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Temporal sequence of changes in rat retina after UV-A and blue light exposure

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Abstract

Two spectral types of retinal light damage were induced in pigmented rats by irradiating small retinal patches at either 380 or 470 nm. The temporal sequence of changes in the retina was followed for up to 2 months by funduscopy and histology. For both damage types, fundus changes were best visible after 3 days. Histology showed that 380 nm specifically damaged photoreceptor cells, particularly the rods. All cell compartments of the rods, including the nucleus were affected already after 3 h. In the next days, damaged rods degenerated. At high doses (2.5 × the funduscopic threshold dose) all rods in the irradiated area were lost, resulting in a local photoreceptor lesion, which was still present at 2 months after the irradiation. At 470 nm, damage occurred both in the photoreceptor layer and in the pigment epithelium. Acute changes, at 1 h after irradiation, consisted mainly of damaged mitochondria in these layers. Next, the pigment epithelium showed swelling, an altered melanin distribution and, at high doses (2.5 × threshold), interruptions of the monolayer. Degeneration of photoreceptor cells was initially limited to a few scattered cells, but 3 days after high doses focal areas of massive degeneration were seen. At late stages, the cells of the pigment epithelium recovered and the photoreceptor layer showed a loss of cells. The results show that the spectral damage types are distinct in the early phases, indicating that different mechanisms are involved. Yet, the end effect of both damage types after exposure at doses up to 2.5 × the funduscopic threshold is remarkably similar and consists of local photoreceptor lesions.

Keywords: Light damage; Photoreceptor cells; Retinal pigment epithelium; Blue light; Ultraviolet light

1. Introduction

The retinal toxicity of light at levels below the threshold for thermal damage was first described by Noell, Walker, Kang and Berman (1966). They found damage in the retina of rats after exposing them for several hours to light at relatively low intensity. Later studies on retinal light damage can be divided into two categories with regard to the exposure conditions employed: (1) Long exposures of free running laboratory animals to relatively low levels of light. (2) Exposure of small retinal fields of anesthetized animals to short, intense irradiations. The latter setup is used as a model for clinical examples of phototoxicity such as solar retinitis and photic maculopathy during eye surgery (Tso, 1989; Byrnes, Antoszyk, Mazur, Kao & Miller, 1992). The present study adheres to the second type of exposure conditions. Animal studies have indicated that this type of exposure also results in photochemical, rather than thermal damage (Friedman & Kuwabara, 1968; Tso, Fine & Zimmerman, 1972; Ham, Mueller, Rufolo & Clarke, 1979). The wavelength dependency of light damage in these exposure conditions was investigated by Ham and colleagues. They showed that damage sensitivity increases for shorter wavelengths, with a maximum in the ultraviolet (Ham et al., 1979; Ham, Mueller, Ruffolo, Guerry & Guerry, 1982). Subsequent histological studies from Ham's group suggested that two spectrally defined damage types add up to this sensitivity curve: Exposure at 350 nm damaged, in aphakic monkeys, predominantly the photoreceptors (rods and cones) (Ham et al., 1982) at 441 nm, damage was found to be most prominent in the RPE (Ham,

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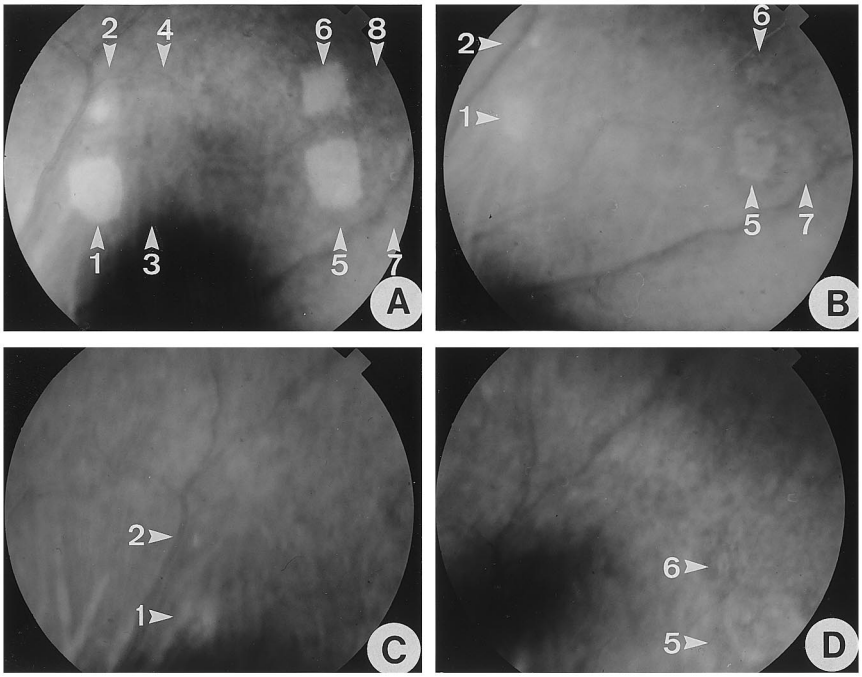

Fig. 1. Fundus photographs of the same eye taken 1 (A), 7 (B) and 21 (C, D) days after irradiation at 470 and 380 nm. During an irradiation four rectangular retinal patches were exposed at different doses. At 470 nm, doses of 1327 (1) and 858 (2) J/cm^2 caused bright spots at day 1, which became smaller and less bright at day 7, and were reduced to white speckles at day 21. Lower doses of 448 (3) and 264 (4) J/cm^2 caused no fundus changes. At 380 nm doses of 2 (5), 1.3 (6), and 0.74 (7) J/cm^2 were visible as grey spots at day 1. They became less bright at day 7. At day 21, a faint, mottled appearance remained. A dose of 0.46 (8) J/cm^2 caused no visible changes.

Ruffolo, Mueller, Clarke & Moon, 1978). These data were limited, however, to a very small number of monkeys. Specific photoreceptor damage was also found after 325 nm laser irradiations (Schmidt & Zuclich, 1980). In contrary, the RPE was the site of primary injury by repeated near UV irradiations in aphakic and pseudophakic monkeys (Li, Tso, Jampol, Miller & Waxler, 1990). After exposure to visible wavelengths, damage was generally found in the RPE and photoreceptors (Friedman & Kuwabara, 1968; Tso et al., 1972; Tso & Woodford, 1983). Lawwill (1982) described damage to mitochondria in all retinal layers after monochromatic irradiations at various wavelengths in the range of 457.9–514.5 nm. Spectral differences in retinal light damage after short, intense irradiations were also found in other species. In the squirrel (Collier & Zigman, 1989) 441 nm damaged both the RPE and the photoreceptors; exposure to 366 nm affected at threshold only the photoreceptors, and rods appeared more severely affected than cones. In the rat, two different pathologies were found at 3 days after monochromatic irradiations at various wavelengths: Photoreceptor damage was found at wavelengths in the range of 320–440 nm; wavelengths of 470–550 nm damaged most prominently the RPE (Gorgels & van Norren, 1995). However, another study on rats comparing broad-band green and UV-A exposures found no morphologic differences (Rapp & Smith, 1992).

From the above it is apparent that there are strong indications for the existence of at least two spectral damage types. Yet, several important questions remain unresolved. First, the distinction between the types is not clear, because the reports vary in the relative susceptibility of the various retinal elements, in particular that of RPE, cones, and rods. Species differences may account for some of the described variation. Also, with increasing dose progressively more retinal elements become affected (Gorgels & van Norren, 1995) and in time, secondary damage may spread to other layers. Following this reasoning, a study employing different doses and focusing on the acute effects may clarify the distinction between the types. Second, only limited data are available on the (sub)cellular localization of the first manifestations of damage, as most studies start analysis at 1 or more days after the exposure. The acute effects are important in order to pinpoint the site of damage initiation. Third, the long term effects of light damage of small retinal patches are uncertain: Ham et al. reported in the monkey a remarkable capacity for repair of mild photochemical lesions of both damage types: The RPE damage at 441 nm recovered in time (Ham et al., 1978) and the limited data on the UV-A induced lesions in the photoreceptor layers suggested that small lesions can be closed in by neighboring photoreceptors (Ham et al., 1982). However, in the squirrel retina no evidence was found for filling in of

Table 1
Funduscopic changes following irradiation at 380 and at 470 nm

Wavelength (nm)	Dose (J/cm^2)	Acute	After 1–7 days	After 3–9 weeks
380	Threshold 0.5–0.7	—	Grayish, vague spot, best visible at 3 days	—
	Moderate 0.8–1.0	—	Sharp-edged bright spot	—
	High 1.3–1.7	First no changes; after 2 h slightly brighter	Sharp-edged bright spot maximum brightness at 3 days	Barely visible, mottled spot
470	Threshold 400–600	—	Small spot, slightly brighter than surround	—
	Moderate 650–850	—	Vague, white spot	—
	High 1200–1350	In two out of seven experiments: hazy patch visible after 1 h	Bright spot, best visible at 3 days	Vaguely visible spot

—, no difference with control.

photoreceptor lesions (Collier, Waldron & Zigman, 1989). In the present study we wanted to address these questions by comparing the pathologies caused by separate wavelengths at various doses and at various time points after irradiation. Based on our previous study (Gorgels & van Norren, 1995), we chose to irradiate at 380 nm for the damage type with emphasis on the photoreceptors, and at 470 nm for the other damage type. The ocular media of the rat readily transmit 380 nm (Gorgels & van Norren, 1992). Damage was assessed by funduscopy and light microscopy at various time points ranging from 1 h to 2 months after irradiation. In addition, electron microscopy was performed at the acute stage in order to examine the early radiation effects at the ultrastructural level.

2. Methods

2.1. Animals

Male Long Evans rats were obtained from Harlan CPB, Zeist, the Netherlands, at 30 days of age. They were kept in a 12–12 h light–dark cycle, at 10–60 lux by white fluorescent light. The animals used in the experiments were 60–130 days old.

2.2. Optics and irradiation

Irradiation of a 450 W Xenon arc was projected onto a rectangular patch of the retina (16×18^{o}) with optics basically consisting of three lenses. The first, condenser, lens at focal distance of the Xenon arc provided a parallel bundle. In the parallel bundle, interference filters were placed to select wavelength. We used filters with bandwidth 10 nm centered around 380 and 470 nm. A filter with peak transmission at 517 nm was used for aiming of the beam. In addition, the parallel beam passed a heat filter (Spindler and Hoyer, KG 3, 3 mm) and a water bath. Neutral density filters could be inserted to reduce irradiance. The second lens ($f = 120$ mm) focused the beam. The focus was conjugated with the pupil plane and a diaphragm was placed here to reduced the size of the entrance pupil. The third lens ($f = 53$ mm) focused the beam in the pupil plane of the rat eye, ensuring Maxwellian view conditions. In a plane conjugate to the retina, a rectangular diaphragm was placed with neutral density filters separated by black bars. The beam was thus divided into four bundles with intensities of about 100, 45, 25, and 10%. Thus, four retinal patches of $6 \times 7°$ were irradiated at different intensities in one exposure. The irradiance provided by this configuration was measured and retinal irradiance was calculated as described previously (Gorgels & van Norren, 1995). Retinal irradiance ranged from 0.5 to 8 mW/cm^2 at 380 nm and from 25 to 250 mW/cm^2 at 470 nm. Exposure time was 6–8 min at 380 nm and 60–90 min at 470 nm. Within these ranges of exposure duration and retinal irradiance, the dose, i.e. irradiance $\times$ exposure duration, determines damage morphology and threshold (Gorgels & van Norren, 1995). The small fraction of light reflected from the fundus, was deflected by a mirror at a pupil plane and was used to observe and monitor the retinal position. If the position changed during irradiation, the experiment was discarded.

For irradiation the rat was sedated with ether, anesthetized with pentobarbital (50 mg/kg) by intraperitoneal injection, and kept anesthetized during the irradiation by intravenous infusion with saline (1 ml/h) containing pentobarbital (15 mg/kg per h). Atropine sulfate (0.1 ml of a solution of 0.5 mg/ml) was injected subcutaneously. Pupils were dilated with drops of phenylephrine 5% and cyclopentolate HCl 1%. Body temperature was measured by a rectal thermometer and kept between 37.5 and 38.5°C by an electrical blanket.

Table 2
Light microscopic changes in the retina following irradiation at 380 and at 470 nm

Dose (T)	Acute (after 1–3 h)		After 1–7 days		After 3–9 weeks	
(nm)	380	470	380	470	380	470
$0.75 \times T$	—	—	PR: dark staining of a few PRs (<1%)	—	—	—
T	—	—	PR: dark staining of a few PRs (<1%)	PR: dark staining of a few PRs (<1%); RPE: swelling, altered melanin distribution	—	—
$1.5 \times T$	PR: dark staining of 5–15% of PRs	—	PR: Dark staining of 10–30% of PRs	As above	PR: 30–40% loss of rods	PR: up to 5% loss of PRs
$2.5 \times T$	PR: most rods stain dark RPE: many phagosomes	PR: light vesicles in IS; a few dark staining rod and cone nuclei (<1%); RPE: light vesicles	PR: all rods degenerate relative sparing of cones	PR: focal massive PR degeneration; RPE: as above and locally interrupted monolayer	PR: total loss of rods relative sparing of cones	PR: focal massive PR loss; RPE: dark staining cells

—, no difference with control.
T, Threshold dose for funduscopic damage at 3 days after irradiation; PR, photoreceptors; RPE, retinal pigment epithelium.

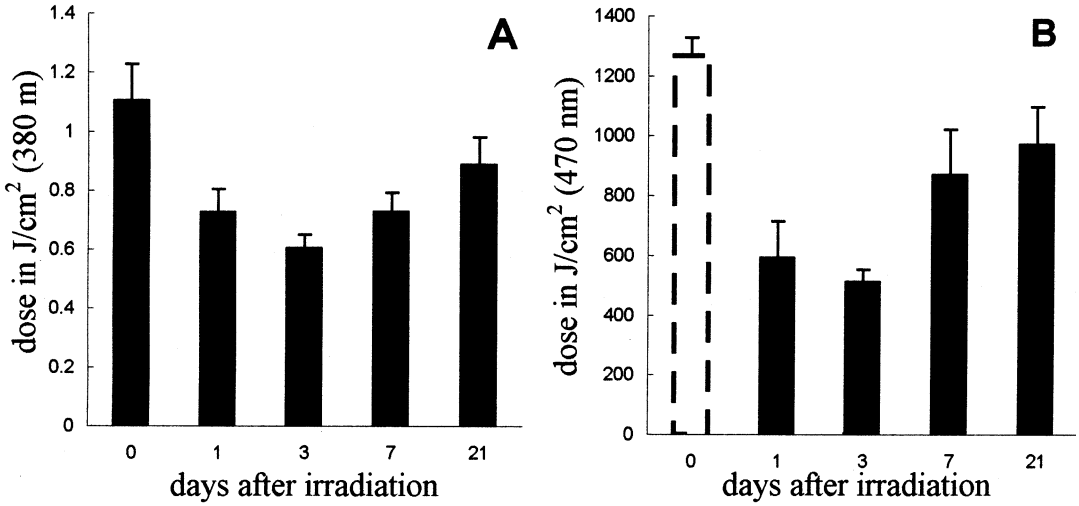

Fig. 2. Damage threshold doses (± S.E.M.) as assessed by funduscopy at different time points after irradiation at 380 nm (A) and at 470 nm (B). Only the data of the experiments with survival of at least 21 days were used in the histogram ($n = 7$). Analysis at day 0 was performed at 2–3 h after irradiation. Threshold dose for 470 nm at day 0 is the average of only two experiments (dotted bar); the other five experiments showed no fundus changes at day 0.

The rat was placed in a holder and the head was fixed at three points: both acoustic ducts and the upper front teeth. The cornea was moistened during the experiment by a continuous flow of saline (4 ml/h). The rat was positioned in the bundle using green light (517 nm) of low retinal irradiance (5–10 mW/cm^2). Both irradiations, at 380 and at 470 nm, were performed in the same eye. They were placed close to each other (20–25° distance) in the central, slightly superior part of the retina. The 380 nm irradiation was done first, followed by the 470 nm irradiation at a slightly more temporal location. Control experiments ($n = 3$) with the 380 nm irradiation as the second irradiation at the more temporal position revealed no influence of this procedure on damage sensitivity and morphology (analyzed at 3 days after irradiation). During the preparation of the animal for the irradiation the lighting conditions were dim white fluorescent light of 50 lux, which was turned off as the irradiation started. After irradiation the animals recovered in the dark for 2–3 h and were placed back in the cage in the cyclic environment of dim white light.

2.3. Analysis

Data were obtained during the first hours and at 1, 3, 7, 21, and 63 days after irradiation. The effect of the irradiation was analyzed by funduscopy and histology. The rats were anesthetized and the pupils were dilated as described above. Indirect funduscopy was performed at a low level of retinal irradiance (< 50 mW/cm^2). A filter was used to block wavelengths shorter than 500 nm. Next, the animal was either allowed to recover or was fixed for histology. The fixation procedure consisted of perfusion through the heart with phosphate buffered saline, quickly followed by the fixative of 2% paraformaldehyde and 2% glutaraldehyde in sodium cacodylate buffer, pH 7.4. The eye was removed and immersed in the same fixative overnight at 4°C. After opening the posterior chamber of the eye, the funduscopically visible damage sites could be seen using a stereo microscope. The position of the patches without visible changes were determined using the retinal vasculature as landmarks. The irradiated parts with adjacent control retina were resected and postfixed in 1% OsO_4 in buffer for 1 h. The tissue segments were dehydrated in ethanol and embedded in Epon. Semithin sections (1 μm) were collected, stained with toluidine blue and examined light microscopically. Ultrathin section were counterstained with uranylacetate and leadcitrate and examined with a Jeol electron microscope. The effects of the irradiation were detected by comparing irradiated area with adjacent control tissue within the same section.

3. Results

Data were obtained of in total 33 animals. For each time point of analysis at least three to four irradiated eyes were evaluated with histology. The animals used for the longer survival experiments were usually also examined funduscopically at earlier time points.

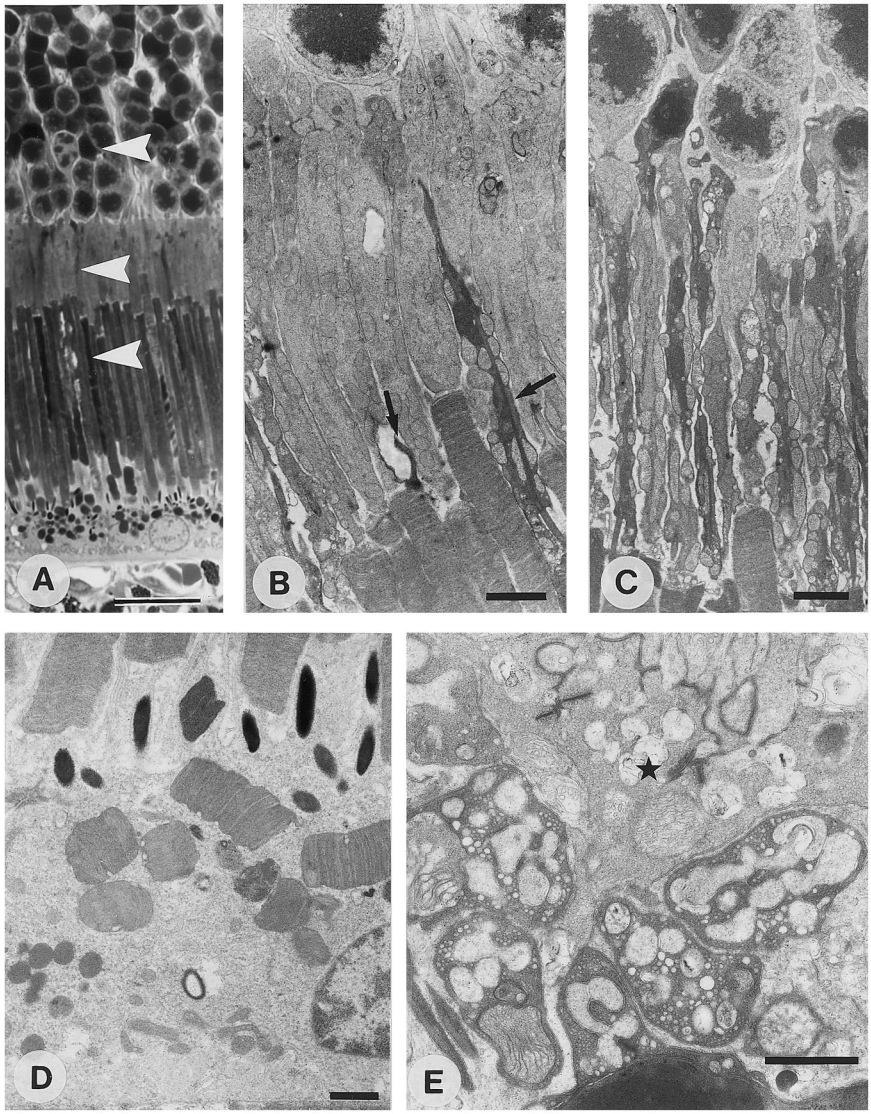

Fig. 3. Acute effects of irradiation at 380 nm. Material was collected at 3 h after exposure to 1.5 × the threshold dose (A,B) and at 5 × threshold (C,D,E). (A) Light micrograph showing dark staining (arrowheads) of a few nuclei, inner and outer segments of photoreceptor cells. (B) Electron micrograph of the inner segment layer. A few inner segments (arrows) are darkly stained, thin and have swollen mitochondria. (C) Practically all photoreceptor elements are damaged. (D) Retinal pigment epithelium is loaded with phagosomes, but appears undamaged. (E) Rod spherules have an electron-dense cytosol. Besides synaptic vesicles, several larger clear vesicles are present. Cone pedicles (*) have a normal appearance. Bars: 20 μm (A), 2 μm (B,C), 1 μm (D,E).

3.1. Funduscopy

Irradiations at 380 nm were performed at doses of 0.25–3 J/cm^2. At 470 nm much higher doses are required for funduscopic lesions (Gorgels & van Norren, 1995) and the employed dose range was 250–1350 J/cm^2. At both wavelengths fundus changes took time to develop (Fig. 1, Table 1). There were no changes in fundo immediately after exposure. A maximal brightness was reached at 3 days after irradiation and thereafter the visibility declined. In Fig. 2 the average dose for a just visible change in fundo is plotted as a function of the time after irradiation at 380 nm (Fig. 2A) and at 470 nm (Fig. 2B). The plot illustrates that for both wavelengths the lowest threshold dose is reached at 3 days after irradiation.

3.2. Histology

The histologic manifestations of both damage types were studied at various doses and time points. In the presentation of the results we focused on the development of the damage over time. The differences between the spectral types were emphasized in a previous paper (Gorgels & van Norren, 1995). Yet, to also facilitate a comparison of the damage types in the present material the following precautions were made: (1) Doses were expressed as multiples of the funduscopic threshold

dose (T). $T = 0.61$ J/cm^2 for 380 nm and 513 J/cm^2 for 470 nm. (2) The morphologic features of both damage types are summarized in Table 2. (3) In two figures the morphology of the two damage types at equal relative doses are presented side by side.

3.2.1. Acute effects at 380 nm

Histologic material was collected at 3 h after the irradiation. With light microscopy, changes were seen in the photoreceptor cells. The number of cells affected depended on the dose. No changes were seen at T (0.5–0.7 J/cm^2). At doses of $1.5 \times T$, some scattered photoreceptor cells (5–15%) were damaged (Fig. 3A,B, Table 2). At doses of $4 \times T$ practically all photoreceptors were damaged (Fig. 7A). Damaged photoreceptors had darkly stained nuclei, inner segments (IS) and outer segments (OS). Increased staining was also noticed in the outer plexiform layer (OPL). Interestingly, in severely damaged retina with all rod nuclei affected, there were still normal appearing cone nuclei present in the ONL, approximately in the same numbers as in control retina (i.e. $< 2\%$). Cone nuclei were distinguished from rod nuclei by their larger size, ovoid shape, and the multiple small clumps of heterochromatin (LaVail, 1976). In addition, the RPE showed a conspicuous increase in number of phagosomes (Fig. 7A). Other retinal layers appeared normal.

Ultrastructural analysis was performed on retina exposed to $1.5 \times T$ (1 J/cm^2; $n = 2$) and to $5 \times T$ (3 J/cm^2; $n = 3$). The irradiated area was compared with adjacent control tissue. In agreement with the light microscopic observations, the irradiation had caused damage in rods. At $1.5 \times T$ a small fraction of the rods was damaged (Fig. 3B), whereas at $5 \times T$ all were affected (Fig. 3C). Changes consisted of condensation of nuclear chromatin and cytosol. The electron-dense appearance of the cytosol was noticed in the spherules, around the nucleus and in IS. Rod spherules contained besides synaptic vesicles also several larger electron-lucent vesicles (Fig. 3E). Mitochondria often had an electron-lucent ground substance and broken cristae. Damaged IS were thin and contained many free ribosomes. The OS surface often was undulating. At the base of the OS the disks were separated by large spaces. The epithelial cells had a heavy load of phagosomes but appeared otherwise normal (Fig. 3D). Between the damaged rods in the tissue irradiated at $5 \times T$, normal appearing cones were seen with normal nuclei, cytosol and axon terminals (Fig. 3E).

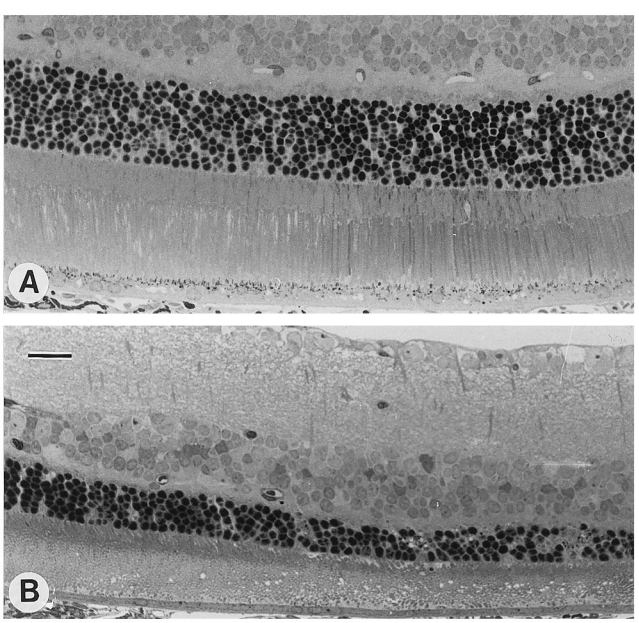

Fig. 4. Light micrographs of retina after exposure to 380 nm at $1.5 \times$ threshold. The irradiated area is on the right; control retina is on the left. (A) 3 h after irradiation. Several photoreceptors are darkly stained. (B) 2 months after irradiation: The irradiated area shows a loss of photoreceptors. Bar: 25 μm.

3.2.2. Progression of damage at 380 nm

The acute changes in the rods, appeared to indicate irreversible cell damage: In the next days light microscopy showed progressive degeneration and loss of rods. In contrast, the increase in phagosomes in the RPE was a transient feature. After 1 day the irradiated RPE did not deviate from the control. The progression of the damage was mainly studied at three doses: Low (T), moderate ($1.5 \times T$) and high ($2.5 \times T$).

3.2.2.1. Low dose (T). At the funduscopic threshold dose (0.5–0.7 J/cm^2), damage was manifested as a few scattered photoreceptors ($< 1\%$) with dark staining of the nucleus and OS at day 1–7 after irradiation. At subthreshold doses of 0.3–0.5 J/cm^2, occasionally a dark staining photoreceptor cell was also observed.

3.2.2.2. Moderate dose (1.5 T). At doses of 0.8–1.0 J/cm^2, the percentage of damaged photoreceptors rose from 5 to 15% at 3 h (Fig. 3A, B) to 10 to 30% of the irradiated photoreceptor cells after 3 days. At day 7, a loss of photoreceptor cells was apparent. At 21 days and 2 months after irradiation, the irradiated area had lost 30–40% of the photoreceptors when compared to the surrounding control retina (Fig. 4B).

3.2.2.3. High dose (2.5 T). A day after doses of 1.3–1.7 J/cm^2 most ONL nuclei had a homogeneously dark staining and the IS appeared crumbly (Fig. 5A). Phagocytes had appeared in the ONL. After 3 days, all photoreceptors except for a few cones were damaged (Fig. 6C). Debris and phagocytes were numerous in the photoreceptor layer. The RPE appeared normal or slightly swollen. After 7 days, the OS and IS had virtually all disappeared (Fig. 5B). In the ONL, the inner part still contained photoreceptor nuclei which

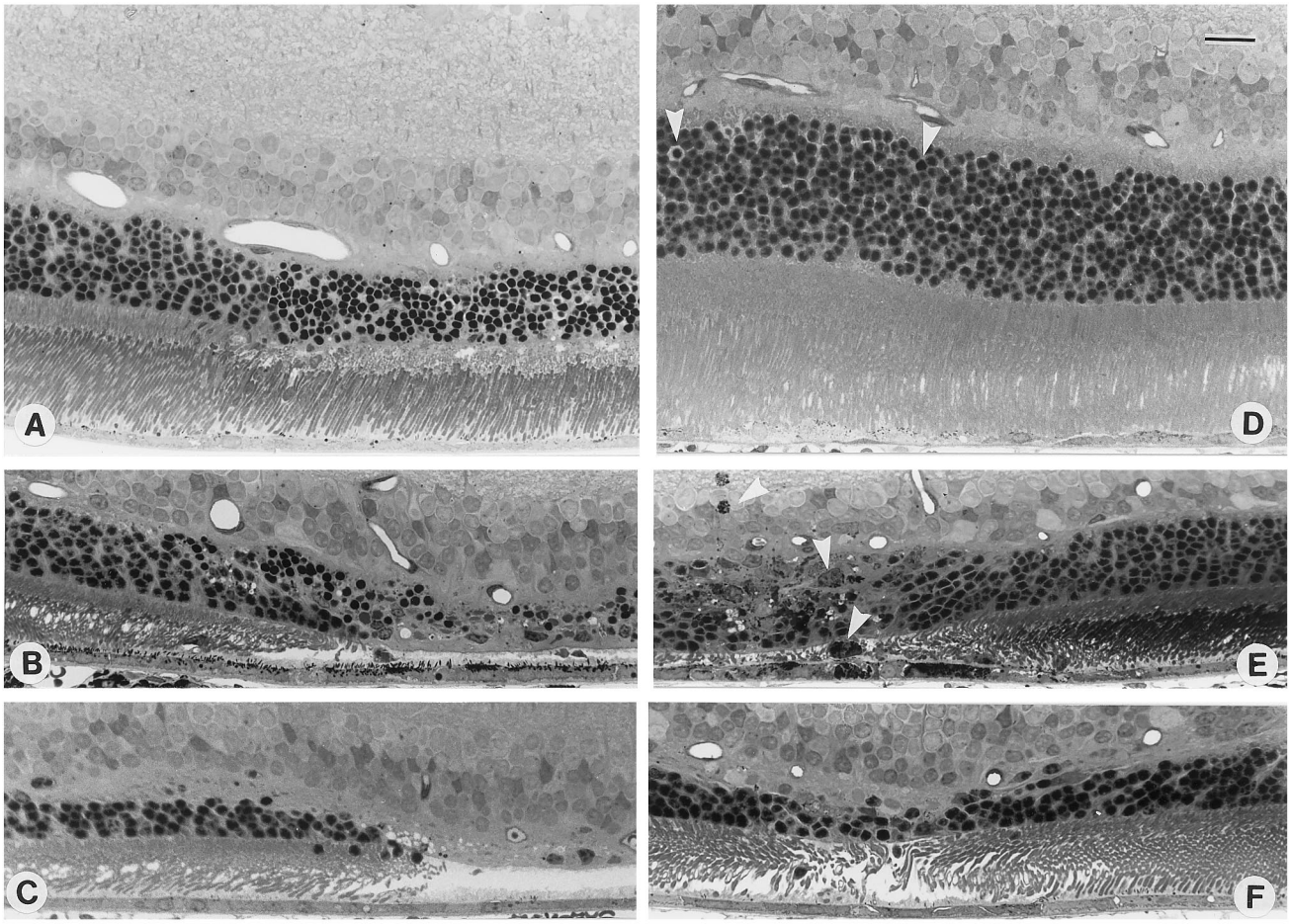

Fig. 5. Comparison of damage by 380 (A,B,C) and 470 nm (D,E,F) at doses of 2.5 × threshold. Micrographs show damage at 1 day (A,D), 7 days (B,E), 3 weeks (C) and 2 months (F) after exposure. Irradiated retina is on the central part of the figure, control retina is on the outside. After 1 day: exposure to 380 nm (A) caused dark staining of practically all photoreceptors and the photoreceptor layer is thinner than in control. The RPE is normal. Exposure to 470 nm (D) resulted in a few pyknotic nuclei (arrowheads) in the ONL. The OS layer is thicker than control. The RPE cells are swollen and lightly stained. After 1 week: exposure to 380 nm (B) caused cell loss in the outer part of ONL and dark staining of nuclei in the inner part of ONL. On the sides the outer rows of neighboring ONL nuclei have shifted into the lesion. The area irradiated at 470 nm (E) shows cell loss and an oblique orientation of columns of ONL nuclei on the sides. Debris and cigar-shaped melanin granules are found in cells in the photoreceptor layer and inner retina (arrowheads). The RPE is swollen and contains dark inclusions. At 3 weeks after irradiation at 380 nm (C) only a few ONL nuclei, possibly of cones, are left; (F) 2 months after irradiation at 470 nm, the photoreceptor layer shows cell loss in the center and oblique orientation of the columns of ONL nuclei on the sides. RPE cells stain darker than control. Bar: 25 μm.

were pyknotic, whereas in the outer part most nuclei had been removed. The columns of nuclei of the adjacent unexposed photoreceptors had obtained an oblique orientation with the outer rows of nuclei pointing into the lesion, suggesting that these nuclei had shifted into the lesion area. At 21 days after irradiation (Fig. 5C) nearly all photoreceptors had been removed. After 2 months, a lesion specific for the photoreceptors remained. The lesion area measured approximately 175 μm in diameter, whereas the irradiated area had been approximately 350 μm in diameter (Fig. 7). A few photoreceptors with cone morphology and a few degenerating photoreceptor nuclei were still present in the lesion area. On the sides the columns of neighboring photoreceptor nuclei had an oblique orientation. No damage was observed in other retinal layers.

3.2.3. Acute effects at 470 nm

Material was collected at 1 h after the irradiation. Light microscopy revealed changes in both the RPE and the photoreceptor cells in retina irradiated at 2.5 × T (1200–1350 J/cm^2; Fig. 8A): Most RPE cells and IS contained small light vesicles. A few photoreceptor nuclei showed dark staining of their chromatin. Some of these had the morphology of cone nuclei. Ultrastructural analysis of retina irradiated at 1.5 × T (900 J/cm^2; $n = 2$) and at 2.5 × T (1300 J/cm^2; $n = 2$) showed mitochondrial changes in RPE and photoreceptor cells as the most conspicuous effects. The number of cells affected depended on the dose. At 1300 J/cm^2, practically all epithelial and photoreceptor cells were affected (Fig. 8B–D). Mitochondria were swollen and had broken cristae. In addition, the RPE contained vacuoles and

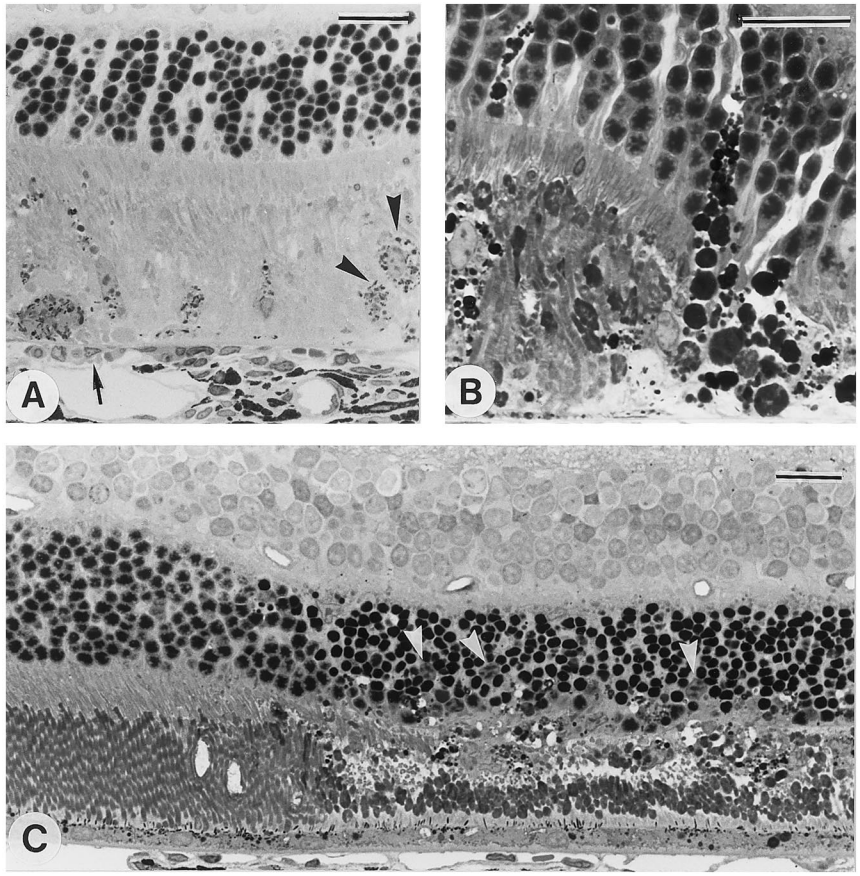

Fig. 6. Comparison of damage by 470 (A,B) and 380 nm (C) at 3 days after irradiation at a dose of 2.5 × threshold. (A) Phagocytes with cigar-shaped melanin granules (arrowheads) are present between the OS. Leukocytes are located at Bruch's membrane (arrow). (B) Columns of debris are present in the ONL and the subretinal space. On the right RPE cells are absent. (C) In the area irradiated at 380 nm (on the right) all ONL nuclei show dark staining except for cone nuclei (arrowheads). The OS are shortened. The RPE appears unaffected or slightly swollen. Bars: 25 μm.

the cytosol was slightly more condensed than in the control RPE. In the photoreceptor cells, damaged mitochondria were noticed in the IS, ONL and in the OPL, both in rod spherules and in cone pedicles. In other aspects, the cones and rods generally appeared normal. Rarely, a swollen, empty IS was seen and a few nuclei in the ONL had dense chromatin.

3.2.4. Progression of damage at 470 nm

3.2.4.1. Low dose (T). At the funduscopic threshold dose of 400–600 J/cm^2, damage was found in the RPE and in the photoreceptor layer. The RPE showed an altered distribution of the melanin granules after 1 day. The granules were not seen in villi, but had accumulated at the apical side of the cells. At day 3, RPE cells were swollen and their melanin granules were spread out in the cytoplasm. Some RPE cells contained many dark inclusions. At later time points the RPE cells did not deviate from the control. Photoreceptor damage consisted of a few ($< 1\%$) dark staining nuclei and OS at 1 and 3 days after irradiation.

3.2.4.2. Moderate dose (1.5 T). Doses of 650–850 J/cm^2 caused similar, but more severe, changes in the RPE cells and involved more photoreceptors. The RPE cells often contained small light vesicles after 1 day (Fig. 9A) and numerous dark inclusions after 3 days (Fig. 9B). In the following weeks the appearance of RPE cells returned to normal (Fig. 9C, D). Photoreceptor damage consisted of a few ($< 1\%$), scattered, dark staining photoreceptors at day 1 and 3. Phagocytic cells containing debris and typically also melanin granules were present in the OS layer. After 1 week, pyknotic cells and debris became scarce. After 2 months the photoreceptor layer in the center of the exposed area was slightly thinner than in the surrounding control retina, corresponding to a loss of up to 5% of photoreceptors.

3.2.4.3. High dose (2.5 T). At 1 day after exposure at 1200–1350 J/cm^2, some RPE cells had lost their cuboidal shape (Fig. 5D). After 3 days, the RPE monolayer was interrupted (Fig. 6B). Mitotic figures were occasionally observed in RPE cells. In the choroid, there were leucocytes at Bruch's membrane in the irra-

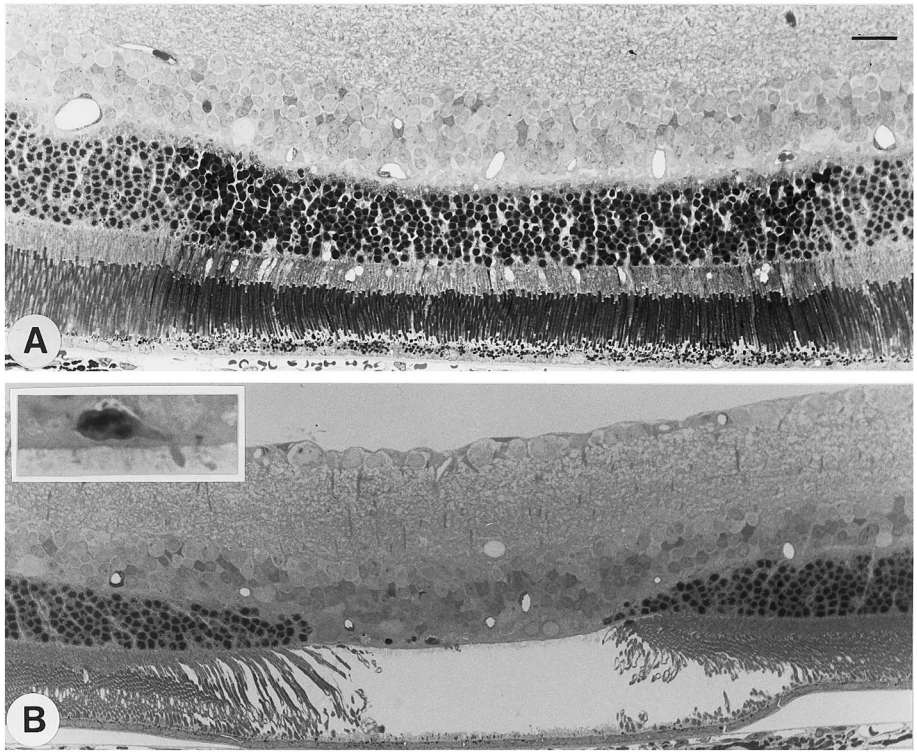

Fig. 7. Micrographs of irradiation at 380 nm, illustrating the size of the damaged area after 3 h (A) and 2 months (B): the photoreceptor lesion after 2 months is smaller than the damaged area after 3 h. Inset: Surviving ONL nucleus in the lesion shows morphology of cone nucleus. The large space between the OLM and the RPE is a preparation artifact. Dose: 4 × threshold (2.5 J/cm^2). Bar: 25 μm.

diated area (Fig. 6A). After 7 days, RPE cells were still swollen but a continuous monolayer was present (Fig. 5E). At late stages, the RPE cells had regained a normal shape, but the cytoplasm stained darker than control and some cells were hypopigmented (Fig. 5F).

The number of degenerating, dark staining photoreceptor cells was still modest (1–3%) at 1 day after irradiation. The thickness of the IS and OS layers was increased (Fig. 5D). After 3 days, there were still scattered dark staining photoreceptors, but in addition, we often observed columns of debris, suggestive of degeneration of entire columns of photoreceptor nuclei (Fig. 6B). This was observed where the underlying RPE cells were absent. Phagocytes with debris and melanin were prominent in the photoreceptor layer (Fig. 6A). Occasionally, mitotic figures were seen in the phagocytes. After 1 week, cell loss was evident, especially in the center. On the sides the columns of photoreceptor nuclei had an almost tangential orientation, suggesting that the outer rows of nuclei had moved towards the center of the irradiation (Fig. 5E). After 3 weeks, most of the pyknotic nuclei had been removed. In the center of the irradiated area, the ONL contained none or just a few photoreceptor nuclei. Towards the sides the number of photoreceptors increased as the columns of nuclei did not stand upright but were bent towards the center of the lesion. Probably these nuclei had occupied the area vacated by degenerated photoreceptors. This configuration made counts of the photoreceptor cell loss at this stage unreliable. In the inner retina, no damage was seen. Cells containing melanin granules were observed in the OS, ONL and INL. A similar picture was seen 2 months after irradiation, with a further decline in phagocytes (Fig. 5F).

4. Discussion

In photochemical processes wavelength is an important parameter. The action spectrum depends on the absorption spectrum of light energy by the chromophore. Different chromophores with different absorption spectra will cause spectrally distinct damage types. In the present study on retinal light damage we compared the effects of irradiations at 380 and 470 nm. Marked differences were found in the early course of the development of damage, while at late stages the damage types become remarkably similar in appearance. As will be discussed later in detail, both damage types resemble damage types previously reported in monkey, thus allowing for an important generalization between rat and monkey.

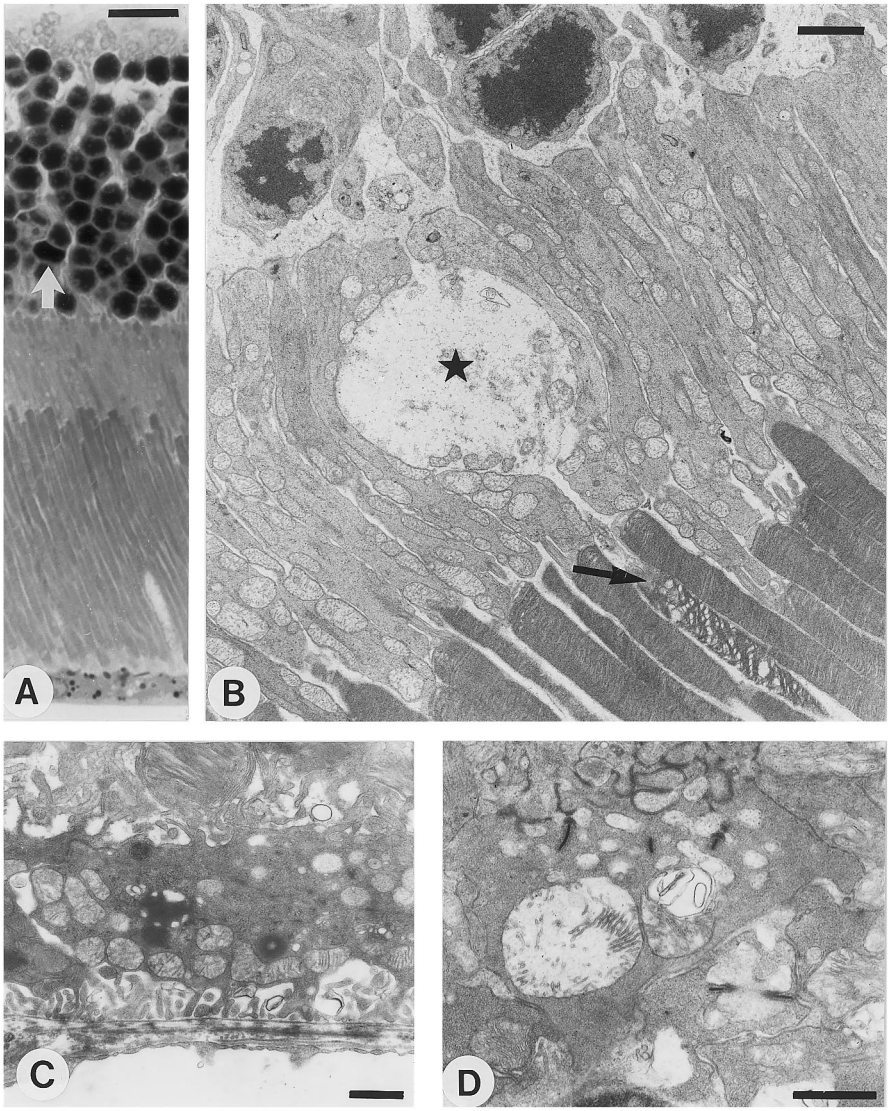

Fig. 8. Acute effects of exposure to 470 nm. Material was collected at 1 h after irradiation at a dose of 2.5 × threshold (1300 J/cm^2). (A) Light micrograph showing a darkly stained photoreceptor nucleus (arrow) and light vesicles in inner segments and pigment epithelium. (B,C,D) Electron micrographs showing swelling and disruption of internal structure in mitochondria in inner segments (B), pigment epithelium (C) and outer plexiform layer (D). In addition, a large, swollen inner segment (*) and a vesiculated outer segment (arrow) are present in B. Bars represent 20 μm (A), 2 μm (B), 0.5 μm (C), 1 μm (D).

4.1. Damage at 380 nm

At 3 h after the irradiation, the first time point of analysis, rods showed signs of damage with no damage at the other cell types in the retina. At later timepoints we witnessed the complete loss of the local rod population without distinct changes in the other retinal layers. A remarkable finding was that the small population of cones in the rat retina ($< 2\%$ (Szel & Rohlich, 1992)) was far less susceptible to intense radiation.

Other studies in rat and squirrel also described that 1 day after threshold doses of UV-A the damage was confined to the photoreceptor cells (Collier & Zigman, 1989; Rapp & Smith, 1992). For RPE damage higher doses were required (Collier et al., 1989; Rapp & Smith, 1992; Gorgels & van Norren, 1995). A clear specificity for rods has not reported before, but a study on the squirrel mentioned that at the border of the lesion, only rods were affected at day 1 (Collier & Zigman, 1989). In the monkey, Ham et al. (1982) found that irradiation at 350 nm caused degeneration of rods and cones accompanied by mild damage to the RPE. Most likely their dose was higher, relative to threshold, than our highest dose, since a funduscopic lesion was visible immediately after exposure. It leaves room for speculation that at lower doses a different susceptibility of rods, cones and RPE cells will also show in the monkey. A relative sparing of cones was also reported in light damage studies using a much different setup, i.e. long exposures of free running rats to low levels of

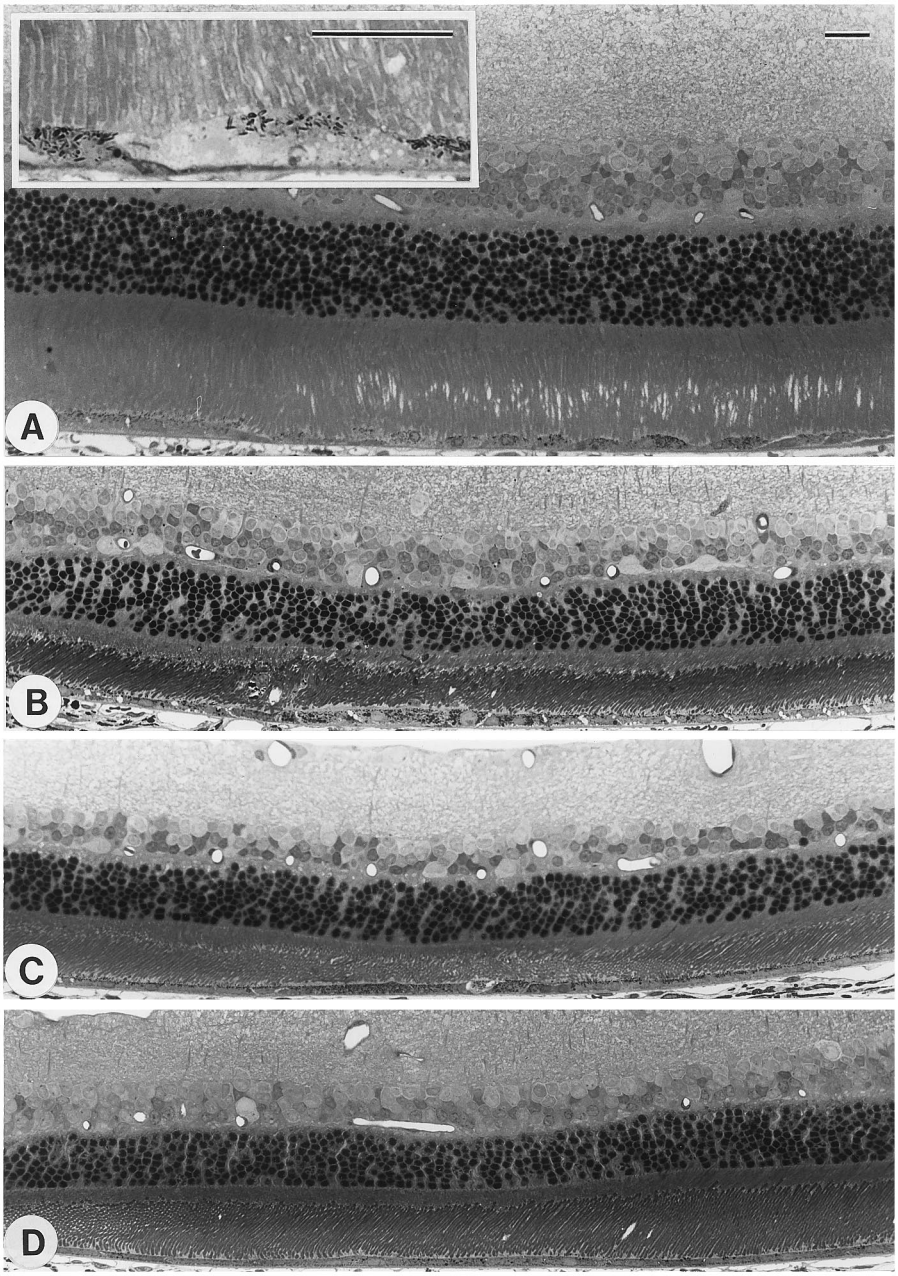

Fig. 9. Development of damage by 470 nm at a dose of 1.5 × threshold (700–900 J/cm^2). The irradiated area is in the center with control retina on the sides. (A) After 1 day: the apical RPE surface is undulating. The cells show no melanin in villi and often contain many light vesicles (see inset). (B) After 3 days: the RPE is swollen and contains many dark inclusions; phagocytes have appeared between the OS and a few photoreceptors are darkly stained. (C) after 1 week: The swelling in the RPE is reduced; OS layer is thinner than control. (D) after 2 months: The RPE shows marginal swelling. The thickness of the photoreceptor layers is slightly decreased. Bars: 25 μm.

white fluorescent light (Cicerone, 1976; LaVail, 1976). These conditions may well induce yet another type damage, but it is important to note that fluorescent light contains UV-A, which in rats readily passes the ocular media to reach the retina (Gorgels & van Norren, 1992).

With electron microscopy the damage was localized at the subcellular level. At 3 h after irradiation all cell compartments were affected and clearly the nucleus was already involved, showing condensation of the chromatin. Other studies on acute UV-A effects are scarce. Schmidt and Zuclich (1980) examined monkey retinas 1 h after high doses of 325 nm. Their basic observation of changes in the nucleus, IS and OS of photoreceptor cells agrees well with our findings.

Our findings are clearly at odds with reports (Rapp, Fisher & Dhindsa, 1994) showing that mild UV-A exposures cause mainly changes in outer segments: Damaged OS appeared pale and showed increased spacing between the disks. The damaged photorecep-

tors apparently can recover as the damaged disks are gradually replaced by normal appearing disks. In our material, damage was found in all cell compartments including OS. In areas with just a few scattered dark photoreceptors, the others appeared normal. None of the dark staining rods appeared to survive. Possibly, the origin of this discrepancy lies in a difference in exposure conditions, affecting the concentration of visual pigment. The conditions that led to specific OS changes consisted of dark adaptation before exposure and, compared to our study, a low retinal irradiance and a long exposure duration. In these conditions retinal light damage probably is mediated by rhodopsin (Noell et al., 1966; Organisciak & Winkler, 1994). Alternatively, the different findings on OS morphology may also originate from differences in fixation of the tissue. A recent study showed that the appearance of OS vesiculations in light damaged retina depends on the fixation method (Szczesny, Walther & Muller, 1996).

When the damaged photoreceptors have been removed, the irradiated area shows a thinning of the photoreceptor layer or, at high doses, a photoreceptor lesion with the other retinal layers intact. Ham et al. (1982) suggested, on the basis of their observations in the monkey, that small lesions (500 μm) might be closed in by photoreceptors from surrounding retina within a month. In the squirrel, much larger lesions of 29° were not covered by photoreceptors at 30 days after irradiation (Collier et al., 1989). In our study using a spot size of 350 μm, clearly a photoreceptor lesion persisted after 2 months. Yet, there were indications that to some extent, filling in from the sides had occurred. After 2 months, the lesion appeared smaller than the originally irradiated retinal area. In addition, there were morphological indications that as soon as damaged photoreceptors were removed, neighboring photoreceptor cells shifted into the lesion. In an ongoing study, we are currently investigating this possible capacity for redistribution of photoreceptor cells in more detail.

4.2. Damage at 470 nm

The most important finding at 470 nm was that damage involved both RPE and photoreceptors, even at threshold levels. In the photoreceptor layer, acute radiation effects (after 1 h) consisted of extensive damage in a few scattered photoreceptors, both in rods and cones. Less extensive damage, limited to the mitochondria, was observed in many of the photoreceptors cells in the irradiated area. In the RPE, similarly mitochondrial changes were prominent. These results agree well with observations of Lawwill (1982) in monkey retinas after irradiations at various visible wavelengths. He concluded that mitochondria are the structure most sensitive to anatomic change.

At low and moderate doses, the RPE cells are probably not irreversibly damaged, because in a few weeks the RPE recovers completely. At high doses, the RPE monolayer was interrupted, suggesting that some RPE cells had degenerated. Alternatively, the gaps in the RPE monolayer could also have developed by detachment of RPE cells to become phagocytic cells in the outer retina. Melanin granules were often observed in the phagocytic cells and the occasional mitoses in phagocytic cells suggest that some of them are actually epithelial cells. At late stages a continuous monolayer was observed, which may indicate that small gaps in the monolayer can be closed in by RPE cells. Yet, the RPE monolayer still showed subtle differences from control RPE: The cells were hypopigmented and had a dark staining cytoplasm.

Degeneration of photoreceptor cells by 470 nm was initially limited to a few cells scattered in the irradiated area, but 3 days after high doses, there was focally massive photoreceptor loss: an entire column of nuclei in the ONL could be degenerating with neighboring columns unaffected. We propose that there are two kinds of photoreceptor damage at 470 nm: First, there is acute damage to a few photoreceptors scattered in the irradiated area, resulting from a direct radiation effect. Second, there is local massive photoreceptor degeneration, occurring later when RPE cells are missing in the monolayer.

Whereas during the first days after the irradiation, the RPE changes were the most prominent features of damage, during the later stages the loss of photoreceptors becomes the most important feature: The RPE recovers, while a lesion or a thinning of the photoreceptor layer remains. Much similar to our findings at 380 nm, we observed that neighboring photoreceptors tend to occupy the space vacated by the degenerated photoreceptors. But again, as with 380 nm lesions, the retina showed only a limited capacity to redistribute the photoreceptors.

This damage type with prominent RPE changes has not been reported before in rat, apart from our previous study with analysis at three days after irradiation (Gorgels & van Norren, 1995). In other species a similar pathology has been described: In monkeys (Ham et al., 1978) irradiation at 441 nm caused RPE damage followed by recovery, and also a few damaged cones and rods were encountered at early stages. These findings resemble our findings at low and moderate doses. Many features of the damage we found at high doses were also induced by white light in monkeys and rabbits (Friedman & Kuwabara, 1968; Tso et al., 1972; Tso & Woodford, 1983; Hoppeler, Hendrickson, Dietrich & Reme, 1988). Apparently, white light causes in these species predominantly this type of damage, rather than the damage without RPE involvement found at 380 nm. The low transmittance of the ocular media at

short wavelengths, probably protects the retina from the latter damage type (Gorgels & van Norren, 1995).

4.3. Mechanism of damage

The early manifestations of damage at the different wavelengths were clearly distinct, indicating that different mechanisms are involved. The acute changes in the rods after 380 nm suggest that rods are directly targeted by the irradiation and that the chromophore may well be a rod specific substance. This might be rhodopsin (Noell et al., 1966) or, rather one of its photoproducts. Using data on the relation between light intensity and bleaching in the rat (Cone, 1963; Perlman, 1978), we calculated that rhodopsin was already fully bleached before the start of the irradiation by the green light that we used for aiming the beam. Electron microscopy at 3 h after irradiation, revealed no subcellular localization of the source of damage, as signs of damage found were found throughout the cell. The further degeneration and removal of the damaged rods follows in the next days without distinct changes in the other retinal layers. The RPE remained intact and no indications of invasion of leucocytes were noticed. The phagocytes involved may well be resident macrophages since they first appear in the ONL.

At 470 nm, both the RPE and the photoreceptors (rods and cones) were affected. While not excluding other possibilities (Noell et al., 1966; Ham & Mueller, 1989; Rozanowska, Jarvis Evans, Korytowski, Boulton, Burke & Sarna, 1995), the acute changes in mitochondria lend support to previous suggestions that a mitochondrial enzyme, such as cytochrome *c* oxidase is the chromophore for this type of damage (Lawwill, 1982; Pautler, Morita & Beezley, 1990). After a few days, we observed a distortion of RPE shape, the monolayer was interrupted at high doses and probably an influx of leucocytes occurred. The massive degeneration of photoreceptor cells is somewhat delayed as compared with 380 nm and it occurs where the RPE is severely damaged. The disturbance of RPE may thus be one of the factors which cause this degeneration of photoreceptor cells. At late stages, as the RPE recovers and the photoreceptor death takes its effect, the morphology of 470 nm damage becomes remarkable similar to that of 380 nm, i.e. a local loss of photoreceptor cells. Differences in details, such as presence of melanin granules in phagocytic cells and hypopigmentation and dark staining cytoplasm of RPE cells, become apparent only at close examination. In albino retina these details probably will not show at all (Rapp & Smith, 1992).

The present findings again emphasize the sensitivity of funduscopy as a means of detecting retinal damage (Gorgels & van Norren, 1995). The threshold dose for funduscopic changes was the same as or only slightly higher than that for light microscopic changes in all stages of damage development. Thus, funduscopy is a useful tool in studies of retinal light damage for instance when evaluating the protective efficacy of pharmaca. It might save animals, effort and time. It should be noted, however, that discrimination between the damage types is not possible by funduscopy alone, since the fundus appearance of the two damage types is very similar. In addition, fundus lesions are best visible during the first days. Structural damage and misalignment of photoreceptors seem to contribute substantially to the brightness of the fundus appearance. During the first days after irradiation at 380 nm, a bright fundus lesion corresponds to specific damage to the photoreceptor cells. At 470 nm, with additional RPE changes and edema, the fundus appearance is chalk-white. In late stages, when damaged photoreceptors have been removed and none or just a few are left, fundus changes are barely visible and could easily be overlooked.

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