

Small research grant application for £6706~ approx. \$9126.

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**Astrocytes as drivers of pathogenicity in Leber's Hereditary Optic Neuropathy:
"Stars in LHON"**

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Summary

Accumulating evidence indicates that Leber's Hereditary Optic Neuropathy (LHON) is characterised by profound mitochondrial dysfunction and altered cellular metabolism in retinal ganglion cells. However, this neuron-centric model does not fully explain disease onset, progression, or the striking inter-individual variability in visual recovery. Astrocytes are critical regulators of neuronal metabolism and inflammatory tone, and emerging evidence suggests that chronic metabolic stress can drive astrocytes towards a maladaptive pro-inflammatory state. Our preliminary data demonstrate significant bioenergetic impairment and increased glycolytic reliance in LHON patient-derived astrocytes, particularly in individuals who fail to recover vision.

Background

Leber's hereditary optic neuropathy (LHON) is a hereditary neurodegenerative condition affecting approximately 1/30,000 people worldwide, associated with three primary "common" mitochondrial DNA mutations, designated m.11778G>A, the m.14484T>C and m.3460G>A. LHON is strongly linked to compromised metabolic function in retinal ganglion cells (RGCs). LHON generally manifests between the ages of 20-70+ years, and following onset, patients rapidly lose vision in both eyes. There is currently no cure and only one approved intervention for LHON, idebenone, although gene therapy trials are underway.

Despite the role of RGCs in LHON having been well characterised it fails to fully explain the disorder, suggesting that other cells of the visual system may also be affected or play a part in the pathogenesis. Glia, non-neuronal cells populating the central nervous system including the retina and optic nerve, provide critical support instrumental for maintaining neuronal health, and a role for glia is increasingly being recognised in many neurodegenerative conditions. Despite this, few if any studies have to date explored the possibility of glial cell dysfunction in LHON. There is a further fascinating and important point, in that those patients with the m.14484T>C mutation have the greatest capacity for some spontaneous visual recovery, and this is not understood.

Astrocytes are a heterogeneous class of glial cells which play key roles in maintaining CNS homeostasis and neuronal health through a variety of means. These functions are facilitated by the metabolic flexibility of astrocytes, which are typically reliant on aerobic glycolysis with some oxidative metabolism¹⁻³. During inflammatory responses, astrocytes show a transient rapid metabolic shift to aerobic glycolysis, followed by upregulation of oxidative metabolism during the resolutive phase of inflammation^{3,4}.

Emerging evidence suggests that during chronic inflammation (such as seen prodromally to many neurodegenerative conditions) this process is impaired: continuous re-activation of pro-inflammatory responses leads to an increase in glycolysis without progression to the appropriate resolutive phase of the inflammatory response, resulting in glial asthenia or glial paralysis^{2,3} thus preventing astrocytes from fulfilling their role as key homeostatic mediators, contributing to and exacerbating neurodegeneration (Fig 1.).

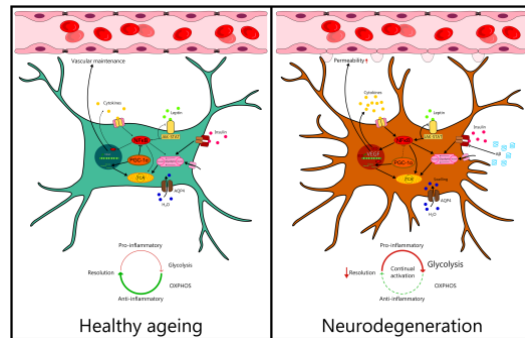


Figure 1. Astrocytes in health and neurodegeneration. Adapted from: Firth, Pye, Potter. Clin Sci (Lond) 24 April 2024; 138 (8): 515–536 (publication by named PDRA)

Our preliminary data

In summary- our preliminary data indicate significant metabolic perturbation in astrocytes from patients with LHON, which may be indicative of the adoption of a pro-inflammatory state as part of LHON pathology, and this may represent a new therapeutic target.

We have been studying this using induced human pluripotent stem cells (iPSCs) derived from a healthy control donor (WT), and m.14484T>C LHON-affected patients, one line with some visual recovery within 5 years from vision loss (designated recovery, R), and one line without any signs of recovery (designated no recovery, NR). We have refined a protocol to differentiate the iSCs into astrocytes (Fig 2).

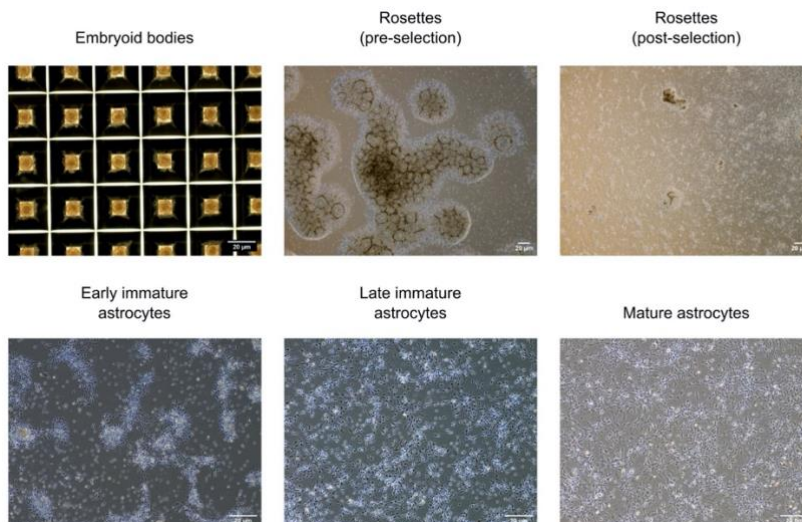


Figure 2: Human patient derived astrocyte differentiation. iPSCs generated from either healthy control or m.14484T>C LHON patients± visual recovery were differentiated into astrocyte-like cells using a commercially available ~60 day protocol (Stem Cell Technologies). (Briefly, embryoid bodies were formed from iPSCs, exposed to dual SMAD inhibition and plated out to generate neural rosettes. CNS-type neural

progenitor cells were selectively isolated via dissociation. Neural progenitor cells were driven to an astrocyte-like fate and matured until ~DIV60 before use in assays. Mature astrocytes were maintained in culture until >DIV90.)

Immunocytochemistry was used to confirm astrocyte identity. Mean fluorescence intensity (MFI) was measured and corrected for cell area using an automated pipeline. Live extracellular metabolic flux analyses were performed to interrogate changes to astrocyte respiration (Fig 3).

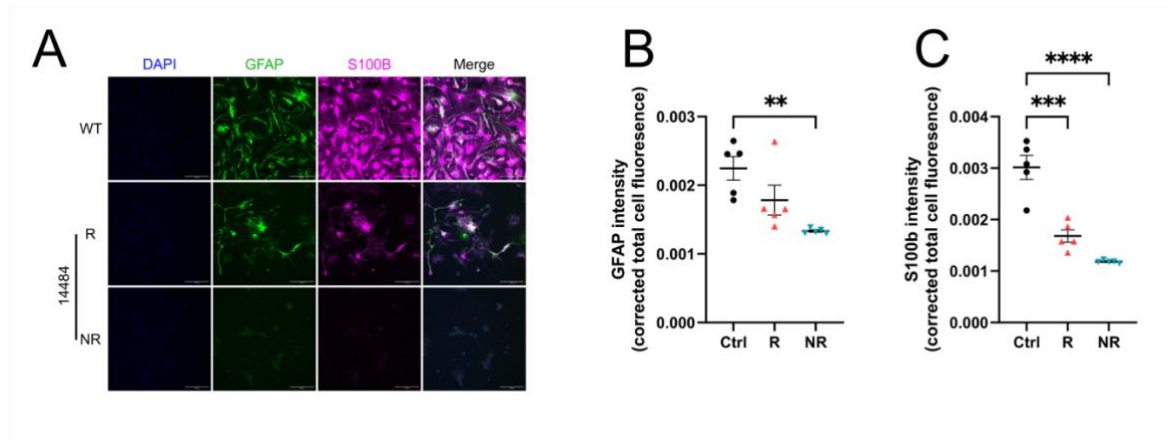


Figure 3: Astrocyte identity confirmation. 1×10^5 astrocyte-like cells were seeded onto coverslips, fixed 24h later, and stained for the canonical *in vitro* astrocyte markers GFAP (green) and S100B (magenta). The nuclear stain DAPI was used to confirm signal colocalization. 100% of cells were GFAP or S100B positive. A custom CellProfiler pipeline was used to determine corrected total cell fluorescence for GFAP and S100B; preliminary analyses (n=1, 5 fields of view/coverslip, data are mean FOV \pm SEM) indicate reduced GFAP and S100B positivity in LHON astrocytes.

$\geq 99\%$ of cells were immune-positive for the astrocyte markers glial fibrillary acidic protein (GFAP) and S100B, indicating astrocyte fate specification across all genotypes. GFAP MFI was significantly reduced in LHON-NR astrocytes relative to WT, whereas LHON-R did not differ significantly from either WT or LHON-NR. S100B MFI was significantly reduced in both LHON phenotypes relative to WT with no significant difference between LHON phenotypes.

Bioenergetic analyses indicated significantly reduced mitochondrial respiration in LHON-NR astrocytes and reduced coupling of oxygen consumption to ATP production relative to both WT and LHON-R astrocytes (Fig 4). Oxygen-ATP coupling was also significantly reduced in LHON-R astrocytes relative to WT. Both LHON phenotypes exhibited a compensatory increase in proton efflux rate, a proxy of glycolysis, relative to WT.

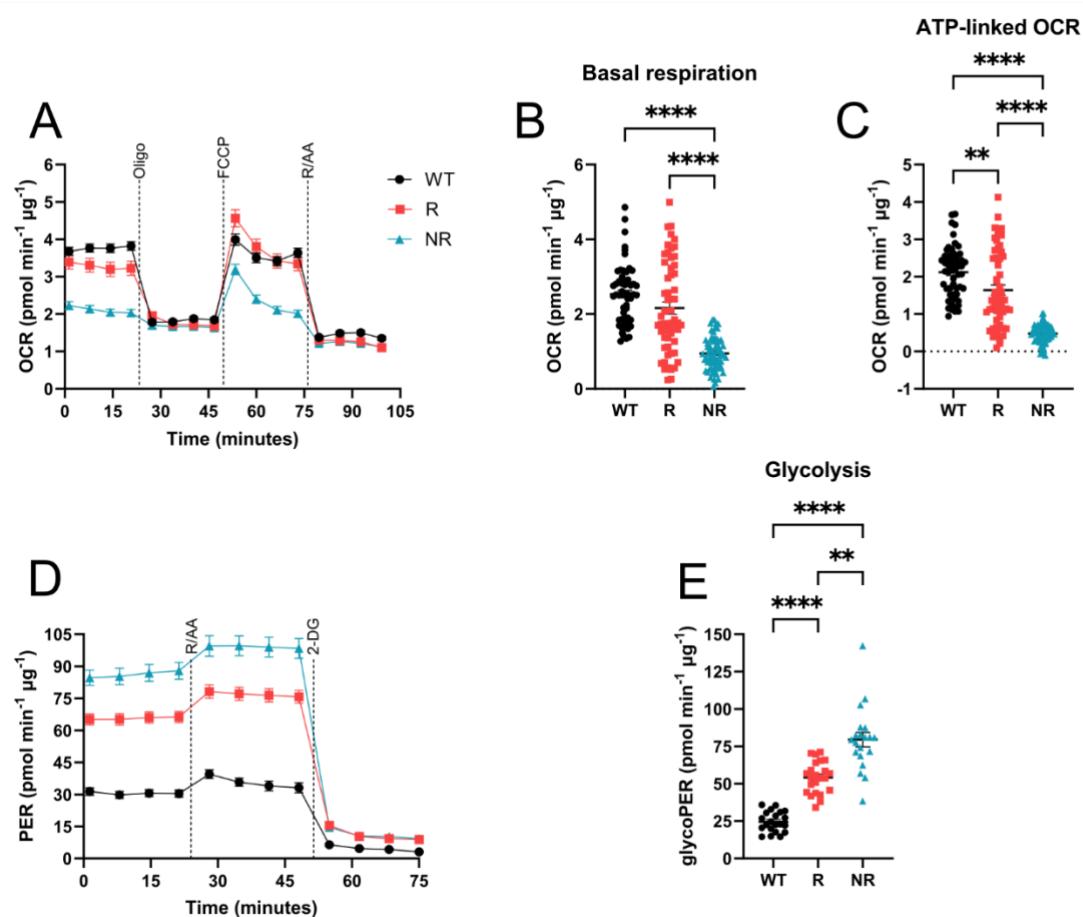


Figure 4: LHON impairs astrocyte bioenergetics and is suggestive of a pro-inflammatory phenotype. 2×10^4 astrocytes were seeded per well for live cell bioenergetic interrogation using the Seahorse XFe⁹⁶ bioanalyser (mitochondrial stress test, **A**, or glycolytic rate assay, **D**). Basal mitochondrial metabolism (**B**) and oxygen consumption linked to ATP production (**C**) were significantly reduced in LHON-affected astrocytes, with the ‘non-recovery’ phenotype showing greater deficits than the ‘recovery’ phenotype. In line with these findings, the glycolytic rate assay (**D**) indicated elevated glycolysis in both LHON phenotypes. $n=57-69$ wells per group (**A-C**), ordinary one-way ANOVA with post-hoc Tukey (**B, C**), $n=21-23$ wells per group (**D, E**), Kruskal-Wallis test with Dunn’s multiple comparisons (**E**). Data are pooled from 2 independent Seahorse plates and presented as mean \pm SEM. ** $p<0.01$, **** $p<0.0001$.

References: 1. PMID 29351512, 2. PMID 25125026, 3. PMID 38652065, 4. PMID 32277522, 5. PMID 35863905.

Aims of proposed research

We hypothesise that astrocyte dysfunction in LHON is characterised by coupled metabolic and inflammatory dysregulation, and that retention of astrocytic homeostatic capacity distinguishes patients with visual recovery from those without.

To test this hypothesis, we will break this down into the following concrete aims:

Aim 3: Characterise the cytokine and transcriptional output of LHON astrocytes under basal conditions.

Using cytokine profiling arrays and targeted transcriptomic analysis, we will define the secretory and gene expression signatures of LHON astrocytes. These data will identify candidate mediators through which astrocytes may influence retinal ganglion cell vulnerability and provide molecular markers associated with visual recovery.

Aim 2: Define the inflammatory signalling phenotype of LHON astrocytes and determine how this differs between recovery and non-recovery genotypes.

We will assess activation of key pro-inflammatory pathways, focusing on NF- κ B and STAT3 signalling, in patient-derived astrocytes from LHON individuals with and without visual recovery and healthy controls. These studies will establish whether LHON astrocytes adopt a sustained inflammatory state and whether this is modulated by recovery status.

Aim 3: Determine whether inflammatory activation in LHON astrocytes is coupled to altered metabolic sensing and oxidative stress pathways.

We will examine the relationship between inflammatory signalling and cellular energy regulation by probing AMPK/ACC signalling and expression of TSPO, a regulator of astrocyte mitochondrial metabolic flexibility. This aim will test whether inflammatory activation in LHON astrocytes occurs alongside a maladaptive catabolic and oxidative stress profile, consistent with impaired immune-metabolic resolution.

Research plan

We will use differentiated sets of astrocytes from our patient-derived induced pluripotent stem cells m.14484T>C LHON patient genotypes (LHON \pm visual recovery, and unaffected healthy control).

We already have these cells prepared and ready to use.

The funding application is for the use of the nCounter Glial Profiling Panel and SDS-PAGE immunoblotting and can begin as soon as funding is available.

The research plan breaks down into a series of experiments/work packages:

WP1:

We will probe the **cytokine release profile** using cell-free astrocyte condition media via a commercially-available array. To characterise the changes to gene expression in these cells, we will use the nCounter Glial Profiling Panel to assess changes to 757 genes related to glial cell function and differentiation of the glial cells and their precursors.

WP2:

We will confirm the **activation of intracellular inflammatory mediators** NFkB and STAT3 in LHON-affected astrocytes via SDS-PAGE immunoblotting.

WP3:

We will probe the existence of an **immune-metabolic link in LHON** by relating NFkB and STAT3 activation to expression and activation of key cellular energy sensors (AMPK, ACC) and a regulator of astrocyte mitochondrial metabolic flexibility (TSPO) via SDS-PAGE immunoblotting.

Replication and sampling

All analyses will be performed across a minimum of 3 independent patient-derived lines per condition, with differentiation batch included as a covariate where appropriate. Differentiation batches will be balanced across genotypes. All experiments will therefore have 3 biological and 3 technical replicates. All experiments will include a control, unaffected line.

Lay summary and clinical application

LHON is a rare disease with devastating consequences. It is a primary mitochondrial disease associated with mitochondrial Complex I dysfunction, reduced ATP and elevated ROS production. LHON clinically manifests as acute consecutive vision loss presenting as central scotoma, and optic nerve thinning associated with the loss of retinal ganglion cells. Despite the approval of idebenone some patients are still significantly impaired and face a life-time of vision loss. If we could understand more about why vision is lost new interventions may be developed which could be used in the NHS either in combination with idebenone or alone.

More specifically, if there is low grade inflammation and astrocytes are involved there may be additional therapeutic options for patients.

This project presents the opportunity to assess changes to glial cell function in LHON for the first time, using LHON patient-derived stem cells which will be differentiated into glia. As a metabolic disorder, many aspects of glial cell function are likely compromised in LHON, which may contribute to the loss of RGCs and visual function in patients. This pilot data may provide hints that guide us to a more holistic understanding of LHON pathology, and potentially open the door to novel treatment avenues in conjunction with existing therapy.

Impact and Significance

LHON is a devastating inherited optic neuropathy with limited therapeutic options and highly variable clinical outcomes. While mitochondrial dysfunction in retinal ganglion cells has been extensively studied, the contribution of glial pathology to disease progression and recovery remains largely unexplored. This represents a critical gap in our understanding of LHON biology.

This project will, for the first time, directly interrogate astrocyte immunometabolic dysfunction in LHON using patient-derived stem cell models stratified by visual recovery. By identifying distinct inflammatory and metabolic phenotypes associated with recovery versus non-recovery, this work has the potential to redefine LHON as a disorder of impaired neuron–glia homeostasis rather than isolated neuronal failure.

The anticipated outcomes of this study are therefore to:

- *Establish astrocytes as active contributors to LHON pathogenicity.*
- *Provide mechanistic insight into why some patients retain the capacity for visual recovery.*
- *Identify candidate inflammatory or metabolic pathways that may be amenable to therapeutic modulation.*

In the longer term, these findings could support the development of adjunctive treatment strategies targeting glial immunometabolism, either alongside existing therapies such as idebenone or as standalone interventions. Moreover, the results will generate strong preliminary data to underpin future mechanistic grant applications and may inform biomarker-led patient stratification approaches in LHON.

In summary

The proposed work if successful would support a **larger mechanistic grant**.

Recovery versus non-recovery insights could guide **patient stratification in future therapeutic interventions**.

Costings

TOTAL REQUESTED: £6706 ~approx. \$9126

WP 1: Bruker Spatial Biology Glial profiling panel Total = £4786

Item	Supplier	Cost (£)
Human Glial Profiling Panel XT Hs CSO	Bruker Spatial Biology	3026
Master Kit 12 reactions x2	Bruker Spatial Biology	286
S&H 1-8 kits/ slides	Bruker Spatial Biology	382
Shipping	Bruker Spatial Biology	341
RNeasy Plus Mini Kit	Qiagen	285
Training & access charges	Internal	346
RNA QC Agilent Bioanalyser	Internal	120

WP 2 & 3: Immunohistochemistry & SDS-page immuno-blotting Total = £1920

Consumable	Supplier	Cost (£)
NFκB (p65) antibody	Cell Signalling Technologies	147
pNFκB (p65) antibody	Cell Signalling Technologies	163
pSTAT3 antibody	Cell Signalling Technologies	180
STAT3 antibody	Cell Signalling Technologies	161
TSPO antibody	Abcam	170
Shipping	Abcam	50
XL Proteome Profiler Array	R&D Systems	879
IRDye® 800CW Streptavidin	Li-COR Biosciences	170

Gantt chart

2026		May	Jun	Jul	Aug	Sept
Activity	Funding application					
	Aim 1					
	Aim 2					
	Analysis - Aim 1					
	Aim 3					
	Analysis - Aim 2					
	Analysis - Aim 3					
	Manuscript, grant application, fellowship preparation & submission					