Targeting neuronal mitochondria for neuroprotection in glaucoma

James R Tribble[1], Amin Otmani[1], Melissa Jöe[1], Peter G Fuerst[2], Robert W Burgess[3], Pete A Williams[1,*]

[1]Division of Eye and Vision, Department of Clinical Neuroscience, St. Erik Eye Hospital, Karolinska Institutet, Sweden, [2]Department of Biological Sciences, University of Idaho, Idaho, U.S.A., [3]The Jackson Laboratory, Maine, U.S.A., *pete.williams@ki.se, petewilliamslab.com

Introduction

Glaucoma is characterized by progressive dysfunction and loss of retinal ganglion cells (RGCs). RGCs sit on a metabolic knife-edge during times of stress that may be exacerbated by aging and genetic impairment. During these periods the viability of RGCs is reliant on mitochondrial regulation to maintain cellular homeostasis and bioenergetic status. Emerging research suggests that a systemic vulnerability to mitochondrial abnormalities exists in glaucoma patients. Genomic analysis has demonstrated increased mitochondrial DNA content and a spectrum of mitochondrial DNA mutations in glaucoma patients [1]. These abnormalities are also present in leukocytes, suggesting a systemic susceptibility to metabolic defects (as opposed to mitochondrial changes in the eye as a consequence of high intraocular pressure; IOP). Such systemic susceptibility is expected to increase glaucoma susceptibility with age. However, the role of mitochondria and metabolic health in glaucoma is yet to be fully elucidated.

We have previously discovered metabolic dysfunction and mitochondrial abnormalities occurring prior to neurodegeneration in glaucoma (in glaucoma patients and animal models) [2,3]. These studies identified that NAD (nicotinamide adenine dinucleotide; an essential REDOX cofactor and metabolite) declines in the retina in an age-dependent manner and renders RGCs susceptible to IOP-related stress, driving glaucomatous neurodegeneration. Preventing NAD depletion via administration of nicotinamide (NAM; the amide of vitamin B_3, an NAD precursor) or through gene therapy (*Nmnat1*; a terminal enzyme for NAD production) robustly protects from age-related neuronal metabolic decline and prevents glaucoma in chronic animal models [2]. Supporting a hypothesis in which pathogenically low NAD leads to glaucoma susceptibility, glaucoma patients have been demonstrated to have systemically low levels of nicotinamide (in sera) [4], and we, as part of a multi-national collaborative team, have completed the first-in-man clinical trial providing compelling preliminary evidence that nicotinamide administration can restore visual function in existing glaucoma patients [†]. Nicotinamide's excellent safety profile, combined with good tolerance and affordability, facilitates its rapid translation into further long-term clinical trials. Our continued work with nicotinamide exemplifies the lab's commitment to developing translational neuroprotective strategies, from bench-to-bedside. Nicotinamide's translation from animal to human studies highlights the importance of understanding the effects that nicotinamide and elevated NAD-processing have on normal, disease, and treated RGCs / eyes.

[†] Hui *et al.* 2020. *medRxiv*, doi: 10.1101/2020.01.28.20019075

Methods

Mice: MitoY (Tg*(Eno2-Yfp/Cox8a)*YRwb/J, founder line 1819), was generated as one of a number of unscreened mutants as previously published. These mice carry YFP fused in-frame with the mitochondrial targeting sequence of *Cox8a* under the control of a rat *Eno2* (neuron specific). We retrieved new founders from cryopreservation and backcrossed onto a clean C57BL/6J background.

Axotomy model: Adult, male, C57BL/6J mice were euthanized, eyes enucleated, and retinas prepared as tissue culture in retinal explant media [2]. In this system, RGCs degenerate over 0-7 days; with 3 days *ex vivo* (DEV) representing ~50% RGC loss from control.

Rats: Ocular hypertension was induced in adult, male Brown Norway rats by intracameral injection of paramagnetic beads targeted to the trabecular meshwork by a hand-held rare earth magnet [‡]. IOP was recorded by hand-held rebound tonometry. 14 days post injection was used as an endpoint for this study.

Nicotinamide treatment: Nicotinamide (NAM; PanReac AppliChem) was administered orally in normal drinking water at the doses shown. For the highest dose in rat (800 mg/kg/d) nicotinamide was delivered in water and food in custom feed (Special Diet Services).

Microscopy: For mitochondrial image analysis in the MitoY mouse, retinas were labelled with antibodies against RBPMS (RGCs) and GFP (YFP) and counterstained with DAPI. Retinas were imaged at 63x with 0.4x optical zoom at 35 nm/px on a Zeiss LSM800 Airyscan confocal microscope and reconstructed and analyzed using Imaris and FIJI.

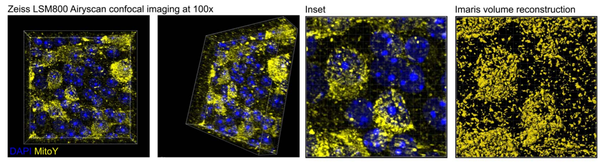
NAD assays: NAD was measured in whole retina or optic nerve homogenate following the manufacturer's instructions (NAD/NADH-Glo; Promega).

[‡] Tribble *et al.* 2019. *bioRxiv*, doi: 10.1101/853275
[1] Williams *et al.* 2017. *J Glaucoma*, doi: 10.1097/IJG.0000000000000767
[2] Williams *et al.* 2017. *Science*, doi: 10.1126/science.aal0092
[3] Tribble *et al.* 2019. *Brain Comms*, doi: 10.1093/braincomms/fcz035
[4] Kouassi Nzoughet *et al.* 2019. *IOVS*, doi: 10.1167/iovs.19-27099

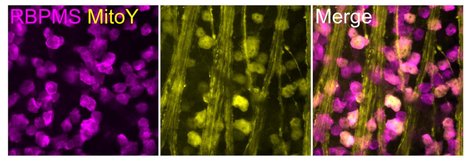
Karolinska Institutet
Department of Clinicial Neuroscience
St. Erik Eye Hospital
Pete Williams
Assistant Professor, Research Group Leader

Pete A Williams
pete.williams@ki.se
ki.se/en/people/petewi
petewilliamslab.com
@pete_the_teapot

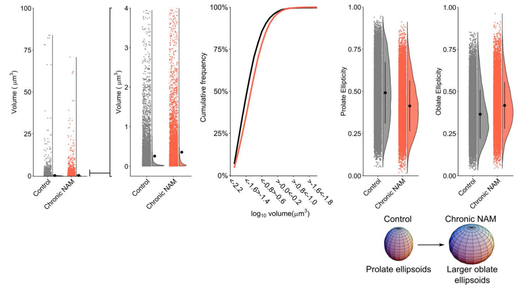
1 **Figure 1: Establishing MitoY imaging protocols.** *Left:* Retinas from naïve mice were flatmounted, fixed, and counterstained with DAPI. Retinas were imaged at 63x with 0.4x optical zoom on a Zeiss LSM800 Airyscan confocal microscope. *Right:* An inset around a RGC was selected, the YFP channel isolated, and volume reconstructed in Imaris. This allows the detailed analysis of volumes metrics (volume, surface area, ellipticity).



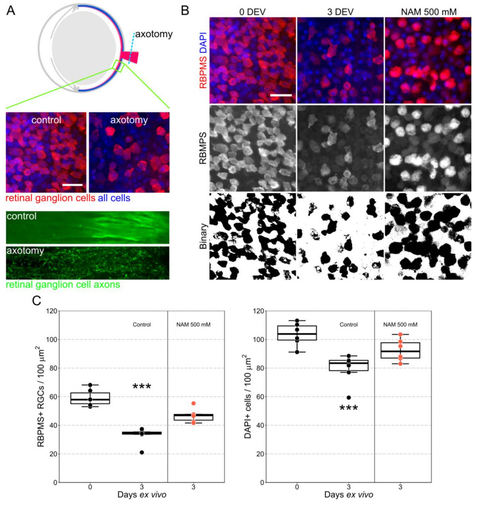
2 **Figure 2: MitoY is retinal ganglion cell specific.** MitoY+ retinas were stained with antibodies against RBPMS (RGCs) and Prox1 (amacrine cells). In the inner retina, MitoY is specific to RGCs. There are MitoY+ rods and rod bipolar cells in the outer retina (*data not shown*).



3 **Figure 3: Chronic nicotinamide administration changes RGC mitochondrial morphology.** MitoY+ retina were flatmounted and imaged following 1 week of oral NAM administration (500 mg/kg/d). *From left*: Following NAM administration mitochondrial volume increases (demonstrated by the rightwards shift in the cumulative frequency plot), and mitochondria become larger and more oblate (see schematic shape below plot). Rainclouds plot represent >15,000 reconstructed mitochondria per group.

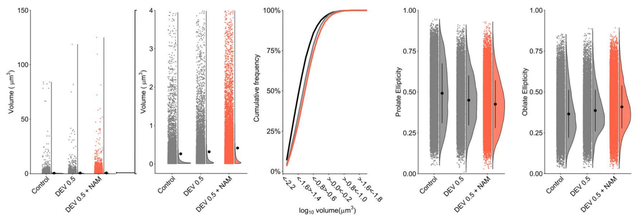


4 **Figure 4: Nicotinamide is robustly protective against axotomy induced insults.** (**A**) Schematic of axotomy explant model. (**B**) Example of retinas at control (day 0), 3 days post axotomy, or 3 days post-axotomy + NAM treatment. (**C**) Boxplots showing RGC density (*left*) and all ganglion cell layer cell density (*right*).

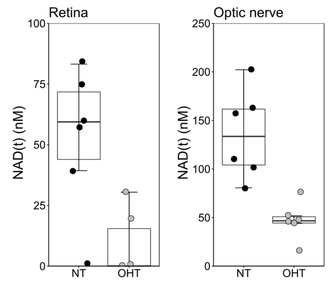


Results

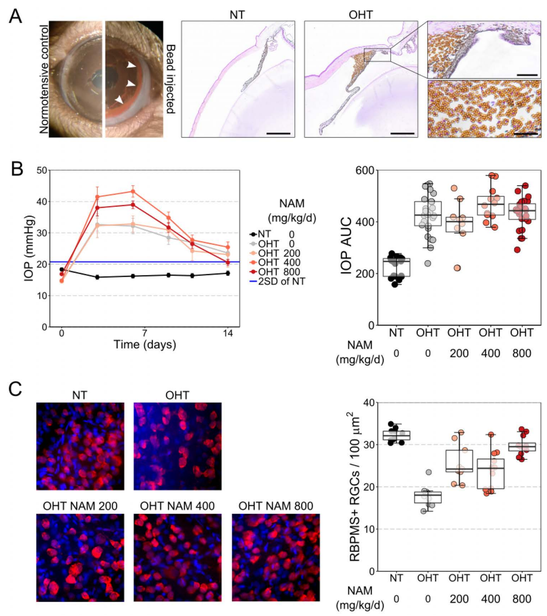
5 **Figure 5: Mitochondrial morphology changes follow axotomy.** Axotomy results in the rapid neurodegeneration of RGCs. Within 12 hours of axotomy (DEV 0.5) mitochondrial morphology changes and this is altered by NAM administration to the media. Rainclouds plot represent >15,000 reconstructed mitochondria per group.



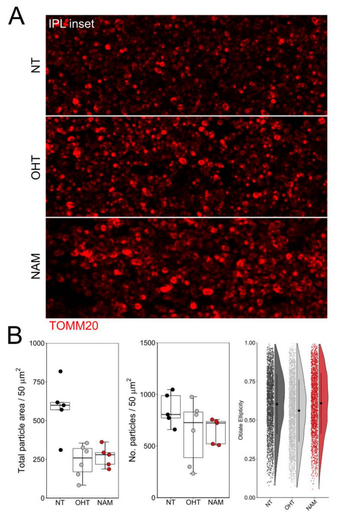
6 **Figure 6: Retinal and optic nerve NAD is depleted following ocular hypertensive insults.** Ocular hypertension was induced in rats (*see Figure 7*), and 14 days post-ocular hypertension whole retinas and optic nerves were processed for NAD assays. Total NAD (NAD(t); NAD^+/NADH) was reduced in both retina and optic nerve.



7 **Figure 7: Nicotinamide protects from ocular hypertensive glaucoma.** (**A**) Brown Norway rats underwent intracameral injections of paramagnetic beads bilaterally and beads were pulled into the drainage structures of the eye via a rare earth magnet (5 mm tip). Histology demonstrate that beads remained within the drainage structures of the trabecular meshwork and Schlemm's canal. This resulted in significant and robust IOP increase that was sustained until euthanasia (**B**). (**C**) 14 days of OHT results in significant RGC atrophy which is robustly protected by nicotinamide treatment. AUC = area under the curve, NT = normotensive, OHT = ocular hypertensive. Blue line in **B** represents 2 standard deviations above the NT IOPs.



8 **Figure 8: Inner plexiform layer (IPL) mitochondrial content declines following ocular hypertensive insults.** Retinas were sectioned and labelled with antibodies against TOMM20 (a mitochondrial marker), the IPL optically cropped out, and mitochondria reconstructed and analyzed. (**A**) Example IPL crops from control, glaucoma, and treated rats. (**B**) Mitochondrial coverage of the IPL declines following ocular hypertensive insults, however total number of mitochondria is unchanged. Following these insults, mitochondria become less oblate and this is prevented by NAM treatment. TOMM20 is a marker of all mitochondria and the IPL will contain mitochondria from glia and amacrine cells as well which may mask degenerative or neuroprotective effects. Rainclouds plot represent ~2,500 reconstructed mitochondria per group.



Conclusions

- Retinal ganglion cells are a particularly vulnerable to metabolic and physical stressors
- Metabolism and mitochondrial health decline with age and is exacerbated by periods of elevated intraocular pressure
- Retina and optic nerve NAD$^+$ declines following ocular hypertensive stress
- Increasing NAD$^+$ by nicotinamide treatment prevents retinal ganglion cell degeneration following two glaucoma-related insults; axotomy and ocular hypertension
- Nicotinamide alters mitochondrial size and morphology in normal and stressed retinal ganglion cells
- Targeting NAD$^+$ decline via nicotinamide has amazing potential as a cost effective, potent neuroprotective for glaucoma with limited side effects.
- These treatments may be even more efficacious in combination with intraocular pressure lowering strategies.

Acknowledgements

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