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TITLE: P2X7 and A_{2A} receptor endogenous activation protects against neuronal death caused by $CoCl_2$-induced photoreceptor toxicity in the zebrafish retina

Running title: P2X7R and A_{2A}R avoids retina cell death

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ABSTRACT

Injured retinas in mammals do not regenerate and heal with loss of function. The adult retina of zebrafish self-repairs after damage by activating cell-intrinsic mechanisms, which are regulated by extrinsic signal interactions. Among relevant regulatory extrinsic systems, purinergic signalling regulates progenitor proliferation during retinogenesis and regeneration and glia proliferation in proliferative retinopathies. ATP-activated P2X7 (P2RX7) and adenosine (P1R) receptors are involved in the progression of almost all retinopathies leading to blindness. Here, we examined P2RX7 and P1R participation in the retina regenerative response induced by photoreceptor damage caused by a specific dose of $CoCl_2$. First, we found that treatment of uninjured retinas with a potent agonist of P2RX7 (BzATP) provoked photoreceptor damage and mitotic activation of multipotent progenitors. In $CoCl_2$-injured retinas, blockade of endogenous extracellular ATP activity on P2RX7 caused further neurodegeneration, Müller cell gliosis, progenitor proliferation and microglia reactivity. P2RX7 inhibition in injured retinas also increased the expression of *lin28a* and *tnfα* genes, which are related to multipotent progenitor proliferation. Levels of *hif1α*, *vegf3r* and *vegfaa* mRNA were enhanced by blockade of P2RX7 immediately after injury, indicating hypoxic like damage and endothelial cell growth and proliferation. Complete depletion of extracellular nucleotides with an apyrase treatment strongly potentiated cell death and progenitor proliferation induced with $CoCl_2$. Blockade of adenosine P1 and A_{2A} receptors ($A_{2A}R$) had deleterious effects and deregulated normal timing for progenitor and precursor cell proliferation following photoreceptor damage. ATP via

P2RX7 and adenosine via $A_{2A}R$ are survival extracellular signals key for retina regeneration in zebrafish.

Keywords: BzATP, P2X7 receptor, adenosine A_{2A} receptor, progenitor proliferation, microglia activation, A740003, photoreceptor death, RRID:AB_514483, RRID:AB_628110, RRID:AB_2109952, RRID:AB_2315387, RRID:AB_2168560, RRID:AB_2738589, RRID:AB_2338840, RRID:AB_2338685, RRID:AB_2338000

1. INTRODUCTION

The adult retina of zebrafish can self-repair following injury throughout not yet completely understood mechanisms regulated in part by injury-induced extracellular signals which interact directly or indirectly regulating multipotent progenitor reprogramming, mitotic activation, and cell migration and differentiation. Regenerative mechanisms induce the reestablishment of interactions among neurons, glia and blood vessels in coherent units to achieve functional repair of the retina (Gemberling *et al*., 2013; Lenkowski & Raymond, 2014; Powell *et al*., 2016; Ail & Perron, 2017).

We have previously described a model of retinal degeneration that primarily involves photoreceptor death across the surface of the retina of zebrafish (Medrano *et al*., 2018). The injury model consisted in injecting a single dose of $CoCl_2$ within the vitreous cavity. We have found that $CoCl_2$ injury effect was mainly cytotoxic first for cones, later for rods and secondarily induced death of 30% of bipolar cells in the zebrafish retina. The majority of cells –including the majority of photoreceptors- died due to cytotoxic effects triggered by $CoCl_2$ and mediated by glutamate ionotropic receptors chiefly of the NMDA kind (Medrano *et al*., 2018). As described with many other types of injuries studied so far – mechanical, surgical, chemical, phototoxic, and mutational- (Vihtelic & Hyde, 2000; Wu *et al*.,

2001; Mensinger & Powers 2007; Fimbel *et al*., 2007; Sherpa *et al*., 2008; Ramachandran, Fausett, & Goldman, 2010; Weber *et al*., 2013; Tappeiner *et al*., 2013) damaging the outer retina with $CoCl_2$ activates the proliferative response of multipotent retinal progenitors and the regeneration of the mature tissue.

Our laboratory has analyzed the effect of purinergic signalling on the regeneration processes in the retina of zebrafish. To that end, we have previously used a model of injury with a low dose of ouabain that mainly killed inner retinal cells or an intermediate dose of ouabain that provoked cell death in all retinal layers but spared a significant amount of retinal cells (Battista *et al*., 2009, Medrano *et al*., 2017). We have described that extracellular ADP acting on metabotropic purinergic receptor Y_1 ($P2RY_1$) activates the proliferative activity of neural progenitors in intact and lesioned retinas of zebrafish (Ricatti *et al*., 2011; Battista *et al*., 2009) demonstrating that purinergic signalling is necessary to fully induce reprogramming of progenitor Müller glia and amplification of Müller glia-derived progenitors to achieve retina repair after injury (Medrano *et al*., 2017).

Furthermore, several groups of research have demonstrated that degenerative processes in many retinopathologies -such as retinal detachment, proliferative vitreous retinopathy, age-related macular degeneration, ischemic-hypoxic degeneration, diabetic retinopathy, retinitis pigmentosa and glaucoma- are at least in part mediated by ATP release and activation of ionotropic (P2RX) and metabotropic (P2RY) purinergic receptors including adenosine receptors (P1R) (Bringmann *et al*., 2001; Zhang *et al*., 2007; Niyadurupola *et al*., 2013; Sugiyama, 2014; Liu *et al*., 2017; Ventura *et al*., 2018). P2X7 receptor (P2RX7) activation by ATP can induce retinal and vascular cell death by inducing influx of extracellular calcium to cells (Zhang *et al*., 2005; Notomi *et al*., 2011; Yang *et al*.,

2011; Sugiyama, 2014; Vessey *et al.*, 2014; Xue *et al*., 2016). It has been described that ATP released in pathological conditions can cause oscillatory changes in the membrane potential, cell swelling, gliosis, hypertrophy and proliferation of Müller cells as well as cell degenerative processes propagation from focal injury regions to uninjured zones (Uhlmann *et al*., 2003; Wurm *et al*., 2011; Reichenbach & Bringmann, 2016).

Adenosine has been described as an important extracellular signal in the nervous system including the retina and can be released by nucleoside transporters or produced from extracellular ATP via enzymatic dephosphorylation (Fredholm *et al*., 2001). Adenosine acting on P1R can protect neurons from ischemia-hypoxia and metabolic and osmotic unbalances (Housley, Bringmann, & Reichenbach, 2009; Melani *et al*., 2014; Rivera-Oliver & Díaz-Ríos, 2014). Adenosine can increase glutamate-mediated excitotoxicity, neurodegeneration, inflammation and pathological angiogenesis (Reece *et al*., 2004; Boia *et al*., 2017; Liu *et al.*, 2017). The extracellular adenine nucleoside can provoke these deleterious effects via either activation or inactivation of A_{2A} receptors ($A_{2A}R$), which involves paradoxical effects of adenosine in the central nervous system (CNS) (Cunha, 2016).

Here we examined the effects of activating and blocking P2RX7 on the intact and injured retina of zebrafish. Because the retina of zebrafish is a self-repairing tissue, we studied the effect of a selective P2RX7 antagonist on multipotent progenitor mitotic activity and degenerative process at relatively short intervals after injury induction. We also examined the effect of an agonist and two antagonists of P1R on the proliferation process of retina progenitors and GFAP expression induced by damage with $CoCl_2$. Additionally, we analyzed the effect of depleting endogenous nucleotides on tissue damage and cell proliferation in the injured retina. We found that P2RX7 activation had a

neuroprotective action on the injured retina. Treatment of intact retinas with BzATP caused photoreceptor injury via powerful activation of P2RX7. Adenosine acting on $A_{2A}R$ exerted neuroprotective actions in the damaged retinal tissue.

2. MATERIALS AND METHODS

2.1. Materials

Paraformaldehyde; 137 mM NaCl, 2.7 mM KCl, 5.7 mM phosphate, pH:7.4 (PBS); Tris base 20 mM, NaCl 140 mM, pH: 7.5 (TBS); Tween-20; MS-222 (tricaine); sucrose; cresyl violet; 5-bromo-2'-deoxyuridine (BrdU); cobalt (II) chloride hexahydrate; and dimethyl sulfoxide (DMSO) (SIGMA-Aldrich, St Louis, MO). Tissue freezing medium (Biopack; Buenos Aires, Argentina). DABCO (2.5%) in PBS:glicerol (1:9) (SIGMA-Aldrich) and normal goat serum (NGS) (Natocor, Córdoba, Argentina). Random primers; Moloney Murine Leukemia Virus reverse transcriptase (MMLV-RT); dNTPs; 1,4-dithiothreitol (DTT); ribonuclease inhibitor (RNAsin); and RNA extraction reagent RNAzol (Genbiotech, Buenos Aires, Argentina). Sense and antisense primers (IDT, Coralville, IA). DNAse-I (Promega, Madison, WI). Real-time PCR mix (Solis Byodine, Tartu, Estonia).

2.2. Animals

Zebrafish (*Danio rerio*, Singapore strain) were purchased from a local breeding farm, in La Plata, Buenos Aires, Argentina. Zebrafish were acclimatised to the laboratory facility conditions for at least one month. They were maintained at 28°C in a 14:10 h light:dark cycle, and they were fed with *Arthemia sp*. and dry food twice a day. The adult zebrafish used in the experiment were an average of three cm in length. Animals were euthanized by immersion in ice-cold MS-222 anaesthetic solution (0.02% w/v), decapitated and enucleated on ice. The Committee on Animal Research at University of Buenos Aires (CICUAL) approved protocols for ethical animal use and care.

2.3. Anaesthesia and intraocular injections

Zebrafish were deeply anesthetized in the anaesthetic solution prepared with fish tank water and placed on a humid surface under a dissecting microscope. For performing retina damage -on day zero (0) for the majority of assays- the left eyes of the experimental zebrafish were given a single injection of 0.6 µl of 6.5 mM $CoCl_2$ hexahydrate diluted in sterile saline solution into the vitreous cavity. The zebrafish were injected during ten to fifteen sec under a dissecting microscope. Zebrafish fully recovered from anaesthesia in five to ten sec in fish tank water. Recovery rates were 100%. The intravitreal concentration (ivc) of $CoCl_2$ was estimated to be 1.3 mM on the basis of a vitreous volume of approximately 3 µl. This approximate value was calculated from the volume difference between the posterior chamber of the eye and the lens radius (Cunvong *et al*., 2013). A bevelled 33 gauge needle (0.375", PT2) and 10 µl syringe (701 RN) (Hamilton Company, Reno, NV) were used to deliver appropriate volumes.

2.4. List of agonists and antagonists of P receptors and other molecules injected *in vivo* in the vitreous cavity of zebrafish

ATPɣS: Adenosine-5'-(γ-thio)-triphosphate tetralithium salt is a non-selective P2 receptor agonist and slowly hydrolysable analogue of ATP. Injected concentration: 5 mM; ivc: 1.0 mM.

BzATP: (3'-O-(4-benzoyl) benzoyl adenosine-5'-triphosphate); potent agonist of P2X7 receptors with a potency over five to ten times higher than ATP. EC50 = 0.7 µM in HEK 293 cells; EC50 = 3.6 and 285 µM for P2X7 of rat and mouse, respectively. Partial agonist of P2X1 (pEC50 = 8.7) and P2Y1 receptors. Agonist specificity for P2X7: BzATP >>> ATPɣS > ATP. Injected concentration: 2 mM; ivc: 400 µM.

A740003: competitive and high affinity antagonist for P2RX7. Selective for P2RX7 over P2X1/3/2/4 and P2Y receptors up to concentrations of 100 µM. IC_{50}: 18 and 40 nM for rat and human P2RX7, respectively (Jacobson & Müller, 2016). Injected concentration: 100 µM, ivc: 20 µM.

8SPT: 8-(p-sulfophenyl) theophylline hydrate is a competitive and non-selective adenosine P1 receptor antagonist (Ki: 1.2 µM). Injected concentration: 100 µM; ivc: 20 µM.

SCH58261: potent and highly selective adenosine $A_{2A}R$ competitive antagonist (Ki = 1.3 nM). Displays 323-, 53- and 100-fold selectivity over A_1, A_{2B} and A_3 receptors, respectively (compound datasheet, Tocris). Injected concentration: 3 µM and 0.5 µM; ivc: 0.6 µM and 100 nM.

CGS21680: Potent adenosine $A_{2A}R$ agonist (Ki = 23-27 nM). Selective for $A_{2A}R$ over A_1R (Ki = 290 nM), A_3R (Ki = 67 nM) and $A_{2B}R$ (Ki = > 10 µM) in humans (Fredholm *et al*., 2001). It has affinity for A_1 and A_3 receptors but can be used to distinguish $A_{2A}R$- and $A_{2B}R$-mediated effects (compound datasheet, Tocris). Injected concentration: 30 µM and 1.5 µM; ivc: 6 µM and 300 nM.

Apyrase: Ecto-nucleotidase triphosphate diphosphohydrolase type I (isolated from potato), which removes β and ɣ phosphates from nucleotides producing monophosphate nucleotides. Injected concentration: 20 U/ml; ivc: 4 U/ml.

2.5. Injection protocol of the different compounds into the vitreous cavity

For experiments depicted in Figures 1 and 2, different zebrafish were injected with saline solution unilaterally into the vitreous cavity containing dimethyl sulfoxide (DMSO 1:50; ivc: 1:250) alone (vehicle) or with: A740003; ATPɣS; ATPɣS + A740003; Bz-ATP; or Bz-ATP + A740003. Injections with ATPɣS or Bz-ATP were performed once daily for two or three days, respectively. Zebrafish were euthanized at 48 or 72 h after the first injection of ATPɣS or Bz-ATP, respectively.

For experiments in Figure 3 and 4a-e, eyes were injected once with $CoCl_2$ at zero (0) hours after lesion (hpl) and with vehicle or A740003 at 0 and 24 hpl. For assays in Figure 4e-r, eyes were injected once with $CoCl_2$ (0 hpl) and with vehicle or A740003 at 0, 24 and 48 hpl. Zebrafish were euthanized and enucleated at 72 hpl. For experiments in Figure 5, groups of five zebrafish per group were bilaterally injected with $CoCl_2$ and with vehicle or A740003 at 0, 24 and 48 hpl and zebrafish were euthanized and enucleated at 72 hpl.

For assays in Figure 6, eyes were unilaterally injected with saline solution, apyrase, $CoCl_2$, $CoCl_2$ + apyrase, or $CoCl_2$ + inactive apyrase on day 0 and then, vehicle, apyrase, or inactive apyrase injections were repeated at 24 hpl. Zebrafish were euthanized and enucleated at 50 hpl. For assays in Figure 7, eyes were injected with $CoCl_2$ on day 0 and apyrase or inactive apyrase at 0 and 24 hpl. Zebrafish were euthanized and enucleated at 50 hpl. For experiments in Figure 8, eyes were injected with $CoCl_2$ or saline solution (vehicle) on day 0 and with vehicle, 8-SPT, SCH58261, or CGS21680 at zero, one, two, three and four days after lesion (dpl). One group of zebrafish was injected within the vitreous once daily during five days with CGS21680. All groups of zebrafish were euthanized and enucleated ten dpl. All injection protocols are described in each figure by means of a table.

Either the left eye or both eyes of zebrafish in control groups were injected with an equivalent volume of vehicle, and an equal number of injections were performed into the vitreous cavity at the same intervals as the experimental groups of zebrafish.

2.6. 5-Bromo-2'-deoxyuridine administration

Groups of five to six zebrafish were injected into the vitreous cavity with 0.6 µl of a solution containing 20 µg/µl BrdU. For assays in Figures 2, 3 and 6, zebrafish were euthanized four hours

after BrdU injection. For assays in Figure 4e-r, zebrafish were euthanized 24 hours after BrdU injection. BrdU intravitreal concentration was estimated to be 4 μg/μl.

2.7. Tissue processing and immunocytochemistry

To prepare eyecups, the cornea, lens and vitreous were removed. Eyecups were fixed with 4% paraformaldehyde for two hours at room temperature. For proliferating cell nuclear antigen (PCNA) staining, eyecups were fixed with ethanol:formaldehyde (9:1) for two hours. Eyecups were then incubated in 5%, 10% (30 min) and 20% sucrose (overnight) and embedded in tissue-freezing medium. Eyecups were cut into 16-μm cryosections in horizontal planes so that the sections that were collected on slides contained temporal and nasal ciliary marginal zones (CMZ). Each slide contained sections representing different portions of the eyecup. Tissue sections were incubated in PBST (PBS plus 0.1% Tween-20) and then in 5% NGS (in PBST) at room temperature for one hour. For BrdU staining, retinas were pre-incubated in 2N HCl at 37°C for 25 min and washed with PBST. Sections were then incubated at 4°C overnight with a primary antibody (diluted in 3% NGS). Slides were washed and incubated in darkness at room temperature for two hours with a fluorescent secondary antibody (1:1000 in 3% NGS). For double-labelling assays, sections were incubated in 3% NGS containing both primary antibodies, washed extensively and then incubated with both fluorescent secondary antibodies at room temperature for two hours. Finally, sections were washed with PBST and stained with Hoechst (1:1000; SIGMA-Aldrich) diluted in PBST for 15 min, washed with PBS and mounted. Omission of either the primary or secondary antibodies was performed as a negative control.

2.8. Detection of microglial cells

To detect microglia in the zebrafish retina, 0.5–0.6 µl of 1 mg/ml FITC-conjugated *Griffonia simplicifolia* lectin 1 (GSL1) (Vector Labs) was injected directly in the vitreous cavity 24 or four hours before euthanasia (Figures 4a-d and 4e-r, respectively). Zebrafish were euthanized 72 hpl. Then, eyecups were fixed with 4% paraformaldehyde for two hours and whole flat retinas were prepared and mounted for direct microscopic observation. For double labelling assays, GSL 1-injected eyes were enucleated, eyecups were fixed with 4% paraformaldehyde and BrdU immunostaining was performed on whole-mount retinas, as previously described (Zou *et al*., 2013).

2.9. Determination of retina surface occupied by microglial cells

Whole retinas were excised from five eyes per experimental or control group of different zebrafish. The retinal surface occupied by microglial cells was determined by quantifying the integrated optical density (IOD) from total fluorescence signals of GSL-1-positive cells over background fluorescence on images. The IOD was determined in the periphery and the optic disc area of whole-mount retinas in two-dimensionally reconstructed confocal images of a $(316\ \mu m)^2$ microscopic field. We analysed ten fields per retina and five eyes from different zebrafish.

2.10. Counting of proliferative nuclei and active caspase 3- and protein kinase C (PKC)-positive cells

We counted BrdU- or PCNA-positive nuclei in all retinal layers under direct observation with an epifluorescence microscope, which allowed us to focus through the section and identify individual nuclei on the retina layers. We did not count cells in areas near or on the optic disc or the ciliary marginal zone that were not layered. We counted BrdU-positive nuclei throughout five different retina sections from each eye (using five to six eyes from different zebrafish) in a double-blind assay. BrdU was present within the eye over a four-hour period before euthanasia, so it labelled nuclei of progenitors in S-phase within this time window. Therefore, BrdU-positive nuclei belonged to progenitors that were in S- or G2-M-phases of the cell cycle. PCNA detection indicated progenitor nuclei principally in G1-, S- or G2-M-phases (Ersoy *et al.*, 2009). In assays shown in Figure 4, BrdU was injected and zebrafish were euthanized 24 hours after. So, BrdU-positive nuclei represented nuclei within the cell cycle and postmitotic nuclei.

Active caspase 3-positive cells were counted when co-localized with a single Hoechst stained nuclei. PKC- and active caspase 3-positive cells were counted in nuclear layers within a $(212\ \mu m)^2$ microscopic field, in five to six retina sections from five to six eyes of different zebrafish.

Serial confocal images throughout the z-axis were taken for several sections of different retinas. Two-dimensional reconstructions were performed, and cells were counted to confirm the number of cells we computed under direct observation or in epifluorescence images.

2. 11. Primary antibodies

Antibodies were diluted in 3 % NGS from stocks prepared according to manufacturers´ instructions.

1. Mouse monoclonal anti-BrdU antibody (1:400; Cat# 11170376001, RRID:AB_514483, Roche Applied Sciences) was used to detect 5-bromo-2'-deoxyuridine, a thymidine analogue that incorporates into DNA during the S phase of the cell cycle.

2. Mouse monoclonal anti-PCNA antibody (1:500; Cat# sc-56, RRID:AB_628110, Santa Cruz Biotechnology, CA, USA). PCNA is a nuclear protein which is expressed during S-phase and G2 phase of the cell cycle.

3. Rabbit polyclonal anti-glial fibrillary acidic protein (GFAP) antibody (1:500; Cat# M0761, RRID:AB_2109952, Agilent, Santa Clara, CA, USA) was used to stain Müller glia (particularly after injury).

4. Mouse monoclonal anti-synaptic vesicle protein 2 (SV2) antibody (1:750; RRID:AB_2315387, Developmental Studies Hybridoma Bank, IA, USA) was used to stain presynaptic terminals containing vesicles in plexiform layers.

5. Rabbit polyclonal anti-PKC antibody (1:350; Cat# sc-10800, RRID:AB_2168560, Santa Cruz Biotechnology) was used to stain α, β, γ, δ and ε-subunits expressed by on- and off-BC.

6. Rabbit polyclonal anti-active caspase 3 antibody (1:350; Cat# 564096, RRID:AB_2738589, BD Biosciences, San Jose, CA, USA) was used to stain active caspase 3-dependent apoptotic cells.

2. 12. Secondary antibodies

Goat anti-mouse ALEXA 488 (Cat# 115-545-003, RRID:AB_2338840) or Cy3-conjugated (Cat# 115-165-062, RRID:AB_2338685) and goat anti-rabbit Cy3-conjugated (Cat# 111-165-003, RRID:AB_2338000) antibodies (Jackson ImmunoResearch Labs, West Grove; PA, USA). Secondary antibodies (1 mg/ml) were diluted 1:1000 in 3 % NGS.

2. 13. Microscopy

Counting was performed using a BX50 epifluorescence microscope (Olympus, Japan) with 40x and 60x objectives and numerical apertures (NA) of 0.65 and 1.35, respectively. All microphotographs were captured with a FV1000 Fluoview confocal spectral microscope with SAPO-60x-oil or SAPO-40x-oil objectives and NA of 1.35 and 0.9, respectively (Olympus). Images from double- or triple-labelled retinas were taken with adequate laser beams and spectral filters with a maximum depth of 1.0 μm in the z-axis and in a fixed x–y-plane of the same microscopic field. Two- or three-dimensional reconstructions were performed with Fluoview Software (Olympus). Images were adjusted for brightness and contrast, combined and labelled with Adobe Photoshop CS5 extended (12.0). The autofluorescence of photoreceptor segments were observed in two-dimensional reconstructions of confocal images from Z-stacks or with the epifluorescence microscope.

2. 14. Quantitative real-time polymerase chain reaction

Zebrafish were euthanized and enucleated at 72 hpl. Eight neural retinas were homogenized in the RNA extraction reagent RNAzol and were considered to be one sample. Three independent samples were examined for each treatment. RNA was quantified with a NanoDrop 3300 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and treated with DNAse-I. Complementary DNA (cDNA) was reverse transcribed from RNA with random primers. One-hundred nanograms of RNA were used for quantitative real-time polymerase chain reaction (qPCR; Applied Biosystems 7500, Thermo Fisher Scientific). Specific primers were selected from the zebrafish genome reported in the Ensembl database (Table 1). Quantitative PCR products were checked by electrophoresis in 2% agarose gels. No-RT controls, in which the MMLV-reverse transcriptase was omitted, and cDNA-containing samples were run in triplicate. Elongation factor 1-α (ef1-α) expression showed no significant

variations among groups and was amplified as the reference gene. Data analyses were performed using the "Gene expression's CT Difference" (GED) method, which considered individual amplification efficiencies (Schefe *et al*., 2006). Amplification efficiencies for all samples were between 0.95 and 1.0. Results were also calculated by the 2-ΔCT (cycle threshold) method, which considers 100% reaction efficiencies (E = 1.0), and values were not significantly different from values obtained with GED analysis.

2. 15. Statistical analyses

Data are expressed as mean values ± standard error of the mean (SEM) from *n* independent experiments or animals. Statistical analyses were performed using one-way analysis of variance (ANOVA), with the number of positive cells as a factor, followed by Newman-Keuls multiple comparison test. For relative changes in gene expression (relative levels of mRNA) statistical analyses were performed using one-way ANOVA followed by Dunnett´s multiple comparison test. Two-tailed Student's t test with Welch´s correction was performed when two groups of data were compared. Significance levels (alpha values) indicated in figure legends were obtained with Graphpad Prism software.

3. RESULTS

3.1. Nuclear and plexiform layer morphology of the retina treated with Bz-ATP

In order to determine whether the selected *in vivo* dose of Bz-ATP -injected within the vitreous cavity of zebrafish- caused damage to retinal cells, a morphological analysis of sections of retinal tissue was performed after treating the retina with three unilateral injections of Bz-ATP at 0, 24 and 48 h (Figure 1). At 72 h after first injection (hpi), a concentration of 400 µM Bz-ATP provoked a specific lesion of the photoreceptor layer without any apparent effect on the INL cells (Figure 1d-f). Damage to photoreceptors was heterogeneously observed across the surface of the outer retina. Particularly, nuclear staining with Hoechst showed that double-cone photoreceptor nuclei were significantly disrupted by 72 hpi. Additionally, BzATP treatment provoked a reduced density of ganglion cell (GC) nuclei in the inner retina (Figure 1d).

SV2 immunoreactivity, which labelled presynaptic terminals in the plexiform layers, was also disrupted at the OPL level. Nuclei in the inner nuclear layer (INL) also showed a noticeable level of disorganization, especially horizontal cell nuclei. When the P2X7 receptor antagonist A740003 was injected together with Bz-ATP (see protocol of injections in Figure 1) nuclear and synaptic layer morphology was indistinguishable from control, saline solution + DMSO-treated retinas (Figure 1g-i and a-c, respectively).

3.2. ATPγS and Bz-ATP effects on the proliferative activity of the multipotent retinal progenitors and the gliotic response of Müller cells

The *in vivo* treatment with Bz-ATP induced strong proliferative activity in the ONL and INL at 72 hpi (Figure 2a-f; see protocol of injections in Figure 1). Moreover, Bz-ATP treatment strongly

increased GFAP immunoreactivity in horizontal and vertical processes of Müller cells in the OPL and INL, respectively, as well as in their end feet. The image in (j) shows BrdU-positive nuclei in close proximity with GFAP staining likely belonging to progenitor Müller cells.

The gliotic response of Müller cells and increase of the proliferative activity of retina progenitors were completely abrogated by the antagonist of P2RX7 (A740003) indicating prevention of tissue damage (Figure 2g-i). Other groups of zebrafish were treated *in vivo* with two intravitreal injections of 5 mM ATPγS (1 mM within the vitreous cavity) at 0 and 24 hpi and euthanized at 48 hpi as depicted in Figure 2.

We did not observe significant morphological changes in retinal sections obtained from ATPγS-injected eyes respect to saline solution-treated retinas (data not shown). ATPγS treatment significantly increased BrdU-positive cell number in the ONL. Treatment with the antagonist of P2RX7 did not affect ATPγS effect on cell proliferation in the ONL (Figure 2k-m). The antagonist by itself did not show significant effects on retinal tissue morphology or cell proliferative activity respect to vehicle-treated retinas (Figure 2o).

3.3. Effect of blocking P2RX7 on cell proliferation in retinas injured with $CoCl_2$

Next, we treated zebrafish with one intraocular injection of $CoCl_2$ to damage retina parenchyma, which showed mitotic activation of retina progenitors in the INL at 50 hpl (Figure 3). The increment in the number of BrdU-positive nuclei induced by injury with $CoCl_2$ was significantly enhanced by blockade of P2RX7 (Figure 3a, d, g and j). The number of Müller cell processes that expressed GFAP was also enhanced in injured retinas treated with the antagonist of P2RX7 (Figure 3b, e, h).

The number of BrdU-positive nuclei closely associated with processes of Müller glia was also enhanced (c, f, i). The transcriptional expression of *lin28a* -which is thought to regulate stem cell self-renewal- was induced by $CoCl_2$ injury as described elsewhere (Medrano *et al.*, 2018; data not shown). Treatment with A740003 of $CoCl_2$-injured retinas for three days further induced *lin28a* expression level (Figure 3l).

The relative level of P2RX7 mRNA was significantly increased by 72 hpl with $CoCl_2$. The transcriptional expression of this receptor was not further modified by treating injured retinas with A740003 (Figure 3m).

3.4. Effect of blocking P2RX7 on microglia cells in retinas injured with $CoCl_2$

We also quantified microglia cells in whole-mounted injured retinas (Figure 4). Retinal areas occupied by microglia cells (GSL1-positive cells) were significantly increased by injury whereas the uninjured tissue showed a low number of microglia cells located in the retina inner surface (data not shown; Medrano *et al.*, 2018). When retinas were injured and treated with the antagonist of the P2RX7, the area of the inner retina occupied by microglia cells was further increased at the periphery and around main branches of the central artery in the optic disc (Figure 4o, p). Microglia cells showed less ramified and amoeboid morphology and were more abundant after treatment with A740003 when examined at 72 hpl. Moreover, treatment with the P2RX7 antagonist reduced the number of microglia cells with BrdU-positive nuclei that were mainly observed in the optic disc region (Figure 4m, n). Transcriptional expression of *Tnf-α* -which was detected as a molecular marker of microglia cell activation- was significantly induced by $CoCl_2$ and further increased by treatment with the antagonist of the P2RX7 (Figure 4q).

3.5. Effect of antagonizing P2RX7 on injury-related gene expression in retinas damaged with $CoCl_2$

A hallmark of tissue response to hypoxia and hypoxia-like damage is the intracellular accumulation of HIF1α as well as the induction of vascular growth. In our experimental conditions, one intraocular injection of $CoCl_2$ was not able to increase *hif1α* transcriptional expression as we have previously demonstrated (Medrano *et al*., 2018). In this study, relative levels of *hif1α* mRNA were significantly increased at 72 hpl by treating $CoCl_2$-injured retinas with A740003 (Figure 5). Relative levels of *vegfab* but not of *vegfaa* mRNA were significantly induced by injury with $CoCl_2$ as previously shown (Medrano *et al*., 2018). Treatment of injured retinas with A740003 provoked the transcriptional induction of *vegfaa* and an additional increase of *vegfab* mRNA at 72 hpl. Relative levels of *vegf* receptor (*vegfr*) *1*, *2/4* and *3* mRNA were increased by injury with $CoCl_2$ as previously shown. Relative levels of *vegfr1* and *2/4* mRNA were not modified by blocking P2RX7 (Figure 5d, e). In contrast, A740003 treatment of damaged retinas significantly enhanced relative levels of *vegfr3* mRNA (Figure 5f).

3.6. Effect of treatment with apyrase on retinal tissue injured with $CoCl_2$

Our previous work demonstrated that the absence of extracellular nucleotides causes a significant reduction of progenitor cell proliferation as well as an important enhancement of tissue damage, which completely inhibits tissue repair after retina injury induced with ouabain.

In this study we also found a significant neuroprotective role of ATP-activated P2RX7. Consequently, we examined the effect of depleting *in vivo* di- and tri-phosphate nucleotides from retina extracellular milieu immediately after injury with $CoCl_2$. To this end, we injected an excess of apyrase into the vitreous cavity of zebrafish eyes injured with $CoCl_2$ (Figure 6).

Treatments of non-injured retinas with either active or inactive apyrase did not induce significant changes in the number of BrdU-positive cells (j), Müller glia reactivity, or morphological appearance (image not shown). Heat-inactivated apyrase did not modify progenitor proliferation or the gliotic response in $CoCl_2$-lesioned retinas (Figure 6d-f and j). On the other hand, injured retinas treated with apyrase showed further enhancements in the number of BrdU-positive cells (Figure 6j) and a very strong gliotic response of Müller glia when analyzed at 50 hpl (Figure 6g-i).

3.7. Effect of apyrase on caspase-3 activation and PKC expression in the retina injured with $CoCl_2$

Next, we investigated the effect of exogenously administrated apyrase on caspase-dependent apoptosis in the injured tissue at 50 hpl. To this end, we performed the immunodetection of active caspase 3 in the injured retina (Figure 7).

Intact retinas obtained from saline solution-injected eyes did not show active caspase-3 immunoreactivity (data not shown) whereas injury with $CoCl_2$ induced the appearance of active caspase 3-positive cells on the retinal tissue that was observed at least until 72 hpl (Medrano *et al*., 2018). Apoptotic cells were localized almost exclusively in the outer nuclear layer (ONL) and just a few ones were localized in the INL of all retina sections analyzed (Figure 7a-c).

$CoCl_2$-injured and apyrase-treated retinas showed an additional increase in the number of cells expressing active caspase-3. Furthermore, apoptotic cell distribution was clearly modified and the number of active caspase 3-expressing cells was more abundant in the INL, ganglion cell layer (GCL) and fibre layer (Figure 7d-f).

As described by Medrano *et al*. (2018), $CoCl_2$ caused a significant but limited reduction in the number of bipolar cells (BC) (Figure 7j-l). Active apyrase treatment drastically reduced the number of BC observed in $CoCl_2$-injured retinas (M-O). Only big-sized ON-BC which receive double input from rod and cones survived to treatment with apyrase (Figure 7n).

3.8. Effect of endogenous adenosine on progenitor cell proliferation and Müller cell gliosis at ten days after injury with $CoCl_2$

Adenosine acting via P1R limits apoptotic cell death in inflammatory processes in the CNS. Previous work of our laboratory demonstrated that treatment with 8-SPT, a non-selective antagonist of P1R, and a relatively high dose of adenosine (5-25 µM) did not significantly modify progenitor cell proliferation in the zebrafish retina damaged with a low dose of ouabain (Battista *et al*., 2009).

So, we examined whether P1R were involved in the regeneration of photoreceptor cells in the injury paradigm performed with $CoCl_2$. We quantified the number of proliferative progenitors by detecting PCNA expression on the tenth day after injury.

Retinas were injured and, beginning immediately after injury induction, treated for five days with 8-SPT or vehicle. Vehicle-treated damaged retinas examined at ten dpl exhibited a high number of proliferative nuclei which were distributed mostly within the ONL, with a small number of proliferative progenitors located in the INL (Figure 8a-c). The gliotic response of Müller cells at ten

dpl was higher in injured retinas than in uninjured retinas whereas it was significantly reduced compared to the gliotic response detected by 96 hpl.

Damaged retinas treated with 8-SPT showed a higher degree of gliosis in comparison with injured retinas not treated with the antagonist (Figure 8d-f). Parenchyma of injured retinas treated with 8-SPT was weaker and more difficult to manipulate indicating a more severe damage than the one observed in untreated $CoCl_2$-injured retinas. Injured retinas treated with 8-SPT also showed a significant enhancement of the number of proliferative cells that were distributed in both the ONL and INL at ten dpl (Figure 8q).

Injured retinas were treated immediately after damage and for the next four days with the selective antagonist of $A_{2A}R$ SCH58261. At 10 dpl, retinas treated with the antagonist depicted a strong gliotic response (Figure 8g-i). Retinal parenchyma showed further damage than injured retinas treated with 8-SPT, including damage of the inner layers that were not affected by $CoCl_2$ cytotoxic effect. Retinas treated with a relatively high (0.6 µM) and low (100 nM) intraocular concentration of SCH58261 showed a significant increment in the number of PCNA-positive nuclei within the ONL and INL at ten dpl (Figure 8g, m, n and r).

Next, we examined the effect of exogenously activating adenosine receptors. Injured retinas were treated with the agonist of $A_{2A}R$ CGS21680 immediately after damage and for the next four days. Retinas treated with a high dose of CGS21680 (6 µM) examined ten dpl depicted a significant decrease in the number of PCNA-positive nuclei -which were orderly distributed mostly in the ONL- compared with the number of proliferative nuclei observed in $CoCl_2$-injured retinas without other treatment (Figure 8j-l and s).

In contrast, treatment with a low dose of CGS21680 (300 nM) did not affect the number of PCNA-positive nuclei observed in the ONL of damaged retinas (Figure 8o, p and s). Treatment of uninjured retinas with the $A_{2A}R$ agonist (6 μM) for five days showed no effect on the number of PCNA-positive nuclei detected 10 days after the first injection of CGS21680 (Figure 8s).

4. DISCUSSION

We have demonstrated for the first time a regulatory and neuroprotective role for P2X7 and adenosine receptors during the regeneration process of the retina of adult zebrafish.

4.1. Two analogues of ATP induce proliferation of different populations of progenitors throughout differential activation of purinergic receptors in intact retinas

Activation of P2RX7 with BzATP in non-injured retinas induced cell death as well as gliotic and proliferative responses of Müller cells and Müller glia-derived multipotent progenitors. We corroborated that these retinal changes were due to the activation of P2RX7 because they were abrogated by treatment with a specific dose of a selective antagonist of these receptors.

Our findings indicated that a strong activation of P2RX7 causes deleterious effects on retinal cells, which in turn induces the proliferative activation of multipotent progenitors. This activation was of similar magnitude and features that the mitotic response of multipotent progenitors provoked by photoreceptor cell death which is induced by damage with light or chemical agents (Weber *et al*., 2013; Medrano *et al*., 2018). Moreover, we identified that several proliferative nuclei co-localized with GFAP staining in Müller glia suggesting that multipotent Müller glia re-entered the cell cycle.

In many pathological conditions, high extracellular concentrations of ATP induce transmembrane pore formation, Ca^{2+} entry and apoptotic cell death throughout P2RX7 activation in several tissue and cell types (Surprenant *et al*., 1996). Cruz *et al*. (2013) have found that activation of P2RX7 with high concentrations of ATP for 12-24 h causes the death of zebrafish larvae. P2RX7 activation with BzATP or high extracellular concentrations of ATP or ATPγS induces high levels of reactive gliosis and death of retinal cells in rodent retinas (Hu *et al*., 2010; Notomi *et al*., 2011). In mammalian

retinas, the cell type affected by P2RX7 activation may be directly related with the cell or layer that chiefly expresses this receptor (Brändle, Kohler, & Wheeler-Schilling, 1998; Pannicke *et al*., 2000; Wheeler-Schilling *et al*., 2001; Puthussery & Fletcher, 2004; Zhang *et al*., 2007; Vessey *et al*., 2014; Sugiyama, 2014). However, there is no previous evidence describing P2RX7 localization in the retina of zebrafish. Our data demonstrated a conspicuous death of nuclei in the ONL; chiefly cones but also rods were affected at 72 h after the first injection of BzATP. So, P2RX7 might be principally expressed in the outer layers of the adult retina of zebrafish. We have also observed a diminished density of GC nuclei so our data suggest that at least some types of GC express P2RX7 (Hu *et al*., 2010). Moreover, P2RX7 might be expressed by pigment epithelial and vascular cells as has been described in the mammalian retina (Kawamura *et al*., 2003; Yang *et al*., 2011). P2RX7 upregulation in retinal or vascular cells might be induced by different kinds of injuring stimulus (Sugiyama *et al*., 2013) and the activation of P2RX7 has been involved in hypoxia-induced death of retinal neurons (Sugiyama *et al*., 2010). So, choroid cell death will cause first cone photoreceptor death because of their major dependence on oxygen and nutrients provided by vascular cells. Nonetheless, these hypotheses require further investigation.

Like it was described for the mammalian retina by using high concentrations of ATP, BzATP injection within the vitreous cavity of the adult zebrafish can be used as a retina injury paradigm that induces the death of photoreceptor cells (Aplin *et al*., 2014).

In rodents, treatment with 1 mM ATP for one hour killed virtually all types of GC in cultured retinas via P2RX7 activation (Resta *et al*., 2007). In our assays, *in vivo* treatments with 1 mM ATPγS likely induced the proliferative response of rod precursors without causing cell death. In fact, we did not observe injury of the retinal tissue, Müller cell gliosis or activation of neural progenitors in the INL.

ATPγS effect on the ONL precursors was not abrogated by A740003. So, ATPγS effect on cell proliferation -at a relatively high dose that causes cell death via P2RX7 activation in the mammalian and chicken retina (Zhang *et al.*, 2005; Resta *et al*., 2007; Hu *et al.*, 2010; Anccasi *et al*., 2013)- might be mediated by P2RY, likely $P2RY_1$, in the adult retina of zebrafish (Battista *et al*., 2009; Ricatti *et al*., 2011; Medrano *et al*., 2017). In addition, it has been demonstrated that activation of $P2RY_1$ by extracellular adenine nucleotides is necessary for the proliferation of multipotent retinal progenitors during perinatal development of rat retinas (de Almeida-Pereira *et al*., 2017).

4.2. Blockade of P2RX7 exacerbated deleterious effects induced by injury with $CoCl_2$

We have previously described that 1.3 mM of $CoCl_2$ injected within the vitreous cavity causes death of virtually all photoreceptor cells. So, it is plausible that considerable amounts of ATP and other nucleotides are released by lytic cells to the extracellular milieu when the retina is exposed to $CoCl_2$. It has been described that lytic release of ATP can cause cell death to neighbouring cells via activation of P2RX7 (Adinolfi *et al*., 2005; Sperlágh & Illes, 2014). Therefore, photoreceptors or interneurons that were not directly affected by $CoCl_2$ could indirectly die by elevated concentrations of ATP acting on P2RX7. In fact, a reduced degree of damage was found in injured retinas of rodents when treated with P2RX7 antagonists or in knockout mice for this receptor (Notomi *et al*., 2011). However, our findings indicated that endogenous activation of P2RX7 protected retina cells against cytotoxic effects of $CoCl_2$ since blockade of this receptor generated more damage and a strong Müller glia gliotic response. In this regard, previous studies have demonstrated that P2RX7 blockade provokes cell death in the lacrimal gland (Dartt & Hodges, 2011) and its activation induces cytokine and neurotrophic factor release protecting retinal ganglion cells from dying (Lim *et al*., 2016; Lu *et al*., 2017).

Activation of P2RX7 as well as other P2X and P2Y receptors by physiological concentrations of extracellular ATP is involved in diverse homeostatic processes such as regulation of cell volume and vasomotor tone. P2RX7 activation in the retina of zebrafish injured with $CoCl_2$ might be necessary to compensate cell volume changes and osmotic imbalances (Jun *et al*., 2007). Our findings suggest that cells of the retina of zebrafish can exert paracrine protective effects via activation of P2RX7.

4.3. Blockade of P2RX7 in retinas injured with $CoCl_2$ increased *lin28a* and *tnfα* transcriptional expression in coincidence with multipotent progenitor proliferation and microglia reactivity

Our findings indicated that blockade of P2RX7 provoked further activation of multipotent progenitors in $CoCl_2$-injured retinas. Accordingly, it has been reported that P2RX7 activation by ATP prevents cyclin expression that controls cell entry and progression through the cell cycle in the corpus luteum of mice (Wang *et al*., 2015).

Injury with $CoCl_2$ provoked a significant increment in the transcripcional expression of P2RX7 whereas treatment with the antagonist of this receptor did not modify the level of P2RX7 mRNA. These findings suggest that P2RX7 transcriptional expression induced by injury with $CoCl_2$ is not autoregulated. A previous study has demonstrated that P2RX7 expression is induced by a deleterious increase of the intraocular pressure in rats, which was prevented by using a non-selective antagonist of P2RX7 (Sugiyama *et al*., 2013); however, this inhibition could be mediated via other types of P2 receptors. Additionally, P2RX7 transcriptional expression could be regulated by growth factor-, cytokine- or neurotransmitter-mediated pathways activated by injury in the zebrafish retina.

After injury and during reprogramming, Müller glia upregulates pluripotency genes associated to neural stem cells. Among the most studied, *ascl1a* and *lin28a* expression is induced in multipotent Müller glia and Müller glia-derived progenitors in the retina of zebrafish (Gorsuch *et al.*, 2017). The expression of both genes was induced in retinas injured with $CoCl_2$ (Medrano *et al*., 2018). In this study, injured retinas treated with the P2RX7 antagonist exhibited a further increase in the level of *lin28a* mRNA that was temporally coincident with the enhancement of multipotent progenitor proliferation. However, the transcriptional expression of *ascl1a* did not show further enhancements

(data not shown). We do not have a straightforward explanation for the lack of effect of blocking P2RX7 on *ascl1a* expression in the injured retina. Previous studies have shown controversial evidence regarding the expression timing of both factors. One of the studies indicated that Lin28a acted upstream of Ascl1a whilst the second study reported the opposite (Ramachandran, Fausett, & Goldman, 2010; Nelson *et al*., 2012). It has also been suggested that these factors regulate each other in a positive feedback loop (Gorsuch *et al*., 2017). Nonetheless, it is possible that *lin28a* and *ascl1a* expression is regulated by different signalling pathways activated by $CoCl_2$-induced injury. It might be that P2RX7 activation by ATP in the injured retina limits *lin28a* -therefore its expression is further increased by blocking P2RX7- without affecting *ascl1a* expression.

We have previously observed that injury with $CoCl_2$ induced the transcriptional expression of TNF-α (Medrano *et al*., 2018) and, in this study we found that blockade of P2RX7 caused further tissue damage and *tnf-α* expression enhancement. In contrast, it has been described that TNF-α release by microglia is mediated via P2RX7 activation by ATP in neural tissues (Suzuki *et al*., 2004). Nonetheless, it has also been demonstrated that TNF-α release from microglia can be activated by lipopolysaccharides (Kuno *et al*., 2005). Additionally, photoreceptor and Müller cells in retinas injured with light or ouabain release TNF-α (Nelson *et al*., 2013). Therefore, our findings suggest that blockade of P2RX7 in injured retinas increases TNFα production by different kinds of retinal cells intensifying in turn the inflammatory process and progenitor proliferation.

It has been recently described that transgenic depletion of resident microglia from retinas of mice causes microglia proliferation and migration to repopulate the adult neural retina. Microglia cells in the optic nerve migrate to the optic disc region and also macrophages in the ciliary body/iris migrate

and differentiate to microglia in the retina periphery (Huang *et al*., 2018). Similarly, in our studies the surface occupied by microglia in the optic disc and retina periphery was increased after injury with $CoCl_2$ and further enhanced by P2RX7 blockade. Additionally, injury and blockade of P2RX7 promoted a morphological switch of microglia to an activated amoeboid-like shape. Moreover, P2RX7 blockade diminished the number of microglia cells that showed BrdU-labelled nuclei in the injured retinal tissue. This indicates that P2RX7 activation by ATP induces mitotic division of resident microglia in the damaged retina and regenerating microglia might also derive from extraretinal origins as it was described in rodents (Huang *et al*., 2018). Our findings also suggest that injury induced microglia regeneration and repopulation from extraretinal sources was proportional to retinal damage severity.

4.4. HIF1α expression is inhibited and VEGF signalling is limited by endogenous activation of P2RX7 in $CoCl_2$-injured retinas

Hif1α expression was affected by A740003 treatment in injured neural retinas. At 72 hpl, levels of *hif1α* mRNA detected after injury with $CoCl_2$ were not significantly modified. In contrast, treatment with the P2RX7 antagonist significantly increased the level of *hif1α* mRNA detected 72 hpl. Enhanced levels of HIF1α may cause hypoxic damage to the retinal tissue. Our findings suggest that activation of P2RX7 by lytic release of ATP prevents Co^{2+}-induced HIF1α accumulation and the consequent oxidative and metabolic stress. In contrast, other studies demonstrated that activation of P2RX7 by ATP increases HIF1α production provoking deleterious effects in mammalian and neuroblastoma cell cultures (Amoroso *et al*., 2012; 2015). It has also been reported that dopamine neurons are not protected by P2RX7 deletion or inhibition in rodent models of Parkinson´s disease

(Hracskó *et al*., 2011). In fact, extracellular ATP can generate neuroprotection or cell death and disease depending on the intensity and duration of P2RX7 activation (Savio *et al*., 2018).

Injury with $CoCl_2$ induces significant increments in the transcriptional expression of *vegfr*1, 2, and 3 as well as *vegfab* but not *vegfaa* in the adult neural retina of zebrafish indicating a limited pro-angiogenic profile (Wild *et al*., 2017; Medrano *et al*., 2018). We have found in this study an additional enhancement of the transcriptional expression of *vegfab* and *vegfr3* and also an induction in the expression of *vegfaa* when P2RX7 was selectively blocked in injured retinas at 72 hpl. It has been described that VEGFAa and VEGFAb isoforms regulate vasculogenesis and angiogenesis and their expression is spatiotemporally regulated during embryo development. Our findings indicated that both isoforms of VEGFA are differentially stimulated according to injury severity in the regenerating adult retina of zebrafish. VEGFR3 activity has been associated to lymphangiogenesis in embryo development and maintenance of lymphatic endothelium in adult animals. Nonetheless, it has been recently reported that VEGFR3 is necessary for angiogenic and vasculogenic processes in zebrafish embryo development (Bower *et al*., 2017). Therefore, these findings could indicate further angiogenic induction likely mirroring aggravated vascular damage generated by blocking P2RX7 in the injured retina of zebrafish.

4.5. Lack of nucleotide signalling amplifies retinal cell death and damage intensity

Tri- and di-phosphate nucleotide depletion from the extracellular milieu of the injured retina further increased proliferative progenitor number and Müller glia reactive response. Additionally, BC number was further reduced by the apyrase treatment compared to retinas that were only injured with $CoCl_2$. As previously reported, $CoCl_2$-treated retinas lost 30 % of BC and among them OFF-BCs

were chiefly affected (Medrano *et al*., 2018). We found in this study that, after nucleotide depletion following injury, only survived big-sized ON-BC receiving input from cones and rods.

Moreover, whereas retinas injured with $CoCl_2$ showed that active caspase 3-positive cells were almost exclusively located in the outer nuclear layer, depletion of extracellular nucleotides caused cell death also in the INL, GCL and vascular endothelial cells that usually survive in retinas treated with the dose of $CoCl_2$ utilized in this study. So, these cells likely died due to the lack of survival signals or protective mechanisms that are endogenously stimulated via nucleotide action on P2 receptors.

These findings are in agreement with the deleterious effect observed by blocking P2RX7 in the injured retina described in this study and with previous findings indicating that extracellular nucleotide depletion and $P2RY_1$ blockade originated the emergence of apoptotic nuclei in retinal layers that were not affected by the injury itself, as it was observed in retinas damaged with a low dose of ouabain (Battista *et al*., 2009). Apyrase treatment for seven days after injury induced with ouabain caused virtually total loss of retinal cells including Müller-glia derived progenitors, which prevented the regenerative process (Medrano *et al.*, 2017). In the assay reported here, retinas were treated with apyrase only for two days after injury with $CoCl_2$. This treatment with apyrase had reversible effects which intensified injury severity and further stimulated re-entry of progenitor Müller glia to the cell cycle. However, a treatment with apyrase for two days did not cause excessive cell death or inhibition of the regenerative process.

4.6. Adenosine role in regulating cell regeneration after injury of photoreceptors induced by $CoCl_2$

Adenosine activity via P1R has been scarcely studied in the zebrafish CNS. Adenosine can be released from cells by nucleoside transporters or produced extracellularly from ATP hydrolysis by NTPDases, ecto-5'-nucleotidases and nucleotide pyrophosphatase phosphodiesterases (Bollen *et al*., 2000; Hunsucker, Mitchell, & Spychala, 2005; Zimmermann, 2006). There are evidences indicating that adenosine via P1R can directly regulate cell proliferation (Merighi *et al.*, 2002; Liu *et al.*, 2017; Borea *et al*., 2018). For instance, activation of A_1R and $A_{2A}R$ provokes proliferation of neural progenitors isolated from mouse embryo brain cortex (Lv, Shao, & Gao, 2018). However, in our study an agonist of $A_{2A}R$ caused a long term decrease in neural progenitor proliferation. A decreased proliferative activity detected ten dpl might be consequence of an enhanced mitotic activity at the time of peak proliferation (four dpl) or high rate of survival of progenitor cells. In the same way, blockade of adenosine receptors might have caused a reversible mitotic arrest or an enhanced death rate of multipotent progenitors provoking a delayed peak of proliferative activity in the INL at ten dpl. Nevertheless, these hypotheses require further examination.

In our assays, exogenous activation of $A_{2A}R$ significantly reduced tissue damage induced by $CoCl_2$. In rat retinas, the specific activation of $A_{2A}R$ protects neurons from ischemia (Li & Roth, 1999; Melani *et al*., 2014). Moreover, different agonists of $A_{2A}R$ decrease cell death and inflammatory cytokine release in a murine model of diabetic retinopathy (Awad *et al*., 2006) and block glutamate excitotoxicity in cultures of avian retinal neurons (Ferreira & Paes-de-Carvalho, 2001). In this regard, we have previously demonstrated that photoreceptor death induced by $CoCl_2$ was strongly prevented by blocking NMDA -and in a lesser degree AMPA/Kainate- glutamate receptors in the adult retina of zebrafish (Medrano *et al*., 2018). This suggests that adenosine might exert

neuroprotective actions via $A_{2A}R$ by limiting glutamate excitotoxicity in the injury paradigm described herein.

The neuroprotective effect of CGS21680 was only observed with high intraocular levels of this agonist which are not selective for $A_{2A}R$. These findings suggest that the neuroprotective effect could be also mediated via A_1 and A_3 receptors and requires a powerful activation of P1R. Moreover, the lack of effect of the relatively low concentration of CGS21680 examined suggests that levels of extracellular adenosine may be elevated in the injured tissue. So, endogenous extracellular adenosine could prevent further effects of relatively low and selective levels of any kind of P1R agonist, including treatment with exogenous adenosine, as we have previously observed by using another paradigm of lesion (Battista *et al*., 2009).

We have also found that blockade of P1R induced an increased degree of tissue damage and Müller cell gliosis. Likewise, it has been reported that blockade of P1R causes microftalmia, accelerated cell death, and retinopathy development in the avian and mammalian retina (Ma *et al*., 2014; Boia *et al*., 2017). However, it has also been described that blockade of P1R with caffeine reduces neuro-inflammation and prevents ganglion cell death in rats with intraocular hypertension (Chen *et al*., 2017). Given that blocking all types of adenosine receptors can cause simultaneous opposite effects, we selectively blocked $A_{2A}R$. Our findings in the injured retina suggest that specific blockade of $A_{2A}R$ was conspicuously more deleterious than inhibiting all kinds of P1R with a high dose of 8-SPT. At odds with our findings, treatment with an antagonist of $A_{2A}R$ caused ganglion cell protection and inflammatory prevention in the rodent retina (Madeira *et al*., 2016). Reported findings regarding $A_{2A}R$ role are controversial since high or low doses of SCH58261 can cause completely

opposite effects in the rodent retina (Pintor *et al*., 2001). Moreover, agonists and antagonists of $A_{2A}R$ might have paradoxical effects (Rivera-Oliver & Díaz-Ríos, 2014). We have used a relatively high and low dose of the antagonist SCH58261, which caused neurodegeneration and had similar effects on regulating the proliferative activity of retinal progenitor cells. In agreement, a high non-selective dose of an agonist for $A_{2A}R$ showed a neuroprotective action in the zebrafish retina.

4.7. Conclusion

Purinergic signalling controls cell death generated by $CoCl_2$ cytotoxic damage. Our findings indicated neuroprotective roles of endogenous extracellular ATP and adenosine chiefly acting on P2RX7 and $A_{2A}R$, respectively, after injury induction. Short term extracellular nucleotide depletion generated significant increases of cell death, gliosis, microglial reactivity and TNF-α production. We found that purinergic signalling may be indispensable to limit and counterbalance neurodegeneration, gliosis, HIF1α accumulation, VEGF/VEGFR overexpression, as well as other cytotoxic mechanisms induced by Co^{2+} such as glutamate excitotoxic neuronal death. Our findings also suggest that adenosine regulates progenitor and precursor cell proliferation which might be mediated by adenosine effect on neuronal protection and multipotent progenitor survival.

Finally, studies on purinergic signalling molecules can be extremely useful to understand how these molecules and their natural and synthetic analogues can be used as pharmacological tools in therapies that limit or counterbalance neuronal death, excessive glia proliferation -like in proliferative retinopathies- or inflammatory responses that drive to blindness.

All authors had full access to the data and take responsibility for the integrity of the results and the accuracy of the data analysis. M.P.F. and M.P.M. designed research, M.P.M and M.P.F. performed experiments and analysis, A.P.F. and R.O.B. helped conduct experiments and analysis, M.P.F and R.O.B. drafted the manuscript.

Data Availability Statement:

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of Interest statement:

The authors have no conflict of interest to declare.

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TABLE 1. Specific primers for detecting relative transcriptional expression of different genes by RT-quantitative real time PCR in the adult neural retina of zebrafish

Gene symbol / Gene ID	**Forward 5'-3'**	**Reverse 5'-3'**
efl1-α (ENSDARG00000020850)	cagcagctgaggagtgatct	gtagatcagatggccggtgg
p2rx7 (ENSDARG00000042440) *(ENSDARG00000042440)*	acaccgagaggaagtttgagg	aggggttttgtctcctgtagtc
lin28a (ENSDARG00000016999)	tttctgtccatgaccaccg	tcagactgcgaaaaccctc
tnf-α1 (ENSDARG00000009511)	tttcacttccaaggctgcca	agaaaaggcctggtcctggt
hif1ab (ENSDARG00000034293)	caggcgacgccattatctct	gataggctgagtggcagctt
vegfaa (ENSDARG00000103542)	gtacatcccgtcctgtgtgg	gcgatacgcgttgcttgac
vegfab (ENSDARG00000034700)	tgttggtggaaattcagcag	caccctgatgacgaagaggt
vegfr1 (ENSDARG00000019371)	ctgctgagtggaactccagg	tgcgtttgctgataatggcg
vegfr2/4 (ENSDARG00000105215)	ccatgtgaccccagtgctaa	tcaacgttttcaatgggaagagg
vegfr3 (ENSDARG00000104453)	cagagctcagaggacgatgg	caaacggcacgcagttgtaa

Primers were selected from the zebrafish genome reported in Ensembl database and checked for specificity by using Blast database. Forward and reverse primers were selected in different exon sequences separated by a long intron and used for determining relative levels of the mRNA coding for the proteins indicated in this table in the adult zebrafish neural retina. *Efl 1-α* was used as an internal reference gene.

FIGURE LEGENDS

Figure 1. Morphological analyses of the adult retina layers of zebrafish after treatment with a potent extracellular ATP agonist of P2RX7: Bz-ATP

Confocal images of retina sections depict double labelling assays in which cell nuclei were labelled with Hoechst (magenta) and plexiform (synaptic) layers were detected with an antibody directed against SV2 which is a protein localized in the membrane of presynaptic vesicles (green). Retina sections were obtained from zebrafish injected *in vivo* into the vitreous cavity once daily with sterile saline solution + DMSO (vehicle; a-c), 400 μM BzATP (d-f), or 400 μM BzATP + 20 μM A740003 (g-i) diluted in vehicle. Retinas were analysed 72 hours after the first injection (hpi). The injection protocol for each experimental group is indicated in panel (j).

BzATP: 3′-O-(4-benzoyl)benzoyl-ATP, a potent agonist of P2RX7. A740003: a specific antagonist of P2RX7. DMSO: dimethyl sulfoxide. Saline sol: saline solution. BrdU: 5-bromo-2´-deoxyuridine. DCN: double cone nuclei, ONL: outer nuclear layer, HCN: horizontal cell nuclei; OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer. Scale bar: 55 μm.

Figure 2. BrdU and GFAP double immunolabelling assays in retina sections after *in vivo* treatments with BzATP, A740003 and ATPγS

Confocal images of retina sections depict BrdU-positive nuclei in green. BrdU was injected into the vitreous cavity four hours before euthanasia. Müller glia activation was detected by using a specific antibody against GFAP, whose immunoreactivity is depicted in magenta. Retina sections were obtained from zebrafish injected *in vivo* into the vitreous cavity once daily with sterile saline solution

+ DMSO (vehicle; a-c), 400 µM BzATP (d-f) diluted in vehicle, or 400 µM BzATP + 20 µM A740003 diluted in vehicle (g-i). Retinas were analysed 72 hours after the first injection (hpi). The injection protocol for each experimental group for images (a-i) is indicated in Figure 1j. Other groups of zebrafish were injected into the vitreous cavity once daily for two days with saline solution + DMSO (image not shown), A740003 + DMSO (k), 1 mM ATPγS + DMSO (l), or 1 mM ATPγS + 20 µM A740003 + DMSO. BrdU was injected into the vitreous cavity four hours before euthanasia and zebrafish were euthanized at 48 hpi. The protocol of injections for each experimental group for images (g-m) is depicted in (p). The image in (j) shows a magnified portion of image (f). Arrowheads depict BrdU-positive nuclei closely associated with GFAP-immunoreactive vertical processes.

Both graphs show BrdU-positive cell number per retinal section after each treatment indicated in the abscissa axis. ***$p<0.001$; **$p<0.01$; *$p<0.05$ by Newman-Keuls multiple comparison test after one-way ANOVA (n= four to five zebrafish per group). BzATP: 3′-O-(4-benzoyl)benzoyl-ATP, a potent agonist of P2RX7. A740003: a specific antagonist of P2RX7. ATPγS: slowly hydrolysable ATP analogue. DMSO: dimethyl sulfoxide. Saline sol: saline solution. BrdU: 5-bromo-2´-deoxyuridine. GFAP: glial fibrillary acidic protein. PRS: photoreceptor segments, ONL: outer nuclear layer, OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer, ILM: inner limiting membrane. Scale bars: 30 µm (c) and 25 µm (j).

Figure 3. BrdU and GFAP double immunolabelling assays in retina sections after *in vivo* treatments with $CoCl_2$ and A740003

Confocal images of retina sections show BrdU and GFAP immunoreactivity in green and magenta, respectively (a-i). Control zebrafish were injected *in vivo* with saline solution + DMSO into the vitreous cavity (a-c). A second group of zebrafish suffered retina damage via a single injection of 1.3 mM $CoCl_2$ + DMSO (vehicle) within the vitreous cavity (d-f). The third experimental group of zebrafish was treated with vehicle containing 1.3 mM $CoCl_2$ and A740003 injected into the vitreous cavity of each fish (g-i). BrdU was injected into the vitreous cavity four hours before euthanasia, which was performed 50 hours after lesion (hpl). The protocol of intraocular injections is shown in (k). The graph in (j) depicts the total number of proliferative cells per retinal section for each experimental group. ***$p<0.001$; **$p<0.01$ by Newman-Keuls multiple comparison test after one-way ANOVA (n= six zebrafish per group).

The graph in (l) shows relative levels of *lin28a* mRNA in neural retinas of $CoCl_2$-injured and $CoCl_2$ + A740003-treated zebrafish. Zebrafish were euthanized 72 hpl. Relative levels of *lin28a* mRNA in saline solution-treated retinas are not shown because *lin28a* mRNA was undetectable in the uninjured tissue. The graph in (m) depicts relative levels of *p2rx7* mRNA in neural retinas of saline solution-treated, $CoCl_2$-injured and $CoCl_2$+ A740003-treated zebrafish that were euthanized 72 hpl. Fold change represents the relative expression ratio from three independent pools of retinas for each experimental condition. *$p<0.5$ by two-tailed unpaired Student´s t-test with Welch´s correction and **$p<0.01$ by Dunnett's multiple comparison test after one-way ANOVA (n= three independent assays). A740003: specific antagonist of P2RX7. *Lin28a*: pluripotency gene. DMSO: dimethyl sulfoxide. Saline sol: saline solution. BrdU: 5-bromo-2´-deoxyuridine. GFAP: glial fibrillary acidic protein. ONL: outer nuclear layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer, ILM: inner limiting membrane. Scale bar: 50 µm.

Figure 4. Microglia response in the injured retina treated with a specific antagonist of P2RX7

Confocal images of whole-mount retinas show Griffonia simplicifolia lectin 1 (GSL1) bound to plasma membrane of microglia cells in green and 5-bromo-2´-deoxyuridine (BrdU) nuclear localization in magenta (a-n). Zebrafish retinas were treated *in vivo* with saline solution + DMSO (vehicle), 1.3 mM $CoCl_2$ (a-c, g-i, m), (data not shown), or with 1.3 mM $CoCl_2$ plus 20 µM A740003 (d-f, j-l, n) diluted in vehicle. All the solutions were injected into the vitreous cavity of each zebrafish. Zebrafish of all experimental groups were euthanized at 72 hours after lesion (hpl). BrdU and GSL1 were injected in the vitreous cavity 24 and four hours before euthanasia, respectively**.** The protocol of daily injections is summarized in (r).

Images in (a-f) depict portions of the surface of the inner retina where capillaries join the circumferential vein (CV) at the retina periphery whereas images in (g-n) show the optic disc (OD) area containing the main branches of the optic artery. Images (m) and (n) show magnified portions of images (i) and (l), respectively. Arrowheads show double staining co-localized in microglia cells that incorporated BrdU in the optic disc area. Long arrows indicate central (c) to peripheral (p) retina directions. Scale bars: 150 µm (c) and 25 µm (m).

Scatter plots depict the integrated optical density (IOD) from total fluorescence signals of GSL-1-positive cells over background fluorescence on confocal images of ten microscopic fields of (316 $\mu m)^2$ per retina. The IOD was determined in microscopic fields containing peripheral capillaries (o) and the OD area (p) in control injured retinas or injured retinas treated with A740003. *$p<0.05$ by two-tailed unpaired t-test with Welch´s correction (n= four to five zebrafish per group).

The graph in (q) depicts relative levels of *tnfα* mRNA in neural retinas of saline solution-treated, $CoCl_2$-injured and $CoCl_2$+ A740003-treated zebrafish that were euthanized 72 hpl. Fold change represents the relative expression ratio from three independent pools of retinas for each experimental condition. ***p<0.001; **p<0.01 by Dunnett's multiple comparison test after one-way ANOVA (n= three independent assays).

Figure 5. Relative expression levels of *hif-1α, vegf* and *vegfr* genes at 72 hours after damage with $CoCl_2$

Seventy two hours after lesion (hpl) RNA was purified from independent pools of eight retinas each obtained from zebrafish whose retinas had been treated with saline solution, 1.3 mM $CoCl_2$, or 1.3 mM $CoCl_2$ + A740003. Complementary DNA was obtained by reverse transcription and real time quantitative PCR reactions were performed with primers for *ef-1α* (as the reference gene) and *hif-1α* (a); *vegfaa* (b); *vegfab* (c); *vegfr1* (d); *vegfr2/4* (e); *vegfr3* (f) (as target genes) mRNA. Fold change represents the relative expression ratio from three independent pools of retinas from each experimental group. Three independent pools of saline solution-treated retinas were used as calibrator samples. The fold change value for control saline solution-treated retinas equalled one. ***p<0.001, **p<0.01,*p<0.05 by Newman-Keuls multiple comparison test after one-way ANOVA (n= three independent assays).

Figure 6. Apyrase effect on progenitor cell proliferation and GFAP expression in injured retinas

Confocal images of retina sections depict proliferative progenitor cell nuclei labelled with BrdU (green) and GFAP immunoreactivity in Müller glia (magenta). Zebrafish were treated with a single dose of 1.3 mM $CoCl_2$, $CoCl_2$ plus inactive apyrase, or $CoCl_2$ plus 4 U/ml apyrase and euthanized 50 h after lesion (hpl). BrdU was injected within the vitreous cavity four hours before euthanasia. Arrowheads signal BrdU-positive nuclei in close association with GFAP-expressing vertical processes of Müller cells. The graph in (j) shows the number of BrdU-positive nuclei for each experimental group as indicated in the abscissas. The injection protocol for each experimental group is summarized in (k). *$p<0.05$ ($CoCl_2$- and apyrase-treated retinas *vs*. $CoCl_2$-injured retinas), ***$p<0.001$ (*vs*. saline solution- or apyrase-treated uninjured retinas) by Newman-Keuls multiple comparison test after one-way ANOVA. Apyrase: ecto-nucleotidase triphosphate diphosphohydrolase type I. BrdU: 5-bromo-2´-deoxyuridine. GFAP: glial fibrillary acidic protein. PRS: photoreceptor segments, ONL: outer nuclear layer, OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer, GCL: ganglion cell layer; ILM: inner limiting membrane. Scale bar: 35 µm.

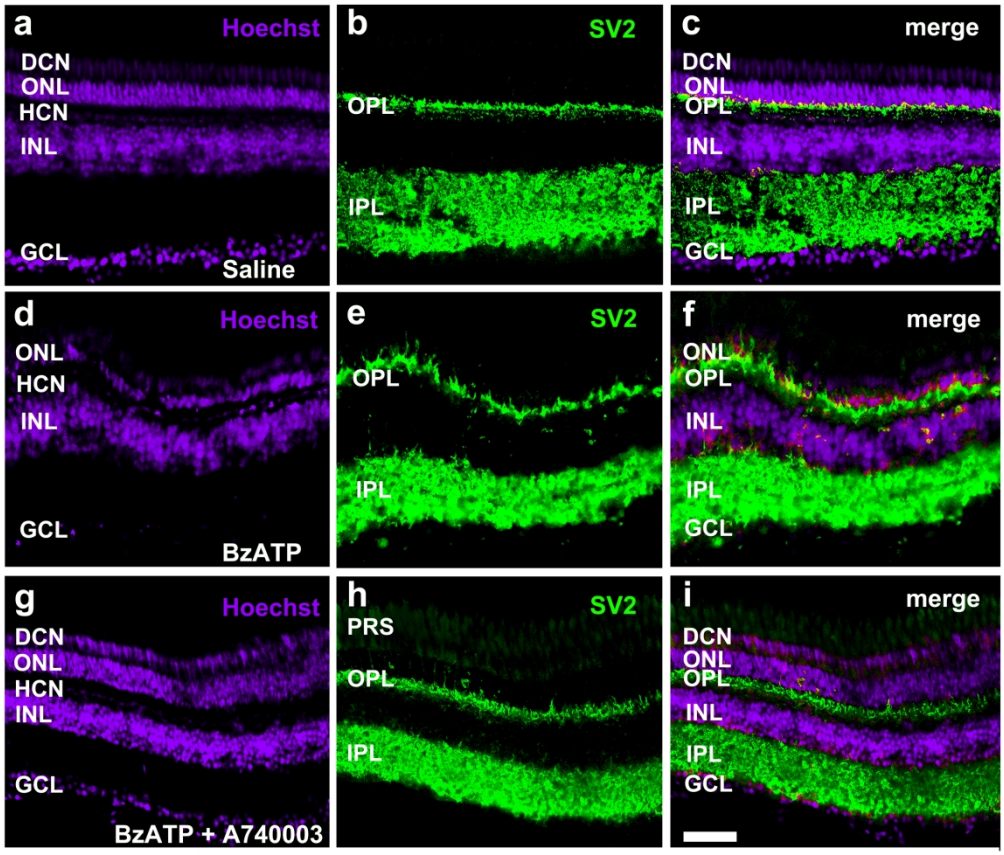
Figure 7. Apyrase effect on apoptotic cell death and PKC expression in retinas injured with $CoCl_2$

Zebrafish eyes were injected with saline solution, a single dose of 1.3 mM $CoCl_2$ plus 4 U/ml inactive apyrase, or $CoCl_2$ plus 4 U/ml apyrase and euthanized 52 h after lesion (hpl). Confocal images of retina sections (a-f) depict apoptotic cells that were detected with an antibody directed against active caspase 3 (red). Images depicted in (g-o) show bipolar cells depicted in red that were detected by using a polyclonal antibody against α, β, δ, γ and ε subunits of protein kinase C (PKC). Cell nuclei labelled with Hoechst are shown in blue on retina sections. Dotted lines depict the synaptic lamina where off-BC terminals synapse with ganglion and amacrine cells in the IPL (b, e, i). Double arrows in (g, i) indicate the INL and the proliferative nuclei migrating to the outer retina.

The graphs in (q) and (r) depict the average number of active caspase 3- and PKC-positive cells per field of $(212\ \mu m)^2$, respectively. The number of cells was examined 52 hpl in saline solution-, $CoCl_2$-, $CoCl_2$ plus apyrase-, and $CoCl_2$ plus heat-inactivated apyrase-treated retinas. $^{**}p<0.01$; $^{***}p<0.001$, Newman-Keuls multiple comparison test after one-way ANOVA (n= four to six zebrafish per experimental group). RPE: retinal pigmented epithelium; PRS: photoreceptor segments; OFF-BC: off-centre bipolar cell synaptic terminals; ON-BC: on-centre bipolar cell synaptic terminals; ONL: outer nuclear layer; PS-OPL: presynaptic outer plexiform layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Scale bar: 25 µm.

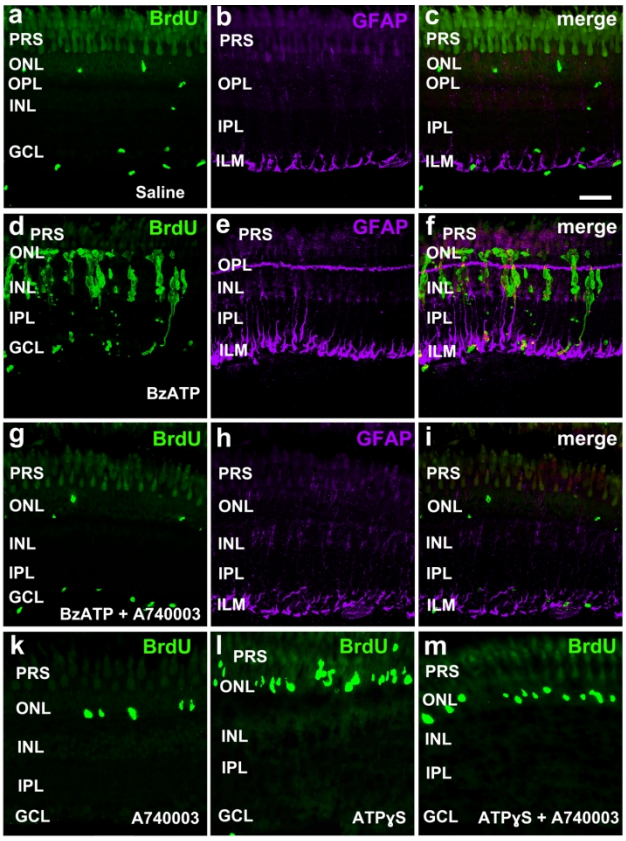
Figure 8. Agonist and antagonist of adenosine receptor effect on PCNA and GFAP double immunolabelling in retina sections after damage with $CoCl_2$

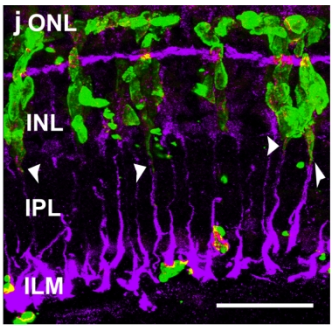
Confocal images depict PCNA-immunoreactive cells in green or red and GFAP expressed by Müller glia in magenta on retina sections lesioned with $CoCl_2$. Zebrafish eyes were injected into the vitreous cavity with a single dose of 1.3 mM $CoCl_2$ or saline solution (day zero) and during the following four days with saline solution, 8-SPT (20 µM), SCH58261 (0.6 µM and 100 nM, high and low concentration, respectively), or CGS21680 (6 µM and 300 nM, high and low concentration, respectively) once daily. One group of uninjured zebrafish was injected during five days with 6 µM CGS21680. All groups of zebrafish were euthanized ten days after lesion (dpl) with $CoCl_2$ or the first intraocular injection. Graphs show the number of PCNA-positive cells per retinal section ten days after $CoCl_2$ or saline solution treatments (q-s). The table in (t) summarizes injection protocols for all groups. ***$p<0.001$; **$p<0.01$; *$p<0.5$ by Newman-Keuls multiple comparison test after one-way ANOVA (n= four to nine zebrafish per group). PCNA: proliferating cell nuclear antigen; GFAP: glial fibrillary acidic protein; 8-SPT: non-selective antagonist of adenosine P1R; SCH58261: selective antagonist of adenosine $A_{2A}R$; CGS21680: agonist of adenosine $A_{2A} > A_3 >> A_1$ receptors. PRS: photoreceptor segments, ONL: outer nuclear layer, OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer, ILM: inner limiting membrane. Scale bar: 25 µm (b) and 20 µm (o).

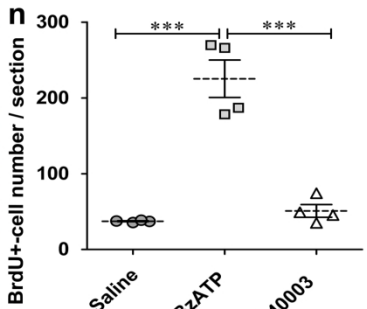


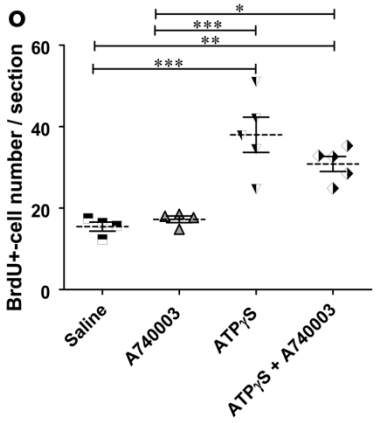
j

0 hpi	24 hpi	48 hpi	68 hpi	72 hpi
Saline sol + DMSO	Saline sol + DMSO	Saline sol + DMSO	BrdU	Euthanasia
BzATP + DMSO	BzATP + DMSO	BzATP + DMSO	BrdU	Euthanasia
BzATP + A740003 + DMSO	BzATP + A740003 + DMSO	BzATP + A740003 + DMSO	BrdU	Euthanasia



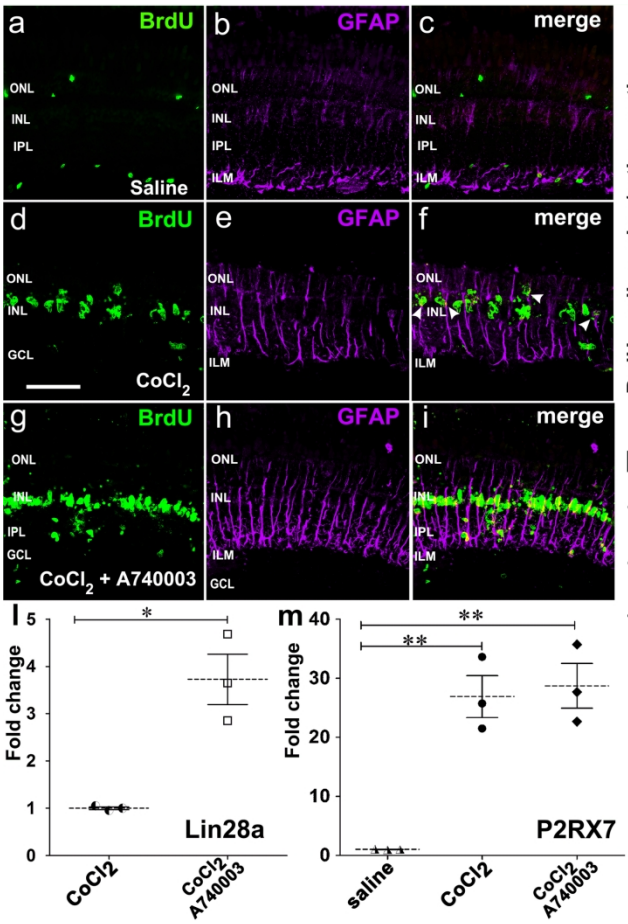


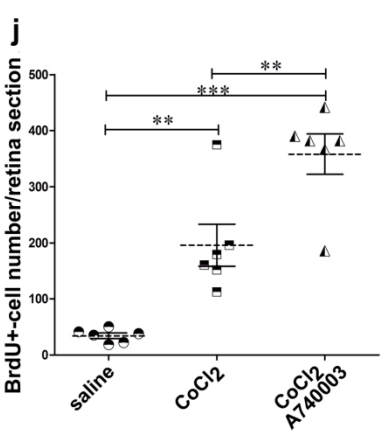




p

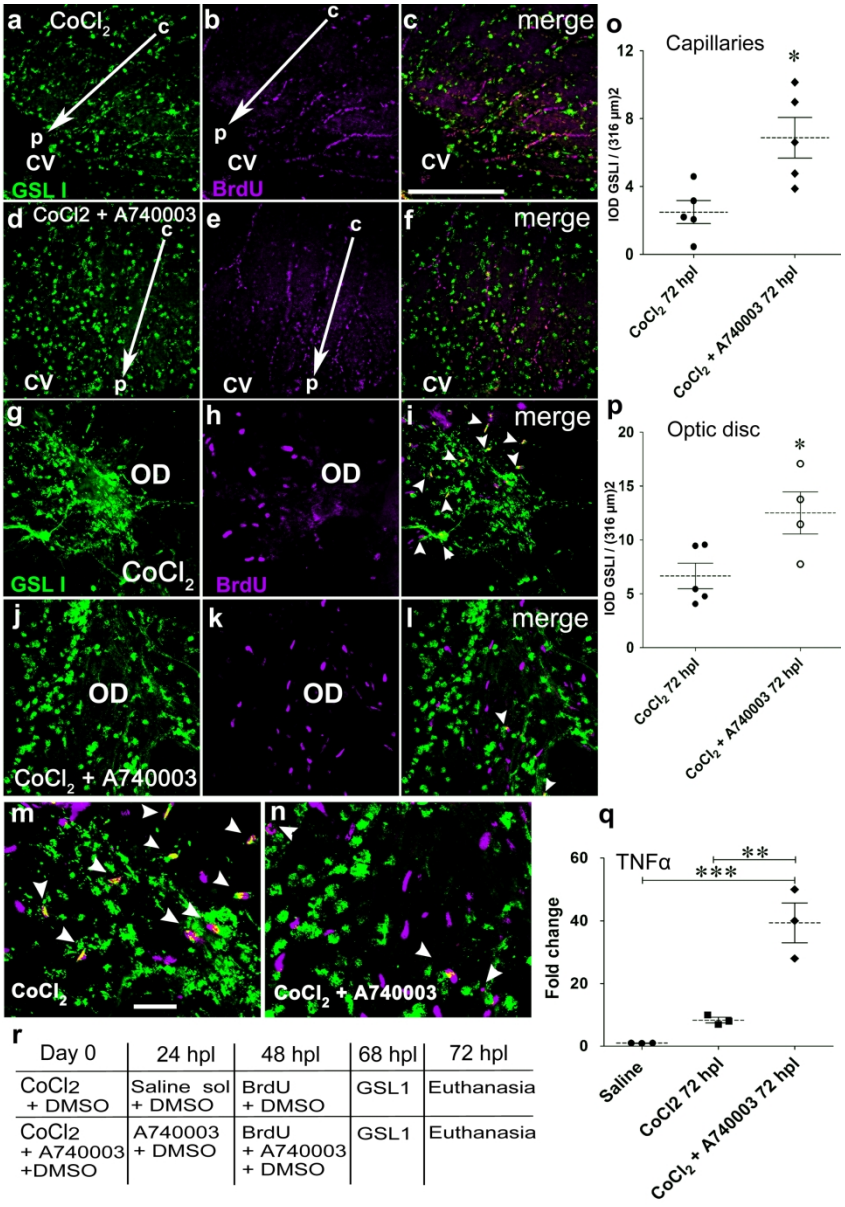
0 hpi	24 hpi	44 hpi	48 hpi
saline sol + DMSO	saline sol + DMSO	BrdU	Euthanasia
ATPγS + DMSO	ATPγS + DMSO	BrdU	Euthanasia
A740003 + DMSO	A740003 + DMSO	BrdU	Euthanasia
ATPγS + A740003 + DMSO	ATPγS + A740003 + DMSO	BrdU	Euthanasia





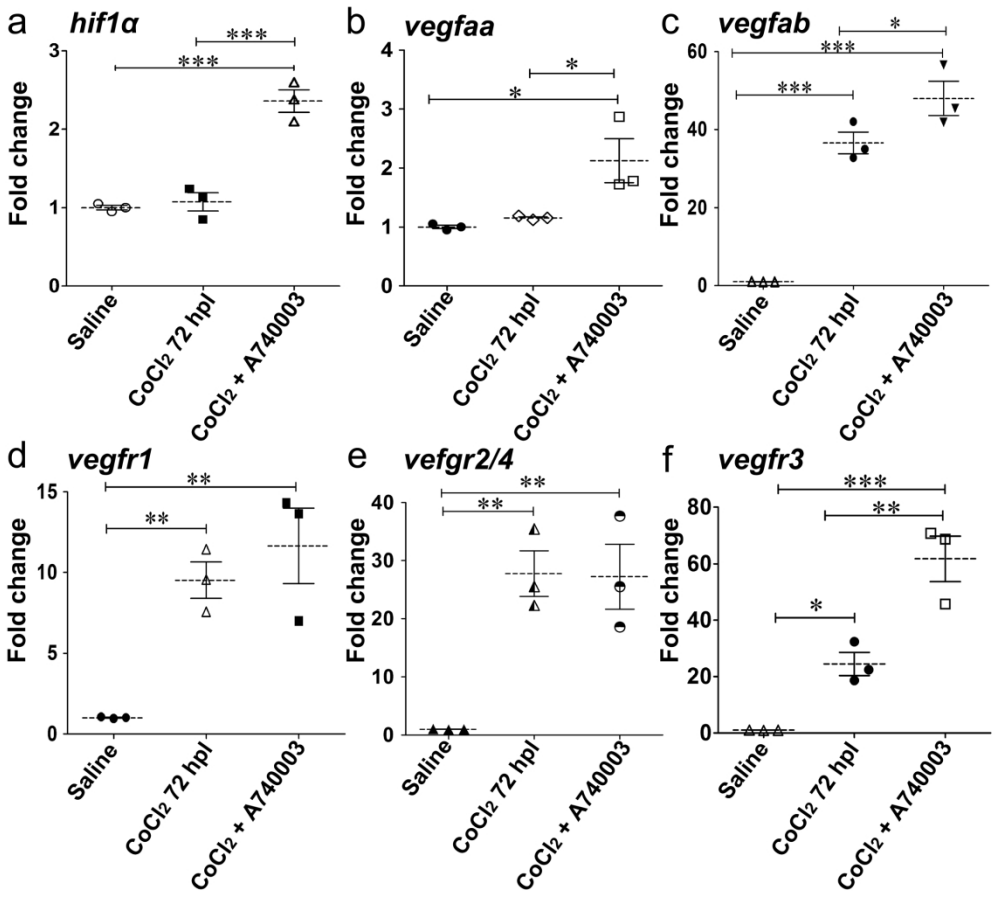
k

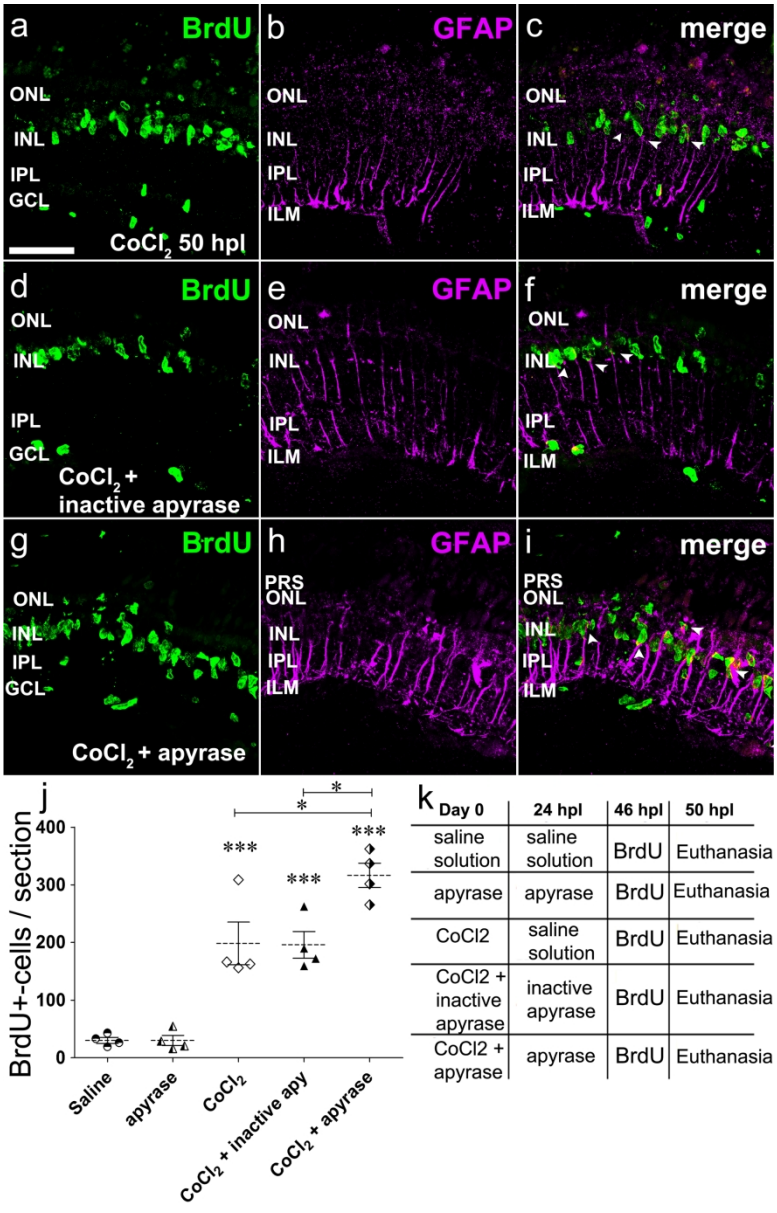
Day 0	24 hpl	46 hpl	50 hpl
saline sol + DMSO	saline sol + DMSO	BrdU	Euthanasia
$CoCl_2$ + DMSO	saline sol + DMSO	BrdU	Euthanasia
$CoCl_2$ + A740003 + DMSO	A740003 + DMSO	BrdU	Euthanasia



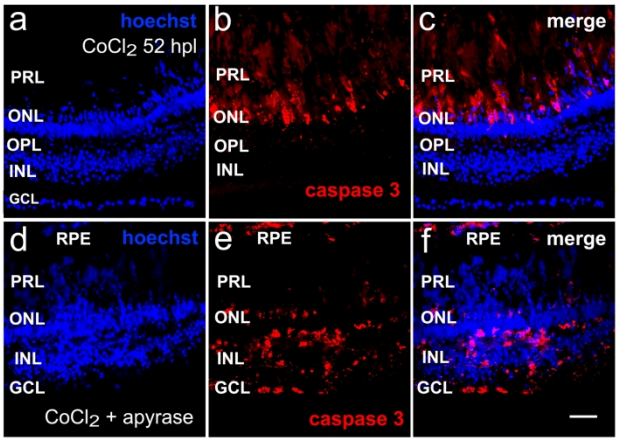
r

Day 0	24 hpl	48 hpl	68 hpl	72 hpl
$CoCl_2$ + DMSO	Saline sol + DMSO	BrdU + DMSO	GSL1	Euthanasia
$CoCl_2$ + A740003 +DMSO	A740003 + DMSO	BrdU + A740003 + DMSO	GSL1	Euthanasia



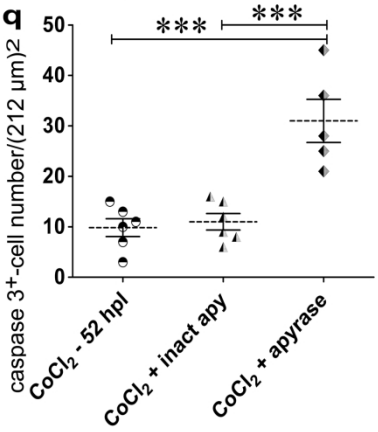


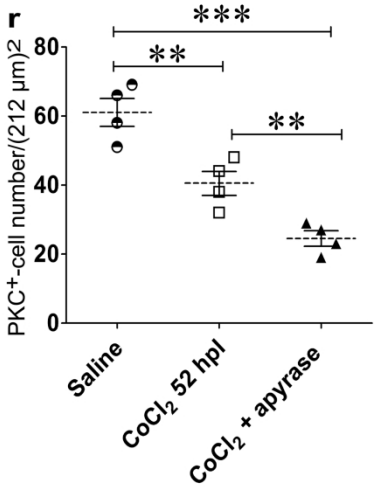
Day 0	24 hpl	46 hpl	50 hpl
saline solution	saline solution	BrdU	Euthanasia
apyrase	apyrase	BrdU	Euthanasia
CoCl2	saline solution	BrdU	Euthanasia
CoCl2 + inactive apyrase	inactive apyrase	BrdU	Euthanasia
CoCl2 + apyrase	apyrase	BrdU	Euthanasia

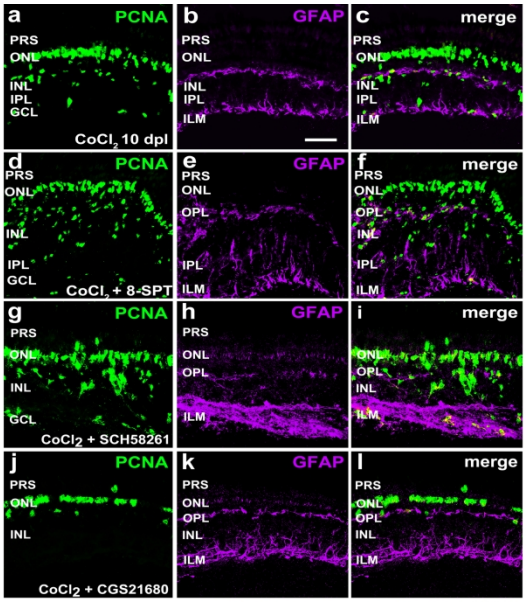


p

Day 0	24 hpl	52 hpl
saline sol	saline sol	euthanasia
$CoCl_2$	saline sol	euthanasia
$CoCl_2$ + inactive apy	inact apy	euthanasia
$CoCl_2$ + apyrase	apyrase	euthanasia







t

Day 0	1dpl	2 dpl	3 dpl	4 dpl	10 dpl
saline solution	saline solution	saline solution	saline solution	saline solution	euthanasia
CGS21680	CGS21680	CGS21680	CGS21680	CGS21680	euthanasia
$CoCl_2$	saline solution	saline solution	saline solution	saline solution	euthanasia
$CoCl_2$ + 8-SPT	8-SPT	8-SPT	8-SPT	8-SPT	euthanasia
$CoCl_2$ + SCH58261	SCH58261	SCH58261	SCH58261	SCH58261	euthanasia
$CoCl_2$ + CGS21680	CGS21680	CGS21680	CGS21680	CGS21680	euthanasia