Characterization of a Neuroprotective Retinal Synapse After Inducing Glaucoma in Long Evans Rats

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Introduction

Glaucoma is a group of degenerative retinal diseases characterized by progressive loss of retinal ganglion cells (RGCs) and their axons leading to irreversible loss of vision. Neuroprotection has been defined as any intervention that prevents optic nerve damage or RGC death. The neurotransmitter, acetylcholine (ACh), has been linked to neuroprotection against excitotoxic cell death and neurodegenerative diseases of the central nervous system (CNS). Results from our lab have demonstrated that ACh provides neuroprotection against glutamate-induced excitotoxicity in isolated pig and rat RGCs *in vitro*. Additional studies using an *in vivo* rat model of glaucoma have shown that intravitreal injections or eye drop application of the α7 nAChR selective agonist, PNU-282987, triggers neuroprotection against the normal loss of RGCs through activation of α7 nAChRs. A specific population of cells in the retina, the starburst amacrine cells (SACs), are the only known retinal cells to release ACh in the mammalian retina. During early retinal development, SACs release ACh which is necessary for the production of retinal waves. However, the role of ACh released from SACs in the mature retina is unclear.

If ACh is neuroprotective to RGCs *in vitro* and SACs are known to release ACh onto the α7 nACh receptors in RGCs of the mammalian retina, do SACs provide endogenous neuroprotection to RGCs? What happens to this transmission of ACh from displaced SACs under glaucoma conditions? Using a well-developed hypertonic glaucoma model in adult rats, this study analyzes changes that occur in the cholinergic synapse between SACs and RGCs in induced glaucoma conditions. It is proposed that the transmission of ACh from SACs onto RGCs provides endogenous neuroprotection to RGCs in the mammalian retina and that this transmission is compromised in glaucoma-like conditions.

Methods

Hypertonic Saline Injection to Induce Glaucoma-like Conditions
Adult Long Evans rats, 3-6 months old, were anesthetized with an IP injection of KAX at 0.1ml/100 gm. The episcleral vein of right eyes were injected with 50 µl of 2M NaCl using a beveled glass microinjection needle. The left eye acted as an untreated internal control.

Retina Removal and Preparation
At various time points after the procedure, animals were sacrificed, eyes were enucleated, and retinas were removed. Whole retinas were laid flat and cut into four quadrants. Retinas were pinned flat with cactus needles in sylgard coated petri dishes. Retinas were then fixed overnight with 4% PFA.

Immunohistochemistry
RGCs were labeled with antibodies against the cell specific marker, Thy 1.1. SACs were identified with an antibody against the enzyme, choline acetyltransferase (ChAT). Briefly, fixed and flat-mounted retinas were permeabilized and blocked with 1% Triton-X 100 with 1% FBS in PBS. Each retina was incubated in mouse primary antibody anti rat-Thy 1.1 and sheep anti rat-ChAT overnight. The following day, after rinsing, retinas were incubated in secondary antibody with goat anti-mouse Alexa Fluor 594 and donkey anti-sheep Alexa Fluor 488 overnight. The following day, retinas were liberally rinsed and mounted on microscope slides for viewing.

Confocal Microscopy and Data Analysis
All retinas were visualized with a Nikon C2+ scanning laser confocal microscope. Using the z-stack acquisition function, a minimum of four high resolution z-stacks were obtained, 4 mm from the ONH, from each of the 4 quadrants of each retina. ImageJ software was used to scroll beneath axon bundles. A fixed 200 x 200 µm^2 grid was applied to all images and the Thy 1.1-positive RGCs and ChAT-positive SACs within the grid for each retinal quadrant were counted blindly. The counts from all quadrants were averaged for each retina.

IOP Measurements
A handheld rebound tonometer was applied daily to experimental eyes to obtain IOP measurements before and after hypertonic injections were performed. On each day, 3 IOP measurements were averaged from each animal.

LC/MS/MS Analysis
After sacrifice, retinas were removed, rinsed in PBS, weighed, and immediately delivered to the Michigan Innovation Center of Kalamazoo for LC/MS/MS analysis and quantification of ACh. LC/MS/MS was performed on a Waters Quattro Micro triple quadrupole mass spectrometer using positive ion electrospray ionization. Each sample was done in triplicate. ACh concentration data was obtained for each sample. The average for each experimental condition was calculated and compared to controls.

ELISA Assay
After sacrifice, all retinas were removed and extensively homogenized according to kit instructions. Quantification of α7 nAChRs was performed using the rat α7nAChR ELISA kit according to kit instructions. Measurements of absorbance at 450 nm were obtained with a microplate reader. The average absorbance was normalized to the blank. The adjusted absorbance was then used to calculate the average optical density in terms of concentration in ng/gm of retinal tissue.

Agonist Administration
Rats received daily eye drops of either DMP-543 or Donepezil at various concentrations in the right eye only for three days before and for two weeks after glaucoma induction. All animals were sacrificed at four weeks after the procedure. Retinas were removed, fixed, flat-mounted, stained and quantified as described above.

mRNA Sequencing
After sacrifice and retinal removal, an RNEasy Plus Mini Kit was used to extract total RNA according to kit instructions. Samples were sent to GENEWIZ, Inc. for differential gene expression and bioinformatics analysis. Pathway enrichment analysis was then performed on selected genes known to be involved in cell survival pathways.

Statistical Analysis
Statistical analyses were performed using one-way ANOVAs in all experiments with Tukey post-hoc analysis. P≤0.05 was considered statistically significant for all results.

Results

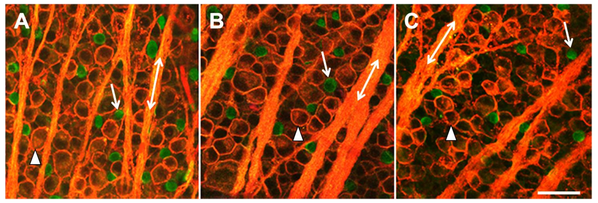


Figure 1. SAC survival in glaucomatous retinas. This figure shows a confocal control untreated retina (A) glaucomatous retina one week after the procedure to induce glaucoma (B), and four weeks post-procedure (C). RGC bodies (arrow head) are shown in red, using anti-Thy1.1 antibody. Double arrows represent axon fascicles. Displaced SACs are shown in green, using anti- choline acetyltransferase (ChAT) antibody. The scale bar represents 50 µm.

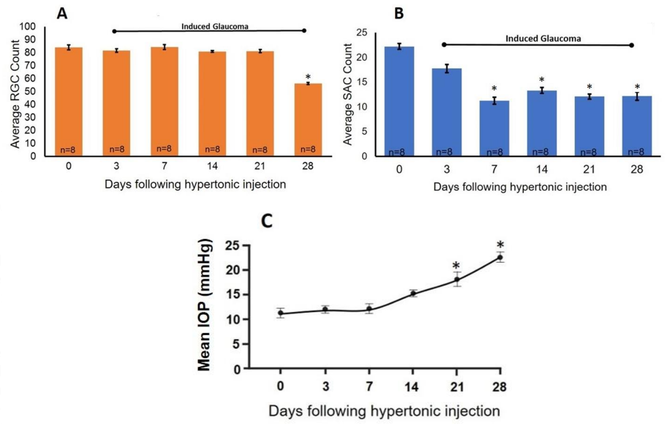


Figure 2. Quantification of RGCs and SACs in glaucoma-induced retinas. This figure summarizes RGC count data (A) and SAC count data (B) at various time points post-surgery to induce glaucoma. Cells were immunostained with antibodies against Thy.1.1 and ChAT. * indicates a significant change as compared to the untreated control condition. (C) represents average IOP measurements obtained before after inducing glaucoma at different time points. Error bars report the standard error.

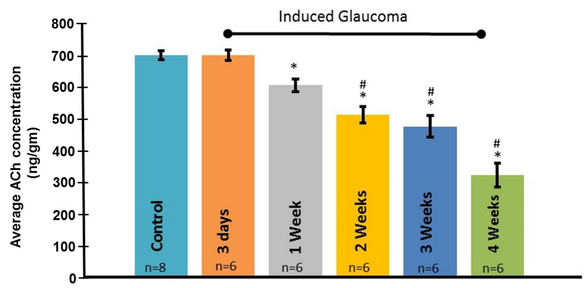


Figure 3. LC/MS/MS analysis for ACh content in experimental retinas. This figure summarizes the average ACh concentration measured in ng/gm of tissue for untreated control and glaucomatous retinas at different time points post-surgery. * indicates a significant decrease in ACh concentration compared to the untreated control condition. # indicates a significant decrease as compared to the 1 week glaucoma condition. Error bars report the standard error.

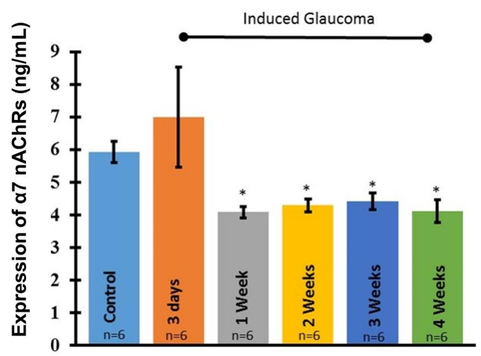


Figure 4. Expression of α7 nAChRs in glaucoma-induced retinas. This figure summarizes the expression of α7 nAChRs at multiple time points post-glaucoma-inducing procedure using a sandwich ELISA kit specific to the α7 nAChR. * indicates a significant decrease as compared to the untreated control condition. Error bars report the standard error.

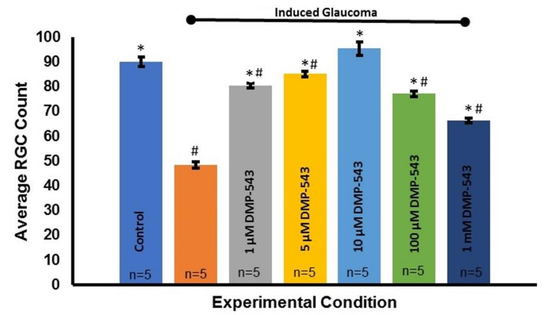


Figure 5. Quantification of RGC survival with treatment of DMP-543. This figure summarizes RGC count data at various time points before and after the procedure to induce glaucoma. Rats were given daily eye drops with the indicated concentrations of DMP-543 for three days before glaucoma induction and for two weeks after. The bar graph represents the average RGC counts for each experimental condition. # indicates a significant change as compared to the untreated control condition. * indicates a significant change as compared to the glaucoma condition. Error bars represent standard error.

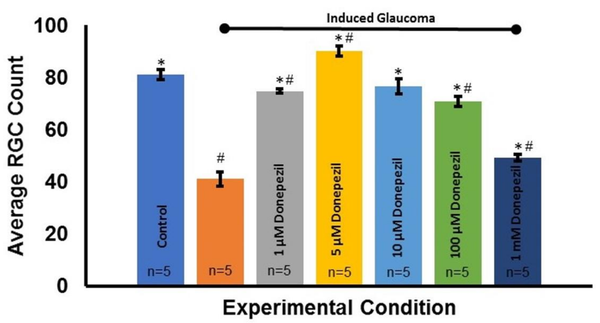
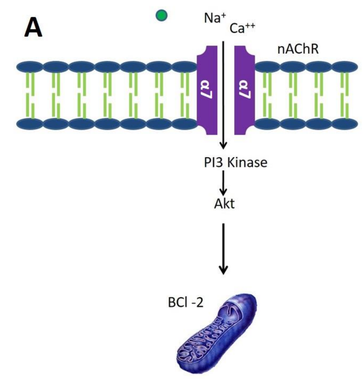


Figure 6. Quantification of RGC survival with treatment of Donepezil. This figure summarizes RGC count data at various time points post-injury. Rats were given daily eye drops with the indicated concentrations of Donepezil for three days before glaucoma induction and for two weeks after. Cells were immunostained with an antibody against Thy 1.1. A minimum of four high -resolution confocal z-stacks were taken per retina, one from each quadrant 4 mm from the ONH. ImageJ analysis was then used to count Thy 1.1-positive RGCs within a fixed sized grid. Cells were counted and averaged for each retina (N=1). The bar graph represents the average RGC counts for each experimental condition. # indicates a significant change as compared to the untreated control condition. * indicates a significant change as compared to the glaucoma condition. Error bars represent standard error.

Results Continued



B

Common Name	Gene Name	Fold Change from Control
α7nAChR	Chrna7	-1.233
Pi3k	Pik3cb	-1.550
AKT	Akt1	-1.256
Bcl-2	Bcl-2	-1.551

Figure 7. Proposed neuroprotective pathway. (A) This figure shows a proposed neuroprotective pathway linking the activation of the α7 nAChR to a known cell survival pathway. (B) reports mRNASeq data corresponding to the proposed neuroprotective pathway. Specifically, fold change values for mRNA targets are listed.

Conclusions

- SAC bodies are lost one week after glaucoma induction, preceding significant RGC loss.
- ACh content decreases significantly one week after glaucoma induction, preceding RGC loss.
- α7nAChR expression on RGCs is lost one week after glaucoma induction, preceding RGC cell body loss.
- The application of 10 µM DMP -543, an ACh release enhancer, is able to promote RGC survival in the glaucoma condition.
- The application of 5 µM Donepezil, an acetylcholinesterase inhibitor is able to promote RGC survival in the glaucoma condition.
- The Pi3K/AKT/Bcl-2 cell survival pathway is shown to be downregulated in the glaucoma condition.
- The results presented here suggest that cholinergic transmission between SACs and RGCs in the mature mammalian retina promotes RGC cell survival and that this transmission is compromised in glaucomatous conditions.
- Providing cholinergic neuroprotection is a previously uncharacterized role for SACs.
- Restoration of this possible neuroprotection can provide a new avenue for effective treatment of glaucoma and other central nervous system diseases.

Acknowledgements

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