Neurochemistry International 125 (2019) 187–196

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/neuint

Blue light exacerbates and red light counteracts negative insults to retinal ganglion cells *in situ* and R28 cells *in vitro*

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ARTICLE INFO

Keywords:
Ganglion cells: R28 cells
Mitochondria
Glaucoma
Visual light
Neuroprotection

ABSTRACT

Neurones are dependent on their mitochondria to produce the necessary amounts of ATP for survival. Retinal ganglion cells (RGCs) have a particularly large number of mitochondria which—unlike neurones in the brain—are exposed to visual light of 400–850 nm. Here we demonstrate that short wavelength visual blue light negatively affects mitochondrial function, causing oxidative stress and decreased cell survival. In contrast, long wavelength red light enhances mitochondrial function to increase survival of cultured R28 cells and reduce the effects of blue light. Induction of retinal ischemia for 60 min in dark conditions caused a reduction in ATP levels accompanied by decreased RGC numbers in all areas of the retina. These effects were diminished when ischemia was induced with concomitant delivery of red light, and exacerbated when blue light was used. We conclude that while the levels of blue light that reach the human retina will be a fraction of those used in the present study, the chronic nature might, on a theoretical basis, be detrimental to RGC mitochondria which are already affected by conditions such as glaucoma. Our findings also show that exposing the retina to red light may be a therapeutic approach to supporting healthy mitochondrial functions as part of the treatment for retinal diseases in which these organelles are affected.

1. Introduction

The survival of retinal ganglion cells (RGCs) is critical for visual processing, perception and other processes, as they transfer information of the visual environment to the brain (Dhande and Huberman, 2014; Martersteck et al., 2017). However, RGCs are susceptible to damage that can occur in the case of acute disease such as ischemic optic neuropathy and optic neuritis, or chronic diseases such as glaucoma (Osborne et al., 2001; Quigley, 2016; Sharif, 2018; Vidal-Sanz et al., 2012). Retinal ischemia and retinal artery or vein occlusions can cause damage to RGC cell bodies in the optic nerve head (ONH). Enhancing the viability of RGCs in order to maintain their function is a major goal of basic and translational research. Current approaches have focused on the development of drugs to reduce RGC loss and prolong vision in diseases such as glaucoma (Chidlow et al., 2007; Doozandeh and Yazdani, 2016; Hanumunthadu et al., 2014). Consequently, candidate drugs have been identified from studies using animal glaucoma models and various cell culture systems (Hanumunthadu et al., 2014; Osborne et al., 2014; Sena and Lindsley, 2017). Although valuable information has been generated, the intended neuroprotection is yet to be achieved, and determining target sites and appropriate delivery of drugs for the treatment of chronic retinal diseases remain a challenge.

Like all neurons of the central nervous system, RGCs depend on mitochondrial-generated ATP in order to survive (Chan, 2006; Osborne et al., 2014; Schon and Manfredi, 2003). It has been suggested that RGC mitochondrial dysfunction accounts for their susceptibility to damage that ultimately results in the loss of vision in glaucoma (Casson et al., 2012; Flammer et al., 2002; Osborne, 2010; Osborne et al., 2006; Pache and Flammer, 2006; Weinreb and Khaw, 2004). This dysfunction can be initiated *in vitro* by the inhibition of enzyme processes which affect oxidative phosphorylation, leading to reduced ATP formation and increased oxidative stress, to finally result in cell death (Boveris and Navarro, 2008; Dietrich and Horvath, 2010; Harman, 1981; Osborne et al., 2014). Experimental studies have also shown that light of different wavelengths can directly affect oxidative phosphorylation when it is absorbed by mitochondrial chromophores (Bell and Hall, 1981; García and Silva, 1997; Gorgidze et al., 1998; Hockberger et al., 1999; Mellerio, 1994; Ortiz de Montellano, 1995; Wataha et al., 2004). This could be key to the survival of RGCs because of their high numbers of mitochondria (Bristow et al., 2002; Carelli et al., 2004; Wang et al., 2003) and exposure to visual light (400–900 nm) where their mitochondrial proteins are directly affected by this light. Studies have shown that blue light (400–480 nm) causes reduced ATP formation, indicating its potential to further damage RGCs *in situ,* as is likely to be

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https://doi.org/10.1016/j.neuint.2019.02.018
Received 12 November 2018; Received in revised form 22 February 2019; Accepted 24 February 2019
Available online 27 February 2019

the case in glaucoma subjects (Osborne, 2010; Osborne et al., 2017, 2014, 2006, 2001). In contrast, red light (680–900 nm) has been reported to be absorbed by cytochrome-c-oxidase (COX) and enhance mitochondrial function thereby reducing the damage caused by a variety of conditions (Albarracin et al., 2013; Begum et al., 2013; Beirne et al., 2017; del Olmo-Aguado et al., 2016; Eells et al., 2003; Gkotsi et al., 2014; Karu, 1999; Osborne et al., 2016; Wong-Riley et al., 2005). Thus, increasing normal energy levels of red light to reach the retina offers a non-invasive approach to enhance mitochondrial functions for treatment of glaucoma subjects where RGC mitochondria are compromised.

Here we report that visual blue light induces mitochondrial damage to RGCs and cells originating from an R28-derived retinal precursor cell line (Seigel, 2014). In contrast, visual red light mitigates the damage to mitochondria caused by blue light. Additionally, exposure of healthy tissues to red light, as opposed to being in the dark, elicits protective endogenous influences. We conclude that the rate of RGC loss in glaucoma might be slowed by reducing and elevating normal levels of visual blue and red light, respectively that normally impinges on the retina.

2. Methods

Cell culture: The R28 cells (Kerafast, Inc. Boston, MA, USA) were grown in high glucose and pyruvate Dulbecco's Modified Eagle Medium (DMEM; Gibco, catalogue number: 41966) supplemented with 3.3% v/v sodium bicarbonate solution (Gibco, catalogue number: 25080), 10% FBS, 1% MEM non-essential amino acids (Gibco, catalogue number: 11140), 1% MEM vitamins (Gibco, catalogue number: 11120), 1% L-glutamine and 1% gentamicin, in an humidified atmosphere of 5% CO_2 at 37 °C.

From a suspension of cultured R28 cells (80,000 cells/ml), 0.1, 0.2 or 1.5 ml were placed in 96, 48 or 6-well plates respectively and allowed to settle for 24 h before subjecting the cells to various treatment regimes. For example, cells were exposed to defined concentrations of the mitochondrial toxins (all from Sigma-Aldrich) rotenone (inhibitor of complex 1) (catalogue number:R8875), 3- nitroproprionic acid (3-NP, inhibitor of complex II) (catalogue number: N5636), antimycin A (inhibitor of complex III) (catalogue number: A8674), sodium azide (inhibitor of complex IV) (catalogue number: S2002), carbonyl cyanide m-chlorophenyl hydrazine (CCCP an inhibitor of oxidative phosphorylation) (catalogue number: C2759), cobalt chloride (to mimic experimental hypoxia) (catalogue number: C8661) or glutamate (to mimic oxidative stress) (catalogue number: 49621) for 24 h in either darkness or the presence of red light (range between 580–575 nm with a peak of 630 nm, 6.5 W/m^2). In addition, cells exposed for 24 h to different intensities of red or blue light. Moreover, cells were incubated for 12 h in either darkness, blue light (range 410–530 nm with peak of 470 nm, 12.08 W/m^2) or red light (630 nm, 6.5 W/m^2) and then exposed to a further 12 h of darkness, blue light or red light. Red or blue light was delivered by LEDs (Electro DH SL, Barcelona, Spain) and the temperature maintained at 37 °C throughout. The intensity of light emitted from LEDs was checked by use of a spectrophotometer bought from Konica Minolta CL-70F.

Cell viability: Assessment of cell viability was carried out using the 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay (Mosmann, 1983) with some modifications. Briefly, cells in 96-well plates were subjected to the appropriate treatment and then MTT was added at a final concentration of 0.5 mg/ml and the cells incubated for 75 min at 37 °C. Medium was then removed and reduced MTT (blue formazan crystals) solubilised by adding 100 µl dimethyl sulfoxide (DMSO) to each well. After agitation of the plates for 10 min, the optical density of the solubilised crystals at 570 nm was measured using an automated microplate reader. The average absorbance of controls in each experiment was defined as 100% and related the average absorbance of cells subjected to various treatment regimes.

Mitochondrial depolarisation: Medium was removed from the cultures (96-well plates) and cells incubated cells with JC-1 dye (2 µg/ml) for 30 min to assess mitochondrial membrane depolarisation. The dye accumulates in mitochondria appearing as red/orange fluorescence (detected at 590 nm) in healthy organelles, or green (detected at 530 nm) when the membrane is depolarised (Perelman et al., 2012).

Production of ROS: Medium was removed from the cultures (96-well plates), washed twice with fresh medium, and then incubated with dihydroethidium (40 µM) for 20 min. The solution was removed, washed twice with fresh medium. Images were recorded immediately using phase fluorescence/contrast microscopy. The formation of ROS was assessed by the presence of red fluorescent chromatin in the nuclei.

Immunocytochemistry: The R28 cells were cultured in 6-well plates and fixed with paraformaldehyde followed by washing with phosphate buffer saline (PBS). After incubation with goat serum (10% in PBS) for 60 min and further washing with PBS, cultures were incubated with anti-HO-1 (ADI-SPA895 Enzo LS, 1:500) at 4 °C overnight. Cells were washed with PBS then exposed to a rabbit secondary antibody conjugated to Alexa Fluor 488 (1:300) for 3 h, followed by washing with PBS. Next, DAPI (0.2 µg/ml) was added to the wash solution. Images were obtained using a Leica DMI6000B fluorescence microscope (Leica Microsystems).

Studies on rat retinas: Induction of ischemia in rats was carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmology and Vision Research. The procedures used for anaesthesia and euthanasia (a blow to the head/neck region and decapitation) were approved by the regional animal oversight committee (University of Oviedo (Spain), Principado de Asturias, Spain) and are described elsewhere (del Olmo-Aguado et al., 2016). A total of 81 rats were given ischemia and maintained in the animal facility at constant humidity and temperature under a 12 h dark-light cycle. Access to food and water was allowed *ad libitum*. For the induction of experimental ischemia, rats (9–11-week-old male Wistar rats, approximately 300 g) were anaesthetised with ketamine/xylazine 80/10 mg/kg. A second injection of 1/3 of the initial anaesthetic solution was used to maintain rats slept for 60 min. Then, rats were and placed in a stereotaxic frame. Drops of a commercial mix of oxybuprocaine hydrochloride (4 mg/ml) and tetracaine hydrochloride (1 mg/ml) (Alcon, USA) were applied to both eyes causing mydriasis and reducing discomfort due to drying of the cornea. One eye of each animal was used as a control and the pupil of the other eye was cannulated with a 30-gauge needle and connected to a raised 5% glucose/saline reservoir, causing the intraocular pressure (IOP) to increase to 160 mmHg. The elevation of IOP was maintained for 60 min, and ischemia confirmed by whitening of the fundus. Ischemia was induced either in near darkness (less than 20 lux room light), in the presence of blue (range between 410–530 nm with peak of 470 nm) or in red (range between 580–575 nm with peak of 630 nm) light. The corneal intensities of blue and red light were 26.3 W/m^2 and 16.5 W/m^2, respectively. Previous studies have established that red light delivered to cells in culture at this intensity for 60 min caused no harmful effects (del Olmo-Aguado et al., 2016). Care was taken to maintain a normal body temperature during procedures. The analgesia included the use of buprenorphine at 0.05 mg/kg that was administrated two days, once the same day of the ischemia and another injection 24 h later. Rats were monitored twice daily and none exhibited signs of discomfort or abnormal behaviour. Rats were sacrificed around 15 days after ischemia. Previous studies have shown that optimum measurable changes in RGC death can be observed at such a time (Sellés-Navarro et al., 1996).

Ganglion cell number analysis: Ganglion cells (Brn3a-positive cells) were determined in whole retinas dissected from ischemic and control fixed eyes (n = 8) where temporal regions were clearly marked. Fixed retinas were placed in 0.01% $NaBH_4$ for 2 min and washed in PBS. Retinas were incubated with 10% donkey serum to block non-specific binding for 1 h, then incubated with agitation in a solution of either antiserum for Brn3a (sc-31984 SantaCruz Biotech, 1:100), GFAP

(Z0334 Dako, 1:500) or isolectin IB4 (L2895 Sigma, 1:1000) containing 0.1% Triton-X and 1% donkey serum for 4 days at 4 °C. After washing with PBS, Bn3a- and GFAP-exposed retinas were exposed to secondary antibodies conjugated to Alexa Fluor 594 (1:300) for 24 h. The isolectin IB4 antibody (Sigma-Merck, Merck KGaA, Darmstadt, Germany) was purchased conjugated with FITC. Retinas were flat mounted, ganglion side up, in Dako mounting medium. Images were recorded using a Leica DM6000B fluorescence microscope (Leica Microsystems) at 20× magnification, and Brn3a-positive cells in 0.3 mm^2 retinal areas at a distance of 335 μm from the temporal to nasal region were determined using ImageJ software (ImageJ 1.46r, NIH, Bethesda, MD, USA).

Western blot analysis: Retinas were placed in a lysis buffer (approximately 1 ml/10 mg tissue) containing phosphatase and protease inhibitors (Sigma-Aldrich). Extraction of proteins was carried out by homogenisation followed by freeze/thawing and homogenisation (in the case of ocular tissues) and sonication. The protein content was determined using a bicinchoninic acid protein kit (Sigma-Aldrich). Protein extracts were mixed with sample buffer (2 M Tris/HCl, pH 6.8, containing 8% SDS, 40% glycerol, 8% mercaptoethanol and 0.002% bromophenol blue) and immediately heated for 5 min at 95 °C. Proteins were fractionated by electrophoresis using 10% polyacrylamide gels containing 0.1% SDS, as described by Laemmli (1970). Proteins were transferred to nitrocellulose membranes and incubated overnight at 4 °C with anti-actin (MAB1501 Millipore, 1:4000), anti-Brn3a (1:500; Santa Cruz, Dallas, TX, USA) which was followed by detection with appropriate biotinylated secondary antibodies. Finally, nitrocellulose blots were developed using a 0.016% w/v solution of 3-amino-9-ethylcarbazole (AEC) in 50 mM sodium acetate (pH 5.0) containing 0.05% (v/v) Tween-20 and 0.03% (v/v) H_2O_2. The colorimetric reaction was stopped with 0.05% sodium azide/PBST solution and the density of the individual bands quantified using ImageJ software (NIH). N refers to the number of experimental animals subjected to western blot analysis. No pools or homogenate were done.

Analysis of ATP levels: Dissected whole retinas were assayed for ATP content using an assay kit (CLS II, Bioluminescence kit; Roche Applied Sciences) by precisely following the manufacturer's instructions.

3. Results

All chemicals applied to R28 cells caused a reduction in cell viability at a defined concentration (Fig. 1A). Irradiation with blue light caused increased cell death at specific intensities, while red light had no significant effect on viability at the tested intensities (Fig. 2A). The concentration of each chemical that was required to reduce cell viability by 30–35% after 24 h was significantly lower when cells were incubated in red light at 6.5 W/m^2 compared with dark conditions (Fig. 1B). The decreased cell viability caused by blue light at 12.08 W/m^2 was attenuated by exposure to red light either before or after irradiation with blue light (Fig. 2B). This observation was noted when cells were maintained in alternating 12 h cycles of darkness and red or blue light over 24 h (Fig. 2B). These conditions also revealed that red light led to increased cell proliferation after 12 and 24 h compared with dark conditions, thereby increasing the amount of viable cells.

Results derived from cells exposed to regimes of light conditions (Fig. 2) stained to reveal mitochondrial function with JC-1 (Fig. 3) or oxidative stress (using dihydroethidium or DHE; Fig. 4) paralleled each other. Green JC-1 dye indicates de-energised or unhealthy affected mitochondria (Fig. 3), while DHE appears as a bright red fluorescent nuclear stain when nuclei have been submitted to oxidative stress (Fig. 4). Following 24 h exposure to blue light few cells remained attached to the culture plates (cell death causes cell detachment) and their mitochondria appear green with JC-1 staining. Mitochondria in R28 cells that had been exposed solely to red light for 12 or 24 h were unaffected (JC-1 stain remains red in colour), which was also observed for cells maintained in the dark (Fig. 3). Blue light-induced toxicity caused increased production of ROS. In contrast, red light caused no increase in ROS, and it counteracted the effect of blue light whether given before or after the insult (Fig. 4).

Cells exposed to similar regimes of light (Fig. 2) showed blue light to increase cytoplasmic levels of the stress protein HO-1. This was not observed in cells maintained in dark or red-light conditions, in which little staining for HO-1 was observed. Exposure to red light reduced the effects of blue light in terms of HO-1 expression when provided either before or after irradiation with blue light (Fig. 5).

Ischemia of the rat retina (induced in near-darkness and with 5 min reperfusion) caused a significant decrease in the ATP content compared with retinas from control eyes (Fig. 6). This decrease was greater when blue light was present during ischemia, but was reduced when red light was used. Moreover, retinas from rats exposed to blue light as compared to darkness (where their pupils was dilated as when given ischemia), showed a decrease in ATP levels. In contrast, red light slightly elevated the level of ATP compared with dark conditions (Fig. 6).

Representative images of the distribution of Brn3a-positive cells in rat retinas of similar eccentricities (with reference to defined blood vessels) from eyes that underwent ischemia/reperfusion for 15 days in darkness, blue or red light (n = 8) are shown in Fig. 7. The average density of Brn3a-positive cells in the peripheral retina was lower than that observed in the medial and central parts. Quantification of Brn3a-positive cells in retinal areas of 0.3 mm^2, 6 mm along the temporal/nasal direction (and through the optic nerve region) (Fig. 8) revealed that ischemia/reperfusion caused decreased numbers of ganglion cells in all retinal areas compared with control retinas. These numbers were further decreased in all retinal areas when blue light was applied through the pupil, but elevated when red light was used during ischemia. Quantitative analysis of 0.3 mm^2 areas from the peripheral to nasal parts (Fig. 8) revealed that ischemia/reperfusion caused an approximately 30% loss of ganglion cells. This loss was significantly reduced when red light was applied during ischemia, but increased when blue light was used. These results were confirmed by western blot analysis of retinal extracts (Fig. 9), which revealed that the relative amount of Brn3a protein in the retina decreased following ischemia in the dark, exacerbated when blue light was used and reduced when ischemia was in presence of red light.

Images processed for GFAP and isolectin IB4 immunoreactivities in flat mount retinas from rats where ischemia was delivered to the eyes in conditions of no light, red light or blue light are shown in Fig. 10. Isolectin IB4-immunoreactivity provides a means for viewing the retinal vasculature (Ernst and Christie, 2006). Compared with control dark conditions, blue light caused a general increase in GFAP immunoreactivity in retinal glia (Müller cells, astrocytes and microglia). GFAP was largely unaffected by red light but appeared slightly elevated with glia (astrocytes/microglia?) close to blood vessels. Ischemia (in the dark), significantly elevated GFAP immunoreactivity in all retinal areas but this did not occur when red light was used during ischemia. Interestingly, ischemia in the presence of blue light resulted in intense patchy areas of GFAP immunoreactivity.

4. Discussion

It is generally accepted that RGC mitochondria are affected in the initiation of glaucoma (primary open-angle glaucoma) and for various reasons lose function further, which explain why RGCs die at different times (Gueven et al., 2017; Hondur et al., 2017; Kamel et al., 2017; Lee et al., 2011, 2012; Osborne, 2010; Osborne et al., 2017, 2006). A goal of our study was to highlight the potential negative effect of visual blue light to enhance progression of glaucoma and to provide evidence that increasing the level of visual red light reaching the retina, will be of benefit to glaucoma subjects.

Mitochondria perform a number of tasks such as maintaining homeostasis and carrying out metabolic functions including oxidative energy metabolism, control of intracellular calcium levels and the

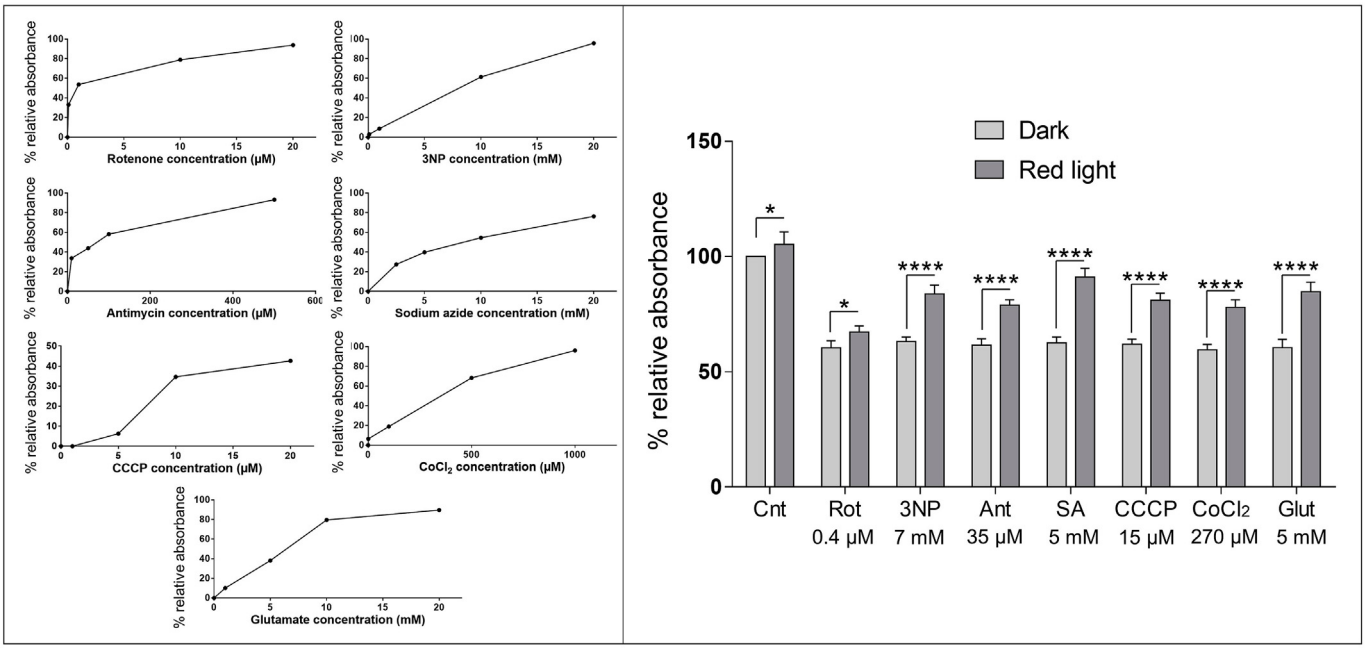

Fig. 1. (A) Dose-response curves of different toxins over 24 h on the survival of R-28 cells in the dark. (B) Comparative negative effect of selected toxins that reduces relative absorbance (indexed as a measure of cell viability) of MTT by 30–35% in the present of darkness or red light (630 nm 6.5 W/m^2). 3NP, 3- nitroproprionic acid; Ant, Antimycin A; SA, sodium azide; CCCP, carbonyl cyanide m-chlorophenyl hydrazine; $CoCl_2$, cobalt chloride; Glut, glutamate. Data are mean values ± SEM, n = 8. ****p < 0.0001 Statistics was determined by a two-way ANOVA with Sidak's multiple comparisons test where.

regulation of neuronal excitability and synaptic transmission (Chan, 2006; Moreira et al., 2007, 2010; Schon and Manfredi, 2003; Albers and Beal, 2000). One likely cause for RGC mitochondrial dysfunction in glaucoma is impaired regulation of optic nerve head (ONH) blood flow. This can be caused by raised intraocular pressure (IOP), diurnal fluctuations in IOP, arterial hypertension, low systolic perfusion pressure, low diastolic perfusion pressure, reduced blood pressure in hypertensive patients, cardiovascular disease, migraines, vasospastic disorders, arteriosclerosis or cerebral spinal fluid pressure changes (Caprioli et al., 2010; Hayreh, 2001; Leske et al., 2008; Osborne, 2010; Osborne et al., 2017, 2006). Any compromised blood supply will cause ischemia (defined as a reduction in the supply of nutrients and oxygen) and consequently affect the mitochondrial function of RGCs located within the ONH (Andrews et al., 1999; Bristow et al., 2002; Carelli et al., 2004).

Recent studies have shown that mitochondrial function can be modulated by light, as it affects chromophores that are associated with ATP synthesis (Bell and Hall, 1981; García and Silva, 1997; Gorgidze et al., 1998; Hockberger et al., 1999; Mellerio, 1994; Ortiz de Montellano, 1995; Wataha et al., 2004). This is of significance to retinal central nervous tissue because it is directly exposed to visual light (400–900 nm). For example, visual blue light (400–480 nm) can affect

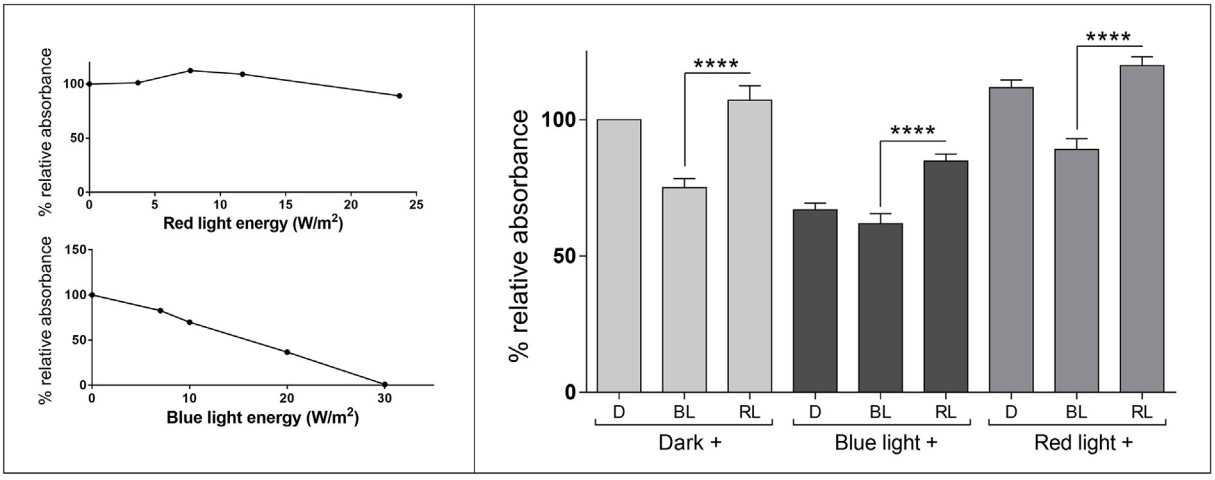

Fig. 2. (A) Comparative effect of different intensities of red and blue light on R28 relative absorbance of MTT (indexed as a measure of cell viability) over 24 h. Results are mean values from eight independent experiments. (B) Effect of 12 h in darkness, blue light (470 nm, 12.08 W/m^2) or red light (630 nm 6.5 W/m^2) and then for 12 h in either darkness or blue light or red light, on R28 cell viability. It is clear that red light enhances the amount of viable cells (possibly because of increased proliferation) after 12 h and even more after a period of 24 h compared with dark conditions. Data are mean values ± SEM, n = 8, ****p < 0.0001. Statistics was determined by a one-way ANOVA with Tukey's multiple comparisons test where. D = dark, BL = blue light, RL = red light.

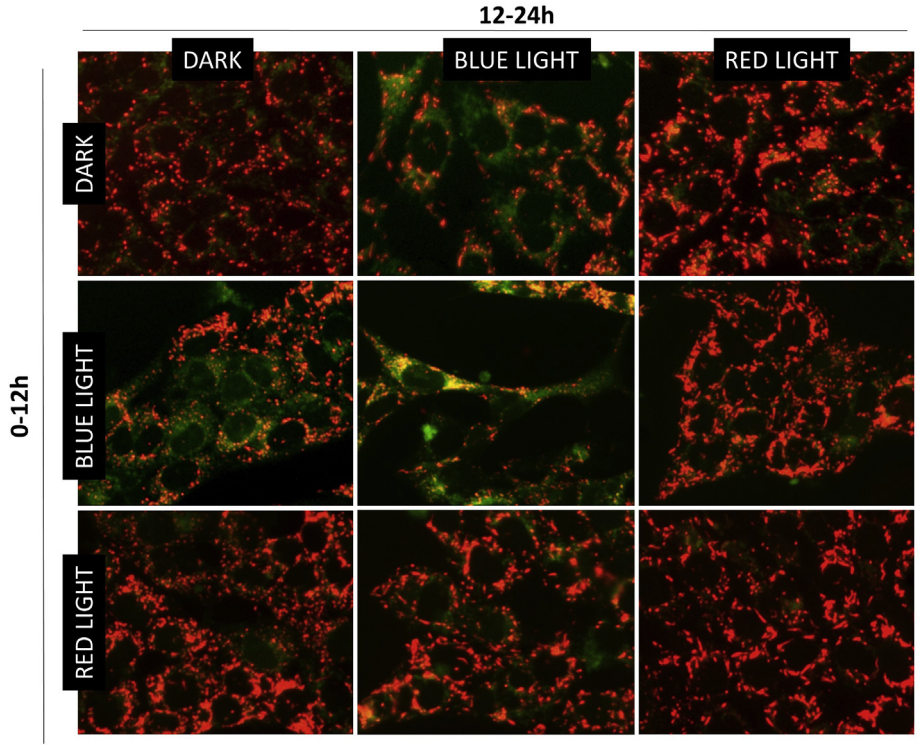

Fig. 3. Representative images of R28 cells stained with JC-1 dye following exposure to different combinations of 12 h of blue (12.08 W/m^2) or red (6.5 W/m^2) light. JC-1 located in mitochondria appears red in colour in healthy cells but green when in an unhealthy state.

mitochondrial flavin and cytochrome constituents of the electron transport chain to reduce ATP synthesis, increase oxidative stress to cause cell dysfunction and death (Osborne, 2010; Osborne et al., 2017, 2014, 2006, 2001). This leads us to question whether the blue light emitted not only from solar energy but also from digital screens and LEDs could impact the RGC mitochondria and be of importance in glaucoma. In contrast, visual red light (650–900 nm) acts on mitochondrial complex IV (or cytochrome *c* oxidase) to stimulate ATP and

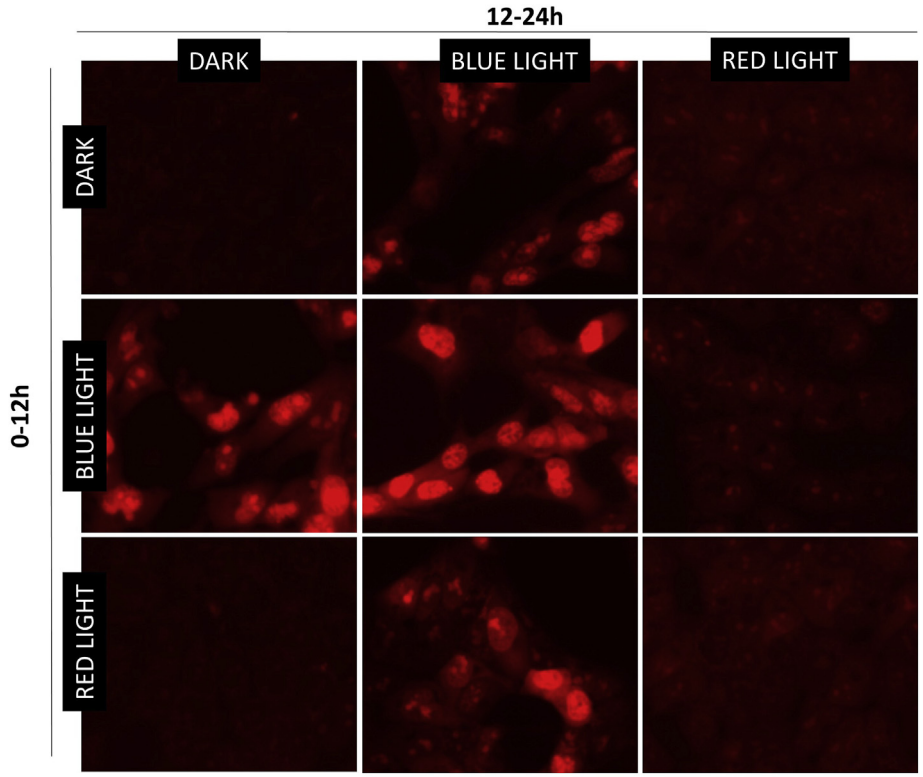

Fig. 4. Representative images of R28 cells stained with DHE following exposure to different combinations of 12 h of blue or red light. Only blue light stimulates ROS formation where the generated red product is translocated into the nucleus. It can be seen that the influence of blue light is counteracted by red light.

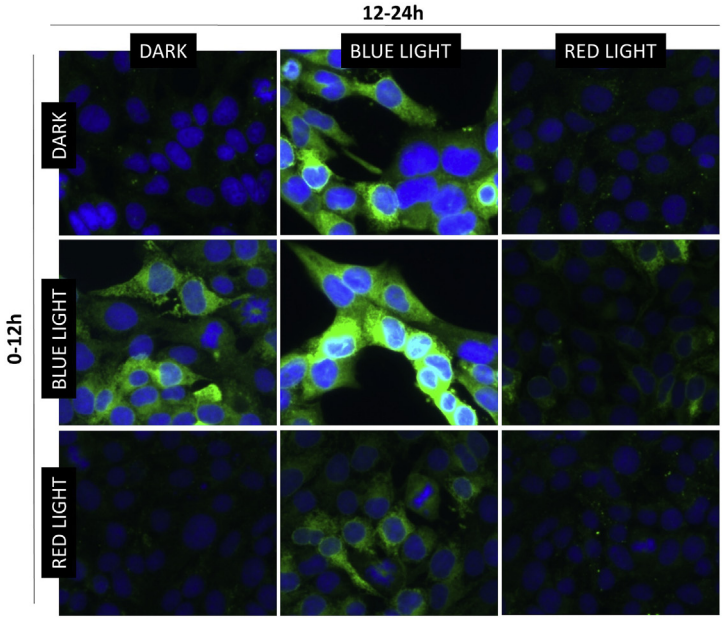

Fig. 5. Representative images of R28 cells stained for the localisation of cell nuclei (DAPI, blue) and cytoplasmic HO-1 (green) following exposure to different combinations of 12 h of blue or red light. Cells in the dark or red light, show little staining for HO-1 but is significantly up-regulation by blue light. Red light nullifies the increase in HO-1 immunoreactivity.

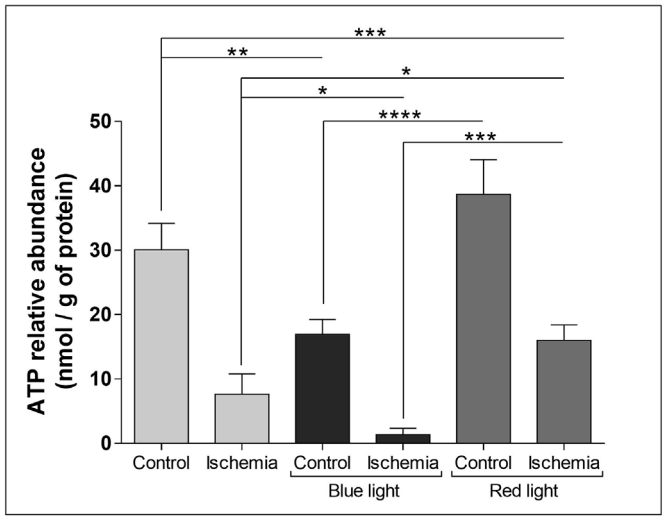

Fig. 6. Relative amount of ATP in rat retinas following ischemia in the dark or in either blue (26.3 W/m^2) or red (16.5 W/m^2) light. Ischemia in the dark causes a significant decrease in ATP compared with control retinas. This decrease in ATP was enhanced when the dilated pupil was exposure to blue light during ischemia but reduced when carried out in red light. Moreover, delivery of blue or red light into eyes not given ischemia, respectively, caused a significant decrease and increase of retinal ATP energy levels compared with from retinas from control eyes in the dark. Data are mean ± SEM, n = 4, ****p < 0.0001. Statistics was determined by one-way ANOVA with Tukey's multiple comparisons test.

nitric oxide production positively, causing an induction of various beneficial factors to enhance cell survival (see, for example, (Gkotsi et al., 2014)). This indicates that delivery of extra visual red light to the retina may be a therapeutic approach for the treatment of mitochondrial dysfunctions as in glaucoma.

In this study we show that blue light, like various mitochondrial poisons (rotenone, 3-NP, antimycin A and sodium azide) dose-dependently reduces cell (R28 cells) survival (Figs. 1A and 2A). The R28 cell line is derived from an immortalised postnatal rat retinal culture, and often used to model retinal cells (Seigel, 2014). Although red light had no effect on R28 cell viability at any intensity tested (Fig. 2A), it significantly attenuates the negative influences of mitochondrial toxins and of blue light (Figs. 1B and 2B). These results support the hypothesis that blue light negatively affects mitochondrial function and can be attenuated by use of appropriate amounts of red light. The intensity or irradiance of the blue light LEDs (range 410–530 nm with peak at 470 nm, 12.08 W/m^2) used to negatively affect cells in culture is low and approximately twice that emitted from a computer screen (range 380–740 nm with peak at 470 nm, 6 W/m^2). However, the intensity of solely the 470 nm wave length of blue light from the LEDs is 0.14 W/m^2, only a fraction of the 470 nm of light emitted on an average sunny day (1.79 W/m^2). It is thus proposed that the gradual impact of blue light from the sun and also artificial lighting needs consideration in relation to the retina when neuronal mitochondria are already compromised as would be the case for individuals with glaucoma. The intensity of the red light LEDs (range between 580–575 nm with a peak of 630 nm, 6.5 W/m^2) used in the experimental studies is non-toxic and attenuates insults to cells by acting on their mitochondria. The irradiance of solely of the 630 nm wavelength of red light from the LEDs is 0.22 W/m^2. The intensity of the same peak (630 nm) of red light from a computer screen or on a sunny day is respectively, 0.007 W/m^2 and 1.62 W/m^2. This argues a case for suggesting that increasing the amount of red light impacting retina especially at night and in artificial environments would benefit the unhealthy retina.

The mechanism by which blue light negatively affects R28 cell survival involves loss of mitochondrial status, oxidative stress and HO-1 formation (Figs. 3–5). The protein HO-1 is a stress-response protein which plays a vital role in maintaining cellular homeostasis and is known to be overexpressed in conditions such as pathological iron deposition, oxidative stress and mitochondrial damage (Himori et al., 2014; Schipper et al., 2000). The level of HO-1 expression may therefore be important in the antioxidant defence systems of organisms (Amersi et al., 1999; Ursu et al., 2014).

In a previous study it was shown that ischemia/reperfusion of the rat retina causes decreased RGC numbers throughout the retina, and that red light delivered via the dilated pupil attenuates this loss (del Olmo-Aguado et al., 2016). The present study was designed to investigate whether blue light can potentiate the loss of RGCs during ischemia. Antibodies to Brn3a—a transcription factor specifically present in RGC nuclei—were used to quantify ganglion cells and determine the distribution of RGCs in flat mounts of the retina (Iwamoto et al., 2014; Lee et al., 2014; Nadal-Nicolás et al., 2009). We confirm other studies reporting RGC density to vary across the retina, becoming lowest in peripheral regions (Danias et al., 2002; del Olmo-Aguado

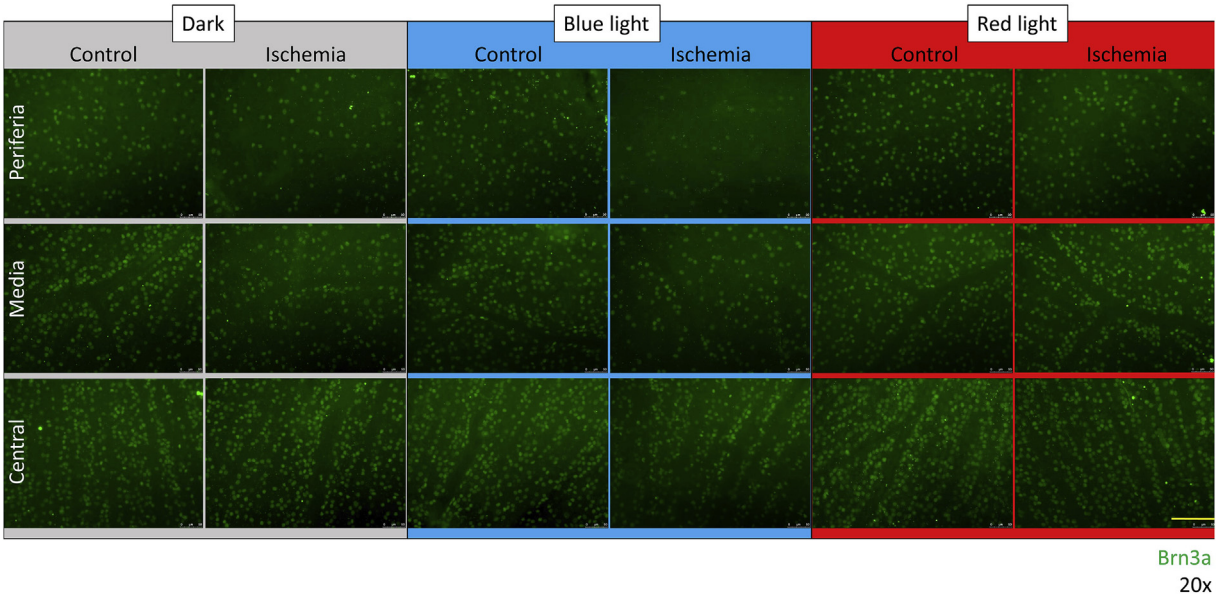

Fig. 7. Representative retinal fields of flat mount retinas stained for Brn3a in central, medial and peripheral parts of the retina. The density of RGCs is least in the peripheral and greatest in the central regions of the retina. Ischemia (in darkness) caused the numbers of Brn3a cells in all areas of the retina to be decreased, reduced even more in blue light (26.3 W/m^2) but blunted in red light (16.5 W/m^2). Scale bar = 100 μm.

et al., 2016; Lee et al., 2014) (Figs. 7 and 8). When analysing RGC numbers, it is essential to either determine the total number of Brn3a-positive cells per retina or, for more practical reasons, restrict the analysis to a defined retinal region. In the present study, we restricted our analysis of Brn3a-positive cells to a 6 mm^2 region spanning from the temporal to the nasal region across the optic nerve. Ischemia/reperfusion caused a statistically significant decrease in RGC numbers in all regions that were analysed (Figs. 7 and 8). The application of blue light exacerbated this loss while red light attenuated the loss, which was confirmed by quantification of Brn3a in whole retinal extracts (Fig. 9). Our results therefore support our hypothesis that blue light may influence the progression rate of glaucoma negatively (Osborne et al., 2014, 2006) and that supplying the retina with defined energy levels of red light may reduce this effect (del Olmo-Aguado et al., 2016; Osborne et al., 2017).

Current evidence indicates that raising the IOP above the systolic blood pressure (as used in our studies) causes an almost complete loss of blood supply to the retina. Depending on the time of reperfusion, the whole of the retina may be affected, with the outer retina being most susceptible (Chidlow et al., 2011; Hernandez et al., 2009; Osborne et al., 2004a, 2004b; Osborne and Larsen, 1996). We demonstrate here that retinal ischemia in the dark for 60 min (5 min reperfusion) results in retinal ATP levels decreasing to about 25% of the normal amount, and decreasing further in blue light conditions. However, this decrease is significantly smaller when ischemia is given in red light (Fig. 6). These changes in ATP levels are in line with studies which show that blue and red light affect mitochondrial function in opposite ways, the former reducing and the latter promoting ATP formation (Gkotsi et al., 2014). The data shown in Fig. 6 also suggest that red light might elevate tissue ATP levels, which is consistent with the preconditioning nature of red light as reported (Albarracin et al., 2013; Begum et al., 2013; Beirne et al., 2017; del Olmo-Aguado et al., 2016; Eells et al., 2003; Gkotsi

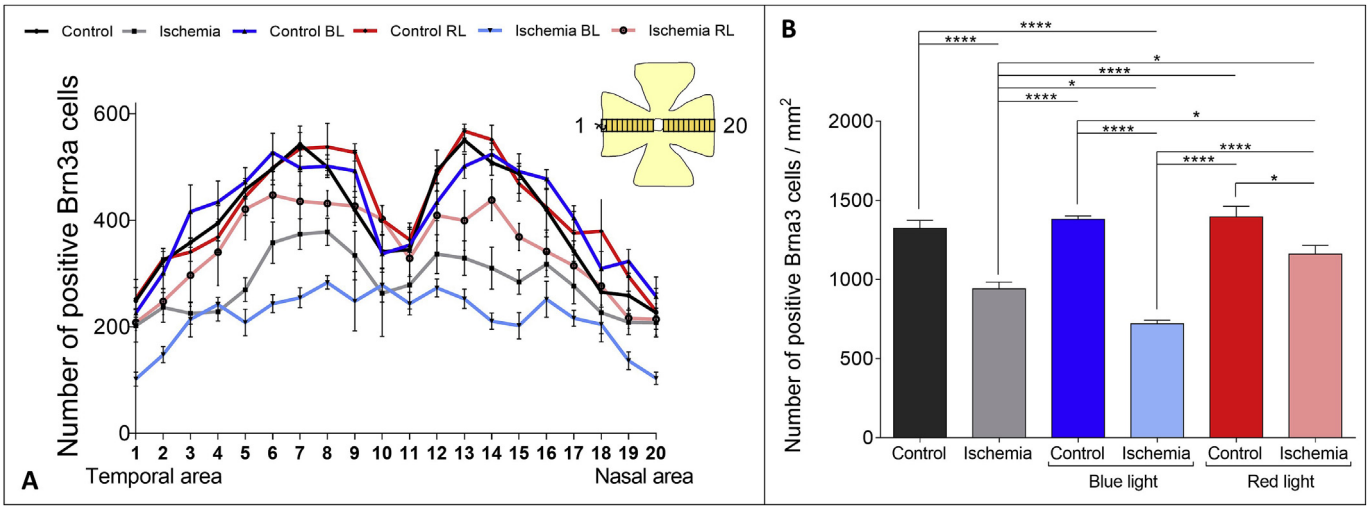

Fig. 8. Quantitative analysis of the number of Brn3a cells in (A) 20 retinal areas of 0.3 mm^2, 6 mm along the temporal/nasal direction and through the optic nerve region, and (B) combined 0.3 mm^2 sectors from the temporal to nasal parts of the retina. The decrease in numbers of Brn3a cells following ischemia is clearly blunted by red light (RL), but significantly elevated by blue light (BL). Data are mean ± SEM, n = 5, ****p < 0.0001. Statistics was determined by a one-way ANOVA with Tukey's multiple comparisons test.

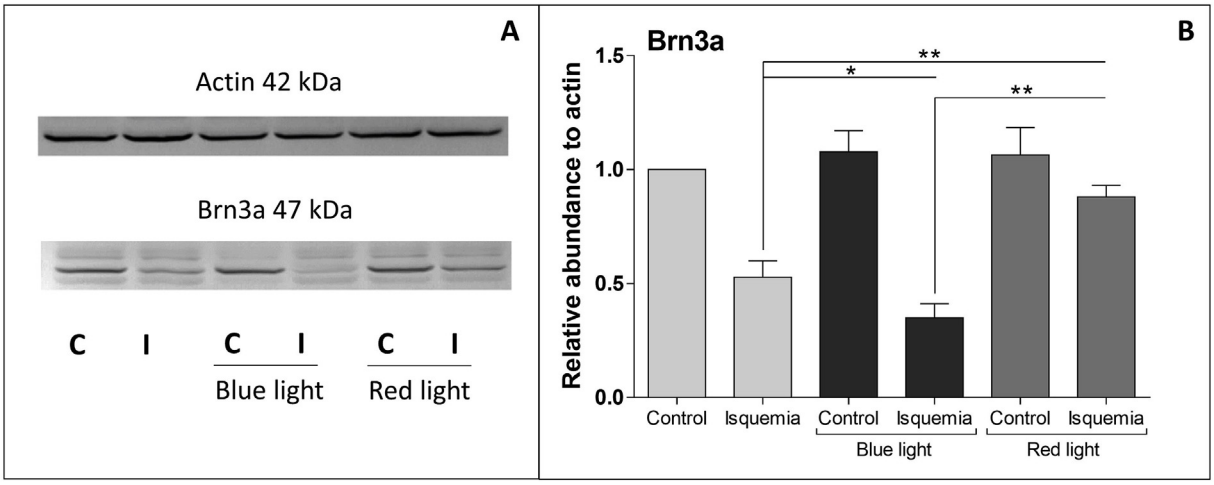

Fig. 9. Comparative ratios of Brn3a and actin proteins in retinal extracts obtained from control rat eyes (C) or where ischemia (I) was administered either in no light, blue light or red light. Western blots of extracts are shown in (A) and (B) the relative intensities of Brn3a related to actin. Data are mean ± SEM, n = 4. Significant differences were determined using a Kruskal-Wallis combined with the Dunn's multiple comparison test, $\alpha = 0.1$.

et al., 2014; Karu, 1999; Osborne et al., 2016; Wong-Riley et al., 2005) and consistent with the finding that red light counteracted an insult of blue light to R28 cells whether given either before or after the insult (Fig. 2B). The finding that blue light reduced normal levels of ATP (Fig. 6) highlights the experimental conditions in which acute high levels of blue light can elicit a negative response after only 1hr of exposure. These studies provide proof of the principle that chronic low amounts of blue light reaching healthy retinal mitochondria will probably not be harmful, but when the organelles are affected negatively, as in glaucoma, it becomes a potential risk factor and exacerbates mitochondrial dysfunctions.

Opposing effects of red and blue light on retinal glia (Müller cells, astrocytes and microglia) were detected in regard to the localisation of GFAP (Fig. 10). Ischemia and to a lesser extent blue light caused a significant up-regulation of retinal GFAP. However, in combination activated GFAP appeared patchy in appearance. We suggest that this is due to loss of tissue cells caused by the combined insults of ischemia and blue light. The decrease in retinal ATP levels to barely detectable amounts following exposure to both insults is consistent with this notion (Fig. 6). While red light counteracted the up-regulation of GFAP caused by ischemia, it also appeared to slightly stimulate the activation of GFAP associated with vascular located microglia/astrocytes. Astrocytes/microglia are known to have a complex association with blood vessels, both morphologically and functionally (Checchin et al., 2006; Gariano et al., 1996; O'Sullivan et al., 2017; Zhao et al., 2018). This observation is of interest as it has been reported that flickering red light stimulates ocular blood flow (Garhöfer et al., 2004; Noonan et al., 2015). Red light stimulates nitric oxide production (Begum et al., 2013; Beirne et al., 2017; Eells et al., 2003; Karu, 1999; Osborne et al., 2016; Wong-Riley et al., 2005) and nitric oxide is known to act as a vasodilator of blood vessels.

In the present study, the intensity of blue light was optimised to have a measurable experimental effect on RGC function when delivered for only 60 min. We do not expect that human retinas would be exposed to such acute amounts of blue light. It is more likely that only a fraction of the blue light used in the present study normally reaches the human retina. However, this blue light would reach RGC mitochondria in a chronic way over long periods of time and so contribute to mitochondrial dysfunction when the organelles are already compromised, as in glaucoma subjects. Importantly, while the intensity of the red light used in the rat studies had no negative influence on cells in culture, it clearly mitigated the effects of blue light. This indicates that use of appropriate amounts of red light may provide a potential non-invasive procedure to treat RGC mitochondrial dysfunctions. The alternative approach of

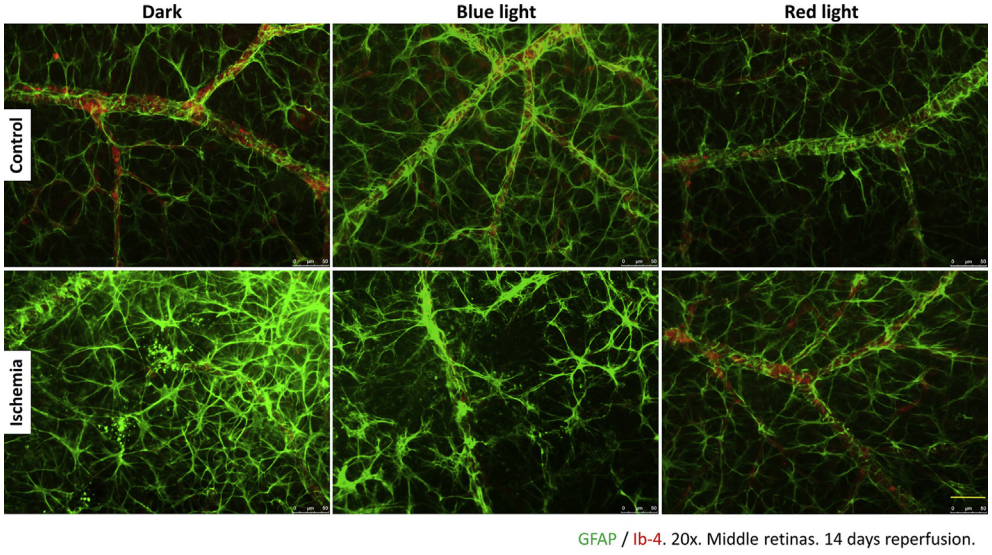

Fig. 10. Flat mount views of retinas processed for the co-localisation of green GFAP immunoreactivity located to Müller cells and astrocytes, the latter being highly associated with blood vessels that appear red using a conjugated antibody for isolectin-IB-4. Compared with control retinas (A), both ischemia (B) and blue light (C) causes a general increase in GFAP immunoreactivity throughout the tissue. Blue light and ischemia (D) enhances GFAP staining even further but it is patchy in appearance suggesting a loss of tissue. Red light also causes an increase in GFAP immunoreactivity that appears more to be associated with blood vessels (D) and this is enhanced when even further when combination with ischemia (E). Scale bar = 100 μm.

using pharmacological agents has been considered over many years, but difficulties associated with delivery and toxicity remain (Hanumunthadu et al., 2014; Osborne et al., 2014; Sena and Lindsley, 2017). Devising procedures for delivery of extra red light to protect RGCs in chronic diseases like glaucoma (Weinreb and Khaw, 2004; Kern and Barber, 2008) remains to be established. One potential idea is to produce fluorescent spectacle filters that convert solar UV light directly into extra visual red light (Menéndez-Velázquez et al., 2018).

A number of preclinical research trials have reported the beneficial influence of red-light therapy in ocular diseases such as age-related macular degeneration and diabetic retinopathy (Ivandic and Ivandic, 2008; Merry et al., 2017; Tang et al., 2014), including the TORPA (NCT00940407) and LIGHTSIGHT (NCT02725762) trials. Such trials rely on exposing individuals to specific environments of red light for defined periods. However, the applicability of red light for the treatment of eye/retinal disorders in mainstream medicine remains to be established (Fitzgerald et al., 2013; Huang et al., 2013) and, importantly, the underlying biochemical mechanism needs to be further clarified. The reported technical approaches of different groups vary considerably, and a biphasic dose-response has been reported, suggesting that optimum levels of light may be more effective than high levels (Karu, 2013; Passarella and Karu, 2014). Variations in the nature of the irradiation source (laser or light-emitting diode), mode of delivery (pulsed or continuous) and chosen wavelength as well as the rate, duration and frequency of treatments that were used are not always disclosed (Fitzgerald et al., 2013; Quirk and Whelan, 2011). In addition, little attention has been given to the potential negative effects of red-light therapy, especially when the treatment necessitates the use of very high intensities to penetrate tissues of different thicknesses and types.

Acknowledgements

We are grateful to Enol Artime, Joanna Serrano and Carlota Suárez-Barrio for their technical help and the financial from the Fundación BBVA. NNO and is a Cátedra de Biomedicina (Chair in Biomedicine).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2019.02.018.

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