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Visible light affects mitochondrial function and induces neuronal death in retinal cell cultures

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Abstract

The aim of this study was to provide "proof of principle" for the hypothesis that light would have a detrimental influence on ganglion cells in certain situations, like in glaucoma, by directly impinging on the many mitochondria in their axons within the globe. In this study primary rat retinal cultures and freshly isolated liver mitochondria were exposed to light (400–760 nm; 500–4000 lux) as entering the eye. For culture assessment, 3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 4-[3-(-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetzolio]-1,3-benzene disulfonate (WST-1) reduction assays were used to assess cell and mitochondrial viability, respectively. Furthermore, cultures were stained for reactive oxygen species (ROS), DNA breakdown, numbers of GABA-immunoreactive (IR) cells and caspase-3 content to provide information concerning the effect of light on neuronal survival. Uptake of ^{3}H-GABA by autoradiography was also used, to assess the effects of light on the energy status of neurons. Light, in an intensity-dependent and trolox-inhibitable manner, reduced cell viability, affected mitochondrial function, increased the number of TUNEL-positive cells, decreased the numbers of GABA-IR neurons and enhanced labelling for ROS. These effects were all exacerbated by the absence of serum. There was also an increased caspase-3 protein content and a reduction of ^{3}H-GABA uptake in light- compared with dark-treated cultures. These findings support the hypothesis that light can affect mitochondria which could lead to neuronal apoptosis if the energetic status of these neurons is already compromised.
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Keywords: Visible light; Mitochondria; Retinal neurons; Apoptosis; Glaucoma

1. Introduction

The human retina is protected from short-wavelength light radiation by the cornea and lens, which between them absorb wavelengths below 400 nm (Margrain, Boulton, Marshall, & Sliney, 2004). The retina is therefore exposed mainly to the "visible component" of the electromagnetic spectrum from 400 to 760 nm (Marshall, 1985). Importantly, this includes blue light which is known to be capable of causing damage to the retina (Algvere, Marshall, & Seregard, 2006; Putting, Zweypfenning, Vrensen, Oosterhuis, & van Best, 1992; Seiler et al., 2000; Sparrow et al., 2002; van Norren & Schellekens, 1990). Noell, Walker, Kang, and Berman (1966) were amongst the first to recognise that light, and in particular, blue light, can induce photoreceptor/retinal pigment epithelial (RPE)[1] damage by a photochemical process leading to a generation of reactive oxygen species (ROS). The levels of ROS are maintained at appropriate levels in healthy cells by antioxidants (e.g. vitamins E and C) and a variety of enzymes [e.g. superoxide dismutase (Sandbach et al., 2001), catalase (Moragon,

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[1] *Abbreviations used:* FITC, fluorescein isothiocyanate; IR, immunoreactive; MEM, minimal essential medium; MTT, 3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; ROS, reactive oxygen species; RPE, retinal pigmented epithelium; RRC, rat retinal culture; SDS, sodium dodecyl sulphate; TdT, terminal deoxynucleotidyl transferase; TUNEL, terminal deoxynucleotidyl transferase dUTP-linked nick-end labelling; WST-1, 4-[3-(-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetzolio]-1,3-benzene disulfonate.

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De Lucas Garcia, Encarnacion Lopez Fernandez, Rodriguez-Manzaneque, & Jimenez Fraile, 2005), glutathione peroxidase (Kortuem, Geiger, & Levin, 2000)], but when cells are unable to keep the formation of these species in check, toxicity can result. In photochemical reactions, ROS are formed by the interaction of excessive light with photosensitizers. A number of studies have shown that blue light in particular can interact with the RPE-associated photosensitizers, lipofuscin (Boulton, Docchio, Dayhaw-Barker, Ramponi, & Cubeddu, 1990) and melanin (Margrain et al., 2004), and photoreceptor-associated photosensitizers such as opsins and retinoids (Boulton, Rozanowska, & Rozanowski, 2001), to damage these cells. Recent studies also suggest that excessive light can initiate photochemical reactions in melanopsin-containing retinal ganglion cells, possibly resulting in excessive ROS production and subsequent damage (Semo, Lupi, Peirson, Butler, & Foster, 2003).

The idea that light-induced damage to the retina arises exclusively from an interaction with specific chromophores (e.g. retinoids, opsins, melanin, lipofuscin, and melanopsin) has been questioned (Osborne, Lascaratos, Bron, Chidlow, & Wood, 2006). This is based on recent findings which have proven that mitochondrial respiratory chain enzymes, such as flavins (Egorov, Krasnovsky, Bashtanov, Mironov, & Ludnikova, 1999) and cytochrome oxidases are able to absorb light maximally around 440–450 nm, and as a consequence, can also contribute to the generation of ROS in cells (Chen, Vazquez, Moghaddas, Hoppel, & Lesnefsky, 2003; Godley et al., 2005; Hockberger et al., 1999; Jung, Kim, & Cho, 1990; King, Gottlieb, Brooks, Murphy, & Dunaief, 2004; Putting, Van Best, Vrensen, & Oosterhuis, 1994; van Best, Putting, Oosterhuis, Zweypfenning, & Vrensen, 1997) and in the process induce oxidative stress. Importantly, oxidative stress and mitochondrial function are interrelated and are involved in the promotion and regulation of apoptosis (Fink & Cookson, 2005).

Osborne et al. (2006) have argued that ganglion cells, in contrast to other retinal neurons, might therefore be particularly susceptible to light-induced injury in certain situations. This is because ganglion cells have a particularly rich supply of mitochondria, associated with their axons within the globe (Wang, Dong, Cull, Fortune, & Cioffi, 2003). It might be that in the healthy retina, ROS, generated by light acting on such mitochondria, are removed by the ganglion cells' scavenging mechanisms. However, when the ganglion cells are in a state of energetic compromise, as might occur because of reduced optic nerve head blood flow caused by raised intraocular pressure (Grunwald, Piltz, Hariprasad, & DuPont, 1998; Kerr, Nelson, & O'Brien, 1998; Yamazaki, Inoue, & Yoshikawa, 1996) or because of a genetic defect (Votruba, 2004), the normal adequate scavenging mechanism of ROS becomes overburdened. The proposition has therefore been made (Osborne et al., 2006) that ROS, produced by light acting on mitochondrial photosensitizers, can become a risk factor to retinal ganglion cells, but only when the cells are in a compromised state, as in optic neuropathies like glaucoma (Flammer & Orgul, 1998; Osborne et al., 1999) and Leber's Hereditary Optic Neuropathy (LHON) (Carelli, Ross-Cisneros, & Sadun, 2004; Riordan-Eva et al., 1995). Weak genetic mitochondrial abnormalities have now been shown to be associated with glaucoma patients (Abu-Amero, Morales, & Bosley, 2006) which is supportive of this idea.

This study was undertaken to provide support for our hypothesis (Osborne et al., 2006) that light entering the eye can interact with neuronal mitochondria to induce dysfunction and apoptosis. Studies were conducted on enriched mitochondrial fractions and on primary rat retinal cultures. In these cultures, certain retinal cell types (e.g. GABA-containing neurons) are particularly abundant and can be quantified easily by immunohistochemistry. Other neuron-types like ganglion cells which can be identified by Thy-1 immunostaining are few in number and are in contrast difficult to specifically assess for viability in culture. Thus, no attempt was made to focus on ganglion cells. Instead we focused more on GABA neurones because they could be identified easily by immunohistochemistry and for uptake of ^{3}H-GABA by autoradiography. The study was therefore designed to provide "proof of principle" that white light (400–760 nm) as impinging on the retina *in situ* is detrimental to non-dividing neurons in culture, and in particular when they are in an energetically compromised state. Moreover, the aim was to show that mitochondrial function is affected by light and as a consequence neuronal survival is influenced.

2. Materials and methods

Procedures used in the present study conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Home Office in the United Kingdom. Rat pups were delivered from animals housed in a 12-h light–dark cycle and food and water were provided *ad libitum*.

2.1. Materials

Cell culture media and reagents (including foetal calf serum; FCS) were obtained from Invitrogen (Paisley, UK). Culture vessels and CellPlus charge-coated 96-well plates were from Sarstedt (Leicester, UK). Polyclonal anti-GABA and FITC-labelled anti-rabbit antibodies were obtained from Sigma Chemical Company (Poole, UK). Anti-actin antibody was from Chemicon International (Temecula, CA) and mouse anti-caspase-3 was from Becton–Dickenson (Cowley, UK). The terminal deoxynucleotidyl transferase (TdT) dUTP-linked nick-end labelling (TUNEL) system was obtained from Promega (Southampton, UK) and 2′,7′-dihydroethidium (DHE) and 4-[3-(-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetzolio]-1,3-benzene disulfonate (WST-1) were from Roche Diagnostics (Lewes, UK). The 2C-UV filters used to exclude certain wavelengths of light were from Lee Filters (Andover, UK). ^{3}H-GABA was from Amersham Biosciences (Amersham, UK). Where not specified, all other chemicals, including 3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), were from Sigma Chemical Company (Poole, UK).

2.2. Isolation of mitochondria

Mitochondria were isolated from fresh rat liver precisely as described elsewhere (www.bio.com/protocolstools/protocol). One hundred microliters aliquots of a suspension of mitochondria were placed in individual wells of 96-well plates for further analysis.

2.3. Rat retinal cell cultures

Primary mixed rat retinal cultures (RRCs) containing neurons and glia were generated from retinas of 3- to 5-day-old rat pups using a modified trypsin digest procedure (Beale, Nicholas, Neuhoff, & Osborne, 1982c; Wood et al., 2003). Briefly, retinas dissected freshly from 6 to 10 Wistar rat pup littermates were incubated for 10 min at 37 °C in a shaking waterbath in sterile solution 1 containing 0.1 mg/ml trypsin (Solution 1: 5.4 mM KCl, 116 mM NaCl, 0.096 mM $NaH_2PO_4 \cdot 2H_2O$, 19.5 mM D-glucose, 0.15 mM $MgSO_4$, 23.8 mM $NaHCO_3$, 3 g/l bovine serum albumin, and 10 mg/l phenol red). After leaving for an additional 5 min at room temperature to settle, the trypsin solution was removed and replaced by solution 1 containing DNAse (bovine pancreas, type II, 1000 U/7.5 ml), soybean trypsin inhibitor (type I-S, 5 mg/7.5 ml) and 0.19 mM $MgSO_4$. The cell mass was gently but extensively triturated with a sterile flame-rounded Pasteur pipette and the mixture centrifuged (180*g*/5 min/4 °C). The resulting pellet was resuspended in minimal essential medium (MEM) containing 10% foetal bovine serum, 91 mg/l gentamicin sulphate, 2.3 mg/l amphotericin B and glucose (final concentration, 25 mM). Suspended cells (1×10^6 cells/ml) were placed either in 24-well plates on borosilicate glass coverslips at 0.5 ml/well or in 96-well plates at 0.1 ml/well. The coverslips had previously been coated by incubating with 10 μg/ml poly-L-lysine in sterile phosphate buffered saline (PBS; 137 mM NaCl, 5.4 mM KCl, 1.28 mM NaH_2PO_4, and 7 mM Na_2HPO_4; pH 7.4) for 15 min. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 and the medium was changed every 3 days. Cultures were generally used after 5 days at which time all major retinal cell types were present (Beale, Hall, & Osborne, 1982a, 1982b; Wood, Chidlow, Graham, & Osborne, 2005).

For serum-deprivation experiments, cells were washed three times with sterilised phosphate buffered saline (PBS) and then serum-free MEM (with all other additives present as outlined previously) was added for the appropriate amount of time (48 h).

2.4. Light treatment regime

A normal culture incubator was equipped with two 8 W strip-lights (spectral irradiance patterns shown in Fig. 1) that could be adjusted to be 30–60 cm directly above a tray containing 24- or 96-well plates with established retinal cells or mitochondria in suspension. The strip-light bulbs were completely covered with 2C-UV filters which excluded light wavelengths below 400 nm (Fig. 1) to mimic the situation that occurs for retinal cells *in situ*. The intensity of light directed onto the cells was determined by use of a digital lux meter (LX-101, Luton, UK).

Well-plates exposed to light or dark were present in the same incubator for 1–2 day periods. White paper covers were placed over well plates to provide dark conditions. In this system any slight increase in the temperature in the incubator caused by the constant light source affected equally cells or mitochondria in the dark or light conditions. We specifically used white paper covers rather than foil so as not to provide insulation for the dark maintained cultures. We also measured the temperature of culture medium and found no differences after being maintained in the light or dark conditions for 2 days.

2.5. Immunohistochemistry

Retinal cultures on coverslips were fixed with 4% paraformaldehyde for 20 min and washed in PBS containing 0.1% Triton X-100 (PBS-T). The cells were then exposed to anti-GABA antibody (1:500) for 4 h at room temperature before washing in PBS-T and then labelling with secondary anti-rabbit IgG-FITC conjugate (1:100). Microscopy was used to count the number of GABA-containing neurons in five randomly chosen visual fields (0.4 mm^2) on each coverslip.

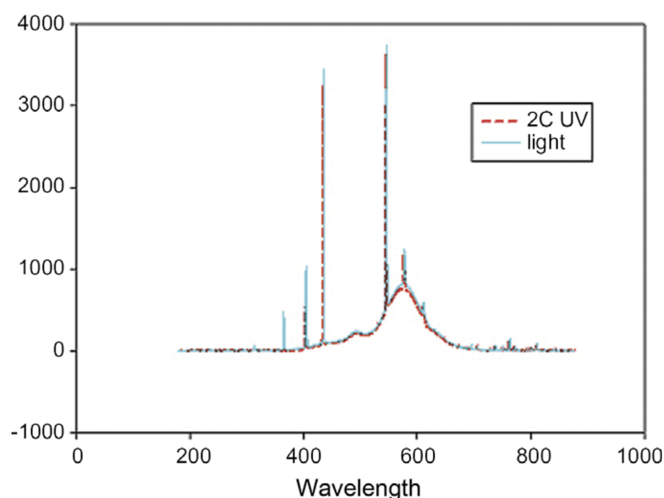

Fig. 1. Emission spectrum of the light source used in the present studies with (red) and without (blue) the covering 2C-UV filter. The *y*-axis is in arbitrary units of wavelength intensity and the *x*-axis is the wavelength in nm.

2.6. MTT assay

The assay used to assess cell viability was the MTT reduction assay modified from that of Mosmann (1983). Briefly, cells or mitochondria (in 96-well plates) were subjected to the appropriate treatments and then MTT was added to wells at a final concentration of 0.5 mg/ml for 1 h at 37 °C. After this time, the medium was removed and reduced MTT (blue formazan product) was solubilized by adding 100 μl of dimethyl sulfoxide (DMSO) to each well. After agitation of the plates for 15 min, the optical density of the solubilized formazan product in each well was measured using an automated microplate reader (Titertek Plus MS212; ICN Flow, Thame, UK) with a 570 nm test wavelength and a 690 nm reference wavelength.

2.7. WST-1 assay

Cells or mitochondria (in 96-well plates) were analysed for mitochondrial dehydrogenase activity using the WST-1 assay. WST-1 is a tetrazolium dye containing an electron-coupling agent that is cleaved by mitochondrial dehydrogenases to a formazan dye with an absorbance at 490nm (Toimela & Tahti, 2004). Mitochondrial dehydrogenase activity is directly related to mitochondrial energy production: a fall in activity suggests a reduced production.

2.8. Assessment of DNA breakdown with the TUNEL procedure

For the TUNEL procedure, treated cells on coverslips were fixed for 30 min with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) and then washed in PBS containing 0.1% Triton X-100 (PBS-T). The labelling procedure was carried out exactly as described previously (Wood & Osborne, 1997). Fixed cells were washed initially in Tris buffer (10 mM Tris–HCl, pH 8.0) for 5 min and then exposed to 1% (v/v) H_2O_2 for 5 min to remove endogenous peroxidase activity. Following a further 5 min wash in Tris buffer, cells were pre-incubated in TdT buffer (30 mM Tris–HCl, pH 7.2, containing 140 mM sodium cacodylate, and 1 mM cobalt chloride) for 10 min at 37 °C. Subsequent to this the transferase reaction was performed by incubating for 60 min at 37 °C with TdT buffer containing 0.25 U/μl of TdT and 40 μM biotin-16-dUTP. As a posi-

tive control for the labelling methodology some cells were treated with DNase 1 (0.1 mg/ml) for 10 min prior to the transferase reaction. The reaction was stopped by incubation in sodium citrate buffer (300 mM NaCl, 30 mM sodium citrate) for 2×15 min before blocking with 2% (w/v) bovine serum albumin (BSA) in PBS and washing in PBS. The coverslips were then incubated with avidin–biotin–peroxidase complex solution in PBS for 60 min at 37 °C. Apoptotic nuclei that had been labelled were visualized with 3′,3′diaminobenzidine (0.5 mg/ml) in 0.1 M sodium phosphate buffer (pH 7.4) containing 0.1% (v/v) H_2O_2. The labelled cells on coverslips were then washed in PBS, mounted on glass slides and visualised with a Zeiss light microscope. For analysis of culture density, coverslips were dipped in solution of toluidine blue (0.5% toluidine blue, 0.5% thionine, 1% sodium tetraborate) for 30 s and washed with PBS to remove excess stain. Microscopy was used to count the number of TUNEL-positive cells in five randomly chosen visual fields (0.4 mm^2) on each coverslip.

2.9. SDS–PAGE and Western blotting

Cells were grown to confluence in six-well plates, washed and harvested in PBS (35.5 °C) and then collected by scraping. After centrifugation (100*g*/5 min/4 °C) and removal of the supernatant, cells were resuspended and then lysed in homogenization buffer [20 mM Tris–HCl, pH 7.4, containing 2 mM EDTA, 0.5 mM EGTA, 0.1 mM phenylmethylsulphonyl fluoride (PMSF) and 140 mM β-mercaptoethanol] using an ultrasonicator (Burkard Scientific Ltd, Uxbridge, UK). An equal volume of sample buffer was added [62.5 mM Tris–HCl, pH 7.4, containing 4% sodium dodecyl sulphate (SDS), 10% glycerol, 10% β-mercaptoethanol and 0.002% bromophenol blue] and the sample was boiled for 3 min prior to use. Protein determination was by the method of Bradford (1976). Electrophoresis was performed by the method of Laemmli (1970) using an LKB midget vertical slab-gel system; 1.5 mm thick 10% polyacrylamide gels containing 0.1% SDS in 360 mM Tris–HCl were used for the electrophoretic separation of the samples.

Transfer of proteins from gel to nitro-cellulose was basically performed by the method of Towbin, Staehelin, and Gordon (1979), exactly as described previously from our laboratory (Osborne, Wood, & Groome, 1994). The blots were immunoprobed with the anti-caspase-3 (1:400) and anti-actin (1:2000) primary antibodies for 3 h at room temperature, and, respectively, secondary anti-goat and anti-mouse antibodies (1:100) conjugated to horseradish peroxidase were subsequently employed. Nitro-cellulose blots were developed with 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. Colour reaction on the blots was stopped with 0.005% sodium azide solution and photography was undertaken using Epson Perfection 3490 Photo.

2.10. Assessment of reactive oxygen species production

Cells were assessed for the production of reactive oxygen species using the dye 2′,7′-dihydroethidium (DHE) according to Carter, Narayanan, and Robinson (1994). DHE is a reduced form of ethidium bromide, which is non-fluorescent and can passively cross the membrane of live cells. In the cell DHE can be oxidized by superoxide anions or hydrogen peroxide to ethidium bromide, which binds to DNA and, when excited, emits red fluorescence that is proportional to the intracellular superoxide anion/H_2O_2 level. After different treatments, cells on coverslips were stained with DHE (10 μg/ml) by incubating for 30 min at 37 °C in culture medium in a humidified chamber and then fixed with 4% paraformaldehyde for 20 min. After washing in PBS-T, coverslips were mounted in PBS containing 1% glycerol and red fluorescence was detected using a Zeiss epifluorescence microscope.

2.11. ^{3}H-GABA uptake assay

Cultures on coverslips (given a treatment of light or dark) were incubated with 10^{-7} M GABA, 10^{-4} M aminooxyacetic acid and ^{3}H-GABA (2μl ^{3}H-GABA; specific activity 88 Ci/mmol/ml incubation solution) in MEM containing 10% foetal bovine serum, 91 mg/l gentamicin sulphate, 2.3 mg/l amphotericin B and glucose (final concentration of 25 mM) for 30 min. Cells were then washed thoroughly in PBS, fixed with 4% paraformaldehyde for 10 min, washed again in PBS, fixed with 1% glutaraldeyde for another 10 min and finally washed sequentially with PBS, distilled water and ethanol. The coverslips were then mounted on glass slides and dipped into emulsion. After approximately 10 days the slides were developed and visualized for the uptake of ^{3}H-GABA by light microscopy.

2.12. Statistics

All data are expressed as mean percentage of control value plus standard error of the mean (SEM) for the indicated number of experiments. Statistical significance was determined by Bonferroni and Games-Howell post-hoc tests after ANOVA using SPSS for Windows, version 12.0 (SPSS Inc., Chicago IL.). For the western blotting data the densitometric reading of the protein of interest was expressed as a percentage of the control protein (actin). In all cases, $p < 0.05$ was considered statistically significant.

3. Results

Fig. 1 shows the spectrum of the light source with and without the covering with 2C-UV filters. The light source emits a number of peaks of wavelengths of light with one being below 400 nm. The 2C-UV filter excludes this wavelength of light but allowing all other peaks of wavelength generated by the light source to be transmitted.

The effects of increasing light intensities (250, 500, 1000, and 4000 lux) on the viability of 5-day-old cultured rat retinal cells, as assessed by the MTT assay, are summarized in Fig. 2A. It can be seen that 2 days of light (250 lux) had no influence on cell survival. In contrast, light of 500 lux for 2 days caused an approximate reduction of 40% in cell viability. Increasing the intensity of light to 1000 and 4000 lux for the same period caused a greater negative effect on cell viability, in an intensity-dependent manner (Fig. 2A). The negative influence of 1000 lux on 5-day-old retinal cultures as revealed by the MTT assay was significantly attenuated by trolox, a well known derivative of vitamin E (Fig. 2A).

Support for the MTT assay data which showed that 1000 lux of light significantly affected retinal cell function was confirmed by measuring mitochondrial dehydrogenase activity in retinal cultures. As shown in Fig. 2B, mitochondrial dehydrogenase activity was significantly reduced in cultures exposed to light (1000 lux) for 2 days.

Fig. 3 shows the effect on GABA-IR labelling of cultured retinal cells after their incubation either in the light (1000 lux; Fig. 3B and D) or dark (Fig. 3A and C), in normal medium (Fig. 3A and B) or medium lacking serum (Fig. 3C and D), for 2 days. There was an evident decrease in the number of neurons labelling for GABA in cultures after either deprivation of serum in the dark (Fig. 3C) or after incubation in the light (Fig. 3B) when compared with control cells (Fig. 3A). Importantly, the detrimental effect of light on the reduction in the number of GABA-IR neurons was particularly enhanced in the serum-free medium (Fig. 3D). Fig. 3E demonstrates quantification of this effect: reductions in the number of GABA-IR neurons caused by depriving cells of serum ($P < 0.05$), by incubat-

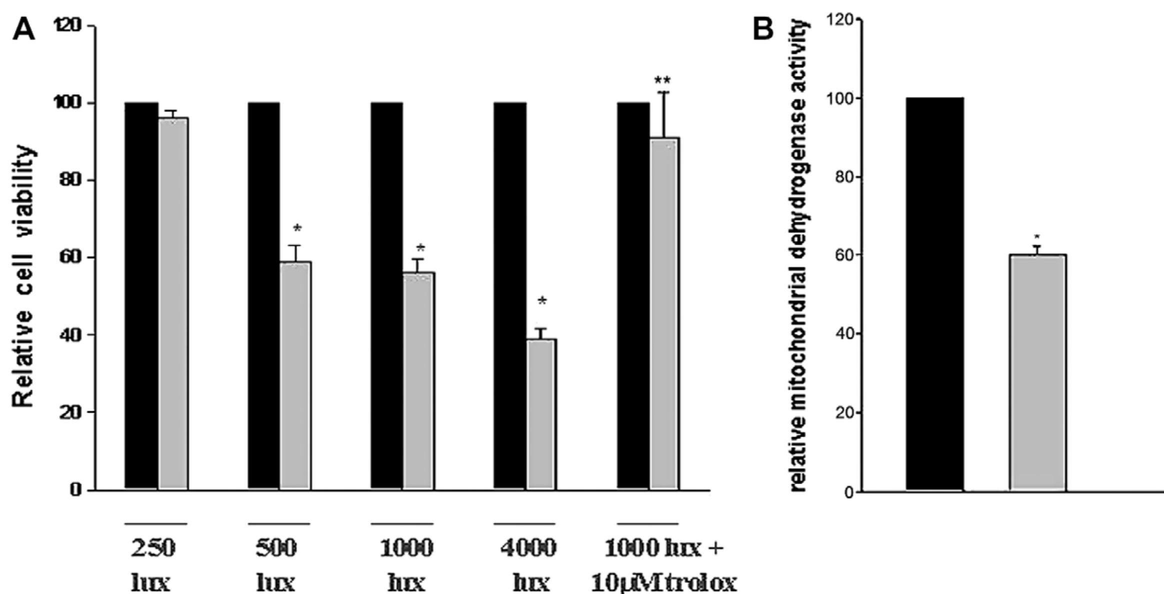

Fig. 2. (A) The effect of increasing light intensities (250, 500, 1000, and 4000 lux) and the influence of trolox (10 μM) on rat retinal cell viability as assessed by the MTT assay. In these studies, 5-day-old rat retinal cultures were placed for 2 days in an incubator with different light intensities (grey bars); some cultures were covered with white paper to avoid the light (black columns). It can be seen that compared with dark conditions, light caused a decrease in the amount of MTT reduction in an intensity-dependent manner and this was significant with light intensities greater than 500 lux (* $P < 0.01$). Moreover, in the presence of 10 μM trolox the reduction of cell viability caused by 1000 lux over 2 days was attenuated significantly (** $P < 0.01$). (B) The effect of 2 days of 1000 lux (grey columns) as compared with dark maintained cultures (black columns) on cell mitochondrial dehydrogenase activity determined by the dye WST-1. Light caused a significant reduction (* $P < 0.01$) in the capacity of WST-1 to be cleaved in cultures when compared with the dark conditions. Values are expressed as percentage of control (dark conditions) and are the means ± SEM of six individual cultures, each analyzed at least in triplicate.

ing in the light ($P < 0.05$) or by combining the two insults were quantifiable and, in all cases, statistically significant ($P < 0.001$).

Evidence that light or serum-deprivation induced cellular DNA breakdown characteristic of apoptosis in retinal cultures is shown in Fig. 4. After 2 days incubation of 5-day-old cultures in light (1000 lux; Fig. 4B), or in the absence of serum (Fig. 4C), a number of cells exhibited varying degrees of TUNEL labelling associated with their nuclei, as compared with cells incubated in the dark with serum present in the medium (Fig. 4A), where nuclear labelling was lacking. This contrasted with cultures exposed to light which were concurrently deprived of serum (Fig. 4D) where the TUNEL positive-nuclei were generally numerous and very darkly labelled. Quantification of the number of clearly defined dark TUNEL-positive cells was conducted in three separate experiments (Fig. 4E). The results showed that [a] placing cells in serum free medium causes a significant increase in TUNEL-positive cells (from approximately 2.9 ± 1.8 to 31.8 ± 5.8 cells per field), [b] light significantly increased the number of TUNEL-positive cells in normal medium (to 24.5 ± 4.1 cells per field) and [c] light had a drastic effect in increasing the number of intense TUNEL-positive cells in serum free medium (to 61.3 ± 1.5 cells per field).

Using the dye DHE, ROS stimulation is indicated by an intense yellow fluorescence where the background is of a red colour (Fig. 5). The yellow fluorescence in cultures in normal medium was barely visible when cells had been kept in the dark (Fig. 5A) but was clearly enhanced when they had been exposed to light for 2 days (1000 lux; Fig. 5B). However, in serum free medium the fluorescence was significantly increased and this was most evident in the light conditions (Fig. 5D) when compared with dark (Fig. 5C). The effect was not quantified.

Further evidence for light having a detrimental effect on neuronal survival was provided by analysing ^{3}H-GABA uptake (Fig. 6; experiment repeated on four separate cultures). Certain neurons readily took up ^{3}H-GABA which was easily demonstrable (Fig. 6A). It was also evident from the autoradiograms that the uptake of ^{3}H-GABA, indicated by the intensity of photographic grains, into GABAergic neurons was significantly greater for cultures kept in the dark than for cultures exposed to light for 2 days (1000 lux; Fig. 6B). In the dark maintained cultures the processes of GABA cells were much more evident in length because of the intensity of the radioactive grains than in cultures exposed to light. No attempt was made to count the number of ^{3}H-GABA neurones or measure the length of their radioactive grain processes in these cultures.

In order to determine whether light could directly affect mitochondrial activity, studies were carried out on isolated liver mitochondria. It can be seen in Fig. 7A and B that mitochondrial dehydrogenase activity and the ability of mitochondria to reduce MTT, respectively, were significantly affected by light. Fig. 7B also shows that when mitochondria were disrupted by sonication or were metabolically insulted with malonic acid (Koeppen &

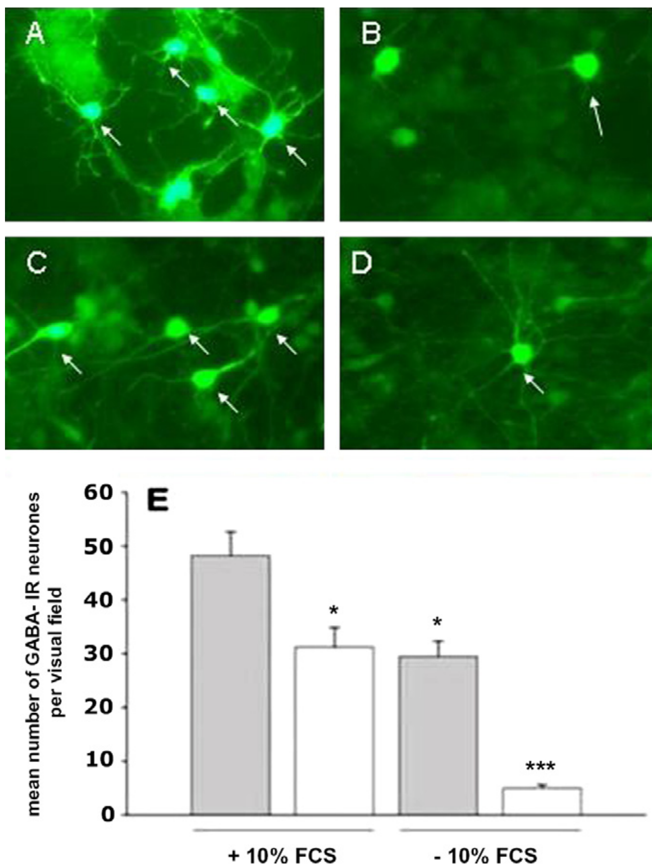

Fig. 3. Representative fields of GABA-IR neuron labelling in 5-day-old mixed rat retinal cultures exposed to light (1000 lux) for 2 days (B and D) or kept in the same incubator in the dark (A and C). Some cultures were also concurrently deprived of serum (C and D). The number of GABA-IR neurones in dark-treated cultures (arrows) was decreased in serum free medium (C) compared with control medium (A). In addition, light reduced the number of GABA-IR neurones compared with dark conditions both in normal serum (B) and serum free (D) cultures. Light also affected glial cell survival (B and D), seen by gaps in the cultures. Scale bar, 20 μm. Quantification of the effect of light/serum-deprivation on numbers of GABA-IR neurons in the retinal cultures (E) revealed that light (white bars) significantly reduced the number of GABA neurones, relative to the dark (grey bars), both in normal culture medium (*$P < 0.05$) and in the absence of serum (***$P < 0.001$). Moreover, the numbers of GABA neurons were reduced in the dark when serum was absent (*$P < 0.05$). The numbers of GABA-IR neurons were averaged from five randomly chosen visual fields. Results are expressed as numbers of GABA-IR neurons per visual field; data were taken from three separate cultures ± SEMs.

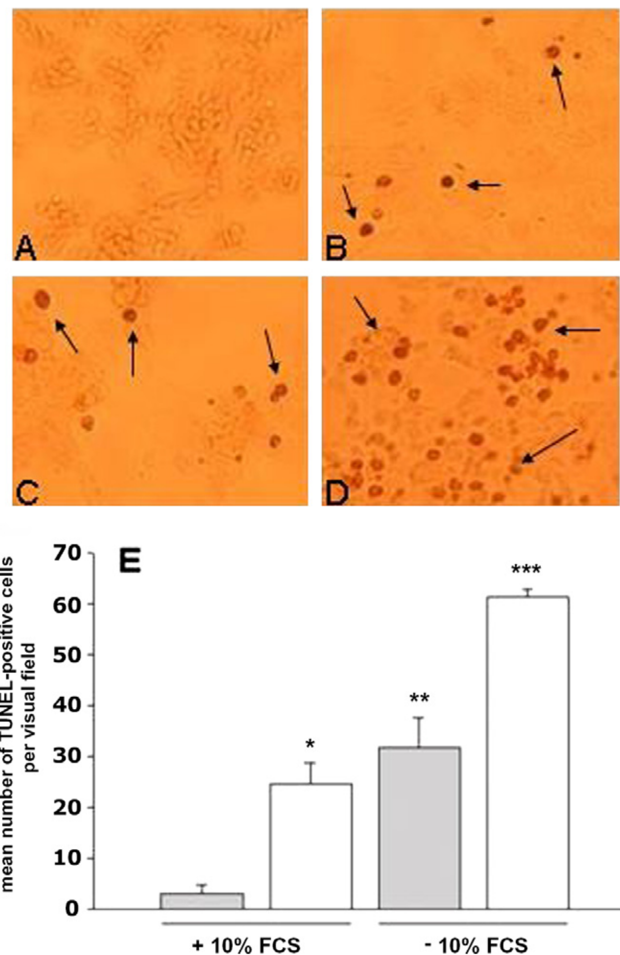

Fig. 4. Occurrence of DNA breakdown, as indicated by labelling for TUNEL, in retinal cultures subjected to light and/or serum-deprivation. Cultures were exposed to 1000 lux of light for 2 days (B and D) or kept in the same incubator in the dark (A and C). Some cultures were also concurrently deprived of serum (C and D). Cultures exposed to light (B and D) or in the dark in serum-free medium (C) demonstrated positive-labelling for TUNEL with varying degrees of intensity (see arrows) when compared with cultures in the dark (A). Particularly dark labelled nuclei were most evident in serum-free cultures in the light (D). Scale bars = 50 μm. Quantification of this effect (E) shows that light (white columns) caused a significant (*$P < 0.05$) enhancement in the numbers of clearly defined TUNEL-positive cells when compared with similar cultures in the dark (grey columns). Moreover, in the absence of serum (−10% FCS) the numbers of TUNEL-positive cells were significantly increased (**$P < 0.001$) relative to cultures in normal conditions (+10% FCS) in the dark. The effects of both treatments were amplified when they were concurrent (***$P < 0.001$). Results are means ± SEMs, for three separate cultures.

Riley, 1987) the effect of light was no longer apparent. This provides support for the view that light affects only intact mitochondria.

Western blotting also provided evidence that light enhanced cell death in retinal cultures. Fig. 8 shows results from three separate cultures where each was exposed to light (1000 lux) for 2 days. It can be seen that, relative to actin levels, light causes a significant enhancement in active, cleaved (17 kDa) caspase-3 protein levels. It is known that pro-caspase-3 protein is cleaved and the active form is elevated in neurons that have been triggered to die by apoptosis (Wang, 2001).

4. Discussion

The aim of this investigation was to provide support for the idea that when retinal neurons exist in a compromised energetic state, as is thought to occur for ganglion cells in glaucoma, then light as entering the eye can increase the likelihood of their demise (Osborne et al., 2006). In the present study we used primary rat retinal cultures where the neurons, as opposed to glia, are in an energetically

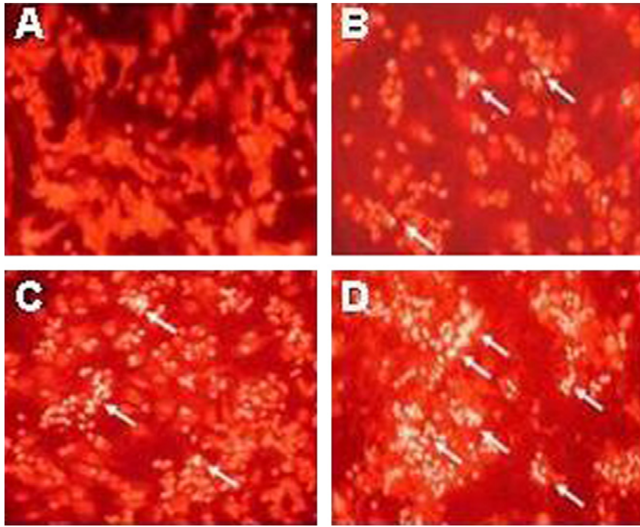

Fig. 5. Labelling for ROS in cultures subjected to the same conditions as in Figs. 3 and 4, using the dye, DHE. In normal culture conditions, in the dark, DHE produced a red fluorescent staining associated with all cells (A). In all other conditions a more intense yellow fluorescent labelling was also evident, indicating an enhancement of ROS production. Positive labelling was particularly enhanced by serum-free conditions, either in the light (D) or the dark (C). Light also caused an increase in ROS labelling in the presence of serum (B). These findings were repeated in three separate experiments. No attempt was made to quantify the labelled ROS. Scale bar, 50 μm.

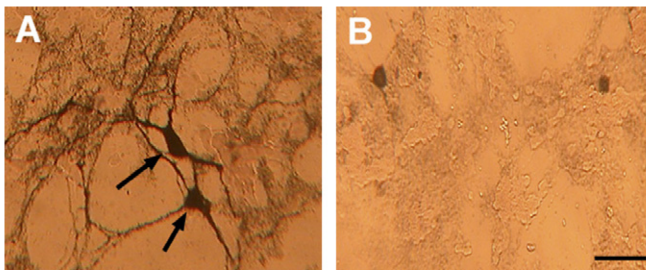

Fig. 6. Autoradiographic evidence for the uptake of ^{3}H-GABA into neurons in a retinal culture subjected in medium plus serum to either 7 days of dark (A) or to 5 days of dark plus 2 days of light (1000 lux; B). At the end of each of these incubations, an additional day was required, in the dark, for exposure of cultures to ^{3}H-GABA and their subjection to autoradiography as described. The results show very clearly that ^{3}H-GABA uptake by neurones (arrows) is less in the cultures exposed to light for 2 days when compared to the cultures maintained constantly in the dark. Scale bars, 50 μm.

compromised state reflected by the fact that they survive for no longer than 20 days (Wood et al., 2005). We found that light (400–760 nm) could induce neuronal death in these cultures and that this process is exacerbated when the energetic state is further compromised by serum deprivation. Moreover, the vitamin E derivative, trolox a known antioxidant significantly blunted the negative influence of light. These data provide strong support for the idea that light can act as a risk factor to already affected neurons, *in situ* (see Osborne et al., 2006).

In the present study, a number of procedures were employed in order to examine the influence of light on retinal neurons in culture. Cell viability was initially assessed by monitoring the capability of the cells to reduce MTT to a formazan product (Mosmann, 1983). MTT reduction is induced by succinate dehydrogenase, a component of complex II in oxidative phosphorylation, and is therefore an indicator of the mitochondrial redox state (Berridge & Tan, 1993; Lippold, 1982). Only living cells in a given cell population reduce MTT. The data shown in Fig. 2A therefore suggest that light significantly affects the redox state of cultured retinal cells and, by implication, their mitochondrial activity.

Support for this is shown in Fig. 2B where it can be seen that light actually reduced the mitochondrial dehydrogenase activity of cultured retinal cells. Moreover, studies on isolated mitochondria demonstrated that light significantly altered both the redox state of these organelles (MTT assay) and their mitochondrial dehydrogenase activity. When mitochondria were disrupted by sonication or their metabolism affected by malonic acid (a mitochondria poison) (Koeppen & Riley, 1987) then light no longer had an effect as determined by the MTT assay in comparison to the dark (Fig. 7B), demonstrating that light only influences intact and metabolically functional mitochondria. Thus, the MTT (redox state) and WST-1 (mitochondrial dehydrogenase activity) experiments on cell cultures and isolated mitochondria provide strong support for the idea that light is detrimental to retinal cells by affecting mitochondrial function.

By counting the number of GABA-IR neurons or TUNEL-positive nuclei and viewing the intensity of staining for ROS, it was concluded that light exposure had a significant negative effect on all of these parameters. Light reduced the number of GABA-positive neurons, increased the number of TUNEL-positive cells and intensified the labelling of ROS (compared with dark conditions). The combined studies thus support the notion that light causes neuronal death by a mechanism that involves the production of ROS. This is in agreement with the observation that trolox, a well known antioxidant, counteracts the influence of light (Fig. 2A). Also, and importantly, the negative effect of light is further enhanced when the neurons are placed in serum-free medium which will necessarily reduce their energetic state even further. These observations provide support for the view that neurons in a stressed state, *in situ*, will be particularly susceptible to the effects of light (Osborne et al., 2006).

In apoptosis, DNA is broken down in a defined manner to allow for appropriate detection by the TUNEL procedure (Gavrieli, Sherman, & Ben Sasson, 1992; Wood & Osborne, 1997). In addition, various cellular proteins, such as caspase-3, are specifically cleaved to an active form in the process of apoptosis (Kroemer, Petit, Zamzami, Vayssiere, & Mignotte, 1995; McConkey, Orrenius, & Jondal, 1990; Wyllie, Kerr, & Currie, 1980). The finding that light caused an elevation of the cleaved, active form of caspase-3

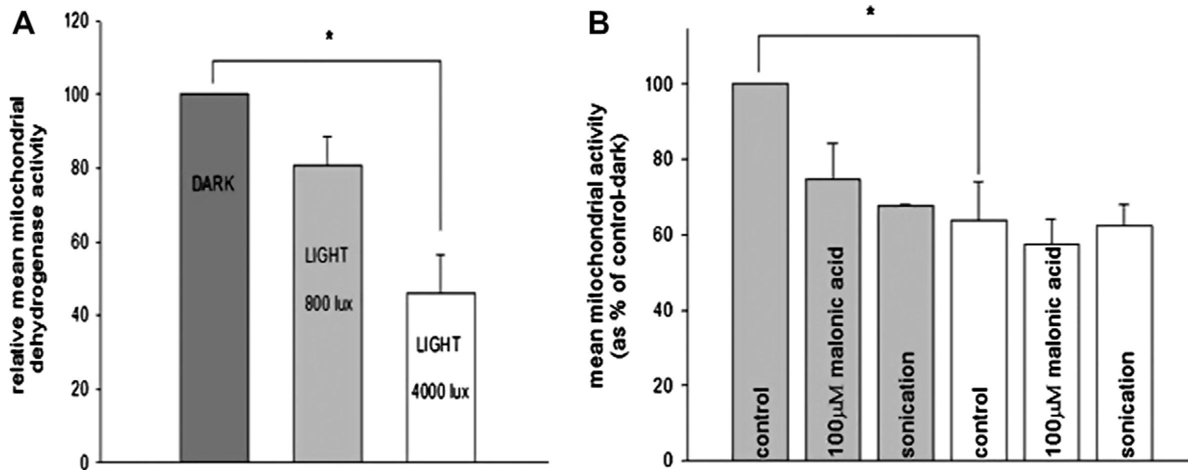

Fig. 7. Relative mitochondrial dehydrogenase activity (measured with WST-1) in freshly isolated liver mitochondria that were subjected to light for 12 h under different conditions. It can be seen that 800 lux of light did not significantly ($P = 0.148$) change the dehydrogenase activity relative to dark conditions. (A) However, when the intensity of light was 4000 lux a significant ($^*P = 0.014$) reduction of enzyme activity occurred (A). The comparative effects of dark (grey bars) and light (white bars: 4000 lux for 12 h) on mitochondrial dehydrogenase activity under different conditions is also shown (B). In some cases the mitochondria were sonicated before incubation. In other cases 100 μM malonic acid was added to the mitochondria. Light caused a significant reduction in the capacity of mitochondria to reduce MTT when compared with dark controls ($^*P < 0.01$). However, reduction of MTT was not significantly different between light and dark mitochondrial suspensions that had either been sonicated or to which malonic acid was added. Results are from six different liver mitochondrial suspensions, each analysed in quadruplets ± SEMs.

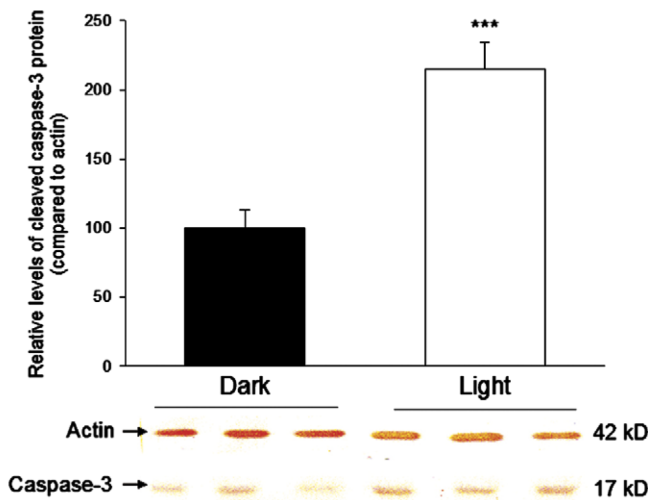

Fig. 8. The relative amount of caspase-3, compared to actin, in three separate rat retinal cell cultures in cultures either grown continuously in the dark for 7 days, or having been exposed on days 6 and 7 to light (1000 lux). Because of the different molecular weights of actin (42 kDa) and active, cleaved caspase-3 (17 kDa) the same blotted sample could be cut and the separate parts immunostained for each of the proteins. Relative to actin, cleaved caspase-3 was significantly increased in these cultures by light, when compared with cultures kept in the dark. It can be seen from the insert that actin levels in each of the triplicate samples appeared visually to be identical yet the caspase-3 staining was elevated in the samples exposed to light. Analysis of the relative differences in the two proteins by densitometry shows this to be of significance ($^{***}P < 0.05$). Results are means of three experiments ± SEMs.

and ROS as well as an increase in the number of TUNEL-positive cells, strongly supports the view that light enhances neuronal apoptosis. Two main pathways (extrinsic and intrinsic) lead to caspase-3 activation and apoptosis (Mancini et al., 1998). The intrinsic pathway is mediated by mitochondria, and can be initiated by a range of stimuli. Apoptosis can also be caused by caspase-independent pathways. The present data, thus, support the idea that light might initiate the intrinsic pathway to cause apoptosis by acting on mitochondria via a generation of ROS but at the same time do not exclude other possible mechanisms. Evidence that light causes a reduction in cellular ATP because of an action on mitochondria is also suggested by studies shown in Fig. 6. The uptake of GABA by neurons is known to be an energy-dependent mechanism (Hell, Maycox, & Jahn, 1990). It was therefore predicted that if light was to reduce cellular ATP levels then the uptake of GABA would be attenuated as compared with cells that had not been exposed to light. This prediction was shown to be correct by using autoradiography to assess the intensity of radioactive grains as an indicator for uptake of ^{3}H-GABA.

The combined experimental data show that, [a] light enhances cell death in rat retinal cultures, [b] the negative light effect on neuronal survival is exacerbated when present in serum-free culture medium, [c] light affects neuronal mitochondrial function, [d] neuronal death caused by light is via apoptosis and [e] that the antioxidant trolox blunts the negative effect of light. These data therefore demonstrate that light, with characteristics similar to that which impinges on the retina *in situ*, has the capacity to stimulate neuronal death in culture. Moreover, it is the mitochondria that appear to be directly affected by light to eventually cause neuronal apoptosis. These findings are consistent with reports that show light can interact with mitochondrial flavin and cytochrome *c* oxidases to subsequently induce a generation of ROS (Godley et al., 2005).

To our knowledge this is the first study to show that light (400–760 nm) can be detrimental to neurons. However, various non-neuronal cell-types, including retinal pigment epithelial cells, have been shown to be affected by light, especially in the blue region of the spectrum (400–500 nm) (Chen, 1993; Chen, Soderberg, & Lindstrom, 1992; Putting et al., 1994; van Best et al., 1997). In many of these studies, mitochondria have been identified as the site affected by light, with ROS being eventually generated, to cause apoptosis (Godley et al., 2005; Hockberger et al., 1999; Jung et al., 1990; King et al., 2004). The present neuronal results are of importance in relation to glaucoma where retinal ganglion cells die (Osborne et al., 1999). Importantly, retinal ganglion cell axons are laden with mitochondria when compared with other retinal neuron-types. Logic therefore suggests that retinal ganglion cells are more at risk from any negative influence of light than other neurons in the CNS because of their large numbers of mitochondria and their direct exposure to the visible component of the electromagnetic spectrum. While photoreceptor inner segments contain many mitochondria they are to a large extent also protected from light by the macular pigments.

This idea was not, however, testable in the present cell culture experiments. This is because all retinal neuron-types in culture have similar morphology which do not reflect their form *in situ.* It is also difficult to specifically examine the influence of light on retinal ganglion cells in such cultures as used in the present study, because they are few in number and are difficult to identify with antibodies that localise the ganglion cell antigens Thy-1 and neurofilament light.

In the present studies on primary rat retinal cultures, "proof of principle" has been provided to show that light can interact with neuronal mitochondria to cause a reduction in energy levels and a generation of ROS. This leads to neuronal death by apoptosis with the process being exacerbated in culture medium lacking serum. We particularly focused on GABAergic neurones because they could be easily identified in culture. It is important to note that GABAergic neurones and other retinal neurones are probably equally susceptible to light injury in culture conditions. However, this is probably not the case in the intact retina. We would argue that neurones heavily dependent on mitochondrial function *in situ* (e.g. ganglion cell with their large numbers of mitochondria in their axons) will be more susceptible to light injury than neurones containing less mitochondria when in an energetically compromised state. It is important also to note that we did not consider the influence of light on glial cells in this study. Glial cells, unlike neurones are heavily dependent on glycolysis and generally respond to a mild injury insult by proliferating rather than dying. We found little evidence for glial cell proliferation but did not investigate this in detail. Our experimental data only allow us to conclude, that light is damaging to neurones in culture, this process is exacerbated when the energy status of neurones is reduced and that the light effect on neurones is by a direct influence upon their mitochondria. Light is known to be able to directly stimulate mitochondrial flavin and cytochrome oxidases to cause oxidative stress (see Section 1). The results therefore support the contention that light is a risk factor to ganglion cell survival *in situ* in certain situations. Ganglion cell axons in the globe contain many mitochondria and will be directly affected by light before reaching the photoreceptors. In glaucoma, retinal ganglion cells probably exist initially at a lower energetic status because of, for example, raised intraocular pressure and ischemia to their axons (Ernest, 1976; Grunwald et al., 1998; Kerr et al., 1998; Yamazaki et al., 1996) reduced neurotrophin support (Sievers, Hausmann, Unsicker, & Berry, 1987) or a specific genetic defect (Abu-Amero et al., 2006). Our hypothesis (Osborne et al., 2006) is that ganglion cells in such circumstances only, i.e. when at an energetically compromised state, be susceptible to light interacting with their axonal mitochondria. We view the presented data as providing support for this hypothesis.

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