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Visual opsins: Physiological alteration promoted by led light

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ABSTRACT

Rods are the most sensitive cells to light present in the retina, being therefore responsible for dim light vision. Light photons captured by the retina stimulate rhodopsin, promoting phototransduction mechanisms that end up sending the information to the brain. However, overexposure to light and continuous receptor stimulation may promote retinal damage. Thus, artificial light might have harmful effects on the retina, most particularly in rods. Light-emitting diodes (LEDs) are nowadays the most used devices, and therefore their potential damage to the visual system should be evaluated and considered as a potential environmental factor in retinal degeneration. Particularly in Wistar rats, tonic receptors stimulation under constant light exposure (LL) produces retinal remodeling, inducing classical photoreceptors death and a re-location of non-classical opsins.

This work aims to show the effects of LED devices on rat retinas under intermittent stimulation. Wistar rats were exposed to white LED under 12:12 light/dark cycles for seven days (LD) to finally analyze the number of photoreceptors nuclei, electroretinograms (ERGs) activity, and glial activation. Our findings demonstrate that animals exposed to LED devices, even when they have intermittent periods of rest in darkness, present early retinal injury after seven days, compared with animals maintained in housing conditions (LDR) or darkness (DD). Altogether, these results suggest that extended LD conditions might induce retinal damage as constant light exposure (LL) does.

1. Introduction

The retina is the specialized part of the central nervous system that captures light photons and sends the information to the brain [1]. Rods are more sensitive to light than cones, being therefore responsible for dim light vision. Photons activate rhodopsin, a G-protein coupled receptor, resulting in a light-evoked response called phototransduction [2]. It has already been shown that overexposure to light and the consequent continuous receptor stimulation promotes retinal damage [3]. Retinal degeneration depends on the wavelength, intensity, and time of exposure used, and therefore it is difficult to compare the results obtained with different models [4]. As early as 1966, Noel and coworkers observed retinal deterioration induced by low-intensity white light [5]. Later work demonstrated almost the same effects in different low-intensity light exposure protocols, observing progressive retinal injury with deterioration of rod ultrastructure, changes in ERG signals and a reduction of the photopigment rhodopsin [6–8].

Within the visible spectrum, blue light is the most damaging wavelength for the retina, promoting retinal damage with induction of oxidative stress, photoreceptors death, and ERG changes at shorter times than those necessary to produce the same effects under white light exposures [9–13].

Although LED devices are cheap and energy-efficient, they have a great component of blue light (wavelengths between 460 and 500 nm), and because of this, they can be harmful to human vision [14,15]. In addition, LEDs are the major domestic and public light sources and are also present in computers, tablets, cell phones, and game consoles, resulting in high artificial exposure of the visual system. Altogether, this means that nowadays we are far more exposed to blue light than twenty years ago, and their users are not instructed about the possible harmful effects of new lighting devices. For this reason, we are convinced that this potential damage to the visual system should be studied since artificial light exposure could be an important environmental factor that might be influencing physiological processes and promoting retinal aging, or accelerating genetic diseases such as retinitis pigmentosa.

On the other hand, LED devices promote retina free radical

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production, apoptosis, and necrosis of photoreceptor cells under high-intensity stimuli [16,17]. Because we are mostly interested in studying the effects of low-intensity light, we have previously developed a retinal damage model by exposing adult Wistar rats to constant white LED sources for 1 to 8 days at 200 lux (LL1 to LL8). Using this model, we found significant photoreceptors cell death after 7 days of constant light exposure (LL7). Under these conditions, the levels of a phosphorylated form of the photopigment Rhodopsin (phospho-Ser ^{334}) are higher than in control animals, indicating changes in the regulation of the phototransduction cascade [1]. Furthermore, we found increased levels of reactive oxidative species (ROS) and superoxide generation in the outer nuclear layer (ONL) after 5 days of exposure, indicating that one of the involved mechanisms in retinal degeneration may be mediated by oxidative reactions. We have concomitantly demonstrated that after five days (LL5) there is a reduction of the fatty acid docosahexaenoic acid (DHA), a major component of the external segment of rod cells, suggesting that the outer membrane segment of rod cells was exposed to DHA oxidation [18]. All these results suggest a dysregulation of physiological processes in photoreceptors cells, rods, and cones, which may lead to death after 6–7 days of constant light exposure (LL6-LL7). Conversely, in the inner retina, more specifically in the retinal ganglion cell layer (RGCs) and inner nuclear layer (INL), cell survival was not affected by LED sources. However, we found significant changes in the localization and protein expression of melanopsin (OPN4) and neuropsin (OPN5) after constant light exposure, indicating a compensatory mechanism of protection against the excess of light [19]. All these findings suggest that tonic receptors stimulation produces retinal remodeling, characterized by classical photoreceptors death and a re-location of non-classical opsins. This short communication aims to report retinal injury under alternating day/night exposure to LED sources at 7 days of stimuli, a time in which we have previously described a significant reduction in ONL accompanied by functional and tissue alterations under constant exposure conditions [1,19].

2. Methods

2.1. Animals

All animal procedures were performed in accordance with the ARVO statement for the use of animals in ophthalmic and vision research, which was approved by the local animal committee (School of Chemistry, UNC, Exp. 0007526/2018). Male adult Wistar rats (12–15 weeks), inbreed in our laboratory for 5 years, were exposed to 12:12 h light-dark cycle at 50 lux of fluorescent lamp; from zeitgeber time (ZT) 0 to 12 from birth until the day of the experiment. Food and water were available *ad libitum*.

2.2. Light exposure

Wistar rats were exposed to 12:12 h light-dark cycle (LD) at 200lux of intensity in boxes equipped with LED lamps (EVERLIGHT Electronic Co., Ltd. T-13/4 3294-15/T2C9-1HMB, color temperature of 5500 K) located in the inner upper surface and temperature-controlled at 24 °C. At rat's eyes level the intensity of light in lux was measured with a light meter (Model 401036; Extech Instruments Corp., Waltham, MA, USA). After 7 days of light treatment the animals were euthanized in a CO_2 chamber at ZT 6. As control groups we used animals maintained in housing conditions to 50 lux fluorescent light (LDR) or kept in constant darkness (DD) for 7 days. For high-intensity exposure experiments, rats were exposed to 3000 lux for 3 h. Light intensity level was measured as mentioned before.

2.3. Outer nuclear layer analysis

The retinal fixation method, sectioning and nuclear quantification were done as previously described [18]. Briefly, rat's eyes were fixed overnight at 4 °C in 4% (W/V) paraformaldehyde in 100 mM sodium phosphate buffer (PBS, pH 7.3), cryoprotected in sucrose and mounted in an optimal cutting temperature compound (OCT; Tissue- Tek Sakura). Retinal sections were cut along the horizontal meridian axis (nasal--temporal). The sections were stained with 1% Hoechst (33258 Sigma Aldrich) for 5 min and photographed using a confocal microscope (Olympus FV1200, Japan) at 40x magnification. The number of ONL nuclei was counted in 4 sections per retina. In each section, 4 pictures were taken in 4 different areas: – left, middle left, middle right and right –. 10 different animals per treatment were analyzed. Quantitative ONL analysis was performed in each picture on the entire ONL (width and length of the micrograph), using the software ImageJ (v. 1.45), and the plugin "Automatic Nuclei Counter" [1].

2.4. Electroretinograms (ERGs)

The scotopic ERGs analysis were performed as previously [18] using an ERG equipment (Akonic BIOPC, Buenos Aires, Argentina). Briefly, three-month-old animals were anesthetized with an intraperitoneal injection of xylazine hydrochloride (2 mg/kg König, Argentina) and ketamine hydrochloride (150 mg/kg, Brouwer, Argentina) and adapted to dark for 20 min. Pupils were dilated with tropicamide (1% Alcon Laboratories) and, in order to prevent eye dehydration and allow electrical contact activity when the electrode is recording, the cornea was irrigated with proparacaine hydrochloride (0.5%, Alcon Laboratories). Both eyes were recorded simultaneously after applying flashes of white light (5 ms, 0.1 Hz) from a photostimulator setting at maximum brightness (3 cd s/m^2 without filter). Then, the recordings were amplified and filtered (1.5 Hz low-pass filter, 300 Hz high-pass filter, notch filter activated). An average of 10 responses for each eye was recorded. The amplitude of the a-wave was measured from the baseline to the peak of the a-wave, and the amplitude of the b-wave was measured from the a-wave valley-to-the peak of the b-wave. The mean of a-wave and b-wave peak latencies and amplitudes of the responses from LD and LDR groups were compared. 15 different animals per treatment were analyzed

2.5. Western blot

From each rat, the whole retinas were dissected, placed in 200 µl PBS buffer containing protease and phosphatases inhibitors, lysed by repeated cycles of ultra-sonication and the total protein content was determined by the Bradford method [20]. Then, the homogenates were resuspended in sample buffer [(SB: 62.5 mM Tris HCl pH 6.8; 2% (W/V) SDS; 10% (V/V) glycerol; 50 mM DTT; 0.1% (W/V) bromophenol blue)] and heated at 90 °C for 5 min. The proteins (25 µg) were separated by SDS-gel electrophoresis on 10% polyacrylamide gels, transferred onto PVDF membranes, blocked for 1 h at room temperature with blocking buffer consisting of 5% (w/v) skim milk in washing buffer (PBS containing 0.1% Tween-20; Sigma, P1379), and then incubated overnight at 4 °C with antibody against Glial Fibrillary Acidic Protein (GFAP, Cat. No. G9269, Sigma–Aldrich Co., St. Louis, MO, USA, dilution 1:500); anti-α-Tubulin (T6199, Sigma) or rhodopsin antibodies (Ret-P1 Sigma) antibody diluted 1:1000 in blocking buffer in the incubation buffer (2.5% (W/V) skim milk and 0.1% (V/V) Tween-20 detergent in PBS). Then membranes were washed three times (15 min each wash) in washing buffer and incubated with the corresponding secondary antibody (Goat antirabbit IRDye ® 700CW or Goat anti-mouse IRDye ® 800cW, Odyssey LI-COR) in PBS for 1 h at room temperature, followed by three washes (15 min each wash) with washing buffer. Membranes were scanned using an Odyssey IR Imager (LI-COR Biosciences) and the quantification of the protein bands was performed by densitometry using the FIJI / Image J program (NIH).

2.6. Immunohistochemistry

The methods for fixation, embedment, sectioning, and histological analysis of eyes were done as previously described [1]. Briefly, after exposure, whole rat eyes were fixed in 4% (w/v) paraformaldehyde (PFA) in phosphatebuffered saline (PBS, pH 7.4) overnight at 4 °C, cryoprotected in sucrose and mounted in optimal cutting temperature compound (OCT; Tissue- Tek® Sakura). 20 μm-thick retinal sections were obtained along the horizontal meridian (nasal-temporal) using a cryostat (HM525 NX-Thermo Scientific). Sections were washed in PBS and permeabilized with PBS 0.2% (v/v) Triton X-100 (Sigma Chemical Co, St. Louis, MO, U.S.A.), 40 min at room temperature. Then, they were blocked with blocking buffer [PBS supplemented with 0,05% Triton X-100; 3% (w/v) BSA, 2% (w/v) horse serum and 0.2% (w/v) Sodium Azide; (Sigma-Aldrich Co., St. Louis, MO, USA) for 2:30 h at room temperature with continuous gentle shaking. After that, sections were incubated with anti-GFAP antibody or goat polyclonal anti-ionized calcium binding adaptor molecule 1 antibody (Iba1, Cat. No. ab107159, Abcam, Cambridge, UK, dilution 1:500) both diluted in blocking buffer, overnight (ON) at 4 °C in a humidified chamber. Samples were then rinsed three times by 5 min in PBS 0.05% (v/v) Triton X-100 and incubated with Goat anti-Rabbit IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 and Donkey anti-Goat IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (Invitrogen-Molecular Probes, Eugene, OR, USA, cat. # A-11037# and A-11055, respectively), and 3 μM DAPI, for 1 h at RT. Finally, they were washed 3 times in PBS and mounted in Mowiol (Sigma-Aldrich. Co., St. Louis, MO, USA). Images were collected using a confocal microscope (Olympus FV1200, Japan).

2.8. Microglia cells number analysis

To analyze the number of microglial cells, vertical cryosections of retina immunostained with Iba1 were used. The quantifications of Iba1-positive cells were made counting the number of cells, by hand, along all retinal cell layers in two areas; central and peripheral. Four images (at 20x magnification) per section were taken in fields on both sides of the optic nerve and close to ora serrata area and three non-consecutive sections per animal from each experimental group were analyzed. Three different animals per treatment were analyzed.

3. Statistical analysis

Statistical analysis was carried out using the Infostat software (Version 2017, InfoStat Group, FCA, National University of Córdoba, Argentina). The normality and homogeneity of the variance assumptions were proved with Shapiro-Wilks and Levene tests, respectively. A non-parametric Kruskal-Wallis test was performed when the data did not comply with the assumptions of the ANOVA. Peer comparisons were made using *t*-test or Wilcoxon signed-rank test, when the normality assumption was violated. Data are expressed as mean ±SE, the number of animals used varies between 3 and 10 according to experimental design and the number of groups were between 3 and 5 independent experiments. In all cases, a p-value < 0.05 was considered statistically significant.

4. Results

Photoreceptors cells have different photopigments. In classical photoreceptors, rod's rhodopsin is more sensitive to light than S, M, and L opsins in cones; thus, rhodopsin might be most greatly affected by light exposure. Rhodopsin protein undergoes several post-translational modifications which are necessary to sustain the protein's structure and the visual transduction mechanism function [21,22]. Among all the alterations caused by light, one of them may be during rhodopsin post-translational modifications. Therefore, to determine differences in its migration that would indicate differential post-traductional modifications under different light conditions, we exposed animals for 1 and 6 days (LL1 and LL6) to 200 lux (low intensity). We also evaluated these possible modifications in retinas of animals exposed for 3 h to 3000 lux (high intensity). Rhodopsin was analyzed by western blot under denaturing conditions (see mat. and methods). Control groups were maintained under housing conditions (LDR) or constant dark (DD). As it is shown in Fig. 1, changes in protein mobility were observed in animals exposed to LED light with respect to control animals, showing more shifts at higher light intensities, indicating post-traductional modifications of rhodopsin protein after LED light exposure. Hence, to continue investigating the effects of low-intensity LED light (200 lux), we evaluated photoreceptor cells in rat retinas exposed to the LD cycle (intermittent phototransduction stimuli under LED). As Fig. 2 shows, the number of photoreceptors in the outer retinas of animals maintained in LD conditions for 7 days was significantly decreased in comparison to the animals kept in darkness (DD), suggesting that LD retinas presented signs of injury. Although no significant differences were observed between the retinas of animals maintained in cycles of LED (LD) and housing condition (LDR), the photoreceptors quantification showed a decrease in the first group in comparison to LDR animals, indicating the beginning of a deleterious process due to the effects of light during this exposure period.

As the ERG has been considered a sensitive method to detect retinal injury [23], we implemented it to evaluate the functional state of the retinas of animals maintained under LD conditions. As it is shown in Fig. 3, no significant differences in the amplitudes of a- and b-waves were observed between animals maintained in LD cycle vs. LDR. However, the latency times of a- and b-waves from LD were significantly lower than control animals, indicating that LED-exposure induces retinal functioning alterations.

Retinal glial cells provide homeostatic and metabolic support to photoreceptors and other neurons [24]. Under pathological conditions, a reactive gliosis response is induced in order to either protect the retina against further damage [24] or to produce cytotoxic effects [25]. Reactive gliosis has been described in different retinal pathologies, including age-related macular degeneration, diabetic retinopathy, glaucoma, retinal detachment, and retinitis pigmentosa [24]. Because we have previously demonstrated glial cell response associated with constant light damage [26], and in order to explore the glial response in the LD cycle of LED stimuli, we analyzed glial activation after 7 days of alternating exposure. Immunostaining for GFAP in retina showed labeling in GCL (ganglion cells layer) in LDR animals (Fig. 4C). This morphology and localization of GFAP positive cells was indicative of retinal astrocytes presence. After seven days in LD condition, the expression of GFAP increased and the labeling extended along the retinal thickness forming an elaborate filamentous structure, indicative of Müller cells activation (Fig. 4D, white arrows). Western blot analysis showed an increased expression of GFAP and the presence of breakdown products (BDPs) in LD animals after 7 days of exposure (Fig. 4B). Data quantification demonstrated a significant increase of GFAP expression at LD 7, compared to LDR (Fig. 4A).

All these findings indicate cell stress conditions in animals exposed to LED lights in alternating cycles with darkness 12:12 (LD). These results may have great implications in the establishment of potential harmful effects of LEDs under light and dark phases, similar to those induced by constant stimulation.

5. Discussion

Retinal degeneration is one of the consequences of overexposure to artificial light. Both domestic and public light sources and different devices such as cell phones, tablets, computers, among others, may be harmful to the retina. Due to this, there is a growing and worrying risk of retinal diseases, such as age-related macular degeneration or retinitis pigmentosa, becoming a serious issue in the next decade for our society.

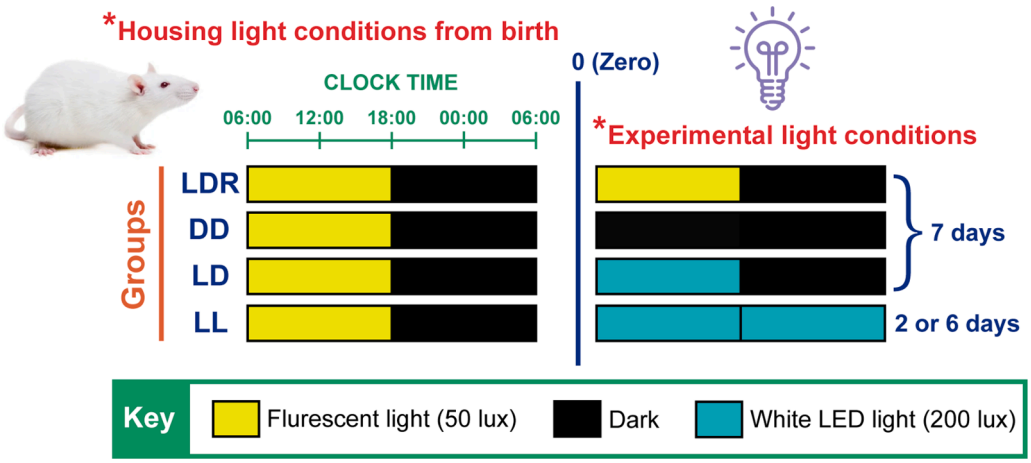

Fig. 1. Western blot analysis of Rhodopsin protein expression in retinas of animals exposed to light: **DD:** constant dark; **LDR:** light-dark cycles in housing condition (12–12 h); **low light:** 24 h and 6 days of constant light (LL, 200 lux); **high light:** 3 h of light (LL, 3000 lux).

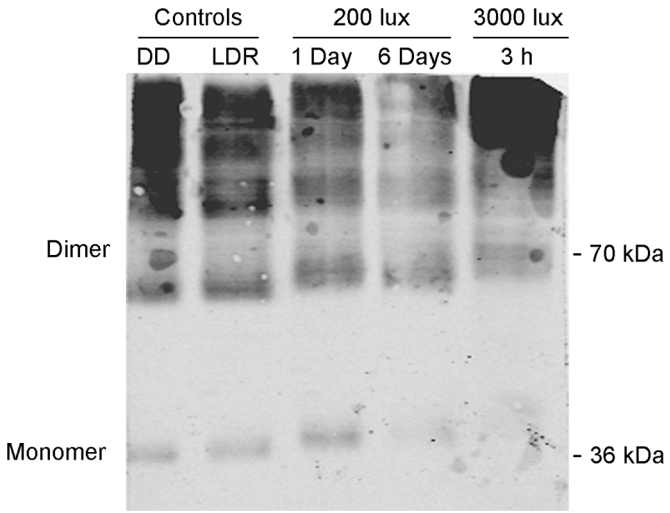

Fig. 2. Outer Nuclear layer analysis. **(A)** Numerical quantification of the number of ONL nuclei. Data are mean ± standard error (SE), $n = 2$ animals/group from five independent experiments ($n = 10$), $^{**}p < 0.01$ *vs* DD by Kruskal Wallis test. **(B)** Representative images of retinas' nuclei under different light exposure conditions. Blue: nuclear DAPI staining. Scale bar indicates 30 μm. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

For centuries, life on earth has been exposed to sunlight alternating day/night and, consequently, the visual system from invertebrates to vertebrates evolved under these laws of nature. With the arrival of artificial light, we have been first exposed to incandescent lamps, considered reliable because of their spectrum similarity to sunlight. However, as the human population increased it became necessary to reduce energy usage, promoting a gradual replacement by low energy lamps. Initially, this substitution was made by compact fluorescent lamps (CFL) and later by LED sources. This resulted in an overexposure to blue light, which makes it a matter of concern mostly because of the symptoms LEDs' blue light component exposure produces. Among these symptoms are abnormal intolerance to visual perception of light, dry eyes, blepharitis, retinal dystrophy, blepharospasm, traumatic brain injury, depression, anxiety, among others [27].

As we have demonstrated before, the constant LED light exposure induces damage on Wistar rats photoreceptors, with a significant reduction of ONL after seven days. We have also observed that animals maintained in LD cycles showed a slight reduction in retinal thickness in comparison to LDR conditions [18], indicating that there may be a differential effect in retinas exposed to LED. Therefore, the aim of the present brief research report was to show that LED lights have detrimental effects on rat retinas, even when they have intermittent periods of rest in darkness. For this aim, we studied the ONL thickness under the 3 conditions (DD, LDR and LD) using the Kruskal Wallis test. The nuclei count revealed a significant reduction of photoreceptor cells in LD vs. DD (LD: 88,142 ± 13,932, DD: 105,219 ± 18,360), while LDR animals showed similar ONL thickness as in total darkness condition (10,0763 ± 3201) (Fig. 2A). This result indicates that the retinas from animals under

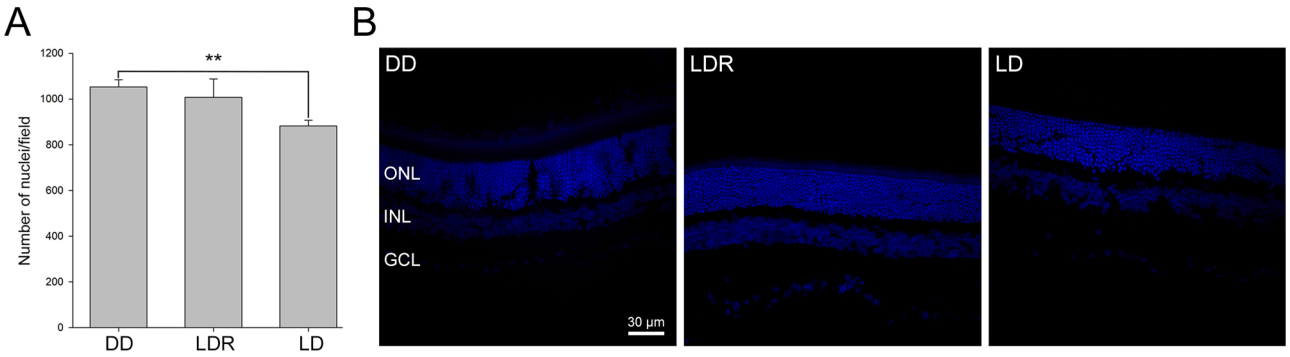

Fig. 3. Scotopic Electroretinogram (ERG) analysis in animals exposed to light-dark cycles (12–12 h) under housing conditions (LDR) or LED (LD, 200 lux) during 7 days. Data are mean ± standard error (SE), $n = 3$ animals/group from five independent experiments ($n = 15$); $^{**}p < 0.01$, $^{*}p < 0.05$ *vs* LDR by Wilcoxon signed-rank test.

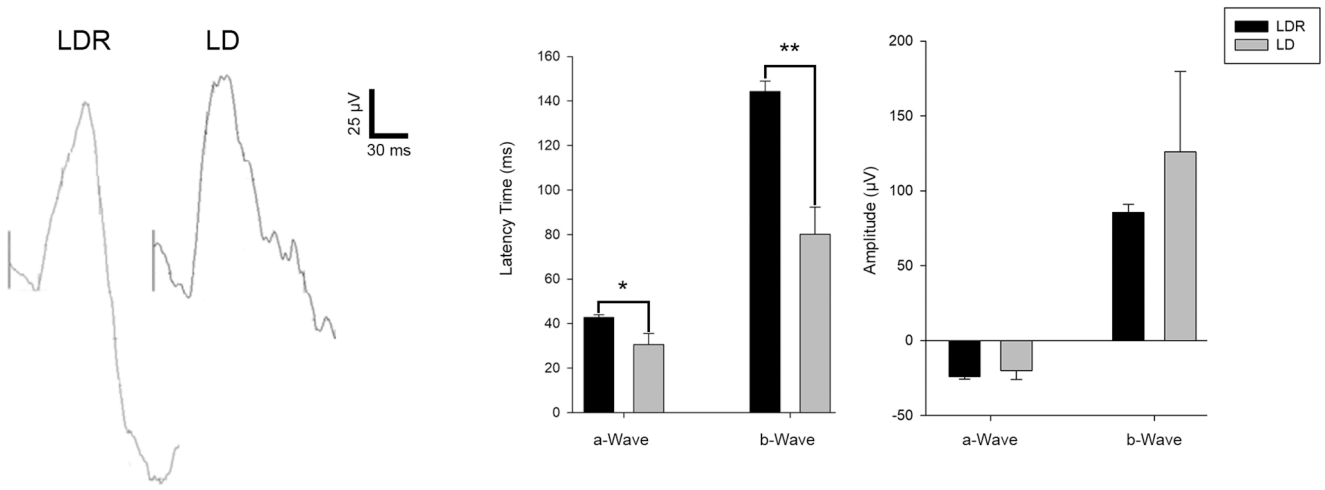

Fig. 4. (A,B) Western blot analysis of GFAP protein expression in retinas of animals exposed to light-dark cycles (12–12 h) with housing conditions (LDR) or LED (LD). Relative expression of GFAP normalized to the content of α-Tubulin. Data are mean ± standard error (SE). $n = 3$ animals/group; $^{*}p < 0.05$ *vs* LDR by T-test. **(C, D)** Analysis of GFAP protein expression by immunohistochemistry in LDR **(C)** or LD **(D)** group**.** The images are representative from 3 different experiments per treatment. Orange: GFAP antibody staining; Blue: nuclear DAPI staining. Scale bar indicates 30 µm. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

LD exposure conditions are affected by LED light, even when they have periods of visual rest (dark). Additionally, we tested the retinal electrical response capacity by ERG in scotopic studies. The analysis of the results obtained after averaging 10 light flashes, allowed us to evaluate the signal intensity (amplitude) and response time (latency) of the a-wave (related to photoreceptors responses) and b-wave (related to internal retina). No significant differences in a- and b- waves' amplitudes signals were observed between LD and LDR (Fig. 3). However, the response times of a- and b-waves from LED exposure retinas were lower with respect to fluorescent exposed animals (Fig. 3). These results indicate that, although there are no changes in the physiology of the retinal responses in LD in contrast to LDR, the retina appears to be more sensitive to flash light stimuli after LED exposure.

We have also demonstrated a significantly increased expression of GFAP by western blot (Fig. 4) in LD retinas, an observation confirmed by immunohistochemistry. Image analysis showed more antibody labeling in the end-foots of Müller cells and astrocytes (Fig. 4), demonstrating a significant macroglial activation in animals exposed to light/dark cycles of LED light. We had already demonstrated microglial cells activation after constant low light exposure [26]. We showed by immunohistochemistry with Iba1 antibody, few and weak-labeled positive cells in GCL, IPL (inner plexiform layer) and OPL (outer plexiform layer) in LDR and DD retinas. However, retinas from animals exposed to constant light for four and six days (LL4 and LL6) showed an increased number of Iba1-positive cells in all these layers as well as in ONL (outer nuclear layer), corresponding to photoreceptors cells. For both periods, cell morphology became ameboid with thicker processes, characteristic of activated cells. Thus, we concluded that the Iba1-positive cells, macrophages and microglia, are activated by constant light exposure. By detecting incremented RNAm levels of CD68, TNF and IL-6 in retinas under constant light conditions, we finally confirmed that the Iba1 positive cells are macrophage and microglia. Altogether, our results support the idea that constant exposure to low-intensity light induces a microglial activation [26]. Microglia analysis by immunohistochemistry shows non-significant differences in the number of Iba-1 positive cells between LD and LDR rat retinas. However, we saw some Iba-1 positive cells next to photoreceptor cells and retinal pigmented epithelium, which suggests a slight activation of microglial cells in LD with respect to LDR or DD (data not shown). Micro and macro glia together are responsible for assembling a pro-inflammatory response in chronic or acute pathological conditions, synthesizing trophic factors necessary for neuronal survival after injury [28]. In retina, macroglial cells maintain a constant communication with other glial cells, and in photoreceptors they are involved in the conversion of all-trans-retinal to 11-cis-retinol in the recycling of photopigments [29]. Thus, in the presence of a stressor, the macroglia activates established protective mechanisms for themselves and the retinal neurons [30]. The results obtained with this model of low light damage, suggest that the exposure to LED light in the LD cycle induces an early activation of microglia that could be functioning as a protective pathway for reversing the injury mechanisms promoted by light.

6. Conclusion

The current study explored the effect of LEDs on retinas from Wistar rats in the context of cycles of illumination. Our findings demonstrate that these animals present early retinal injury after 7 days of exposure, even when they have dark resting periods, strengthening the idea that LED devices may induce visual injury. This study may have important implications on the discovery of potential harmful effects of LED illumination on vision health, and could be potentially used for regulations about domestic lighting and smart technologies. Future evaluations of longer exposure periods must be assessed in order to further characterize the biological mechanism of retinal injury induced by LED illumination, which is nowadays widely used in our society.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Ethics statement

All animal procedures were performed in accordance with the ARVO statement for the use of animals in ophthalmic and vision research, which was approved by the local animal committee: Comité Institucional de Cuidado y Uso de Animales de Laboratorio en el Ámbito de la Facultad de Ciencias Químicas—CICUAL-FCQ-en los proyectos científicos -fs. 6-EXP-UNC:0007526/201 8 Number 740.

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