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The effects of age on mitochondria, axonal transport, and axonal degeneration after chronic IOP elevation using a murine ocular explant model

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ABSTRACT

The purpose of this study was to compare younger and older mice after chronic intraocular pressure (IOP) elevation lasting up to 4 days with respect to mitochondrial density, structure, and movement, as well as axonal integrity, in an *ex vivo* explant model. We studied 2 transgenic mouse strains, both on a C57BL/6J background, one expressing yellow fluorescent protein (YFP) in selected axons and one expressing cyan fluorescent protein (CFP) in all mitochondria. Mice of 4 months or 14 months of age were exposed to chronic IOP by anterior chamber microbead injection for 14 h, 1, 3, or 4 days. The optic nerve head of globe–optic nerve explants were examined by laser scanning microscopy. Mitochondrial density, structure, and movement were quantified in the CFP explants, and axonal integrity was quantified in YFP explants. In control mice, there was a trend towards decreased mitochondrial density (# per mm2) with age when comparing younger to older, control mice, but this was not significant (1947 ± 653 vs 1412 ± 356; $p = 0.19$). Mitochondrial density decreased after IOP elevation, significantly, by 31%, in younger mice ($p = 0.04$) but trending towards a decrease, by 22%, in older mice ($p = 0.82$) compared to age matched controls. Mitochondrial mean size was not altered after chronic IOP elevation for 14 h or more ($p \geq 0.16$). When assessing mitochondrial movement, in younger mice, 5% were mobile at any given time; 4% in the anterograde direction and 1% retrograde. In younger untreated tissue, only 75% of explants had moving mitochondria (mean = 15.8 moving/explant), while after glaucoma induction only 24% of explants had moving mitochondria (mean = 4.2 moving/explant; difference from control, $p = 0.03$). The distance mitochondria traveled in younger mice was unchanged after glaucoma exposure, but in older glaucoma explants the distance traveled was less than half of older controls ($p < 0.0003$). In younger mice, mitochondrial speed increased after 14 h of elevated IOP ($p = 0.006$); however, in older glaucoma explants, movement was actually slower than controls ($p = 0.02$). In RGC-YFP explants, axonal integrity declined significantly after 4 days of IOP elevation to a similar degree in both younger and older mice. Older mice underwent greater loss of mitochondrial movement with chronic IOP elevation than younger mice, but suffered similar short-term axonal fragmentation in C57BL/6J mice. These transgenic strains, studied in explants, permit observations of alterations in intracellular structure and organelle activity in experimental glaucoma.

1. Introduction

Glaucoma is the most common preventable cause of blindness worldwide (Quigley and Broman, 2006). Elevated intraocular pressure (IOP, Anderson and Hendrickson, 1974, Quigley et al., 1981) causes vision impairment by death of retinal ganglion cells (RGC) and remodeling of the optic nerve head (ONH). Older age (Burgoyne and Downs, 2007) and myopia (Boland and Quigley, 2007) are known risk factors for open angle glaucoma. Glaucoma animal models been used to further our understanding of RGC loss in glaucoma. This includes mouse lines that have been genetically modified to have fluorescent astrocytes (Nguyen et al., 2017), axons (Johnson et al., 2016;

Abbreviations: ONH, Optic Nerve Head; ON, Optic Nerve; IOP, Intraocular pressure; YFP, Yellow Fluorescent Protein; CFP, Cyan Fluorescent Protein; ATP, Adenosine Triphosphate; RGC, Retinal ganglion cells; RGC-YFP, Mouse strain expressing YFP in selected axons; Mito-CFP, Mouse strain expressing CFP in all mitochondria; Thy1, Thymus cell antigen 1, theta promoter; NB, Neurobasal Solution; LSM 710, Zeiss 710 Confocal Laser Scanning Microscope

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Kalesnykas et al., 2012; Li et al., 1999; Leung et al., 2011), and mitochondria (Kang et al., 2008; Miller and Sheetz, 2004; Shen and Cai, 2012). These tools permit assessment of initial effects after chronic IOP exposure. We developed (Kimball et al., 2017) an *ex vivo* explant of the eye and optic nerve to study acute and chronic effects of IOP elevation in mice. In the present research, we studied explants from the two mouse strains, one with yellow fluorescent protein (YFP) in a select number of RGC and a second strain with cyan fluorescent protein (CFP) in all mitochondria. The explant model permits the study of living RGC and mitochondria in their natural environment, though without an active blood supply. By comparison to research with cultured RGC (Welsbie et al., 2013), retinal explants (Johnson et al., 2016; Bull et al., 2011) or eye cup (Ishikawa et al., 2014; Howell et al., 2007; El-Danaf and Huberman et al., 2015), the axons of our model system are in their original configuration at the ONH. Tissues imaged in this manner are subject to the biomechanical effects of IOP, which can be controlled during observations by maintaining set levels.

IOP elevation alters RGC axonal transport in both the anterograde and retrograde directions, in human (Quigley and Green, 1979), monkey (Gaasterland and Kupfer, 1974) and rodent (Morrison et al., 1990) eyes. RGC are provided with energy from adenosine triphosphate, often distributed by mitochondria that travel along the axon or from mitochondria in neighboring astrocytes (Morrison et al., 2013). While it is known that the movement of mitochondria and other organelles along the axon is interrupted at the level of the sclera within the ONH, the precise mechanism(s) that are disturbed are not yet fully elucidated. In addition, the differences in resistance to glaucoma injury by age of the subject (Boland and Quigley, 2007; Chrysostomou et al., 2010) among different strains of experimental mice has been demonstrated (Cone et al., 2010; Myers et al., 2010; Steinhart et al., 2013). The reasons for greater damage in some older animals have not yet been determined.

In a pilot study using the ONH explants, we studied the effects of 1–3 h of acute IOP elevation, along with 4 explants that had undergone prior IOP elevation for 3 days. In the present report, we extensively studied the changes in mitochondrial structure, density and movement, as well as axonal integrity, in explants that had undergone prior IOP elevation for up to 4 days. We compared quantitative alterations in 4 month old mice and 14–17 month old mice of the C57BL/6J strain on which the two transgenics are based.

2. Methods

2.1. Animals

Animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, using protocols approved and monitored by the Johns Hopkins Animal Care and Use Committee. In this study, two transgenic mouse strains were used, one expressing YFP in selected axons (B6.Cg-Tg(Thy-1YFP)HJrs/J, Jackson Catalog Number 3782, RGC-YFP) and a second expressing CFP in all mitochondria (B6.Cg-Tg(Thy-1CFP/COX8A)S2Lich/J, Jackson Catalog Number 7967, Mito-CFP). Both types of mice were bred in a C57BL/6J background, obtained from Jackson Laboratories (Bar Harbor, ME), and expressed YFP or CFP under the control of the mouse thymus cell antigen 1, theta (Thy1) promoter. In YFP mice, the fluorescent marker was expressed in only a small proportion of RGCs. In CFP mice, all mitochondria of many cell types were fluorescent, due to linkage to human cytochrome c oxidase, subunit 8A (ubiquitous), targeting signal fused to the N-terminus. We studied a total of 41 animals, 16 RGC-YFP and 26 Mito-CFP mice, with 31 and 47 explants respectively, as both eyes of most animals were utilized (Table 1). Twenty-nine explants were from 4 month old mice of the Mito-CFP strain, 18 explants were from 14 month old Mito-CFP mice, and 31 explants were from 14 to 17 month old RGC-YFP mice. For comparison to the older RGC-YFP mice, we utilized comparable data on younger (4 months old) mice, published previously (Kimball et al., 2017).

Table 1
Animals in protocols.

Strain	Treatment	Age (months)	Experimental Group	N (explants)
Mito-CFP	Control	4	Younger, Control	8
Mito-CFP	14 Hour Glaucoma	4	Younger, Glaucoma 14Hr	8
Mito-CFP	1 Day Glaucoma	4	Younger, Glaucoma 1Day	9
Mito-CFP	3 Day Glaucoma	4	Younger, Glaucoma 3Day	4
Mito-CFP	Control	14	Older, Control	6
Mito-CFP	14 Hour Glaucoma	14	Older, Glaucoma 14Hr	3
Mito-CFP	1 Day Glaucoma	14	Older, Glaucoma 1Day	5
Mito-CFP	3 Day Glaucoma	14	Older, Glaucoma 3Day	4
			Total Mito-CFP	**47**
RGC-YFP	Control	14–17	YFP- Older, Control	18
RGC-YFP	14 Hour Glaucoma	14	YFP- Older, Glaucoma 14Hr	5
RGC-YFP	1 Day Glaucoma	14	YFP- Older, Glaucoma 1Day	5
RGC-YFP	4 Day Glaucoma	14	YFP- Older, Glaucoma 4Day	3
			Total RGC- YFP	**31**

2.2. Chronic IOP elevation model

Mice were anesthetized with an intraperitoneal mixture of ketamine (Fort Dodge Animal Health, Fort Dodge, IA), xylazine (VedCo Inc., Saint Joseph, MO) and acepromazine (Phoenix Pharmaceuticals, Burlingame, CA) at 50, 10 and 2 mg/kg concentrations, respectively). To elevate IOP, the anterior chamber of the left eye was injected (Cone et al., 2010) with Polybead Microspheres (Polysciences, Inc., Warrington, PA, USA). The protocol consisted of 2 μL of 6 μm diameter beads, then 2 μL of 1 μm diameter beads, followed by 1 μL of viscoelastic compound (10 mg/ml sodium hyaluronate, Healon; Advanced Medical Optics Inc., Santa Ana, CA). The injections were made through a glass cannula with a 50 μm diameter tip, connected to a Hamilton syringe (Hamilton, Inc., Reno, NV). The contralateral eye (right) was used as a control. Among the bead treated RGC-YFP and Mito-CFP animals, eyes were explanted at one of 3 time points post glaucoma induction; 14 h, 1 day, or 3–4 days.

2.3. Preparation of explants

Mice were deeply anesthetized by an intraperitoneal anesthetic injection. IOP measurements were taken with a Tonolab Tonometer (TioLat, Inc., Helsinki, Finland). Each eye, along with a 1.5 mm nerve segment, was enucleated, and the animals were euthanized. A cautery mark on the superior cornea provided subsequent orientation. Eyes were kept in Neurobasal Solution (NB, Thermo Fisher Scientific, Waltham, MA). Axial length and width of the eye were measured using a digital caliper (Instant Read-Out Digital Caliper; Electron Microscopy Sciences, Hatfield, PA, USA). Each globe was glued with cyanoacrylate onto a plastic chuck, superior side facing up. The nerve was placed flat on the chuck surface and 1% agarose (Sigma A4603, St. Louis, MO) that was pre-heated to 37 °C was applied to immobilize the optic nerve. Supplemental NB was added after 1 min to keep the tissue hydrated.

2.4. Imaging of explants

Imaging was performed on a Zeiss 710 Confocal Laser Scanning Microscope (LSM 710, Carl-Zeiss, Oberkochen, Germany) in two-