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Research article

The expression of adenosine receptors changes throughout light induced retinal degeneration in the rat

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ABSTRACT

The modulation of adenosine receptors, A1 (A1R) and A2A (A2AR), is neuroprotective in different models of retinal injury. In order to understand the processes underlying retinal degeneration, we studied the expression of adenosine receptors in the retinas of control and continuously illuminated (CI) rats by qRT-PCR, Western blot (WB) and immunohistochemistry (IHC).

Significant increases of A1R, A2AR, and A2BR mRNAs at 1, 5, and 7 days of CI ($P < 0.0001$) were observed by qRT-PCR. Also, a significant increase of A3R mRNA was detected after 5 and 7 days of CI. WB studies showed a significant rise of A1R on day 1 of CI and on days 5 and 7 ($P < 0.0001$), while A2AR increase was seen from 2 days of CI on ($P < 0.001$).

After 1 day of CI, A1R immunoreactivity (A1R-IR) increased in ganglion cell layer, inner nuclear layer, and in both the outer and inner plexiform layers. After 2 days of CI, the A1R-IR went back to control levels. After 5 days of CI, a second rise in A1R, which persisted until 7 days of CI, was measured ($P < 0.0001$). A significant rise of A2aR immunoreactivity was also observed at day 2 of CI at GCL and INL and subsided at days 5 and 7 ($P < 0.0001$).

The observed up-regulation of A1R after 1 day of CI, corresponds with the peak of oxidative stress; while the rise of A2aR at day 2 of CI, coincides with the massive apoptosis of photoreceptors. We postulate that an early modulation of adenosine receptors could delay or prevent the degeneration of photoreceptors.

1. Introduction

Continuous illumination (CI) of rat retina produces photoreceptor (PH) degeneration characterized by membranous discs disorganization, tubular transformation and increased phagocytosis by the retinal pigment epithelium (RPE). Synaptic degeneration in the outer plexiform

Abbreviations: AMD, age-related macular degeneration; ATP, adenosine triphosphate; A1R, adenosine receptor type A1; A2AR, adenosine receptor type A2A; A2BR, adenosine receptor type A2B; A3R, adenosine receptor type A3; BDNF, brain-derived neurotrophic factor; CI, continuous illumination; CNS, central nervous system; CPA, cyclopentiladenosine; CTL, control; DAB, diaminobenzidine; GABA, gamma-aminobutyric acid; GDNF, glial cell line-derived neurotrophic factor; GCL, ganglion cell layer; IHC, immunohistochemistry; IL, illuminated; IL-2, interleukin-2; IL-1β, interleukin-1β; IL-6, interleukin-6; INF-γ, interferon-γ; INL, inner nuclear layer; IPL, inner plexiform layer; IR, immunoreactive; ISH, in situ hybridization; JMD, juvenile macular degeneration; LIRD, light-induced retinal degeneration; mRNA, messenger ribonucleic acid; NF-κB, nuclear factor kappa-light-chain enhancer of activated B cells; NFL, nerve fiber layer; NIH, National Institutes of Health; NMDA, *N*-methyl-*D*-aspartate; OD, optical density; ONL, outer nuclear layer; OPL, outer plexiform layer; PH, photoreceptor; PKA, protein kinase A; qRT-PCR, quantitative reverse transcription polymerase chain reaction; RGC, retinal ganglion cell; RP, retinitis pigmentosa; RPE, retinal pigment epithelium; RT-PCR, reverse transcription polymerase chain reaction; SFK, Src family tyrosine kinase; TNF, tumor necrosis factor; trk-B, tyrosine kinase B; V, volt; VEGF, vascular endothelial growth factor; W, watt; WB, western blot; Wnt, wingless-related integration site

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layer (OPL) and apoptosis in the outer nuclear layer (ONL) also ocur [1–6]. This model of light induced retinal degeneration (LIRD) resembles many of the characteristics of human retinal degenerative diseases such as age-related macular degeneration (AMD) [7], juvenile macular degeneration (JMD) and retinitis pigmentosa (RP). LIRD has been widely used to study degenerative diseases of the retina [8–10].

In the central nervous system (CNS), adenosine is a neuromodulator that is translocated to the extracellular space by membrane nucleoside transporters or is synthesized by ectonucleotidases. Adenosine binds to P1 purinergic family receptors known as A1, A2A, A2B and A3, which are G-protein coupled receptors that either inhibit (A1R, A3R) or stimulate (A2AR, A2BR) adenylate cyclase [11]. In the retina, adenosine inhibits NMDA receptor-mediated activity and phospholipase C, and increases the activity of GABA receptors and ATP sensitive K^+ channels [12]. In rod photoreceptors, adenosine inhibits calcium influx through L-type calcium channels [13], reduces glutamate release through A2-like receptors [14] and controls the characteristics of the subretinal space [15].

In the retina, adenosine-like immunoreactivity has been found in the ganglion cell layer (GCL) and the nerve fibre layer (NFL), and some moderate staining has been observed in selected cells (displaced cholinergic amacrine cells and GABAergic amacrine cells) of the inner nuclear layer (INL) [16,17].

Receptor binding studies showed that A1R is localized in the inner retina of rabbit, mouse, rat, monkey and human retinas [16–18]. In situ hybridization (ISH) further confirmed the presence of A1R mRNA in GCL cells and in some cells of INL which possibly correspond to acetylcholine containing amacrine cells [19].

On the other hand, A2R was localized in the outer retina using autoradiography in rabbit and mouse retina [17,18], but ISH contradictorily showed that A2AR mRNA expression was prominent in the INL, less intense in GCL and ONL and weak in the retinal pigment epithelium (RPE) [19]. A2AR was also found in photoreceptors of bovines [20], teleost fish [21], zebrafish [22] and salamander [13] using immunohistochemistry. While A2BR showed a diffuse labelling in retinal tissue, A3R expression was not found in rat retinas in the study of Kvanta et al [19], although A3R mRNA was detected in rat retinal ganglion cells using RT-PCR [23].

In recent years, the modulation of adenosine receptors has emerged as a potential neuroprotective strategy to treat retinal degenerations [24]. Adenosine protects the retina from ischemic injury through A1R and/or A2R stimulation [12]. It has been demonstrated that adenosine protects cultured retinal cells from NMDA-induced cell death through A1 receptors [25,26], and that A2A receptor antagonists protect the retina against ischemia [27]. However, little is known about the role of adenosine in the model of LIRD.

The aim of this work was to analyze the changes in the expression of adenosine receptors in the retina of rats subjected to CI by using qRT-PCR, Western Blotting (WB) and immunohistochemistry (IHC). This work would be the first step in developing experimental therapeutic strategies based on adenosine receptor modulation.

2. Materials and methods

2.1. Animals and illumination procedure (animal model of continuous illumination)

A total number of 45 male Sprague Dawley albino rats (body weight 200 g, age 60 days) were used. Rats were obtained from the animal house of the Faculty of Veterinary, University of Buenos Aires. Before the experiment, animals were kept at 12/12 h light/dark cycles (Lighting level: 80 lux during light period). Groups of 4 rats were simultaneously placed in an open white acrylic box of 60 cm x 60 cm x 60 cm with 12 halogen lamps (12 V 50 W each) located on top. Lighting level (12,000 lux) was determined using a digital illuminance meter. Temperature was maintained at 21 °C. The rats were continuously illuminated for either 1, 2, 5 or 7 days (referred as 1d, 2d, 5d and 7d, respectively). In order to obtain at least 3 animals per time point, this procedure was repeated 3 times for immunohistochemical procedures, and another 3 times to obtain tissues for WB and qRT-PCR. In addition, 3 rats were kept at 12/12 h light/dark cycles and were used as control animals (CTL) in each case (IHC, WB and qRT-PCR). All the animals were kept with food and water ad libitum. Animal care was performed in accordance with the NIH Guidelines for the Care and Use of Animals and the principles presented in the Guidelines for the Use of Animals in Neuroscience Research by the Society for Neuroscience. The described animal model was approved by the Institutional Committee for the Use and Care of Laboratory Animals of the Faculty of Medicine, University of Buenos Aires (CICUAL,"Comité Institucional para el Cuidado y Uso de Animales de Laboratorio").

2.2. RNA isolation and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

CTL and illuminated animals (n = 6 per group) were deeply anaesthetized by intraperitoneal injection of Ketamine (40 mg/kg; Ketamina 50®, Holliday-Scott SA, Beccar, Argentina) and Xylazine (5 mg/kg; Kensol®, Laboratorios König SA., Buenos Aires, Argentina) decapitated, and their eyes were enucleated. The posterior segments of the eyes were frozen and stored at −80 °C until used. Tissues were homogenized with TRIzol (Invitrogen, Madrid, Spain) and RNA was isolated with RNeasy Mini kit (Qiagen, Germantown, MD). Three µg of total RNA were treated with 0.5 µl DNAseI (Invitrogen) and reverse-transcribed into first-strand cDNA using random primers and the SuperScript III kit (Invitrogen). Reverse transcriptase was omitted in control reactions, where the absence of PCR-amplified DNA confirmed lack of contamination from genomic DNA. Resulting cDNA was mixed with SYBR Green PCR master mix (Invitrogen) for qRT-PCR using 0.3 µM forward and reverse oligonucleotide primers (Table 1). Quantitative measures were performed using a 7300 Real Time PCR System (Applied Biosystems, Carlsbad, CA). Cycling conditions were an initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. At the end, a dissociation curve was implemented from 60 to 95 °C to validate amplicon specificity. Gene expression was calculated using absolute quantification by interpolation into a standard curve. All values were divided by the expression of the house keeping gene 18S.

2.3. SDS-PAGE and Western-blotting

Retinas of CTL, 1d, 2d, 5d and 7d rats were dissected out (n = 5 per experimental group). Tissues were homogenized (1:3, w/v) in lysis buffer (100 mM NaCl, 10 mMTrisHCL, 0.5% Triton X-100, 50ul Protease inhibitor cocktail (Merck)). All procedures were carried out at 4 °C. Protein concentration was determined by the Bradford method, with bovine serum albumin as standard, using a Beckman Spectrophotometer DU-65. Then, 50–100 µl of each sample were mixed 4:1 with 5X sample buffer (10% SDS, 0315 M Tris−HCl, 25% Beta-Mercaptoethanol, 50% Glycerol, 0.2 ml bromophenol blue 0.1%, pH 6.8) and heated for 10 min at 100 °C. Samples were run (50 µg of

Table 1
List of genes studied and primers designed to determine mRNA levels by qRT-PCR.

A1R	Forward	GTGATTTGGGCTGTGAAGGT
	Reverse	AGGTGTGGAAGTAGGTCTGTGG
A2A	Forward	CGGGAACTCCACGAAGACC
	Reverse	AGCAAAGAGCCCGACGATG
A2b	Forward	TCTTCCTCGCCTGCTTCGT
	Reverse	GGAGTCAGTCCAATGCCAAA
A3	Forward	GAAAGCCAACAATACCACGAC
	Reverse	AGTGCTAGGGAGACGATGAAAT

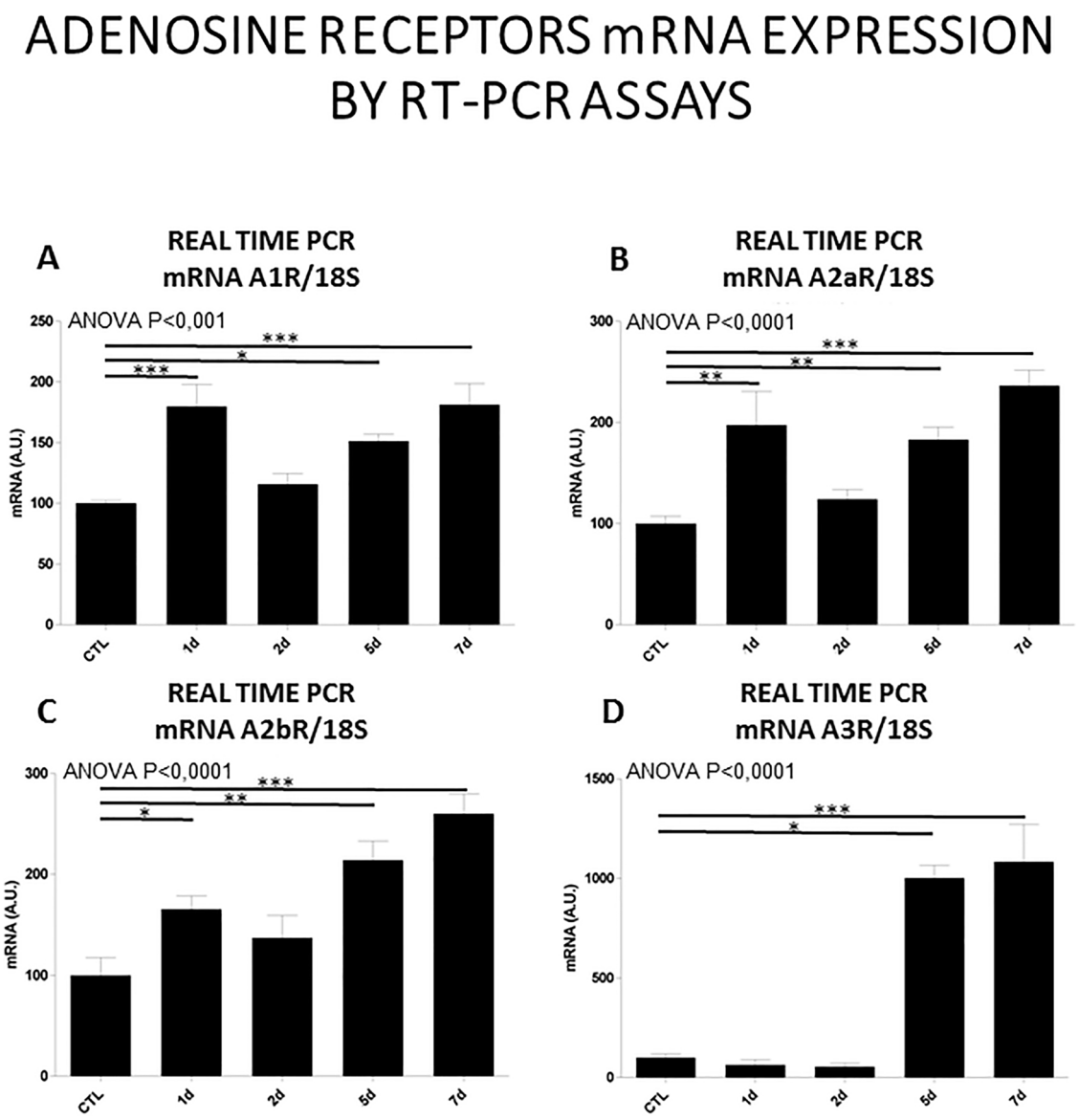

Fig. 1. qRT-PCR of adenosine receptors. Figure shows mRNA expression along continuous illumination: A) A1R, B) A2aR, C) A2bR and D) A3R. Values are compared to control levels, bars represent mean ± SD, * $p \leq 0.05$; ** $p \leq 0.01$; ***$p \leq 0.001$. A.U.: Arbitrary units.

protein per lane) on SDS–polyacrylamide gels (10% or 15% running gels with 5% stacking gel), with 0.24 mMTrizma base, 4.38 mM SDS, 0.19 M glycine, pH 8.3, as the electrolyte buffer. Kaleidoscope Prestained Standards (Bio-Rad) were used as molecular weight markers. For Western blot analysis, proteins were transferred at 100 mV for 1 h onto 0.2-μm polyvinylidenedifluoride membranes (GE healthcare life sciences) in a transfer buffer (15% m/v Glycine, 3% m/v Trisma, 20% v/v ethanol).

For receptor identification, membranes were incubated overnight at 4 °C with either a rabbit polyclonal antibody to A1R (Santa Cruz Biotech. Inc., USA, dilution 1:250), or a rabbit polyclonal antibody to A2AR (Santa Cruz Biotech. Inc., USA; dilution 1:100). To test for protein loading accuracy, a monoclonal anti-β-actin antibody (Sigma, dilution, 1:1000) was used. To visualize immunoreactivity, membranes were incubated with Amersham ECL Rabbit IgG, HRP-linked F(ab)2 fragment (from donkey), and were developed using a chemoluminiscence kit (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific), and exposed to X-ray blue films (Agfa, Argentina). Developed films were scanned with a HP Photosmart scanner and optical density (OD) quantified by Image Light Studio software of Li-Cor. Data were statistically analysed using a one way ANOVA test and Graphpad Software.

2.4. Tissue processing for immunohistochemistry

Control and illuminated animals were deeply anesthetized at the different time points with Ketamine (40 mg/kg; Ketamina 50®, Holliday-Scott SA, Beccar, Argentina) and Xylazine (5 mg/kg; Kensol®, Laboratorios König SA., Buenos Aires, Argentina), and their eyes were removed; the cornea and lenses were cut off, and the remaining tissues with a cup shape were fixed by immersion in a solution containing 4% paraformaldehyde in 0.1 M phosphate buffer for 2 h. Eyes were immersed in a solution containing 30% sucrose in 0.1 M phosphate buffer and were embedded in Tissue Tek. The frozen eyes were cut along a vertical meridional plane at -20 °C using a Lauda Leitz cryostat, and sections (thickness: 20 μm) were mounted on gelatin coated glass slides and processed by immunohistochemistry.

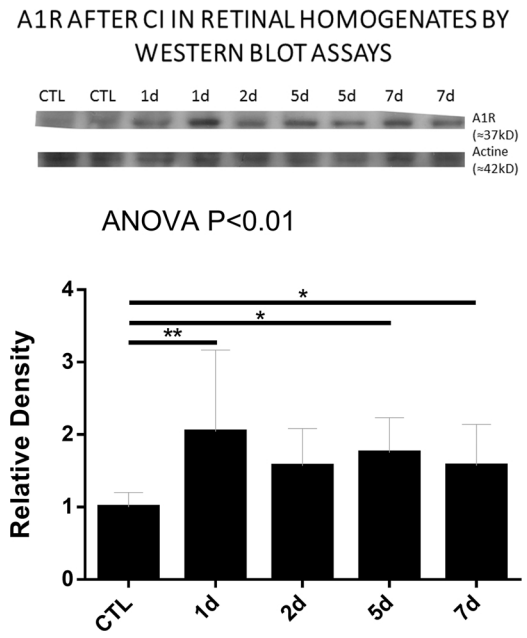

Fig. 2. Western Blot of A1 receptor in control and illuminated rat retinas. Bars represent mean ± SD, significant differences between groups are shown as: * $p \leq 0.05$; ** $p \leq 0.01$.

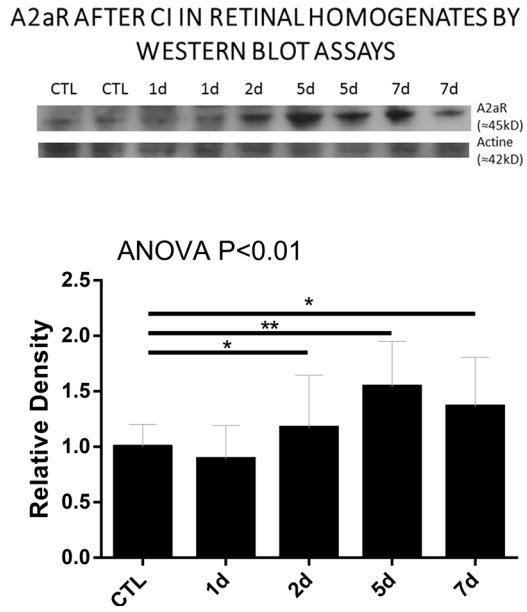

Fig. 3. Western Blot of A2a receptor in control and illuminated rat retinas. Bars represent mean ± SD, significant differences between groups are shown as: * $p \leq 0.05$; ** $p \leq 0.01$.

2.5. Immunoperoxidase technique

In order to inhibit endogenous peroxidase activity, sections were incubated in methanol containing 3% hydrogen peroxide for 30 min. After washes in phosphate buffered saline (PBS), pH 7.4, sections were incubated in 10% normal goat serum for 1 h. Then, sections were incubated either with a polyclonal rabbit antibody to A1R (Santa Cruz Biotech. Inc., USA, dilution 1:500), or a polyclonal antibody to A2AR (Santa Cruz Biotech. Inc., USA; dilution 1:500). Then sections were incubated in goat anti rabbit biotinylated antibody (Sigma Chemical Co.; dilution 1:200) and in ExtrAvidin-Peroxidase® complex (Sigma Chemical Co.; dilution 1:200). All antisera were diluted in phosphate-buffered saline (PBS) containing 0.2% Triton X-100. Incubations with A1R and A2AR antibodies were performed overnight at 4 °C while biotinylated antibody and ExtrAvidin-Peroxidase® complex were performed at room temperature (RT) for 1 h. Controls were performed by omitting primary antibodies. Reaction was developed using the DAB/nickel intensification procedure [28].

2.6. Double labelling technique

Some sections were incubated overnight with a mixture containing a polyclonal rabbit antibody to A1R (Santa Cruz Biotech. Inc., USA, dilution 1:50) and a mouse monoclonal antibody to Brn-3a (Santa Cruz Biotech. Inc., USA, dilution 1:50).Sections were later incubated in a mixture of goat anti rabbit antibody conjugated to Alexa Fluor® 488 (Abcam, dilution 1:50) and goat anti-mouse antibody conjugated to Alexa Fluor® 555 (Abcam, dilution 1:50) at RT for 1 h. and were observed using an Olympus IX-83 inverted microscope. Simultaneously, negative controls were performed by omitting primary antibodies.

2.7. Image analysis of immunoperoxidase sections

Six retinal sections from 4 animals of each experimental group were analyzed. Care was taken on selecting anatomically matched areas of retina among animals before assay. Slides were analysed using a Zeiss Axiophot microscope attached to a video camera (Olympus Q5). Images were taken using Q capture software. The optical density of selected retinal layers were evaluated using the Fiji software (NIH, Research Services Branch, NIMH, Bethesda, MD). The mean background density was measured in a region devoid of immunoreactive cells, immediately adjacent to the analyzed region. OD was calculated using a grey scale of 255 grey levels. To avoid external variations, all images were taken the same day and under the same light conditions.

2.8. Statistical analysis

Values are expressed as mean ± standard deviation. At least 3 similar separate experiments were evaluated in all cases. A1R and A2AR immunohistochemical results were evaluated using ANOVA and non-parametric Kruskal-Wallis test included in the GraphPad software (GraphPad Software, San Diego, CA). Differences were considered significant when $p < 0.05$.

3. Results

3.1. Expression of adenosine receptors mRNAs exposed to continuous illumination

The expression of adenosine receptor genes at various time-points in response to continuous illumination was studied using qRT-PCR. The results showed a significant up-regulation of A1R mRNA on 1d, 5d and 7d. A1R mRNA maximum peaks were observed on 1d and 7d (Fig. 1 A).

A2AR mRNA expression exhibited a similar pattern to that of A1R mRNA. Results showed a significant up-regulation of A2AR mRNA on 1d, 5d and 7d. A2AR mRNA also peaked on 1d and 7d (Fig. 1B).

A2BR mRNA expression exhibited a similar pattern to A1R mRNA and A2AR mRNA expressions. Results showed a significant up-regulation of A2BR mRNA on 1d, 5d and 7d but in this case, mRNA levels were higher on 5d and 7d (Fig. 1C).

A3R mRNA expression was extremely low in retinas of CTL, 1d and 2d but it presented a significant increase on 5d and 7d (Fig. 1D).

3.2. Effect of continuous illumination on adenosine receptors determined by western blot

The WB studies showed significant increases of A1 receptor levels in illuminated rat retinas on 1d, 5d and 7d. The peak was observed on day

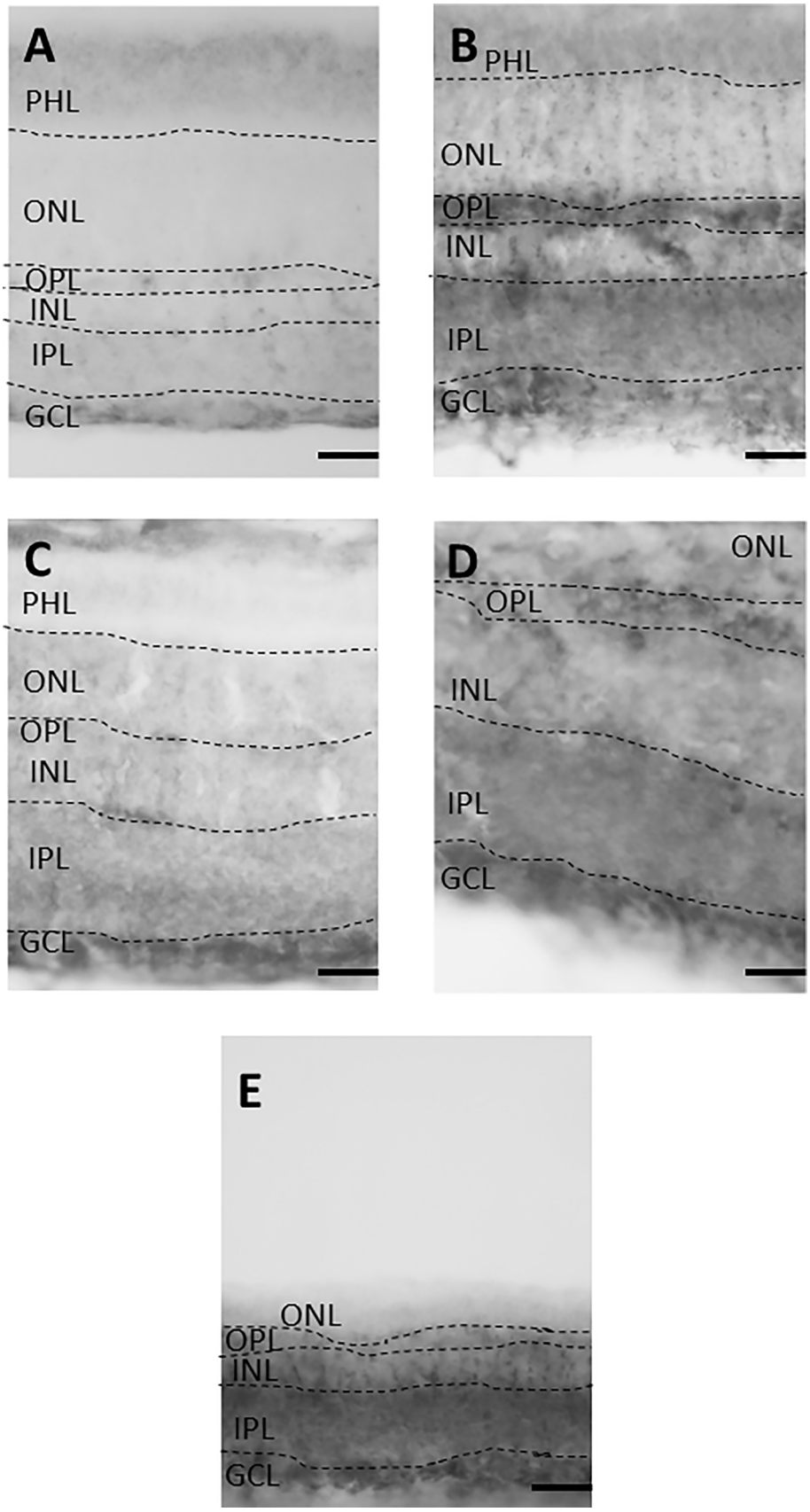

Fig. 4. A1R immunoperoxidase staining. Retinal sections from control (A) and illuminated rats (B–E) as follows: B) 1d, C) 2d, D) 5d and E) 7d. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer, PH, photoreceptor layer. Scale bar: 50 µm.

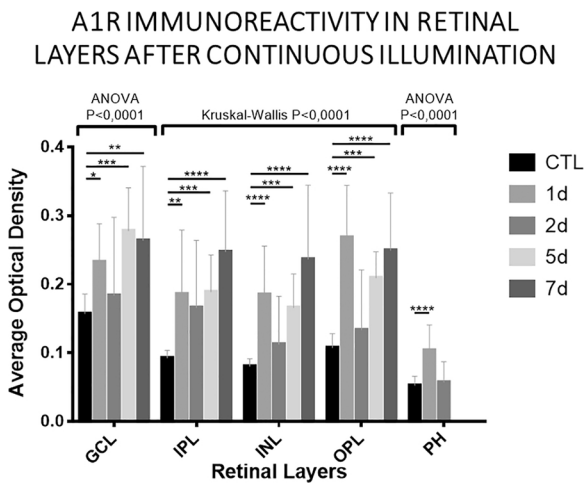

Fig. 5. Optical density of A1 Receptor immunoreactivity of control and illuminated rat retinas. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer, PH, photoreceptor layer. In every group, the first column corresponds to the A1R OD of CTL retinas and the following columns correspond to 1d, 2d, and 5d and 7 d, respectively. Bars represent mean ± SD, significant differences between groups are shown as: * $p \leq 0.05$; ** $p \leq 0.01$; ***$p \leq 0.001$; **** $p \leq 0.0001$.

1 (ANOVA, $P < 0.01$) (Fig. 2). They also showed significant increments of A2AR from 2d on. The peak was observed on day 5 (ANOVA, $P < 0.01$) (Fig.3).

3.3. Effect of continuous illumination on the immunohistochemical distribution of adenosine receptors

Control rat retinas showed strong A1R-IR in the NFL and the GCL, and in some cases there was also A1R-IR in the inner portion of the inner nuclear layer (INL). Weaker immunostaining was observed in photoreceptor layer, OPL and IPL (Fig. 4A). At 1d, there was an increase of A1R-IR in the inner retina (NFL, GCL & IPL) and OPL (Fig. 4B) which was maintained up to 7d (Fig. 4C, D and E). An important degeneration of photoreceptors, accompanied by a reduction of ONL, was observed at 5d and 7d (Fig. 4D and E). Quantification by image analysis showed the changes of OD in each layer (Fig. 5). All immunoreactive layers (GCL, INL, INL, OPL) showed a significant increase in OD at 1d ($P < 0.0001$). At 2d, we found a general decrease in A1R-IR back to CTL levels. At 5d, the levels of A1R-IR showed a second rise which was maintained at 7d ($P < 0.0001$). The OD of the photoreceptor (PH) layer could not be determined after 5d and 7d because the photoreceptors degenerate by these times of illumination. However a significant increase of OD was determined in PH layer at 1d (Fig. 5).

Double labelling technique confirmed the existence of A1R on Brn-3a reactive ganglion cells. The coexistence was confirmed in CTL and illuminated retinas although A1R immunoreactivity was more evident in GCL at 5d and 7d (S1).

Our results showed strong A2AR -IR on the GCL, the inner INL, the OPL, and the IPL in CTL rats. A weaker staining was observed in the PH (Fig. 6A). Along illumination, the same immunoreactive pattern was observed although a decrease in A2aR-IR was seen at 1d, and an increase of immunoreactivity was observed thereafter (Fig. 6B-E). As mentioned before, we observed a major degeneration of photoreceptors accompanied by a reduction of ONL in retinas after 7 days of CI (Fig. 6E). Quantification of OD by image analysis showed increments in all layers at 2d of CI although it was significant in the GCL, and the INL. The GCL, IPL and INL also showed a significant rises in 5d and 7d. The OPL showed a significant rise in the OD only in 5d. The OD of PH layer could be accurately measured solely for the first 2 days due to the degeneration seen after. In this period of time, there was a significant drop in OD in 1d which came back to CTL levels in 2d (Fig. 7).

Other adenosine receptors as A2BR and A3R were only analysed using qRT-PCR in the present study because their distribution has been reported as diffuse (A2BR) or negative (A3R) in the rat retina [19].

4. Discussion

The techniques of qRT-PCR and WB demonstrated a peak of A1R expression at 1d. Furthermore, IHC demonstrated a peak of A1R in all the immunoreactive layers as well as in photoreceptors. This is the moment of maximal oxidative stress previously determined in the model by us [10]. Probably, the pathological activation of the phototransduction pathway, and/or the oxidative stress, turn on the expression of A1R gene; and the increase of A1R mRNA is translated to an increase of A1R protein levels in retinal tissue as shown by WB and IHC.

We postulate that the present changes of A1R could enable the retina to resist light-induced oxidative stress as several reports from the literature have demonstrated the protective role of adenosine transmission. In fact, adenosine levels increase after glutamate-induced toxicity, as well as in glaucoma animal models, playing a neuroprotective role through A1 or A2A receptors [29]. A1R protective action is probably due to the presynaptic inhibition of glutamate release and the modulation of NMDA receptor activity [30]. In our hands, A1R agonist N-Cyclopentiladenosine (CPA), administered before CI, diminished apoptosis and Müller cell activation in LIRD while the A1 receptor antagonist (DPCPX) does the opposite [31].

Double labelling technique confirmed that A1R immunoreactivity was more evident in GCL at the moment 5d and 7d (S1). These results are consistent with the second increase detected of A1R.

Contrary to A1R-IR, A2AR -IR decreased significantly in photoreceptors at 1d, the moment of maximal oxidative stress, and normalized in 2d. However, a rise of A2AR expression was observed in 2d by qRT-PCR (in total retina), by IHC in many immunoreactive layers and on 3d by WB (also in total retina). This occurs at the moment of the massive apoptosis of photoreceptors in the model of LIRD. Our hypothesis is that Light induced damage probably releases adenosine to the extracellular space inducing a downregulation of A2AR after 1d of continuous illumination. Once Adenosine binds to the receptors and/or is degraded in the extracellular space, there is a bounce of A2AR in 2d because the cells express the receptor on their cell surface again. An alternative explanation is that other inflammatory signals trigger the expression of A2AR.

We postulate that the modulation of A2AR could delay or prevent photoreceptor degeneration if the retina is treated at this precise moment in order to stimulate intrinsic protective molecular pathways before the death program has been triggered.

It was demonstrated that A2AR regulates BDNF levels and activates trkB receptor [32]. The distribution of trkB receptor overlaps with the distribution of A2AR s in RGC and in a subpopulation of amacrine cells in rodent retinas [33,34] reinforcing our hypothesis that A2AR activation could be neuroprotective.

A complex crosstalk between IL-6, A1R and A2AR stimulates BDNF production and protects RGCs. The stimulation of RGC with IL-6 upregulates A2AR and A1R playing an important role on the levels of BDNF and other neurotrophins (GDNF, VEGF, etc) that are protective in models of LIRD [35].

A2AR has also been described in retinal microglial cells [36] where it controls microglial functions such as proliferation, synthesis of inflammatory enzymes, (e.g. COX2) and inflammatory mediators (INF-γ, IL-1β, IL-2, IL-6 and TNF) [37]. Studies of glaucoma animal models showed increases of microglial inflammatory mediators and; retinal function was recovered by modulating A2A receptors [27,36].

Other adenosine receptors, including A2BR and A3R, were only analysed using qRT-PCR in the present study because their distribution has been reported as diffuse (A2BR) or negative (A3R) in the rat retina [19]. A2BR mRNA surprisingly showed a similar pattern to that

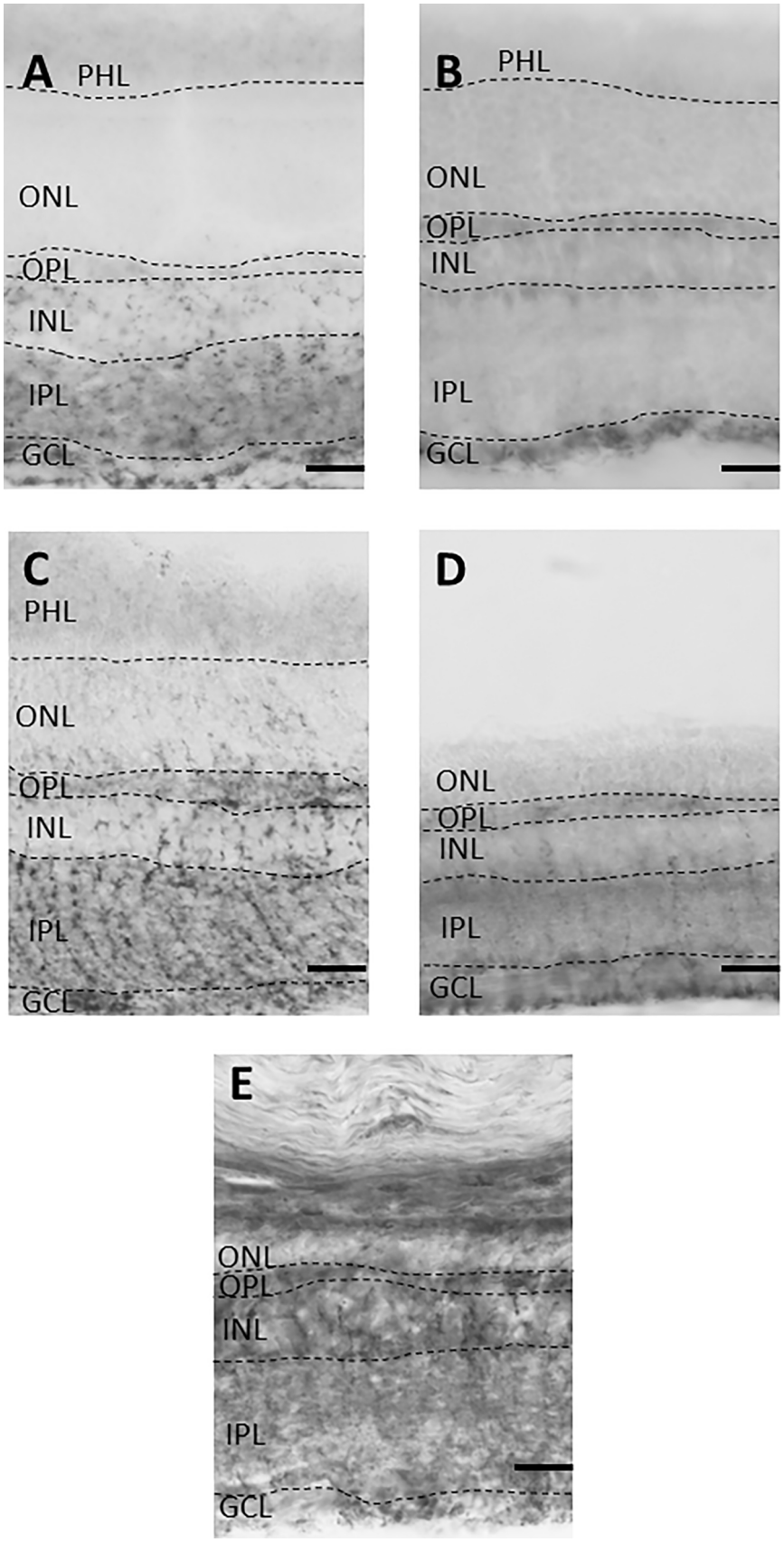

Fig. 6. A2AR immunoperoxidase staining. Retinal sections from control (A) and illuminated rats (B–E) as follows: 1d, C) 2d, D) 5d and E) 7d. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer, PH, photoreceptor layer. Scale bar: 50 μm.

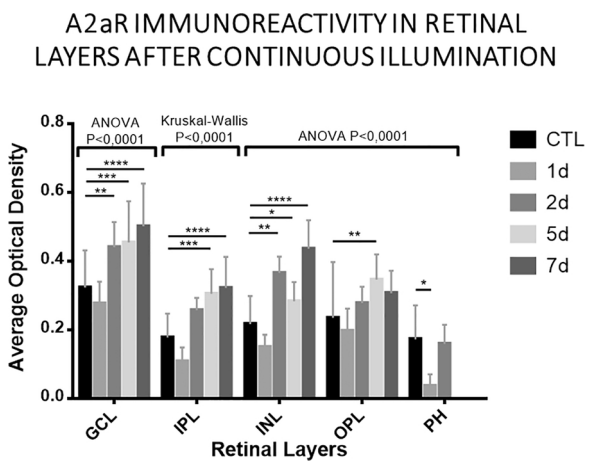

Fig. 7. Optical density of A2a Receptor of control and illuminated rat retinas. GCL, ganglion cell layer; IPL, inner plexiform layer; OPL, outer plexiform layer; ONL, outer nuclear layer, PH, photoreceptor. In every group, the first column corresponds to the A2aR OD of CTL and the following columns correspond to 1d, 2d, 5d and 7d respectively. Bars represent mean ± SD, significant differences between groups are shown as: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** ≤ 0.0001.

observed with A2AR in our work. Although little is known about this receptor in the retina, it is possible that some of the actions mediated by A2AR are also mediated by A2BR.

The low expression of A3R mRNA in retinas of CTL, 1d and 2d is in accordance with reports that showed A3R mRNA occurs only in RGC of rat retina [38]. Surprisingly, light stimulation did not increase A3R expression in the first two days of CI but a huge up-regulation of A3R mRNA was observed in 5d and 7d tissue when the maximal retinal damage was reported [6]. Perhaps A3R increment is a signal of extreme tissue damage and/or inflammatory reaction and, in that case, it could be used as a potential marker of this condition. A3R is a GPCR associated with an inhibitory Gi subunit. It is known that A3R is overexpressed in inflammatory cells and mediates anti-inflammatory, anticancer and anti-ischemic effects through a modulation of Wnt and NF-κB pathways [39]. The beneficial effect of A3 agonist drugs has been explored in experimental models of glaucoma as they reduce intraocular pressure (IOP) and attenuate calcium rise triggered by NMDA receptors in RGCs [39,40]. In addition, A3R agonists have been used to prevent the apoptosis of RGCs in experimental autoimmune uveitis [41], in retinal models of ischemia-reperfusion, after partial optic nerve transection, and in *in vitro* models of induced retinal degeneration [42].

In conclusion, knowing that adenosine receptor agonists and antagonists have been shown to have beneficial effects in various animal models of degenerative diseases of the retina, our results reveal a number of potential targets in our model of LIRD. In this model, there are two different time windows for potential therapeutic intervention: an early one, at days 1–2 of CI, when A1 and A2A receptors are up-regulated; and a late window, at days 5–7 of CI, when in addition to A1 and A2 receptors increments, A3 receptor mRNA is also up-regulated. We hypothesize that the modulation of adenosine receptors (probably the activation of A1R or the inhibition of A2AR) at the first window could be an important tool to prevent photoreceptor death, because photoreceptor apoptosis occurs mainly at the first 2 days of illumination. The second window may be responsible for the reorganization of the inner retina and the modulation of adenosine receptors in this period and could protect inner retinal neurons after the loss of photoreceptors. Further work is needed in order to know if the present findings are indicative of a general response to light which occurs in different species and in human retinal pathologies.

Contributions

MS., EML., MV., NM., MRF & IML. conducted the research, analyzed and interpreted data.

MS., EG., AM. and JJLC. designed the research.

MS. and JJLC. wrote the manuscript.

All authors reviewed and approved the manuscript.

Competing interest

The authors declare that they do NOT have competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.neulet.2018.09.053.

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