**Supplementary file: Detailed Materials, Methods & Statistic Analysis**

**Reagents**

The anaesthetic substances propofol, midazolam and ketamine were purchased from Fresenius (Bad Homburg, Germany), B.Braun (Melsungen, Germany) and Pfizer (Ketanest S, Berlin, Germany), respectively. For adequate dosing, substances were pre-diluted in sterile water to achieve stock solutions for the various cell culture dose response experiments.

**Cell culture and treatment**

SH-SY5Y neuroblastoma cells (ATCC No. CRL-2266) were cultivated in RPMI 1640 medium (GIBCO Life Technologies, Darmstadt, Germany), Penicillin-Streptomycin, 1% glutamate and 10% FBS in standard cell culture conditions until 80 % confluence was achieved. Cells were seeded in 6-wellculture plates at a density of 3 x 105 for individual treatment 24h prior to experiments. For neuronal injury through combined hypoxia and glucose deprivation (OGD), medium was changed to glucose-free RPMI 1640 medium (GIBCO Life Technologies) and cells were transferred to an air sealed sub-culture chamber connected to separate gas supplies (Oxycycler C42, Biospherix, Lacona, USA) with automated maintenance of the desired oxygen concentration of 3.5% for 16 h. Following hypoxia, re-oxygenation without medium change was performed for 2 h before cells were harvested for analysis. For post-treatment, anaesthetics were administered at the beginning of the re-oxygenation period at the indicated concentrations. For pre-treatment experiments, substances were applied immediately prior to transfer of cells to the hypoxia chamber. For vector transfections necessary for luciferase assays, medium was changed to penicillin/streptomycin and fetal bovine serum free medium prior to transfection. Cells were cautiously detached by trypsination for flow-cytometry measurements. Experiments were repeated six times.

**TLR4 flow-cytometry**

Staining for TLR-4 surface expression and flow-cytometry was done following standard procedures for antibody incubation and flow-cytometric detection (anti-TLR4 antibody, #ab45126, Abcam, Cambridge, USA; concentration 1:100). Relative changes in surface expression were determined in relation to unstained control cells.

**Quantification of mitochondrial membrane potential**

Mitochondrial membrane potential (ΔΨm) was quantified using the Mito-Probe JC-1 Assay (Molecular Probes, Darmstadt, Germany). SH-SY5Y cells were stained with 2 µmol/L JC-1 for 15 min prior to cell collection after OGD or treatment as indicated in the individual sets of experiments. Cells were analysed using flow-cytometry. The red fluorescent signal was recorded in FL2, whereas the green fluorescence was recorded in FL1. Mitochondrial depolarization is indicated by a decrease in the ratio of red/green fluorescence, therefore the ratio between red and green fluorescence intensity was calculated (FL2/FL1 ratio).

**Lactate dehydrogenase (LDH) release assay**

LDH released from SH-SY5Y cells was analysed in cell culture supernatants using a LDH release detection assay (Cytotoxicity Detection Kit, Roche Diagnostics, Mannheim, Germany). 50 μl of the reaction mixture containing the LDH assay catalyst and the dye solution were added to 50 μl of cell culture supernatants and incubated for 15 min at room temperature. The absorbance at 490 nm was measured on a plate reader (Spectramax plus384, Molecular Devices, Biberach, Germany) using the reference wavelength of 690 nm. LDH release of treated cells was compared to LDH release of untreated cells. Relative results are expressed as the fold change versus naïve. Cells treated with Triton X-100 served as positive controls.

**NF-**κ**B p65 and HIF-1α DNA-binding ELISA**

DNA-binding activity of NF-κB p65 and HIF-1α were analysed using a DNA-binding ELISA following the manufacturer’s instruction (NF-κB p65, HIF-1α TransAM Kit, ActiveMotif, Rixensart, Belgium). OD at 450 nm was analysed on a plate reader (Spectramax plus384) to determine DNA-binding activity of NF-κB and HIF-1α.

**Luciferase gene expression assay**

Cells were transiently transfected with a dual luciferase reporter gene construct of inducible firefly luciferase under control of the NF-κB transcriptional response element and a construct with constitutive renilla luciferase expression (Cignal NFκB Reporter Kit, Hilden, Germany). Transfections were performed using Lipofectamin 2000 (Invitrogen, Darmstadt, Germany). Cells were transfected 24 h prior to treatment with anaesthetics or OGD and were harvested using a commercial lysis buffer (ReporterLysis Buffer, Promega, Mannheim, Germany). Positive controls were obtained by incubating cells with PMA (15 ng.ml-1) and Ionomycin (1 µg.ml-1) for 16 h. Luciferase activity was measured using a dual-luciferase assay system (Dual-Glo Luciferase Assay System, Promega) on a microplate luminometer (EG & G-Berthold, Bad Wilbach, Germany) by measuring light emission over an interval of 10 s. Results were normalized to the renilla luciferase activity measured in the same well and were expressed as relative luminescence units [RLU].

**Animals**

Adult male and female Sprague-Dawley rats (1:1, 280-350 g body weight, Charles River, Sulzfeld, Germany) were fed with standard rodent diet *ad libitum* while kept on a 12-h light/12-h dark cycle. All procedures involving animals concurred with the statement of the Association for Research in Vision and Ophthalmology for the use of animals in research and were approved by the Committee of Animal Care of the University of Freiburg (Permit No. 35-9185.81/G-14/123, Chairperson Dr. Schwarzmeier) on January 28 2015. All types of surgery and manipulations were performed under general anaesthesia with isoflurane/O2 for retrograde labelling with fluorogold or a mixture of intraperitoneally administered ketamine 50 mg.kg-1 (Ceva-Sanofi, Germany) and xylazine 2 mg.kg-1 (Ceva-Sanofi) for the ischemia and reperfusion experiments. Body temperature was maintained at 37 ± 0.5°C with a heating pad controlled by a rectal thermometer probe. After surgery, buprenorphine (50 µg.kg-1; Essex Pharma, Germany) was applied subcutaneously to treat pain. While recovering from anaesthesia, the animals were placed in separate cages and gentamicin ointment (Refobacin®; Merck, Darmstadt, Germany) was applied on ocular surfaces and skin wounds. The number of animals used for retinal ganglion cell (RGC) quantification and molecular analysis was 6 per group.

**Retrograde labelling of RGC**

Rats were anaesthetised with isoflurane, placed in a stereotactic apparatus (Stoelting, Kiel, Germany) and retrograde RGC-labelling was done as described previously 7 days prior to retinal ischemia.[29](#_ENREF_29)

**Retinal ischemia/reperfusion injury (IRI) and treatment with propofol**

Rats were anaesthetised intraperitoneally with xylazine and ketamine. To evaluate the effect of propofol on neuronal damage, animals were randomised to receive treatment with propofol or PBS immediately following retinal reperfusion. Retinal IRI was performed as described previously.[30](#_ENREF_30) Briefly, the anterior chamber of the left eye was cannulated with a 30-gauge needle connected to a reservoir containing 0.9% NaCl. Intraocular pressure was increased to 120 mm Hg for 60 minutes and ocular ischemia was confirmed microscopically by interruption of the retinal circulation. Reperfusion was initiated by removing the needle tip promptly. Rats without immediate recovery of retinal perfusion at the end of the ischemic period or those with lens injuries were excluded from the investigation, since the latter prevents RGC death and promotes axonal regeneration.[31](#_ENREF_31) Propofol (20 mg.kg-1 per hour) was administered intravenously via a tail vein catheter (Gauge 26) for 4 hours. In order to achieve a higher flow rate propofol 1% was diluted with sodium chloride 0.9% to a final concentration of 5 mg.ml-1.

**RGC quantification**

Animals were euthanized by CO2-inhalation 7 days after ischemia. Retinal tissue was immediately harvested, placed in ice-cold Hank´s balanced salt solution and further processed for whole mount preparation and RGC quantification as described previously.[32](#_ENREF_32)

**Western Blot**

Retinal tissue was harvested 24 h after ischemia and the retinal layer was lysed in TRIS-Buffer. Equal amounts of protein were separated on a 13% sodium dodecyl sulfate polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA), and the membranes were blocked with 5% skim milk in Tween20/PBS and incubated in the recommended antibody dilution (cleaved Caspase-3 #9665, Caspase-3 #9664, Cell Signaling Technology, Danvers, USA) overnight at 4°C. After incubation with a horseradish peroxidase-conjugated anti-rabbit secondary antibody (GE Healthcare, Freiburg, Germany), proteins were visualized using the ECL plus Chemiluminescence Kit (GE Healthcare). Densitometries are expressed as the fold change in the IRI ±propofol retina vs. the individual, non-ischemic control retina

**RT-PCR**

Retinal tissue was harvested 24 h after retinal ischemia. For RT-PCR, the retinal layer was dissected and homogenized in RNA-stabilizing Buffer. Homogenates were further processed via spin-column purification (RNeasy Mini Kit, Qiagen, Hilden, Germany) and gene expression for TLR-4 and NF- κB was analysed by RT-PCR as previously described.[33](#_ENREF_33) The following TaqMan Probes were used (Life Technologies, Darmstadt, Germany): 1. TLR-4: #Rn00578225\_m1, 2. NF- κB: #Rn01399565\_m1, 3. Caspase-3: #Rn00563902\_m1, 4. GAPDH: #Rn01775763\_g1.

Gene of interest expression was normalized against GAPDH. Data are expressed as the fold change in the IRI ±propofol retina vs. the individual, non-ischemic control retina.

**Statistical analysis**

All data are graphed as boxplots with median, 1st and 3rd quartiles and max/min for whiskers. In the text, mean ±SD is used. The following stepwise approach was used to test the statistical assumptions for ANOVA testing: 1. Distribution: normality was tested using Shapiro-Wilk test. 2. Homogeneity of variances: homogeneity of variances was evaluated by Brown-Forsythe test. All p values for Brown-Forsythe test were >0.05 except for 1B, 2B, 3B and 3C. When p values were <0.05 for Brown-Forsythe, we relied on the robustness of ANOVA against inequality of population variances in the case of equal samples sizes. 3. Independence: independence of observation was given per study design, since no repeated measurements on the same sample or animal were done and experimental repetitions were true replications.

Data with normal distribution were compared using one-way ANOVA (α=0.05) for between-group comparisons with post hoc Bonferroni multiple comparison. Each ANOVA was run across the whole dataset per experiment. Multiplicity adjusted (exact) p values are reported for all groups tested statistically different. For groups reported “not statistically different” the exact p value is provided. In addition, the 99% confidence interval of differences is reported for comparisons with p values between 0.05 and 0.15. Non-parametric Kruskal Wallis one-way ANOVA on ranks with post hoc Newman-Keuls test was used for data with lack of normal distribution. Kruskal Wallis ANOVA on ranks with post hoc Newman-Keuls test was used for: 1C, 2B, 4A and 4B.

For the *in vivo* studies, we wished to detect a 25% decrease in RGC death by propofol intervention. Assuming an expected SD of 10% in RGC density counts from our previous usage of this technique and based on previously published data and power analysis,[32](#_ENREF_32),[34](#_ENREF_34)an a priori power analysis (α=0.05 with two-sided hypothesis, power 80%) indicated that a sample size of six animals per group would be sufficient to detect such a difference. One-way ANOVA with post hoc Bonferroni test was used for between-group comparison of the RGCquantificationdata. Two groups (RT-PCR data) were compared using unpaired, two-tailed t-test. Data were analysed with a computerized statistical program (GrapPad Prism 6.0, GraphPad Software). *P*<0.05 was considered statistically significant.

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