Bright cyclic rearing protects albino mouse retina against acute light-induced apoptosis

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Purpose: Previous studies have shown that albino rats born and raised in bright cyclic light are protected from light-induced apoptosis. The present study was designed to determine if bright cyclic rearing provides protection against retinal degeneration caused by acute light exposure in albino mice.

Methods: BALB/c mice were born in dim cyclic light (5 lux, 12 h ON/OFF). At 1 week of age, half of the litters were moved into 400 lux cyclic light. At 5 weeks of age, mice raised in the dim or bright cyclic conditions were divided into two groups. One group was placed in constant light (3,000 lux for 72 h) and the other was maintained in its original cyclic light environment. Control and constant light-stressed mice were dark-adapted for 24 and 48 h, respectively, after which their eyes were removed immediately for morphologic evaluation or preparation of rod outer segment (ROS) membranes. ROS lipids were extracted and fatty acid methyl esters were analyzed by gas-liquid chromatography. Eyes used for TUNEL (terminal deoxynucleotidyl transferase mediated dUTP nick end labeling) and DNA fragmentation assays were enucleated immediately after the 72 h light exposure.

Results: Measurement of outer nuclear layer (ONL) thickness indicated there was no difference in the number of viable photoreceptor cells in the dim-reared controls compared to bright-reared controls. Constant light exposure significantly reduced the ONL thickness in dim- and bright-reared groups, with the largest change occurring in the dim-reared mice. TUNEL assay showed no apoptotic photoreceptor cells in either control group; however, apoptotic nuclei could be detected in both exposed groups, with the largest number found in the dim-reared mice. After light exposure, DNA fragmentation was prominent in dim-reared mice, but not present in bright-reared animals. There was no significant difference in the fatty acid composition of ROS membranes in the dim- and bright-reared control mice. However, constant light exposure resulted in a greater loss of docosahexaenoic acid (22:6n-3) in the ROS of dim-reared animals.

Conclusions: Mice raised in a bright cyclic light environment are protected against light-induced apoptosis. We suggest that the protection is due to the up-regulation of cell survival pathways or the down-regulation of pathways that are vulnerable to acute cell stress.

In 1966, Noell et al. [1] discovered that albino rats exposed to constant illumination underwent dramatic retinal degeneration. In this animal model, as well as in inherited retinal degenerations, the “final common pathway” of photoreceptor cell death is apoptosis [2-4]. For this reason, the animal light damage model is widely used to study the mechanisms of stress-induced photoreceptor cell death, with the goal of obtaining insight into the mechanisms of cell death in retinitis pigmentosa, a heterogeneous group of progressive hereditary retinal degenerations in humans [5].

There are a number of factors that determine the degree of retinal damage caused by light exposure [6]. First are the parameters of the light used, such as intensity, wavelength, and the duration of exposure [7,8]. Next are intrinsic factors, such as age [9], genotype [10], diet [11-14], body temperature [1,15], eye pigmentation [16,17], time of exposure relative to the light cycle [18], location of affected retinal area [16], level of stress and sex hormones [19,20], and the pre-exposure light history [21,22]. Endogenous cytokines [23-25] and other retinal proteins [26,27] are increased in the retina during acute damaging light exposure. Finally, a variety of exogenous molecules that fall roughly into the categories of growth hormones/cytokines [28-32] and anti-oxidants [33-37] have been shown to protect retinal photoreceptors from light-induced apoptosis.

It has been known for many years that the light history of an animal affects its susceptibility to light-induced apoptosis. The early studies by Noell and Albrecht [11] found that the retinas of albino rats raised in darkness were more heavily damaged than those from animals raised in cyclic light. Later, Penn et al. [38] reported that albino rats born and raised in bright cyclic light (800 lux) were protected from light damage, whereas rats born and raised in 5 lux cyclic light were not. Examination of biochemical and morphological parameters in these two groups of rats revealed that bright rearing caused the up-regulation of the anti-oxidants vitamins C and E and of three glutathione (GSH) enzymes, GSH peroxidase, GSH S-transferase, and GSH reductase [22]. Also, the level of docosahexaenoic acid (DHA, 22:6n-3), the major polyunsaturated fatty acid in retinal rod outer segment (ROS) membranes, was greatly reduced in the bright-reared animals, as was the level of rhodopsin and the rhodopsin packing density.
in the lipid bilayer [22]. There were also significant morpho-
logical changes in the retinas of the bright-reared animals. Outer segments were shorter and much more disorganized in the bright-reared animals, and had the appearance impending cell death [38,39]. However, these were adaptive responses, which could be reversed in a few weeks if the animals were returned to dim cyclic light [40]. These findings support the “photostasis” hypothesis of Penn and Williams [41], which states that the retina of animals can adapt biochemically and morphologically to catch a constant number of photons each day.

Several years ago, we proposed our “metabolic stress” hypothesis to explain the neuroprotection found in animals raised in bright cyclic light or the relative longevity of retinal photoreceptor cells that carry a potentially lethal mutation in a gene specifically expressed in the retina [42,43]. Simply stated, any stress to the retina that is potentially damaging to photoreceptor cells elicits an endogenous response that results in the up-regulation of endogenous neuroprotective mecha-
nisms or the down-regulation of pathways that are susceptible to the stress. Examples of this response include the changes in anti-oxidant molecules and proteins noted above [22,26,27], reduction in light-induced apoptosis in mice made transiently hypoxic before light challenge [44], and up-regulation of bFGF [45,46] and CNTF [46] in retinas of albino rats moved from dim to bright cyclic environments several days before acute light challenge.

While much research in retinal degeneration has been done in rats, mice have some distinct advantages. Their genome is easily manipulated and there is a vast amount of information available through the Mouse Genome Project. There are also several genetic mouse models of retinal degeneration and, at the present time, microarray analyses are more informative for mice than for rats. It is for these reasons that we have turned to the present time, microarray analyses are more informative for mice than for rats. It is for these reasons that we have turned to the present time, microarray analyses are more informative for mice than for rats. It is for these reasons that we have turned to the present time, microarray analyses are more informative for mice than for rats. It is for these reasons that we have turned to the present time, microarray analyses are more informative for mice than for rats. It is for these reasons that we have turned to the present time, microarray analyses are more informative for mice than for rats. It is for these reasons that we have turned.

METHODS
Animals: BALB/c mice were born in dim cyclic light (5 lux, 12 h ON/OFF) in our animal facility. At the age of 1 week, half of the litters were moved to bright cyclic light (400 lux, 12 h ON/OFF) and maintained under these light conditions until they were used in an experiment. In our experience, breeding was more consistent and litters were larger if the mice were bred in dim cyclic light and maintained there until the pups were at least 1 week old. The mice were fed laboratory chow ad libitum and had free access to water. The animal care strictly conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the University of Oklahoma Health Science Center (OUHSC) Guidelines for Animals in Research. All protocols were reviewed and ap-
proved by the Institutional Animal Care and Use Committees of the OUHSC and the Dean A. McGee Eye Institute.

At 5 weeks of age, mice in each rearing environment were divided into two groups containing equal numbers of males and females. The light-stressed group was exposed to 72 h of constant illumination in a box with reflective white surfaces. Three fluorescent tubes (cool white, 34 W) gave an illumination of 3,000 lux 2 cm off the floor of the cage (mouse eye level). The control group was maintained under the original light condition, either in dim or bright cyclic light. After constant light stress, mice from dim and bright cyclic light reared groups were placed in the dark for 48 h. Control (non-light-stressed) animals from both groups were dark-adapted for 24 h.

Tissue harvest: For all groups, mice were killed under general red light and eyes enucleated and placed immediately on ice in vials containing phosphate buffered saline (PBS). Dissections were performed at room light under a dissecting microscope. After removing the cornea and the lens, the retinas from each pair of eyes were bluntly dissected from the pigment epithelium/choroid/sclera, frozen in liquid nitrogen, and stored together at -80 °C until processed.

Histology: Eyes were removed, placed into paraformaldeh
de, and processed for histology. Five µm thick paraffin sections were cut along the vertical meridian, through the optic nerve head (ONH), and stained with hematoxylin and eosin for viewing. The thickness of the outer nuclear layer (ONL) and the length of the rod inner segment plus rod outer segment (RIS + ROS) were measured at 0.33 mm intervals from the ONH to the inferior and superior ora serrata.

Terminal dUTP nick end labeling (TUNEL) and DNA ladder ing: Apoptosis of retinal cells was determined by TUNEL assay, using the S7101 ApoTag plus Peroxidase In Situ Apoptosis Detection Kit (Intergen Company, Purchase, NY), according to the manufacturer’s instructions, on 5 µm thick paraffin embedded sections. In the light-stressed groups, eyes were enucleated immediately after the 72 h exposure. DNA laddering was determined as described previously by Donovan et al. [47] with some modification. Briefly, retinas were homogenized in lysis buffer (50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5% SDS, and 0.5 mg/ml proteinase K). The resultant homogenates were extracted with phenol/chloroform to remove the redundant protein and the contaminated RNA was digested by incubating with 20 µg/ml of RNase A for 2 h at 37 °C. Finally, the genomic DNA was run on a 2% agarose gel containing 0.5 µg/ml ethidium bromide.

Fatty acid measurement: ROS were prepared from each pair of retinas by discontinuous sucrose gradient centrifuga-
tion as previously described by Papermaster and Dryer [48] and modified by Wiegand and Anderson [49]. Lipids were extracted in chloroform/methanol following the procedure of Bligh and Dyer [50] and converted to methyl esters as previously described [51]. The fatty acid composition of ROS total lipids was determined by gas-liquid chromatography on an Agilent Technologies 6890N gas chromatograph equipped with a model 7683 autosampler (Palo Alto, CA), using a DB-225 capillary column (30 m x 0.53 mm I.D. x 0.5 µm film thick-
ness; J & W Scientific, Palo Alto, CA). The samples were dissolved in 20 µl of chromatographic grade nonane and 3 µl were injected at 250 °C with the split ratio set at 5:1. The oven temperature at the time of injection was 160 °C, but was increased linearly at 1 °C/minute until it reached 220 °C, and was held at this temperature for 5 min. Helium carrier gas flowed at a linear velocity of 39 cm/sec (4.2 ml/min) and the flame ionization detector was maintained at 270 °C. The detector output was collected on a computer and the areas under each peak were integrated and processed using Chemstation software (Agilent Technologies).

**Statistical analysis:** Results are presented as mean and standard deviation. ONL thickness is the average of about 12 measurements made on each mouse retina at 0.33 mm intervals in a sagittal plane passing through the ONH. Unpaired t-test was used for assessing significant differences across groups for the histology results. Values below p<0.05 are reported as significant. The Scheffé test was used for the analysis of fatty acid results.

**RESULTS**

**Effect of light history on retinal structure:** There were no apparent morphological differences in the retinas of 5 week old albino mice born and raised in 400 or 5 lux cyclic light, when examined at the light microscope level (Figure 1A,C, respectively). The sections presented here were taken about 1 mm from the ONH in the superior retina, the region of greatest light sensitivity in rats [16]. There are 11-12 rows of nuclei in the ONL and the RIS and ROS appear to be healthy and organized. Quantitative analysis of the ONL layer of these two groups of animals showed no significant differences in the average ONL (p=0.44) or RIS + ROS (p=0.13) thickness measured at 0.33 mm intervals from the ONH to the inferior and superior ora serrata (Figure 2). Exposure to 3,000 lux light for 72 h resulted in significant damage to the retinas of both light history groups, with the dim-reared group being the most affected. There was modest reduction in ONL thickness and the appearance of shortened rod outer segments in the bright-
reared group (Figure 1B). Compared to non-exposed bright-reared controls, the average ONL thickness (Figure 2) was reduced by 17% (p<0.0001) and the average RIS + ROS thickness was reduced by 28% (p<0.0001). In the section taken from the same retinal region of a dim-reared animal (Figure 1D), there was a dramatic loss of photoreceptor cell nuclei with many of those remaining appearing to be pyknotic. The outer segments were also greatly attenuated. Compared to dim-reared controls, there was a 59% reduction (p<0.0001) in average ONL thickness (Figure 2) and 71% reduction in the average RIS + ROS thickness (p<0.0001). Comparing the two light-stressed groups, in the dim-raised animals, the average ONL thickness was 50% less (p<0.0001) and the average RIS + ROS thickness was 56% less (p<0.0001) than that in bright-reared, light-stressed animals.

Effect of light history on apoptosis: To provide further data on the number of apoptotic cells in the four experimental groups, we performed TUNEL assay on eyes enucleated immediately after the 72 h of light exposure (Figure 3). There were no TUNEL-positive cells observed in the retinas of dim- or bright-reared control mice (Figure 3A,C, respectively). In contrast, TUNEL-positive cells were found in retinas of both groups that were exposed to constant light, with the greatest number occurring in the dim-reared animals (Figure 3D). Similar results were obtained in 3-4 animals in each group. DNA fragmentation patterns determined by neutral gel electrophoresis confirmed the TUNEL assay findings (Figure 4). DNA from animals raised in dim cyclic light showed greater laddering than DNA from bright cyclic reared mice.

Effect of light history on docosahexaenoic acid composition of ROS: Previous studies in rats born and raised in dim or bright cyclic light showed that bright-reared animals had significantly lower levels of docosahexaenoic acid (DHA, 22:6n-3) in their ROS compared to dim-reared rats [22,52]. Figure 5 shows the DHA content of ROS of the four groups of mice. These membranes were prepared after 48 h of dark-adaptation of light exposed mice and after 24 h of dark-adaptation of control mice. The highest levels of DHA were found in the two control groups and there was no significant difference between the dim-reared (36 mole percent) and bright-reared (35 mole percent) animals. Following 72 h of constant light exposure, the DHA content was significantly reduced in both
group compared to controls (Figure 5), with the greatest loss occurring in the dim-reared animals (16 vs. 23 mole percent after light stress).

**DISCUSSION**

Structural and biochemical evidence presented in this study show that preconditioning albino mice with bright cyclic light prevents light-induced apoptosis. Thus, albino mice are capable of undergoing adaptive responses to environmental lighting conditions, as we [22,38,39,52] and others [11,18,21,40,41,46,53,54] have previously demonstrated in albino rats. We propose that bright cyclic rearing provides a sub-lethal stress for photoreceptor cells that provokes a neuroprotective adaptive response that either up-regulates protective mechanisms or down-regulates metabolic activities that are susceptible to exacberation or damage by acute light challenge.

Genetic factors affect the susceptibility of both albino rats and mice to light-induced apoptosis. LaVail et al. [6] examined the light sensitivity of 7 different inbred strains of albino mice and compared those data to the sensitivity of BALB/c mice, and found that A/J, AKR/J, and N2W/Lac J strains were equally sensitive to BALB/c mice. However, Ma/Mj J and RF/J strains were more sensitive than BALB/c, and RI/J/J was the most sensitive strain. These results clearly show that albino mice with the same phenotype (albinism), but a different genotype can show a wide range of light sensitivity. Comparison of genetic differences between these mouse strains could give information on the factors that affect susceptibility to light damage. However, it is also likely to generate a large number of false positives, since some differences will not be related to neuroprotection. Our approach, which examines the adaptive response of mice of the same genetic background, should eliminate most false positives. In fact, results of the present study suggest that mice may have some advantages over rats for these types of studies. In rats raised from birth [21,22,38-40] or adapted to bright cyclic light as adults [45,55], there is a slow loss of photoreceptor cells compared to rats raised in dim cyclic light. In both cases, bright cyclic rearing protects the retinas from light damage, but a proteomic or genomic analysis will be complicated by two processes, adaptation and apoptosis. In mice raised from 7 to 35 days of age in bright cyclic light, there was no loss of photoreceptor cells (Figs. 1 and 2) and no evidence of apoptosis (Figs. 3 and 4). Therefore, any differences between dim and bright cyclic reared mice should reflect adaptive responses to their different light histories.

We recently used a differential display-PCR approach to examine retinas of albino rats raised under identical conditions to those used in the present study, and found a reduced expression of cytochrome C oxidase-III (CO-III) RNA in the bright-reared animals [56]. In situ hybridization showed a reduction in CO-III RNA in all retinal layers. Western blots of retinal homogenates from these animals showed less immunoreactive protein in the bright-reared rats, and cytochrome C oxidase enzyme activity was also correspondingly lower in this group. Since CO-III is a mitochondrial protein, we suggested that adaptation served two purposes. First, there is a shift in retinal energy production from oxidative phosphorylation to substrate-linked phosphorylation. This reduces the production of reactive oxygen species, which have been implicated as causal in light-induced apoptosis [57-63]. Second, lower cytochrome C oxidase enzyme activity may reduce the level of oxidized cytochrome C that can be released from the mitochondria and thus prevent apoptosis, since only the oxidized form stimulates apoptosis [64,65].

![Figure 4](http://www.molvis.org/molvis/v9/a47)  
**Figure 4.** Effect of cyclic light environment and light stress on DNA fragmentation in the retina. DNA fragmentation patterns in dim and bright cyclic reared mice. The light history and exposure times are shown at the top of gel. The dim cyclic reared mice without constant light exposure served as control, and its genomic DNA was loaded in the left lane. Lane M contains 100 bp DNA marker.

![Figure 5](http://www.molvis.org/molvis/v9/a47)  
**Figure 5.** Effect of cyclic light environment and light stress on the fatty acid composition of rod outer segment membranes. Fatty acid composition of rod outer segments of the four experimental groups. Measurements were done on ROS prepared from both retinas of a mouse (n=8).
Penn and Williams [41] showed that retinas albino rats raised in bright cyclic light environments have lower amounts of rhodopsin than dim-raised rats, and suggested that the retina responded to high or low photon flux by altering the content of rhodopsin in the ROS, to assure capture of a constant number of photons each day. They named this phenomenon photostasis. Later studies by Penn and Anderson [22] showed that the packing density of rhodopsin in the ROS lipid bilayer was less in bright cyclic-raised rats and that their ROS were shorter. These and the other changes discussed in the Introduction were proposed to be anatomical and biochemical adaptation of the retina to bright light stress, to reduce the susceptibility to light-induced apoptosis. The process responds rapidly to changes in environmental light intensity and shows remarkable plasticity. Rats moved from 800 lux to 5 lux showed increase in both their retina rhodopsin level and ERG response [21].

Similar data were found by Schremser and Williams [53,54] in rats raised at 3 or 200 lux. Rats moved from 3 lux to 200 lux, decreased the rhodopsin level by lowering the disc packing density rhodopsin content per disc, and shortening their ROS. Although we did not measure rhodopsin levels in our mice, this seems to be a reasonable explanation and suggests that mice exhibit a photostasis response similar to that described for rats [41].

The retina contains high concentrations of long chain polyunsaturated fatty acids (PUFA), particularly docosahexaenoic acid (DHA, 22:6n-3) [66]. DHA is important in the early development of the human infant retina [67,68] and is necessary for optimal retinal function in rats [69,70], guinea pigs [71,72], and monkeys [73,74]. Animals cannot synthesize the n-3 double bond, so DHA must be obtained in the diet [75]. In dietary n-3 deficiency, the retina conserves DHA by recycling from retinal pigment epithelium (RPE) to the retina [76]. The strong preference of the retina for DHA over n-6 PUFA is somewhat of an enigma, since it is the most susceptible fatty acid in animals for lipid peroxidation and ROS provide an ideal environment for peroxidation (light, high oxygen flux). Although difficult to reduce by dietary manipulations, the DHA content of ROS is lower in two circumstances, bright cyclic rearing [22,52] and hereditary retinal degeneration [77-81]. We hypothesized that both conditions reflect an oxidant stress environment for peroxidation (light, high oxygen flux). Although difficult to reduce by dietary manipulations, the DHA content of ROS is lower in two circumstances, bright cyclic rearing [22,52] and hereditary retinal degeneration [77-81]. We hypothesized that both conditions reflect an oxidant stress environment for peroxidation (light, high oxygen flux). Although difficult to reduce by dietary manipulations, the DHA content of ROS is lower in two circumstances, bright cyclic rearing [22,52] and hereditary retinal degeneration [77-81]. We hypothesized that both conditions reflect an oxidant stress environment for peroxidation (light, high oxygen flux).

In the present study, the levels of DHA were not different between dim- and bright-cyclic reared mice. However, following light challenge, there was a greater loss of DHA in the dim-reared animals, suggesting that their anti-oxidant defenses were less effective in preventing lipid peroxidation. This is consistent with our findings in rats, where bright cyclic rearing led to the up-regulation of glutathione antioxidant enzymes (reductase, peroxidase, and S-transferase) and increased levels of small molecule antioxidants (vitamins C and E) [22].

A number of endogenous responses to chronic light rearing conditions or to acute light stress have been reported for retinas of mice and rats, and can be roughly divided into three categories. (1) Structural and molecular changes that reduce the efficiency of photon capture and visual transduction, (2) Up-regulation of endogenous pathways that protect against apoptosis, and (3) Down-regulation of endogenous pathways that are sensitive to stress-induced apoptosis. Identifying the molecular etiology of these responses and learning how to control their expression could provide a rational basis for treatment of a variety of inherited retinal degenerations. In the studies described herein, we establish that the albino mouse provides some distinct advantages over albino rats for these types of investigations.

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