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LABORATORY INVESTIGATION

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Selective photoreceptor damage in albino rats using continuous blue light

A protocol useful for retinal degeneration and transplantation research

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Abstract *Purpose:* To develop a retinal degeneration model with selective photoreceptor loss and RPE sparing, to be used as recipient for evaluating retinal transplants. *Methods:* Albino rats were exposed to blue light, continuously, for 1–7 days (24–168 h) in a specially designed cage. Eyes were histologically analyzed at periods between 2 h and 8 months after the light exposure. Electroretinograms (ERGs) were recorded from some rats at 12–216 days after exposure. Using behavioral methods, visual thresholds of some rats were determined before exposure and re-measured between 18 and 52 days following exposure. *Results:* Apoptotic nuclei appeared exclusively in the photoreceptor layer after 1–5 days exposure to blue light. Light microscopy revealed that 2–4 days of light exposure reduced the outer nuclear layer (normally eight to ten rows) to 1 row of cells in the central retina and to two to three rows in the periphery, both in the superior and the inferior retina. Average ERG a- and b-wave amplitudes of light-damaged rats were both reduced by about 98%. Visual performance in the behavioral test was substantially impaired. *Conclusions:* Continuous exposure of albino rats to moderate blue light for 2–5 days selectively eliminates most of the photoreceptors while leaving the RPE initially intact.

Introduction

The aim of the present study was to develop and characterize a model of photoreceptor degeneration which could be used as a host for evaluating the function of retinal transplants. Since healthy RPE (retinal pigment epithelium) is needed for the development of organized transplant photoreceptors with outer segments [39], such a model would require a nearly uniform degeneration of rod photoreceptors throughout the retina, without initially damaging the RPE. The RPE must remain functional for several weeks after the initial damage to the photoreceptors. Although photoreceptor degeneration can be induced by various drugs (e.g., [9,47]), there are likely to be systemic side effects, uneven damage to the retina and introduction of unknown factors in the model that can affect transplanted cells. Therefore, photoreceptor damage by light exposure [23,24,29,33,34] (review in [25,35, 49]) was chosen to develop the model. The setup of the light damage procedure had to be simple, easy to handle and not require special optical instrumentation. At the same time, damage to the cornea or lens by UV light [49] had to be prevented because this would interfere with subsequent functional testing of the transplanted tissue. The existing light damage models did not fulfill these requirements. Either the damage was not uniform (e.g., [19,31,44], or the damage occurred too fast so the RPE showed secondary damage early [10,19,24], or the damage was not efficient enough (e.g., [18,19,21,42, 45]).

In general, there are two different experimental models of light damage: short-term exposure of anesthetized animals to high-intensity light [12,15,24], which requires an optical system to focus the light on the retina; and a longer-term, continuous exposure of freely moving animals to lower-intensity light [24,33]. The type and extent

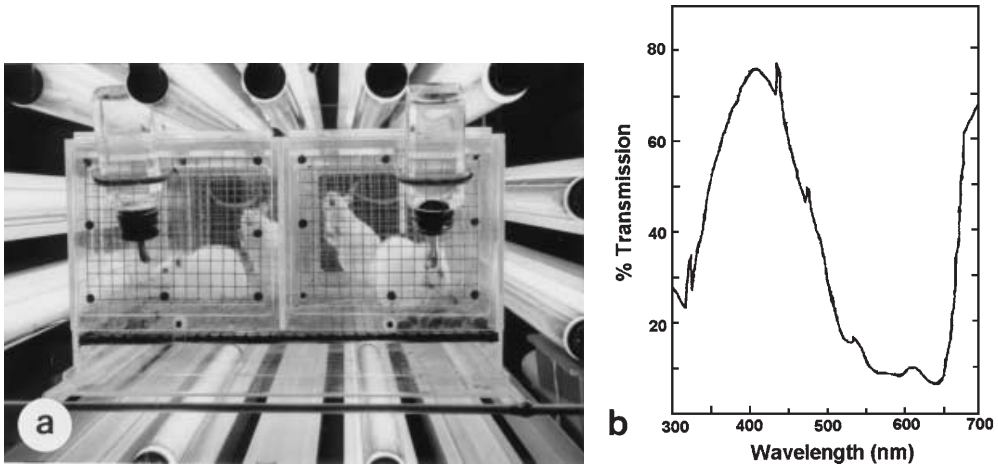

Fig. 1 **a** Light damage cage. The cage is divided into four individual compartments by acrylic plastic along the longitudinal axis, and by wire mesh in the center. The doors at both ends are also made of wire mesh, so air can be blown through the cage to regulate the temperature. **b** Transmission spectrum of "Alice Blue" Filters (Lee #197 and Roscolux #378). The transmission peak is at 420 nm, the bandpass width between 320 and 500 nm. The acrylic plastic of the cage blocks all wavelengths below 385 nm

of damage depends on the experimental paradigms, e.g., light intensity, exposure time, spectral composition of the light, external and body temperature, and on the intensity and cycles of light the animals were exposed to before light damage (e.g., [13,23,25,31]). Different regions of the retina have different sensitivities to light, with the most sensitive region usually being the central to midperipheral superior retina in the rat [31,45].

The effects of the light wavelength on the retina are related to the spectral sensitivity of photoreceptor visual pigments and non-visual pigment chromophores [17,25]. Because rhodopsin absorbs green light most efficiently, this portion of the spectrum has been shown to be especially damaging for the rod photoreceptors [24,25,31]. However, blue light has been shown in a model of continuous light exposure to be more damaging than green light at the same luminance [2], and in anesthetized animals, blue light causes photoreceptor damage at lower irradiances than green light [12,31,32,43]. Short-duration exposure of anesthetized rats to monochromatic laser light of different wavelengths in the UV to blue range (320–440 nm; 130 μW/cm^2 to 22.6 mW/cm^2) specifically damages the rod photoreceptors while monochromatic light of different wavelengths in the green range (470–550 nm; 46–182 mW/cm^2) mostly damages the RPE [12]. The specific effect of blue light on rod photoreceptors has been recently confirmed in another light damage system using blue light of similar irradiance (403 nm; 300 μW/cm^2 to 33 mW/cm^2) in anesthetized rats [36,46]. However, it was not known whether the same effect would be seen using continuous exposure of freely moving rats to blue light of lower intensity. This would be preferable because the time course of damage would be expected to be slower than with exposure of anesthetized rats with high-intensity light, leaving a longer time window with intact RPE for retinal transplantation. Several animals can be exposed at the same time under the same light conditions.

The present study characterizes the different stages of degeneration in a model of freely moving animals exposed to continuous blue light. To identify which cells are primarily affected by blue light, the TUNEL label (terminal deoxynucleotidyl-transferase mediated dUTP nick end labeling) for apoptotic cells was used [11,14,36]. Retinal function was evaluated with electroretinography, and behavioral estimations of visual thresholds were obtained.

Part of this study has been published as an abstract [40].

Material and methods

Light damage

Seventy-five female adult albino Sprague-Dawley rats (weight 200–250 g) were used for this study (59 were light-damaged, 16 served as normal controls). The animals were treated according to the regulations in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

On the day of arrival from the supplier (Harlan Sprague Dawley, Indianapolis, Ind.), or 1–2 days later, the animals were housed in specially designed acrylic plastic/wire cages (Fig. 1a), surrounded by 48″ (122 cm) daylight fluorescent tubes (Sylvania F40/DX) (five on top, two on each side and two on the bottom) covered with blue filters (Lee filter #197 or Roscolux #378, transmission peak at 420 nm; band pass with more than 30% transmission 320–500 nm; Axxis, Louisville, Ky.) (Fig. 1b). The acrylic plastic of the cage blocked wavelengths below 385 nm. The illuminance in the cage was 678 lux (at bottom of cage) to 1291 lux (at top of cage) measured with a 371R Optical Power Meter (Graseby Optronics, Orlando, Fla.).

The size of the frame supporting the light tubes and the cage was 122×68.5×52 cm, the size of the cage inside the frame 86.5×44.5×21.5 cm (length × width × height). The cage was designed so it could be easily cleaned. Four rats could be housed simultaneously in individual compartments (length 41 cm, width and height 21.5 cm). The doors at both ends (not facing the light tubes) were open to outside air and covered with wire mesh to which the water bottles were attached (see Fig. 1a). The temperature in the cage was kept between 22° and 26°C by using fans to circulate air through the open sides of the cage and the wire mesh separating the compartments in the center. The bottom of the cage consisted of a wire mesh with 8 mm squares, and of a removable acrylic plastic tray underneath that was washed every 2 days. A small amount of food pellets, sufficient for 2 days, was placed on the bottom wire mesh.

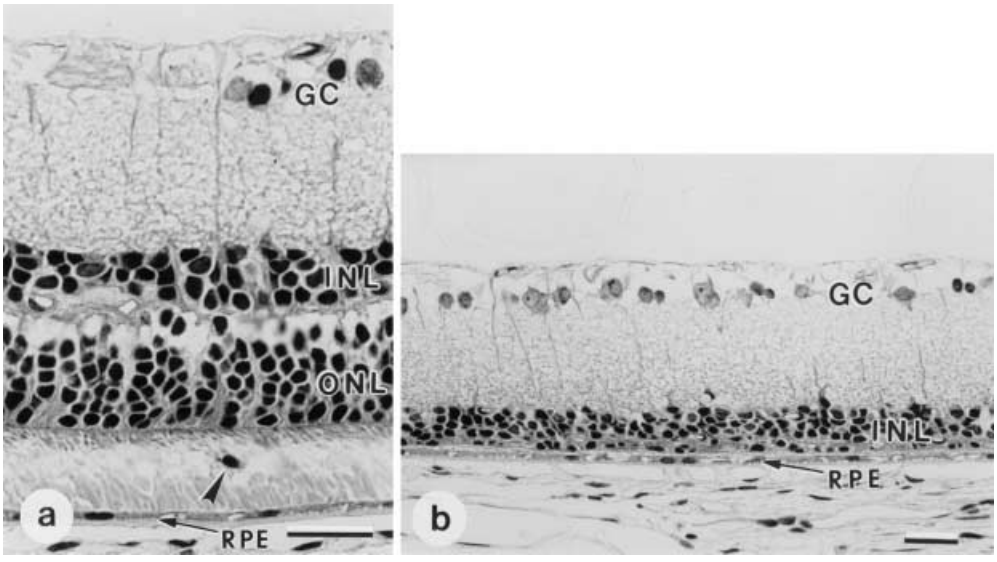

Fig. 2a,b One-day and 2-day exposure, superior retina. **a** One-day exposure, mid-peripheral retina, ca. 1.45 mm from optic disc, 1.89 mm from ora serrata. Disruption of outer segments. Macrophage in subretinal space (*arrowhead*). The thickness of the outer nuclear layer is only slightly reduced. This type of damage was seen throughout the retina. **b** Two-day exposure. Central retina (0.68– 0.9 mm from optic disc): most photoreceptors are gone. The outer nuclear layer is reduced to a single row of nuclei. *Bars* 20 µm. *RPE* Retinal pigment epithelium, *ONL* Outer nuclear layer, *INL* Inner nuclear layer, *GC* ganglion cell layer

To maximize the retinal area affected by the light, the rats' pupils were dilated once with 1% atropine sulfate (which kept the rats' pupils open for 7 days) before they were exposed to blue light for 1–7 days (24–168 h). No weight loss was noted in light-exposed animals compared to controls. After the light exposure, the rats were returned to normally lighted cages in the animal facilities (12 h light on/off; light intensity 8–320 lux).

Histology

Animals were killed by an overdose of sodium pentobarbital (300 mg/kg) for histological evaluation. Most animals were perfusion-fixed with 4% paraformaldehyde with 0.18% picric acid in 0.1 N sodium phosphate buffer. The eyecups were dissected and postfixed in cold Bouin's fixative (Sigma) for at least 18 h, and embedded in low-melting paraffin or in histocryl plastic (Electron Microscopy Sciences, Fort Washington, Pa.). Other animals were perfusion-fixed with 4% paraformaldehyde. Their eyecups were washed with phosphate-buffered saline (PBS), infiltrated with sucrose and frozen in Tissue-Tek on dry ice. Paraffin (8-µm), frozen (8-µm) or plastic (2-µm) sections were cut and stained with hematoxylin/eosin. Selected sections were processed for TUNEL (see below).

Visualization of apoptotic cell death

Nicked DNA was labeled with biotinylated nucleotides (TUNEL) to detect apoptotic cells. The TUNEL-method was carried out as described [11] with some modifications using an "in situ cell death detection kit" (Boehringer Mannheim, Indianapolis, Ind.). Cryostat sections of retinas from rats killed at different times after being exposed to blue light were rinsed with PBS (pH 7.4) and incubated in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 5 min. After being washed with PBS, the sections were covered by 50 µl TUNEL reaction mixture (containing fluorescent-labeled nucleotide mixture and terminal deoxynucleotidyl-transferase) in a humidified chamber for 60 min at 37°C. After being rinsed with PBS, the sections were analyzed with a fluorescence microscope and photographed. The experiments were repeated 4 times. Each experiment included as negative controls one slide with sections of normal retina, incubated with TUNEL reaction mixture and one slide with sections of light-damaged retina incubated in the same reaction solution lacking the deoxynucleotidyl-transferase.

Electroretinography

Full-field electroretinograms (ERGs) were recorded under dark-adapted conditions from both eyes of 13 light-damaged rats, 12–216 days after 4 days of exposure to blue light, and from 2 normal (control) rats not exposed to blue light. Following overnight dark adaptation, rats were anesthetized with 45 mg/kg sodium pentobarbital (i.p.) and had their pupils dilated with 2% phenylephrine hydrochloride and 0.2% cyclopentolate hydrochloride under dim red light illumination. ERGs were elicited with 10-µs flashes of white light (4.6 log footlambert) presented at intervals of 15 s. Responses were monitored with a chlorided silver wire loop placed on the cornea topically anesthetized with proparacaine hydrochloride; a saline cotton wick was placed in the mouth as reference electrode and a subdermal electrode in the neck served as ground. ERG responses were differentially amplified at a gain of 10,000 (–3 dB at 2 and 300 Hz), digitized at a sampling rate of 1302 Hz, and summed with customized software on a personal computer [20]. The software provided the capability of detecting signals as small as 1–2 µV through the use of a "smart" artifact reject window and signal averaging tailored to the signal amplitude. All recordings from the light-damaged animals, whether or not discernible from noise, were assigned cornea-negative ("a-wave") and cornea-negative to cornea-positive ("b-wave") amplitudes for quantitative comparison with the responses from the normal control animals.

Behavioral tests

Ten normal Sprague-Dawley rats were trained in a modified Skinner box to touch one stimulus panel (out of 3) that was illuminated on a display monitor using a Vision Research Graphics display system. Correct responses were reinforced by fruit juice. Following training to an 80% correct criterion, visual thresholds were assessed under very low (<1 lux) ambient illumination. Rats were observed with the aid of an infra-red light source and CCTV system. The onset of each trial was accompanied by an audible tone. Visual thresholds for detection of the 2-pixel white stimulus were obtained from frequency-of-seeing curves generated for each rat (see example in Fig. 7a) using a modified staircase/method-of-limits paradigm [27,28]. The intensity of the stimulus was reduced in 0.05-log steps following each correct response, and increased by 0.05 log following an incorrect response. Two consecutive incorrect responses resulted in an increase of 20 steps to prevent loss of stimulus control of the rat's responding near threshold.

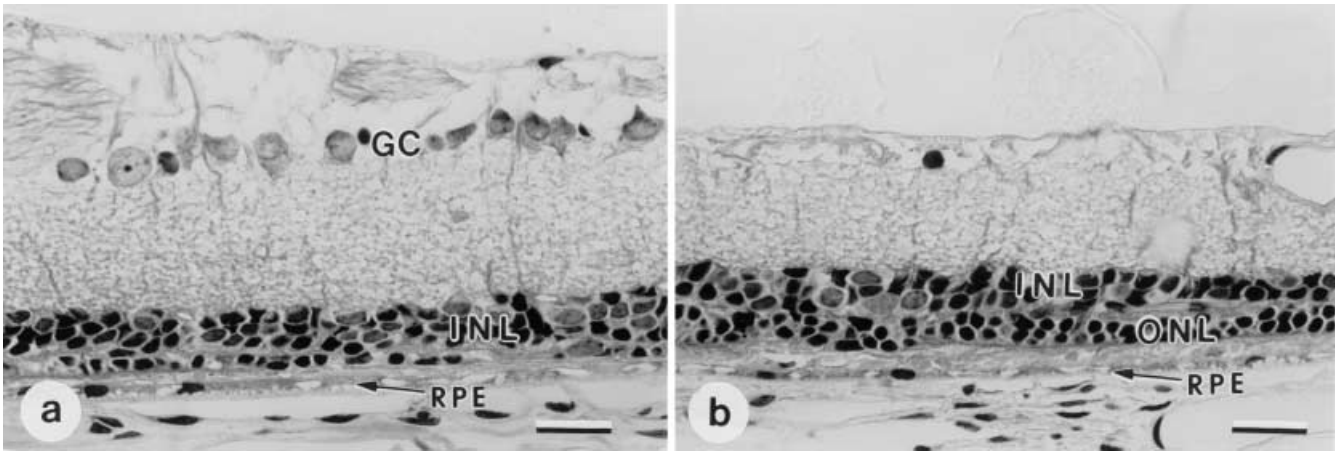

Fig. 3a,b Four-day exposure, superior retina. **a** Central retina (0.09–0.26 mm from optic disc). Less than one continuous row of photoreceptors left. **b** Peripheral retina (2.89–3.06 mm from optic disc). Two to three rows of photoreceptors with remnants of outer segments. *Bars* 20 µm. *RPE* retinal pigment epithelium, *ONL* Outer nuclear layer, *INL* Inner nuclear layer, *GC* ganglion cell layer

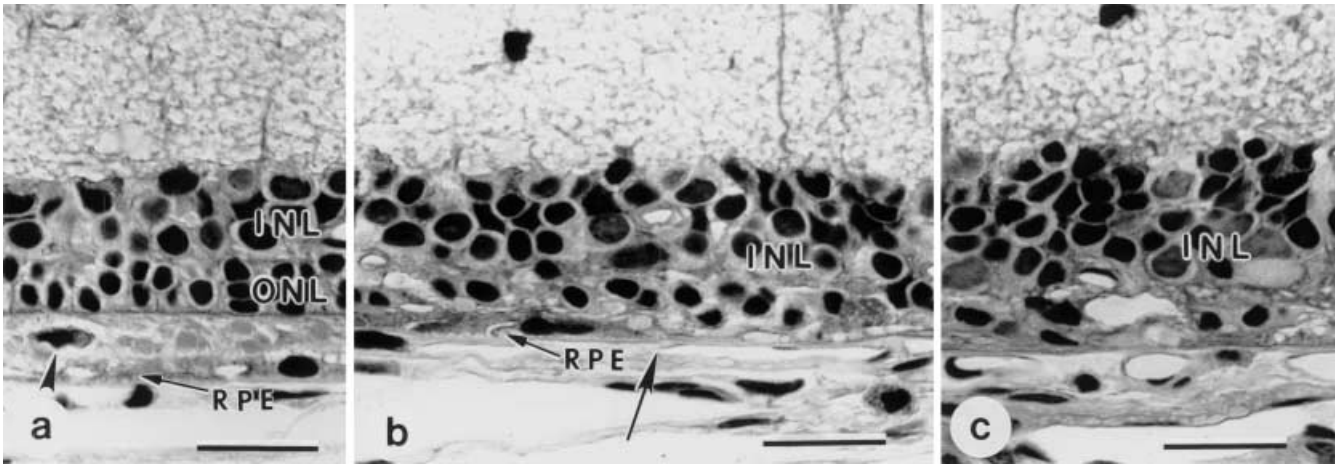

Fig. 4a–c Seven-day exposure, superior retina. **a** Peripheral retina (3.3 mm from optic disc). Two to three rows of photoreceptors in outer nuclear layer, remnants of outer segments. *Arrowhead* Macrophage. RPE apparently intact. **b** Transition to area with RPE damage, indicated by arrow (2.35 mm from optic disc). The area on the left with RPE is towards the periphery. **c** Midperipheral retina (1.32 mm from optic disc). RPE damaged and no longer recognizable. Retina is apparently directly apposed to Bruch's membrane. *Bars* 20 µm. *RPE* Retinal pigment epithelium, *ONL* Outer nuclear layer, *INL* Inner nuclear layer, *GC* Ganglion cell layer

Following visual threshold assessment, the rats were light damaged and thresholds were re-measured beginning 18 days the light damage period ended. Testing was concluded after 52 days following the light exposure.

Results

Histology at 10 days after a 1–7 day blue light exposure period

Normal unexposed retinas contain about 8–10 rows of photoreceptor nuclei (data not shown). One day (24 h) of light exposure (evaluated at 10 days after the end of the light damage period) produced extensive damage to outer segments (OS) throughout the retina, but there were still four to seven rows of photoreceptor nuclei left in the outer nuclear layer (ONL) in the superior retina (Fig. 2a). Little difference was evident between the superior and inferior halves of the retina (data not shown). In contrast, a light exposure period of 2 days (48 h) caused a substantial reduction of the ONL to one row of nuclei in the central retina (up to 1.9 mm from the optic disc) and to three rows in the periphery (2.9–3.6 mm from the optic disc) of the superior retina (Fig. 2b). Similar, less extensive damage was observed in the inferior retina (data not shown). Remnants of OS were seen only in the peripheral retina where more photoreceptors had survived. A light exposure period of 4 days (96 h) resulted in photoreceptor destruction which extended still further into the periphery of the retina, both in the superior (Fig. 3a,b) and the inferior half (data not shown). In the su-

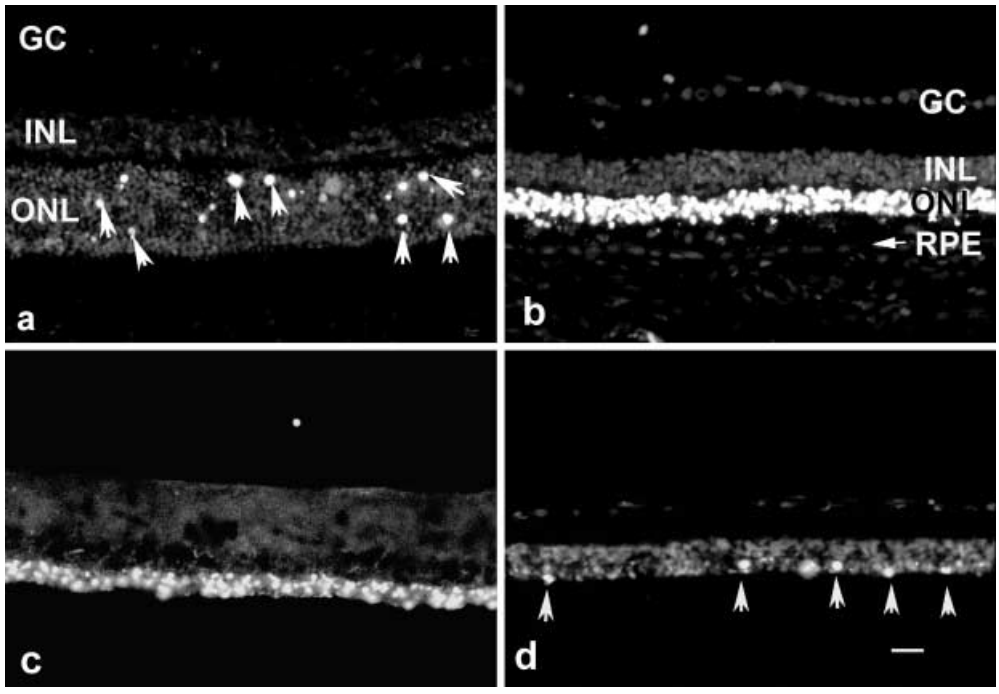

Fig. 5a–d Apoptotic cell death in the retina **a** After 1-day blue light exposure; **b** after 5-day blue light exposure; **c** 1 week following 4-day blue light exposure; **d** 2 weeks following 4-day blue light exposure. Note that all of the labeled cells are located in the ONL and no positive staining was observed in the RPE or the inner part of the retina. *ONL* Outer nuclear layer, *INL* Inner nuclear layer, *GC* Ganglion cell layer. *Arrows* indicate fluorescently labeled apoptotic cells. *Bar* 20 µm

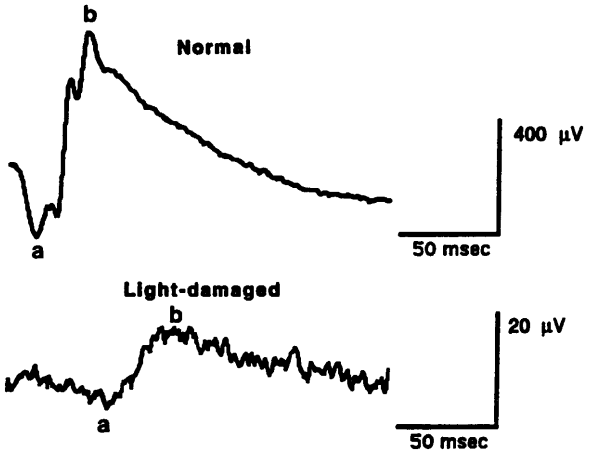

Fig. 6 Example of dark-adapted full-field electroretinograms (ERGs) from a normal rat not exposed to blue light and a light-damaged rat previously exposed to 4 days of constant blue light. Note the difference in vertical scale for the calibration marker at right. The response from the light-damaged rat has markedly reduced, but detectable, a- and b- waves ("a-wave": cornea-negative amplitude; "b-wave": cornea-negative to cornea-positive amplitudes, designated by *a* and *b*)

perior retina, up to 2.35 mm from the optic disc, only one row of photoreceptors was seen. At this time, damage of a small area of RPE could be seen in the mid-superior retina, ca. 1.1 mm from the optic disc (not shown). A light exposure period of 7 days (168 h) caused extensive RPE damage in a large area of the mid-superior retina, extending from 1.06 to 2.35 mm from the optic disc (Fig. 4b,c). Outside this area, the RPE appeared light microscopically intact (Fig. 4a,b), and the peripheral retina still contained 2–3 rows of photoreceptors in the ONL (Fig. 4a). Based on these results, a light exposure period of 4–5 days of exposure was chosen for most of the following experiments.

TUNEL label

No TUNEL labeling was detectable in non-exposed control rat retinas or when the light damaged retinal sections were incubated with reaction solution lacking the deoxynucleotidyl-transferase (data not shown). In rats that were killed immediately after a light exposure period of 1 day, scattered labeled apoptotic cells were found in the ONL of the retina (Fig. 5a). In contrast, after a light exposure period of 5 days there was extensive cell death in the ONL (Fig. 5b). No apoptotic cells were found in the RPE, inner nuclear layer or ganglion cell layer. After a light exposure period of 4 days, the ONL gradually became thinner with fewer potentially apoptotic cells. This progressive phenomenon was evident at 1 week (Fig. 5c) and 2 weeks (Fig. 5d) following a 4-day exposure.

Electroretinography

Based on the best responses from each of the 13 light-damaged animals, the reduction in ERG amplitude averaged approximately 98% for the a-wave (mean, 5.0±0.7 µV; normal mean 238 µV) and the b-wave (mean, 14.9±2.0 µV; normal mean, 716 µV). Representative examples of recordings are shown in Fig. 6.

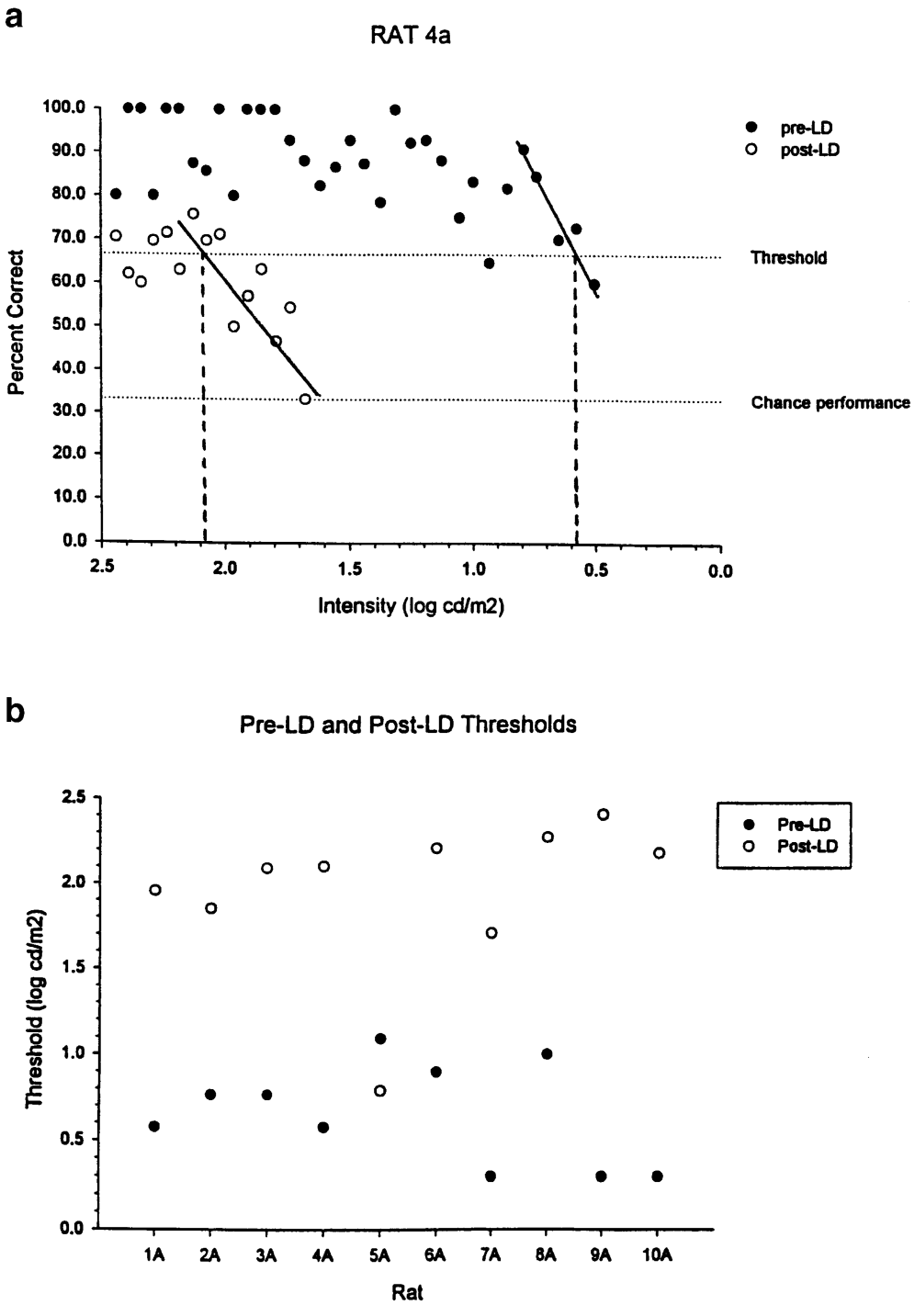

Fig. 7a,b Behavioral tests. **a** Example of "frequency-of-seeing curve" of rat 4a before (*filled circles*) and after light exposure (*open circles*). The threshold of this rat, calculated at 0.6 log cd/m^2 before light exposure, increased to 2.1 log cd/m^2 after light exposure. **b** Thresholds before and after 4 days of light exposure. In 9 of 10 rats, the thresholds increased (range 1.2–2.2 log units) after light exposure. Rat 5A did not show an increase in threshold

Behavioral tests

After light exposure, 9 of 10 rats displayed severe visual deficits on the behavioral test. As shown in the sample case in Fig. 7a, deficits included an elevation of visual threshold and also poorer performance at the highest luminances tested. Figure 7b plots pre- and post-light damage thresholds for the ten rats tested. Nine of the ten rats showed a substantial 1.2–2.2 log unit elevation of visual threshold. These deficits remained for 3–7 post-exposure weeks in which the animals were tested. Rats not exposed to the blue light did not show such changes in threshold when tested over a similar time period.

Discussion

Effectiveness of this light damage model

This study shows that significant rod photoreceptor damage throughout the entire retina can be achieved in albino rats with moderately intense blue light (700–1300 lux) after a continuous exposure time of only 2–4 days. By comparison, exposure to white light of 1240–1400 lux for 7 days reduces the photoreceptor layer to one to three nuclei in the superior midperipheral retina [18,21,42]. Other light damage studies using continuous white light have reported exposure times of 2–4 weeks to achieve reduction of the photoreceptor layer to one row of photoreceptors in spite of using a higher light intensity, 1900 lux [41] to 3500 lux [7,8]. In addition, a 4-week exposure to 3500 lux of white light also leads to a local destruction of the RPE [7]. The extent of retinal damage in our experiments might have been more extensive if the experimental animals had been dark-adapted prior to light exposure [22,25]. This is because dark-adaptation increases the rhodopsin content of the retina [3]. However, a period of dark-adaptation might also result in RPE cell damage at earlier time points following blue light exposure. This would create the undesired effect of a narrower time window for prospective transplantation experiments.

UV-A (320–400 nm) [31] and blue light (400–480 nm) [12,43] are damaging at much lower irradiance levels than light of longer wavelengths. Anderson et al. [2] showed that continuous exposure to blue light (437–538 nm) [of a much lower intensity (~65 lux) than used in our study] reduced the outer nuclear layer to one row of nuclei after about 4 weeks of exposure, whereas with the same intensity of green light (545–580 nm) there were still four rows of photoreceptor nuclei left after 4 weeks.

Moderate blue light exposure affects rod photoreceptors first

Recently, in two models of photoreceptor damage, either by intense white light (3000 lux) [14] or green light (3400 lux) [1], the loss of photoreceptor cells in light-induced retinal degeneration was shown to occur by an apoptotic mechanism. This was later also shown with exposure to intense blue light (403 nm; 300 μW/cm^2 to 33 mW/cm^2) [36] (review in [37]). Our data with Tunel labeling support these findings.

It was important to avoid exposing the animals to light below 400 nm because UV-A light has been shown to produce damage in all retinal layers at an intensity of 2.8 mW/cm^2 in rats [32], and damage to both photoreceptors and RPE cells at lower intensity (100–200 μW/cm^2) [30,31].

Mechanism of damage

Since exposure to blue light caused apoptosis of the photoreceptors first, it can be assumed that a photochemical reaction with photoreceptor-specific proteins caused the apoptotic cell death. For example, rhodopsin-mediated damage by 403-nm blue light (33 mW/cm^2) has recently been demonstrated by Williams et al. [46].

However, blue light can be damaging to RPE when higher light intensities are used. At a dose of 110–380 kJ/m^2 (11–38 mW/cm^2×1000 s), blue light (404 nm) inhibited cytochrome oxidase and thus inhibited the mitochondrial metabolism in photoreceptors and RPE [5,6]. This effect of blue light was dependent on the presence of oxygen, and reversibility or irreversibility of the damage in photoreceptors depended on the light intensity (the damage to RPE was reversible under both conditions). In pigmented rabbits, blue light (412–425 nm) at a threshold intensity of 18 J/cm^2 (180 kJ/m^2) led to a significant increase in permeability of the blood retinal barrier as shown by vitreous fluorophotometry, and to loss of melanin granules in the RPE [29]. Similarly, exposure of primate retinas with intense blue light (440 nm; 11–36 J/cm^2, corresponding to 110–360 mW/cm^2 for 100 s) led to specific lesions in the RPE which were intensified by the presence of oxygen [38].

In this light damage model, RPE cells were not initially affected. This difference in the susceptibility of the RPE to light exposure appears to be due to the lower intensity of light in our experiments.

Electroretinography shows extensive damage and no recovery

Electroretinography has been used to study the extent of light damage (e.g., [16,24,30]) and retinal degeneration [4,26], and to investigate treatments aimed at reducing the severity of light damage (e.g., [21]). The severity of photoreceptor damage in our model, as assessed beginning 12 days after the light damage period, was clearly demonstrated by the electrophysiological data. The average response was only 2% of control. The magnitude of the photoreceptor dysfunction resulting from 4 days' continuous exposure to blue light might make it possible to detect very small light responses of single or multiple retinal transplants in a light-damaged retina, as has been shown for RPE transplants in the RCS rat [48].

Behavioral tests show severe reduction of visual performance

Our tests of visual performance consisted of a simple detection of light. Although there was some variation in the

pre-exposure visual thresholds for individual rats (which may have been due to different visual abilities or behavioral performance), 9 of the 10 light-damaged rats showed a marked elevation of visual threshold following exposure. As is evident in Fig. 7b, except for one case (rat 5A), there was no overlap between the distributions of pre- and post-exposure thresholds. Histological analysis of the retinas of Rat 5A revealed some remaining scattered rod photoreceptors (mainly in the inferior retina), but the amount of surviving photoreceptors was not more than in the other light-damaged rats (data not shown). For transplantation purposes, this provides an appropriate baseline for assessment of transplant function.

This model is useful for studies of retinal degeneration and transplantation

In summary, we have shown that exposure of freely moving rats to continuous blue light resulted in damage to rod photoreceptors throughout the retina. The damage to the retina was irreversible and grew worse with time. The RPE cells were directly affected only if the exposure to blue light was longer than 4 days. While RPE damage has also been detected in longer-term survival after light exposure (data not shown), it is likely, albeit not proven that such damage is caused secondary to photoreceptor death. In either case, in the experimental paradigm described here, there is evidently a period of at least several weeks in which the RPE cells are apparently healthy. We have shown that RPE cells of light-damaged rat recipients can support photoreceptors of fetal intact-sheet transplants for up to 10 months if the transplantation is done within 4 weeks after light damage [39]. This indicates that RPE cells can be rescued from degeneration by the presence of a neural retinal transplant. This RPE–photoreceptor trophic interaction demonstrates the possible existence of two-way support mechanism for cell survival and cell function.

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