Glaucoma is one leading cause of irreversible blindness worldwide, characterized by progressive loss of retinal ganglion cells (RGCs) and damage to the optic nerve head. Elevated intraocular pressure (IOP) is the major risk factor of glaucoma and also the only risk factor that can be targeted and treated in current clinical practice. However, as the underlying pathogenesis of glaucoma remains elusive, controlling IOP is not enough to halt the progress of the loss of visual function in a significant proportion of patients. Besides elevated IOP, aging is an important risk factor with the incidence of glaucoma increasing significantly in the aged population. Molecular processes leading to this age-related RGC susceptibility to neurodegeneration are largely unknown.

Crystallins might play a pivotal role in this context. Crystallins, comprised of the alpha-, beta-, and gamma subclasses in all vertebrate classes, are the predominant structural proteins in the lens, evolutionarily related to stress proteins. More recently, crystallins were found to be abundant in the retina. They are associated with neurodegenerative diseases within the retina, such as ischemia–reperfusion injury, glaucoma, and diabetic retinopathy.
Besides that, mutations of crystallins are related to age and neurodegenerative diseases, including Parkinson’s disease, Alzheimer’s disease, multiple sclerosis, and Huntington’s disease.17–19

We have shown that expression of the crystallin proteins within the retina is upregulated in response to elevated IOP. Furthermore, we could show that this reaction is age dependent. Alpha-crystallins show a high homology with small heat shock proteins (HSPs) and tend to function as distinct anti-apoptotic regulators, with a chaperone-like activity upon external stimuli. Alpha-B crystalline has been shown to be neuroprotective.20–22 Beta- and gamma-crystallins form their own superfamily and have shown neuroprotective and neuroregenerative properties on retinal neurons both in vitro and in vivo, whereas the underlying mechanism cells and interaction partners remain unclear.24–26

Increased levels of α-crystallins have been found in glial cells in several models of neuroinflammation13,27,28 in association with activation of astrocytes. It is assumed that α-crystallins regulate astrogliosis in response to inflammatory signals.29,30 Interestingly, crystallins of the β/γ superfamily are reported to upregulate the expression of both CNTF and STAT3 in the retina, in cultures, and in vivo.31,32 However, the exact mechanisms are still unknown.

Müller glial cells (MGCs) have a fundamental role for the maintenance of retinal homeostasis and their interaction with most other retinal cells is well documented.33 Müller cells are not only essential for structural reasons but also for the role they have in the complex molecular network of activation/response of the sensory neuroretina under stressful conditions as it occurs in traumas or diseases characterized by the cascade of inflammation.34 In response to injury or disease, Müller cell’s stress reaction is primarily thought to be protective through secretion of growth factors and regulation of other pro-survival effects.35 Via secretion, retinal Müller cells act as an endogenous source of neurotrophic factors, such as ciliary neurotrophic factor (CNTF) or brain-derived neurotrophic factor (BDNF).35,36 Although there is a link that crystallins affect gliosis and play a role in several models of neuroinflammation including glaucoma, there exact role of action has not been described.

Therefore, we aimed to analyze the age-dependent expression of specific crystallins, unravel the neuroprotective potential of the crystallins in more detail by understanding their distribution in Müller cells, and their potential mode of action.

METHODS

Animal Treatment and Ethical Statement

All experiments were conducted in accordance with the Association of Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. The study was approved by the ethics committee of animal research Rhineland-Palatinate (permit number: 14-1-085). Exclusively female Sprague Dawley rats (n = 22) were used in this study. All animals were housed in the Translational Animal Research Center (TARC) of the Johannes-Gutenberg University Mainz. The animals are kept in groups and provided with litter, nesting material, and nest boxes under a regular 12-hour day-night cycle. Food and water were provided ad libitum. The animals are examined daily for changes of the eye and abnormal behavior by trained staff members of the TARC. During all interventions, minimizing pain and indisposition for the animals was at highest priority. In total, 26 rats were included in this study. Different aged animals aged 10 days, 12 weeks, and 44 weeks at the time of the first intervention were used (10 days: n = 7, 12 weeks: n = 7, and 44 weeks: n = 7). Another 5 animals at the age of 8-weeks were used for in vitro retinal culture.

Experimental Glaucoma Induction and Intraocular Pressure Determination

The experimental glaucoma model was carried out through episcleral vein occlusion (EVO) of only the left eyes to create a constant IOP elevation for period of 7 weeks, the right eyes of the animals were left untouched and used as control. The EVO technique in rats was first described by Shareef et al. in 1995. In brief, the animals were under general anesthesia, with intraperitoneal Ketamin (Ketamin Inresa, 100 mg/kg; Inresa Arzneimittel GmbH, Germany) plus medetomidine hydrochloride (Xylazin, 10 mg/kg; Dorbene, Zoetis, Germany) injection. Oxybuprocaine eye drops (Novesine, OmniVision, Germany) were applied topically onto the ocular surface. The conjunctiva and tenon was opened carefully and three of the five episcleral vein-trunks were cauterized using a medical cauteryization device (Bovie Medical Corporation, USA). After the surgery, novaminsulfon (Novalgin, Ratiopharm, Ulm, Germany) was added to water tanks to minimize postoperative pain for the following 3 days. The IOP was measured right before performing EVO and on a weekly basis postoperatively using a Tonolab device (iCare, Finland). The animals were awake and not anesthetized during the IOP measurements. Topical anesthesia was applied (Novesine, OmniVision, Germany) and only slightly fixated through handhelding. Ten IOP readings were taken per measurement and subsequently averaged. Animals with fluctuating IOPs or no signs of IOP elevation were excluded from the study (10 days: n = 2, and 44 weeks: n = 2).

Preparation of Retinal Explants

Five Sprague-Dawley rats were euthanatized by CO2. Eyes were enucleated immediately postmortem and transferred to a petri dish containing ice-cold betaisodona solution (Braunol, Braun, Germany) for 3 minutes. The eyes were then dissected in ice cold sterile Hank’s Balanced Salt Solution (HBSS; Gibco BRL, Eggenstein, Germany). The anterior segment of the eye was detached, and the intact retina was separated from the optic cup and flat mounted on Millipore filters (Millipore; Millicell, Cork, Ireland), with the ganglion cell layer facing upward. Vitreous body was removed carefully subsequently.

Retinal Sample Preparation for Liquid-Chromatography Electrospray Ionization Mass Spectrometry

For protein extraction, retinal tissue was shock frozen with liquid nitrogen and disintegrated to powder in a mortar. The samples were incubated in lysis buffer (0.5% n-dodecyl-β-D-maltoside in 20 mM Tris-buffered saline) at 4°C for 1 hour and another hour in ultrasonic bath on ice to ensure a maximum of cellular breakdown. Protein concentration of each sample was determined by standard bicinchoninic acid
(BCA) protein assay kit (Pierce, Rockford, IL, USA) following the instruction of the manufacturer. From each retinal elute, 80 μg of protein was loaded onto a NuPAGE Novex 12% Bis-Tris Protein Gel (Thermo Fisher, USA) and was subjected to polyacrylamide gel electrophoresis (PAGE). Each gel lane was divided into 15 individual pieces and de-stained using ammonium bicarbonate (Sigma Aldrich, USA) and acetonitrile (AppliChem, Germany), reduced, alkylated, dehydrated, and digested with sequence grade trypsin (Promega, USA) at 37°C overnight. The extracted peptides were further purified by C-18 ZipTips (Merck, Germany) as per the manufacturer's protocol. The samples were then dehydrated and stored at −20°C until further mass spectrometry (MS) measurement.

**MS Measurement**

The MS measurement was conducted as previously published. The continuum MS data were collected by an ESI-ITQ Orbitrap XL-MS system (Thermo Scientific, Bremen, Germany) and searched against UniProt database with MaxQuant software version 1.5.3.30 (Max Planck Bremen, Germany) and searched against UniProt database. A target-decoy-based false discovery rate (FDR) was set to 0.01 for identification of peptides and proteins, the minimum peptide length was 6 amino acids and the minimum unique peptides were set at 2. Fold-changes of the label-free quantitation (LFQ) intensities were calculated and then extrapolated to RGCs/mm², the mean values were compared between groups. Primary antibodies used were Brn3a (C-20; 1:125; Santa Cruz Biotechnology; sc-31984). Secondary antibodies used were donkey anti-goat conjugated with Alexa Flour 598 (1:500; Life Technologies, A1507).

**Cultivation of Retinal Explants With Crystallins**

To study the neuroprotective effects of the crystallins, retinal explants of untreated rats (n = 5) were cut equally into 4 pieces and each piece was transferred into culture medium in a Lucimex dish 35 (Sarstedt, Nümbrecht, Germany). The culture medium contains Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F12; Gibco BRL, Eggenstein, Germany), supplemented with 15 mM HEPES (Sigma Aldrich, Germany), 100 U/mL penicillin, 100 μg/mL streptomycin medium with or without the addition of 200 μg/L crystallins, α-crystallin B (CRYAB), β-crystallin B2 (CRYBB2), and γ-crystallin B (CRYGB; Polyclonal Antibody, Biossua). The Lucimex dishes were then placed into a high-pressure incubation chamber and incubated pressurized by 60 mm Hg for 48 hours. The retinal tissue was cultured i, and aerated with humidified 5% CO₂, balance air, at 37°C. The home-made pressure chamber allows accurate increase of hydrostatic pressure at 37°C, with sufficient turnover of CO₂ and air, more details can be found in our previous study. The retinas explanted from the adult Sprague Dawley rat were cultivated in flat mount under different conditions. Retinal explants without cultivation served as a control. As further control groups, explants were cultivated for 48 hours with or without 60 mm Hg hydrostatic pressure. Furthermore, explants were cultivated with additional CRYAB, CRYBB2, and CRYGB for 48 hours under 60 mm Hg pressure.

**Quantification of RGCs**

RGCs in retinal explants were labeled by the brain-specific homeobox/POU domain protein 3A (Bmn3a, C-20 goat polyclonal IgG; Santa Cruz Biotechnology, USA) immunohistochemical staining. Briefly the retinal explants were washed in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde (PFA; pH = 7.4; Carl Roth), and then in 30% sucrose solution for 12 hours, subsequently snap-frozen in methyl butanal (Merck, Germany). The explants were stained as previously described. Immunofluorescent RGCs were visualized with a fluorescent microscope (Axioskop Carl Zeiss, Germany). Six pictures of different regions in the quarter of the retina were taken (20-fold magnification). Total numbers of the RGCs were countered using an ImageJ macro (ImageJ Fiji version 1.51, total = 6 counts/quadrant, and 6 retinal quadrants per group), an average value was calculated and then extrapolated to RGCs/mm², the mean values were compared between groups. Primary antibodies used were Brn3a (C-20; 1:125; Santa Cruz Biotechnology; sc-31984). Secondary antibodies used were donkey anti-goat conjugated with Alexa Flour 598 (1:500; Life Technologies, A1507).

**Immunodetection of Crystallins in Human and Rat Retina**

Immunohistochemistry staining against α-crystallin B (CRYAB), β-crystallin B2 (CRYBB2), and γ-crystallin B (CRYGB) on frozen sections of retina from human donor eyes and Sprague-Dawley rats was performed. Human donor eyes (n = 1) were obtained through the University eye clinic eye bank after full consent by family members and in accordance with the Declaration of Helsinki. Briefly, sections were incubated with PBS containing 5% goat or donkey serum for 30 minutes, prior to overnight incubation with primary antibody at 4°C. Following the incubation, sections were washed in PBS, incubated with secondary antibody for 1 hour at room temperature, washed, drained, and mounted using DAKO Paramount (DAKO Corporation, Carpenteria, CA, USA). Primary antibodies used were CRYAB (1:200; Enzo Life Sciences, ADI-SPA-223); CRYBB2 (1:200; Cloud Clone; PAF346Mu01); CRYGB (1:200; Abgent, San Diego, CA, USA, AP16201c). Secondary antibodies used were goat Anti-Rabbit IgG H&L (1:1000; Alexa Fluor 488). Immunohistochemical staining in paraffin sections of rat retina is performed using VECTASTAIN ABC AP Kits (AP, Vector Laboratories, Burlingame, CA, USA) following the manufacturer’s introduction.

**Isolation and Culture of Müller Cells From Porcine Eyes**

The ocular bulbs of pigmented pigs (3–6 months, Piétrain breed) were obtained from a local slaughter house within 1 to 2 hours after slaughtering and kept in PBS on ice. The retinae were harvested under sterile conditions, no later than 3 hours after slaughtering. The method was described in detail in a previously published study. The rationale for using pig eyes for this study was multifold. The pig eye not only has a similar size to the human eye, it also has a visual streak that is comparable to the human macula. In addition, it was important for us to use Müller cells derived from adolescent animals. The number of retinala needed to perform studies on Müller cells in vitro would have also meant using a substantial number of rats or mice to obtain these cells. We therefore decided to reduce animal usage with the additional benefits mentioned above and use adolescent pig retinae. Müller cells were isolated from dissociated retina with the Papain Dissociation Kit from Worthington (PDS LK003150L).
as per the manufacturer’s protocol. The culture flasks were pretreated with 2 μg/cm² poly-D-lysine for 1 hour and with 1 μg/cm² laminin at 37°C for 2 hours in advance. The isolated cells were resuspended in DMEM/Ham F-12 supplemented with 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin in three 75 cm² culture flasks. The first medium change took place 24 hours after isolation to remove nonadherent and foreign cells. Subsequently, the medium was replaced every 2 days. The cells were cultured for at least 7 days until they had completely differentiated and reached a confluence of over 90%, in order to be able to be passaged and used for further experiments. Immunohistochemistry staining against Müller cell marker, glutamine synthetase, and RGC marker, Brn3a, was performed to analyze the number of Müller cells in the percentage and RGCs in the culture. After 7 days in vitro (DIV), we obtained a pure Müller cell culture, which was positive for glutamine synthetase and negative for Brn3a and other retinal markers.

Detection of Endogenous Crystallins in Müller Cells

For the detection of endogenous crystallins present in Müller cells, the Müller cells were seeded in 48-well plates of 15,000 cells in each well, incubated overnight at 37°C in the incubator to allow adherence, and stained immunohistochemically the next day at a confluence of 70% to 80%. Briefly, the cells were washed 3 times with PBS, fixed with 4% PFA, permeabilized with 0.1% Triton X-100 (Sigma Aldrich) for 5 minutes and blocked with 1% bovine serum albumin (BSA; Sigma-Aldrich) and 0.05% Tween-20 (Sigma Aldrich) in PBS for 45 minutes at room temperature. The cells were incubated with primary antibodies against crystallins at 4°C overnight. Primary antibodies used were CRYAB (1:200; Enzo Life Sciences, ADI-SPA-223); CRYBB2 (1:200; Cloud Clone; PAF346Mu01); and CRYGB (1:200; Abgent; AP16201c).

After washing with PBS, the Müller cells were further incubated with HRP-conjugated goat anti-rabbit secondary antibody at room temperature. Secondary antibodies used were goat anti-rabbit, conjugated with HRP (1:800; Sigma Aldrich; 12-348). The cell nuclei are labeled by DAPI. The Müller cells and the labeled uptake crystallins were visualized with a fluorescent microscope (Axiophot; Carl Zeiss, Germany). Images through the DAPI channel and the TRITC channel were obtained in the defined position from each well.

Analyzing Crystallin Uptake Via Western Blot

After 1 hour of incubation with basic medium or with additional CRYAB, CRYBB2, or CRYGB, the Müller cells were lysed as follows. The cells were washed twice with cold PBS and incubated in 70 μL lysis buffer, 4,4'-diaminodiphenylmethane (DDM; 1% +1% protease inhibitor), on ice. After 5 minutes of incubation, the cells were detached from the bottom of the well plate. The cell lysates were sonicated for 1 minute in an ultrasonic bath on ice and then centrifuged at 1500 g for 5 minutes, the supernatants were collected for further analysis.

Protein concentration of each lysate was determined by BCA Protein Assay Kit (Pierce, Rockford, IL, USA) as per the manufacturer’s instructions. From each Müller cell lysate, 30 μg of protein was loaded onto a Novex NuPAGE 12% Bis-Tris polyacrylamide gel (Thermo Fisher, USA). The gel electrophoresis was ran using NuPAGE running buffer MES at 4°C with a voltage of 100 V and increased to 200 V after 10 minutes.

After electrophoresis, the proteins were blotted onto a nitrocellulose membrane using a wet transfer system (Bio-Rad, Hercules, CA, USA) and standard transfer buffer (with 20% methanol), a voltage of 20 V was applied for 15 minutes. Brief Ponceau red staining was performed to assess quality of the transfer as well as whole protein content. The signals of the crystallins were set in relation to the charge control and are presented in relative signal intensity.

Microarray Analysis of Müller Cells Secretome

Of the Müller cells, 45,000 were seeded into 24-well plates and allowed to grow and adhere to the surface overnight. The cells were pre-incubated with the proteins CRYAB (Abcam), CRYBB2 (Cloud Clone), and CRYGB (CUSABIO) or control medium for 4 hours. Crystallins used in the cell culture medium were with a final concentration of 1.7 μg/mL (n = 3).

After incubation, the cells were washed twice with PBS and incubated with DMEM/Ham-F12 supplemented with reduced concentration of fetal calf serum (2%) for 16 hours. This medium is referred to as being the conditioned medium. Additional control medium was collected without conditioning through the cells (n = 10 for every experimental group).

Commercially available antibodies against different growth factors, comprising BDNF, CNTF, interleukin 6 (IL-6), transforming growth factor-beta 2 (TGFB2), transforming growth factor-beta 1 (TGFB1), pigment epithelium-derived factor (PEDF), apolipoprotein E (ApoE), nerve growth factor beta (NGFB), vascular endothelial growth factor A (VEGFA), clusterin (CLU), complement Factor H (CFH), and matrix metalloproteinase 9 (MMP-9; detailed information can be seen in Table 1) were used. These factors are known to be secreted by Müller cells and have an impact on the survival...
TABLE 1. Antibodies Used in Microarray Analysis

| Antibody                                      | Distributor/Manufacturer                                      |
|-----------------------------------------------|---------------------------------------------------------------|
| Rabbit anti-human BDNF antibody               | Ab6201, Abcam, Cambridge, United Kingdom                      |
| Anti-ciliary neurotrophic factor (CNTF)        | ABIN1598530, Antibodies Online GmbH, Aachen, Germany          |
| Anti-interleukin 6 (IL6)                      | GTX79381, Enzo Life Sciences, Israel                          |
| Anti-transforming growth factor-beta 1 (TGFβ1)| ABIN2998227, Antibodies Online GmbH, Aachen, Germany          |
| Anti-transforming growth factor-beta 2 (TGFβ2)| ABIN2992615, Antibodies Online GmbH, Aachen, Germany          |
| Anti-pigment epithelium-derived factor (PEDF)  | ABIN4237861, Antibodies Online GmbH, Aachen, Germany          |
| Anti-apo lipoprotein E (ApoE)                 | ABIN258785, Antibodies Online GmbH, Aachen, Germany           |
| Anti-nerve growth factor beta (NGFβ)          | ORB11126, Biorybt, Cambridge, United Kingdom                  |
| Anti-vascular endothelial growth factor A (VEGFA)| Ab39250, Abcam, Cambridge, United Kingdom                    |
| Anti-clusterin (CLU)                         | A91250, Abcam, Cambridge, United Kingdom                     |
| Anti-matrix metalloprotease 9 (MMP9)          | ABIN1077938, Antibodies Online GmbH, Aachen, Germany         |

FIGURE 1. Working hypothesis and workflow overview.

The antibodies were diluted in PBS and spotted in triplicate on a nitrocellulose slide using a non-contact array spotter (Scienion GmbH, Berlin, Germany) to create an antibody microarray. The conditioned medium (n = 10 per group) was labeled with Dylight 649 (1:10 in PBS, Thermo Fisher Scientific, Waltham, MA, USA) for 1 hour in the dark and quenched with Tris-HCl for 1 hour. As a negative control, PBS was used. The slides were blocked with 5% BSA in 0.5% Tween-PBS for 1 hour, washed 3 times with 0.5% Tween-PBS, and subsequently incubated with the labeled conditioned medium for 2.5 hours. After washing the slides three times the microarrays were digitalized with an array scanner (Aviso GmbH, Aachen, Germany). For data analysis, spot intensity was quantified with ImagiGene 5.0 Software (BioDiscovery, El Segundo, CA, USA). Defective spots were manually excluded from further evaluation. The fluorescence intensities were then determined, with the median signal of the respective triplicates being averaged. Fold-changes of the fluorescence intensities were calculated, a cutoff of 1.5 was used to determine the significantly differentially expressed secretome, as described before.45–47 The overview of the workflow carried out in this study is presented in Figure 1.

RESULTS
Crystallin Expression in the Glaucoma Model of Chronical Elevated IOP at Different Ages

The results from the mass-spectrometric based approach were statistically evaluated with a special focus on crystallin subtypes known to have an age-related reduction in abundance and potential neuroprotective properties based on literature research and on our own previous findings. In our study, we found these crystallins alpha-crystallin B (CRYAB), beta-crystallin B2 (CRYBB2), and gamma-crystallin B (CRYGB) to be significantly more abundant in the retina of animals in response to chronically elevated IOP and changed throughout aging (Fig. 2) (Supplementary Data S2).
Crystallins Protect Retinal Neuron in Glaucoma

Figure 2. Abundance changes in alpha-crystallin B (CRYAB), beta-crystallin B2 (CRYBB2), and gamma-crystallin B (CRYGB). CRYAB, CRYBB2, and CRYGB show a clear increase in abundance within aging in response to chronically elevated IOP, the increase in the 10-day-old animals is particularly significant. The rate of the increase in all three crystallins decreases with ageing (10 days: \( n = 5 \), 12 weeks: \( n = 7 \), and 44 weeks: \( n = 5 \); vertical bars represented means ± SEM, fold-change cutoff = 2).

Neuroprotective Effects of CRYAB, CRYBB2, and CRYGB on Retinal Ganglion Cells In Vitro

The effects of CRYAB, CRYBB2, and CRYGB as being most interesting in part one were selected for this part of the study to further elucidate the mechanism of action in neuroprotection. Representative fluorescence microscopy of Brn3a staining after 48 hours of cultivation with or without elevated hydrostatic pressure conditions (60 mm Hg), and with additional CRYAB are given in Figures 3A, 3B, and 3C.

In retinal explants without cultivation (baseline), an average number of 1684/mm² was determined. A reduction in RGC numbers to 918 ± 39.5/mm² (**P < 0.0001) and an additional 60 mm Hg of hydrostatic pressure further reduced the number of RGC to 678 ± 61.4/mm² (**P < 0.0005). All three crystallins significantly improved the RGC survival against elevated pressure, whereas CRYGB showed the most significant protective effect in vitro.
FIGURE 4. Immunodetection of α-crystallin B (CRYAB), β-crystallin B2 (CRYBB2), and γ-crystallin B (CRYGB) in the retina from humans and Sprague-Dawley rats. The left panel shows expression profile of the crystallins in the human retina. The middle and right panels represent expression profile of the crystallins in the rat retina. Corresponding expression profile of the crystallins can be seen in both human and rat retina. CRYAB, CRYBB2, and CRYGB are expressed in both human and rat retina and is predominantly detected in the ganglion cell layer (GCL) and weakly noted inner nuclear layer (INL) and outer nuclear layer (ONL). In both human and rat retina, immunoreactivity for CRYGB is weaker compared to CRYAB and CRYBB2 (scale bars = 20 μm).

of incubation time further reduced the number of RGC to 678 ± 61.4/mm² (**P < 0.005). Incubating the explants with CRYGB significantly improved the RGC survival to 1519 ± 115.1/mm², CRYAB and CRYBB2 increased the survival to 1203 ± 115/mm² and 1040 ± 93.3/mm², respectively (Fig. 3D, ****P < 0.0001, ***P < 0.005, and *P < 0.05;

FIGURE 5. Detection of endogenous crystallin in primary Müller cells. Primary Müller cells were fixed and then stained immunohistochemically against CRYAB, CRYBB2, and CRYGB. Cell nuclei are marked by DAPI (blue) and endogenous crystallins are shown in TRITC channel (red). All three crystallins can be detected in Müller cells. CRYAB showed the strongest fluorescent among all and a distinguishable diffuse distribution pattern throughout the Müller cells. The endogenous CRYBB2 and CRYGB showed weaker fluorescent and are distributed in the cytoplasm.
**FIGURE 6. Intracellular uptake of crystallins in primarily isolated Müller cells from pig eyes.** (A, B) Representative microscopy images of Müller cells incubated with labeled **CRYAB**. (A) Müller cells under light microscope, **CRYAB** in the TRITC channel, and cell nuclei in the DAPI channel. (B) Merged TRITC and DAPI channels, **CRYAB** can be seen accumulated in cytoplasm around nuclei. (D, E) Representative microscopy images of Müller cells incubated with labelled **CRYBB2**. (A) Müller cells under light microscope, **CRYBB2** in the TRITC channel, and cell nuclei in the DAPI channel. (B) Merged TRITC and DAPI channels, **CRYBB2** can be seen accumulated in cytoplasm around nuclei. (C, F, G) The signal intensity of all crystallins are increased in the Müller cells incubated with the respective crystallin. The intracellular abundance of **CRYAB** and **CRYGB** in the Müller cells are both significantly increased compared to the control, whereas the abundance of **CRYBB2** in the Müller cells was not significantly changed (**P < 0.01, n = 3; vertical bars represent means ± SD; scale bar = 50 μm).
**Crystallins Protect Retinal Neuron in Glaucoma**

**FIGURE 7.** The effect of α-crystallin B (*CRYAB*), β-crystallin B2 (*CRYBB2*), and γ-crystallin B (*CRYGB*) on the secretion of growth factors by primary Müller cells (A–I). The charts represent the median signal corresponding to the proteins bound with the dye Cy5. The growth factors *CLU*, *VEGFA*, *MMP9*, *CRYAB*, *IL-6*, *CNTF*, *NGF*, and *TGFβ2* are significantly upregulated (fold-change cutoff = 1.5; *n* = 5). (J) Protein-protein interaction network (from string-db.org) of the nine genes which are related to the uptake of the crystallins (medium confidence = 0.400).

*n* = 6 quarters of retina/group; vertical bars represented means ± SD).

**Immunodetection of Crystallin in Human and Rat Retina**

To determine the localization of *CRYAB*, *CRYBB2*, and *CRYGB* in the retina, retinal samples from both human and Sprague-Dawley rats were examined. Crystallins shown similar expression pattern in both human and rat retina. *CRYAB*, *CRYBB2*, and *CRYGB* is predominantly detected in the ganglion cell layer (GCL) and weakly noted inner nuclear layer (INL) and outer nuclear layer (ONL). However, the fluorescence signal of *CRYGB* is weaker compared to *CRYAB* and *CRYBB2* (Fig. 4).
**Immunodetection of Endogenous Crystallins in Primary Müller Cells**

To detect the endogenous crystallins in Müller cells, primary Müller cells were stained 1 day after passaging, against α-crystallin B (CRYAB), β-crystallin B2 (CRYBB2), and γ-crystallin B (CRYGB) using specific antibodies and the cell nuclei were labeled by DAPI (Fig. 5). All three crystallins were detected in primary Müller cells. Endogenous CRYAB has shown the strongest fluorescent and a distinguishing diffuse distribution pattern throughout the cells, whereas the endogenous CRYBB2 and CRYGB have shown weaker fluorescence and were distributed in the cytoplasm (Scale bar = 50 μm).

**Intracellular Uptake of Crystallins in Primarily Isolated Müller Cells From Porcine Eyes**

Primary Müller cells were incubated for 1 hour with basic medium or with additional CRYAB, CRYBB2, or CRYGB labeled with Mix-n-Stain CF555 Antibody Labeling Kit (Sigma Aldrich, St. Louis, MO, USA). The Müller cells were then washed, fixed, and analyzed by a fluorescence microscope. Müller cells incubated with labeled crystallins showed red fluorescence in scattered areas within the cytoplasm, whereas all cell nuclei were marked by DAPI in blue. Merged images confirm the crystallins were uptaken by Müller cells and accumulated in small vesicles in the cytoplasm around the nuclei (Figs. 6B, 6E).

Furthermore, the uptaken crystallins by Müller cells were quantified by Western blot analysis. The crystallins CRYAB, CRYBB2, and CRYGB could be detected in the control cells as well as in the Müller cells incubated with the different crystallins. The molecular weight (MW) of CRYAB, CRYBB2, and CRYGB are 24 kDa, 23 kDa, and 21 kDa and they were detected in the Western blot at the expected MW (Supplementary Material S3). The signals of the crystallins were set relative in charge control and are given below as the relative signal intensity.

An average signal intensity of CRYAB is detected at 14.75 ± 2.14 in the control group and 23.72 ± 2.01 in the treated sample (**P < 0.01, n = 3; see Fig. 6C). In accordance with the immunocytochemistry, the signal for CRYBB2 was also weak in the Western blot. The mean signal intensity of the samples incubated with this crystallin (0.044 ± 0.015) showed a slight but nonsignificant increase in comparison to the control cells (0.028 ± 0.01, P = 0.18, n = 3; see Fig. 6F).

**Secretome Analysis by Microarray**

The fluorescence intensity of clusterin (CLU), VEGFA, MMP9, and transforming growth factor beta 2 (TGFb2) was significantly increased after incubation with CRYAB, CRYBB2, and CRYGB, TGFb2 showed a more than 15-fold increase in the cells incubated with CRYAB and CRYBB2 (Figs. 7A, 7B, 7D, 7I). Incubation with CRYGB increased the secretion of CRYAB (Fig. 7E) and PEDF is increased in both the cells incubated with CRYAB and CRYGB. CNTF was exclusively increased in cells incubated with CRYAB, whereas IL-6 and nerve growth factor (NGF) were exclusively increased in cells incubated with CRYGB. Protein-protein interaction network (from string-db.org) of the nine respective genes of the proteins which are related to the uptake of the crystallins (medium confidence = 0.400; Fig. 7J).

**Functional Enrichment in the Network**

To get better understanding and insight into the functional role of the crystallin regulated secretome, a functional enrichment analysis by the biological functions (gene ontology [GO]) classification of the differentially crystallin upregulated targets was performed. CRYAB, VEGFA, MMP9, and TGFb2 are the proteins with higher abundance in the secretome of cells incubated by all three crystallins. The top GO terms for biological functions of these proteins comprise blood vessel remodeling, trabecula morphogenesis, outflow track morphogenesis, tissue remodeling, and negative regulation of apoptotic signaling pathway (Table 2).

Some proteins were exclusively higher abundant in the secretome of cells incubated with CRYAB or CRYGB. The respective functional enrichment analyses can be found in Tables 3 and 4. The top GO terms exclusively regulated in the CRYAB network are negative regulation of amyloid fibril formation.
fibril formation, glial cell activation, and positive regulation of axon extension. In the CRYGB network, the top exclusively regulated GO terms are negative regulation of amyloid fibril formation, positive regulation of nitric oxide biosynthetic process, and negative regulation of intrinsic apoptotic signaling pathway.

**DISCUSSION**

The data obtained in this study provides further evidence for a potential age-related and also neuroprotective effect of CRYAB, CRYBB2, and CRYGB. The protective effect of crystallins could be mediated by potential endocytotic uptake and involvement of Müller cells in a precise mode of action for each selected crystalline member, respectively.

In the animal model used in this study, it leads to glaucomatous injury, going along with loss of RGCs and their axons, thinning of the retinal nerve fiber layer (RNFL) and functional changes. In the presented study, crystallins of all subclasses were found in the present study, crystallins in retinal samples of juvenile animals in response to IOP, whereas the upregulation of crystallins was less distinctive in adult animals and almost entirely lost in the aged animal group. The ability of increasing endogenous crystallin expression might therefore decrease throughout aging, correlated with an age-related increase of RGC susceptibility to IOP. CRYAB, CRYBB2, and CRYGB were mostly significantly correlated with aging.

CRYAB, CRYBB2, and CRYGB addition to culture reduced RGC loss against elevated pressure in vitro. Neuroprotective effects of CRYAB and CRYBB2 on RGCs in glaucoma animal models has been previously described, and CRYBB2 additionally also demonstrated axonal regeneration properties after optic nerve injury (ONI). Comparing staining for CRYAB, CRYBB2, and CRYGB in human and rat retina, we had a similar expression profile in human and rat retina that was found, although the human staining was of significantly worse quality. The GCL showed the most intense crystallin staining, weaker staining was found in the INL and ONL. Müller cells are the principal glial cells of the retina with an extended funnel shape, radiating from the soma in the inner nuclear layer. We showed that the crystallins are expressed endogenously in primary Müller cells.

Müller cells support the function and metabolism of retinal neurons and are active players in normal retinal function and retinal degeneration. Müller cells provide trophic substances to neurons and remove metabolic waste and can be considered a major contributor to the retinal secretome. Secreced trophic factors are key to maintain the structural and functional integrity of the retinal neurons, as well as mediating inflammatory responses in the retina. They regulate synaptic activity through neurotransmitter uptake and recycling, and providing neurons with neurotransmitter precursors. The factors secreted by Müller cells, such as CNTF, BDNF, ApoE, CRYAB, and PEDF, exert beneficial effects on survival of RGCs and neurite regeneration in vitro and may constitute effective agents for neuroprotection of RGC.

We showed that external crystallins, especially CRYAB and CRYGB, can be incorporated into the cytoplasmic space of Müller cells, which went in hand with an increased secretion of CRYAB, CRYGB, and PEDF. The most obvious increase of secretion was detected for PEDF.

PEDF is a secreted protein known as a multifunctional cytokine with important implications in morphogenesis, cell differentiation, and tissue remodeling, as well as an important regulator of neuronal survival. Although it is not neurotrophic itself, PEDF can drastically increase the potency of neurotrophins, such as fibroblast growth factor-2, CNTF, and glial cell line-derived neurotrophic factor. Furthermore, CRYAB, CRYGB, and PEDF have all shown neuroprotective potentials.

Clusterin plays a prosurvival role during cell death and confers resistance against cytotoxic agents. Upregulation of CLU is documented in neuronal injuries and degenerations, including Alzheimer’s disease, spinal cord injury, neovascular age-related macular degeneration, and retinal degeneration induced by ischemia, diabetes, and light.

VEGFA has recently been recognized as an important neuroprotectant in the central nervous system (CNS), it has been shown to influence not only the maintenance of the normal vasculature but also neuronal growth, maintenance, and survival. Within the context of the retina, the receptors for VEGFA are present in normal retinal neuronal cells and RGCs were found to be exquisitely sensitive to VEGFA depletion compared with other tissues. Depressed VEGFA levels directly impact RGC survival.

The expression of several MMPs is altered in patients with glaucoma and in experimental animal models. The MMP9 is a secreted gelatinase, which can cleave inflammatory cytokines, and may modify cytokine signaling during the injury-induced proliferation.

The secretion of CRYAB and PEDF from Müller cells is significantly increased by both CRYAB and CRYGB. It is impossible to tell if the CRYAB in the culture medium of the primary Müller cells incubated with CRYAB is the residual of added CRYAB or the actual secretion from Müller cells. The expression of CRYGB was also increased in the rod outer segments and the retinal pigment epithelium in light induced retinal degeneration, as well as in the retina upon glaucomatous injury and mechanical injury. As alpha-crystallins demonstrate a chaperone-like activity upon external stimuli and we have shown in this study the strong expression in CRYAB in primary Müller cells, it is
reasonable to assume that Müller cells regularly contain a sufficient intracellular pool of CRYAB, and is able to release it upon stress stimulus. In addition, interestingly CRYGB seems to be one of the regulator of CRYAB secretion from Müller cells.

PEDF is a multifunctional protein, besides its demonstrable neurotrophic action, PEDF also exhibits antiangiogenic activity, such as inhibition of retinal and choroidal neovascularization. In mice, murine, and human retina, a reduced PEDF level is correlated with retinal degeneration. Furthermore, CNTF is a well-known neurotrophic factor secreted by retinal Müller cells. Crystallins seem to upregulate the expression of CNTF. In this study, significant upregulation of CNTF is seen exclusively in the CRYAB group.

In the CRYAB group, NGF and IL-6 are significantly increased. NGF is a polypeptide member of the small family of evolutionarily well-conserved neurotrophins, such as BDNF and neurotrophin-3 (NT3). Increased availability of mature NGF is shown to improve RGC survival and axonal growth.

IL-6 is involved in a variety of the CNS pathologies, including injury, infection, and neurodegeneration. The effect of IL6 on neuronal health is variable. In the retina of rodent glaucoma models, IL-6 and its mRNA are upregulated surrounding the RGCs and their axons. Application of IL-6 protects cultured RGCs from pressure-induced apoptosis and promotes axon regeneration following optic nerve crush. There are also studies suggesting that IL-6 deficiency protects RGCs in models of glutamate excitotoxicity and optic nerve crush and prevents both structural degeneration of the optic nerve and vision loss in a high-IOP glaucoma model in mice.

There are some limitations in the present study we would like to highlight. First, in the exploratory proteomics experiment, contralateral eyes of the animals were used as control to reduce the number of the animals in this study. The contralateral eyes are not always the perfect control group due to sympathetic inflammatory responses in the eyes. As we were looking at the changes in the crystallins, the abundance of the crystallins in the contralateral eyes would likely alter in the same direction, thus leading to a less significant result over all. Therefore, using the contralateral eye as the control does not influence this study. Second, it is always beneficial to use more than one technique to confirm the findings from label-free quantification. Due to the limitation of sample material and the plethora isoforms of crystallin, we cannot confirm all differentially expressed crystallins by a second technique in the present study design. The main focus of this study was to explore the neuroprotective effects of crystallins and the underlying mechanism. Building on these results, different techniques will be used to further study the crystallins’ role in aging and glaucoma. Last, but not least, it is unlikely that all the proteins responsible for the neuroprotective effects of the Müller cells are identified by means of microarray analysis. We also tried to analyze the Müller cell secretome via mass-spectrometry assisted by means of microarray analysis. We also tried to analyze the neuroprotective effects of the Müller cells are identified not least, it is unlikely that all the proteins responsible for the crystallins’ role in aging and glaucoma. All evidence of blindness in southern Germany due to glaucoma and degenerative conditions. Future studies are needed to go into more details into the involved signaling pathways and modes of action.

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Crystallins Protect Retinal Neuron in Glaucoma

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