EBERHARD KARLS UNIVERSITÄT TÜBINGEN
MEDIZINISCHE FAKULTÄT
Universitätsklinikum Tübingen

Aptamer-based neuroprotection in retinal oxidative stress

José Hurst, Agnes Fietz, Isabell Schleicher, Sven Schnichels
Universitäts-Augenklinik Tübingen, Elfriede-Aulhorn-Str. 7, Tübingen
Contact: jose.hurst@med.uni-tuebingen.de

Introduction

Diseases such as glaucoma, retinitis pigmentosa, age-related macular degeneration (AMD), and retinal ischemia lead to degenerative processes that result in vision loss and blindness. Apoptosis is the ultimate cause of photoreceptor and retinal pigment epithelial cell loss. Oxidative stress has a causal relationship with the pathogenesis of glaucoma, AMD, and RP. The neurotrophic factor BDNF and its receptor TrκB are promising targets that support neuron survival and promote the growth and differentiation of new neurons and synapses. However, the use of BDNF as a therapeutic application is limited due to its short half-life and side effects. The aim of this project was to test the neuroprotective effect of an aptamer as a partial agonist of TrkB on retinas damaged by oxidative stress.

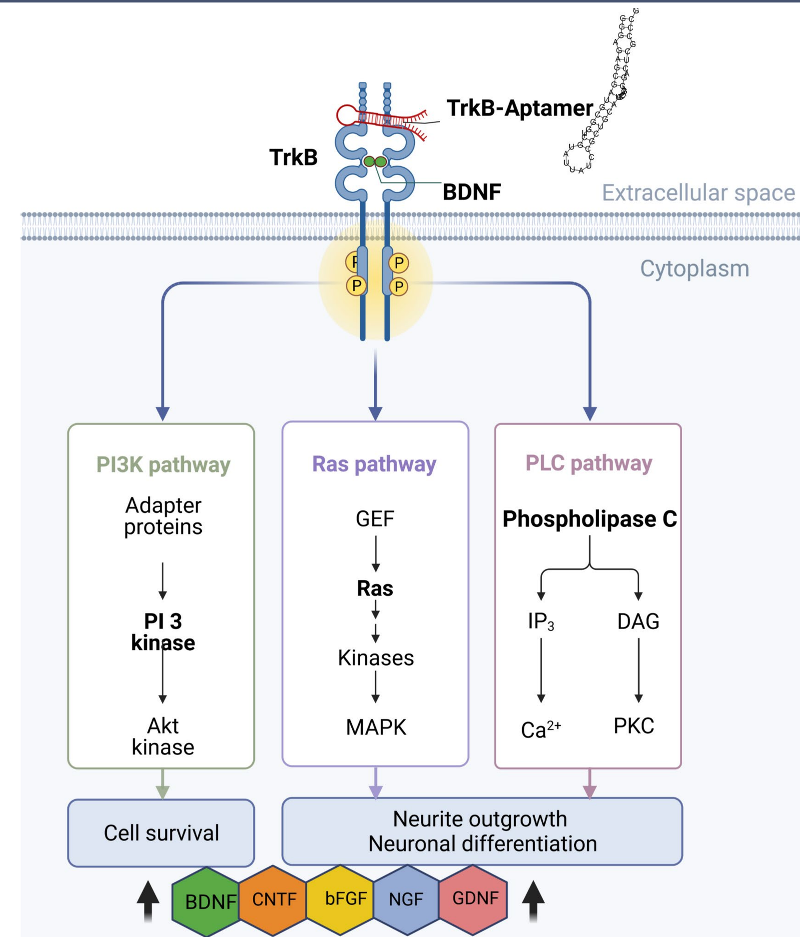

Figure 1: **Mechanism of Activation**. Binding of the TrκB-aptamer or BDNF to the TrκB-receptor induces dimerization, which in turn leads to autophosphorylation of the intracellular membrane domains. This in turn activates three main signaling pathways, including PI3K, Ras and the phospholipase C-pathways. Activation of these pathways result in expression of the growth factors GDNF, NGF, BDNF, CNTF, and bFGF. The last two in particular are very important for photoreceptor survival.

Material & Methods

Isolation and cultivation of porcine retinal explants: After removing the cornea, lens and vitreous a clover leaf-like structure was generated. Using a dermal punch (Ø = 8mm) the retinas were pierced in a circular fashion and transferred into a petri dish containing Neurobasal-A medium (Thermo Fisher Scientific). The retinal explants were carefully removed and placed on a 12-well plate with Millicell culture inserts (Merck, with pore size of 4µm) containing 100µl of retina culture media per insert and 1ml per well with the ganglion layer (GCL) facing up. Explants were cultivated at 5% CO_2 and 37°C. Every second day 80% of the media was replaced. **$CoCl_2$ degeneration model:** porcine retinal explants were stressed with 300µM $CoCl_2$ to induce degeneration for 48h and treated afterwards with the TrκB- aptamer or BDNF for another 48h. **Blue light exposure:** Retinal organ cultures were exposed to BL from above with an intensity of 30mW/cm² (455-465nm) and further cultivated. **qRT-PCR:** RNA isolation and cDNA synthesis of porcine retina explants were performed according to the manufacturer's instructions with a MultiMACS cDNA Kit (Miltenyi Biotec). For specific primer design, Primer3 software, based on the published GenBank sequence (GenBank: *sus scrofa* taxid:9823), was used. RT-qPCR was carried out (CfX 96 System, BioRad Laboratories) using the SYBR Green SsoAdvancedTM Mastermix (Bio-Rad Laboratories). The relative expression of the target genes was expressed as the fold changes in gene expression. The expression levels of the target genes were normalized against the housekeeping genes *Actin-β* and *RLP4*. **Western Blot:** 15µg samples were separated performing gel electrophoresis with Mini-Protean precast gels. Proteins were transferred to Cytiva Amersham Protran Supported NC Nitrocellulose membrane (Fisher Scientific). Membrane was blocked with Intercept Blocking Buffer (LI-COR), incubated with primary Antibodies (Blocking Buffer / 1% Tween) over night at 4 °C. After washing (TBS-T), membranes were incubated for 2h at RT with secondary antibodies (LI-COR) in Blocking Buffer / 1% Tween. Images were generated by Odyssey Fc Imaging System (LI-COR Biosciences). The expression levels of the target proteins were normalized against the housekeeping protein β-Actin.
Statistics: ANOVA and Kruskal-Wallis-post-hoc-test were used to analyze differences between groups. Statistical analysis was performed using Graphpad PIRSM. For all statistical tests, significance with respect to the control group is indicated using the following symbols and significance levels: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Shown are mean values ± SEM.

Results

Specificity of the TrκB-aptamer

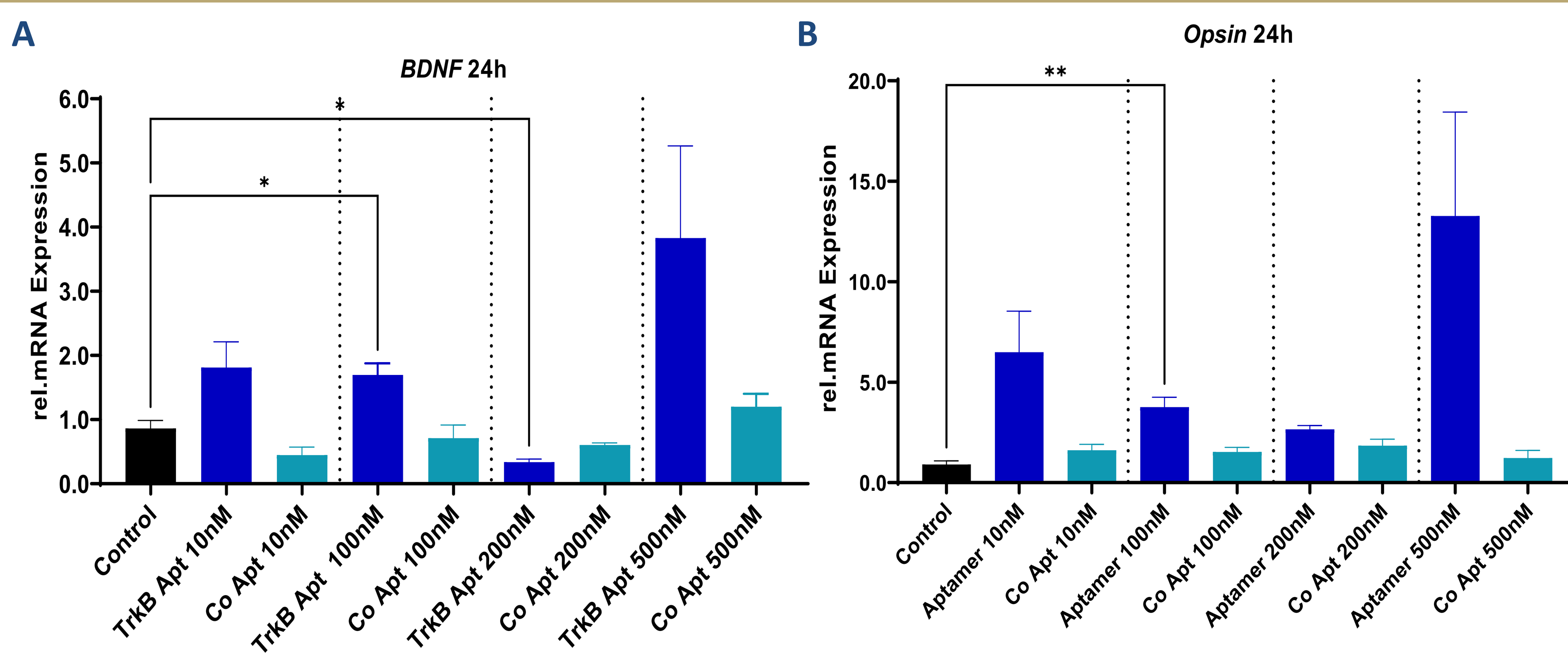

Figure 2: mRNA expression in retinal explants after 24h. **A** *BDNF* mRNA expression was analyzed as it represents the central growth factor. An increase (2- up to 4-fold) was observed in retinal explants through incubation with the TrkB-aptamer (TrkB Apt), whereas the control aptamer (Co Apt) did not have any influence. **B** mRNA expression of *opsin*, one of the cellular markers for cones, was also investigated. Again, a significant increase in mRNA expression was achieved by the TrkB-aptamer, which dose-dependently ranged from 2.5-fold (200nM) to 15-fold (500µM).

Comparison BDNF vs. TrκB-aptamer

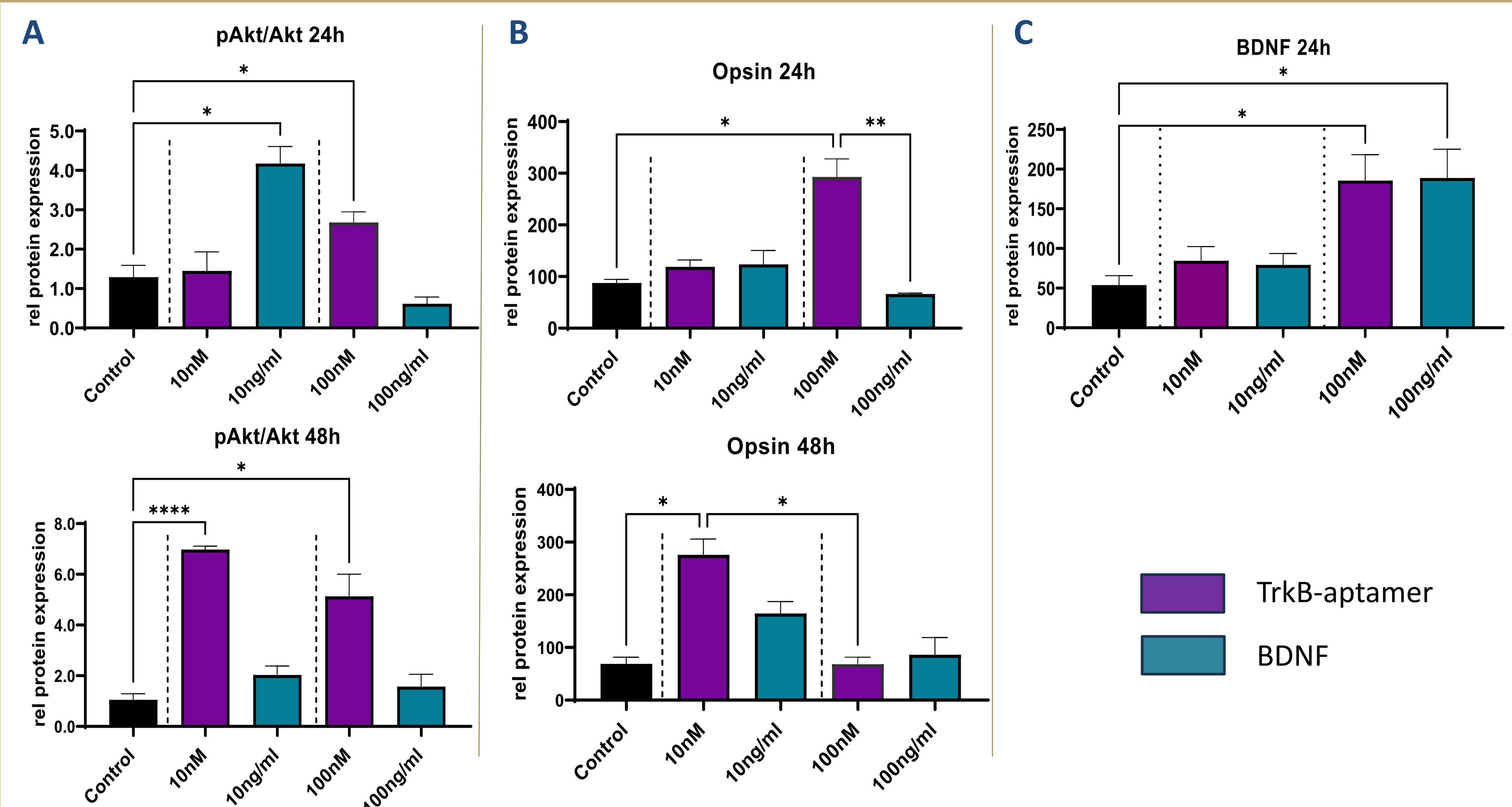

Figure 3: Retinal organ cultures were incubated with TrkB-aptamer (10nM; 100nM) or BDNF (10ng/ml; 100ng/ml) and further cultivated for 24h and 48h. **A** Phosphorylation of Akt is a clear indication of the activation of the TrkB-signaling pathway. Significantly more phosphorylated Akt (pAkt) protein expression was detected after 24h in the BDNF (10ng/ml) and in TrkB-aptamer (100nM) groups. Interestingly, after 48h the strongest increase in the 10nM TrkB-aptamer group was observable, while the effect of BDNF was no longer present. **B** Protein expression of Opsin was significantly induced due to Aptamer treatment after 24h and 48h and barely due to BDNF. **C** A significant increase of BDNF by the TrkB-aptamer (100nM) and by BDNF (100ng/ml) itself was detected after 24h.

Neuroprotective effects of the TrκB-aptamer after blue light

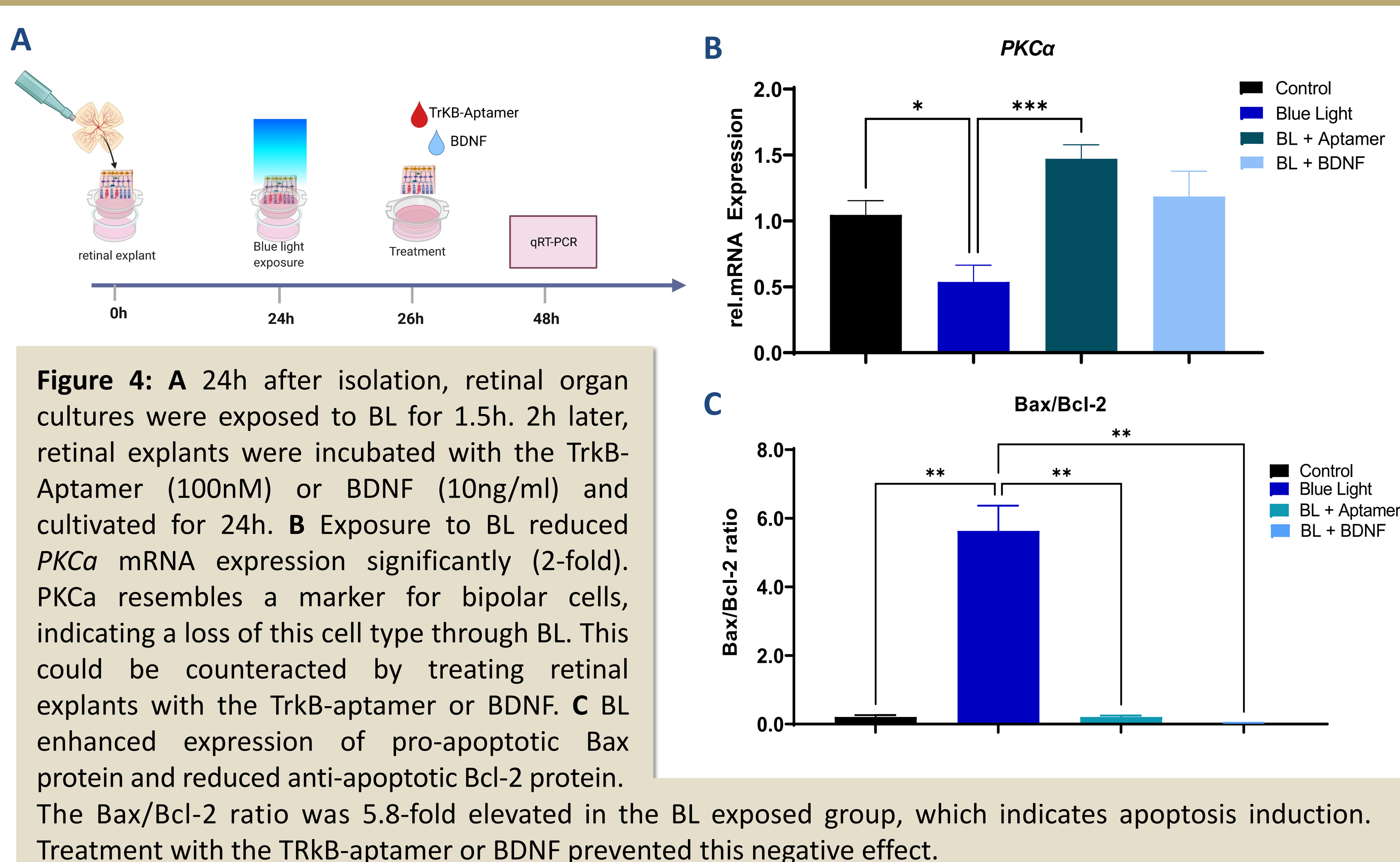

Figure 4: **A** 24h after isolation, retinal organ cultures were exposed to BL for 1.5h. 2h later, retinal explants were incubated with the TrkB-Aptamer (100nM) or BDNF (10ng/ml) and cultivated for 24h. **B** Exposure to BL reduced *PKCα* mRNA expression significantly (2-fold). PKCα resembles a marker for bipolar cells, indicating a loss of this cell type through BL. This could be counteracted by treating retinal explants with the TrkB-aptamer or BDNF. **C** BL enhanced expression of pro-apoptotic Bax protein and reduced anti-apoptotic Bcl-2 protein. The Bax/Bcl-2 ratio was 5.8-fold elevated in the BL exposed group, which indicates apoptosis induction. Treatment with the TRkB-aptamer or BDNF prevented this negative effect.

Neuroprotective effect of the TrκB-aptamer after $CoCl_2$ induced damage

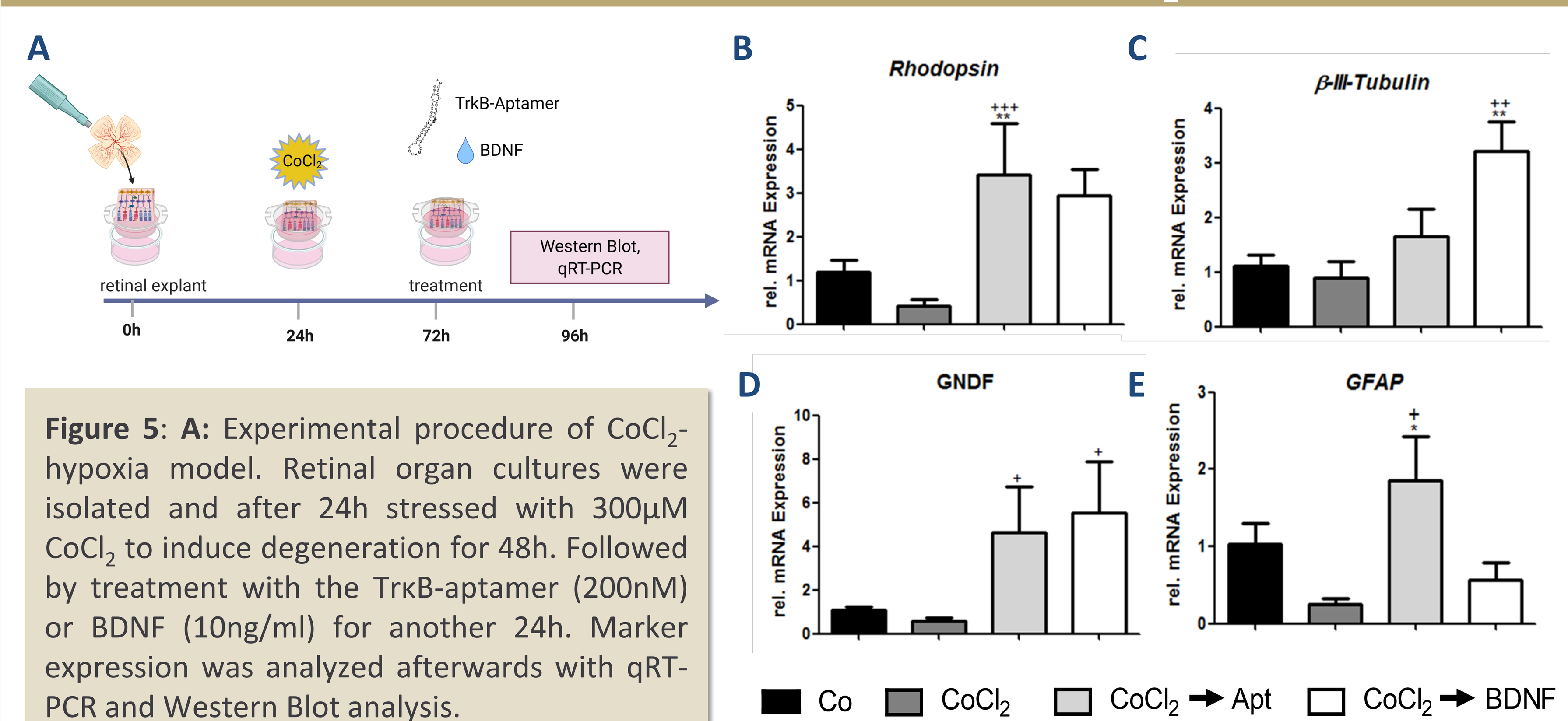

Figure 5: **A:** Experimental procedure of $CoCl_2$-hypoxia model. Retinal organ cultures were isolated and after 24h stressed with 300µM $CoCl_2$ to induce degeneration for 48h. Followed by treatment with the TrκB-aptamer (200nM) or BDNF (10ng/ml) for another 24h. Marker expression was analyzed afterwards with qRT-PCR and Western Blot analysis.

Figure 5: **B** $CoCl_2$ damage was confirmed by a 2.5-fold decrease in *Rhodopsin* mRNA. Treatment with the TrκB-aptamer resulted in 3.6-fold increase of *Rhodopsin* mRNA. Similar results were obtained by subsequent treatment with BDNF (3-fold increase). **C** $CoCl_2$ damage led to a 1.4-fold reduction in *TUBB3* mRNA expression, standing for a reduced amount of retinal ganglion cells. This was counteracted by the TrκB-aptamer (+1.5-fold) or BDNF treatment (+3-fold). **D** $CoCl_2$ reduced *GDNF* mRNA by 2-fold. Treatment with the aptamer or BDNF neutralized this effect significantly. **E** After $CoCl_2$ treatment, *GFAP* mRNA expression decreased 4-fold, probably due to the loss of Müller cells. The neuroprotective effect of the TrκB-aptamer was also demonstrated (1.8-fold increase in *GFAP* mRNA expression). "→" subsequently; "+" with respect to $CoCl_2$,"*" with respect to control.

Summary & Conclusion

- The TrκB-aptamer activated specific and dose dependently TrκB downstream targets
- A longer lasting effect of the TrκB-aptamer compared to BDNF treatment was observed
- Neuroprotective effects of the TrκB-aptamer after BL induced degeneration was demonstrated
- Neuroprotective effects of the TrκB-aptamer on $CoCl_2$ damaged retinas was confirmed

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