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TITLE: *Characterization of proliferative, glial and angiogenic responses after a CoCl₂-induced injury of photoreceptor cells in the adult zebrafish retina*

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ABSTRACT

The adult zebrafish is considered a useful model for studying mechanisms involved in tissue growth and regeneration. We have characterized cytotoxic damage to the retina of adult zebrafish caused by the injection of cobalt chloride (CoCl₂) into the vitreous cavity. The CoCl₂ concentration we used primarily caused injury to photoreceptors. We observed the complete disappearance of cones, followed by rods, across the retina surface from 28-96 h after CoCl₂ injury. The loss of 30% of bipolar cells was also observed by 50 h after lesion (hpl). CoCl₂ injury provoked a strong induction of the proliferative activity of multipotent Müller glia and derived progenitors. The effect of CoCl₂ on retina cells was significantly reduced by treatment with glutamate ionotropic receptor antagonists. Cone photoreceptor regeneration occurred 25 days after injury. Moreover, a single dose of CoCl₂ induced vascular damage and regeneration, whereas three injections of CoCl₂ administered weekly provoked neovascular-like changes 20 days after injury. CoCl₂ injury also caused microglial reactivity in the optic disc, retina periphery and fibre layer. CoCl₂-induced damage enhanced pluripotency and proneural transcription factor gene expression in the mature retina 72 hpl. Tumour necrosis factor alpha, vascular endothelial growth factor (VEGF) and VEGF receptor mRNA levels were also significantly enhanced by 72 hpl. The injury paradigm we have described in this work may be useful for the discovery of signalling molecules and pathways that participate in the regenerative response and it may serve as a model to screen for compounds that could potentially treat aberrant angiogenesis.

INTRODUCTION

Zebrafish (*Danio rerio*) represent an experimentally convenient alternative vertebrate model for the investigation of human diseases. The use of the zebrafish as a model system for studying molecules and pathways involved in health and disease is supported on the basis of evolutionary conservation of cellular, molecular and physiological processes; an average homology of 70% between human and zebrafish genes (Barbazuk *et al.*, 2000); and the finding that 84% of genes that cause diseases in humans have a zebrafish orthologue. Furthermore, the experimental use of zebrafish allows for the validation of new therapeutic targets (Zon & Peterson, 2005), with the advantage of using drugs in an *in vivo* model that can be added directly to the fish water.

Zebrafish have the capacity to regenerate several tissues and organs, such as the heart, kidneys and peripheral and central nervous systems, including portions of the brain (Gemberling *et al.*, 2013). Therefore, zebrafish are valuable experimental animals for the development of tissue and organ injury strategies and for the investigation of tissue and cell regenerative mechanisms. For instance, zebrafish as well as many other teleost fish species, are able to regenerate the whole retina. The retina can be injured with different levels of severity, including different retina layers and cell types, and regeneration originates from intrinsic multipotent progenitors, which are represented by a subpopulation of differentiated Müller glia (Lenkowski & Raymond, 2014). Many paradigms of damage (for example, chemical, surgical, laser, intense light and transgenic ablation) have been extensively investigated, and they range from injury of almost the whole retina to targeted neuronal cell ablation (Vihtelic & Hyde, 2000; Wu *et al.*, 2001; Yurco & Cameron, 2005; Fimbel *et al.*, 2007; Sherpa *et al.*, 2008). Lesion paradigms have been developed and characterized to the

end of studying molecular and cellular mechanisms that regulate and mediate retina regeneration (Mensing & Powers, 2007; Rajaram *et al.*, 2014; Powell *et al.*, 2016; Ail & Perron, 2017). Zebrafish have also been rated as suitable experimental vertebrates—an alternative to rodents—for the investigation of the mechanisms involved in regulating physiological and pathological angiogenesis (Cao *et al.*, 2010; Chávez *et al.*, 2016). Zebrafish embryos can be used for the screening of angiogenic inhibitors, which could lead to the discovery of therapeutic approaches for the treatment of angiogenesis-dependent eye diseases (Cunvong *et al.*, 2013; Rezzola *et al.*, 2016).

A previous study has described injury to the rodent retina using cobalt chloride (CoCl₂) (Hara *et al.*, 2006). The goal of that study was to investigate molecular and cellular targets for neuroprotective and anti-angiogenic therapies that could be used as possible treatments of ocular diseases (Hara *et al.*, 2006). CoCl₂ has also been added to the water of zebrafish embryos to evaluate the effects on the developmental morphology of whole-body vasculature (Wu *et al.*, 2015).

Cobalt ions (Co²⁺) are necessary at trace concentrations as co-factors for some proteins and vitamin B12 synthesis, but it is associated with toxic effects when present at higher concentrations. Co²⁺-induced pathologies include severe cardiomyopathy, vision or hearing impairments, reversible hypothyroidism and polycythaemia (Scharf *et al.*, 2014).

CoCl₂ prevents prolyl and asparaginyl hydroxylase activity and proteasome degradation of the hypoxia inducible factor-1 α (HIF-1 α) (Jaakkola *et al.*, 2001; Semenza, 2001), and it prevents the incorporation of iron for heme production (Maines & Sinclair, 1977), which can lead to chemical hypoxia. Hypoxia and CoCl₂ provoke HIF-1 α intracellular

accumulation, which stimulates vascular endothelial growth factor (VEGF) secretion, angiogenesis and haemorrhages (Goldberg & Schneider, 1994; Ladoux & Frelin, 1994; Minchenko *et al.*, 1994). HIF-1 α accumulation induces oxidative stress, inflammation, mitochondria damage and signalling for cell survival, death, migration and proliferation (Masoud & Li, 2015; Wigerup *et al.*, 2016). Cobalt-associated mechanisms of toxicity are not yet understood, and hypoxic effects are far from being the unique cause of cell death induced by this metal. Co²⁺ can cause a large increase in oxidative stress, DNA breakage, epigenetic changes, histone modifications, inflammation, loss of biological functions and necrotic and apoptotic cell death (Scharf *et al.*, 2014; Uboldi *et al.*, 2016). Additionally, some Co²⁺-associated toxic effects can be due to the deregulation of Ca²⁺ homeostasis and signalling by blocking Ca²⁺ entry through channels and Ca²⁺ binding to intracellular proteins (Krizaj & Copenhagen, 2002; Scharf *et al.*, 2014).

In the present report, we described a paradigm in which retina photoreceptors were injured by injecting a particular dose of CoCl₂ into the vitreous cavity of adult wild-type zebrafish. We characterized the CoCl₂-dependent damage, and we studied its effects on photoreceptor, bipolar cells (BC) and synaptic layer integrity; Müller glia and microglia activation; HIF-1 α , VEGF and VEGFR gene expression; intraretina vessel angiogenesis; and apoptotic cell death at different time intervals following injury. We showed that CoCl₂ deleterious effects were significantly mediated by glutamate. Furthermore, we characterized the CoCl₂ treatment effect on multipotent Müller glia and Müller glia-derived progenitor mitotic induction and on pluripotency and proneural gene expression, which is indicative of retinal repair after injury.

MATERIALS AND METHODS

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). Vectashield and normal goat serum (NGS) (Vector Labs, Burlingame, CA). Random primers (Invitrogen, Carlsbad, CA). 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 (Roche Applied Sciences, Mannheim, Germany).

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 *Artemia sp.* and dry food. 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 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.

Cobalt chloride and ouabain treatments

Zebrafish were deeply anesthetized in the anaesthetic solution prepared in fish tank water and placed on a humid surface under a dissecting microscope. On day 0 for the majority of assays, the left eyes of the experimental zebrafish were given a single 0.6 μ l injection into the vitreous cavity of 6.5 mM CoCl_2 hexahydrate diluted in sterile saline solution. The zebrafish were injected during a 10–15 sec period under a dissecting microscope, and they fully recovered from anaesthesia in fish tank water in 5–10 sec. Recovery rates were 100%.

For the majority of experiments performed the intravitreal concentration (ivc) of CoCl_2 was estimated to be 1.3 mM on the basis of a vitreous volume of approximately 3 μ l. 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 μ l syringe (701 RN) (Hamilton Company, Reno, NV) were used to deliver appropriate volumes. For experiments shown in Fig. 1, different zebrafish were injected once into the vitreous cavity with a solution containing 20 mM (ivc = 4.0 mM), 12 mM (ivc = 2.4 mM) or 6.5 mM (ivc = 1.3 mM) CoCl_2 on day 0. For experiments in Fig. 9N–P, eyes were injected once with 6.5 mM CoCl_2 on day 0, the eyes were then injected with 3 mM (ivc = 0.6 mM) CoCl_2 on days 7 and 15 after the first injection and the zebrafish were euthanized 20 days after lesion (dpl). For assays in Fig. 6A–E, three groups of four zebrafish per group were injected with either 6.5 mM CoCl_2 , 16 μ M ouabain (estimated ivc = 3.2 μ M) or saline solution into both eyes over a period of 20 sec under the dissecting microscope. Zebrafish were euthanized and enucleated 8 h after lesion (hpl). For experiments in Fig. 6F–K and Fig. 7, three groups of five zebrafish per group were injected with 6.5 mM CoCl_2 or saline solution in both eyes, and zebrafish were euthanized and enucleated 72 hpl. 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 in the vitreous cavity at the same intervals as the experimental groups of zebrafish.

5-Bromo-2'-deoxyuridine administration

Groups of five to six zebrafish were injected into the vitreous cavity with 0.6 μ l of a solution containing 20 μ g/ μ l BrdU. At all intervals after saline solution or CoCl₂ injection, the BrdU solution was injected 4 h before euthanizing the fish using the same orifice punctured in the cornea on day 0. BrdU intravitreal concentration was estimated to be 4 μ g/ μ l.

Glutamate receptor antagonist treatment of CoCl₂-injured eyes

Three groups of six zebrafish per group were injected into the vitreous cavity of the left eye with 0.6 μ l of a solution containing 1.5 mM 2-amino-5-phosphonopentanoic acid (APV; estimated ivc = 300 μ M), 250 μ M 6,7-dinitroquinoxaline-2,3-dione (DNQX; estimated ivc = 50 μ M) in dimethyl sulfoxide (DMSO, 0.2% V/V in saline solution; ivc = 0.04%), or vehicle (0.2% DMSO in saline solution; ivc = 0.04%). APV is a selective N-methyl-D-aspartate (NMDA) receptor antagonist that competitively inhibits glutamate binding to this ionotropic receptor. DNQX is a selective non-NMDA ionotropic receptor antagonist that competitively blocks glutamate binding to α -amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)/ kainate receptors. Glutamate receptor antagonist (or vehicle) injections were performed 18 h before and 6 and 30 h after damage with CoCl₂ (or saline solution). Fifty hours after CoCl₂ treatment, zebrafish were euthanized and enucleated, and eyecups and cryosections were prepared. All groups were injected with BrdU 4 h before euthanasia, as explained above. Tissue sections from each eye were used for Hoechst staining to examine

nuclear layer morphology, and other sections from the same eyes were used for BrdU immunolabelling.

Tissue processing and immunocytochemistry

To prepare eyecups, the cornea, lens and vitreous were removed. Eyecups were fixed with 4% paraformaldehyde for 2 h at room temperature. For proliferating cell nuclear antigen (PCNA) staining, eyecups were fixed with ethanol:formaldehyde (9:1) for 2 h. Eyecups were then incubated in 5%, 10% (30 min) and 20% sucrose (overnight) and embedded in tissue-freezing medium. Eyecups were cut into 16- μ m 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 1 h. For BrdU labelling, 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 2 h 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 2 h. 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.

Microglial cell detection and immunostaining for 5-Bromo-2'-deoxyuridine

To detect microglial cells 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 4 h before euthanizing the fish. Then, eyecups were fixed with 4% paraformaldehyde for 2 h and either cryostat sections or whole flat retinas were prepared and mounted for direct microscopic observation. For triple-labelling assays, GSL 1-injected eyes were enucleated, eyecups were fixed with 4% paraformaldehyde and BrdU immunostaining and Hoechst staining were performed on the sections, as previously mentioned (Zou *et al.*, 2013).

Blood vessel detection with tomato lectin in whole-mount retinas

Ten micrograms per millilitre of biotinylated *Lycopersicon esculentum* (tomato) lectin (Vector Labs) and Alexa 488-conjugated streptavidin (1:300; Vector Labs) were diluted in PBST and used to detect blood vessels on whole-mount zebrafish retinas. Retinas were fixed with 4% paraformaldehyde for 2 h, carefully washed and then incubated at 4°C overnight with the tomato lectin, washed with PBST and incubated with the fluorescent streptavidin at room temperature for 2 h and, finally, washed and mounted.

Counting of proliferative nuclei, GSL1- 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 neural retina layers, as well as the CMZ. We did not count cells in areas near or on the optic disc 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-h period before euthanasia, so it labelled the nuclei of progenitors that had initiated S-phase

within this time window. Therefore, the stained nuclei that were observed 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 of the cell cycle (Ersoy *et al.*, 2009).

Active caspase 3-positive cells were counted when co-localized with a single Hoechst stained nuclei. PKC-, active caspase 3-, and GSL1-positive cells were counted in nuclear layers within a $(212 \mu\text{m})^2$ microscopic field, in 5–6 retina sections from 5–6 eyes of different zebrafish. Whole retinas were excised from five eyes per experimental or control group of different zebrafish. The number of microglial cells was also counted in the periphery of whole-mount retinas in two-dimensionally reconstructed confocal images of a $(316 \mu\text{m})^2$ microscopic field. We analysed ten fields per retina and four to six eyes from 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.

Primary antibodies

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

1. Mouse monoclonal anti-BrdU antibody (1:400; 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, Santa Cruz, 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; DAKO, 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; 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; Santa Cruz, CA, USA) 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; BD Biosciences, San Jose, CA, USA) was used to stain caspase 3-dependent apoptotic cells.

Secondary antibodies

Goat polyclonal anti-mouse ALEXA 488- or Cy3-conjugated and anti-rabbit Cy3-conjugated antibodies (Jackson ImmunoResearch labs, West Grove; PA, USA). Secondary antibodies (1 mg/ml) were diluted 1:1000 in 3 % NGS.

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. The autofluorescence of photoreceptor segments

were observed in two-dimensional reconstructions of confocal images from Z-stacks or with the epifluorescence microscope.

Quantitative real-time polymerase chain reaction

Zebrafish were euthanized; 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 with CoCl₂ (ivc = 1.3 mM), ouabain (ivc = 3.4 μM), or saline solution at 8 hpl and with CoCl₂ (ivc = 1.3 mM) or saline solution at 72 hpl. 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 and quantified. 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 were amplified. No-RT and cDNA-containing samples were run in triplicate. Elongation factor 1- α (*efl- α*) and vascular endothelial growth factor-aa (*vegfaa*) gene expression showed no significant variations among groups and were amplified as reference genes. Data analyses were performed using the “Gene expression’s C_T Difference” (GED) method, which considered individual amplification efficiencies (Scheffe *et al.*, 2006). Amplification efficiencies for all samples were between 0.95 and 1.0. Results were also calculated by the 2- Δ CT (cycle threshold) method, which considers 100% reaction efficiencies (E = 1.0), and values were not significantly different from values obtained with GED analysis.

Statistical analyses

Data are expressed as mean values \pm 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 Tukey's post hoc multiple comparison test. For relative changes in gene expression (that is, mRNA relative levels) at 8 hpl, statistical analyses were performed using the non-parametric Kruskal–Wallis test followed by Dunn's multiple comparison test. When two groups of data were compared (that is, control vs CoCl₂-treatment at a particular time point), a two-tailed Student's *t* test was performed, and Welch's correction was applied when the standard deviations between groups were significantly different ($p < 0.05$). Statistic tests and significance levels (alpha values) are indicated in figure legends. *F*- and *p*-values for each statistical comparison were obtained with Graphpad Prism software and are indicated in the Results section.

RESULTS

Histology of the retina treated with CoCl₂ at different intervals after treatment

Morphological analyses of retina sections were performed at different intervals after a single injection of CoCl₂. Different concentrations of CoCl₂ were able to induce cell death in all, several or specific retina layers (Fig. 1). At 96 hpl, a concentration of 4.0 mM CoCl₂ caused complete cell death across the neural retina, the retinal pigmented epithelium (RPE) and the choroid layer (Fig. 1B). A concentration of 2.4 mM CoCl₂ caused damage to the retinas; however, a noticeable number of cells remained alive in the inner nuclear layer (INL) and ganglion cell layer (GCL) (Fig. 1C). A concentration of 1.3 mM CoCl₂ provoked a specific lesion of the photoreceptor layer without any apparent effect on other neural retina layers (Fig. 1D). Damage to photoreceptors was uniformly produced across the surface of the

outer retina. Moreover, a less homogenous but conspicuous damage was observed in the RPE.

Next, we analysed the morphology of the retina at different time points following a single injection of 1.3 mM CoCl₂. At 50 hpl, the photoreceptor inner and outer segments (PRS) were highly disorganized, and many of them had disappeared (Fig. 1F). Double-cone photoreceptor nuclei disappeared shortly thereafter, followed by the complete absence of single cone and then rod nuclei in the outer nuclear layer (ONL) by 96 hpl (Fig. 1G). At 25 dpl with a concentration of 1.3 mM CoCl₂, neural retina and RPE showed an advanced but incomplete degree of regeneration. Newly formed photoreceptor cell nuclei in the ONL and differentiated cone segments were observed (Fig. 1H).

CoCl₂ treatment induced caspase 3-dependent apoptosis in the photoreceptor layer

To further evaluate the effect of 1.3 mM CoCl₂ on photoreceptors, we used a specific antibody directed against active caspase 3, which labelled late apoptotic events. At all time points analysed, the majority of active caspase 3-positive cells were observed in the ONL (Fig. 2). At 28 hpl, cells labelled with active caspase 3 were mainly observed in the double-cone nuclei layer (Fig. 2D–F), whereas no active caspase 3 was detected in saline solution-treated retinas (Fig. 2A–C). At 50 hpl, the remaining cone nuclei were picnotic and their membrane segments were ripped, whereas rod nuclei remained with reduced density near the outer plexiform layer (OPL) (Fig. 2G–I). At 72 hpl, the remaining nuclei in the ONL expressed active caspase 3, whereas the INL and GCL thickness was virtually unchanged (Fig. 2J–L). A very low number of active caspase 3-expressing cells was observed in the INL.

Death of photoreceptors caused by CoCl₂ triggered progenitor proliferation

The intravitreal treatment with CoCl₂ induced mitotic activation of progenitor Müller glia and Müller glia-derived progenitors (Fig. 3). At 50 hpl, nuclei labelled with a 4-h pulse of BrdU were observed across retina sections within a single stratum in the INL (Fig. 3D–F, Q). GFAP expression extended from the feet of Müller glia in the control retinas (Fig. 3A–C) to their projections across inner layers (Fig. 3D–F). At 50 hpl, BrdU-positive nuclei co-localise with GFAP cytoplasmic stain in the vertical processes of Müller glia. At 72 hpl, numerous proliferative clusters that were also labelled with a 4-h pulse of BrdU were observed across the whole retina in the INL (Fig. 3G–I, R). GFAP-positive processes were hypertrophic. The proliferative nuclei in the outer portion of the INL were, in turn, surrounded by horizontal processes of Müller glia on the ventricular side.

At 96 hpl, outer retina layers were completely absent across the whole retina section, but no conspicuous change was observed in the INL or inner plexiform layer (IPL) (Fig. 3J–L, S). The maximum number of BrdU-positive cells was observed at 96 hpl (plot; $F = 272.4$; one-way ANOVA $p < 0.0001$). Severely damaged retinas following exposure to 2.4 mM CoCl₂ also exhibited a large number of proliferative cells distributed in clusters at 96 hpl (Fig. 3P).

We observed Müller glia reactivity in response to injury by GFAP overexpression at 28 hpl (data not shown), with a progressive increase in GFAP expression and thickening (hypertrophy) of its cellular processes and feet until at least 96 hpl. Müller glia-derived progenitor proliferative clusters observed on the fourth day after injury were limited by the inner limiting membrane (ILM) formed by Müller glia hypertrophic feet. On the outer retina, Müller glia processes anastomosed, resulting in limited proliferative progenitors in the region

that would normally occupy the OPL, which was no longer existent. At 10 dpl, many nuclei labelled with PCNA were localized in the regenerating ONL across the retina section (Fig. 3M–O, T and plot). A low number of mitotically active nuclei were observed in the INL in close proximity to GFAP-positive processes. GFAP expression was significantly reduced, and it was mostly detected in the ILM and INL outer border.

Figure 4 shows the effect of 1.3 mM CoCl₂ on synaptic layers. At 50 hpl, the OPL was completely disorganized, as shown by the downregulation of SV2 -a protein in the membrane of presynaptic vesicles- when the PRS and the ONL were also disappearing (Fig. 4E–H). At 96 hpl, the OPL was not longer detected by SV2 immunoreactivity (Fig. 4I–L). SV2 expression in the IPL was not apparently affected by CoCl₂ treatment at 50 and 96 hpl.

Specific PKC-immunoreactive bipolar cells were lost following photoreceptor cell death

BC were detected with a PKC antibody that recognized all of the subunits of this kinase (Fig. 5). At 28 hpl, no significant changes in the number of BC in the INL were observed between saline- and 1.3 mM CoCl₂-treated eyes at the time that the photoreceptor layer began to degenerate (data not shown). From 50 to 96 hpl, the number of BC showed a significant decrease of about 30% in injured retinas (plot in Fig. 5J); $F = 19.42$, one way-ANOVA $p = 0.0005$). PKC staining was undetectable in small soma BC in the INL and in the synaptic contacts of off-BC and on-BC in the IPL (Fig. 5E, F). PKC immunoreactivity detected in the Mb-type BC (rod and cone on-BC) was not affected at any interval after injury (D–I).

Effect of glutamate ionotropic receptor antagonists on CoCl₂-induced retinal damage

To better understand the mechanism of injury induced by CoCl₂, we assessed the effect of treating retinas with two different glutamate ionotropic receptor antagonists several hours before and after injury induction. This was meant as a protective treatment to avoid glutamate excitotoxicity after CoCl₂-induced damage. We found that nuclear layers detected with Hoechst in retinas treated with DNQX or APV showed a significantly improved morphology when analysed at 50 hpl. DNQX-treated retinas exhibited healthy double-cone nuclei, and the ONL itself, including single-cone and rod nuclei, was densely populated by well-formed nuclei. Moreover, the INL was also better preserved compared to the unprotected injured retinas. The APV treatment also caused strong photoreceptor layer protection, and INL integrity was almost completely preserved. INL thickness was normal, and its nuclei were well-organized. Horizontal cell nuclei were also clearly arranged at the outer border of the INL. Principally in injured retinas treated with APV, GFAP expression in Müller cells was significantly reduced and limited to the inner limiting membrane. Nuclei detected with BrdU were observed in all retina sections treated with CoCl₂, indicating a significant injury-induced increase of progenitor proliferative activity (Fig 6F–H). Nonetheless, the number of BrdU-labelled nuclei was significantly reduced in injured retinas by NMDA- or AMPA-receptor antagonist treatment (scatter plot in Fig 6; $F = 77.55$, one-way ANOVA $p < 0.0001$).

Differential expression of HIF-1 α , VEGF and VEGFR mRNA levels in the retina at two intervals after injury

To further characterise the deleterious mechanism of CoCl₂, we analysed its effect on regulating HIF-1 α , VEGF, and VEGFR mRNA levels. We also compared the effects of CoCl₂ treatment on gene expression compared to ouabain treatment. We determined HIF-1 α mRNA levels at an early interval after injury (8 hpl) in different groups of zebrafish that were injured with either 1.3 mM CoCl₂ or 3.2 μ M ouabain, whereas other groups of zebrafish were treated with saline solution. At 8 hpl, we found that HIF-1 α mRNA levels showed a significant decrease to 0.5-fold in the retinas injured with CoCl₂ relative to undamaged retinas; in comparison, ouabain-treated retinas showed a non-significant decrease to 0.8-fold (Fig. 7A; $p = 0.0241$, Kruskal–Wallis statistic = 7.448). We also assessed the relative levels of vascular endothelial growth factor ab (VEGFab) and aa (VEGFaa) mRNA, which did not show significant changes by 8 hpl for either ouabain or CoCl₂ (Fig. 7B, C; $p = 0.1133$ and $p = 0.0794$, respectively; Kruskal–Wallis statistic = 4.356 and 5.067, respectively). At 8 hpl, VEGF receptor-1 (VEGFR1) mRNA levels showed a significant decrease to 0.4-fold in retinas damaged with CoCl₂, whereas ouabain induced a non-significant decrease (Fig. 7D; $p = 0.0273$; Kruskal–Wallis statistic = 7.20). In contrast, shortly following an injury, VEGF receptor-2/4 (VEGFR2/4) mRNA levels did not show changes relative to saline solution- or ouabain-treated retinas (Fig. 7E; $p = 0.1931$, Kruskal–Wallis statistic = 3.289).

At 72 hpl with CoCl₂, relative levels of HIF-1 α mRNA in the mature region of neural retinas (without CMZ) did not show significant changes (Fig 7F; two-tailed unpaired t test with Welch's correction $p = 0.6802$). At the same interval after injury, VEGFab mRNA levels showed a significant increase of about 40-fold, whereas VEGFaa mRNA levels showed no significant changes (Fig. 7G, H; two-tailed unpaired t test with Welch's correction

p= 0.0134, p= 0.3764, respectively). At 72 hpl, relative levels of VEGFR1, VEGFR2/4 and VEGFR3 mRNA showed significant increases of 10-, 30- and 15-fold, respectively, in mature neural retinas treated with 1.3 mM CoCl₂ (Fig. 7I–K; two-tailed unpaired t test with Welch's correction p = 0.0481, p = 0.0394, and p = 0.0354, respectively). Relative levels of HIF-1 α , VEGF and VEGF receptor mRNA in injured retinas were compared with mRNA levels that were identified in undamaged, saline solution-treated mature neural retinas. An increase in HIF-1 α gene expression was expected to occur shortly after injury with CoCl₂. **In contrast, our findings indicated that CoCl₂ deleterious effect on retinal cells was likely not mediated by HIF-1 α gene induction.**

Effects on pluripotency and proneural basic helix-loop-helix (bHLH) gene expression following treatment with CoCl₂

The importance of the two pluripotency genes *ascl1a* and *lin28a* for retinal repair, which are expressed by injury-induced multipotent progenitors, has been extensively described elsewhere (Ramachandran *et al.*, 2010). We determined the relative expression of these genes in mature regions of the neural retina. We found that the relative levels of *ascl1a* and *lin28a* mRNA showed significant increases of about 70- and 3,000-fold, respectively, by 72 hpl when compared with the expression levels of these genes normally observed in uninjured saline solution-treated retinas (Fig. 8A, B; p = 0.0241, p = 0.0413, respectively, two-tailed unpaired t test with Welch's correction). Furthermore, we quantified the relative expression of transcripts of two proneural bHLH factors that are expressed in progenitors of the photoreceptor cell lineage at different stages during development and retinogenesis, as well as in the regeneration of adult zebrafish retina (Poggi *et al.*, 2005; Taylor *et al.*, 2015). The relative transcriptional expression level of *atoh7* (*atoh-7*), determined in the mature region of neural retinas, showed a significant increase of about 35-fold when assessed at 72

hpl (Fig. 8C; two-tailed unpaired t test with Welch's correction $p = 0.0240$). In contrast, the relative expression levels of the mRNA of *neurod* (D) were not affected by cobalt treatment at the early interval of 72 hpl.

The relative mRNA expression levels of two injury-induced genes—GFAP and tumour necrosis factor alpha (TNF α)—in mature neural retinas without the CMZ were significantly increased by about 30- and 15-fold, respectively by 72 hpl (Fig. 8E, F; $p = 0.0328$, $p = 0.0373$, respectively, two-tailed unpaired t test with Welch's correction).

Activation of microglia in specific regions of the mature retina and the CMZ following treatment with CoCl₂

Microglial cells were principally located in the peripheral tissue contiguous to the CMZ and around the collector circumferential vein (CV) (Fig. 9A–D). An injury-induced increase in the number and size of microglial cells was observed in the CMZ and retinal ganglion cell (RGC) differentiating area at 55 hpl (Fig. 9E–H), which temporally coincided with an increase in the proliferative activity of the CMZ (Fig. 9R; two-tailed unpaired t test $p = 0.0001$) and adjacent areas.

A significantly higher number of microglial cells were observed across retina sections in the fibre layer at 55 hpl (Fig. 9M–P and plot in Q; two-tailed unpaired t test with Welch's correction $p = 0.0294$). In contrast, BrdU-positive cell numbers did not change in the GCL or fibre layer.

Fig. 10 A–D depicts an injury-induced increase in the number of microglial cells (see the graph in Fig. 10) and the distribution area of the optic disc blood vessels and around arterial and venous peripheral capillaries in whole-mount retinas (two-tailed unpaired t test with Welch's correction $p = 0.0029$).

The morphology of retina blood vessels at three intervals after injury caused by CoCl_2

At 96 hpl with a single intravitreal injection of CoCl_2 , main artery branches (approximately 6–7 grade I arteries) emerging from the optic disc showed signs of oedema, rupture and leakage (Fig. 10H–J). Erythrocytes were tightly packed inside the swollen vessels (Fig. 10H). Capillary vessels branching at the retina periphery were ripped, and blood cells were spread in the parenchyma, which was also damaged in this zone (Fig. 10I, J). In uninjured retinas (Fig. 10E–G), grade III–V blood vessels were parallel to each other and followed straight paths. In contrast, vessels followed erratic and curvy pathways in CoCl_2 -treated retinas.

Eleven days after injury with a single dose of CoCl_2 main artery branches appeared to be repaired, and their number was slightly increased (8–9), whereas grade II ramifications occurred closer to the optic disc compared to grade II ramifications in the control retinas (Fig. 10K). At 11 dpl, capillary vessels in the retina periphery re-established their connections with the CV (Fig. 10L, M).

A three-dose CoCl_2 treatment administered at 0, 7 and 15 dpl generated severe damage to the retinal tissue (data not shown). At 20 dpl, the main optic artery exhibited 11–12 primary branches, some of which formed anastomosis loops (Fig. 10N). Peripheral blood capillaries also showed a higher degree of branching (grade VI or higher), growth tips, anastomosis bridges and tortuous trajectories (Fig. 10O, P).

DISCUSSION

This work characterized for the first time the use of CoCl_2 to cause specific photoreceptor cell death across the surface of the outer retina in adult zebrafish. Similarly to other types of damage, the intravitreal injection of CoCl_2 induced cell death and Müller glia reprogramming and the subsequent mitotic activation of Müller glia-derived multipotent progenitors.

The induction of photoreceptor cell death following CoCl_2 injury

Several active caspase 3-expressing cells were detected in the ONL at different time points after injury. Apoptotic cells and picnotic nuclei were first observed in the region occupied by double-cone nuclei, then in the region occupied by single-cone nuclei and, finally, in the internal half of the ONL, which was composed only of rod nuclei. These findings and morphological observations of the damaged tissue indicated that photoreceptors died in an orderly sequence after CoCl_2 treatment. Photoreceptors completely disappeared in the following order: 1) their segments, 2) their nuclei, and 3) their end feet, which was concordant with the way photoreceptors die in neurodegenerative eye diseases caused by ischemic, hypoxic and angiogenic disorders (Holopigian *et al.*, 1997; Kern & Berkowitz, 2015; Scarinci *et al.*, 2016). Not every cell in the dying tissue expressed active caspase 3, at least not simultaneously, which may indicate that many cells likely die through caspase 3-independent apoptotic mechanisms. It is possible that necrosis and autophagy are also involved in photoreceptor cell death (Bellot *et al.*, 2009).

Cobalt-associated mechanisms of toxicity are far from being understood. CoCl_2 might induce photoreceptor cell death by inhibiting prolyl hydroxylase activity, which regulates HIF-1 α degradation. The cellular effects triggered by CoCl_2 generate equivalent conditions as

the ones observed in *in vivo* hypoxic microenvironments, and they share identical signal transduction and transcriptional regulation pathways (Bruick, 2003; Li *et al.*, 2006; Dai *et al.*; 2012). It has been suggested that CoCl₂ also affects haemoglobin, preventing an oxygen supply from reaching cells (Maines & Sinclair, 1977). However, we have not observed HIF-1 α transcription induction at early (8 hpl) and late (72 hpl) intervals after injury. *In vitro* experiments have indicated that HIF-1 α mRNA levels are increased from 30 min to 2–3 h after hypoxia or CoCl₂ treatment and they decay significantly over time, whereas the protein content remains elevated (Li *et al.*, 2006; Chamboredon *et al.*, 2011). Our *in vivo* experiments showed that relative levels of HIF-1 α mRNA were decreased by 8 hpl following CoCl₂ treatment, and to a lesser degree following ouabain treatment. At 72 hpl following CoCl₂ treatment, HIF-1 α mRNA retina levels were variable, but on average, they were not different from levels found in non-injured retinas. The initial decrease might indicate a possible feedback mechanism of the accumulated HIF-1 α protein on its transcription, limiting the deleterious accumulation of this protein. Despite extensive attempts, we have not been able to reliably demonstrate whether CoCl₂ intraocular treatment modifies HIF-1 α protein content in the zebrafish retina at 24 hpl (data not shown). Furthermore, VEGF and VEGFR mRNA levels were not changed at 8 hpl, whereas VEGFR1 was reduced by the CoCl₂ treatment, which is also at odds with chemical hypoxia effects.

On the other hand, CoCl₂-induced cell injury might be due to its many and not completely characterized cytotoxic effects. For instance, it may be that photoreceptors die because Co²⁺ blocks Ca²⁺ entry, thereby interfering with intracellular Ca²⁺ homeostasis and signalling (Krizaj and Copenhagen, 2002; Sharf *et al.*, 2014). It has been demonstrated that Co²⁺ is internalized in cells, provoking severe oxidative stress and necrotic, as well as apoptotic, cell death (Uboldi *et al.*, 2016). In the present work, we observed that ouabain,

which blocks Na^+/K^+ -ATPases, affected relative levels of HIF-1 α and VEGFR1 mRNA similarly to CoCl_2 in a short period following injury; however, the ouabain dose we assayed principally damaged inner retina cells (Fimbel *et al.*, 2007; Battista *et al.*, 2009), indicating that their cytotoxic effects involved different mechanisms affecting different types of cells. In our assays, CoCl_2 primarily damaged photoreceptors, and cones were the most sensitive to cobalt-toxicity. In agreement with our results, intense light lesions that primarily kill photoreceptors induced downregulation of HIF-1 α transcripts in Müller glia between 8 and 36 h after light treatment (Qin *et al.*, 2009). Nevertheless, it remains unclear why photoreceptors were the main type of cell affected by exposure to 1.3 mM CoCl_2 .

CoCl_2 may also cause RPE cell death, which may in turn provoke photoreceptor death. This seems plausible given that RPE cells were injured early after CoCl_2 treatment. However, the outer and inner segments of photoreceptors were concomitantly disrupted, followed by cone and then rod nuclei disappearance. More likely, the dose of CoCl_2 that we assessed was directly deleterious to both RPE and photoreceptor cells. Cones present a higher metabolic rate and are more susceptible to hypoxic environments (Kawamura & Tachibanaki, 2008); therefore, their death might be due to CoCl_2 -induced cytotoxicity in choroid vessels, which leads to O_2 deficit. Nonetheless, whether 1.3 mM CoCl_2 kills photoreceptors via cytotoxic mechanisms, hypoxia-like mechanisms (probably indirectly) or both remains unclear.

The extensive activation of multipotent progenitors across the INL following CoCl_2 treatment

Co-localization of BrdU-positive nuclei and GFAP cytoplasmic labelling in the same cells in the INL at 50 hpl indicated that the mitotically active cells were likely reprogrammed Müller glia and some early derived progenitors. At 72 hpl, a strong increase in the number of

proliferative cells was likely due to an exponential production of rapidly dividing progenitors that originated from injury-responsive Müller glia. We observed that the injury following exposure to CoCl_2 , which targeted and killed virtually all photoreceptors, accelerated the induction and rate of progenitor proliferation activity, peaking between 72–96 hpl. In contrast, when the injury was caused with a single injection of ouabain (either at a low, medium or high dose), the Müller glia-derived progenitor proliferative peak was observed at 5–7 dpl (**Fimbel *et al.*, 2007; Sherpa *et al.*, 2008; Medrano *et al.*, 2017**).

A single intravitreal injection of ouabain at low concentrations damages inner retina cells (Fimbel *et al.*, 2007), whereas higher doses of ouabain provoke cell death across all retina layers, including RPE and choroid cells (Sherpa *et al.*, 2008; Medrano *et al.*, 2017). In contrast, injury with intense light causes photoreceptor death as the original and principal effect (Wu *et al.*, 2001; Weber *et al.*, 2013; Rajaram *et al.*, 2014). Results from our research group and from other authors suggest that the injury of photoreceptors induces a more potent damage-induced signalling, which in turn causes a faster activation of the proliferative response across the tissue.

A key fact we observed with CoCl_2 treatment was that photoreceptor cell loss was extensive and homogenous across the retinal surface in almost all sections examined in each eye. Furthermore, CoCl_2 treatment homogeneously increased multipotent progenitor proliferative activity across the whole retina surface, including the CMZ, soon after injury. Therefore, this paradigm model can be used to cause cell death in all photoreceptors in wild-type zebrafish, and it can be used as an alternative to light or laser damage. Light damage is mainly effective on albino zebrafish; very high-intensity light and several weeks of dark adaptation are required to induce damage in wild-type zebrafish retinas. Moreover, light

injury in wild-type zebrafish is restricted to localized portions of the retina. For instance, the periphery is much less affected by intense light than the central part of the retina, and photoreceptors are affected in specific patches (Weber *et al.*, 2013; Rajaram *et al.*, 2014). Previous research characterized a lesion paradigm with N-Methyl-N-Nitrosourea (MNU) in adult wild-type zebrafish retina that preferentially affected rod photoreceptors (Tappeiner *et al.*, 2013). The lesion paradigm we characterized in the present report completely eliminated cone photoreceptors first and then rod photoreceptors by 72 hpl.

Regeneration of photoreceptors

On day 10 after injury, a relatively high number of proliferating progenitors were observed, mostly in the ONL. These mitotically active cells were likely rod and cone precursors that entered the S phase or remained in the G2 phase of the cell cycle when they were detected 10 days after damage (Wu *et al.*, 2001; Lenkowski & Raymond, 2014). The number of proliferating cells was significantly higher than the proliferative activity generally detected in the ONL (rod precursors only) during normal growth. Moreover, a few dividing nuclei of injury-induced Müller glia were detected in the INL, suggesting that novel precursors in the ONL were still being generated by multipotent Müller glia.

At 25 dpl, we observed a conspicuous morphological regeneration of many cone cells, including their outer segments, in the newly formed ONL. The sequential differentiation of cone photoreceptors first, followed by rod photoreceptors, was in agreement with the formation of cells from retinal multipotent progenitors during retinogenesis (Poggi *et al.*, 2005).

Other retina layer effects caused by exposure to CoCl₂

The OPL was dismantled and the end feet of photoreceptors were severely damaged at 50 hpl, whereas the IPL thickness, which was also detected at the presynaptic level, was not significantly damaged. This further suggests that treatment with 1.3 mM CoCl₂ killed mainly photoreceptors. However, around 30% of BC were affected by injury at 50 hpl. Damage of INL cells can be ascribed to cytotoxic effects of CoCl₂, the death of photoreceptors or both. For instance, BC death, which occurs at a later interval after CoCl₂ injury, could be due to the absence of trophic signals released by photoreceptors or the impairment of function (that is, synaptic and electric activity) between outer- and inner-layer neurons. Glutamate released by dying photoreceptors might also be cytotoxic to BC (Anastassov *et al.*, 2014). In fact, almost all off-BC were affected by CoCl₂ treatment, and this cell type expresses excitatory cationic channel-associated glutamate receptors.

Horizontal cells in the INL, which form reciprocal synaptic connections with the end feet of photoreceptors, were also disrupted. Horizontal cell nuclei were affected by the time presynaptic portions of the OPL dismembered. Nevertheless, it was difficult to distinguish horizontal cell nuclei after damage because progenitors were migrating from the INL to the ONL by 72 hpl.

Glutamate activation of NMDA and AMPA/kainate receptors as a mediator of CoCl₂ cytotoxicity

Retinas that were protected against glutamate excitotoxicity, with either NMDA or AMPA-receptor antagonists before and after damage induction, showed diminished progenitor proliferation levels compared with the proliferation levels observed in unprotected injured retinas. DNQX treatment robustly protected cone and rod

photoreceptors and INL nuclei by 50 hpl. APV-treated retinas showed strongly protected nuclei in the ONL and particularly healthy and orderly distributed nuclei in the INL, including horizontal cell nuclei. These findings indicated that CoCl₂-induced second-order neuron death was probably mediated by glutamate cytotoxicity through AMPA and NMDA receptor activation. Moreover, Co²⁺-induced photoreceptor death involved, at least in part, glutamate-mediated cytotoxicity. Enhanced cell survival and improved retinal layer lamination, together with a significantly low degree of progenitor proliferation and a decreased glial reactivity to injury of Müller cells, indicated the protective nature of both antagonists of glutamate effects (Choi *et al.*, 1988). These findings suggest that progenitor mitotic activation in the INL induced by the death of photoreceptors is partially, but significantly, mediated by glutamate binding to NMDA and AMPA/kainate receptors in outer and inner retina cells. Furthermore, our findings suggest that binding of glutamate to these receptors may be crucial for CoCl₂-induced photoreceptor cell death propagation across the whole retina surface (Saade *et al.*, 2013).

Proneural gene induction with CoCl₂ at 72 hpl

Induction of the expression of the proneural bHLH transcription factor *Ascl1a* and the pluripotency factor *Lin-28* is needed for the injury-induced reprogramming of multipotent Müller glia following injury (Ramachandran *et al.*, 2010; Powell *et al.*, 2016). These gene transcripts, principally *lin28a*, are barely detected in undamaged neural retinas. We observed a significant increase in the transcriptional expression of these genes by 72 hpl, in agreement with the time of multipotent progenitor amplification. Furthermore, transcripts of the proneural bHLH factor *Atoh7* were significantly increased in injured retinas by 72 hpl. In developmental retinogenesis of zebrafish, *Atoh7* is required for RGC genesis; however, not

all Atoh7-positive cells differentiate as RGC. This transcription factor is expressed in early multipotent progenitors of other retinal neurons, including photoreceptors (Poggi *et al.*, 2005; Bassett & Wallace, 2012). Therefore, our findings indicate that Atoh7 transcriptional expression is induced early in proliferative multipotent progenitors that will primarily generate photoreceptors to replace lost cells. The transcription factor NeuroD is expressed by progenitors of photoreceptor and amacrine cells in vertebrate retinogenesis, and it induces photoreceptor genesis and differentiation in adult zebrafish retina regeneration (Taylor *et al.*, 2015). In contrast to Atoh7, NeuroD transcriptional expression—which also induces cell cycle exit to initiate photoreceptor genesis—was not yet increased in Müller glia-derived multipotent progenitors by 72 hpl, probably because it was too early to induce photoreceptor genesis in this proliferative phase.

Injury-induced microglia activation and distribution in retina layers

Microglial cell number and distribution were significantly augmented after injury with CoCl₂. However, microglia migration and proliferation were enhanced, albeit in a limited manner. For instance, microglial cells present in the fibre layer across the retina were more numerous after injury, but they remained localized in the same layer in which they were likely associated with the capillary network. Microglia migration to the IPL, INL or outer retina layers was never observed. Microglial cells detected in the ciliary epithelium before damage occupied a wider area adjacent to the CMZ and differentiating RGC region after injury. Activated microglial cells principally remained associated to areas surrounding peripheral vascular vessels. A similar situation was observed in whole-mount retinas; microglial cells occupied an extensive area around capillaries and the CV in the periphery and around the optic artery main branches of the injured retina. A recent report indicated that CoCl₂ kills microglial cells in porcine retina cell organotypic cultures (Kuehn *et al.*, 2017). *In vitro* conditions can be much more stringent than *in vivo* environments, which can trigger

effective protective mechanisms and removal of toxic agents from the eye. Microglia activation releases proinflammatory signals—such as TNF α , among others cytokines—that trigger Müller glia reprogramming and mitotic activation (Nelson *et al.*, 2013). Microglia activation and proinflammatory signal secretion might induce early mitotic activity in retinal areas adjacent to the optic disc and CMZ (at 55 hpl). It has been demonstrated that cultured RGC from rat samples release cytokines, including TNF α and several interleukins, to the extracellular medium (Lim *et al.*, 2016). Our results showed that TNF α transcriptional expression was increased by 72 hpl, supporting the idea of enhanced TNF α release and its involvement in the induction of multipotent cell proliferation, in addition to its known proinflammatory role. Nevertheless, the proliferative activity of retinal tissue might be activated by TNF α in specific zones, as well as by several other characterized extracellular signals provided by lytic photoreceptors (Battista *et al.*, 2009; Lenkowski & Raymond, 2014; Powell *et al.*, 2016; Medrano *et al.*, 2017).

Angiogenic effects of CoCl₂-induced injury

We observed that VEGFab mRNA levels, as well as VEGFR1, VEGFR2/4 and VEGFR3 mRNA levels, were enhanced by 72 hpl, indicating the activation of angiogenic signalling pathways and the regenerative angiogenic response after 3 days of inducing damage with CoCl₂.

Intraretina blood vessels were damaged by 96 hpl, indicating ischemic and hypoxic conditions. We normally observed 6–7 primary branches of the main optic artery in control zebrafish. These branches were significantly swollen and torn. Blood cells leaked out to the parenchyma due to rupture of the peripheral capillary–CV circuitry, which was observed by four days after injury with a single injection of CoCl₂ and which suggested blood supply interruption.

At 11 dpl, primary branches in the optic disc area exhibited unswollen vascular walls, a slight increase in the number of branches (8–9) and secondary branching that occurred closer to the optic disc. Neovascular-like effects were more evident at 20 dpl when the retinal tissue was severely damaged with a three-dose treatment of CoCl_2 . These effects were likely causally related to the several-fold increases in the transcripts of *vegfab* and *vegfr2/4* genes that were observed at 72 hpl. At this interval, parenchyma and endothelial cells regrew and partially recovered from injury. Main artery branches were more numerous (11–12) than in undamaged retinas, and some of them formed loops by anastomosis. Neovascular-like effects were also evident at the retina periphery where the capillaries join the CV. We found that capillary growth showed signs of neovascularization, such as enhanced branching (higher than grade VI); a tortuous morphology; and the presence of angiogenic tips and horizontal bridges between neighbouring capillaries, which formed local loops that prevented normal blood flow. Similar effects were previously described in adult zebrafish in conditions where hypoxic damage occurred following 80–90% O_2 depletion in aquarium water for several days (Cao *et al.*, 2010).

The effect of CoCl_2 treatment on angiogenic gene (*vegf* and *vegfr*) expression and intraretina blood flow interruption (ischemia) observed by 72 and 96 hpl, respectively, could not be ascribed to HIF-1 α -induction, but it was likely a consequence of Co^{2+} toxicity in endothelial cells. Indeed, early effects of cobalt (8 hpl) provoked a decrease of both HIF-1 α and VEGFR1 mRNA levels, and it did not affect VEGFab, VEGFaa or VEGFR2/4 transcription, which could be induced by enhancements in the intracellular level of HIF-1 α . Additionally, HIF-1 α mRNA levels showed no change by 72 hpl, whereas VEGF and VEGFR mRNA levels were significantly enhanced.

Damage with a regulated concentration of CoCl_2 could be appropriate for the investigation of cellular mechanisms underlying the regenerative response in the adult zebrafish retina, with an almost complete absence of differentiated photoreceptors together with damage to blood vessels. Other *in vivo* and *in vitro* models induced by CoCl_2 have been described in mammalian retinas (Hara *et al.*, 2006; Kuehn *et al.*, 2017). CoCl_2 kills inner retina and microglial cells in organotypic cultures of porcine retina. However, *in vivo* models in mouse indicated that controlled doses of CoCl_2 delivered in the vitreous cavity kill mainly photoreceptors, as we observed in the adult zebrafish retina.

Furthermore, an *in vivo* model of the adult zebrafish retina may be useful for the study of changes in the homeostatic interaction of neurons, glial cells and endothelial cells after injury (Chávez *et al.*, 2016; Ail & Perron, 2017). Finally, repeated injections of low doses of CoCl_2 caused a moderate degree of aberrant angiogenesis, which provides an alternative animal model for the study of drugs that can control this process in regenerative retinas in place of neovascularization models induced by hypoxia in the whole animal (Cao *et al.*, 2010; Wu *et al.*, 2015).

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COMPETING INTERESTS

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

MPM: conception and design, acquisition of data, initial draft, analysis and interpretation of data, manuscript revision. APF: acquisition of data, data analysis, contributed to the initial draft. PAS: acquisition of data, data analysis, contributed to the final version of the manuscript. NP: acquisition of data, data analysis, contributed to the final version of the manuscript. ROB: contributions to conception and design, data interpretation; critical review, resources. MPF: conception and design, analysis and interpretation of data, writing/manuscript preparation, resources and funding, manuscript revision, final approval of the manuscript.

DATA ACCESSIBILITY

Data analyzed in this study is available from the corresponding author on reasonable request.

CONFLICT OF INTEREST

The authors declare no competing financial interests.

ABBREVIATIONS

AMPA: α -amino-3-hydroxy-5-methyl-4-isoxazole propionate; APV: DL-2-amino-5-phosphonovalerate; ascl1: achaete-scute homolog 1 or achaete-scute bHLH transcription factor 1; atoh7: atonal homolog 7 or atonal bHLH transcription factor 7; BC: bipolar cell; bHLH: basic helix-loop-helix transcription factor; BrdU: 5-bromo-2'-deoxyuridine; **BV: blood vessel**; CMZ: ciliary marginal zone; Co²⁺: cobalt ion; CoCl₂: cobalt chloride; CV: **collector** circumferential vein; DCN: double cone nuclei; dpl: days after lesion; DNQX: 6,7-Dinitroquinoxaline-2,3(1H,4H)-dione; efl1- α : elongation factor 1 alpha; GCL: ganglion cell layer; GFAP: glial fibrillary acidic protein; GSL1: *Griffonia simplicifolia* lectin 1; HIF-1 α : hypoxia inducible factor 1 alpha; hpl: hours after lesion; ILM: inner limiting membrane; INL: inner nuclear layer, IPL: inner plexiform layer, ivc: intravitreal concentration; NMDA: N-methyl-o-aspartate; LEC: *Lycopersicum esculentum* lectin; mRNA: messenger RNA; neuroD: neurogenic differentiation bHLH transcription factor; OD: optic disc; ONL: outer nuclear layer; OPL: outer plexiform layer; PCNA: proliferating cellular nuclear antigen; PKC: protein kinase C; PRS: photoreceptor segments; **RGC: retinal ganglion cells**; RPE: retinal pigmented epithelium; SV2: synaptic vesicle 2 protein; TNF α : tumour necrosis factor alpha; VEGF: vascular endothelial growth factor; VEGFR: vascular endothelial growth factor receptor.

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FIGURE LEGENDS

Figure 1. Morphological analyses of retina sections after injury with CoCl₂.

Pictures in the upper row depict cresyl violet stain of retina sections obtained from zebrafish injected into the vitreous cavity with sterile saline solution (A), 4.0 mM (B), 2.4 mM (C) or 1.3 mM (D) of CoCl₂ and analysed 96 hours after lesion (hpl). Images in the lower row show cresyl violet stain of retina sections obtained from zebrafish injected *in vivo* into the vitreous cavity with sterile saline solution (E) or with 1.3 mM CoCl₂ and analysed 50 h (F), 96 h (G), and 25 days (H) after injury with CoCl₂. Black arrows in (B) indicate membrane vesicles containing pigmented material. Dpl: days after lesion, RPE: retina pigmented epithelium, PRS: photoreceptor segments, DCN: double cone nuclei, 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: 55 µm.

Figure 2. Analysis of caspase 3-dependent apoptotic cell death after injury induced with CoCl₂.

Confocal images of retina sections depict double labelling assays in which cell nuclei were labelled with Hoechst (blue) and apoptotic cells were detected with an antibody directed against activated-caspase 3 (red). Retinas were treated *in vivo* with saline solution or with 1.3 mM CoCl₂. Arrowheads indicate active-caspase 3-positive cells together with small picnotic nuclei in the region of the double cone nuclei (DCN) at 28 hpl (F), the single cone and the rod nuclei at 50 hpl (I) and the rod nuclei only at 72 hpl (L) in the outer nuclear layer (ONL). Arrows in (I, L) indicate active caspase-3-positive cells detected in the inner nuclear layer (INL). Hpl: hours after lesion, PRS: photoreceptor segments, OPL: outer plexiform layer, IPL: inner plexiform layer, GCL: ganglion cell layer. Scale bar: 30 µm.

Figure 3. BrdU and GFAP double immunolabelling in retina sections after damage with CoCl₂.

Confocal images of retina sections depict 5-bromo-2'-deoxyuridine (BrdU)-positive nuclei in green, except in (M) where green stain depicts nuclei expressing PCNA. BrdU was administered in the vitreous cavity 4 h before euthanasia. GFAP immunoreactivity is depicted in red, except in (T) where red labelling depicts nuclei expressing PCNA. Zebrafish were injected into the vitreous cavity with a single dose of 1.3 mM CoCl₂ or with saline solution (A-C) and euthanized at different intervals after injury. Images in (Q, R, S, T) show whole retina sections digitally reconstructed with 9-10 photomicrographs. The image in (S) depicts a whole retina section dyed with cresyl violet to reveal nuclear and plexiform layer morphology at 96 hpl. Images of retinas were obtained from groups of zebrafish euthanized at: 50 hpl (D-F, Q); 72 hpl (G-I, R); 96 hpl (J-L, S, P); and 10 dpl (M-O, T). The section depicted in (P) was obtained from a retina injured with a single dose of 2.4 mM CoCl₂. The graph shows the number of proliferative cells per retinal section at different intervals after CoCl₂ or saline solution treatment. ***p<0.001; &&&p<0.001 (vs. control) by Tukey's multiple comparison test after one-way ANOVA (n= 5 zebrafish per group). PCNA: proliferating cell nuclear antigen; GFAP: glial fibrillary acidic protein; RPE: retina pigmented epithelium, PRS: photoreceptor segments, ONL: outer nuclear layer, OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer, hpl: hours after lesion, dpl: days after lesion. Scale bar indicates 35 μm (A); 400 μm (Q).

Figure 4. Synaptic and nuclear layers, and GFAP expression in retina sections after injury with CoCl₂.

Confocal images of retina sections depict labelling of SV2 protein in presynaptic terminals at the plexiform layers (green), GFAP immunoreactivity (red) and Hoechst stain of nuclei in the same microscopic field (blue). Zebrafish were treated with a single dose of 1.3 mM CoCl₂ or

with saline solution (A-D) and euthanized at 50 h (E-H) and 96 h (I-L) after injury. Dotted lines in (J) depict the approximate location where the OPL should be found and in (K) a membrane formed by GFAP-positive projections at the outer border of the proliferating INL. GFAP: glial fibrillary acidic protein, SV2: synaptic vesicle protein. PRS: photoreceptor segments, ONL: outer nuclear layer, OPL: outer plexiform layer; PS-OPL: presynaptic outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; PS-IPL: presynaptic inner plexiform layer; GCL: ganglion cell layer; ILM: inner limiting membrane; hpl: hours after lesion. Scale bar indicates 30 μm .

Figure 5. PKC expression in retina sections following damage with CoCl_2 .

Bipolar cells detected by using a polyclonal antibody against protein kinase C (PKC)-($\alpha\beta\delta\gamma\epsilon$ subunits) are depicted in red (B-C, E-F, H-I). Cell nuclei on retina sections from saline solution- (A, C) or 1.3 mM CoCl_2 -treated eyes 50 and 96 hpl are shown in blue (D-F and G-I, respectively). 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. Scale bar: 60 μm .

The graph depicts the average number of PKC-positive cells per microscopic field of (212 μm)² in saline solution- and CoCl_2 -treated eyes, which were examined 50 and 96 hpl. ** $p < 0.01$; *** $p < 0.001$, Tukey's multiple comparison test after one-way ANOVA ($n = 4$ zebrafish). Hpl: hours after lesion; OFF-BC: off-centre bipolar cell synaptic terminals; ON-BC: on-centre bipolar cell synaptic terminals; PRS: photoreceptor segments; DCN: double cone nuclei; ONL: outer nuclear layer; OPL: outer plexiform layer; PS-OPL: postsynaptic outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer.

Figure 6. CoCl₂-injured retinas were treated with glutamate receptor antagonists before and after injury.

Confocal images depict cell nuclei in blue, and 5-bromo-2'-deoxyuridine (BrdU)-positive nuclei and GFAP immunostaining in red in retina sections, which were obtained from saline solution plus DMSO- (A, E), CoCl₂- (B, F), CoCl₂ plus APV- (C, G), or CoCl₂ plus DNQX-treated eyes (D, H). Fifty hours after lesion (hpl) with CoCl₂, zebrafish were euthanized and enucleated to prepare eyecups and perform cryosections. Zebrafish were injected *in vivo* into the vitreous cavity with 300 μM APV or 50 μM DNQX 18 h before, and 6 and 30 h after 1.3 mM CoCl₂ treatment. Control retinas were obtained from eyes that were injected with DMSO (1:500) in saline solution (mimicking antagonist treatment) and with saline solution (mimicking injury) at the same intervals indicated above. ***p<0.001; **p<0.01 (*vs.* control group); &&& p<0.001 (CoCl₂- *vs.* CoCl₂+APV- or CoCl₂+DNQX-treated groups) by Tukey's multiple comparison test after one-way ANOVA (n= 5 zebrafish per group). DMSO: dimethyl sulfoxide; APV: selective antagonist for NMDA glutamate receptors; DNQX: selective antagonist for AMPA/kainate glutamate receptors; PRS: photoreceptor segments; DCN: double cone nuclei; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. Scale bars indicate 20 μm in (A, B-D, and E-H).

Figure 7. Relative expression levels of *hif-1α*, *vegf* and *vegfr* genes at two intervals after damage with CoCl₂ or ouabain.

Eight or seventy two hours after lesion (hpl) RNA was purified from independent pools of 8 retinas each obtained from zebrafish whose retinas were treated with saline solution, 1.3 mM CoCl₂, or 3.2 μM ouabain. 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, F); *vegfaa* (B, G); *vegfab* (C, H); *vegfr1* (D, I); *vegfr2/4* (E, J); *vegfr3* (K) (as target genes) mRNA. Fold change represents the relative expression ratio from three independent pools of CoCl₂-treated retinas or three independent pools of ouabain-treated retinas, which were considered the samples of interest. Three independent pools of saline solution-treated retinas were used as the calibrator sample. The fold change value for control saline solution-treated retinas equalled 1. *p<0.05 by Dunn's multiple comparison test after Kruskal-Wallis (A-E) or by two-tailed unpaired Student's t-test with Welch's correction (F-K) (n= 3 independent assays).

Figure 8. Relative expression levels of *lin28*, *bhlh*, *gfap* and *tnfa* genes at 72 h after damage with CoCl₂.

Seventy two hours after lesion (hpl), RNA was purified from independent pools of 8 retinas each obtained from zebrafish whose retinas were treated once with saline solution or 1.3 mM CoCl₂. Complementary DNA was obtained by reverse transcription and real time quantitative PCR reactions were performed with primers for *vegfaa* (as reference gene), *ascl1a* (A), *lin28a* (B), *atoh7* (C), *neurod* (D), *gfap* (E), *tnfa* (F) (as target genes) mRNA. Fold change represents the relative expression ratio from three independent retina pools treated with CoCl₂, which were considered the samples of interest. Three independent pools of saline solution-treated retinas were used as the calibrator sample. The fold change value for control saline solution-treated retinas equalled 1. *p<0.05 by two-tailed unpaired Student's t-test with Welch's correction (n= 3 independent assays).

Figure 9. Proliferative nuclei and microglial cells in the mature retina and the CMZ after lesion with CoCl₂

Zebrafish retinas were treated *in vivo* with 1.3 mM CoCl₂ or with an equivalent volume of saline solution (A-D) and euthanized at 55 hours after lesion with the cobalt salt. Microglial cells specifically labelled with *Griffonia simplicifolia* lectin 1 (GSL1) are depicted in green,

and cell nuclei labelled with Hoechst and 5-bromo-2'-deoxyuridine (BrdU)-positive nuclei are depicted in blue and red, respectively. BrdU was injected in the vitreous cavity 4 h before euthanasia. Arrows in (B, F) show nuclei labelled with GSL1 in the ciliary epithelium, peripheral blood vessels (BV) and adjacent regions to the ciliary marginal zone (CMZ). Arrows in (J) depicts a low number of GSL1-positive cells in the GCL of saline solution-treated retinas. Arrows in (C) depict BrdU-positive cells in the CMZ and regions of differentiating retina adjacent to the CMZ. Arrowheads in (F, G) show photoreceptor segment debris. The arrowhead in (K, L) likely depicts a proliferating endothelial cell. Arrows in (H, P) indicate co-labelling of GSL1 and BrdU in a microglial cell. Dotted lines approximately delimit BV, CMZ and retinal ganglion cell (RGC) differentiating region.

Scatter plots depict the average number of microglial cells in the GC and fibre layers (Q) and BrdU-positive cells per CMZ (R) in a 16- μm thick retina section obtained 55 hpl from eyes treated with saline solution or 1.3 mM CoCl_2 . * $p < 0.05$ and *** $p < 0.001$ by two-tailed unpaired t-test with Welch's correction ($n = 4$ zebrafish per group) or two-tailed unpaired t-test ($n = 6$ zebrafish per group). Hpl: hours after lesion, PRS: photoreceptor segments, DCN: double cone nuclei, ONL: outer nuclear layer, OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer. Scale bars = 50 μm (E) and 20 μm (P).

Figure 10. Optic artery main branches, peripheral capillary network and microglial cells in whole-mount retinas after injury with CoCl_2

Microglial cells labelled with *Griffonia simplicifolia* lectin 1 (GSL1) are depicted in red. Images (A) and (C) show microglial cells that were present in the optic disc (OD) region of whole-mount retinas 72 hours after the *in vivo* treatment with saline solution or 1.3 mM

CoCl₂, respectively. Images (B) and (D) depict microglial cells in the retina periphery containing the collector circumferential vein (CV) and ciliary marginal zone (CMZ) as well as adjacent areas containing differentiating and mature retinal ganglion cells. The graph depicts the average number of microglial cells per microscopic field of (316 μm)² in whole-mount retinas. **p<0.01, two-tailed unpaired t-test with Welch's correction (n= 6 zebrafish).

Images (E-P) show in green endothelial cells labelled with *Lycopersicum esculentum* (tomato) lectin (LEL) in whole-mount retinas. Optic artery main branches are depicted in images (E, H, K, N) and peripheral capillaries and the CV are depicted in images (F-G, I-J, L-M, O-P). Retinas were treated *in vivo* with saline solution (E-G) or a single dose of 1.3 mM CoCl₂ (H-M) and analysed 96 h or 11 dpl. Images in (N-P) were obtained from whole-mount retinas treated *in vivo* with a single dose of 1.3 mM and two doses of 0.6 mM CoCl₂ that were administered one and two weeks later. Morphological analyses of blood vessels were performed 20 days after the first intraocular injection of CoCl₂. Double arrows indicate central (C) and peripheral (P) retina directions. Dotted lines delineate the CV. The big arrowhead in (N) indicates anastomosis between two main artery branches in the OD region. Small arrowheads in (O, P) signal transversal bridges formed among parallel capillaries and small arrows indicate angiogenic growth tips. Arrowheads in (P) also indicate loops formed by anastomosis between capillaries. Hpl: hours after lesion, dpl: days after lesion, PRS: photoreceptor segments, DCN: double cone nuclei, ONL: outer nuclear layer, OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer. Scale bars= 90 μm in (A) and (E).

Table 1

Gene symbol / Gene ID	Forward 5'-3'	Reverse 5'-3'
<i>efl1-α</i> (ENSDARG00000020850)	cagcagctgaggagtgatct	gtagatcagatggccggtgg
<i>gfap</i> (ENSDARG00000025301)	gagactgaggagtggatcgc	gattccaggtcacaggtcagg
<i>ascl1a</i> (ENSDARG00000038386)	acgaccctctgagtccagaa	gacatcctcccaagcgagtg
<i>lin28a</i> (ENSDARG00000016999)	tttctgtccatgaccaccg	tcagactgcgaaaaccctcc
<i>atoh7</i> (ENSDARG00000069552)	acgagaccctacagatggct	gtctgtaggccgtcgaactg
<i>neurod</i> (ENSDARG00000019566)	accacgaagggcatgaaact	acacttgtcgggtccagttcg
<i>hif1ab</i> (ENSDARG00000034293)	caggcgacgccattatctct	gataggctgagtggcagctt
<i>vegfaa</i> (ENSDARG000000103542)	gtacatcccgtcctgtgtgg	gcgatacgcggttgcttgac
<i>vegfab</i> (ENSDARG00000034700)	tgttggtggaaattcagcag	caccctgatgacgaagaggt
<i>tnf-α1</i> (ENSDARG00000009511)	tttcactccaaggctgcca	agaaaaggcctggtcctggt
<i>vegfr1</i> (ENSDARG00000019371)	ctgctgagtggaactccagg	tgcgtttgctgataatggcg
<i>vegfr2/4</i> (ENSDARG000000105215)	ccatgtgaccccagtgctaa	tcaacgttttcaatgggaagag
<i>vegfr3</i> (ENSDARG000000104453)	cagagctcagaggacgatgg	caaacggcacgcagttgtaa

















