Long-Term Effects of Light Damage on the Retina of Albino and Pigmented Rats

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PURPOSE. To observe the morphology and physiology of the retina in rats 11 weeks after a constant (24-hour) but moderate (500-lux) illumination for 1 week.

METHODS. Levels of aspartate, γ-aminobutyric acid (GABA), glutamate, glutamine, and taurine were measured by high-pressure liquid chromatography (HPLC) in the retina and vitreous humor in rats of both albino and pigmented (Long-Evans) strains. Semithin sections were used to determine retinal morphology. The TUNEL method was used to detect cells degenerating by apoptosis. Because the GABAergic system has been shown to be particularly sensitive to the loss of photoreceptors, an additional immunohistochemical study using anti-GABA, anti-glutamate decarboxylase (GAD)67, and anti-GAD65 antibodies was performed.

RESULTS. No apparent morphologic changes were found in the retina of pigmented rats after constant illumination, whereas in albino rats disappearance of photoreceptors (except in the extreme retinal periphery) and cell bodies was observed. A significant number of TUNEL-positive nuclei also occurred in the remaining nuclear and ganglion cell layers. However, no change in the distribution of GABA, GAD67, and GAD65 immunoreactivities was found in either strain under constant illumination compared with control animals. Constant illumination affected the retinal levels of aspartate, glutamate, glutamine, glycine in both strains, whereas GABA contents did not change but taurine was decreased only in albino rats. A significant increase of vitreal glutamate levels was also found in both strains and of taurine levels only in albino rats.

CONCLUSIONS. Phototoxicity can provoke durable retinal alterations beyond the period of lighting, suggesting progressive and probably continuous modifications of retinal physiology, even in pigmented animals in which the retina seems morphologically normal. (Invest Ophtalmol Vis Sci. 2002;43:813–820)

Constant illumination damages the pigment epithelium and the neural retina.1–3 In severe cases, photoreceptor degeneration by fragmentation of DNA and apoptotic mechanisms occurs.4–6 Damage is thought to be initiated by a dysfunction in the phototransduction cascade after constant illumination2,8 and can appear after short exposure to high light intensities or after exposure to relatively low light intensities over longer periods.7,9 The capacity of photoreceptors to recover from light damage is enabled when only their inner and outer segments are affected,10,11 but when nuclear damage occurs, degeneration becomes definitive. Although it has been suggested that light damage is initiated by overactivation of the phototransduction cascade, the degree of alteration depends on the number of melanosomes concentrated in the epithelial cells of the iris and retina. Ocular melanin may protect the retina, probably by the absorption of light.11–12 Because of this, albino rat retinas are particularly sensitive to the deleterious effects of artificial constant light, whereas several months of light exposure seem unable to produce morphologic damage in the retinas of pigmented animals.13,14 In most cases, such observations have been made immediately or shortly after cessation of illumination. Whether the degenerative processes continue for longer periods has seldom been investigated.

The present study focused on the possible long-term continuation of morphologic and biochemical damage in albino and pigmented rat retinas after exposure to moderate (500 lux) but continuous illumination for 1 week. Biochemical damage was assessed by variation, at the retinal and vitreous levels, of several neuroactive amino acids: aspartate, glutamate, glutamine, glycine, taurine, and γ-aminobutyric acid (GABA). We observed that rats killed 11 weeks after the end of constant illumination showed dramatic morphologic and/or biochemical retinal modifications. We confirmed findings in previous studies that demonstrated the disappearance of photoreceptors, over a large percentage of the retinal surface. We show that, almost 3 months after the light exposure, cellular degeneration persisted, as suggested by the presence of TUNEL-positive nuclei in inner retinal layers in albino rats, and a dysfunction in amino acid metabolism, not only in albino but also pigmented rats in which the retinas seem morphologically normal.

MATERIALS AND METHODS

Animals

All experimental procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of the Institut National de la Santé et de la Recherche Médicale (INSERM) committee on Animal Research. Two-month-old rats (five males and five females) of both Wistar and Long-Evans strains, were purchased from Janvier Breeding Facilities (Le Genest St-Isle, France). On arrival, they were housed separately in clear plexiglas cages and exposed to continuous light by two 20-W white fluorescent bulbs (Oslam L; Osram Sylvania, Munich, Germany) suspended 30 cm above the cages at an illumination of 500 lux. Animals had free access to food and water. After continuous illumination, rats were kept in a temperature-controlled room in 12 hours of light (25–30 lux) and 12 hours of dark for 11 weeks. The results were compared with those obtained in rats of the same age, sex, and strains maintained under the latter conditions for 12 weeks.

All rats were killed by decapitation between 10 AM and noon to avoid possible circadian fluctuations in the metabolism of amino acids.
One eye was collected for biochemical measurements and the other for semithin sectioning, immunohistochemistry, or demonstration of presumptive apoptotic cells by the TdT-mediated dUTP-digoxigenin nick-end labeling (TUNEL) method.

**Amino Acid Assays**

Five animals for each strain and sex were used. One retina (vitreous body as retinal pigment epithelium was carefully peeled from the retina) and vitreous body were collected separately from each animal. Taurine, glutamate, aspartate, glutamine, glycine, and GABA concentrations were determined within the retina and corresponding vitreous body by high-pressure liquid chromatography (HPLC) with a fluorescence monitor (Merck, Darmstadt, Germany). The amino acids were chosen for their major function in the retinal physiology and preferential localization in retinal cells. Glutamate, and possibly aspartate, are neurotransmitters located in various retinal cells, including photoreceptors; glutamine, which is a precursor and a degradative product of glutamate, is found in Müller cells; taurine, with a function that remains poorly known (it may be involved in the control of osmolarity and in the protection of photoreceptor membrane), is synthesized in cells located in the inner retina and taken up by photoreceptors; and GABA, which is an inhibitory neurotransmitter located preferentially in amacrine and inner plexiform cells.15-16 Individual retina and vitreous bodies were homogenized in 10 M hydrochloric acid (HCl): 1 mg sample in 100 mL HCl, centrifuged at 15,000 g for 10 minutes at 4°C. Twenty microliters of supernatant of vitreous bodies and 10 mL of retinas were used for the determination of amino acid concentrations. The stock reagent contained 27 mg of amino acid concentrations. The stock reagent contained 27 mg o-phthalaldehyde (OPA), 1 mL methanol, 5 mL β-mercaptoethanol, and 9 mL 0.1 M sodium tetraborate. The working solution was prepared by diluting the stock solution with 0.1 M sodium tetraborate (1:5 vol/vol). Precolumn derivation was performed by mixing 20 mL (vitreous bodies) or 10 mL (retina) sample volumes with working strength OPA. Separation of the different amino acids was achieved by reverse-phase liquid chromatography (column AccQTag, Waters Chromatography, Guayancourt, France). The mobile phase (pH 7.8), with a flow rate of 1 mL/min, consisted of water-acetonitrile-methanol containing 0.1 M dibasic sodium phosphate buffer. Excitation and emission wavelengths were set at 340 and 450 nm, respectively. Results were expressed in nanomolar per milligram protein estimated by the Lowry method.17

Variations in the extracellular pool of amino acids and changes in their retinal reuptake mechanisms due to the lighting conditions were estimated by the ratio of amino acid content in the vitreous and retina.

Descriptive statistics were provided by Icare (Massy, France). They were computed on the original variables—that is, concentrations in the retina and the ratio between concentrations in the vitreous humor and retina of each amino acid. The original measurements were log transformed to stabilize and to equalize the variances of the studied variable. Cross analyses of variance for 2 X 2 X 2 factorial experiments were used to estimate the second- and third-order interactions and the effects of rat strain, sex, and light. When second- and third-order interactions were not significant, they were removed from the model, and then the principal factors were tested. Statistical significance was concluded when P < 0.01.18

**Immunohistochemical and TUNEL-Staining Procedures**

With both techniques, eyes were collected and fixed by immersion in 0.1 M phosphate-buffered saline (PBS, pH 7.4), containing 4% paraformaldehyde. For immunohistochemistry, two eyes for each experimental condition were rinsed in PBS containing 20% sucrose and embedded in optimal cutting temperature (OCT) compound (Tissue-Tek; Sakura Finetechnical, Zoeterwoude, The Netherlands). Sections 10-μm thick were cut with a cryostat (Bright Instrument Co., Huntington, UK) at −20°C and mounted on gelatin-coated slides. Sections were incubated in 1:1 methanol-H2O2 containing 3% H2O2 mixture for 5 minutes to remove endogenous peroxidases. They were then incubated in PBS containing 0.01% Triton X-100 and 0.2% gelatin (PBSTG) for 30 minutes. The sections were incubated with either a rabbit polyclonal anti-GABA antibody (Sigma-Aldrich Chimie, Lyon, France) diluted 1:9000, or antibodies direct against its synthesizing enzymes: glutamate decarboxylase (GAD67) and GAD65. The rabbit polyclonal anti-GAD67 (Chemicon, Euromedex, Souffleweserheim, France) was diluted 1:2500, and the rabbit polyclonal anti-GAD65 (Chemicon, Euromedex) was diluted 1:3000. All antibodies were diluted in PBSTG and incubated for 24 hours at 4°C. The immunoreactivity was demonstrated using 3,3′ diaminobenzidine (DAB) as a chromogen. The sections were dehydrated and mounted in prehardened medium (Eukitt; Kindler; Freiburg, Germany). Control experiments were performed by omitting the primary antibody; the other steps of the procedure were the same. In this case, no labeling was observed.

For the TUNEL method, two eyes for each experimental condition were embedded in paraffin, and 1-μm thick mid sagittal sections were obtained. TUNEL-positive nuclei were detected by a kit (Apoptag; Oncor, Gaithersburg, MD). Endogenous peroxidases were inactivated by incubating sections in H2O2. Sections were preincubated in the equilibration buffer for 30 seconds at room temperature and were then treated with terminal deoxynucleotidyl transferase (TdT) and digoxygenin deoxyuridine triphosphate (dUTP) for 1 hour at 37°C. They were rinsed in buffer for 30 minutes at 37°C. Retinas were incubated with a peroxidase-coupled anti-digoxigenin antibody for 30 minutes at room temperature. The 3′-OH DNA tail was detected by incubating retinas with a DAB-H2O2 solution and were stained with methyl green for 10 minutes. Control samples were made by omitting TdT during the first step of the procedure. No labeling was observed in control sections.

**RESULTS**

**Presumptive Apoptotic Cells**

TUNEL-labeled nuclei were rarely found in the retinas of control and light-exposed Long-Evans and control Wistar rats. Far more numerous TUNEL-positive nuclei were observed in the retinal nuclear layers of light-exposed Wistar rats. Most cells were found either in the remaining outer nuclear layer or in the distal and proximal parts of the inner nuclear layer. More rarely, some labeled nuclei were present in the ganglion cell layer. Apoptotic cells were observed only occasionally in control rat retina (Fig. 1).

**Immunohistochemical Studies**

GABA-, GAD67-, and GAD65-immunoreactive profiles were observed in the inner nuclear, inner plexiform, and ganglion cell layers in control albino and pigmented rats (Fig. 2). In such animals, the distribution and the intensity of immunolabeling varied according to the retinal area observed and, in part, to methodologic considerations. However, when compared with light-exposed animals, no apparent changes in the distribution of GABA-, GAD67-, and GAD65-immunoreactive profiles were observed in spite of the loss of photoreceptors in albino rats (Fig. 3).

**Biochemical Assays**

Because for each amino acid except taurine, neither the rat strain nor sex was a significant factor, results were pooled, and light effects were tested on the totality of the animals (i.e., on control and light-exposed groups). For taurine, because the second-order interaction for rat strain × light was significant, analysis was conducted separately for pigmented Long-Evans and albino Wistar rats.

The retinal content of amino acids tended to decrease in continuously light-exposed animals, compared with control rats, except for GABA and taurine. The level of GABA (Fig.
4a) was not significantly changed by the light conditions, and the level of taurine (Fig. 4b) was decreased only in albino rats.

Because the vitreous body is thought to be the recipient of neuroactive substances not taken up by the retina,19 it seemed appropriate to determine the ratio between retinal and vitreal levels for each amino acid. Except for glutamate, for which an increase in the ratio was observed in continuously light-exposed animals (Fig. 5a), and taurine, for which an increase of the ratio was observed in light-exposed albino rats (Fig. 5b), no significant difference was observed between experimental and control animals.
Exposure to constant illumination has been used as a model to study the effects of photoreceptor loss in retinal diseases, such as vitamin A deprivation or retinitis pigmentosa. It is well known that exposure to constant illumination, even at moderate intensities, can provoke retinal damage, often accompanied by the destruction of photoreceptors. However, whether the deleterious effects cease immediately after the animals are replaced in normal lighting conditions has seldom been studied. We showed that at least 11 weeks after the end of a period of moderate illumination, retinas continue to present important morphologic and/or biochemical changes. As observed by others, we found that the outer nuclear layer is almost totally lacking in the retina of albino rats, although several photoreceptors persist at the extreme periphery. We also show that cellular degeneration (probably by apoptosis, as suggested by the TUNEL method) continues to occur within the remaining inner layers. A decrease in the number of nuclei in the inner nuclear layer has already been reported in the rat retina under constant illumination. Although the type of degenerating cells remains to be determined, it can be suggested that they are residual photoreceptors and/or second-order neurons. Furthermore, such a loss of second-order neurons has already described in the degenerative retina in Royal College of Surgeon (RCS) rats. In this case, the degeneration could be explained, either by the loss of synaptic input because of the disappearance of photoreceptors and its consecutive failure to release trophic factors and neurotransmitters, or by the fact that the lighting conditions in which the rats were maintained...
after the experiment (12 hours of light at 25–30 lux and 12 hours of dark) do not alter the retina in control animals but provoke cellular degeneration in more fragile RCS retinas. The retinas of pigmented rats exposed to the same continuous illumination did not exhibit visible morphologic changes. It is possible that cellular degeneration may occur after constant light exposure, although it was not visible after 11 weeks. Obviously, some protection exists in the retina of pigmented rats, probably due to melanin pigment in the iris and retinal pigment epithelium (RPE). The pigmented iris is thought to limit the effect of constant illumination, and pupillary dilatation is generally necessary to induce cellular degeneration in the retina of pigmented species. However, the protective role of melanin in RPE is controversial. It seems dependent on the light’s intensity, because it becomes cytotoxic by producing free radicals at high light intensities. We cannot exclude the influence of genetic factors in explaining the protection against light, because the degree of retinal susceptibility varies between two strains exposed to the same light intensity.

Except for GABA in both strains and taurine in pigmented rats, the exposure to light provoked a reduction in the content of retinal amino acids examined, not only in albino but, more surprising, in pigmented rats. Aspartate and glutamine retinal levels are decreased, whereas their proportional levels in the vitreous body do not vary, suggesting that their turnover remains unchanged. Aspartate is an amino acid located in almost all retinal cells (including rods), predominantly in ganglion cells. Its function remains unclear, but it is often accumulated in the same cells as glutamate. A neurotransmitter action has sometimes been proposed but, even if it is able to activate a subpopulation of glutamate receptors (N-methyl-D-aspartate [NMDA]-sensitive), it seems more likely to be involved in the cellular intermediary metabolism. The reason for the reduction in aspartate levels remains to be determined, but it cannot be fully explained by the disappearance of rods, because the percentage reduction remains approximately the same in illuminated albino rats (without photoreceptors) as in pigmented rats.

Figure 4. Comparison of amino acid concentrations (in nanomoles per millimeter protein) in the retina of control and 500-lux-exposed animals. (a) Because there was no significant difference between strains (albino and pigmented) and sexes, results were pooled for aspartate, glutamate, glutamine, glycine, and GABA (20 animals compared for each condition). The concentrations of all amino acids except GABA, were decreased in 500-lux-exposed animals. (b) Light-exposure and rat-strain effects were demonstrated for taurine; a significant decrease in content was found only in 500-lux-exposed Wistar rats (10 animals compared for each condition). **P < 0.01, ***P < 0.001.
which a loss of photoreceptors is not evident). Glutamine is synthesized by glial Müller cells and used as a precursor and indirect product for glutamate. In a previous study, the existence of Müller cell gliosis was observed in the retina of light-exposed albino rats.\textsuperscript{34} Although such gliosis was not determined in the current study, alterations in Müller cells may exist and influence the functional and biochemical equilibrium of the retina. The decrease of glutamine may also be explained by dysfunction of the glutamatergic metabolism. Indeed, glutamate is the neurotransmitter used in the principal retinal chain including photoreceptors,\textsuperscript{15,35} and it is also neurotoxic when its receptors are overstimulated, as in ischemia, for instance.\textsuperscript{56} In light-exposed animals, these cells cannot use this amino acid, provoking in turn a reduction in the synthesis of its precursor-degradation product glutamine, with no change in its turnover, as suggested by the steadiness of its proportional level in the vitreous humor. Retinal glutamate levels are also decreased by light exposure. Such a decrease may be explained, at least partially, by the loss or by the alteration of photoreceptors, which become unable to synthesize it. Moreover, as suggested by the increased glutamate levels in the vitreous humor (except in light-exposed male Long-Evans rats), the release and/or reuptake is probably also modified.

Light provoked a significant decrease in retinal taurine levels, only in light-exposed Wistar rats in which photoreceptors clearly degenerated. Taurine is present in high concentration

![Figure 5](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933592/)
in the retina\textsuperscript{15–17} and appears to be synthesized mostly in cells located in the inner retina (probably in the ganglion cells) before being transported to the photoreceptors.\textsuperscript{58} The decrease in taurine previously reported in light-exposed albino rats\textsuperscript{59} can be easily explained by the loss or by the alteration of photoreceptors. However, its synthesis in the cells of the inner retina may continue, as demonstrated by the increased levels in the vitreous.

Glycine and GABA are inhibitory neurotransmitters in the retina.\textsuperscript{15} They are located in interneurons in the inner nuclear and ganglion cell layers, and glycine is particularly involved in rod circuitry.\textsuperscript{60} The decrease observed in retinal glycine levels suggests that light induces neuronal dysfunction in the retina in addition to photoreceptors. Why the alteration in the glycergic system was particularly dramatic in light-exposed male rats remains to be determined, but because no significant changes in glycine levels were observed in the vitreous humor, the regulation in glycine metabolism may be only slightly altered (as for aspartate and glutamine). GABA levels were statistically unchanged by light exposure, even when photoreceptors had disappeared. Immunohistochemical studies confirmed the biochemical results: no apparent changes in immunolabeling patterns were observed in light-exposed animals. Consequently, the GABAergic system may be very resistant to light damage and not modified by the loss of photoreceptors. Thus, it is possible that the disappearance of GABA immunoreactivity in dystrophic Abyssinian cats\textsuperscript{41} may have no direct correspondence with loss of photoreceptors, but rather with the disease itself.

Finally, we showed that even though the photoreceptors were not necessarily massively destroyed by constant light exposure, as in pigmented rats, retinal physiology was durably, perhaps permanently, affected. These alterations, which are probably due to cellular degeneration that is less extensive than in albino rats, could also reflect a biochemical adaptation to the initial lighted conditions.

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References


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