Axonal transport of mitochondria: developing an in vivo imaging assay for glaucoma research.

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Introduction: The earliest reported evidence to support the idea that the pathophysiology of glaucomatous damage to retinal ganglion cell axons is obstruction of axonal transport head included observations on swollen and distorted mitochondria accumulated within the non-human primate retina after ischemia, particularly along its posterior boundary. More recent evidence similarly suggests that abnormalities of mitochondrial function and transport are among the earliest events in glaucomatous pathogenesis (Chrysostomou V, et al 2013 & Osborne NN, et al 2016). Nevertheless, nearly all of these studies were based on histological material (i.e., a temporal static view).

Purpose: To develop a reliable in vivo assay of mitochondrial transport in the Brown-Norway rat eye. Such an assay would benefit future studies that seek to investigate the role of this critical function during the early sequence of pathophysiological events of glaucomatous axonal damage, as well as to assess novel therapeutic interventions.

Methods:

- Subjects: Adult Brown-Norway rats (Ratts norvegicus; Charles River Laboratories Inc. Wilmington, MA).
- All injections used DMSO as the solvent and vehicle.
- Anterograde transport studies utilized intravitreal injections performed by inserting the needle through the sclera superiorly, approximately 1 mm behind the limbus, at an angle of 45° to avoid contact with the relatively large crystalline lens of the rodent eye.
- Retrograde transport studies utilized stereotaxic injections into the right, or left superior colliculus (SC) depending on experiential assignment.
- CSLO images of in vivo retina and post-mortem brain structures were obtained (Spectralis HRA + OCT; Heidelberg Engineering GmbH, Heidelberg, Germany).
- The infrared and BluePeak™ blue laser (488 nm) autofluorescence imaging modes were used with 100 images averaged using the automatic real-time (ART) eye tracking software.
- Custom rigid gas-permeable contact lenses (3.5 mm posterior radius of curvature, 5.0 mm optical zone diameter, and +5 diopter back vertex power) were used to maintain corneal hydration and clarity during imaging.

In order to quantify in vivo labeling results obtained across tracer concentration and time courses, all CSLO-FL images were exported from the instrument as raw TIFF files without contrast manipulation and analyzed with ImageJ. The parameter Integrated Pixel Density (IPD, mean pixel intensity per unit area) was measured for a region of interest that was 80% of the total area of the image centered on the optic nerve head (to exclude perimeter of all images).

For these in vivo histological studies tissue was post-fixed in a solution of 4% paraformaldehyde and permeabilized with 1% Triton for three hours.
- Primary antibodies incubated for 16 hours in 1:200 RBPMS (Phosphosolutions), 1:1000 Neurofilament (Convance), and 1:250 GS (Abcam).
- Secondary antibodies incubated for 16 hour with 1:300 (Alexa488 & Alexa633 Thermofisher).
- Whole mount retina images where taken using a Leica DMi8 Confocal Microscope with 20x and 63x objective lenses.

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Results Anterograde Transport:

The MitoTracker dyes we evaluated, MitoTracker Green, MitoTracker Orange, and MitoTracker Orange-Redox, each have their own potential advantages and also clear limitations. With MitoTracker Green (Figure 1) only axon bundles arising near the injection site are labeled consistently despite varying concentration between 1 and 150µM and imaging at 4, 24 hours, 72 days and 5 days post-injection.

Results Retrograde Transport:

Retrograde transport studies utilized stereotaxic injections into the right, or left superior colliculus (SC) depending on experiential assignment (Figure 6).

MitoTracker labeling was observed by CSLO-FL in both eyes 4-hours after injection (Figure 6) in six different animals. This is both faster than would be predicted for mitochondrial bulk transport rate and unexpected insofar as only 3-5% of axons were predicted to be labeled by MitoTracker in the contralateral eye by active retrograde axonal transport mechanisms given that only 3-5% of RGC axons cross at the optic chiasm to project to the contralateral hemisphere in pigmented rat. This fascinating finding suggests an alternative route of delivery, perhaps via subarachnoid space, gliocytes, or another systemic pathway. However, with the limitations created by the need to normalize a signal to detect the fluorescent marker, we were unable to continue with the aim of creating a viable assay that would be reliable under experimental conditions with this dye.

Conclusions: The MitoTracker dyes are not selective enough to support an assay of axonal mitochondrial transport in the live rat retina using commercial CSLO instrumentation. Future experiments will seek to determine if labeled mitochondria are translocating the entire length of the axon, from the superior colliculus to the retinal ganglion cell soma, or if there is a non-mitochondrial, sulfhydryl-rich molecule to which MitoTracker also binds and undergoes transport. The latter pathway will be carried out by attaching an EM contrast agent, such as a gold nanoparticle, to the MitoTracker dye, that will allow for verification that the dye is being transported by mitochondria and not another sulfhydryl-rich organelle.

While we were able to carry out anterograde and retrograde transport studies, multiple limitations led us to conclude that with further development of methodology to overcome critical barriers, the MitoTracker dyes are not good candidates for use in a reliable assay of axonal mitochondrial transport.