P2X7 and A2A receptor endogenous activation protects against neuronal death caused by CoCl2-induced photoreceptor toxicity in the zebrafish retina

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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 signaling 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 CoCl2. 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 CoCl2-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 tnfa genes, which are related to multipotent progenitor proliferation. Levels of hif1α, vegf3r, and vegfαa 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 CoCl2. Blockade of adenosine P1 and A2A receptors (A2AR) had deleterious effects and deregulated normal timing for progenitor and precursor cell proliferation following photoreceptor damage. ATP via P2RX7 and adenosine via A2AR are survival extracellular signals key for retina regeneration in zebrafish.

KEYWORDS
A740003, adenosine A2A receptor, BzATP, microglia activation, P2X7 receptor, photoreceptor death, progenitor proliferation, RRID:AB_2109952, RRID:AB_2168560, RRID:AB_2315387, RRID:AB_2338000, RRID:AB_2338685, RRID:AB_2338840, RRID:AB_2738589, RRID:AB_514483, RRID:AB_628110
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 (All & Perron, 2017; Gemberling, Bailey, Hyde, & Pass, 2013; Lenkowski & Raymond, 2014; Powell, Cornblath, Elsaehidi, Wan, & Goldman, 2016).

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 CoCl₂ within the vitreous cavity. We have found that CoCl₂ injury effect was mainly cytotoxic first for cones, later for rods and secondarily induced death of 30% of bipolar cells (BCs) in the zebrafish retina. The majority of cells—including the majority of photoreceptors—died due to cytotoxic effects triggered by CoCl₂ and mediated by glutamate ionotropic receptors chiefly of the N-methyl-D-aspartate (NMDA) kind (Medrano et al., 2018). As described with many other types of injuries studied so far—mechanical, surgical, chemical, phototoxic, and mutational (Fimbel, Montgomery, Burket, & Hyde, 2007; Mensinger & Powers, 2007; Ramachandran, Faussett, & Goldman, 2010; Sherpa et al., 2008; Tappeiner et al., 2013; Vihtelic & Hyde, 2000; Weber et al., 2013; Wu et al., 2001), damaging the outer retina with CoCl₂ activates the proliferative response of multipotent retinal progenitors and the regeneration of the mature tissue.

Our laboratory has analyzed the effect of purinergic signaling 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, Ricatti, Pafundo, Gautier, & Faillace, 2009; Medrano et al., 2017). We have described that extracellular ADP acting on metabotropic purinergic receptor Y₁ (P2RY₁) activates the proliferative activity of neural progenitors in intact and lesioned retinas of zebrafish (Battista et al., 2009; Ricatti, Battista, Zorrilla Zubilete, & Faillace, 2011) demonstrating that purinergic signaling 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 retinopathies—as 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) 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 A₂₅ receptor exerted neuroprotective actions in the damaged retinal tissue.
2.2 Animals

Zebrafish (*Danio rerio*, Singapore strain) were purchased from a local breeding farm, in La Plata, Buenos Aires, Argentina. Zebrafish were acclimatized to the laboratory facility conditions for at least 1 month. They were maintained at 28°C in a 14:10 hr light : dark cycle, and they were fed with *Artemia* sp. and dry food twice a day. The adult zebrafish used in the experiment were an average of 3 cm in length. Animals were euthanized by immersion in ice-cold MS-222 anesthetic solution (0.02% wt/vol), 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 Anesthesia and intraocular injections

Zebrafish were deeply anesthetized in the anesthetic solution prepared with fish tank water and placed on a humid surface under a dissecting microscope. For performing retina damage—on Day 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 CoCl₂ hexahydrate diluted in sterile saline solution into the vitreous cavity. The zebrafish were injected during 10–15 s under a dissecting microscope. Zebrafish fully recovered from anesthesia in 5–10 s in fish tank water. Recovery rates were 100%. The intravitreal concentration (ivc) of CoCl₂ 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 (Cunyong, Huffmire, Ethell, & Cameron, 2013). A beveled 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

APTS: Adenosine-5′-(γ-thio)-triphosphate tetrallithium salt is a nonselective 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 5–10 times higher than ATP. EC50 = 0.7 μM in HEK 293 cells; EC50 = 3.6 μM 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 >> ATPS > 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. IC50: 18 nM 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 nonselective adenosine P1 receptor antagonist (Ki: 1.2 μM). Injected concentration: 100 μM; ivc: 20 μM (Tocris Bioscience, Bristol, UK).

SCH58261: potent and highly selective adenosine A2AR competitive antagonist (Ki = 1.3 nM). Displays 323–53- and 100-fold selectivity over A1, A2B, and A3 receptors, respectively (compound datasheet, Tocris Bioscience, Bristol, UK). Injected concentration: 3 μM and 0.5 μM; ivc: 0.6 μM and 100 nM.

CGS21680: Potent adenosine A2AR agonist (Ki = 23–27 nM). Selective for A2AR over A1R (Ki = 290 nM), A2B-R (Ki = 67 nM) and A2B (Ki ≥ 10 μM) in humans (Fredholm et al., 2011). It has affinity for A1 and A2B receptors but can be used to distinguish A2AR- and A2B-mediated effects (compound datasheet, Tocris Bioscience, Bristol, UK). Injected concentration: 30 and 1.5 μM; ivc: 6 μM and 300 nM.

Apyrase: Ecto-nucleotidase triphosphate diphosphohydrolase type I (isolated from potato), which removes β and γ 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 DMSO (1:50; ivc: 1:250) alone (vehicle) or with: A740003; APTs; ATPS + A740003; Bz-ATP; or Bz-ATP + A740003. Injections with ATPS or Bz-ATP were performed once daily for 2 or 3 days, respectively. Zebrafish were euthanized at 48 or 72 hr after the first injection of ATPS or Bz-ATP, respectively.

For experiments in Figure 3 and 4a–e, eyes were injected once with CoCl₂ at 0 hr after lesion (hpl) and with vehicle or A740003 at 0 and 24 hpl. For assays in Figure 4e–r, eyes were injected once with CoCl₂ (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 CoCl₂ 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, CoCl₂, CoCl₂ + apyrase, or CoCl₂ + 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 CoCl₂ on Day 0 and apyrase or inactive apyrase injections were repeated at 24 hpl. Zebrafish were euthanized and enucleated at 50 hpl. For experiments in Figure 8, eyes were injected with CoCl₂ or saline solution (vehicle) on Day 0 and with vehicle, 8-SPT, SCH58261, or CGS21680 at 0, 1, 2, 3, and 4 days after lesion (dpl). One group of zebrafish was injected within the vitreous once daily during 5 days with CGS21680. All groups of zebrafish were euthanized and enucleated 10 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 4 hr after BrdU injection. For assays in Figure 4e–r, zebrafish were euthanized 24 hr 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 2 hr at room temperature. Sections were cut with a scalpel and stained with antibodies against SV2, which is a protein localized in the membrane of presynaptic vesicles.
temperature. For proliferating cell nuclear antigen (PCNA) staining, eyecups were fixed with ethanol : formaldehyde (9:1) for 2 hr. 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

FIGURE 2  BrdU and GFAP double immunolabeling assays in retina sections after in vivo treatments with BzATP, A740003, and ATP_\gamma S. Confocal images of retina sections depict BrdU-positive nuclei in green. BrdU was injected into the vitreous cavity 4 hr 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 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 analyzed 72 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 4 hr 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 < .001; **p < .01; *p < .05 by Newman–Keuls multiple comparison test after one-way ANOVA (n = 4–5 zebrafish per group). Scale bars: 30 μm (c) and 25 μm (j). A740003, a specific antagonist of P2RX7; ANOVA, analysis of variance; ATP_\gamma S, slowly hydrolysable ATP analogue; BrdU, 5-bromo-2 0-deoxyuridine; BzATP, 3'-O-(4-benzoyl)benzoyl-ATP, a potent agonist of P2RX7; DMSO, dimethyl sulfoxide; GCL, ganglion cell layer; GFAP, glial fibrillary acidic protein; ILM, inner limiting membrane; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer; PRS, photoreceptor segments; Saline sol, saline solution [Color figure can be viewed at wileyonlinelibrary.com]
FIGURE 3  BrdU and GFAP double immunolabeling 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 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 4 hr before euthanasia, which was performed 50 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 < .001; **p < .01 by Newman–Keuls multiple comparison test after one-way ANOVA (n = 6 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 < .5 by two-tailed unpaired Student’s t test with Welch’s correction and **p < .01 by Dunnett’s multiple comparison test after one-way ANOVA (n = 3 independent assays). Scale bar = 50 μm. A740003, specific antagonist of P2RX7; ANOVA, analysis of variance; BrdU, 5-bromo-2'-deoxyuridine; DMSO, dimethyl sulfoxide; GCL, ganglion cell layer; GFAP, glial fibrillary acidic protein; ILM, inner limiting membrane; INL, inner nuclear layer; IPL, inner plexiform layer; Lin28a, pluripotency gene; ONL, outer nuclear layer; Saline sol, saline solution [Color figure can be viewed at wileyonlinelibrary.com]
FIGURE 4  Microglia response in the injured retina treated with a specific antagonist of P2RX7. Confocal images of whole-mount retinas show GSL1 bound to plasma membrane of microglia cells in green and BrdU nuclear localization in magenta (a–n). Zebrafish retinas were treated in vivo with saline solution + DMSO (vehicle), 1.3 mM CoCl2 (a–c, g–i, m), (data not shown), or with 1.3 mM CoCl2 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 hpl. BrdU and GSL1 were injected in the vitreous cavity 24 and 4 hr 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 colocalized 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 IOD from total fluorescence signals of GSL-1-positive cells over background fluorescence on confocal images of 10 microscopic fields of (316 μm)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 < .05 by two-tailed unpaired t test with Welch's correction (n = 4–5 zebrafish per group). The graph in (q) depicts relative levels of tnfα mRNA in neural retinas of saline solution-treated, CoCl2-injured and CoCl2 + 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 < .001; **p < .01 by Dunnett's multiple comparison test after one-way ANOVA (n = 3 independent assays). ANOVA, analysis of variance; BrdU, 5-bromo-2′-deoxyuridine; DMSO, dimethyl sulfoxide; GSL1, Griffonia simplicifolia lectin 1; IOD, integrated optical density [Color figure can be viewed at wileyonlinelibrary.com]
marginal zones (CMZs). Each slide contained sections representing different portions of the eyecup. Tissue sections were incubated in PBS plus 0.1% Tween-20 (PBST) and then in 5% NGS (in PBST) at room temperature for 1 hr. For BrdU staining, retinas were preincubated 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 2 hr with a fluorescent secondary antibody (1:1,000 in 3% NGS). For double-labeling 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 2 hr. Finally, sections were washed with PBST and stained with Hoechst (1:1,000; Sigma-Aldrich, St Louis, MO) 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 fluorescein isothiocyanate (FITC)-conjugated Griffonia simplicifolia lectin 1 (GSL1) (Vector Labs, Burlingame, CA) was injected directly in the vitreous cavity 24 or 4 hr before euthanasia (Figure 4a–d,e–r, respectively). Zebrafish were euthanized 72 hpl. Then, eyecups were fixed with 4% paraformaldehyde, and BrdU immunostaining was performed on whole-mount retinas, as previously described (Zou, Tian, Ge, & Hu, 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 μm)² microscopic field. We analyzed 10 fields per retina and five eyes from different zebrafish.

2.10 | Counting of proliferative nuclei and active caspase 3- and protein kinase C-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 CMZ 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 4-hr period before euthanasia, so it labeled 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, Bunyak, Chagin, Cardoso, & Palaniappan, 2009). In assays shown in Figure 4, BrdU was injected and zebrafish were euthanized 24 hr after. So, BrdU-positive nuclei represented nuclei within the cell cycle and postmitotic nuclei.

Active caspase-3-positive cells were counted when colocalized with a single Hoechst stained nuclei. Protein kinase C (PKC)- and active caspase-3-positive cells were counted in nuclear layers within a (212 μm)² microscopic field, in five to six retina sections from five to six eyes of different zebrafish.

**FIGURE 6** Apyrase effect on progenitor cell proliferation and GFAP expression in injured retinas. Confocal images of retina sections depict proliferative progenitor cell nuclei labeled with BrdU (green) and GFAP immunoreactivity in Müller glia (magenta). Zebrafish were treated with a single dose of 1.3 mM CoCl₂, CoCl₂ plus inactive apyrase, or CoCl₂ plus 4 U/mL apyrase and euthanized 50 hpl. BrdU was injected within the vitreous cavity 4 hr 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 < .05 (CoCl₂- and apyrase treated retinas vs. CoCl₂-injured retinas), **p < .001 (vs. saline solution- or apyrase-treated uninjured retinas) by Newman–Keuls multiple comparison test after one-way ANOVA. Scale bar: 35 μm. ANOVA, analysis of variance; Apyrase, ectonucleotidase triphosphate diphosphohydrolase type I; BrdU, 5-bromo-2′-deoxyuridine; GCL, ganglion cell layer; GFAP, glial fibrillary acidic protein; ILM, inner limiting membrane; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer; PRS, photoreceptor segments [Color figure can be viewed at wileyonlinelibrary.com]
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.

**FIGURE 7** Apyrase effect on apoptotic cell death and PKC expression in retinas injured with CoCl2. Zebrafish eyes were injected with saline solution, a single dose of 1.3 mM CoCl2 plus 4 U/ml inactive apyrase, or CoCl2 plus 4 U/ml apyrase and euthanized 52 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 PKC. Cell nuclei labeled 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 μm)², respectively. The number of cells was examined 52 hpl in saline solution-, CoCl2-, CoCl2 plus apyrase-, and CoCl2 plus heat-inactivated apyrase-treated retinas. **p < .01; ***p < .001, Newman–Keuls multiple comparison test after one-way ANOVA (n = 4–6 zebrafish per experimental group). Scale bar: 25 μm. ANOVA, analysis of variance; GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; OFF-BC, off-center bipolar cell synaptic terminals; ON-BC, on-center bipolar cell synaptic terminals; ONL, outer nuclear layer; OPL, outer plexiform layer; PKC, protein kinase C; PS, photoreceptor segments; PS-OPL, presynaptic outer plexiform layer; RPE, retinal pigmented epithelium [Color figure can be viewed at wileyonlinelibrary.com]
FIGURE 8  Agonist and antagonist of adenosine receptor effect on PCNA and GFAP double immunolabeling in retina sections after damage with CoCl₂. Confocal images depict PCNA-immunoreactive cells in green or red and GFAP expressed by Müller glia in magenta on retina sections lesioned with CoCl₂. Zebrafish eyes were injected into the vitreous cavity with a single dose of 1.3 mM CoCl₂ or saline solution (Day 0) and during the following 4 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 5 days with 6 μM CGS21680. All groups of zebrafish were euthanized 10 days after lesion (dpl) with CoCl₂ or the first intraocular injection. Graphs show the number of PCNA-positive cells per retinal section 10 days after CoCl₂ or saline solution treatments (q–s). The table in (t) summarizes injection protocols for all groups. ***p < .001; **p < .01; *p < .5 by Newman–Keuls multiple comparison test after one-way ANOVA (n = 4–9 zebrafish per group). Scale bar: 25 μm (b) and 20 μm (o). 8-SPT, nonselective antagonist of adenosine P1R; ANOVA, analysis of variance; CGS21680, agonist of adenosine A₂a > A₂ > A₁ receptors; GCL, ganglion cell layer; GFAP, glial fibrillary acidic protein; ILM, inner limiting membrane; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer; PCNA, proliferating cell nuclear antigen; PRS, photoreceptor segments; SCH58261, selective antagonist of adenosine A₂AR [Color figure can be viewed at wileyonlinelibrary.com]
1. Mouse monoclonal anti-BrdU antibody (1:400; Cat# 11710376001, RRID:AB_514483, Roche Applied Sciences, Penzberg, Germany) 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, Santa Cruz, CA). PCNA is a nuclear protein which is expressed during S-phase and G2 phase of the cell cycle.

3. Rabbit polyclonal anti-GFAP antibody (1:500; Cat# M0761, RRID: AB_2109952, Agilent, Santa Clara, CA) 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, Iowa City, IA) was used to stain presynaptic terminals containing vesicles in plexiform layers.

5. Rabbit polyclonal anti-PKC antibody (1:350; Cat# sc-10800, RRID:AB_2738589, BD Biosciences, San Jose, CA) was used to stain active caspase-3-dependent apoptotic cells.

6. Rabbit polyclonal anti-active caspase-3 antibody (1:350; Cat# 564096, RRID:AB_2338685) and goat anti-mouse ALEXA 488 (Cat# 115703, RRID:AB_2338000) were used to detect 5-bromo-2'-deoxyuridine, a thymidine analogue that incorporates into DNA during the S phase of the cell cycle.

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). Secondary antibodies (1 mg/ml) were diluted 1:1,000 in 3% NGS.

2.13 Microscopy

Counting was performed using a BX50 epifluorescence microscope (Olympus, Tokyo, Japan) with ×40 and ×60 objectives and numerical apertures (NAs) 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, Tokyo, Japan). Images from double- or triple-labeled 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 using Fluoview Software. Images were adjusted for brightness and contrast, combined, and labeled with Adobe Photoshop CS5 extended (12.0). The autofluorescence of photoreceptor segments was 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 RNeasy 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 (Applied Biosystems 7500, Thermo Fisher Scientific, Waltham, MA). 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, Lehmann, Buschmann, Unger, & Funke-Kaiser, 2006). Amplification efficiencies for all samples were between 0.95 and 1.0. Results were also

### 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’ |
|---------------------|---------------|---------------|
| ef1-α (ENSDARG00000020850) | cagcagctgaggagttgct | gtagatcagatgctgcgg |
| p2x7 (ENSDARG00000042440) | acacccagagagaattgtagg | aagggttttgcctctgtagtc |
| lin28a (ENSDARG0000016999) | tttctgtccatgaccacg | tcacagctgcaaaaccccct |
| tnfa1 (ENSDARG0000009511) | tttcatccaaaggtgcaca | agaaagctgcctgtctgtg |
| hif1α (ENSDARG00000034293) | caggcgcagcctattcct | gtagctgtaagcagcctt |
| vegfaa (ENSDARG00000103542) | gtacatccgctcctgtgctg | gcgaacacgtgttgcgtg |
| vegfαb (ENSDARG00000034700) | ttgtgcgaatatattcgcag | caccctgtagcaagacctg |
| vegfr1 (ENSDARG00000019371) | ctgctgtgtagaaaaatccag | tgcgtttcccctgttagagcc |
| vegfr2/4 (ENSDARG000000105215) | ccatgtgaccccagtgctaa | tcaagctttttcatttggagag |
| vegfr3 (ENSDARG000000104453) | cagagctcagagagttgag | caaacggcagctgattgtaaa |

Note: 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. Ef1-α was used as an internal reference gene.

Abbreviations: PCR, polymerase chain reaction; RT, reverse transcription.
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 ± 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 hr (Figure 1). At 72 hr 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 inner nuclear layer (INL) cells (Figure 1d–f). Damage to photoreceptors was heterogeneous 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 labeled presynaptic terminals in the plexiform layers, was also disrupted at the outer plexiform layer (OPL) level. Nuclei in the 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). 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 1.

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).

#### 3.2 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.

Next, we treated zebrafish with one intraocular injection of CoCl2 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 CoCl2 was significantly enhanced by blockade of P2RX7 (Figure 3a,d,g,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 (Figure 3c,f,i). The transcriptional expression of lin28α—which is thought to regulate stem cell self-renewal—was induced by CoCl2 injury as described elsewhere (Medrano et al., 2018; data not shown). Treatment with A740003 of CoCl2-injured retinas for 3 days further induced lin28α expression level (Figure 3).

The relative level of P2RX7 mRNA was significantly increased by 72 hpi with CoCl2. 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 CoCl2

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 CoCl2 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 CoCl2**

A hallmark of tissue response to hypoxia and hypoxia-like damage is the intracellular accumulation of hypoxia-inducible factor 1-alpha (HIF1α) as well as the induction of vascular growth. In our experimental conditions, one intravitreous injection of CoCl2 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 CoCl2-injured retinas with A740003 (Figure 5). Relative levels of vascular endotelial growth factor ab (vegfab) but not of vegfa mRNA were significantly induced by injury with CoCl2 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 CoCl2 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 CoCl2**

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 di- and tri-phosphate nucleotides from retina extracellular milieu immediately after injury with CoCl2. To this end, we injected an excess of apyrase into the vitreous cavity of zebrafish eyes injured with CoCl2 (Figure 6).

Treatments of noninjured retinas with either active or inactive apyrase did not induce significant changes in the number of BrdU-positive cells (Figure 6j), Müller glia reactivity, or morphological appearance (image not shown). Heat-inactivated apyrase did not modify progenitor proliferation or the gliotic response in CoCl2-lesioned retinas (Figure 6d–f,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 CoCl2**

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 CoCl2 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).

CoCl2-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 fiber layer (Figure 7d–f).

As described by Medrano et al. (2018), CoCl2 caused a significant but limited reduction in the number of BCs (Figure 7j–l). Active apyrase treatment drastically reduced the number of BC observed in CoCl2-injured retinas (M–O). Only big-sized ON-BCs 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 10 days after injury with CoCl2**

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 nonselective 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 CoCl2.

We quantified the number of proliferative progenitors by detecting PCNA expression on the 10th day after injury. Retinas were injured and, beginning immediately after injury induction, treated for 5 days with 8-SPT or vehicle. Vehicle-treated damaged retinas examined at 10 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 10 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 CoCl2-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 10 dpl (Figure 8q).

Injured retinas were treated immediately after damage and for the next 4 days with the selective antagonist of A2AR 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 CoCl2 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 10 dpl (Figure 8g,m,n,r).

Next, we examined the effect of exogenously activating adenosine receptors. Injured retinas were treated with the agonist of A2AR CGS21680 immediately after damage and for the next 4 days. Retinas treated with a high dose of CGS21680 (6 μM) examined 10 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 CoCl2-injured retinas without other treatment (Figure 8j–l).

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,s). Treatment of uninjured retinas with the A2AR agonist (6 μM) for 5 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 noninjured 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 (Medrano et al., 2018; Weber et al., 2013). Moreover, we identified that several proliferative nuclei colocalized 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, Ca2+ entry, and apoptotic cell death throughout P2RX7 activation in several tissue and cell types (Surprenant, Rassendren, Kawashima, North, & Buell, 1996). Cruz, Lin, Chao, and Hwang (2013) have found that activation of P2RX7 with high concentrations of ATP for 12–24 hr causes the death of zebrafish larvae. P2RX7 activation with BzATP or high extracellular concentrations of ATP or ATPS 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; Puthussery & Fletcher, 2004; Sugiyama, 2014; Vessey et al., 2014; Wheeler-Schilling, Marquardt, Kohler, Guenther, & Jabs, 2001; Zhang et al., 2007). 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 hr 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 1 hr 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 ATPS 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 (Anncasi, Ornelas, Cossenza, Persechini, & Ventura, 2013; Hu et al., 2010; Resta et al., 2007; Zhang et al., 2005)—might be mediated by P2RY, likely P2RY1, in the adult retina of zebrafish (Battista et al., 2009; Medrano et al., 2017; Ricatti et al., 2011). In addition, it has been demonstrated that activation of P2RY1 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 CoCl2

We have previously described that 1.3 mM of CoCl2 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 CoCl2. It has been described that lytic release of ATP can cause cell death to neighboring cells via activation of P2RX7 (Adinolfi et al., 2005; Sperlágh & Illes, 2014). Therefore, photoreceptors or interneurons that were not directly affected by CoCl2 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 CoCl2 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 GCs from dying (Lim, Lu, Beckel, & Mitchell, 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 CoCl2 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 CoCl2 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 CoCl2-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 CoCl2 provoked a significant increment in the transcriptional 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 CoCl2 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 CoCl2 (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 while the second study reported the opposite (Nelson et al., 2012; Ramachandran et al., 2010). 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 signaling pathways activated by CoCl2-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 CoCl2 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 tnfα 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 TNF-α 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
endothelial growth factor (VEGF) signaling is limited in the retina (Savio, de Andrade Mello, da Silva, & Coutinho-Silva, 2018). 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-labeled 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 vascular endothelial growth factor (VEGF) signaling is limited by endogenous activation of P2RX7 in CoCl2-injured retinas

HIF1α expression was affected by A740003 treatment in injured neural retinas. At 72 hpl, levels of hif1α mRNA detected after injury with CoCl2 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 ATP increases HIF1α production provoking deleterious effects in mammalian and neuroblastoma cell cultures (Amoroso et al., 2015; Amoroso, Falzoni, Adinolfi, Ferrari, & Di Virgilio, 2012). 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, de Andrade Mello, da Silva, & Coutinho-Silva, 2018).

Injury with CoCl2 induces significant increments in the transcriptional expression of vegfr1, 2, and 3 as well as vegfa but not vegfab in the adult neural retina of zebrafish indicating a limited pro-angiogenic profile (Medrano et al., 2018; Wild et al., 2017). We have found in this study an additional enhancement of the transcriptional expression of vegfa 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 vasculosgenesis 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 signaling 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 CoCl2. As previously reported, CoCl2-treated retinas lost 30% of BCs 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-BCs receiving input from cones and rods.

Moreover, whereas retinas injured with CoCl2 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 CoCl2 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 P2RY1 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 7 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 2 days after injury with CoCl2. 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 2 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 CoCl2

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, Gijsbers, Ceulemans, Stalmans, & Stefan, 2000; Hunsucker, Mitchell, & Spychala, 2005; Zimmermann, 2006). There are evidences...
indicating that adenosine via P1R can directly regulate cell proliferation (Borea, Varani, Gessi, Merighi, & Vincenzi, 2018; Liu et al., 2017; Merighi et al., 2002). For instance, activation of A1R and A2AR provokes proliferation of neural progenitors isolated from mouse embryo brain cortex (Lv, Shao, & Gao, 2018). However, in our study, an agonist of A2AR caused a long-term decrease in neural progenitor proliferation. A decreased proliferative activity detected 10 dpl might be consequence of an enhanced mitotic activity at the time of peak proliferation (4 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 10 dpl. Nevertheless, these hypotheses require further examination.

In our assays, exogenous activation of A2AR significantly reduced tissue damage induced by CoCl2. In rat retinas, the specific activation of A2AR protects neurons from ischemia (Li & Roth, 1999; Melani et al., 2014). Moreover, different agonists of A2AR 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 CoCl2 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 A2AR 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 A2AR. These findings suggest that the neuroprotective effect could be also mediated via A1 and A3 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 microtialmia, accelerated cell death, and retinopathy development in the avian and mammalian retina (Boia et al., 2017; Ma et al., 2014). However, it has also been described that blockade of P1R with caffeine reduces neuroinflammation and prevents GC 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 A2AR. Our findings in the injured retina suggest that specific blockade of A2AR 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 A2AR caused GC protection and inflammatory prevention in the rodent retina (Madeira et al., 2016). Reported findings regarding A2AR role are controversial since high or low doses of SCH58261 can cause completely opposite effects in the rodent retina (Pintor, Quarta, Pèzzola, Reggio, & Popoli, 2001). Moreover, agonists and antagonists of A2AR 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 nonselective dose of an agonist for A2AR showed a neuroprotective action in the zebrafish retina.

5 | CONCLUSION

Purinergic signaling controls cell death generated by CoCl2 cytotoxic damage. Our findings indicated neuroprotective roles of endogenous extracellular ATP and adenosine chiefly acting on P2RX7 and A2AR, 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 signaling may be indispensable to limit and counterbalance neurodegeneration, gliosis, HIF1α accumulation, VEGF/vascular endothelial growth factor receptor (VEGFR) overexpression, as well as other cytotoxic mechanisms induced by CoCl2 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 signaling 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 glial proliferation—like in proliferative retinopathies—or inflammatory responses that drive to blindness.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

AUTHOR CONTRIBUTIONS

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.

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