Elongation of axons during regeneration involves retinal crystallin beta 
b-2 (crybb2)

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Abstract

Adult retinal ganglion cells (RGCs) can regenerate their axons in vitro. Using proteomics, we have discovered that the supernatants of cultured retinas contain isoforms of crystallins, with crystallin beta-b2 (crybb2) being clearly upregulated in the regenerating retina. Immunohistochemistry revealed the expression of crybb within the retina, including in filopodial protrusions and axons of RGCs. Cloning and overexpression of crybb2 in RGCs and hippocampal neurons increased axonogenesis, which in turn could be blocked with antibodies against beta crystallin. Conditioned medium from crybb2-transfected cell cultures also supported the growth of axons. Finally, real-time imaging of the uptake of GFP-tagged-crybb2 fusion protein showed that this protein becomes internalized. These data are the first to show that axonal regeneration is related to crybb2 movement. The results suggest that neuronal crystallins constitute a novel class of neurite-promoting factors that likely operate through an autocrine mechanism, and that they could be used in neurodegenerative diseases.
Introduction

Adult retinal ganglion cells (RGCs) exhibit only a short and transient sprouting reaction after injury, and they fail to extend axons throughout the interior of the optic nerve (1). The failure of regeneration is commonly attributed to inhibitory factors associated with myelin components and/or the glial scar that includes cells and extracellular matrix proteins (2-7). The inhibitory myelin proteins have been shown to include NogoA, myelin-associated glycoprotein, and oligodendrocyte-myelin glycoprotein, all of which act through the Nogo receptor, NgR (8-11). Expanding on this pathway of inhibition, blockage of signaling through NgR, applying antibodies against NogoA, and inactivation of RhoA (a downstream effector of NgR) signaling result in only modest axonal regeneration (12-14).

There are several experimental conditions that permit the regrowth of RGC axons, including (I) replacing the distal segment of the cut optic nerve with a sciatic nerve segment (15), (II) injuring the optic nerve and delayed culturing of retinal explants in vitro (16) or dissociation of RGCs and culturing of primary cells mainly in the postnatal retina (17), and (III) injuring the lens (18-21) or implanting a peripheral nerve fragment directly into the vitreous (22). Expanding on the mutual inflammatory mechanism of lens injury, stimulation of ocular macrophages with intravitreal injections of zymosan (19, 21, 23) also supports axonal growth. Explorations of alternative noninflammatory mechanisms have revealed that coculturing dissociated RGCs with injured lens tissue devoid of macrophages also improves the growth of axons (24). In an attempt to localize the growth factors within the lens, a coculture of retinal stripes with intact lens epithelium cells was shown to also support axonal growth in vitro (25), suggesting that independent mechanisms can result in the successful growth of axons. Consequently, axon regeneration in RGCs may require multiple approaches, such as (I) inactivation of growth-inhibiting signals, since this naturally occurs in the aforementioned models; (II) activation of the intrinsic growth state of neurons, as this occurs by injuring the lens; and (III) adjusting the microenvironment to permit the formation of growth cones, since this occurs in vitro. The gene cohort that is activated when mature RGCs are transformed from a degenerative to a regenerative state in the lens injury model in vivo was recently investigated in fluorescently prelabeled, dissociated, and enriched RGCs (26).
Here we demonstrate that regrowth of axons \textit{in vitro} is associated with the release of intraretinal factors that in turn trigger growth. This was accomplished using either optic nerve crush (OC) or combined optic nerve crush with lens injury (OC-LI) \textit{in vivo} to transform RGCs into a regenerative state (26). The retinas were then explanted and cultured \textit{in vitro} to allow them to regrow their axons, thus transforming them into a state of axonogenesis. After quantifying successful axonal regeneration, the culture supernatants were collected, concentrated, and subjected to proteomic analysis to obtain the profiles of proteins that were released from the retina during axonal regeneration. Several proteins were reproducibly identified and analyzed, among which retinal crystallins showed a remarkable abundance. Examination of the most-upregulated crystallin, beta-b-2 (crybb2), revealed that it promotes axonal growth. This is the first study that indicates that the process of axonal regrowth from RGCs is associated with releasable neuronal crystallins that operate through an autocrine mechanism facilitating axonal growth from explants and dissociated neurons from the retina and the hippocampus.

\textbf{Methods}

\textbf{Treatment of animals.} Experiments were performed on 46 adult rats of the Sprague-Dawley strain that weighed 200–230 g (i.e., 90–150 days old). The care of the animals conformed to the Statement for the Use of Animals of the Association for Research in Vision and Ophthalmology, and was authorized by the local ethical committee. Animals were anesthetized intraperitoneally with a mixture of 12.5 mg of ketamine sulfate (50 mg/ml, Parke-Davis) and 0.1 ml of xylazin (2%, Bayer) per 100-g body weight. To perform OC, the left optic nerve was surgically exposed intraorbitally, and after a longitudinal incision of the meninges the nerve was mechanically crushed for 10 s with the aid of jeweler’s forceps under a microscope. This surgery was performed under visual control to avoid damaging the blood supply, which remained untouched by the procedure. Lens injury was induced through a retrolenticular approach, in which the lens capsule was punctured with the fine tip of a microcapillary (19). Control rats received no treatment.

In order to produce interspecies controls, eyes from marmosets (\textit{Callithrix jacchus}) aged 3 months ($n=6$) were obtained postmortem from the Institute of Reproductive Medicine, University of Muenster (Dr. Lütjens). These eyes were enucleated immediately after euthanasia and transferred to the laboratory for preparing retinal cultures.
**Retinal explants and quantification of growth.** Rats were killed 4 days following OC-LI. The eyes were enucleated and the retinas prepared as whole mounts on nitrocellulose filters (pore size 45 µm, Sartorius, Göttingen, Germany). Retinas were cut into eight radial pieces and cultured in poly-D-lysine- and laminin-coated Petriperm dishes (Bahr et al., 1988) in a chemically defined, serum-free, S4 growth medium (PromoCell, Heidelberg, Germany). Explants were incubated at 37°C in 5% CO$_2$ and 55% O$_2$ for 3 days. The numbers of axons extending from the explants in culture were quantified after 72 h using phase-contrast optics (x200 magnification, Axiovert, Carl Zeiss, Oberkochen, Germany). Monkey eyes were obtained within 20 min after death and were processed in the same way as the rat retinas.

In the experiments designed to test whether identified crybb2 exert neurite growth-promoting activity, conditioned medium (CM) of crybb2-transfected RGC-5 cells was also added to the culture medium of untreated retinas at the time of explantation ($n=5$ retinas). Control experiments were performed without CM ($n=4$). The onset and the rate of axonal growth were determined by observing the explants at regular intervals or by time-lapse videomicroscopy. Whenever more than five axons were present at the onset of outgrowth, the five longest axons were measured. The mean growth rate was obtained from data collected over a period of 72 h.

Axonal growth was monitored by computer-assisted phase contrast microscopy over different time periods (live cell imaging) using an Axiovert microscope and Axiovision software (Zeiss). The images were quantified with the help of software (Image Tool 3.0, [http://ddsdx.uthscsa.edu/dig/itdesc.html](http://ddsdx.uthscsa.edu/dig/itdesc.html), University of Texas Health Science Center at San Antonio) by measuring the advancing axonal growth cones over several hours. Velocity plots and the final lengths of fibers were determined, and videos were generated in AVI and QuickTime formats.

**Cell culture of the RGC-5 cell line and primary hippocampal neurons.** The immortalized RGC line (RGC-5) was a gift from Prof. Agarwal (UNT Health Science Center, USA) (27). Cultures of the cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO$_2$ at 37°C. The cells were trypsinized and then propagated using a 1:20 split after 3 days of culture. Primary
hippocampal neurons from rats at embryonic day 18 (E18) were prepared as described (28).

Briefly, the hippocampus was dissected from E18 rat embryos, dissociated, and neurons were plated onto glass coverslips coated with poly-ornithine (Sigma) at a density of $8 \times 10^5$ cells per coverslip. After the neurons attached, they were used for transfections 2 h after plating using a transfection reagent (Lipofectamine 2000, Invitrogen) according the manufacturer’s instructions. After incubation for 2 h, the transfection medium was replaced by Neurobasal medium (supplemented with B27, 0.5 mM glutamine, and 100 U/ml penicillin/streptomycin; Invitrogen). The cells were detached after the transfection by moderate pipetting and then replated onto new coverslips at a lower density (4–6 x $10^4$ cells per coverslip) in a 24-well plate. Neurons were fixed after 3 d.i.v. with 4% paraformaldehyde and 15% sucrose in phosphate-buffered saline (PBS) for 20 min at 4°C.

**Vitality test by calcein-AM live cell assay.** Vital RGC-5 cells were identified using the acetoxymethyl ester of calcein (calcein-AM) (Invitrogen, Karlsruhe, Germany). The stock solution (1 µg/µl calcein-AM in DMSO) was diluted 1:200 to produce a final concentration of 5 µM calcein-AM in the culture medium (DMEM). Cells were incubated for 45 min with this solution under normal culture conditions. Calcein-AM passively crosses the cell membrane of viable cells and is converted by cytosolic esterases into green fluorescent calcein, which is retained by cells with intact membranes and inactive multidrug resistance protein (29).

**Two-dimensional electrophoresis analysis by MALDI-MS.** CM of cultivated retinas and cells was harvested, purified, and concentrated by an estimated factor of 20 by ultrafiltration (cutoff 3 kDa; Vivaspin 4, Vivasience, Hannover, Germany). For buffer exchange and denaturation, samples were diluted in 8 M urea lysis buffer, concentrated again, and stored in liquid N$_2$. Two-dimensional electrophoresis was applied to a concentrated volume of 300 µl according to the manufacturer’s instructions (Amersham Biosciences, USA). For each run, 18-cm strips with a pH range from 3 to 10 were used. Gels were stained with Coomassie brilliant blue. Spots were analyzed by MALDI-MS at the Department of Integrated Functional Genomics, University of Münster (30).
Western blots. RGC-5 cells and retinal tissue were lysed in 1% Triton X-100 in PBS (with 20 mM Tris/HCl, 0.1% mercaptoethanol, and complete protease inhibitor) and solubilized by sonication (Sonifire, Branson, Danbury, CT, USA) at 4°C for 30 min. The samples were separated by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schüll). All of the blots were developed in accordance with the manufacturer’s instructions (ECL Kit, Amersham Biosciences). An anti-rat-β-crystallin antibody raised in rabbit (1:1000 dilution) (31) was used to detect β-crystallin. The second antibody was a horseradish-peroxidase-conjugated goat-anti-rabbit antibody (1:50,000 dilution) (Sigma, Germany). Loading of comparable amounts of protein was confirmed with a mouse-anti-β-actin antibody (1:1.000 dilution) (Sigma-Aldrich). Gel run and blot was influenced by high contents of salt and urea due to concentration of samples (see 2D-PAGE). Same amounts of protein were loaded per lane, shown by actin staining (data not shown). Densitometry was performed with ImageJ software National Institute of Health (NIH) (data not shown).

cDNA generation and real-time PCR. Total RNA extracted from retinas using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol was eluted in RNase-free water. Reverse transcription was performed using the Omniscript reverse transcription kit and the T7-(dT)24 primer (Qiagen).

RT-PCR was performed using the TaqMan® optimized gene expression assay including the Universal PCR Master Mix (Applied Biosystems) according to the manufacturer’s instructions. Crybb-2 expression was determined using the crybb2-TaqMan assay (ID Rn00564035_m1). All samples were analyzed by a sequence detection system (GeneAmp 5700, Applied Biosystems), with all analyses performed in triplicate. The levels of gene expression were normalized to those of nonregenerated control retinas. 18sRNA (ID Hs99999901_s1) was used as an endogenous control (housekeeping gene). To determine the relative change in gene expression, system software was used to compare the number of cycles (c). The relative-quantification strategy was employed, which measures the relative change in mRNA expression and is based on the expression level of the target gene versus that of the endogenous control, expressed as the change relative to the predefined internal standard of mRNA and calculated according to the formula: Ratio=$2^{-ΔΔct}$. Evaluation of data based upon this ΔΔct-method. Results displayed as changes in expression compared to samples from non-treated animals as control.
**PCR cloning and transfection.** Full-length crybb2 was cloned into the pEGFP-N2 vector (Clontech, Palo Alto, CA, USA) coding for a fusion protein with the GFP at the C-terminus. The cDNA generated by PCR with following primers: forward, 5´-ACG AAT TCA TGG CCT CAG ACC ACC AGA-3´; and reverse, 3´-ACGGAT CCA GCT GGA GGG GTG GAA GG-5´. The amplified cDNA fragment was inserted into the pEGFP-N2 vector. Control cells were transfected with a pEGFP-N2 vector without any insert. RGC-5 cells and primary embryonic hippocampal neurons were transfected with a transfection reagent (Lipofectamine 2000, Invitrogen).

**Immunofluorescence.** Retinal explants were removed from the petri dishes, embedded in TissueTek (Tekura Finetek), and frozen in liquid N2. Cryosections (10–12 µm thick) were cut and collected on gelatinized glass slides. The sections were fixed in ice-cold methanol for 10 min, washed three times for 5 min each in PBS (pH 7.4), and blocked with 10% fetal calf serum (FCS) for 30 min. The FCS was removed and the sections were incubated with the primary antibody diluted in FCS in a humid chamber at 4°C overnight. Following rinsing in PBS three times for 5 min each, the sections were incubated with the secondary antibody and diluted in FCS for 1 h at room temperature in the dark. Sections were rinsed in PBS three times for 5 min each and coverslipped with the anti-fade Mowiol containing 10 µg/µl Hoechst 33258 in order to label the cell nuclei. The sections were then viewed under a fluorescence microscope equipped with the appropriate filters (Axiophot, Zeiss), and documented digitally.

Cells (RGC-5 and hippocampal neurons) were fixed in 4% PFA/sucrose in PBS for 15 min at 37°C and then quenched with 25 mM glycine in PBS. Blockage and staining were performed as described for the retinal tissue. The primary antibody was either rabbit-anti-crybb (rat) (gift from Dr. Sashidar, Hyderabad, India; dilution 1:400) (31) or monoclonal mouse-anti-GFP (BD-Biosciences, Heidelberg, Germany; dilution 1:200). Calmodulin, synaptotagmin, and tau-1 were detected with sheep antiserum (Chemicon, Limburg, Germany; dilution 1:400), goat antiserum (Santa-Cruz, Heidelberg, Germany; dilution 1:200), and monoclonal mouse antibody (Chemicon; dilution 1:200), respectively. The secondary antibodies were conjugated with cy2 or cy3 for performing double-labeling fluorescence. Control slides were treated only with secondary antibodies. Sections of control and experimental eyes were stained simultaneously in order to avoid variations in the immunohistochemical staining.
**Confocal imaging of crybb2 uptake.** The cells \( n=26 \) were imaged in real time with a confocal microscope (Velocity Grid, Improvision, Tübingen, Germany) and associated software (Velocity Acquisition, Improvision). RGC-5 cells received CM from GFP-crybb2-transfected cultures containing the secreted fusion protein. After 30 h the cells were fixed and then stained with antibodies to GFP, in order to enhance the fluorescence associated with uptake of the fusion protein. Multiple images were obtained along the \( z \)-axis in order to determine the distribution of the recombinant crybb2 throughout the cell.

**Results**

Axons regenerated in both species from which retinas were obtained. The onset of axonal growth from rat retinas differed between the various experimental groups. After 72 h of culture, virtually no axonal regeneration was evident in control retinas that were explanted without any treatment of the optic nerve or lens (Fig. 1A). OC and subsequent explantation 3 days later resulted in vigorous regeneration of axons after 72 h of culture (Fig. 1B). Combined pretreatment \textit{in vivo} in the OC-LI group resulted in a further increase in the number of regenerating axons (Fig. 1C). The differences in the regenerative growth between all three groups (Fig. 1D) were highly significant \( P<0.01 \).

The juvenile monkey retina (used here as an interspecies control) regenerates axons without being pretreated (32), and the growth of axons was numerically comparable to that observed with OC pretreatment in rats (similar to Fig. 1B; data from monkey retina not shown).

**Proteomic examination of culture supernatants.** Several protein spots were reproducibly detected throughout the two-dimensional electrophoresis gels (Fig. 2A). Landmark protein spots that appeared with consistent staining intensity in all groups of explants were first mapped and identified. Among the proteins mapped, neuron-specific enolase (spots 18–20), recoverin (spots 22 & 23), and enzymes such as malate dehydrogenase (spot 21) were seen across the gel (Fig. 2A).

In addition to the landmark spots, a conspicuous group of proteins appeared in the middle range of molecular masses (20–30 kDa) at slightly basic pH values (Fig. 2A). This area
(within the rectangular frame in Fig. 2A) also contained several enzymes (spots 1–7; also listed in Table 1) whose positions did not change substantially between the experimental groups. However, the intensity of staining of some spots varied, indicating differences in regulation between the groups (Fig. 2B–F).

Peptide mapping confirmed that the spots corresponded to crybb (Table 1). In the nonregenerated control retina, only one crybb spot (no. 8) appeared in the gel within the marked area, in addition to the aforementioned enzymes (Fig. 2B). The pattern of crystallin expression and release into the culture medium for the retinas pretreated with OC-LI is shown in Fig. 2C, from which it appears that eight isoforms corresponding to crybb2 (Table 1) and one spot corresponding to isoform crybb3 (spot 11, Table 1) were released into the medium. The pattern of crystallin expression in the supernatant obtained from the OC group is shown in Fig. 2D. The RGC-5 cell line supernatant exhibited the pattern of crystallin expression shown in Fig. 2E. Finally, cultured monkey retinas released a very similar group of enzymes and crystallins, as shown in Fig. 2F. Mapping revealed that most of these monkey protein spots corresponded to crybb2. A typical MS profile of crybb2 is shown in Fig. 2G. A comparison of the different groups of retinas revealed that all regenerating groups (Fig. 2A, C–F) produce and release several isoforms of crybb2 (spots 10–17), whereas the control nonregenerating control retina only releases one isoform (spot 8). However, the isoform corresponding to spot 8 showed similar staining throughout the groups examined (Fig. 2A–F). Table 1 summarizes the protein spots that were identified by MALDI-MS. The low heterogeneity (22-23 kDa) of crybb2 spots most likely resulted from different modifications within the samples, e.g. glycosylation, acetylation, esterification and/or results from the 2D-PAGE method, e.g. caboxymethylation of cystein residues.

**Detection of beta crystallin within the retinal tissue.** In order to examine whether retinal tissue contains beta-crystallins and thereby confirm it as the source of the supernatant crystallins, immunoblotting was performed with an antibody staining all isoforms of rat-crybb, including crybb2. Fig. 2H shows immunohistochemical detection of rat crybb in control and regenerating retinal tissue. It appeared that crybb with a molecular mass of 23 kDa is upregulated in the regenerated retina compared with nonregenerating control retina. Additional bands at higher molecular masses of oligomer and other isoforms were also detected, and probably correspond to the extremely stable covalent bonds of oligomers of β-crystallins, which are known to be stable even after hard
denaturation such as induced by heat, β-mercaptoethanol, and urea (8 M) (33). Also a 48 kDa band of beta crystallin was detected by Western blotting of regenerated retinal tissue samples. Due to the molecular weight and the effusive portion of crybb2 of all beta crystallin isoforms, it is most likely that this band represents a dimer of crybb2.

To confirm that the increase in protein expression is also reflected at the level of mRNA, we cultured treated and untreated retinas under the same conditions, and used the crybb2-TaqMan® assay for quantitative real-time PCR after reverse transcription of isolated RNA. Compared to nonregenerated retinas, the gene activity in OC and OC-LI retinas was upregulated by up to four- and tenfold, respectively (Fig. 2J).

**Cellular localization of beta crystallin.** We also used immunohistochemistry to examine whether certain isoforms of beta crystallin are expressed within adult RGCs and embryonic hippocampal neurons from E18. Explanted retinas (Fig. 3A) and axons grown therefrom (Fig. 3B) were positively stained for crybb. Within the tissue, the area of strongest axonal outgrowth was the same as the area of highest beta crystallin expression (Fig. 3A, B). It is most likely that beta crystallin is anterogradely transported to axons, to accumulate within growth cones (Fig. 3B, insert). In addition to the explants, the distribution of expressed crybb is similar in dissociated primary hippocampal neurons and retinal neurons, with both the cell bodies and the processes including growth cones being stained (Fig. 3C, D). In order to confirm that RGCs produce beta crystallins, as suggested by analyzing the supernatant of RGC-5 cells, this was analyzed by immunocytochemistry. As Fig. 3E and F show, all filopodial and lamellipodial protrusions stained positive for crybb, indicating a strong accumulation of the protein within structures thought to represent the motile elements of the cells. Finally, the addition of antibody to crybb did not interfere with the viability of RGC-5 cells in culture, as detected with calcein-AM (Fig. 3G), but it did result in a marked reduction of the attachment and growth of the cells (Fig. 3H) compared to controls (Fig. 3I).

In order to determine whether crybb expression changes within retinal tissue exposed to different treatments and culture explantations, the explants were processed for staining with the anti-rat-beta-crystallin antibody. Virtually no crybb expression was detected in controls consisting of untreated (and thus nonregenerated) retinal tissue (Fig. 4A–C). In contrast, regenerated retinal explants showed strong staining within the ganglion cell layer (GCL) and to some extent on individual cells inside the inner nuclear layer (Fig. 4D–F).
These data support the view that RGCs express crybb during the process of axonal growth and confirm the data obtained with RGC-5 cells (Fig. 3E, F).

**Crybb2 enhances fiber outgrowth and is transported to the growth cones.** To directly analyze the effect of crybb2 on neuronal outgrowth, we cloned crybb2 cDNA into an expression vector for enhanced green fluorescence protein N2 (EGFP tagged) for use in transfection assays. Transient transfections were performed with RGC-5 cells and primary hippocampal neurons from E18 rat embryos, with control experiments performed with EGFP-N2 vector without the insert (Fig. 5A). The formation of elongated fibers in RGC-5 cells was evident after transfection with crybb2 (Fig. 5B). Crybb2-EGFP fluorescence accumulated at the mostly distal tips of axons, resembling growth cones (Fig. 5B). Quantification of the fiber length revealed that significantly longer processes grew out from the transfected cells compared to controls expressing only EGFP (Fig. 5C). In analogy to RGC-5 cells, hippocampal neurons were successfully transfectable (Fig. 5D) and also responded to transfections with crybb2 with extensive axonal sprouting (Fig. 5E). Crybb2-EGFP fluorescence was pronounced within the growth cones (Fig. 5E). Comparison of the lengths of these sprouts revealed that the processes were significantly longer in hippocampal neurons (Fig. 5F).

**Blockage of axonal growth with anti-rat-crybb antibody.** After establishing that crybb2 is expressed in RGCs and supports the growth of axons, the next step was to examine the effects of blockage of crybb with antibodies directed at the protein. For this purpose anti-beta-crystallin rat antibody was added to the culture medium of the regenerating retina, which significant decreased the rate of axonal movement when monitored in live tissue culture over a period of 72 h. Fig. 6A–C shows three time points of a typical recorded series. After 24 h of regeneration *in vitro*, the retinas exhibited a uniformly distributed population of axons, with the longest axons being about 200 µm in length (Fig. 6A). After 35 h *in vitro*, more axons had grown and formed a denser fiber carpet (Fig. 6B). The addition of the anti-rat-crybb antibody at this time point virtually stopped further advancement of the fibers (Fig. 6C). The binding of the crybb antibody to the axons and explants was examined by staining only with the fluorescent secondary antibody in blocked retinas, which confirmed that crybb was bound to the explants (Fig. 6D) and axons (Fig. 6D, insert).
The mean rate of growth of the fastest axons was determined from the onset of outgrowth before adding the antibody to the culture medium. Measurements after 24 h of culture revealed that the growth rate was about 40 µm/h (Fig. 6E). After 42 h of culture the antibody was added to the medium, which decreased the rate of growth to around 10 µm/h; this subsequently partially recovered after 65 h to an average growth rate of 25 µm/h. A second addition of antibody stopped the growth of axons within few hours (Fig. 6E). A video of this experiment is available on the Internet at [http://www.experimentelleophthalmologie.de/70654613610.htm](http://www.experimentelleophthalmologie.de/70654613610.htm) [A. Blocking of retinal axonal outgrowth]

These experiments demonstrated that crybb is involved in the movement of growth cones.

**Crybb2 CM induces axonal regeneration.** Crybb2 was shown to accumulate in the culture medium of regenerated retinas and RGC-5 cells (Fig. 2), indicating that it is released during the process of regeneration. We subsequently transfected RGC-5 cells with GFP-tagged crybb2 to overexpress and hence oversecrete crybb2, and then harvested the CM, purified and concentrated it, and investigated whether it supports axonal growth in cell and tissue cultures. The addition of this conditioned-transfection-crybb-2 (CTC) medium to RGC-5 cells resulted in the formation of lengthy, axon-like processes (Fig. 7A) that were significantly longer than the processes formed without CTC medium or medium transfected with only the GFP vector (Fig. 7B). The addition of CTC medium to untreated control retinal explants resulted in vigorous regeneration of axons (Fig. 7C), indicating that this medium also supported axonal growth in explants. Counting of the numbers of axons revealed a five- to sixfold increase compared to control retina (Fig. 7D). RGC-5 cells and retinal explants showed spontaneous GFP fluorescence without being processed for immunohistochemistry, thus indicating that the GFP-tagged crybb2 bound to the RGC-5 cells (Fig 7A), retinal explants (Fig. 7C), and axons (Fig. 7C, insert). This experiment indicated that transfection resulted in the secretion of crybb2 into the medium, which in turn stimulated the growth of axons when used as a CM. Western Blot analysis revealed an signal enhancement of 8-fold about 23 kDa indicating that the post-transfection conditioned medium contained more crybb (Fig. 7I).

High-resolution confocal microscopy was used to investigate the uptake and intracellular localization of recombinant crybb2 protein with RGC-5 cells (n=26) cultivated in the CTC medium. The fluorescence of GFP-tagged crybb2 was enhanced with anti-GFP staining. Fig. 7E-H shows different views of an RGC-5 cell with an elongated fiber. The GFP-crybb2
staining can be seen throughout the fiber (i.e., it is not restricted to the outer cell membrane). The intracellular distribution of the crybb2 was elucidated in a video of the series of confocal images, which is available on the Internet at http://www.experimentelleophthalmologie.de/70654613610.htm. [B. Uptake of beta-b2-crystallin]

The series of consecutive images of the RGC-5 cell in the z-axis outlines the distribution of crybb2 in all compartments of the cell soma and fiber, and demonstrates that the internalized protein is distributed throughout neurons.

Double-labeling immunocytochemistry was used to examine the intracellular colocalization of further functionally relevant molecules. Double staining of RGC-5 cells with crybb and calcium-binding calmodulin revealed strong colocalization in the filopodial cell processes (Fig. 8A–C), suggesting either the binding of crybb to calmodulin or its direct involvement in the calcium homeostasis. In contrast, double-labeling immunofluorescence with exocytosis-triggering synaptotagmin showed a strong colocalization in the cell body but not in the cell processes, suggesting the presence of release into the medium from the cell body (Fig. 8D–F), which probably involves the secretory pathway of synaptotagmins. Finally, double staining of beta crystallin and tau-1 revealed the localization of tau-1 within the cell body partially and within only one process (Fig. 8G–I), whereas beta crystallin was localized outside the cell body, outlining all processes including the axon (Fig. 10G–I), suggesting that crybb shows in these cells a different distribution than tau-1.

Discussion

We have examined the supernatants of retinal pieces regenerating axons in vitro, and have identified proteins that are released into the culture medium. Isoforms of beta crystallins were reproducibly observed, with crybb2 and crybb3 being specifically upregulated within the culture medium of regenerating retinas, suggesting that this protein plays a role during axonogenesis. Further characterization of the most abundant protein (i.e., crybb2) revealed that it is expressed in filopodial processes of RGCs and their axons, as well as in primary hippocampal neurons and neurites. In addition to its expression and release into the medium, crybb2 can be taken up by neurons and support the growth of axons both in RGCs and hippocampal neurons. This effect of crybb2 on axonogenesis
and growth cone movement is blockable with antibodies directed at the protein. To our knowledge, this is the first description of releasable neuronal beta crystallins during axonal regeneration in adult CNS tissue, and in particular of crybb2, which is the first intrinsic neuronal crystallin that can move between the cytoplasm and surroundings of the neurons during the process of adult axonogenesis, thus assigning it a crucial role in the regrowth of axons after injury.

Crystallins were originally defined as a group of structural proteins of the vertebrate lens (34, 35) and of some extralenticular tissues, including the nervous system and the retina (36) Within the lens they are synergistically responsible for refractive functions such as maintaining the transparency of the lens throughout life via hydration, and preventing opacification and the formation of cataracts (37). The ubiquitous occurrence of crystallins in several tissues and cell types (including RGCs), and their homology and relation to heat-shock proteins, has resulted in some of them being classified as stress proteins, although they are also vital to normal tissue differentiation (34-39). One of the functions ascribed to α-crystallins is molecular chaperoning (40-42) – binding and stabilizing less stable proteins or preventing incorrect folding and interactions within and between proteins. However, no neuronal function has yet been attributed to the β- and γ-crystallins that are expressed in the postnatal RGCs of mice (43-45). Different crystallins are also temporarily expressed within the rat retina after injury (46), indicating their involvement in post injury repair. The neuronal survival and neurite growth-promoting effects of eye lens puncture on adult and early postnatal rat RGCs (18-21, 24) have lead to suggestions of a role of crystallins. However, the exact mechanism for these effects may involve either inflammatory events and activation of macrophages, as shown by examinations of macrophage factors (18, 21), or noninflammatory effects postulated from the results of coculturing lens and retinal tissue (24, 25).

Crystallins are very stable proteins due to their characteristic “Greek key motif” structure, comprising four antiparallel β-strands, which is also believed to be responsible for their function (35). Some components of this βγ-superfamily of proteins can undoubtedly prevent phase separation of the cytoplasm of lens fiber cells (47, 48). It is likely that crystallins play a similar role within the neuronal cytoplasm and the lens (47). However, such a mechanism would require internalization of the crystallins, and the uptake investigations reported here have yielded data supporting the transfer of crybb2 into the cytoplasm of RGCs. Alternatively, the beta crystallins may act as ligands inhibiting or downregulating apoptotic receptors such as FAS/APO-1, as shown with human αB-
crystallin in vitro (48). Further experimental investigations are essential to demonstrating such ligand-receptor binding and to unraveling the signal transduction pathway that leads to the rescue of neurons.

β-crystallins are also thought to be involved in senile cataract formation by inducing either disulfide bond formation at cys-residues or by N-terminal cleavage and subsequent loss of the native structure (33, 35). In addition, β-crystallin in dissociated nerve cells responds to stress by translocation from the nuclear region to the cytoplasmic compartment (51), thus ensuring storage of cytoplasmic Ca$^{2+}$.

Crybb2 protein has been detected within the retina, brain, and testis (44, 50), and crybb2 mRNA has been reported in various tissues including the retina (50). Crybb2 is involved in both cAMP-dependent and cAMP-independent phosphorylation (50) within the lens. This coupling to phosphorylation pathways indicates important functional roles in retinal tissue. The results of the present study are consistent with the above studies, and show that various isoforms of crybb2 are present in the adult retinal tissue. In addition, this study has revealed the localization of crybb2 within RGCs and their regenerating axonal processes, especially in filopodia and growth cones. This is the first study to show a nonrefractive role of crybb2 in regenerative axonogenesis and to suggest that crybb2 moves from the RGCs into the extracellular space and backwards into the cells, although the underlying mechanisms remain to be elucidated. Its colocalization with synaptotagmin 1 – which is a leading calcium sensor within the retina and likely triggers exocytosis (52) – may point to a similar mechanism of secretion into the culture medium. Colocalization of crybb2 with calmodulin – which is the major calcium-binding protein in the retinal GCL (53) and in the RGC-5 cell line (54) – provides further evidence that crybb2 also operates through calcium binding by its greek key motifs (55). The double staining with crybb2 and tau-1 protein which is a specific marker for axons showed that crybb2 was translocated into the processes while tau-1 remained confined to the cell body (56). Finally, the similarity between RGCs and hippocampal neurons indicates that similar functions may be attributed to β-crystallins within the CNS. Interactions between these and other proteins involved in the growth of axons may be possible, but they remain to been demonstrated experimentally.
In conclusion, we have shown that β-crystallins represent a new class of substances that are secreted by the retina and RGCs into culture media. One member of these crystallins, crybb2, exerts remarkable neurite-promoting activity in cultures of retinal stripes, the RGC-5 cell line, and primary hippocampal neurons. This is the first study that shows a function of crystallins within regenerating nervous tissue.

Acknowledgements

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Bibliography


51.


Figure legends

**Figure 1:** Regeneration of retinal ganglion cells (RGCs) after different surgical pretreatments of the retina 4 days before explantation. (A–C) Retinal explants from female Sprague-Dawley rats after 72 h of culture: (A) control retina, no previous treatment; (B) optic nerve crush (OC) only; (C) OC and lens injury (OC-LI). (D) Quantification of growing axons per explant. Scale bar, 50 µm.

**Figure 2:** (A-G) Two-dimensional PAGE image (Coomassie brilliant blue staining) of conditioned culture medium, where the numbers indicate altered and excised protein spots. (A) Whole gel (pH 3–10) of OC-LI sample. (B–F) Focused gel areas of different samples corresponding to boxed area in A: (B) control retina, (C) OC-LI (rat), (D) OC (rat), (E) RGC-5 cell culture, (F) monkey retinal culture, (G) MALDI-MS spectrum of protein spot 8, which corresponded to beta b2-crystallin. (H) Expression of rat beta crystallin in retinal tissue. Immunohistochemical detection in Western blots with anti-crybb antibody. Retina: nonreg., no treatment; reg., OC-LI retina after 3 days of culture; Lens: sample of lens material served as positive control. Actin: β-actin staining for comparing protein loading. (J) Enhancement of crybb2 transcription by retinal regeneration. mRNA-expression levels of crybb2 measured in real-time PCR from samples of OC and OC-LI regenerated retina relative increase change of crybb2 expression (fold-change) in comparison to control, measured by RT-PCR and calculated by the ∆∆ct-method. (K) Amino acid sequence of crybb2 protein.

**Figure 3:** (A-F) Endogenous expression of rat beta crystallin protein in tissue and cell culture. Immunostaining with rabbit anti-rat-crybb and cy2(green)-anti-rabbit antibodies (dilution 1:1000). (A) regenerating retinal explant after 3 days of culture (OC-LI). (B) Higher magnification of the brightest area of explant from A. (C) Hippocampal neuron from E18 rat 3 days after seeding. (D) Higher magnifications of C. (E) RGC-5 cell line. (F) Higher magnifications of cells from E. Scale bars: A, 100 µm; C and E, 20 µm. (G-I) Adherence and growth of normal RGC-5 cells was prevented by anti-crybb antibody. Antibody was added directly after plating the cells. Green fluorescence shows calcein-AM staining of living cells: (G) 12 hours after plating, (H) 20 hours after plating, (I) control cells (no antibody). Controls were performed with addition of heat-inactivated anti-crybb and anti- anti-actin antibodies which did not exhibit any detectable effects (data not shown). Scale bar, 20 µm.
Table 1: List of all excised two-dimensional-gel protein spots analyzed by MALDI-MS.

Figure 4: Enhanced expression of beta-crystallin after retinal regeneration. Retinas were fixed after 3 days of culture and cryosections were stained with anti-rat-crybb antibody (green) and nuclear DAPI (blue). (A–C) Control retina, no treatment: (A) anti-crybb antibody staining, (B) DAPI staining, (C) overlay of A and B. (D–F) Regenerated retinal explant from OC-LI rat: (D) anti-crybb antibody staining, (E) DAPI staining, (F) overlay of D and E. Scale bar, 25 µm. OS, outer segment; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar, 30 µm.

Figure 5: Crybb2 expression promotes axonal outgrowth in retina and fiber elongation in RGC-5 cells. Transport to and accumulation at growth cones. RGC-5 cells and hippocampal neurons were transfected 2 h after plating with expression vectors for EGFP-N2 and crybb2-EGFP. (A–C) Transfection study of RGC-5 cells: (A) EGFP-N2, (B) crybb2-EGFP with inserted area of growth cone at higher magnification, (C) quantification of fiber length at 2 days after transfection. (D–F) Transfection study of hippocampal neurons from E18 stage: (D) EGFP-N2, (E) crybb2-EGFP with boxed area at higher magnification of an axonal growth cone, (F) quantification of axonal length at 2 days after transfection. Scale bars, 20 µm.

Figure 6: Blocking of axonal growth in retinal tissue culture by rabbit anti-rat-crybb antibody. Retinal explant from rat pretreated by OC-LI 4 days previously: (A) after of 24 h in vitro explantation, (B) after 35 hours, (C) after addition of anti-crybb antibody (dilution 1:1000). (D) Staining with cy2-anti-rabbit antibody at 30 h after the addition of the first antibody. Scale bars: C, 100 µm; D, 50 µm. (E) Velocity plot of axonal growth in regenerating retinal explant (OC-LI). Red line: average axonal growth velocity of OC-LI rats; green line: average axonal growth; blue arrows: times at which anti-beta-crystallin antibody was added. Controls were performed with addition of heat-inactivated anti-crybb and anti-actin antibodies which showed no effects (data not shown).

Figure 7: (A–D) Recombinant beta b2-crystallin is taken up from conditioned medium harvested from crybb2-transfected RGC-5 cell culture and effects neurite growth both in RGC-5 cells and retinal explants. (A) White arrow: elongated fiber of a RGC-5 cell. (C)
Margin of retinal explant with inserted area of higher magnification. (B, D) Quantifications of ganglion cell fiber elongation and growth of RGC axons. Scale bars: A, 25 µm; B, 100 µm. (E-H) Recombinant crybb2 protein is taken up intracellularly and distributed in all compartments of the cell, as shown by confocal microscopy imaging. Red fluorescence indicates staining of GFP (recombinant crybb2-GFP) by rabbit-anti-GFP and anti-rabbit cy3 antibodies. E-H are different views of RGC-5 cell with elongated fiber. Scale bar, 20 µm. (I) Secretion of rat beta crystallin by RGC-5 cells into culture medium. Immunohistochemical detection in Western blots with anti-crybb antibody. **Control:** sample of conditioned medium of non-transfected cells **crybb2:** sample of conditioned medium of crybb2-transfected cells. There is a clear signal enhancement of 8-fold compared to the control when signals where measured by densitometry (data not shown).

**Figure 8:** Double-labeling immunofluorescence of RGC-5 cells. (A–C) Colocalization of crybb with calmodulin, in particular along the filopodial processes. (D–F) Colocalization of crybb with synaptotagmin in the cell body, but not along the cell processes. (G–I) Images show that tau-1 is mainly localized in the cell body and partially within one process (arrow), whereas beta crystallin outlines all cell processes. Scale bar, 10 µm.
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Figure 1
Figure 2

Figure 2 contains a gel image with labeled bands and molecular weight markers. The gel shows protein profiles under different pH conditions, with bands marked from 1 to 23. The bottom panels A to E depict close-ups of specific regions. Panel F shows a bar graph with percentage values. Panel G contains a spectrum graph with m/z values. Panels H and J illustrate protein profiles for Retina and Lens. Panel K provides a sequence of amino acids for comparison.
Figure 3

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Scale bars: 100 μm
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control

OC-LI
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**C**

- Fiber length (μm)
  - EGFP: 50 ± 10
  - Crybb2: 200 ± 20

**F**

- Axon length (μm)
  - EGFP: 100 ± 10
  - Crybb2: 200 ± 20
Figure 6
Figure 7

Confocal Imaging RGC-5

Secretion of crybb

I  control  crybb2

23 kDa

> 8.3-fold
Figure 8