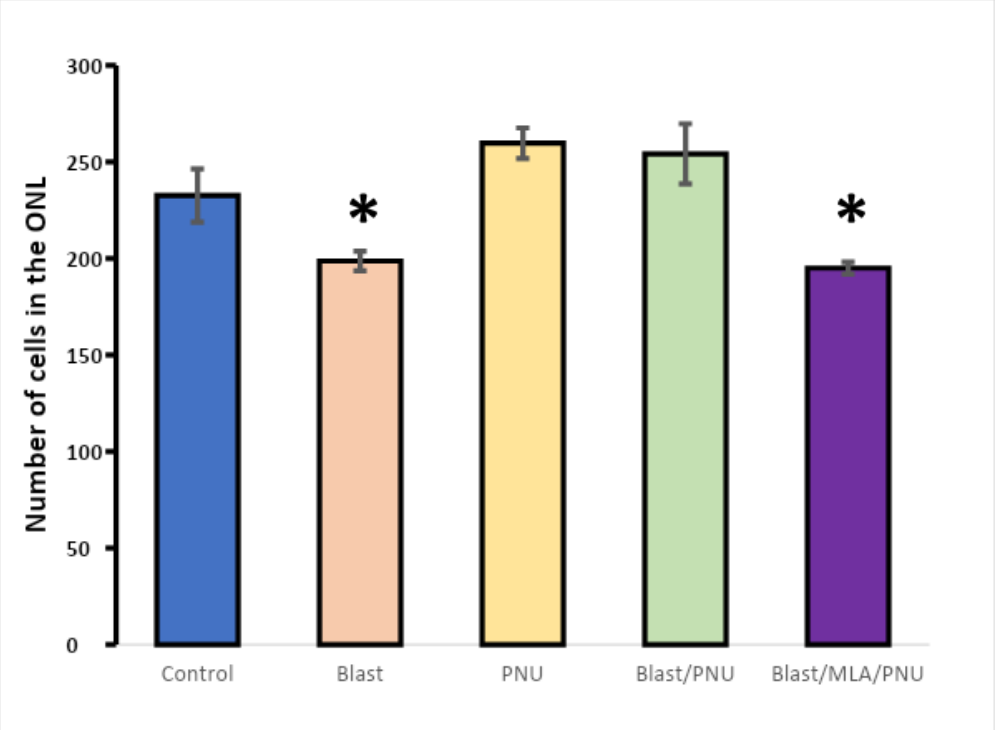


*Table 2: Bar chart visualizing the change in the number of cells in the ONL across different treatments.*

*3.4 Derivation of Retinal Neurons From Müller Glia in the ONL After PNU-282987 Treatment*

In this study, we used transgenic mice to trace the lineage of cells derived from the Müller glia, These cells will appear red from the red fluorescent Müller glia reporter gene (tdTomato). RFP antibodies and Alexa Fluor 594 was used to co-label the transgenic Müller glial cells. The photoreceptors that originated from the Müller glia were labelled with recoverin antibodies and secondarily labelled with Alexa Fluor 488 (green). The retinas were viewed under the confocal microscope, and cell counts were obtained from 200 $\mu m^2$ (60x) images for the control, blast with PNU, and blast with MLA and PNU conditions.

Under control and blast conditions, there was no observation of td-positive photoreceptors in the ONL (Fig. 8A, 8B). While blast conditions exhibited a significant loss of cells in all retinal layers (Fig. 8B), only Müller glial end feet can be seen at the ONL for both conditions.

On the other hand, blast-exposed retinas that were given PNU-282987 treatment for 3 weeks showed tdTomato-positive red photoreceptors co-labelled with recoverin (Fig. 8C). Quantification of this effect is shown in Table 3. When compared to the ONL of the control group (Fig. 8A), a 5.64% increase in the percentage of tdTomato positive photoreceptors was observed in the ONL, from an average cell count of 0 cells (control) to 14.3 cells (Blast/PNU). Unblasted retinas with PNU-282987 treatment for 3 weeks also showed tdTomato-positive red photoreceptors co-labelled with recoverin. When compared to the ONL of the control group, a 6.23% increase in the percentage of tdTomato positive photoreceptors was observed in the ONL, from an average cell count of 0 cells (control) to 16.2 cells (PNU) (Table 3). However, a stark statistical difference is observed when blast-exposed retinas are treated with Methyllycaconitine (MLA) in the form of eye drops an hour before PNU-282987 (Fig. 8D). MLA is a specific antagonist to the alpha 7 subtype of neural nicotinic acetylcholine receptors in the retinal pigment epithelium, which can decrease the neuroregenerative effects of PNU-282987. In the presence of MLA, there is only a 1.1% percentage of tdTomato-positive photoreceptors observed in the ONL, compared to the 6% of cells that are double-labelled after blast exposure with PNU treatment. MLA significantly decreased the effect of PNU, providing strong evidence that PNU-282987's effect is mediated through alpha7 nAChRs.

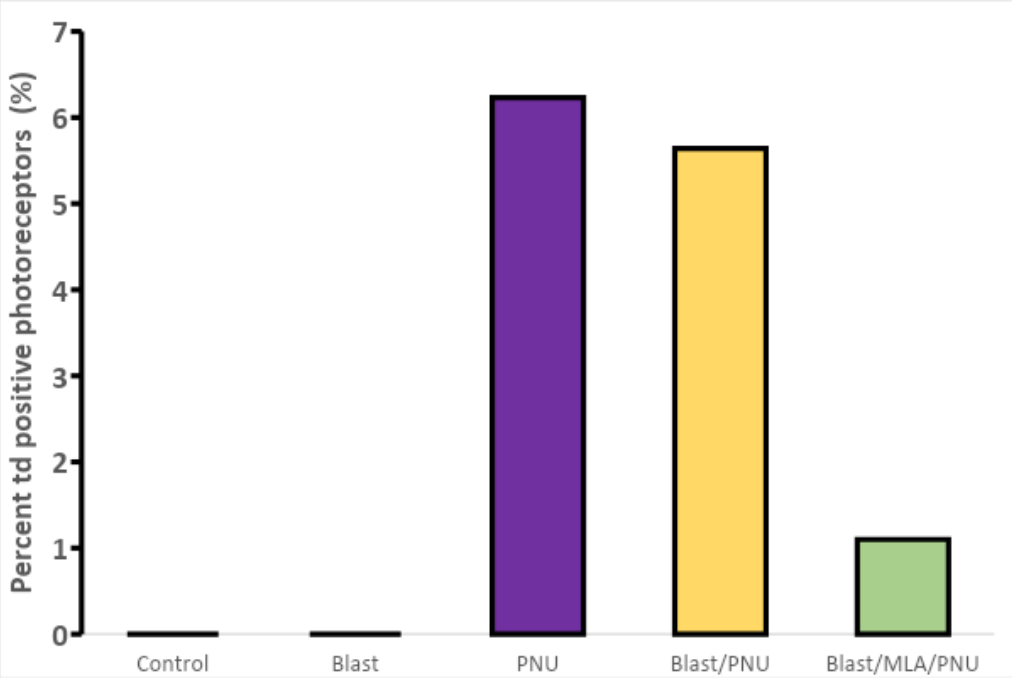


*Table 3: Bar chart visualizing the change in the percent of tdTomato positive photoreceptors in the ONL across different treatments.*

*3.5 Data Tables*

*Table 4: Data Tabulation and Calculation of the Control Group.*

| **Condition** | **Total Number of cells in ONL** | **Total number of double-labelled photoreceptors** |
|---|---|---|
| Control 1 | 215 | 0 |
| Control 2 | 230 | 0 |
| Control 3 | 228 | 0 |
| Control 4 | 176 | 0 |
| Control 5 | 228 | 0 |
| Control 6 | 294 | 0 |
| Control 7 | 257 | 0 |
| **Control (Mean)** | 232.571429 | 0 |
| **Standard Deviation** | 36.349494 | 0 |
| **N** | 7 | 7 |
| **SEM** | 13.7388173 | 0 |
| **Percent of Double Labelled Photoreceptors** | 0.00% | |

*Table 5: Data Tabulation and Calculation of Blast Conditions.*

| **Condition** | **Total Number of cells in ONL** | **Total number of double-labelled photoreceptors** |
|---|---|---|
| Blast 1 | 209 | 0 |
| Blast 2 | 187 | 0 |
| Blast 3 | 212 | 0 |
| Blast 4 | 198 | 0 |
| Blast 5 | 205 | 0 |
| Blast 6 | 181 | 0 |
| **Blast (Mean)** | 198.666667 | 0 |
| **Standard Deviation** | 12.4365054 | 0 |
| **N** | 6 | 6 |
| **SEM** | 5.07718207 | 0 |
| **Percent of Double Labelled Photoreceptors** | 0.00% | |
| **Percent change in cell (increase or decrease)** | -14.578215 | |

*Table 6: Data Tabulation and Calculation of PNU Treatment.*

| **Condition** | **Total Number of cells in ONL** | **Total number of double-labelled photoreceptors** |
|---|---|---|
| PNU 1 | 250 | 15 |
| PNU 2 | 262 | 10 |
| PNU 3 | 275 | 15 |
| PNU 4 | 283 | 18 |
| PNU 5 | 228 | 17 |
| PNU 6 | 260 | 22 |
| **PNU (Mean)** | 259.666667 | 16.16666667 |
| **Standard Deviation** | 19.3975944 | 3.970726214 |
| **N** | 6 | 6 |
| **SEM** | 7.91903473 | 1.621042189 |
| **Percent of Double Labelled Photoreceptors** | 6.23% | |
| **Percent change in cell (increase or decrease)** | 11.6502867 | |

*Table 7: Data Tabulation and Calculation of Blast Condition with PNU Treatment.*

| **Condition** | **Total Number of cells in ONL** | **Total number of double-labelled photoreceptors** |
|---|---|---|
| Blast/PNU 1 | 255 | 12 |
| Blast/PNU 2 | 207 | 14 |
| Blast/PNU 3 | 276 | 12 |
| Blast/PNU 4 | 314 | 16 |
| Blast/PNU 5 | 250 | 14 |
| Blast/PNU 6 | 223 | 18 |
| **Blast/PNU (Mean)** | 254.166667 | 14.33333333 |
| **Standard Deviation** | 38.1335373 | 2.338090389 |
| **N** | 6 | 6 |
| **SEM** | 15.5679514 | 0.954521404 |
| **Percent of Double Labelled Photoreceptors** | 5.64% | |
| **Percent change in cell (increase or decrease)** | 9.28542179 | |

*Table 8: Data Tabulation and Calculation of Blast Condition with MLA & PNU Treatment.*

| **Condition** | **Total Number of cells in ONL** | **Total number of double-labelled photoreceptors** |
|---|---|---|
| Blast/MLA/PNU 1 | 188 | 2 |
| Blast/MLA/PNU 2 | 195 | 3 |
| Blast/MLA/PNU 3 | 210 | 1 |
| Blast/MLA/PNU 4 | 195 | 0 |
| Blast/MLA/PNU 5 | 201 | 3 |
| Blast/MLA/PNU 6 | 187 | 2 |
| Blast/MLA/PNU 7 | 189 | 4 |
| Blast/MLA/PNU (Mean) | 195 | 2.142857143 |
| Standard Deviation | 8.26639785 | 1.345185418 |
| N | 7 | 7 |
| SEM | 3.12440471 | 0.508432298 |
| **Percent of Double Labelled Photoreceptors** | 1.10% | |
| **Percent change in cell (increase or decrease)** | -16.154791 | |

**Discussion**

For this research project, I used Methyllycaconitine (MLA), an antagonist to the alpha 7 subtype of neural nicotinic acetylcholine receptors to inhibit the effect of PNU-282987 in blast-exposed mouse retinas. The objective of this experiment was to determine if Methyllycaconitine (MLA) will inhibit the effect of PNU-282987 in blast-exposed mouse retinas. The hypothesis of this experiment is that PNU-282987 will act on the alpha 7 subtype of neural nicotinic acetylcholine receptors to induce neurogenesis.

Using a modified paintball gun, a blast pressure of 35 PSI was delivered onto the anatomical left eye of the mice to mimic blast injury. The pressure from a single blast was sufficient to cause significant damage to the INL, ONL, and GCL of the retina, with no significant change in retinal thickness when measured at certain points from the optic nerve head. A decreased photoreceptor count in the ONL was observed in blast-exposed retinas compared to the control model.

Retinal neurons in adult mammals were once perceived to lack the ability for neuroregeneration upon damage. In previous studies from the lab, it has been discovered that PNU-282987 has the ability to artificially induce BrdU incorporation in all retinal layers, and neurogenesis when applied at a specific dose in the form of eye drops. When PNU-282987 reaches the retinal pigment epithelium (RPE), it binds to its alpha-7 nACh receptors, resulting in changes in the gene expression of the RPE. Upregulation of genes such as those involved in immune system activation, molecular signalling and transportation, cell differentiation, and gene translation occurs, while downregulated genes include those involved in gliosis and cytokine activity (Webster et al, 2022). The hypothesis our lab has proposed is that changes in gene expression in the RPE will result in the release of signalling molecules from the RPE that reach the endfeet of Müller glia resulting in cell proliferation. This process induces

dedifferentiation and production of progenitor cells, which re-enters the cell cycle and migrates to the retinal layers for the production of retinal neurons in adult mammals (Webster et al, 2022). The results from this experiment support the ability of PNU-282987 to induce neurogenesis in unblasted and blast-exposed mouse retinas, as seen from the significant increase of cell counts in the outer nuclear layer (ONL) of the retina when compared to the control group.

Methyllycaconitine (MLA) is a selective antagonist to the alpha 7 neural nicotinic acetylcholine receptors. In the study by Webster et al. in 2019, MLA was given to transgenic mice to determine the selectivity of PNU-282987 to alpha 7 nicotinic acetylcholine receptors in the retinal pigment epithelium (RPE). RPE cells were treated with PNU-282987 or MLA with PNU-282987 and were cultured for different times (0-72 hours). The cleaned and modified culture media was injected into the vitreous chamber of adult mice that were treated with BrdU eye drops for 2 weeks. Mice injected with PNU-282987 treated culture media (72 hours) exhibited BrdU+ cells in the INL and ONL, while MLA significantly reduced the number of BrdU+ cells. In this experiment, we verified that a significant decrease in cell count occurred in blast-exposed mouse retinas. Typically, PNU-282987 reverses this effect unless MLA was applied along with PNU-282987. Cell counts from MLA/PNU-treated animals were similar to the cell counts associated with the blast effect. These results support the hypothesis that the proliferation of MG is mediated by the α7 nACh receptors located in the RPE when PNU-282987 is applied.

In this study, we used transgenic mice to trace the lineage of cells derived from the Müller glia, These cells will appear red from the red fluorescent Müller glia reporter gene (tdTomato). RFP antibodies and Alexa Fluor 594 were used to label the transgenic Müller glial cells. The new photoreceptors that originated from the Müller glia were labelled with

recoverin antibodies and visualized using Alexa Fluor 488 (green) fluorescent secondary antibodies. Under control and blast conditions, there was no observation of td-positive photoreceptors in the ONL However, unblasted and blast-exposed retinas that were given PNU-282987 treatment for 3 weeks showed tdTomato-positive red photoreceptors double-labelled with recoverin. A decrease in tdTomato-positive photoreceptors was observed when blast-exposed retinas were treated with Methyllycaconitine (MLA) in the form of eye drops an hour before PNU-282987 treatments. These results support the study's objective to verify that methyllycaconitine (MLA) inhibits the effect of PNU-282987 in blast-exposed mouse retinas to support the hypothesis that PNU-282987 induces Müller glia dedifferentiation and the production of progenitor cells through the alpha 7 nicotinic acetylcholine receptors from the RPE.

**Future Studies and Implications**

Although triple labelling was attempted for this study to observe neurogenesis in the Müller glia, ONL, and GCL, antibodies that were left too long during the immunostaining process resulted in the overstaining of the GCL. Further studies are needed to analyze the mechanism and effects of PNU-282987 on the neurogenesis of retinal ganglion cells in blast-exposed retinas. Recent studies by Spitsbergen et al. were able to provide evidence that RGCs affected the electrophysiological function of the retina. They were also capable of directing axonal growth into the optic nerve, which eventually forms synapses in the brain (Spitsbergen et al., 2023). This test was done via retrograde labelling of the axons that lead back to RGCs in the GCL. Such steps are needed to increase our understanding of the functional effects of PNU-282987.

There are also some limitations to the blast model used in this study. Our modified paintball gun delivered a single blast of pressurized air at 35 PSI directly perpendicular to the

anatomical left eye, leaving damages from the pressure isolated at the retina. However, blast waves generated by an explosion come in a single pulse of increased air pressure, which turns from a negative pressure to a positive wave almost immediately (National Academies Press (US), 2014). Field conditions such as the surrounding elements can also change wave characteristics. The wave is followed by a high-velocity blast wind that can accelerate to the speed of a hurricane. Therefore, blast injuries vary from mild traumatic brain injuries to the loss of body parts and even death. Damages to major body systems like the nervous system could change the way PNU-282987 is analyzed.

Despite the limitations, we were able to observe the specificity of PNU-282987 to alpha 7 subtype of nicotinic acetylcholine receptors and to understand its ability to induce neuroprotective and neurogenerative properties in blast-exposed retinas. With further research, the development of potential treatments for neurodegenerative eye diseases like glaucoma or macular degeneration would be made possible, which would greatly improve the quality of life for those affected.

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