In vitro and *In vivo* Response of Retinal Muller Glia to Photobiomodulation

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Müller glial cells extend across the retina, interacting with the retinal neurons and blood vessels, and play a significant role in maintaining normal retinal function. Müller cells become activated (undergo gliosis) when the retina is stressed, for example following bright light exposure, or when cells such as retinal photoreceptors degenerate. Initially, *reactive gliosis* occurs, to induce neuroprotective events. Muller cells, sensing the loss of the neurones, are induced to express chemokines, which recruit macrophages to clear debris in the areas of cell damage. Muller cells also start to release neurotrophic factors to promote the survival of remaining neurons. After the loss of a large number of retinal cells however, activated Müller cells undergo *proliferative gliosis*, and form a seal around the most damaged area, as well as invade the space behind the retina forming subretinal scar tissue. The presence of the scar tissue seriously limits the delivery of nutrients to the photoreceptors from the underlying choroid, thus causing further stress and cell death, leading to the progression of the disease and inhibiting or limiting the potential for retinal remodelling and regeneration.

Photobiomodulation is a non-invasive therapeutic approach where light of a certain wavelength is used to induce a cellular response that promotes tissue regeneration. Near infrared light (NIR), specifically, light at 670nm wavelength, is known to ameliorate light-induced loss of retinal photoreceptors and reduce retinal inflammation. However, its effects on Müller glial activation and gliosis are not well understood. In this project we aimed to investigate the effects of NIR on Müller cells in culture, and also in a model of retinal degeneration (light-induced retinal damage).

Our first aim was to ***test the in vitro effects of 670nm red light on human Müller cell viability, proliferation and migration.*** In the first part of this series of experiments we established the optimal cell densities, media and 670nm dose-response. To assess the effects of 670nm light on Muller cell viability, growth and proliferation, we used a stress paradigm where cells were grown in serum-free media, mimicking starvation, and a scratch wound of Muller cells grown on plates, mimicking physical damage and cell loss. We found that 670nm light did not induce Muller cell death in culture under normal or stressed (serum free) conditions (*Figure 1*).

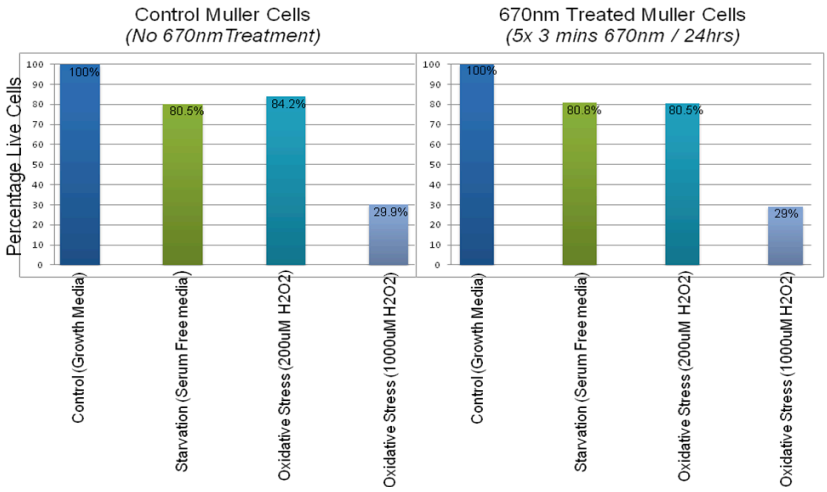

Figure 1: Muller cell viability was not affected by exposure to 670nm red light (5 x 3mins over 24 hours). High levels of oxidative stress killed Muller cells, even with 670nm red light.

We found that 670nm light treatment had significantly reduced Muller cell proliferation and migration of cells in normal growth media or serum free media (*Figure 2*).

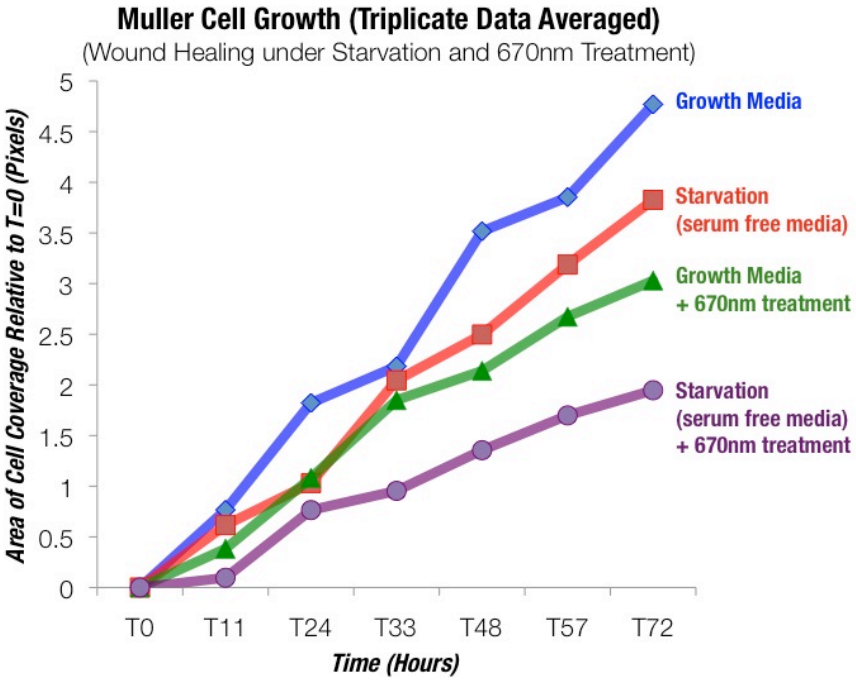

Figure 2: Muller Cell proliferation and migration was quantified using Image J software analysis, to assess cell growth in response to a scratch wound. Cells were grown in standard growth media or starved (serum free), with and without 3 x 670nm light treatments over 72 hours. The graph shows the area of cell growth, relative to the initial area at time = '0' (no cell growth at time of scratch wound)

The analyses of cellular and functional changes in Muller cells under metabolic or physical stress are ongoing and form the basis for the assessment of the effect of 670nm light treatment on the human retinal Muller cells *in vitro*.

Our second aim was to ***characterise the responses of activated Müller glia to NIR treatment in a stressed retina model.*** In these experiments we used the light-induced model of retinal degeneration where rats born and raised in dim (5lux) light until they reach young adulthood, to around 100 days of age. We exposed rats to bright light (1000lux), the equivalent of a well-lit office environment, continuously for 24 hours. This exposure to bright light causes an ***acute*** loss of photoreceptor cells. Interestingly, there is a greater rate of cell loss in the superior retina, when compared with the inferior retina after 24 hours and that 'hot spot' of tissue damage is in the centre of the visual axis of the rats' eye, thus is the functional equivalent of the human macula. Other features generally associated with retinal degeneration can also be seen in the hot-spot, including thickened and tortuous Müller cell processes (gliosis), increased glial fibrillary acidic protein (GFAP) immunoreactivity in Müller cells - a widely accepted sign of retinal stress – retinal pigmented epithelium (RPE) breakdown, and associated leukocyte infiltration into the subretinal space. The area immediately

surrounding the hot-spot – the *penumbra* - showed similar, but less severe structural changes. Significantly, we found that these signs of damage became more severe, and cell loss continued in the penumbra, long after the period of (bright continuous light) BCL exposure had ended, suggesting a ***chronic*** cell death process in the most affected area. Muller cells may play an important role in this process, through the development of glial scarring behind the retina, thereby blocking the delivery of the all-important nutrients from the underlying choroid, and leading to the starvation and death of the remaining photoreceptors (*Figure 3*).

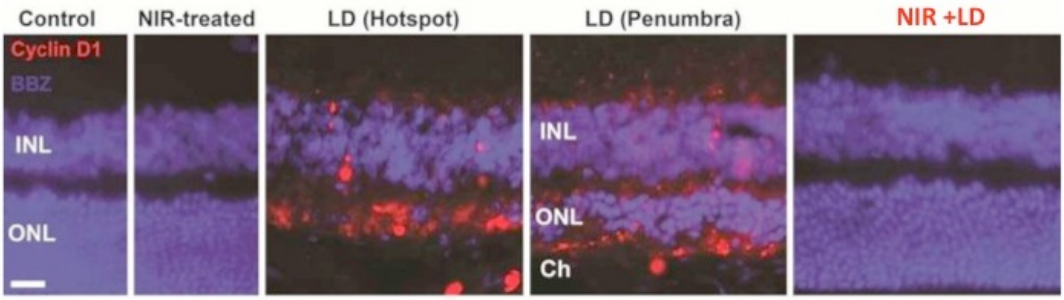

Figure 3: Immunohistochemistry for Cyclin D1 (red), showing proliferating Muller cells invading the damaged outer retina in the hot spot and penumbra in the light damage (LD) rat model. In light damage animals treated with 670nm light fro 3min on 5 consecutive days (NIR+LD), Cyclin D1 expression was not detectable.

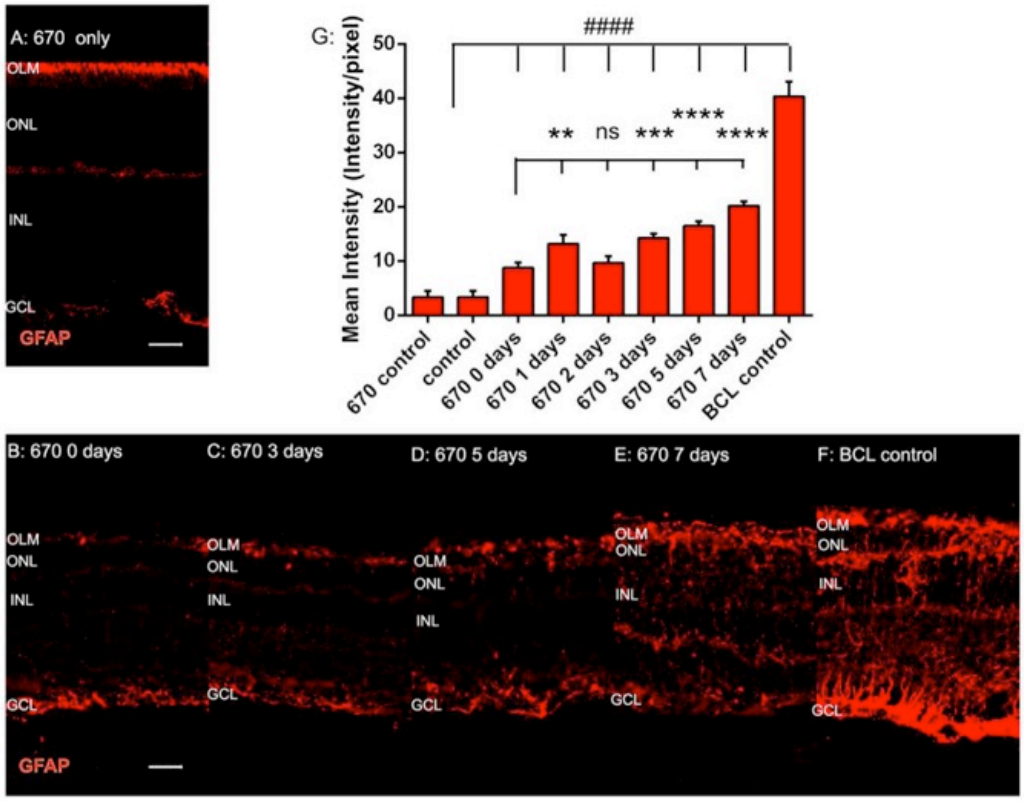

Figure 4: GFAP labelling was used to assess retinal stress. Retinas treated with 670nm light showed significantly lower expression of GFAP (red) when compared to non-treated LD animals (panel on the right in the bottom row).

We have shown earlier, that 670nm light is protective to photoreceptors in this animal model of retinal degeneration, however, the effects of the treatment on Muller cells

has not been assessed. To evaluate the effect of 670nm light on the Muller cells undergoing reactive gliosis, we used animals that were exposed to damaging light (LD) and commenced treatment with 670nm light at 0, 1, 2, 3, 5 or 7 days after LD. We found that retinas treated with the 670nm light showed significantly reduced stress as evidenced by moderate expression of GFAP in Muller cells, when compared to LD animals not treated with 670nm light.

We also investigated the effect of 670nm light on Muller cell proliferation. Using an antibody against Cyclin D1, a cellular proliferation marker, we found that following LD, Muller cells showed strong proliferation in the hot spot and the penumbra regions (*Figure 4*). This proliferation peaked 3 days after damage and if left untreated, scar tissue started to form in the outer retina by 7 days after light damage. Treatment with 670nm light, starting 0, 1, 2 or 3 days after bright light exposure resulted in reduced cell loss and prevention of Muller cell proliferation (*Figure 5*). Delay in 670nm red light treatment by as much as 5 to 7 days after light damage was still beneficial in mitigating Muller cell proliferation significantly.

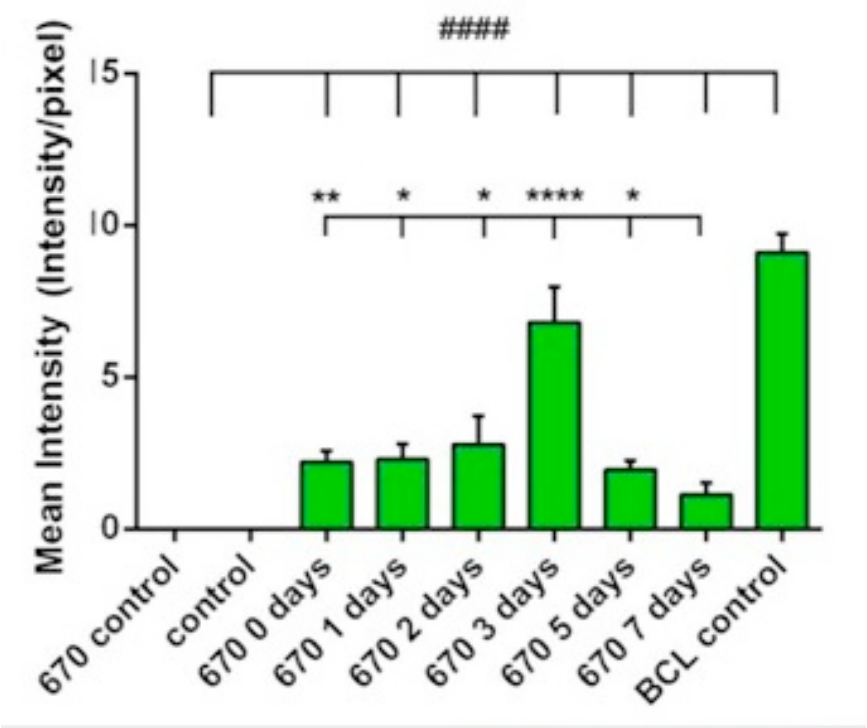

Figure 5: Quantitative summary of Muller cell Cyclin D1 expression. Retinas treated with 670nm light showed significantly less Cyclin D1 expression compared to bright continuous light (BCL) control.