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14. ABSTRACT

The eye is an exposed organ that is particularly vulnerable to injuries that result from blast exposure. Typically, lost or damaged retinal neurons in adult mammals do not proliferate or spontaneously regenerate. However, in this study, the reversal of vision loss and neurogenesis of adult retinal neurons in mice is investigated after blast exposure using a specific alpha7 nicotinic acetylcholine receptor agonist, PNU-282987, which has previously been shown to induce neurogenesis in glaucoma rodent models. In designed experiments, blast exposure is delivered to adult rodents to test the hypothesis that eye drop application of PNU-282987 reverses the loss of retinal neurons associated with blast exposure and recovers visual function. The results of these studies can lead to eye drop treatments that significantly improve visual function for soldiers that experience blast exposure in combat to improve their quality of life. In addition, the results of these studies will challenge current ideas that maintain the adult mammalian CNS is incapable of regeneration.

15. SUBJECT TERMS

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Introduction:

In designed experiments, blast exposure has been delivered to adult mice to test the hypothesis that eye drop application of PNU-282987 will reverse the loss of retinal neurons associated with blast exposure and recover visual function. These experiments will be the first to determine if activation of alpha7 nicotinic acetylcholine receptors in the eye can replace neurons lost to blast exposure in adult mammals and is the first study to link alpha7 nicotinic acetylcholine receptor induced neurogenesis with change of retinal function in adult mammals.

Keywords: Blast exposure, combat trauma, neurogenesis, recovery of visual function, ERG, retina, regeneration, Muller glia, mice, transgenic, PNU-282987, alpha7 nicotinic acetylcholine receptors.

3. Accomplishments:

Two specific aims have been designed to test the hypothesis that eye drop application of the alpha7 nicotinic acetylcholine receptor agonist, PNU-282987, will reverse the loss of retinal neurons associated with blast exposure and recover visual function measured with ERG recordings.

1.To determine that eye drop application of the α7 nAChR agonist, PNU-282987, reverses the loss of retinal neurons typically associated with blast exposure.

2.To demonstrate that an increase of retinal cells after exposure to PNU-282987 affects visual function.

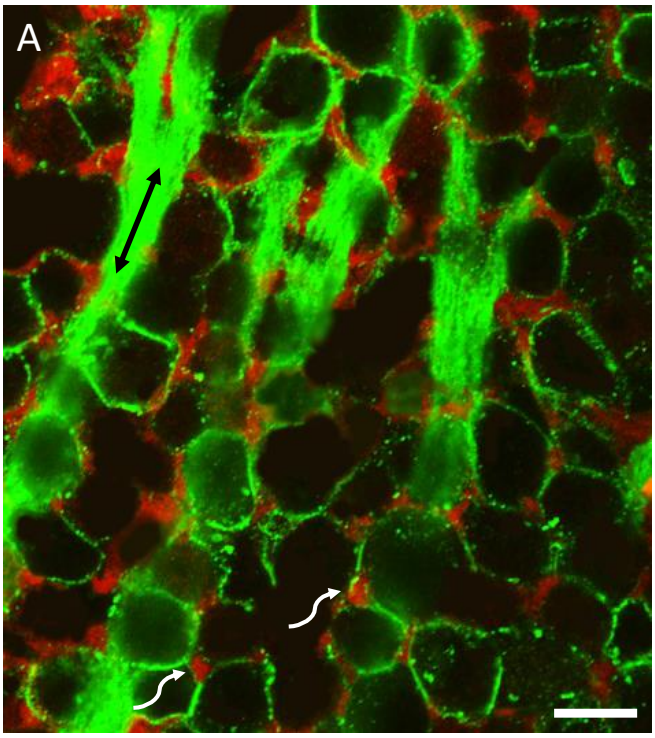
In order to test aim #1, 4 major tasks have been outlined. To test aim #2, two major tasks have been outlined. During this fourth quarterly review, we have made significant progress in the following major tasks:

Aim 1; major task 1: establish the effect of blast exposure on neuronal survival in the retina. This task is now completed. During the first quarter, different blast pressures on neuronal survival in the retina was assessed. During the second quarter, quantification of neuronal survival using different blast pressures was quantified. During the third quarter, we accumulated large enough "N's to complete this major task. "N"s of 5 have now been collected and quantified for blasts between 20 and 38 psi. The data will be presented at the SFN convention in November 2021. Blast exposure using 20 psi was the minimal pressure needed to induce significant damage to retinal neurons. An average of 19% (+/- 4) of neurons were lost throughout all retinal layers using 20 psi compared to control unblasted retinas. 30 psi induced more retinal damage losing an average of 32% (+/- 8) of all neurons in all retinal layers. The maximal amount of damage that still allowed functional recovery occurred when 38 psi was used to blast the mice eyes (N=16). This resulted in a loss of 39% (+/- 8) of neuronal loss in all retinal layers. Blasts greater than 38 psi resulted in animal death a majority of the time and will not be used for the remainder of this study.

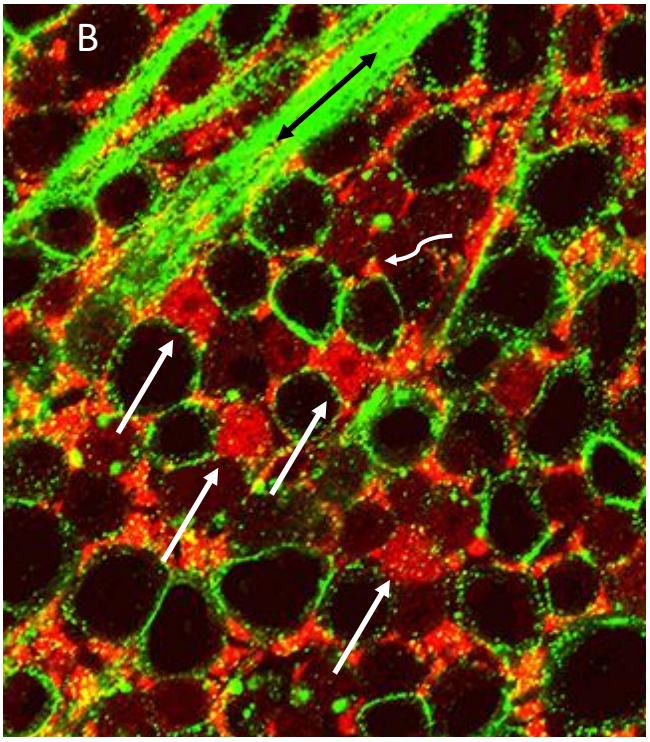
Major task 2 under Aim1: examine the effect of PNU-282987 on neuronal loss after blast exposure in wild type mice and in transgenic mice containing tdTomato Muller glia. We have treated the blast exposed wild type animals to eye drops containing 1 mM PNU-282987. 1 mM PNU-282987 eye drop application was found to elicit the maximal regenerative effect in wild type mice as well as in transgenic animals. Blast exposed animals were treated with PNU-282987 for 1 week, 2 weeks and 4 weeks following the blast. At the end of these times periods, the animals were sacrificed, the retinas were removed and immunostained with antibodies for different retinal neurons in transgenic mice containing tdTomato Muller glia that allows for lineage tracing and cell counting. PNU-282987 significantly restored loss of retinal neurons due to blasts using 38 psi.

Figure 1 illustrates a retinal flat mount confocal image illustrating the RGC layer that was obtained from a transgenic mouse 1 week after receiving a blast exposure (38 psi) (A). Figure 1B illustrates an image obtained from a blast exposed retina that was treated with PNU-282987 eye drops for one month after the blast. All red stained tissue originated from Muller glia. In figure 1A, the Muller glia end feet (red) that extend into the RGC layer are seen outside of RGCs labeled with antibodies against Thy1.2 (green) to label glycoproteins in the RGC plasma membrane. One week after blast exposure, RGC membranes have broken down after blast exposure, resulting in significant loss of cells. In figure 1B, a transgenic mouse was treated with 1 mM PNU-282987 eye drops for one month after blast exposure. As seen in this image, PNU-282987 induced new cells in the RGC layer that originated from Muller glia (arrows). Quantification of the increase in new cells is currently underway as well as immunocytochemical analysis of retinal neurons in other retinal layers that are induced from Muller glia. The double ended arrows represent the axon fascicles that converge as they travel to the optic nerve head. The curved arrows indicate the Muller glia end feet that are typically found outside of RGCs in the RGC layer.

After blast



After blast with PNU-282987 treatment

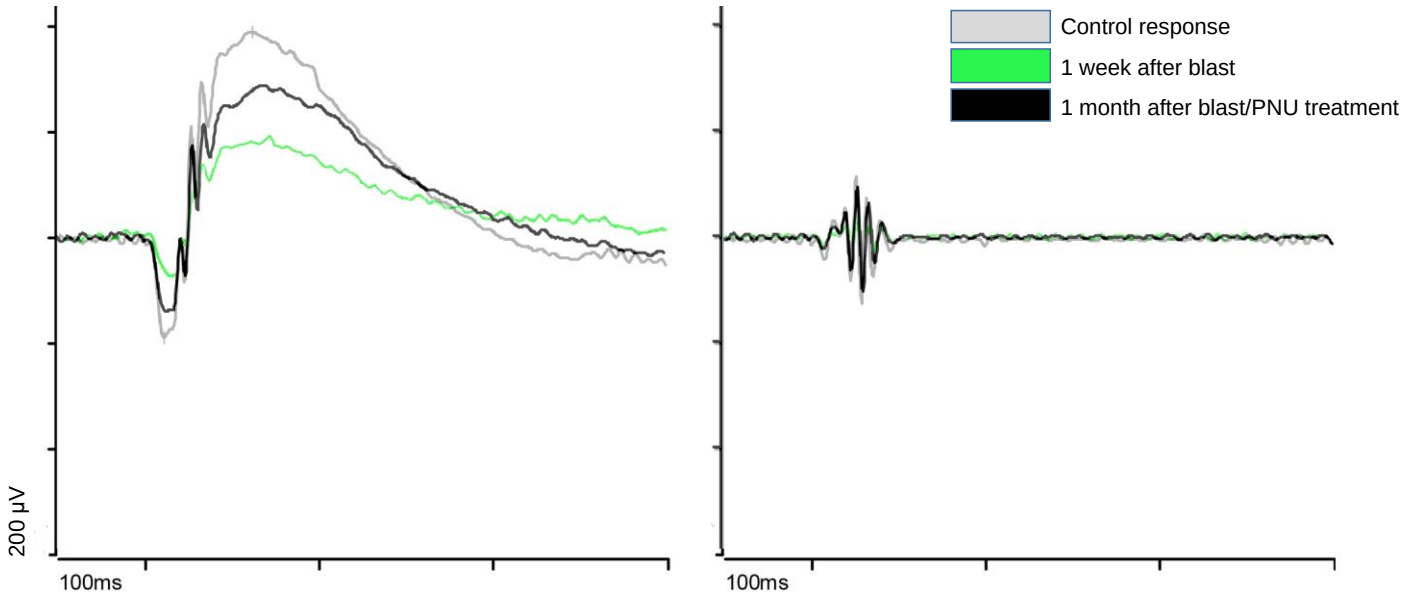


Major task 3 under Aim 1: demonstrate specificity of the ACh receptor involved in PNU-282987 induced neurogenesis. When eyes were treated with 10 micromolar MLA (methyllycaconitine) before PNU-282987, the effect of PNU-282987 was significantly reduced to support the hypothesis that the PNU-282987 effect seen in figure 1B occurs through activation of alpha7nAChRs. This task is complete.

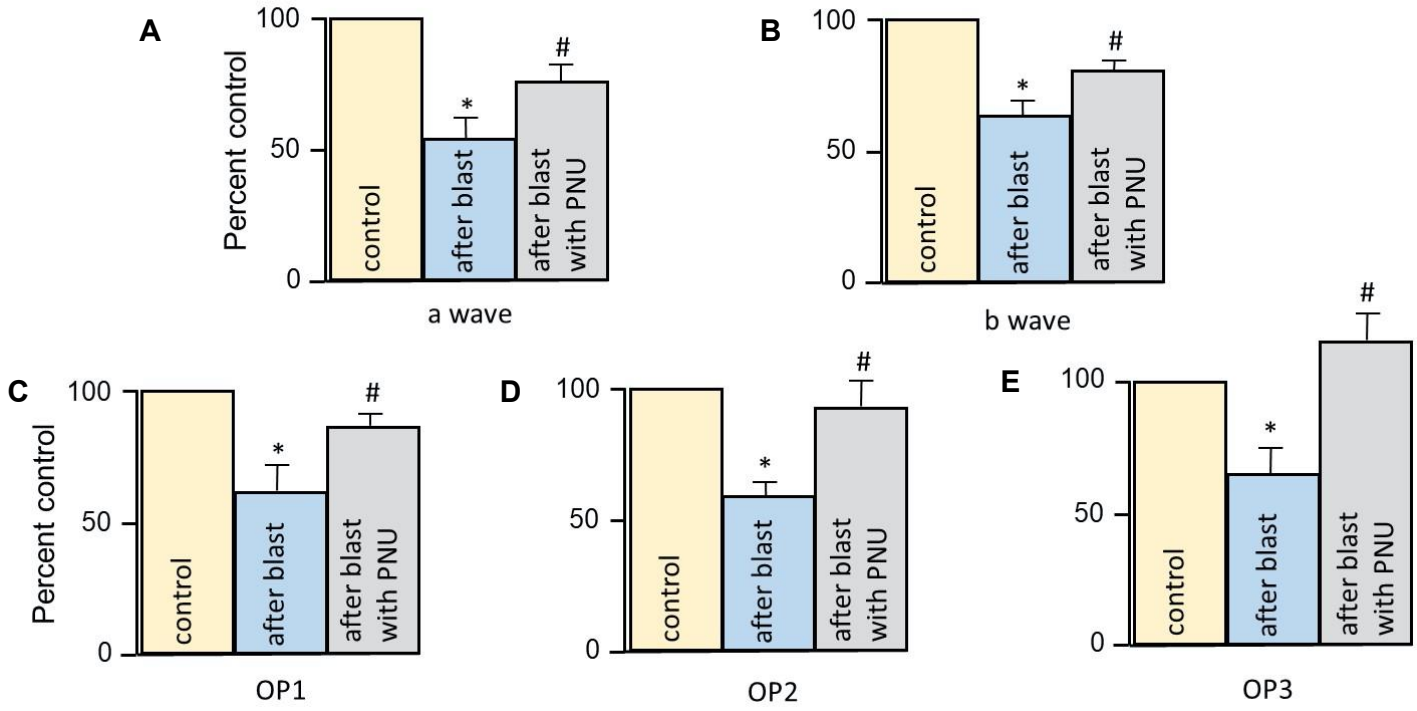
Major task 4 under Aim 1: Quantify morphological changes that occur in the retina and optic nerve after PNU-282987 treatment after blast exposure. Retrograde studies using transgenic tdTomato Muller glia in adult mice treated with PNU-282987 after blast exposure have been conducted. Neuro Vue dye paper was inserted into the optic nerve of PNU-282987 treated transgenic mice with and without blast exposure after being removed from transgenic mice. The retinas and optic nerve remained attached and were placed in 4% PFA for several days to allow the retrograde dye to label RGC bodies in the RGC layer in wild type mice as well as in transgenic mice. In the 3rd quarterly report, images were provided to support the hypothesis that newly regenerated RGCs that originate from Muller glia extend axons into the optic nerve. To complete this major task, measurements of cell layer thickness before and after blast exposure need to be obtained, quantified and compared for statistical differences. We have brought in an undergraduate student to perform this activity.

Aim 2; major task 1: delivery of scotopic stimulation to record rod ERG responses before and after PNU-282987 treatment in control and blast exposed adult mice. We have now completed dark-adapted ERG experiments in animals after switching from a 4 hour dark adapted period of time to a 12 hour dark adapted period of time. This longer time in the dark made a significant different during dark adapted recordings and significantly reduced the standard error between recordings. We have completed the scoptopic ERG studies in wild type mice, after blast exposure and after treatment with PNU-282987 for 1 month after blast exposure.

As seen in the ERG recording shown in figure 2 below, blast exposure significantly decreased all wave components in the scotopic ERG (yellow trace), including the a wave, b wave, as well as oscillatory potentials 1, 2 and 3 (Fig 2A and B). However, 1 month after eye drop treatment with PNU-282987, the ERG wave recovered significantly after the initial blast (black trace).



Quantification of recovery after blast exposure under scotopic conditions has been completed. As seen in figure 3 below, bar graphs summarize the effect of the blast on the different ERG waves. In all instances, a blast exposure of 38 psi reduced ERG waves by approximately 50%. However, 1 month following PNU-282987 eye drop treatment, there was significant recovery of every ERG wave under scotopic conditions. Error bars represent standard error. * represents significance from control ERG waves, while # represents significance from ERG recordings obtained 1 week after blast exposure. $P<0.05$ was considered statistically significant.



Aim 2; major task 2: Delivery of phototopic stimulation to record cone and inner retinal cell ERG responses before and after PNU-282987 treatment in control and blast exposed adult mice. ERGs recorded from light adapted eyes have been significantly consistent and have been recorded in control animals, blast exposed animals and blast exposed animals in the presence of PNU-282987. In the 3rd quarterly report, photopic ERG waveforms were recorded before and after blast exposure. When treated with PNU-282987, recovery did not significantly occur under scotopic conditions when only treated with PNU-282987 for 4 weeks. As a result, we have now obtained photopic ERG recordings for longer periods of time (2-3 months). This data is now being quantified and analyzed.

Plans for next quarter: All scotopic ERG experiments will be quantified and analyzed for statistically significant recovery of the ERG waves. Particular attention will be focused on the phNR wave. In addition, we are also planning on recording C waves from ERG recordings as the C wave indicated retinal pigment epithelium activity. Previous work from this lab has provided evidence that signaling molecules are released from the RPE to induce regeneration of new neurons in adult mammals. As a result, the analysis of C wave activity is necessary. In addition to finalizing the scoptopic ERG studies, immunocytochemistry studies in transgenic mice will be conducted for several retinal neuronal markers to verify that new neurons are generated in all retinal layers and that they are derived from Muller glia. Lastly, in the next quarter, final quantification of retinal neuronal layer thickness will be performed under control conditions, after blast exposure, after PNU-282987 treatment, and after blast exposure with PNU-282987 treatment. These experiments will be conducted over the next 3 months by a new undergraduate student that has been recruited to our lab to perform these studies.

4. Impact

Vision loss is responsible for reduced quality of life and a substantial burden on national healthcare systems. The results of this study could lead to eye drop treatments that significantly improve visual function in soldiers that experience blast exposure in combat to improve their quality of life. In addition, the results of this study will challenge current ideas that the adult mammalian CNS is incapable of regeneration. This proposal directly addresses functional recovery as a result of a traumatic blast event to align with one of the FY19 VRP focus areas.

5. Changes/Problems:

This project is on track to complete the major tasks and milestones outlined in this award. To date, there have been no significant changes and problems encountered that have prevented us from achieving the outlined milestones at this time. Specifically, there have been no significant changes in the approach, prolonged delays, expenditures, use of vertebrate animals, or in the use of biohazards. A minor change included the need to change the time to dark adapt mice for scotopic studies from 4 hours to 12 hours. Another minor change in the tasks included extending the time ERGs need to be measured after blast exposure and PNU-282987 treatment for photopic studies.

6. **Products:**

An abstract to the Society for Neuroscience (SFN) annual convention has been submitted to be presented November, 2021.

Spitsbergen JB, Linn CL. 2021. Neurogenesis and Functional Recovery of Adult Retinal Neurons in Mice using an Alpha7 nAChR Agonist after Blast Exposure. SFN Abstract.

7. Participants and Collaborating organizations:

No collaborating organizations are involved with this project.

The three key personnel involved in this project during the last three months of the award are Cindy Linn (PI), Sarah Webster (graduate student) and Jake Spitsbergen (Graduate Student). In addition, Hope Vanzo-Sparks (graduate student) is working to assist with the data collecting and analysis as outlined in the grant proposal. A new undergraduate student was also recruited to perform the morphological quantification of retinal layers under different experimental conditions.

Name:	*Cindy Linn*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*3*
Contribution to Project:	*Dr. Linn is helping to record all ERG waveforms in control untreated adult mice, as well as manage the grant requirements.*
Funding Support:	*DOD for summer research*

Name:	*Jake Spitsbergen*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*3*
Contribution to Project:	*Jake Spitsbergen has set up the paintball gun to deliver blasts to adult mice eyes that leads to loss of retinal neurons. He has quantified this loss using immunocytochemistry and confocal microscopy and is recording ERGs using the Celeris ERG system.*
Funding Support:	*DOD for summer research*

Name:	*Sarah Webster*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*3*
Contribution to Project:	*Sarah Webster has performed the NeuroVue retrograde optic nerve studies to demonstrate that newly regenerated RGCs send axons down the optic nerve. She also performs immunostaining of retinal tissue using antibodies against retinal cell markers in transgenic mice.*
Funding Support:	*DOD for summer research*

Name:	*Hope Vanzo-Sparks*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	

Nearest person month worked:	*3*
Contribution to Project:	*Hope is assisting with the data collecting and analysis of retinal layers as outlined in the grant proposal. In addition, she is assisting with immunostaining experiments of retinal tissue using antibodies against retinal cell markers.*
Funding Support:	*DOD for summer research*

Name:	*Treyton Boswick*
Project Role:	*Undergraduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*3*
Contribution to Project:	*Treyton will perform the morphological analysis of retinal layers under control conditions, after blast exposre, after treatment with PNU-282987 and after blast exposure with PNU-282987 treatment*
Funding Support:	*DOD for summer research*

8. Special reporting requirements:

Quad chart for year 1 attached

9. **Appendices: Quad chart**