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. Neuronal protection 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 intraocular injections or eye drop application of the α7 nAChR agonist, donepezil, prevents neurodegeneration and decreases the normal loss of RGCs through activation of α7 nAChR. A specific population of cells in the retina, the starburst amacrine cells (SACs), are necessary for the survival of 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 mammalian retina is unclear. If ACh is neuroprotective to SACs in vitro and SACs are known to release ACh onto the α7 nAChR receptors in SACs of the mammalian retina, do SACs provide endogenous neuroprotection in RGCs? What happens to this transmission of ACh from differentiated 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 SACs in the mammalian retina and that this transmission is compromised in glaucomatous 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 episceral veins of right eyes were injected with 50 μl of 2% NaCl using a bevelled glass micropipette. The left eye acted as an untreated control.

Retina Removal and Preparation

At various time points after the procedure, animals were sacrificed, eyes were excised, and retinas were removed. Whole retinas were laid flat and cut into four quadrants. Retinas were pinned flat in syngly 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 amine, choline. Both primary antibodies (Thy 1.1, fixed and flat and fixed and flat-mounted were labeled and blocked with 1% Tinit X 100 with 1% PBS in PBS). Each retina was incubated in the following order: Thy 1.1 and sheep and rabbit-CHY overnight. The following day, rinsing, retinas were incubated in secondary antibody with and without Alexa Fluor 594 and Alexa Fluor 488. The primary antibodies were allowed to incubate overnight. The following day, retinas were rinsed rapidly and mounted on microslides for viewing.

Confocal Microscopy and Image Analysis

All retinas were visualized with a Nikon C2+ scanning laser confocal microscope. Using the 2-μm acquisition function, a minimum of four high resolution stacks were obtained, 4 μm from the ONH, from each of the 4 quadrants of each retina. ImageJ software was used to scroll through the images. A level of 5000 was used to determine the ChAT level. A level 2000 was used to determine the TH level. Thy 1.1- positive SACs and ChAT-positive SACs within the grid for each retinal quadrant were counted. The number of SACs within each quadrant were averages 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 for Kalamazoo for LC/MS/MS analysis and quantification of ACh. LC/MS/MS was performed on a Waters Quattro Micro triple quadruple mass spectrometer using positive ion electrospray ionization mode. 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. Measurement of absorbance at 450 nm was 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/ml of retinal tissue.

Antagonist 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, dried, and quantified as described above.

mRNA Sequencing

After sacrifice and retinal removal, an RNaseq Plus Mtx Kit was used to extract total RNA according to kit instructions. Samples were sent to GENEXIQ, 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 ANOVA 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). SACs (arrow heads) are shown in red, using anti-Thy.1 antibody. Double arrows represent axon fascicles. This study demonstrates 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. SACs were identified with a minimum of four high resolution stacks, 4 μm from the ONH. Each retina was incubated in the following order: Thy 1.1 and sheep and rabbit-CHY overnight. A level of 5000 was used to determine the ChAT level. A level 2000 was used to determine the TH level. Thy 1.1- positive SACs and ChAT-positive SACs within the grid for each retinal quadrant were counted. The number of SACs within each quadrant were averages for each retina.

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

Figure 4. Measurement of SACs. This figure summarizes the expression of α7 nAChRs in glaucomatous retina at various time points post-glaucoma-inducing procedure using a sandwich ELISA kit specific to the α7 nAChR. * indicates a significant decrease 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-glaucoma-inducing procedure. 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 2-μm stacks were taken per retina from each quadrant 4 μm from the ONH. A level 2000 was used to determine the ChAT level. A level 5000 was used to determine the TH level. Thy 1.1- positive SACs were identified with a minimum of four high resolution stacks, 4 μm from the ONH. Each retina was incubated in the following order: Thy 1.1 and sheep and rabbit-CHY overnight. A level of 5000 was used to determine the ChAT level. A level 2000 was used to determine the TH level. Thy 1.1- positive SACs and ChAT-positive SACs within the grid for each retinal quadrant were counted. The number of SACs within each quadrant were averages for each retina.

Conclusions

• SACs 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 SACs is lost one week after glaucoma induction, preceding RGC cell loss.
• The application of 10 μM DMP-543, an ACh receptor agonist, was 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 P30K/AKT/BD-2 cell survival pathway is shown to be downregulated in the glaucomatous 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 unidentified mechanism for SACs.
• Restoration of this possible neuroprotection could provide a new avenue for effective treatment of glaucoma and other central nervous system diseases.

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