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Adenosine A_3 receptor activation is neuroprotective against retinal neurodegeneration

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ABSTRACT

Death of retinal neural cells, namely retinal ganglion cells (RGCs), is a characteristic of several retinal neurodegenerative diseases. Although the role of adenosine A_3 receptor (A_3R) in neuroprotection is controversial, A_3R activation has been reported to afford protection against several brain insults, with few studies in the retina. *In vitro* models (retinal neural and organotypic cultures) and animal models [ischemia-reperfusion (I-R) and partial optic nerve transection (pONT)] were used to study the neuroprotective properties of A_3R activation against retinal neurodegeneration. The A_3R selective agonist (2-Cl-IB-MECA, 1 μM) prevented apoptosis ($TUNEL^+$-cells) induced by kainate and cyclothiazide (KA + CTZ) in retinal neural cultures (86.5 ± 7.4 and 37.2 ± 6.1 $TUNEL^+$-cells/field, in KA + CTZ and KA + CTZ + 2-Cl-IB-MECA, respectively). In retinal organotypic cultures, 2-Cl-IB-MECA attenuated NMDA-induced cell death, assessed by TUNEL (17.3 ± 2.3 and 8.3 ± 1.2 $TUNEL^+$-cells/mm^2 in NMDA and NMDA+2-Cl-IB-MECA, respectively) and PI incorporation (ratio DIV4/DIV2 3.3 ± 0.3 and 1.3 ± 0.1 in NMDA and NMDA+2-Cl-IB-MECA, respectively) assays. Intravitreal 2-Cl-IB-MECA administration afforded protection against I-R injury decreasing the number of $TUNEL^+$ cells by 72%, and increased RGC survival by 57%. Also, intravitreal administration of 2-Cl-IB-MECA inhibited apoptosis (from 449.4 ± 37.8 to 207.6 ± 48.9 annexin-V^+-cells) and RGC loss (from 1.2 ± 0.6 to 8.1 ± 1.7 cells/mm) induced by pONT. This study demonstrates that 2-Cl-IB-MECA is neuroprotective to the retina, both *in vitro* and *in vivo*. Activation of A_3R may have great potential in the management of retinal neurodegenerative diseases characterized by RGC death, as glaucoma and diabetic retinopathy, and ischemic diseases.

1. Introduction

Retinal degenerative diseases, such as glaucoma and diabetic retinopathy, are leading causes of blindness worldwide. These diseases are associated with ischemia (Cherecheanu et al., 2013; Hayreh, 2013; Jaulim et al., 2013; Li et al., 2012; Osborne et al., 2004; Schmid et al., 2014) and axonal damage (Anderson and Hendrickson, 1974; Chua and Goldberg, 2010; Coxon et al., 2010), and glutamate excitotoxicity may also play a role (Gupta and Yücel, 2007; Martin et al., 2002; Moreno et al., 2013; Stem and Gardner, 2013). Moreover, the loss of retinal ganglion cells (RGCs) is a feature of glaucoma and diabetic retinopathy (Ha et al., 2012; Nickells, 2007; Quigley et al., 1995; Yang et al., 2012).

One major challenge in the field of retinal neurodegenerative

Abbreviations: A_3R, A_3 receptor; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CTZ, cyclothiazide; DARC, Detection of Apoptosing Retinal Cells; DIV, days *in vitro*; I-R, Ischemia-reperfusion; KA, kainic acid; pONT, partial optic nerve transection; RGC, retinal ganglion cell; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling.

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diseases is to halt or attenuate vision loss caused by disease progression. Currently, neurodegeneration is not the main focus of therapies for retinal diseases, and the surgical success rates are still not adequate. Therefore, it is of utmost importance that other pharmacologic approaches are developed to prevent progression of retinal neurodegenerative diseases and treat neurodegeneration. In fact, research on neuroprotection in retinal neurodegeneration has increased in the last decades, particularly in glaucoma, and different candidates to protect RGCs have been claimed (Baltmr et al., 2010; Harper et al., 2011).

Adenosine, a purine nucleoside, is a neuromodulator in the Central Nervous System. Its biological effects are mediated through G protein-coupled receptors by inhibition (A_1 and A_3) or stimulation (A_{2A} and A_{2B}) of adenylate cyclase. The adenosine A_3 receptor (A_3R), the last adenosine receptor to be cloned (Zhou et al., 1992), is involved in a variety of different intracellular signaling pathways, from G_q protein coupling activating phospholipase C (PLC), inositol triphosphate (IP3) and intracellular calcium (Ca^{2+}) or inhibition of adenylyl cyclase activity through G_i coupling (Gessi et al., 2008; Jacobson and Gao, 2006). The effects mediated by A_3R agonist are controversial. On one hand, the activation of A_3R was shown to induce or increase apoptosis in cancer cells (Kamiya et al., 2012; Kanno et al., 2012a,b; Morello et al., 2009; Nagaya et al., 2013; Nogi et al., 2012; Otsuki et al., 2012), and optic nerve oligodendrocytes (González-Fernández et al., 2014). On the other hand, in the ischemia-reperfusion (I-R) myocardium model, A_3R activation mediates cardioprotective effects (Headrick and Peart, 2005) and protection after brain ischemia (Gessi et al., 2011).

More direct evidence of the ability of A_3R activation to afford neuroprotection was provided by the observation that A_3R knockout mice are more vulnerable to hypoxia-induced neurodegeneration in the hippocampus than wild-type mice (Fedorova et al., 2003).

Taking into account that RGCs express A_3R (Zhang et al., 2006a) and that its activation attenuates the rise in calcium in RGCs after activation of glutamate and P2X receptors (Zhang et al., 2010, 2006b) we hypothesized that activation of A_3R affords protection to retinal cells, in particular to RGCs, in different models of retinal neurodegeneration.

2. Material and methods

2.1. Animals

Adult male Wistar (250–300 g, Charles River, France) or Dark Agouti (250–300 g, Harlan Laboratories, UK) rats were housed in a temperature- and humidity-controlled environment and were provided with standard rodent diet and water *ad libitum* whilst kept on a 12-h light/12-h dark cycle. All procedures were in agreement with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, the EU Directive 2010/63/EU for animal experiments and Home Office, UK.

2.2. Primary rat retinal neural mixed culture

Retinal neural cell cultures were prepared as previously described (Santiago et al., 2007). Briefly, the retinas were dissected from 3 to 5 day old Wistar rat pups in Ca^{2+} and Mg^{2+} free Hank's balanced salt solution (HBSS; in mM: 137 NaCl, 5.4 KCl, 0.45 KH_2PO_4, 0.34 Na_2HPO_3, 5 glucose; pH 7.4). Retinas were digested with 0.05% trypsin (w/v) for 15 min at 37 °C. The dissociated cells were pelleted by centrifugation and resuspended in Eagle's minimum essential medium (MEM) supplemented with 26 mM $NaHCO_3$, 25 mM HEPES, 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were plated at a density of 2.0×10^6 cells/cm^2 on glass coverslips coated with poly-D-lysine (0.1 mg/ml). The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. These retinal cell cultures are composed of neurons (photoreceptors, bipolar cells, horizontal cells, amacrine cells and RGCs), and glial cells (Müller cells, astrocytes and microglia), as previously described (Santos-Carvalho et al., 2013a).

After 6 days in culture, cells were incubated with 1 μM 2-Cl-IB-MECA (A_3R agonist; Tocris Bioscience, UK), 1 μM MRS1191 (A_3R antagonist; Tocris Bioscience, UK), or both, 30 min before incubation with 100 μM kainic acid (KA) and 30 μM cyclothiazide (CTZ) which prevents α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor desensitization, for 24 h.

2.3. Retinal organotypic culture

Retinas of 8–9 weeks old male Wistar rats were dissected in ice cold HBSS (pH 7.2) and flat-mounted onto 30 mm diameter culture plate inserts with a 0.4 μm pore size (Millicell, Millipore, USA), with the ganglion cell layer (GCL) facing upward. The retinal organotypic tissue was cultured in six-well plates containing Neurobasal-A media supplemented with B27 (1x), L-glutamine (2 mM) and gentamicin (50 μg/ml), and maintained for 4 days *in vitro* (DIV) in a humidified incubator at 37 °C and 5% CO_2.

When present, 2-Cl-IB-MECA (1 μM) was added at day 1 (DIV1) and again 1 h before the incubation with 300 μM N-methyl-D-aspartic acid (NMDA; Sigma–Aldrich, USA) at day 2 (DIV2). MRS1191 (1 μM) was added 30 min before insult.

2.4. Retinal ischemia-reperfusion injury model

Twenty Wistar rats were anesthetized by isoflurane inhalation using a gas anesthetizing system (1.2 ml/min; VetEquip, USA). Then, oxybuprocaine (4 mg/ml; Laboratórios Edol, Portugal) anaesthetic was applied topically to the eyes and the pupils were dilated with tropicamide (10 mg/ml; Laboratórios Edol, Portugal).

Both eyes were injected intravitreally with 2-Cl-IB-MECA (1.2 μM, 5 μl) or with 5 μl of sterile saline solution (vehicle, 0.9% NaCl; Fresenius Kabi, Portugal) 2 h before ischemia. The anterior chamber of the left eye was cannulated with a 30-gauge needle connected to a reservoir infusing a sterile saline solution. Retinal ischemia was induced by retinal blood flow blockade due to an increase in intraocular pressure to approximately 90 mmHg (TonoLab, Icare, Finland) for 60 min (Ishikawa et al., 2012). The contralateral eye served as the non-ischemic control. The observed whitening of the iris and the loss of the red reflex confirmed retinal ischemia. The needle was withdrawn after 60 min, and reperfusion was confirmed by the re-appearance of the red reflex. Fusidic acid (10 mg/g; Leo Pharmaceutical, Denmark) was applied in the conjunctival sac at the end of the experiment. The animals were allowed to recover for 24 h before sacrifice.

2.5. Partial optic nerve transection (pONT) model

Partial optic nerve transection was performed in the left eyes of twenty-four Dark Agouti rats, as previously described (Levkovitch-Verbin, 2003). Animals were anesthetized by intraperitoneal administration of ketamine and medetomidine as described above. Animals were randomly divided into 4 groups: pONT + PBS, pONT + 2-Cl-IB-MECA, 2-Cl-IB-MECA and PBS (control). 2-Cl-IB-MECA (1.2 μM, 5 μl) or vehicle was administered intravitreally immediately before pONT procedure. Briefly, the upper lid was retracted using a sterile suture. To access the optic nerve, a small incision was made on the conjunctiva and the eye was gently retracted forward using forceps, exposing the optic nerve. The optic

nerve was cleaned and was partially transected using a diamond knife, with care not to damage the blood supply.

2.6. In vivo imaging of retinal cell apoptosis

Detection of Apoptosing Retinal Cells (DARC) is an *in vivo* non-invasive imaging technique that uses the optical properties of the eye, and the high affinity binding of annexin-V to phosphatidylserine, to allow direct visualization of apoptotic cells (Cordeiro et al., 2004; Galvao et al., 2013). Annexin-V fluorescently conjugated allows real-time detection of single cells undergoing apoptosis (annexin-V labeled cells) with a confocal scanning laser ophthalmoscope (cSLO). Animals were imaged using the Heidelberg Retinal Angiograph (HRA) Spectralis (cSLO) (Heidelberg Engineering, Germany) 2 h after intravitreal injection of fluorescently labeled annexin-V, as previously described (Cordeiro et al., 2004). To optimize visualization, eye drop lubricant Viscotears® Liquid Gel (Novartis, UK) was regularly applied. Pupils were dilated with 2.5% phenylephrine hydrochloride and 1.0% cyclopentolate hydrochloride (Bausch & Lomb, France). During image acquisition, the HRA Spectralis was focused on the retinal nerve fiber layer, as identified in the reflectance mode. The images were collected and the number of spots (annexin-V positive cells) was counted using the public domain ImageJ program (http://rsb.info.nih.gov/ij/).

2.7. Histological preparation

2.7.1. Preparation of paraffin sections

The eyes of Dark Agouti rats were enucleated immediately after sacrifice, fixed in 4% (w/v) paraformaldehyde (PFA) and kept at 4 °C until further processing. After dissecting out the cornea, lens and vitreous, the eyecups were transferred to a paraffin-embedding machine (Leica TP1020, Leica Microsystems GmbH, Germany) where they were submitted to overnight sequentially cycles of ethanol (70%, 90% and 100%), xylene and paraffin. The paraffin blocks were then cut using a microtome (Leica Microsystems GmbH, Germany) into 7 μm sections. For each eye 3–4 different sections from separate areas of the retina were analyzed. On each retinal slice an average of 20–70 cells were counted.

2.7.2. Frozen retinal sections

Wistar rats, under deep anesthesia (75 mg/kg ketamine and 10 mg/kg xylazine), were transcardially perfused with PBS (pH 7.4), followed by 4% (w/v) PFA in PBS. The eyes were enucleated, washed in PBS and then transferred to 4% PFA for 1 h. The cornea, lens, pupil and iris were removed and the eyecup was further fixed for 1 h in 4% PFA. After washing in PBS, the eyecups were cryopreserved in 15% (w/v) sucrose in PBS for 1 h followed by 30% (w/v) sucrose in PBS overnight at 4 °C. Finally, the eyecup was embedded in tissue-freezing medium (OCT; Shandon, USA). The frozen blocks were cut using a cryostat into 10 μm thickness sections and the cryosections were collected on SuperFrost Plus glass slides (Menzel-Glaser, Germany) and stored at −20 °C.

2.7.3. Immunofluorescence labeling

Paraffin sections were placed on a hot plate to allow sections to adhere to the slide, deparaffinization and rehydration of the sections was performed by sequential incubations in xylene and descending ethanol series (100%, 90% and 70%). Antigen retrieval was achieved by microwaving the sections in 10 mM citrate buffer (pH 6.0) for 5 min. Both paraffin-embedded and cryosections were blocked and incubated with the primary antibody (mouse anti-Brn3a, 1:500, Millipore, USA) overnight in a humidity chamber at 4 °C. The sections were then washed and incubated with the secondary antibody (Alexa Fluor 546 anti-mouse IgG, 1:400, Life Technologies, USA) for 1 h at room temperature (RT). Nuclei were stained with DAPI (1:2500). The sections were then washed and mounted in PBS/glycerol (for paraffin-embedded sections) or Dako mounting medium (for cryosections). Images were acquired using a confocal microscope (Zeiss LSM 710, Germany).

2.8. TdT-mediated dUTP nick-end labeling (TUNEL) assay

TUNEL assay was used to quantify cell apoptosis. TUNEL assay identifies apoptotic cells by the terminal deoxynucleotidyl transferase (TdT)-mediated addition of labeled deoxyuridine triphosphate nucleotides to the 3′-OH end of DNA strand breaks (Gavrieli et al., 1992).

Rat retinal neural cell cultures were fixed in 4% PFA at 4 °C and blocked with 3% BSA and 0.1% Triton X-100 for 1 h at RT. In retinal sections, TUNEL assay was performed after immunostaining. TUNEL assay was performed according to the manufacturer's instructions (Promega, USA). Nuclei were counterstained with DAPI. The preparations were mounted using Glycergel mounting medium (Dako, Denmark) and visualized using a confocal microscope (Zeiss LSM 710, Germany).

For retinal cultures, 7–10 random images were acquired per condition in a total of 7–9 independent cell culture preparations. For the organotypic cultures, from each retina, 3 images of the GCL were randomly acquired per quadrant (12 images per retina), in a total of 5 independent retinal organotypic cultures. Concerning retinal sections, 4 slides comprising 2 retinal sections from the periphery and 2 from the central retina were analyzed. From each slide, 3 images were acquired. Per experimental group, 3–4 eyes from different animals were used. In each image, the number of $TUNEL^+$ cells in the outer nuclear layer (ONL), inner nuclear layer (INL) and GCL was counted. The number of Brn3a-immunoreactive cell nucleus was counted in the same images Table 1.

2.9. Propidium iodide incorporation assay

Retinal organotypic cultures were incubated with propidium iodide (PI; 2 μM) at 48 h (DIV 2) and 96 h (DIV 4) to assess cell death. Random images from the GCL, encompassing the four retinal quadrants of each retina (3 images from each quadrant), were acquired in a fluorescence microscope (Leica DM IRE2, Germany) using the 10x objective. PI^+ cells per retinal quadrant were counted at DIV2 (before incubation with NMDA) and at DIV4. The extent of cell death was expressed as the ratio between PI^+ cells at DIV4 and DIV2.

2.10. Statistical analysis

Data are represented as mean ± SEM. Statistical comparisons between different groups were performed using one-way ANOVA followed by Bonferroni pos hoc test using GraphPad Prism (GraphPAd 6, San Diego, CA, USA). Differences were considered significant for $p < 0.05$.

Table 1
Summary of the number of samples used in the study.

Samples	Images per coverslip/slide	n
Retinal primary cultures	7–10 images/coverslip	7–9
Retinal organotypic culture	12 images (3 per quadrant)/retina	5
Retinal sections	3 images/slide (4 slides/eye analyzed)	3–4

3. Results

3.1. Activation of A_3 receptor protects retinal cells from excitotoxicity-induced cell death in culture

In order to assess the potential neuroprotective effects mediated by A_3R activation, TUNEL assay (Fig. 1A and B) was performed in a primary retinal neural cell culture, well characterized in our lab (Costa et al., 2009; Santiago et al., 2007; Santos-Carvalho et al., 2013b). Retinal cultures were incubated with the selective A_3R agonist (2-Cl-IB-MECA, 1 μM) and/or antagonist (MRS1191, 1 μM), 45 min before the exposure to kainate (KA, 100 μM) in the presence of cyclothiazide (CTZ, 30 μM) to prevent AMPA receptor desensitization (Partin et al., 1993). The incubation with KA + CTZ for 24 h significantly increased the number of $TUNEL^+$ cells, compared with control cells (29.2 ± 4.6 and 86.5 ± 7.4 $TUNEL^+$ cells/field, in control and KA + CTZ, respectively; $^{****}p < 0.0001$). The incubation of cells with 2-Cl-IB-MECA significantly prevented the increase in the number of $TUNEL^+$ cells induced by KA + CTZ (37.2 ± 6.1 $TUNEL^+$ cells/field; $^{**}p < 0.01$). Furthermore, the protective effect of 2-Cl-IB-MECA was lost when MRS1191 was also present (80 ± 9.9 $TUNEL^+$ cells/field), showing the specificity of the protective effects observed. Incubation of cells with 2-Cl-IB-MECA, MRS1191 or both did not significantly change the number of $TUNEL^+$ cells compared with the control (data not shown).

3.2. A_3 receptor activation protects retinal cells against excitotoxicity-induced cell death in retinal organotypic cultures

The neuroprotective effect of A_3R activation against cell death induced by excitotoxicity in retinal organotypic cultures was investigated by exposing the organotypic cultures to NMDA (300 μM) for 48 h, in the presence and/or absence of A_3R agonist and/or antagonist. Retinal structure is preserved in organotypic retinal cultures, allowing interactions between the different cell types, thus providing a very useful model for neuroprotection studies. Cell death was assessed with TUNEL (Fig. 2A and B) and PI incorporation (Fig. 2C) assays. In control conditions, the number of apoptotic cells in the GCL was 1.4 ± 0.6 $TUNEL^+$ cell/mm^2. The incubation with NMDA for 48 h significantly increased the number of $TUNEL^+$ cells in the GCL to 17.6 ± 2.3 $TUNEL^+$ cell/mm^2 ($^{****}p < 0.0001$). The incubation with 2-Cl-IB-MECA (1 μM) prior NMDA attenuated the increase in the number of $TUNEL^+$ cells (8.3 ± 1.2 $TUNEL^+$ cell/mm^2; $^{***}p < 0.001$), indicating a decrease in cell death. Moreover, pre-incubation with the A_3R antagonist abolished the protective effect of 2-Cl-IB-MECA (16.3 ± 1.3 $TUNEL^+$ cell/mm^2).

Similar results were found for PI incorporation. In control condition, the DIV4/DIV2 ratio was 1.4 ± 0.1. When the organotypic cultures were incubated with NMDA for 48 h, there was a significant increase in the PI^+ cells ratio, compared with control (3.3 ± 0.3; $^{****}p < 0.0001$). Pre-treatment with 2-Cl-IB-MECA (1 μM) prevented the increase in the ratio of PI^+ cells induced by NMDA (1.3 ± 0.1: $^{***}p < 0.001$). This protective effect was eliminated when the A_3R antagonist (MRS1191; 1 μM) was also present. Incubation with 2-Cl-IB-MECA or MRS1191 alone does not change the DIV4/DIV2 ratio for PI^+ cells (data not shown).

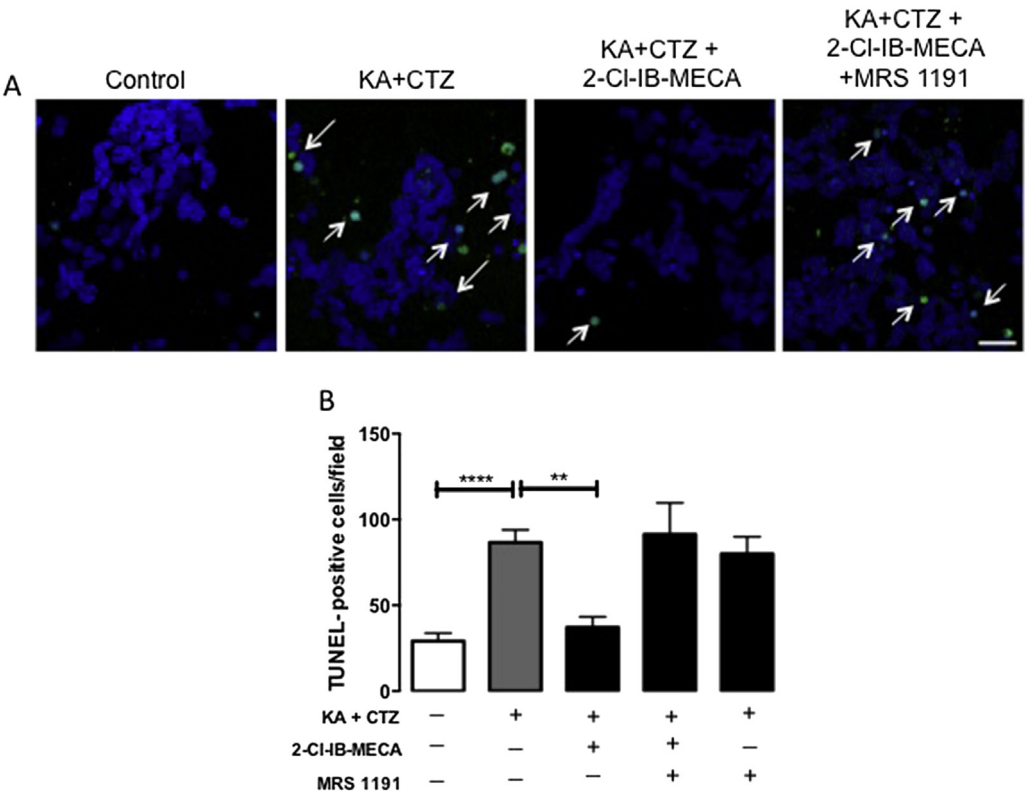

Fig. 1. A_3 receptor activation protects retinal mixed neuronal cell cultures against excitotoxic-induced cell death. Retinal cell apoptosis was quantified using TUNEL assay (green) (A). Retinal cells were pre-treated with 2-Cl-IB-MECA (1.2 μM) 30 min before exposure to KA + CTZ for 24 h. Nuclei were stained with DAPI (blue). Images in the figure represent magnified imaged of each field. B: Quantification of TUNEL + cells in retinal cell cultures (per field). The results represent the mean ± SEM of 7–9 independent experiments. $^{****}p < 0.0001$; $^{***}p < 0.001$, One-way ANOVA test followed by Bonferroni post-test. Arrows indicate TUNEL positive cells. Scale bar 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

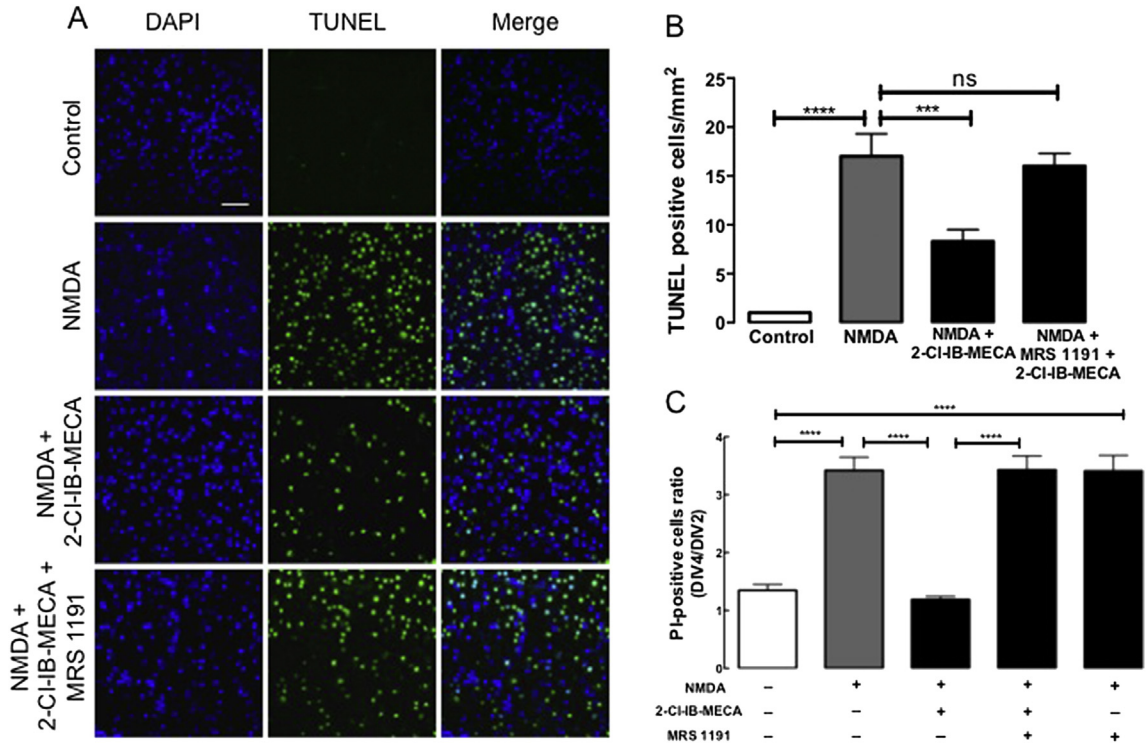

Fig. 2. A_3 adenosine receptor agonist protects retinal cells against excitotoxic-induced cell death in retinal organotypic cultures. TUNEL and PI assays were used to quantify cell death in retinal organotypic cultures. (A) Apoptosis was assessed with TUNEL assay: green staining indicates $TUNEL^+$ cells. (B) The results represent the mean ± SEM, and represent the number of $TUNEL^+$ cells per mm^2 in the GCL; ****$p < 0.0001$; ***$p < 0.001$; one-way ANOVA test followed by Bonferroni post-test. Scale bar is 20 μm. (C) Retinal organotypic cultures were treated with 2-Cl-IB-MECA at DIV1 and DIV2 (prior incubation with NMDA). Cell death was assessed with propidium iodide (PI) staining in live cells at DIV2 (before NMDA) and DIV4. The results represent the mean ± SEM of 5 independent experiments, and represent the ratio of PI^+ cells in the GCL between DIV4 and DIV2; ****$p < 0.0001$; One-way ANOVA test followed by Bonferroni post test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. A_3 receptor agonist protects retinal cells in a model of ischemia-reperfusion injury

We next tested the protective effects of A_3R activation in a model of retinal ischemia-reperfusion (I-R) injury. *In vivo* DARC imaging was not performed in these animals due to the transient corneal oedema associated with this particular model of ischemia-reperfusion, which does not allow visualization of the eye fundus. As reported previously, retinal I-R injury induces the loss of retinal cells (Fernandez et al., 2009). Both eyes were injected intravitreally with 2-Cl-IB-MECA (1.2 μM, 5 μl) or with 5 μl of sterile saline solution 2 h before ischemia (90 mmHg for 60 min). TUNEL assay was performed on retinal sections to evaluate retinal cell apoptosis (Fig. 3A–C). I-R injury caused a significant increase in the number of $TUNEL^+$ cells in the retina compared with the control (0 ± 0.4 and 72 ± 14.4 $TUNEL^+$ cells/mm for control and I-R retinas, respectively; ****$p < 0.0001$, Fig. 3A and B). A single intravitreal injection of 2-Cl-IB-MECA, prior ischemia, significantly inhibited the increase in the number of $TUNEL^+$ cells in the retina (***$p < 0.001$, Fig. 3A and B), when compared with I-R group. When analyzing $TUNEL^+$ cells per nuclear layer (Fig. 3C), we found that the intravitreal injection of 2-Cl-IB-MECA decreased the number of apoptotic cells in all nuclear layers.

To evaluate the potential protective effect of A_3R activation specifically in RGCs, the number of surviving RGCs (Brn3a-immunoreactive cells) was also counted (Fig. 3D and E). Brn3a is a POU domain transcription factor, which is used to label RGCs in the adult rat retina (Nadal-Nicolás et al., 2009). Retinal I-R significantly decreased the number of RGCs (14 ± 6.0 Brn3a-immunoreactive cells/mm; **$p < 0.01$) compared with the control (contralateral) eye (34 ± 10.0 Brn3a-immunoreactive cells/mm). Injection of 2-Cl-IB-MECA significantly inhibited the loss of RGCs (22 ± 4.0 $Brn3a^+$ cells/mm; *$p < 0.05$) triggered by I-R injury. The administration of 2-Cl-IB-MECA to non-ischemic eyes did not significantly affect the number of $Brn3a^+$ cells.

3.4. A_3 receptor activation protects retinal cells after 7 days of pONT

Specific RGC death can be achieved by transecting the axons of the RGCs. The partial optic nerve transection (pONT) model was performed to evaluate the potential protective properties of 2-Cl-IB-MECA against RGC death. The pONT model can be used to model optic neuropathy, such as glaucoma or traumatic optic neuropathy. *In vivo* DARC imaging showed that 7 days after pONT there was a significant increase in the number of annexin-V positive cells (449.4 ± 37.8 cells; ****$p < 0.0001$, Fig. 4A and B), compared with the control (99.0 ± 17.1 cells). Intravitreal administration of 2-Cl-IB-MECA reduced apoptosis when compared with pONT animals (207.7 ± 48.8 annexin-V positive cells, ***$p < 0.001$, Fig. 4A and B). The injection of 2-Cl-IB-MECA to non-operated animals did not significantly change the number of annexin-V positive cells (204.2 ± 41.5 cells) comparing with control.

We next assessed RGC survival in retinal sections of the same animals. The number of RGCs was inferred by counting Brn3a-immunoreactive cells, as before. Consistent with *in vivo* findings, a significant reduction in the number of RGCs was observed in pONT eyes at 7 days (1.2 ± 0.6 $Brn3a^+$ cells/mm, **$p < 0.01$) compared with control (16.8 ± 5.1 $Brn3a^+$ cells/mm) (Fig. 4C and D). Treatment with A_3R agonist inhibited the decrease in the number of RGCs induced by pONT (8.1 ± 1.6 $Brn3a^+$ cells/mm, **$p < 0.01$;

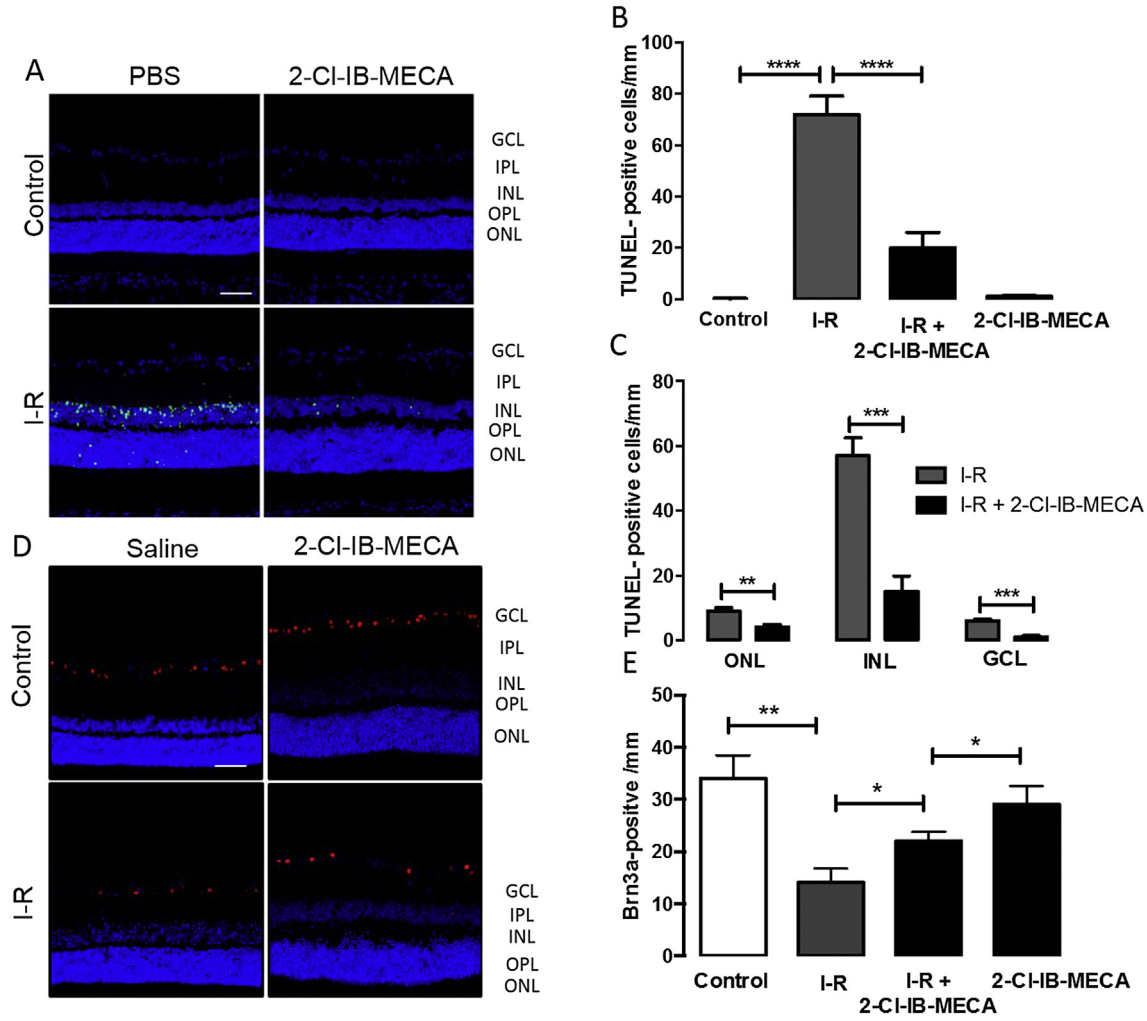

Fig. 3. - A_3 receptor agonist protects retina against ischemia-reperfusion injury. A: TUNEL assay (green) was performed in retinal cryosections. Nuclei were stained with DAPI (blue). B: Quantification of TUNEL$^+$ cells in the retina. The number of TUNEL$^+$ cells was counted in the total retina (B) and in the different retinal layers (C). The results are expressed as mean ± SEM, and represent the number of TUNEL$^+$ cells per retinal slice. ***$p < 0.001$; one-way ANOVA test followed by Bonferroni post-test. Fig. 4B represents the quantification of the total number of TUNEL$^+$ cells in all nuclear retinal layers, while Fig. 3C represents the quantification per nuclear layer. D: Representative images of Brn3a staining (red) in retinal sections. The number of RGCs was evaluated by counting Brn3a$^+$ cells (red) in different retinal sections. Nuclei were stained with DAPI (blue). E: Quantification of Brn3a$^+$ cells. The results are expressed as mean ± SEM of 3–4 independent experiments, and represent Brn3a$^+$ cells/mm; **$p < 0.01$, *$p < 0.05$; one-way ANOVA followed by Bonferroni's multiple comparison test. Scale bar: 20 μm. GCL - Ganglion cell layer; IPL – Inner plexiform layer; INL – Inner nuclear layer; OPL – Outer plexiform layer; ONL – Outer nuclear layer. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4D). Moreover, treatment with the A_3R agonist alone did not significantly affect the number of Brn3a$^+$ cells (11.8 ± 2.8 Brn3a$^+$ cells/mm).

4. Discussion

The results presented herein demonstrate that activation of A_3R protects retinal neurons both *in vitro* and *in vivo*. We show that activation of A_3R with the selective agonist 2-Cl-IB-MECA decreased the number of apoptotic cells and increased RGC survival.

The protective effects mediated by the activation of A_3R are controversial, and may be dependent on the experimental model used (cells, type of tissue, animal model and insult) or on the concentration of the agonists used. A recent study showed that 2-Cl-IB-MECA (10 μM, 100 μM, and 1 mM) leads to concentration-dependent oligodendrocyte cell death, and incubation of *ex vivo* preparations of optic nerve with 2-Cl-IB-MECA activates caspase-3 (González-Fernández et al., 2014). At these concentrations, 2-Cl-IB-MECA was previously shown to induce apoptosis in other cell types. Indeed, 2-Cl-IB-MECA (used in the range 10–200 μM) induces cell death in human leukaemia cells, human A549, Lu-65 and SBC-3 lung cancer cells, A172 human glioma, RCC4-VHL human renal, in thyroid cancer cells, and in 5637 human bladder cancer cells (Kamiya et al., 2012; Kanno et al., 2012a,b; Mlejnek and Dolezel, 2010; Morello et al., 2009; Nagaya et al., 2013; Otsuki et al., 2012). Interestingly, in the latter cells, when provided at 1 μM, 2-Cl-IB-MECA did not significantly increase cell death. When low concentrations were used (in the range 10 nM^{-1} μM), the activation of A_3R is protective against I-R of the heart, lung and brain (Chen et al., 2006; Headrick and Peart, 2005; Lee et al., 2001; Luo et al., 2010; Mulloy et al., 2013). Moreover, there is additional evidence in astrocytes on the dual role of A_3R activation, mediating both protective and cell death depending on the A_3R agonist concentration used (Abbracchio et al., 1997; Di Iorio et al., 2002; Jacobson et al., 1997; Yao et al., 1997). Indeed, the detrimental effects observed for higher concentrations of 2-Cl-IB-MECA are not entirely surprising, knowing that 2-Cl-IB-MECA is a potent A_3R

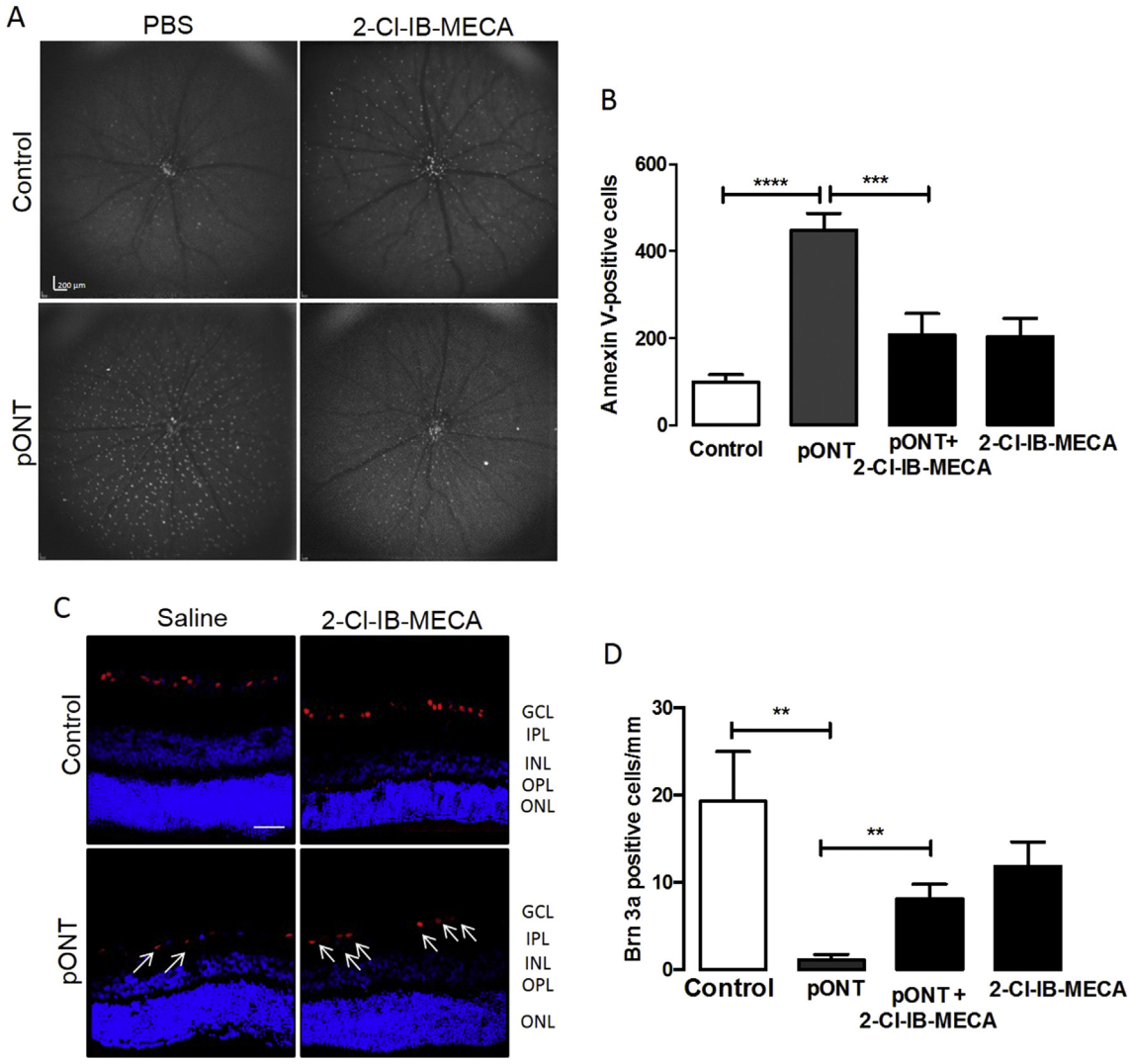

Fig. 4. A_3 receptor activation protects retinal cells against cell death and decreases RGC loss after partial optic nerve transection. *In vivo* DARC images were acquired 7 days after pONT. Representative images are depicted in A. *In vivo* DARC images (A) using HRA Spectralis show cells undergoing apoptosis in the GCL, 2 h following injection of fluorescently-labeled annexin-V. Wide-angle retinal images show white bright spots, which represent single apoptotic retinal cells (A). B: Apoptotic retinal cells (white spots) were counted and the results represent the mean ± SEM of 3–4 independent experiments, of the number of cells labeled with annexin-V; ***$p < 0.001$; ****$p < 0.0001$; one-way ANOVA test followed by Bonferroni post-test. Scale bar 200 µm. The survival of RGCs was assessed 7 days after pONT. C: The loss of RGCs was evaluated by staining RGCs with anti-Brn3a (red). Nuclei were stained with DAPI (blue). D: The number of $Brn3a^+$ cells/mm was counted and the results are expressed as mean ± SEM. **$p < 0.01$; student t-test. Scale bar: 20 µm. GCL – Ganglion cell layer; IPL – Inner plexiform layer; INL – Inner nuclear layer; OPL – Outer plexiform layer; ONL – Outer nuclear layer. Arrows indicate Brn3a-immunoreactive cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

agonist with a Ki value of 0.3 nM for rat A_3R and with good selectivity for the other adenosine receptors (A_3R Ki: 0.3 nM; $A_{2A}R$ Ki: 470 nM; A_1R Ki: 820 nM; Jacobson and Knutsen, 2001). At higher concentrations, 2-Cl-IB-MECA will most likely to activate both A_1 and A_{2A} receptors, which adds increased complexity to analyze the results.

In the retina, the neuroprotective effects of A_3R have not been extensively studied. A few reports have demonstrated that activation of A_3R in immunopurified isolated RGCs attenuates the increase in intracellular calcium concentration after NMDA or P_2X_7 receptors activation (Hu et al., 2010; Zhang et al., 2010, 2006b). In rats, intravitreal injection of the A_3R agonist MRS3558 prevents RGC loss induced by P_2X_7 receptor activation (Hu et al., 2010).

One of the big challenges in neuroprotection is that most neurodegenerative diseases are multifactorial and so the mechanisms by which the diseases progress are uncertain, making it difficult to develop a specific therapeutic strategy (Liu and Pang, 2013). In the present study, trying to overcome this problem, we have tested a different number of insults. One mechanism proposed to retinal neurodegenerative disorders, such as glaucoma (Gupta and Yücel, 2007; Martin et al., 2002) and diabetic retinopathy (Moreno et al., 2013; Stem and Gardner, 2013) is the overactivation of glutamate receptors, leading to increased intracellular calcium concentration culminating in cell death. In particular for glaucoma, some controversy exists regarding the increase of glutamate levels in the vitreous of animals with experimental glaucoma (Carter-Dawson et al., 2002; Wamsley et al., 2005). Nevertheless, glutamate receptors or transporters have previously been shown altered in experimental glaucoma (Gupta and Yücel, 2007; Martin et al., 2002). Our results show that the A_3R agonist is protective against excitotoxicity-induced (KA + CTZ) cell death, using an *in vitro* model, which is in accordance with previous results (Zhang et al., 2010). The protection conferred by the activation of A_3R upon glutamatergic excitotoxicity appears to be dependent on the cell

type, since A_3R activation does not protect cultured cortical neurons from excitotoxicity-induced death, notwithstanding the known receptor expression on these cells (Rebola et al., 2005).

Furthermore, we also used the retinal I-R injury model to test the protective effects of A_3R activation. Retinal ischemia contributes to visual impairment and blindness in diabetic retinopathy (Li et al., 2012), retinal vascular occlusion diseases (Jaulim et al., 2013), ischemic optic neuropathy (Hayreh, 2013) as well as in glaucoma (Cherecheanu et al., 2013). Our results showed that A_3R activation decreased retinal cell death and increased RGC survival. The modulation of A_3R activity, either with allosteric modulators or with A_3R agonists, attenuates I-R injury in lungs (Mulloy et al., 2013), heart (Du et al., 2012), and brain (Chen et al., 2006; Von Lubitz et al., 1994). In the retina, adenosine has been used to protect against I-R injury through the activation of A_1R (Li and Roth, 1999; Li et al., 2000; Wang et al., 1997) although in these studies the A_3R agonists were not tested. Interestingly, the protective effects of 2-Cl-IB-MECA may not be restricted to RGCs. In the I-R injury model, we found that 2-Cl-IB-MECA significantly reduced cell death in the INL. Most probably, this is an indirect effect mediated by RGCs, taking into consideration the lack of evidence of the expression of A_3R in cells present at the INL. Nevertheless, further studies are needed to better clarify the protective mechanisms mediated by A_3R activation in the inner retina, and identification of cells being protected may help elucidate novel therapeutic options for retinal diseases.

Finally, damage to the optic nerve leads to axonal transport impairment which is associated with traumatic optic neuropathy and other degenerative optic neuropathies such as glaucoma (Anderson and Hendrickson, 1974; Chang and Goldberg, 2012; Coxon et al., 2010). Our results, using a pONT model, showed that intravitreal injection of 2-Cl-IB-MECA attenuated the neurodegenerative process. This was shown both *in vivo* (annexin-V positive cells) and histologically (TUNEL- and $Brn3a^+$ cells).

According to the results, we hypothesize that A_3R activation will decrease intracellular calcium concentration, as reported previously (Zhang et al., 2010, 2006a). Indeed, in the models tested there is evidence of intracellular calcium increase upon exposure to NMDA and KA + CTZ (Santiago et al., 2006). Moreover, calcium increase was reported to be involved in I-R injury (Sakamoto et al., 2009).

Due to the increase in life expectancy and because age is a strong risk factor for most of retinal neurodegenerative diseases, visual impairment represents an increasing and substantial burden to society, having a strong economic impact with a significant reduction on quality of life. Therefore, it is of major importance to develop new therapeutic approaches to fight blindness. Research in neuroprotection has identified several neuroprotective drugs, and some promising results have been shown with both *in vitro* and *in vivo* research. Nevertheless, there are just a few clinical trials addressing this issue (NCT01408472, NCT00466479, NCT01544192), due to a number of limitations such as, injury variability between patients, small treatment window after injury and reliable end points (Levin and Peeples, 2008). A neuroprotective drug is, by definition, an agent with a specific receptor on the target tissue, that should have adequate penetration to reach the receptor, and should decrease neuronal damage, promoting neuronal survival in animal models and with proven efficacy in clinical trials (Chua and Goldberg, 2010). The A_3R was previously identified as being present in the retina and particularly in RGCs (Ghiardi et al., 1999; Sanderson et al., 2014; Zhang et al., 2006a). Our results clearly show that treatment with the A_3R agonist 2-Cl-IB-MECA decreased the number of cells undergoing apoptosis as shown by the TUNEL assay (Figs. 1–3) and the DARC technique (Fig. 4), also increasing RGC survival as shown by Brn3a immunelabeling (Figs. 3 and 4). Regarding these results, A_3R appears to be a promising target to pursue in clinical research. In fact, there is an ongoing phase 2 glaucoma clinical trial on the safety and efficacy of daily IB-MECA administration (NCT01033422) in reducing intraocular pressure (major risk factor of glaucoma) in patients with ocular hypertension and/or glaucoma. Although in the present study, the effects of intravitreal injection of 2-Cl-IB-MECA in IOP were not assessed, it was previously reported that adenosine or 2-Cl-IB-MECA lowers intraocular pressure in humans and mice, respectively (Polska et al., 2003; Wang et al., 2010). Nevertheless, the activation of A_3R as strategy to lower intraocular pressure is still controversial. The activation of A_3R was shown to be involved in the regulation of chloride channels in nonpigmented ciliary epithelial cells, suggesting that A_3R agonists would increase aqueous humor secretion and thereby intraocular pressure, while A_3R antagonists would be a potential strategy to treat ocular hypertension (Gessi et al., 2008; Mitchell et al., 1999; Okamura et al., 2004; Schlötzer-Schrehardt et al., 2005). In addition, topical application of A_3R antagonist was reported to reduce intraocular pressure in mice, while topical administration of A_3R agonist increased intraocular pressure (Avila et al., 2001; Yang et al., 2005). Regardless the effects of A_3R activation on intraocular pressure, the protective properties mediated by A_3R activation against retinal degeneration may present a valuable tool.

5. Conclusions

In this study, we showed that A_3R activation protects retinal cells, particularly RGCs, in different models of cell degeneration mainly depicting excitotoxicity, I-R injury and axon damage. Therefore, targeting this receptor might be an important step towards a potential therapy for retinal neurodegenerative diseases, mainly those targeting RGCs particularly optic neuropathies such as glaucoma.

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