Crystallin-β-b2-Overexpressing NPCs Support the Survival of Injured Retinal Ganglion Cells and Photoreceptors in Rats

Michael R. R. Böhm,1,2 Sarah Pfrommer,1 Carolin Chiwitt,1 Matthias Brückner,1 Harutyun Melkonyan,1 and Solon Thanos1,2

PURPOSE. Crystallin β-b2 (crybb2) is known to support the regeneration of retinal ganglion cell (RGC) axons in culture. We investigated whether neuronal progenitor cells (NPCs) overexpressing crybb2 (crybb2-NPC) affect secondary retinal degeneration due to optic nerve crush in vivo.

METHODS. NPCs were produced by dissociation and propagation of rat embryonic neural tube and eye primordial cells at embryonic days 13.5 and 15. Retinal degeneration was induced by injured optic nerve crush (by suture, 20 seconds). Several groups were built: crybb2-NPC were injected into the vitreous body, while the Controls were comprised of recombinant crybb2-injected and PBS-injected groups. The eyes, in particular the retina, were analyzed by immunohistochemistry and Western blotting for different antigens at 2 and 4 weeks after surgery.

RESULTS. At 2 and 4 weeks post surgery, crybb2-NPC resided within the vitreoretinal compartment, and were persistently nestin-positive throughout the experimental period. The cells stained positive for various neurotrophins and acted as “living” cell factories to support the survival of injured RGCs. The crybb2-NPC migrated throughout the eye structures and sometimes became integrated within the tissue. Most of the ocular cells responded to the appearance of crybb2-NPC with marked changes of certain proteins, including Iba-1 (microglia), vimentin (glial cells), and rhodopsin (photoreceptors). Photoreceptors also displayed a better survival after crybb2-NPC injection compared to control groups.

CONCLUSIONS. Crybb2-NPC exert beneficial effects on the vitreoretinal compartment, which suggests that modified crybb2-NPC could be used in a novel strategy for the treatment of degenerative vitreoretinal diseases. However, future studies must determine the safety of in vivo administration of crybb2-NPC.

Retinal degenerative diseases affect millions of people worldwide1 and, hence, methods must be developed to prevent such degeneration and the consequent visual impairment. One of these strategies could involve placing cells inside the eye that produce protective substances.

Neural stem cells and neural progenitor cells (NPCs) have been isolated from different parts of the embryonic and adult central nervous system (CNS) of several mammalian species.2–5 NPCs secrete several neurotrophic factors (NTFs) and exert protective effects on diseased neurons.6–8 NPCs can be manipulated through pretreatment with growth factors or via gene transfection.1,2,5,9 Attempts to replace lost photoreceptors so far has focused on injecting stem cells into the retina or the subretinal space. Although some of the injected cells differentiate into photoreceptors and integrate into the host tissue, the quantity of cells involved generally is low, and in most cases they fail to function.2,10–12 Alternative approaches include the modification of NPCs to rescue visual function through the engineered expression of NTFs, such as glial cell–derived NTF (GDNF)-producing human-derived NPCs. These were shown to have a positive effect after being injected into the degenerating retinas of Royal College of Surgeons (RCS) rats.1

In addition to classical NTFs and differentiation molecules, ocular crystallins appear to have a crucial role in the development of retinal cells, including photoreceptors.13–15 Crystallins, which also are expressed in retinal neurons,16 have been identified in various neurodegenerative disorders, including in those affecting the retina.17 While several studies have demonstrated that crystallin α has a role in retinal disorders, there is little information on the effects of crystallin β/γ.18–21 Adult retinal ganglion cells (RGCs) have an enhanced chance of survival when exposed to lens injury.22–25 Testing of different classes of crystallins has revealed that crystallins of the β/γ superfamily possess a strong neuroprotective and regenerative potential by mimicking the effects of lens injury.16

Crystallins β and γ (cryg) are expressed during the development of the mouse eye.14 Increased levels of cryg have been observed in cultured astrocytes affected by hypoxia,25 neuronal aging processes,24 and neuronal repair.25 Added crystallin β-b2 (crybb2) enhances the growth of axons in retinal explants and in primary hippocampal neurons.26 However, to our knowledge no protective effects of crybb2 on RGCs in vivo or on photoreceptors have yet been reported.

To investigate neuroprotective features of NPCs, we used the optic nerve crush model to induce retinal degeneration and injected transfected NPCs, overexpressing crybb2 intravitreally
after surgery. In controls, NPCs were replaced with intravitreal injection of recombinant crybb2 protein or injection of PBS. The aims of our study were to elucidate the survival and migration of NPCs within the eye, the intravitreal production of NTFs and crystallins, and the effects of overexpressed crybb2 on degenerating RGCs and photoreceptors. Therefore, immunohistochemistry and Western blotting (WB) were used to examine intravitreal distribution of NPCs and production of NTFs that may affect the fate of photoreceptors, glial cells, and microglia.

**METHODS**

**Animals and Drugs**

Pregnant Sprague-Dawley (SD) rats at embryonic days (E) 13.5 and 15 (n = 52) were used as a source of NPCs for these studies. Adult SD rats at approximately 3 months of age (n = 64) of both sexes were used for the surgery. All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were housed in standard animal rooms under a 12-hour light/dark cycle, with food and water provided ad libitum. All surgical experiments were performed under general anesthesia by cesarean section at days E13.5 and E15, and were stored in sterile Hank’s balanced salt solution (HBSS; PAA Laboratories, Pasching, Germany) with 1% PenStrep (Sigma-Aldrich, Hamburg, Germany), on ice until dissection. The brains were removed and homogenized mechanically. The homogenate then was treated with 1% PenStrep, 1% N2 Supplement (PAA Laboratories), 10 ng/mL recombinant human basic fibroblast growth factor (bFGF; RD Systems, Minneapolis, MN), and 10 μl insulin (2.5 mg/ml; PromoCell, Heidelberg, Germany). The suspension was triturated gently and 4.5 × 10^5 cells were plated at a density of 200,000 cells/mL on noncoated Advanced TC Petri dishes (Greiner Bio-One, Frickenhausen, Germany) to inhibit further differentiation. The cells were incubated at 37°C in a humidified atmosphere of 5% CO2. All cell harvesting steps were performed under sterile conditions.

Rat crybb2 was cloned into pIRES-ACGFP (Clontech, Palo Alto, CA) so that rat crybb2 and green fluorescent protein (GFP) could be translated from a single bicistronic mRNA. For transfection of NPCs, cultured cells that formed neurospheres were dissociated mechanically into single cells and centrifuged for 5 minutes at 400 rpm (4°C). The culture medium was removed and approximately 5 × 10⁶ cells were resuspended in 100 μl Amaxa Rat NSC Nucleofector Kit (Lonza Cologne, Cologne, Germany). Vector (3.5 μg DNA) was added, the cells and DNA were mixed gently, and then the cells were electroporated using the Nucleofector device (Lonza Cologne). The transfection protocol used was very similar to that described by the manufacturer, thus resulting in the highest transfection efficiencies. Cells were used for a maximum of 2 weeks after preparation. After finishing this procedure, crybb2-overexpressing NPCs (crybb2-NPC) were placed into the culture medium and cultured for up to 7 days, and the efficacy of transfection was monitored using GFP fluorescence.

Transfected crybb2-NPC were prepared for injection by first washing the cell suspensions with HBSS. Cell numbers then were counted using the trypan-blue exclusion method, and then the cells were transferred to the transplantation medium, HBSS. Surgery and cell injection were performed within 1 hour of cell preparation.

**Preparation and Transfection of NPCs**

To retrieve and culture NPCs, embryos were removed under deep anesthesia by cesarean section at days E13.5 and E15, and were stored in sterile Hank’s balanced salt solution (HBSS; PAA Laboratories, Pasching, Germany) with 1% PenStrep (Sigma-Aldrich, Hamburg, Germany), on ice until dissection. The brains were removed and homogenized mechanically. The homogenate then was treated with 0.1% trypsin in HBSS to obtain single cells. The suspension was transferred into a culture medium comprising Dulbecco’s modified Eagle’s medium-Ham’s (DMEM-F12; PAA Laboratories) supplemented with 1% PenStrep, 1% N2 Supplement (PAA Laboratories), 10 ng/mL recombinant human basic fibroblast growth factor (bFGF; RD Systems, Minneapolis, MN), and 10 μl insulin (2.5 mg/ml; PromoCell, Heidelberg, Germany). The suspension was triturated gently and 4.5 × 10⁵ cells were plated at a density of 200,000 cells/mL on noncoated Advanced TC Petri dishes (Greiner Bio-One, Frickenhausen, Germany) to inhibit further differentiation. The cells were incubated at 37°C in a humidified atmosphere of 5% CO₂. All cell harvesting steps were performed under sterile conditions.

**Unilateral Optic Nerve Crush (ONC) and Intravitreal Treatment**

To generate the primary degeneration of RGCs and a photoreceptor transsynaptic response, we performed unilateral ONC to induce damage of axons followed by the intravitreal injection of crybb2-NPC. After a skin incision near the superior orbital rim, ONC was performed according to the “mini lesion” technique, which involves ligation with a suture (8.0 Ethicon; Johnson & Johnson, St-Stevens-Woluwe, Belgium) for exactly 20 seconds. A 10 μl volume of crybb2-NPC was injected through the sclera via a glass capillary, with care being taken to avoid damage to the lens. To reveal the effects administered by intravitreal injected crybb2-NPC, several groups in the experimental setup were built. In the controls, 10 μl of recombinant crybb2 protein (180 μg/μl) were injected intravitreally to reveal the effects of recombinant crybb2 protein compared to crybb2-NPC due to retinal degeneration. After subcloning into pQE32 (Qiagen, Hidden, Germany), rat crybb2 was expressed and purified: bacterial cultures were centrifuged (1500g for 20 minutes) and subsequently resuspended in buffer 1 (8 M urea, 50 mM NaH₂PO₄, 15 mM imidazole, 10 mM Tris-HCl, and 100 mM NaCl; pH 8.0). After cloning, cell lysates were centrifuged at 20,000g for 30 minutes. The supernatant was purified using immobilized metal affinity chromatography (Clontech); elution was performed using buffer 1 at pH 6.0 supplemented with 100 mM imidazole. The proteins were dialyzed in aqua dest. For control

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**Table 1.** Experimental Groups (In Vivo)

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>N</th>
<th>Weights of SD Rats</th>
<th>Experimental Manipulation</th>
<th>Times, wk</th>
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<tbody>
<tr>
<td>Effects of crybb2</td>
<td>16</td>
<td>180 to 250 g</td>
<td>Left ONC with intravitreal injection of crybb2</td>
<td>2, 4</td>
</tr>
<tr>
<td>IHC</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>WB</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Effects of crybb2-NPC</td>
<td>16</td>
<td>180 to 250 g</td>
<td>Left ONC with intravitreal injection of crybb2-NPC</td>
<td>2, 4</td>
</tr>
<tr>
<td>IHC</td>
<td>4</td>
<td></td>
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<tr>
<td>WB</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Effects of PBS (control)</td>
<td>16</td>
<td>180 to 250 g</td>
<td>Left ONC with intravitreal injection of PBS</td>
<td>2, 4</td>
</tr>
<tr>
<td>IHC</td>
<td>4</td>
<td></td>
<td></td>
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<tr>
<td>WB</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult control</td>
<td>16</td>
<td>180 to 250 g</td>
<td>Untreated</td>
<td>2, 4</td>
</tr>
<tr>
<td>IHC</td>
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<tr>
<td>WB</td>
<td>4</td>
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IHC, immunohistochemistry; WB, Western blotting; wk, weeks.
The procedure used to stain cell nuclei with 4',6-diamidino-2-phenylindole is described above. The slides were viewed with the appropriate filter on a microscope equipped with epifluorescence (Axioskop; Carl Zeiss Meditec, Oberkochen, Germany). Negative controls comprised sections processed without the addition of the primary antibodies. Control and experimental sections were stained simultaneously to avoid variations in immunohistochemical staining. Table 2 lists the antibodies used, including their working dilutions. All stained cells were counted within a region of 0.2 mm² (five randomized regions per eye) at a magnification of ×40.

**Western Blotting**

WB was performed to confirm the immunohistochemical findings and evaluate retinal degeneration. Eyes taken from four animals of each group were enucleated. The extraocular tissues were dissected, the retina was discharged carefully from the sclera, and the vitreous was peeled off. All isolated samples were frozen in liquid nitrogen and kept at −80°C until further use.

Samples were homogenized in SDS sample buffer (62.5 mM Tris-HCl, 2% wt/vol SDS, 10% glycerol, 50 mM dithiothreitol, and 0.01% wt/vol bromophenol blue). The samples were sonicated and heated, and then the protein concentration was determined using the Bradford method.

A total of 50 μg protein from each sample was fractionated on 8%, 10%, or 12% SDS-polyacrylamide gel electrophoresis (depending on the protein) with a protein marker (BioRad, Hercules, CA). After electrophoresis, the proteins were transferred onto a nitrocellulose membrane (Schleicher & Schuell BioScience, Dassel, Germany). The blots were incubated in blocking solution (5% freshly prepared fat-free dried milk and 0.1% Tween-20 PBS) for 1 hour, followed by incubation overnight at 4°C with a variety of antibodies (Table 3). The membrane then was incubated with the secondary antibody conjugated with horseradish peroxidase in blocking solution for 1 hour at RT. Antibodies were detected with enhanced chemiluminescence (Amer sham, Rockville, UK) and documented on X-ray films (Amersham Pharmacia Biotech, Amersham, UK). Loading of comparable amounts of protein was confirmed with anticalnexin (see below). Each gel was run three times. The relative densities of the protein spots were analyzed using Alpha Ease (Alpha Ease FC software 4.0; Alpha Innotech, San Leandro, CA). The protein density of a fixed area was
was observed 4 weeks after ONC (Figs. 3B, 3D). When the immunohistochemistry was performed with sections from crybb2-NPC-treated eyes, most cells stained positive for crybb2 (Fig. 3E).

Intraocular Localization of Intravitreally Injected crybb2-NPC

Nestin was used as a stem-cell marker to identify the distribution of crybb2-NPC at 2 and 4 weeks after intravitreal transplantation. NPCs were observed as follows: arrested crybb2-NPC in the vitreous cavity, arrested and migrating crybb2-NPC in the vitreoretinal compartment, and arrested and migrating crybb2-NPC in the anterior segment, including the anterior chamber, cornea, and ciliary body (Table 4).

Arrested nestin-positive cells were localized in the vitreal cavity, at the vitreoretinal interface (Figs. 4G–I), in most retinal layers (Figs. 4A–C), and in the subretinal space, including the choroid (Figs. 4D–F) 2 weeks after transplantation. They also were found in the anterior chamber, associated with the corneal endothelium (Figs. 4J–L) and epithelium of the ciliary body (Figs. 4M–O) 4 weeks after transplantation. In the host retina, NPCs were detected in the ganglion cell layer (GCL), inner nuclear layer (INL), and outer nuclear layer (ONL). These observations show that NPCs are redistributed within the eye during the first 2 weeks after injection. The distribution of NPCs was similar at 4 weeks post surgery (Fig. 4), with some NPCs having reached quite distant locations, such as the cornea (Figs. 4J–L). These data supported the view that NPCs can migrate within the eye.

Staining of Ocular Tissue after Injection of crybb2-NPC

The marker neurofilament 200 was used to examine the response of RGCs to damage and to the intravitreal injection of NPCs (Figs. 5A–D). A moderate loss of RGCs was found in the crybb2-NPC group compared to the PBS group. Compared to the adult control, the population of RGCs had reduced to 82.63 ± 6.60% in the crybb2-NPC group and to 55.56 ± 8.56% in the PBS group 2 weeks after implantation. After 4 weeks, these values were 52.78 ± 5.73% and 18.75 ± 3.99%, respectively. In the crybb2 group, the population of RGCs had reduced to 58.30 ± 2.41% after 2 weeks and 55.56 ± 3.99% after 4 weeks (Fig. 5E). After 4 weeks the population of RGCs was significantly higher in the crybb2-NPC group compared to the PBS group. Compared to the adult control, the population of RGCs had reduced to 82.63 ± 6.60% in the crybb2-NPC group and to 55.56 ± 8.56% in the PBS group 2 weeks after implantation. After 4 weeks, these values were 52.78 ± 5.73% and 18.75 ± 3.99%, respectively. In the crybb2 group, the population of RGCs had reduced to 58.30 ± 2.41% after 2 weeks and 55.56 ± 3.99% after 4 weeks (Fig. 5E). After 4 weeks the population of RGCs was significantly higher in the crybb2-NPC group compared to the PBS group.

These percentages were confirmed by WB analysis using antibodies detecting brain-specific homeobox/POU domain

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Isotype</th>
<th>Dilution</th>
<th>Molecular Mass</th>
</tr>
</thead>
</table>
| Primary antibodies
| BRN-3b         | Covance (Princeton, NJ)       | Rabbit polyclonal | 1:2,000  | 45 kDa         |
| Rhodopsin     | Millipore                     | Mouse monoclonal  | 1:1,000  | 39 kDa         |
| Vimentin      | Sigma-Aldrich                 | Mouse monoclonal  | 1:500    | 58 kDa         |
| Secondary antibodies
| Secondary rhodopsin | Cell Signaling Technology, Inc. (Danvers, MA) | Horse antimonuse | 1:4,000  |
| Secondary vimentin | Cell Signaling Technology, Inc. | Horse antimonuse | 1:4,000  |
| Secondary BRN-3b | Amersham Pharmacia Biotech   | Donkey antirabbit | 1:10,000 |
| Loading control
| Calnexin       | Sigma-Aldrich                 | Rabbit            | 1:10,000 | 90 kDa         |

BRN-3b, brain-specific homeobox/POU domain protein 3b.
Figure 1. Morphologic and immunohistochemical characterization of NPCs derived from cultured rat fetal neural tissue. (A, B) Cultured NPCs were found in cell clusters, called neurospheres (arrows). (B) Single cells with morphologic features reminiscent of stem cells also were found (arrowheads). (C–Q) Immunohistochemical characterization of cultured NPCs (using nestin, green) and several other markers (red) in vitro. Labeling with antibodies raised against BDNF (C–E), oncomodulin (F–H), calcineurin (I–K), crybb2 (L–N), and cryg (O–Q) revealed that these molecules were expressed in NPCs in vitro. 4’,6-Diamidino-2-phenylindole (DAPI, blue) was used to stain cell nuclei. Scale bars: 20 μm (A, B, E, H, K, N, Q).
protein 3b (Fig. 5F). These data showed that crybb2-producing NPCs support the survival of injured RGCs.

Photoreceptor Response

The marker rhodopsin was used to examine the response of photoreceptors to axotomy due to optic nerve crush and to the intravitreal injection of NPCs. Immunohistochemical evaluation of rhodopsin revealed intense labeling of the photoreceptor outer segments (POS) in adult retinas. A slight decrease in rhodopsin was found in all treated groups after 2 weeks (Figs. 6A–D). Comparison of crybb2-NPC and crybb2 groups with the PBS group revealed less decay of stained POS after 4 weeks (Figs. 6E–H).

Additional WB analysis revealed a massive decay of rhodopsin in the PBS group (89.40 ± 6.4.16% at 2 weeks and 21.06 ± 3.53% at 4 weeks). Comparable rhodopsin was detected in the crybb2-NPC group after 2 weeks (94.95 ± 12.27%), while a smaller decrease in rhodopsin was observed after 4 weeks (66.36 ± 14.88%, P < 0.01; Figs. 6I, 6J). These data showed that photoreceptors respond to the injury-induced loss of RGCs with a reduction in rhodopsin, and that this reduction can be neutralized at least partly by the addition of crybb2-producing NPCs.

Response of Glial Cells

Antibodies detecting GFAP and vimentin were used to detect retinal glial reactivity. A distinct labeling of both proteins in the nerve fiber layer (NFL), ONL, and OPL in the crybb2-NPC group was observed after 2 weeks (Figs. 7E, 7F). The intensity of the vimentin staining in the inner retinal layers was comparable to that in the adult retinal control (data not shown). Increased labeling of vimentin (Fig. 7C) and GFAP (Fig. 7D) in all retinal layers of the crybb2 group was seen after 2 weeks. Compared to the PBS group, the crybb2-NPC animals exhibited reduced staining of vimentin and GFAP in the outer retinal layers, including the NFL, RGC layer, inner plexiform layer (IPL), INL, and the outer plexiform layer (OPL), after 4 weeks. Increased labeling of the OPL was detected. At 4 weeks, the staining profiles of both proteins were similar to those found at 2 weeks (Figs. 7A, 7B). WB analysis revealed lower
levels of vimentin in the PBS group (122.48 ± 48.17%, P < 0.05) and the crybb2-NPC group (144.3 ± 10.3%, P < 0.01) compared to the crybb2 group (204.84 ± 17.42%) after 2 weeks (Figs. 7I, 7J). In contrast, significantly decreased vimentin was observed in the crybb2-NPC group (109.53 ± 55.83%) compared to the crybb2 (205.23 ± 28.46%) and PBS groups (186.66 ± 11.31%) after 4 weeks (P < 0.05; Figs. 7I, 7J).

Retinal Microglia Responses to Intravitreal Injection of crybb2-NPC and crybb2

Immunohistochemical evaluation revealed a significant increase in the recruitment and activation of microglia in the PBS group. Microglia were found in the GCL and NFL, while in the crybb2 and crybb2-NPC groups those cells were observed in the IPL, INL, and OPL (Figs. 8A–D). Quantitatively, after 2 weeks, 22.40 ± 3.36 cells/0.2 mm² of recruited microglia were found in the PBS group, compared to 16.5 ± 3.7 cells/0.2 mm² in the crybb2 group (P < 0.05) and 13.86 ± 1.58 cells/0.2 mm² in the crybb2-NPC group (P < 0.01; Fig. 8E). After 2 weeks, increased numbers of activated microglia were found in the PBS group (13.20 ± 1.79 cells/0.2 mm²) compared to the crybb2 group (7.50 ± 2.38 cells/0.2 mm², P < 0.05) and the crybb2-NPC group (6.00 ± 2.74 cells/0.2 mm², P < 0.01; Fig. 8F). After 4 weeks, microglia were observed in the GCL, NFL, and OPL in the PBS group, while in the crybb2 and crybb2-NPC groups they also were found in the ONL (data not shown).

In summary, fewer microglia were found in the crybb2 group than in the other groups. Significantly increased numbers of microglia were found in the PBS group (18.25 ± 3.60 cells/0.2 mm², P < 0.05) and the crybb2-NPC group (16.75 ± 1.58 cells/0.2 mm², P < 0.01) compared to the crybb2 group (14.50 ± 0.55 cells/0.2 mm²; Fig. 8E). Increased numbers of activated cells were found in the crybb2-NPC group (12.13 ± 1.73 cells/0.2 mm²) compared to the PBS group.

**Table 4. Localization of Grafted NPCs after Transplantation**

<table>
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<th>Localization</th>
<th>NPC – 2 weeks</th>
<th>NPC – 4 weeks</th>
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<tbody>
<tr>
<td>Cornea</td>
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<td>X</td>
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<tr>
<td>AC</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>GCL</td>
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<tr>
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<td>ONL</td>
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<td>X</td>
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<tr>
<td>IPL</td>
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<tr>
<td>INL</td>
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<tr>
<td>RPE</td>
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<td>X</td>
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<tr>
<td>Choroidea</td>
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</table>

Grafted crybb2-NPC were found throughout the retinal layers, and in extraretinal tissue 2 and 4 weeks after grafting. AC, anterior chamber.
group (9.5 ± 1.0 cells/0.2 mm², P < 0.01) and the crybb2 group (8.17 ± 1.33 cells/0.2 mm²) after 4 weeks (P < 0.05; Fig. 8F). The data showed that microglial cells appear to be sensitive indicators of activation in all of the experimental groups of this study.

DISCUSSION

In our study we examined the fate of intravitreally grafted NPCs within the eye and the responses of retinal cells to the grafting of either nontransfected or transfected NPCs exemplified by crybb2-NPC. There were three principal findings in this study:

1. NPCs reside and migrate within the eye and continue to produce NTFs.
2. Crybb2 and crybb2-NPC reduce the axotomy-induced loss of RGCs and changes in photoreceptor rhodopsin. crybb2-NPC reduce glial reactivity, indicating that crybb2 is involved in retinal repair mechanisms.
3. Crybb2 reduces the recruitment and activation of retinal microglia, suggesting a reduced inflammatory response.

Intraocular Characteristics of NPCs

NPCs harvested from cultures and injected into the vitreous cavity were able to produce the same markers used to characterize them in culture. This finding suggested that the host environment does not change the profile of proteins synthesized within the vitreous body. This observation was crucial for setting the basic data for injecting transfected NPCs. We observed a migration of intravitreally applied crybb2-NPC within the entire eye for up to 4 weeks after grafting. A previous study demonstrated an overall integration of NPCs into the neonatal retina of rats after 4 weeks.11 In agreement with our results, previous studies have detected the retina as the main donor for implanted NPCs in cases of injured,2 ischemic,27 or degenerating retinas.10 The preferred location was the ONL, the subretinal debris zone, and the layer of photoreceptors.10 Our observations revealed the detection of transplanted crybb2-NPC in the vitreoretinal compartment, subretinal space, and choroid after 2 weeks, while crybb2-NPC were found in the GCL, INL, and ONL after 4 weeks. Crybb2-NPC were associated with the anterior chamber, corneal endothelium, and ciliary body after 4 weeks. To our knowledge, this is the first demonstration of an overall migration of grafted crybb2-NPC through the entire rat eye. Migration over long distances is not surprising, since it has been shown that NPCs are able to produce extracellular matrix-degrading metalloproteinases.8

Responses of the Ocular Tissue

Crybb2 was found to support the survival of axotomized RGCs due to compression and prevent the dissolution of rhodopsin.
in photoreceptors. Crybb2 usually is produced within the regenerating retina and facilitates the elongation of regenerating axons in retinal explants. Therefore, it is not surprising that significantly fewer RGCs were lost in the crybb2 and crybb2-NPC groups than in the PBS group. These data confirmed the involvement of crybb2 in the repair of RGC metabolism after injury to retinal axons, although the mechanisms underlying the signal transduction remain to be elucidated. Liedtke et al. have shown that GFP-labeled crybb2 is internalized by RGCs in culture. It is most likely that such uptake takes place in vivo and is responsible for the survival effects observed in our study.

Reduction of rhodopsin and its delocalization within the POS is an early indication of degenerative onset. We found that crybb2-NPC delayed rhodopsin decay.

It has been assumed that growth-factor failure is one reason for photoreceptor death in inherited retinal photoreceptor degenerations. The external supply of growth factors, such as bFGF, protects photoreceptors. Higher levels of growth factors, such as bFGF, ciliary NTF (CNTF), and BNDF, were found to be associated with photoreceptor death in RCS

**Figure 5.** (A-F) Effects of crybb2 and crybb2-NPC on RGCs in the retina. Expression of neurofilament 200 (NF-200, red) shown by immunohistochemical staining of slices (10 μm) of samples from animals treated with PBS (A, B) and crybb2-NPC (C, D) after 2 and 4 weeks. Cell nuclei are marked blue with DAPI. Cy-3 was used for negative control as a secondary antibody (data not shown). (E) Number of persistent RGCs (per 0.2 mm²) in retinal slices (10 μm) in relation to that of adult control tissue (in %). The number of RGCs decreased in the PBS group (P < 0.001) and crybb2 group (P < 0.01) compared to crybb2-NPC-treated animals after 2 weeks. Decreased numbers of RGCs were observed in the PBS group compared to the crybb2 (P < 0.001) and the crybb2-NPC (P < 0.001) groups after 4 weeks. (F) WB analyses of retinas. Lysates of retinas treated as described were prepared and tested for brain-specific homeobox/POU domain protein 3b (BRN-3b, 45 kDa). Calnexin was used as the loading protein control. Less protein was detected in the PBS group than in the crybb2 and crybb2-NPC groups after 2 and 4 weeks. Scale bar: 20 μm (A-D). Statistical differences: **P < 0.01, ***P < 0.001.
FIGURE 6. (A–D) Expression of retinal rhodopsin (green) shown by immunohistochemical staining of slices (10 μm) of samples from animals treated with PBS (A, E), crybb2 (B, F), and crybb2-NPC (C, G) after 2 and 4 weeks. The negative control is shown with Cy-2 (D, H) as the secondary antibody. (I) WB analyses of retinas were prepared and tested for rhodopsin expression. The expression of calnexin verified the amount of protein that loaded per lane. (J) Corresponding densitometric analyses of the WB results (n = 4) in PBS-, crybb2-, and crybb2-NPC–treated animals 2 and 4 weeks after ONC. The rhodopsin expression was lower in the crybb2-NPC group than in the crybb2 group after 2 weeks (P < 0.05). A significant reduction in rhodopsin expression was observed in the PBS group compared to the crybb2 (P < 0.001) and crybb2-NPC groups (P < 0.01) after 4 weeks. The rhodopsin expression was lowest in the PBS-treated group. Scale bar: 20 μm (A–H). Statistical differences: *P < 0.05, **P < 0.01, ***P < 0.001.
FIGURE 7. (A–H) Expression of retinal vimentin and GFAP shown by immunohistochemical staining of slices (10 μm) of samples from animals treated with PBS (A, B), crybb2 (C, D), and crybb2-NPC (E, F) 2 weeks after ONC. Glial cells are marked red with vimentin and green with GFAP. The negative control is shown with Cy-3 (G) and Cy-2 (H) as secondary antibodies. (I) WB analyses of retinas. Lysates of retinas treated as described were prepared and tested for vimentin expression. Calretinin expression verified the amount of protein that loaded per lane. (J) Corresponding densitometric analyses of the WB results (n = 4) in PBS, crybb2, and crybb2-NPC-treated animals 2 and 4 weeks after ONC in relation to control.
The vimentin expression significantly increased in the crybb2-treated animals compared to the control and the animals treated with PBS \( (P < 0.05) \) and crybb2-NPC \( (P > 0.01) \) after 2 weeks. After 4 weeks there were no significant differences in vimentin in the PBS- and crybb2-treated groups \( (P > 0.05) \), whereas there was less vimentin expression in the crybb2-NPC-treated group \( (P < 0.05) \). Scale bar: 20 \( \mu \)m (A-H). Statistical difference: *\( P < 0.05 \), **\( P < 0.01 \).

**FIGURE 8.** (A–D) Expression of ED-1 and Iba-1 in retinal microglial cells, as assessed by antibody staining of slices (10 \( \mu \)m) from animals treated with PBS (A), crybb2 (B), and NPC (C) 2 weeks after ONC. Microglial cells are marked red for ED-1 and green for Iba-1. ED-1 and IBA-1 positive cells were found only in the NFL and GCL in the PBS-treated animals, whereas they also were found in the INL, IPL, and OPL in the crybb2- and NPC-treated groups. The negative control is shown with Cy-2 and Cy-3 (D) as the secondary antibodies. (E, F) Quantification of retinal microglial cells. (E) Count of ED-1/Iba-1-stained cells in the retinal layers. (F) Count of Iba-1 positive microglia cells. Scale bars: 20 \( \mu \)m (A–D). Statistical differences: *\( P < 0.05 \), **\( P < 0.01 \).
ONC.45 The dynamic relationship between intermediate progenitor cells from apoptosis. 33 BDNF, which is expressed crystallins in vitro.23 Crystallins of the have been shown that RGCs and astrocytes are donors of retinal factors, crybb2.

we have examined closely the overexpression of one of these factors, crybb2. Increased levels of crystallins of the \( \beta/\gamma \) superfamily were found in animal models of retinal diseases, like retinitis pigmentosa, light-induced damage, and diabetic retinopathy.58–60 Compared to observations of increased crybb2 expression in regenerating retinas (e.g., GCL and INL) after ONC, no expression was found in unaffected eyes.26 This line of evidence, together with our own findings, suggests that crybb2 exerts beneficial effects on damaged RGCs and secondarily on photoreceptors. One of the mechanisms operating through crybb2 may be an increase in the amount of neuroprotective CNTF released by astrocytes.10 To our knowledge, no other studies have investigated the neuroprotective effects of intravitreal crybb2 in vivo.

An early and significant increase in glial cell activity due to crybb2 administration was found along with decreased vimentin expression in the crybb2-NPC group. A complex glial network, including Müller cells and astrocytes, has neuroprotective effects, releasing NTFs, such as bFGF, CNTF, and antioxidants after retinal injury.16,41,42 Early upregulation of vimentin and GFAP occurs in several retinal disorders, such as retinopathy pigmentosa, macular dystrophies, or secondary retinal degenerations.43,44 and after ONC.45 The dynamic relationship between intermediate filaments and retinal glial cells is the key mechanism underlying the rapid modification of the Müller cell structure in response to changes in the retinal environment.46–47 It has been shown that RGCs and astrocytes are donors of retinal crystallins in vitro.23 Crystallins of the \( \beta/\gamma \) superfamily are released during periods of critical stress in the retina.16,23 Increased retinal GFAP expression induces glial cells to switch into a regenerative state after the intravitreal injection of crystallin \( \beta/\gamma \).16 Our observations confirmed the finding of a direct association between glial reactivity and crybb2. The crybb2-NPC–mediated effects on glial cells differ considerably. We concluded that additionally secreted NTFs by crybb2-NPC may inhibit retinal glial activity.

NPCs and NTFs

While the exact mechanism underlying the effects of NPCs within the retina are not understood fully, it is likely to be based on growth-factor production.68 Retinal NTFs exert protective effects on neighboring host neurons.49,50 Several NTFs delay RGC death and photoreceptor loss: fibroblast growth factors (e.g., bFGF), CNTF, brain-derived NTFs (e.g., BDNF), and insulin-like growth factors. RGCs appear to be better protected by BDNF, while photoreceptors may be better protected by bFGF and CNTF.34 In our study, modified crybb2-NPC overexpressed crybb2 in addition to NTFs for up to 4 weeks after transplantation in vivo. Nestin is expressed during CNS stem-cell and progenitor-cell proliferation, and neuronal migration,51 and protects progenitor cells from apoptosis.52 BDNF, which is expressed by glial cells and neuronal cells,53,54 prolongs the survival of RGGs in vitro, reduces RGC death during development, and exerts survival effects on injured adult neurons.53,54,55 Calcineurin is expressed in the RGCs of rat retinas and exerts important cellular effects in the immune and nervous systems.55,56 It is involved in acute and chronic disorders resulting from glaucoma.56 Oncomodulin has a high affinity for RGCs, exerting axon-promoting effects on these cells in mature optic nerves in vivo,57 and has a central role in inflammation-induced regeneration.58

Microglia

Microglial recruitment and activation are associated with retinal degeneration, as shown in the RCS rat.59 These cells respond to several proinflammatory factors, including lipopolysaccharides and cytokines.60,61 On the other hand, they produce and secrete NTFs, such as CNTF, GDNF, and nerve growth factor, to protect photoreceptors from cell death.62

The number of activated microglial cells was highest in the PBS group after 2 weeks, suggesting an increased response to dying neurons and inflammatory disorders. Inflammatory cytokines may promote the transformation of ramified to active microglia. The effect is more predictable compared to that of crybb2, which we attribute to increased cell death within the retina. An increased activation and recruitment of microglia in the crybb2-NPC group after 4 weeks was another interesting finding. An attenuated loss of RGCs and photoreceptors is indicative of a lower rate of retinal cell death in this group. Furthermore, several NTFs secreted by NPCs have inhibitory effects on the cellular immune response. We suggest a microglial reaction to the cellular response of NPCs.

CONCLUSIONS

In conclusion, to our knowledge this is the first study to show the migration of intravitreally injected NPCs through the entire rat eye. This finding implies that NPCs are able to interact with different types of ocular tissue. Crybb2-NPC prevents RGC loss and decay of rhodopsin in the injury-induced model of retinal degeneration. In addition to their survival and migration within the eye, crybb2-producing NPCs increase the chances of survival of RGCs and photoreceptors. The expression of further survival-promoting factors may be introduced in these cells, thus assuring a permanent and approximately constant delivery within the eye. This strategy of creating factor-producing “cell-factories” in the neighborhood of areas of degeneration may become a fruitful approach for the treatment of retinal disorders by providing bioavailable growth factors.

Acknowledgments

M. Wissing and M. Langkamp-Flock provided skillful technical assistance, and M. Reis typed the manuscript. English Science Editing (ESE) provided native linguistic editing of the manuscript.

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