Double Cone Dystrophy and RPE Degeneration in the Retina of the Zebrafish gnn Mutant

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PURPOSE. To characterize morphologic alterations in the retina of the visual mutant zebrafish gantenbein (gnn) and to examine whether these alterations correlate with those present in human hereditary eye diseases.

METHODS. The gnn mutant was isolated by behavioral and macroscopic screening. Retinas of gnn zebrafish larvae were examined at different developmental stages from 2 to 9 days postfertilization (dpf) by standard histologic staining techniques and by immunocytochemistry. Ultrastructural alterations were examined by electron microscopy. The genetic map position of the induced mutation was identified by mapping with two candidate primer pairs on single larvae.

RESULTS. The gnn mutant exhibited shortened outer photoreceptor segments and altered RPE morphology. In the photoreceptor layer of the mutant, the total number of lectin-labeled cones was reduced in all developmental stages from 2 to 7 dpf, whereas the amount of rhodopsin-positive cells remained at the wild-type (WT) level. Labeling with zebrafish opsin antibodies revealed dystrophic red cones at 5 dpf, whereas the morphology of all other cone types was largely unaffected. Electron microscopy unveiled electron-dense deposits between the discs of the double cone outer segments. In addition, the onset of progressive RPE degeneration was observed at this stage of development. At later stages, all cone types and the RPE became degenerative. The morphology of distinct sections remained largely unaffected by the mutation. The gnn mutation was located approximately 4.3 cM from the simple sequence length polymorphism (SSLP) marker Z15453 on linkage group 16.

CONCLUSIONS. In gnn mutant zebrafish, cones, and especially red cones, are dystrophic in early retinal development. Subsequent to this cone dystrophy, the RPE becomes dysfunctional and starts to degenerate in later stages of development. Thus, the early developmental morphology of gnn exhibits similarities to cone dystrophies most commonly seen in age-related macular degeneration (AMD) among humans, whereas the later stages of degeneration in gnn resemble RPE alterations in retinitis pigmentosa (RP) in humans. The gnn zebrafish mutant may therefore be a useful model for examining the possible interplay and connection between cone dystrophy and RPE degeneration. (Invest Ophtalmol Vis Sci. 2003;44:1287–1298) DOI:10.1167/iovs.02-0363

Hereditary eye diseases in humans (RP1 and AMD2) and in animal models (RCS rat; rd4 and rds mice; prcd5 and rcd17 dogs, and zebrafish9) predominantly affect photoreceptors. Even though these diseases are similar in morphologic pathogenicity, their genetic origin may be due to mutations affecting different proteins, such as the photograph pigment itself, proteins of the transduction cascade, or metabolic products in the retinal pigment epithelium (RPE).29 A growing body of evidence indicates that a mutation expressed in one particular retinal cell type may affect not only these cells autonomously, but also may affect nonautonomously other retinal cells in which the mutation is not expressed.10 Clear evidence for such non–cell-autonomous effects are rod mutations that cause cone and RPE degeneration in RP and similar disorders in experimental animals.3,5 The mechanisms underlying these non–cell-autonomous degenerations remain unknown. They possibly involve trophic or toxic interactions, mediated either by diffusible factors or by contact-mediated cell interactions.

In recent years, several studies of degenerative processes in vision-specific zebrafish mutants have been performed. In all these experiments, mutant larvae were identified by specific behavioral assays, either by testing the optokinetic response (OKR)8,11 or optomotor response (OMR)8 or by inducing an escape response to a threatening object.12 A number of mutants with altered morphology in all retinal layers have been briefly described by Fadool et al.13 Brockerhoff et al.14 identified the red-blind mutant, partial optokinetic response b (pob). Homozygous pob mutants show altered electroretinogram (ERG) thresholds and exhibit a selectively decreased number of red cones, whereas other cone types remain unaffected in these larvae. A non–photoreceptor-cell–specific mutation, night blindness a (nha), was identified by Li and Dowling12 and shows parallels to RP in morphology and physiology (i.e., degenerating rod photoreceptors, a shift in the dark adaptation curve to photopic levels, and a reduced b-wave in the full-field ERG). In a further ERG examination, five mutants with absent or altered OKR and slightly modified ERG were described.15 Allwardt et al.15 described the recessive mutant nrc, which exhibits no OKR but has a highly unusual ERG, in all probability due to abnormal, immature-appearing photoreceptor terminals, which are characterized by “floating” synaptic ribbons, unassociated with postsynaptic processes or arciform densities.

Recently,8 we examined OKRs and OMRs of 450 zebrafish mutants previously isolated on the basis of defects in organ formation, tissue patterning, pigmentation, axon guidance, or other visible phenotypes. Twelve mutant zebrafish with various defects of the visual system and 13 blind mutants with outer retina dystrophy were identified. As in humans and in retinal dystrophies in other animal models, loss of photoreceptors was the single most common cause of inherited blindness in zebrafish. Lakritz (lak), one of the mutants identified in this screening, was described only very recently by Kay et al.17 The mutant shows a clear reduction in the number of ganglion cells (RGCs) but overproduction of inner retinal cells such as bipo-

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Supported by the Graduate School of Neurobiology Tübingen of the German Research Foundation and fortune Grant 882/0-0.

Submitted for publication April 11, 2002; revised August 14 and October 4, 2002: accepted October 11, 2002.

Commercial relationships policy: N.

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lar and Müller cells (MCs). Cloning of the *lak* gene revealed that *lak* encodes atonal homologue 5 (*atb5*), a member of a family of vertebrate basic helix-loop-helix (bHLH) transcription factors that is presumed to play a crucial role in inducing the cell-fate switch in retinal progenitor cells to give rise to RGCs.

Because of its short generation period, its ease of care, and the possibility of mutagenesis, the zebrafish is the only vertebrate animal model that has thus far enabled researchers to perform large-scale mutant screening with a reasonable amount of effort. In addition, the chance of identifying the affected genes of nearly all mutant phenotypes isolated so far increases with advancing knowledge of the zebrafish genome sequence and the many possibilities of genetic intervention in zebrafish development.

Conscious of all these advantages and possibilities of the zebrafish as a model organism, we undertook a search for specific zebrafish mutations that exhibit morphologic analogies to human retinal degenerative diseases and succeeded in isolating visual mutant larvae by screening the offspring of zebrafish at 5 days postfertilization (dpf).

In this article, we describe one of these recessive mutants, gantenbein (*gnn*), which has small eyes and expanded mela-nophores but a normal OKR and is therefore not blind. Morphologic analyses showed that the retinas of *gmn* mutants exhibit a dystrophic cone system and subsequent rod and RPE degeneration.

The pathologic morphology in *gmn* mutants includes cone dystrophy, especially among the red-green-sensitive double cones (DCs), although the rod system develops, normally for the most part. During ongoing development, the dystrophic DCs lead to a consecutive degeneration of all cone types and consequently to degeneration of the rod system and the RPE, with RP-like alterations. In a first mapping approach, we identified the *gmn* mutation on linkage group (LG) 16.

**METHODS**

**Fish Maintenance and Breeding**

Fish were maintained and bred as described elsewhere.

The zebrafish strains used were Tübingen (Tu) and Tübingen Long Fin (TL).20,21 Gantenbein (*gnn*), larvae were obtained by mating of identified heterozygous carriers and sorted according to eye size and body pigmentation phenotype. Embryos were raised at 28°C in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, and 0.33 mM MgSO₄) and staged according to development in days postfertilization.

**Mutagenesis**

All experiments were in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. In a screening for mutations in the visual system, 16 wild-type (WT) males (WIK), females (of Tu or TL background) were prepared and mounted on slides to be used in a weekly interval. We identified the *gmn* mutation on linkage group (LG) 16.

**Screening**

To isolate mutants with visual defects, optokinetic behavior was tested as previously described. Briefly, 5-day-old larvae were placed inside a rotating drum (4–12/sec) with black-and-white stripes (eight stripes of 25° width). Larvae were immobilized in 2.5% methylcellulose to suppress an OMR. Larvae with an appropriate OMR showed a smooth-pursuit movement of the eyes in the direction of the rotating drum followed by a fast saccade to reach the initial position.

**Retinal Histology**

Before fixation, larvae were anesthetized on ice at 4°C. For light microscopy, they were then immediately fixed in 4% paraformalde-hyde in 0.2 M phosphate buffer (PB; pH 7.4) for 1 hour (4°C). Larvae for electron microscopy (EM) were fixed in 1% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M PB overnight.

For standard histology, fixed larvae were dehydrated in a graded series of ethanol-water mixtures and embedded in plastic (Technovit 7100; Kulzer) because this method allows thin sectioning (<5 μm) with a high level of tissue preservation, permitting many sections to be obtained from the small mutant eyes. Furthermore, no overlying cells affected the observation of the labeled cells, and morphometry was easily and reliably feasible on these sections. On the other hand, we used cryostat sections for all the antibodies that failed to label on plastic sections.

**Immunocytochemistry**

We used two different embedding techniques for our immunohisto logic observations. On the one hand, we used plastic sections (Technovit 8100; Kulzer) because this method allows thin sectioning (<5 μm) with a high level of tissue preservation, permitting many sections to be obtained from the small mutant eyes. Furthermore, no overlying cells affected the observation of the labeled cells, and morphometry was easily and reliably feasible on these sections. On the other hand, we used cryostat sections for all the antibodies that failed to label on plastic sections.

**Plastic Sections.** Fixed larvae were carefully dehydrated in a graded series of ethanol-water mixtures and subsequent 100% acetone wash (1 hour) for final dehydration. After overnight infiltration in embedding medium (Technovit 8100; Kulzer) basic solution, larvae were positioned in polymerization medium for at least 3 hours (4°C). After polymerization, the specimens were sectioned at 5 μm and collectedonto poly-L-lysine-coated slides, air dried, and stored at −20°C for further processing.

Slides were thawed, washed three times in PBS (50 mM, pH 7.4), and then incubated in a solution of PBS with 20% normal goat serum (NGS; Sigma) for 1 hour. Sections were then incubated with antipeanut agglutinin (PNA) lectin (Sigma) conjugated with FITC 1:10 for 1 hour. Afterward, slides were washed in PBS and incubated with monoclonal mouse anti-rhodopsin (Biodesign, Saco, ME) in 1:500 PBST at 4°C overnight. Immunoreaction was detected using Cy3-labeled goat anti-mouse IgG diluted 1:1,000. Sections were then overlaid with 4',6-diamino-2-phenylindole solution (DAPI; Vectastain, Vector Laboratories, Burlingame, CA), to counterstain the cell nuclei, and covered with.

**Cryostat Sections.** Fixed larvae were cryoprotected in 30% sucrose for at least 4 hours. The whole larvae were embedded in tissue-freezing medium (Cryomatrix; Reichert-Jung, Vienna, Austria) and rapidly frozen in liquid N₂. Sections (10 μm thick) were cut at −20°C, mounted on gelatinized slides, and air dried at 37°C for at least 2 hours. The slides were stored at −20°C until further use.

For immunohistochemistry, slides were thawed, washed three times in PBS (50 mM, pH 7.4), and incubated in 20% NGS and 2% BSA in 0.3% PBS/Triton X-100 (PBST) for 1 hour. Sections were then incubated overnight in primary antibody in PBST at 4°C. Immunoreac-
Retinal Degeneration in the Zebrafish gnn Mutant

PNAS, March 2003, Vol. 100, No. 5

As in most of the recessive zebrafish mutants described so far, the gnn phenotype seems not to be restricted to the retina but is also expressed in other body parts (e.g., most of the embryos die until 9 dpf). Thus, it is likely that the swim bladder does not form normally and that the embryos are consequently unable to feed. However, up to now, none of the alterations that may occur outside the retina have been described.

Developmental Morphology of the gnn Mutant Eye and Retina

To obtain insight into differences in retinal development between gnn and WT eyes, we analyzed mutant and WT retinas between 3 and 5 dpf with standard histologic techniques (Fig. 1) and quantified the number of cells in the nuclear layers as well as the eye radius, in the different developmental stages (Fig. 2). At the early developmental stage of 3 dpf, only minor differences were observable between WT (Fig. 1A) and gnn (Fig. 1D) retinas; all five retinal layers were fully identifiable. However, the gnn lens (diameter, 65 μm) appeared smaller than in the WT eye (diameter, 74 μm). At this stage, the number of cells in the ganglion cell layer (GCL) and outer nuclear layer (ONL) were at similar levels in WT and gnn retinas, whereas the number of cells in the inner nuclear layer (INL) was already reduced by a third in the gnn retina (Figs. 2C, 2D). However, the eye radius was almost identical in WT and gnn larvae (Figs. 2A, 2B). At 4 dpf, the small lens, and, partially therefore, smaller eye size (Figs. 2A, 2B) of gnn became evident, and the number of cells was reduced in all nuclear layers of the gnn retina (Figs. 2C, 2D). In addition, shortened cone outer segments (OS); Fig. 1E) were identified. The RPE was hypotrophic and pale and started to overgrow the lens, and the difference in the eye radius between WT and gnn larvae had increased (Figs. 2A, 2B). Cells in the GCL and INL of the WT retina were almost twice as numerous as the cells in the gnn retina nuclear layers (Figs. 2C, 2D), whereas the number of cells in the ONL was reduced by one third in the gnn mutant. Thus, the number of cells in all nuclear layers of gnn was reduced by approximately one third, because of ongoing degeneration. The lamination of the inner retina developed normally in gnn mutant embryos. No differences in lamination were observable between gnn and WT retinas at 5 and 5 dpf.

Development and Degeneration of Photoreceptors and RPE in the gnn Mutant Retina

Cone Dystrophy. To identify whether the cone matrix sheath (CMS) is affected by the gnn mutation, we labeled gnn larvae with the lectin PNA, which did not showed any abnormalities in its OKR performance but expressed expanded melanophores and smaller eye size.

RESULTS

We screened approximately 100 homozygous phenotypes with recessive mutations at 5 dpf and uncovered 20 phenotypes with alterations of either the OKR, eye size, or body pigmentation. It turned out that fish with undisturbed OKRs but expanded melanophores and/or small eyes were most likely to express a gradual course of retinal degeneration, because they were not blind a priori. One of these mutants is gnn, which did not show any abnormalities in its OKR performance but expressed expanded melanophores and smaller eye size.

Electron Microscopy

The EM-fixed larvae were washed in 0.1 M PB for 2 hours and postfixed in 1% osmium tetroxide for 1 hour, 20 minutes. After a rinse in 0.1 M PB, specimens were dehydrated in a graded series of ethanol-water mixtures up to 70% ethanol, and then contrasted in 2% uranyl and 70% ethanol acetate at 4°C overnight. On the following day ethanol dehydration was continued to 100%. After preinfiltration in 1:1 100% ethanol/embedding resin (TAAB Laboratories, Aldermastron, UK), larvae were infiltrated in pure embedding resin overnight. Larvae were then positioned in Beem caps with fresh resin and polymerized at 60°C for approximately 16 hours. Ultrathin transverse sections 40 nm thick were prepared and stained with lead citrate. Sections were examined and photographed with a transmission electron microscope (model EM 900; Carl Zeiss, Oberkochen, Germany).

Quantitative Analyses

The radius in the developing eyes of WT and gnn zebrafish was defined as the distance from the center of the lens to Bruch’s membrane and was measured on Richardson-stained plastic sections.

The number of cells in the different retinal layers was quantified on 3-μm-thick Richardson- or DAPI-stained plastic sections. The number of red, green, blue, and UV opsins- and rhodopsin-labeled photoreceptors was quantified on 12-μm-thick radial sections (n ≥ 3 for all opsins and rhodopsin in WT and gnn retinas) and the difference in the number of opsins- and rhodopsin-labeled photoreceptors between WT and gnn was determined with a protected Tukey post hoc test with a 5% level of confidence.

Genetic Map Position

The gnn mutation is affected by the CMS and is expressed in other body parts (e.g., most of the embryos die until 9 dpf). Thus, it is likely that the swim bladder does not form normally and that the embryos are consequently unable to feed. However, up to now, none of the alterations that may occur outside the retina have been described.

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these tissues, which consist mainly of nerve fibers, are labeled by PNA in the WT but not in gnn. Because this labeling has not been described in any other publication concerning PNA labeling so far, it may be zebrafish-specific labeling that should be characterized in future experiments.

To find out whether all four cone types were affected by degeneration or whether the degeneration was specific for a certain cone type, we analyzed the expression of the four different cone opsins (UV, blue, green, and red) by labeling WT and gnn retinas with the respective antibodies at 5, 7, and 9 dpf. The 5-dpf embryonic age was chosen as initial stage for our opsin observations because all four cone types are identifiable by opsin antibodies at this time in WT zebrafish. However, differences in the stage of maturation of the different cone types at this time of development are reflected in the opsin immunolabeling: Rather, immature cones showed diffusely labeled somata and labeled OS (blue cones: Figs. 4A, 4B; green cones: Figs. 4E, 4F), whereas almost mature cones showed distinct opsin labeling in the OS only (UV cones: Figs. 4C, 4D; red cones: Figs. 4G, 4H). In addition, some of the opsin antibodies may label cone terminals (Figs. 4A, 4B, 4E-G). To determine the differences in opsin labeling between WT and gnn larvae, we focused on the differences in the labeling pattern specific to the particular opsin under investigation and ignored the previously described differences in other opsin labeling.

At 5 dpf, UV opsin was identified similarly in WT (Fig. 4C) and gnn (Fig. 4D) retinas as a distinct labeling of the cone OS. Labeling for blue opsin revealed the same number of marked cones in WT (Fig. 4A) and mutant (Fig. 4B) animals 5 dpf. Both siblings showed labeled pedicles; however, blue opsin labeling in the WT retina was restricted to the OS, and the somata were only faintly labeled (Fig. 4A), whereas blue opsin immunoreactivity in gnn was most distinct in the somata, thus indicating an immature or degenerated state of the blue-sensitive cones.

Labeling for green opsin was similar in WT (Fig. 4E) and gnn (Fig. 4F) retinas at 5 dpf, with immunoreactivity restricted to the cone pedicles and the OS in both WT and gnn retinas.

In contrast to UV, blue, and green opsins, only a few cones expressed immunoreactivity for red opsin in the gnn retina (Fig. 4H) at 5 dpf. WT larvae, in contrast (Fig. 4G), showed a large amount of red-opsin-labeled OS over the entire retina. Quantitation of the number of opsin-labeled photoreceptors per examined retinal section (Fig. 5) suggests that the red-sensitive member of the DCs is the most severely affected cone type at the 5 dpf stage, because the number of labeled red cones in the WT differed significantly (P ≤ 0.01) from that in the gnn larvae. Even though we noted a slight decrease in the number of green cones as well, none of the differences between the other cone types in WT and gnn larvae were significant at this developmental stage. Thus, the clear reduction of red cone labeling in the gnn mutant indicates that disruption of the gnn gene leads to cone-specific defects in the retina, most severely affecting the red cones.

In later developmental stages, at 7 and 9 dpf, the immunoreactivity was largely reduced for all types of opsins (data not shown). Thus, the initial defect in red cones shifted to a more generalized disease process that affected all types of cones.

To determine the effects of the gnn mutation on the ultrastructural level, we examined retinal development of mutant retinas between 5 and 9 dpf by means of transmission electron microscopy. In the early developmental stage at 3 dpf we observed normal development of the photoreceptors. Cone inner segments (IS) as well as cone OS were clearly observable in WT (Fig. 6A) and gnn (Fig. 6B) retinas. Two days later (5 dpf), retinal dystrophy became evident in the gnn retina. Unlike the situation in the WT (Fig. 6C) at 5 dpf, where the ongoing elongation of the OS indicated the progress of...
normal retinal development, *gnn* expressed clear retinal dystrophy at this developmental stage. OS were shortened in the mutant retina (Figs. 6D, 6E). In addition, electron-dense debris was identified in the OS (Figs. 6D, 6E, 6G) of the *gnn* cones. This debris was observed only in the OS of the red-green-sensitive double cones (DC-OS), which lay close to the RPE, and never in the OS of blue- or UV-sensitive single cones (SC-OS), which were located more toward the vitreous (Fig. 6E). Figure 6F shows a higher magnification of SC-OS, where no electron-dense debris was identified between the forming discs of the OS. In contrast, a close-up of the DC-OS (Fig. 6G) revealed the location of the electron-dense debris in the intermembrane space of the red-green-sensitive cone. At 6 dpf, there were very few severely shortened OS left in the *gnn* retina (Fig. 7), and the different cone types were no longer distinguishable. It seems that after this stage of development the primarily unaffected SCs (blue and UV) became degenerative as a consequence of the severely dystrophic DCs and the degenerating RPE (see later description), because cone outer segments were virtually absent in the later developmental stages at 7 and 9 dpf.

**Effect on Rods.** To identify whether photoreceptor degeneration was restricted only to the cones or whether the rods were also affected, we labeled WT and *gnn* zebrafish larvae with anti-rhodopsin between 3 and 5 dpf. In the 3 dpf developmental stage, rhodopsin labeling was first present in the far periphery of both WT and *gnn* retinas (Figs. 3A, 3B). These first rod photoreceptors were located near the marginal zone, a special region of the fish retina, where photoreceptors are added to the retina throughout life. At later developmental stages (4–5 dpf), labeled rods were also identified in more central areas of the WT (Figs. 5C, 5E) and *gnn* (Figs. 3D, 3F) retinas. These data, together with the quantitation of the rhodopsin labeling (shown for 5 dpf only in Fig. 5), which showed that there were no significant differences between rhodopsin-labeled rods in WT and *gnn* larvae, showed that rod development was not affected by the *gnn* mutation between 3 and 5 dpf. The number and localization of rods in the mutant retina (Figs. 3B, 3D, 3F) remained similar to that observed in WT siblings (Figs. 3A, 3C, 3E) at the respective developmental stages. Even in the later developmental stages up to 9 dpf, we identified identical rod labeling in WT and *gnn* retinas. Rod development therefore appears to be largely unaffected by the *gnn* mutation, because we observed developing rods in the *gnn* ventral retinal periphery at all developmental stages. Only a few rods were identified in the central retina at all developmental stages examined in WT and mutant retinas. This can be attributed to the fact that the zebrafish retina is cone dominated throughout development until 12 dpf. Because *gnn* mutant larvae rarely survived the 9-dpf stage, we never identified any rods in our electron microscopic observations (Fig. 6, 7) of the central retina.
Effect on the Retinal Pigment Epithelium. We examined the ultrastructure of the RPE at developmental stages between 3 and 9 dpf in the gnn mutant retina. Up to 3 dpf, RPE development was completely normal in gnn larvae, and no differences were identifiable between WT (Fig. 6A) and gnn (Fig. 6B) retinas. In both groups, RPE cells showed normal morphology, and the melanin granules had a rounded shape (Figs. 6A, 6B). Two days later, at 5 dpf, the shape of the melanin granules in the RPE of gnn mutant larvae had changed from round and egglike structures to much smaller and coarser pigments. Furthermore, RPE cell microvilli extended abnormally deep into the retina down to the level of the cone IS (Figs. 6D, 6E, 6F). In the WT retina (Fig. 6C), RPE cell microvilli never extended that far into the retina at this stage of development. However, phagocytosis of the shed discs seemed not to be affected at the 5 dpf stage of the gnn mutant larvae, and no debris was found in the subretinal space (Figs. 6D, 6E). Finally, at 6 dpf, melanin granules showed irregular distribution throughout the gnn mutant RPE (Fig. 7) and were found clustered in swollen RPE cells, which themselves showed fewer mitochondria than RPE cells in the corresponding WT retina. In addition, the swollen RPE cells reached into the outer limiting membrane (OLM) or even protruded farther in the direction of the vitreous toward the ONL. Thus, the morphologic alterations in the RPE observed at the late stages (>6 dpf) of gnn retina degeneration showed impressive analogies to the pathohistology of RP in humans.33

Cone Synapses and Second-Order Neurons

To examine the vertical signal pathway from the cones toward the ganglion cells (GCs), we analyzed the ultrastructure of the cone pedicles at 5 dpf by electron microscopy. In both, the WT and gnn pedicles, classic synaptic structures, were easily identified in the cone terminals. Ribbon synapses, associated with postsynaptic processes or arciform densities, were clearly visible, as were horizontal and bipolar cell dendrites. Thus, synaptic connections between the cone photoreceptors and their second-order neurons developed normally in the gnn mutant.

To obtain further insight into the functionality of these first synaptic connections in the outer plexiform layer (OPL), we labeled the GluR4 subunits of the AMPA-type glutamate receptors at the synapse between photoreceptors and OFF bipolar cells (OFF-BCs) in the OPL. In the 5-dpf zebrafish WT retina, we identified GluR4 labeling in presumptive apical OFF-BC dendrites in the OPL and on MC radial processes and their end feet in the GCL (Fig. 8A). This labeling pattern was identical with the labeling described in goldfish.34,35 In gnn retinas, the GluR4 labeling in the OPL was similar to the intensity and labeling pattern in the WT, whereas the MC radial processes and end feet were only faintly labeled (Fig. 8B), thus suggesting...
an alteration of AMPA-receptor expression and MCs due to ongoing degeneration in the mutant retina.

To assess whether signal transmission occurs normally in the inner retina, we labeled mixed rod-cone bipolar cells (Mb BCs) by using an antibody against PKCβ1 at 5 dpf to visualize the presence of specific neurons involved in the signaling pathway from the photoreceptors to the GCs. Almost no difference was discernible in the PKCβ1 labeling between WT (A) and gnn mutant (B). Blue cone pedicles were equally labeled in WT and gnn. (C, D) UV-opsin immunoreactivity cones showed distinctly labeled OS in both the WT (C) and gnn mutant (D). (E, F) Green opsin immunoreactivity was expressed equally in both WT (E) and gnn siblings (F). Cone pedicles and OS were clearly labeled, and cone somata were diffusely labeled. (G, H) Red-opsin immunoreactivity cones were normally distributed in WT (G) and showed clear labeling of OS and pedicles. In the gnn mutant (H), only a very few red cone OS were labeled. Arrows: cone outer segments; arrowheads: cone pedicles; (*) cone somata. Scale bar, 10 µm.

**Gnn Genetic Map Position**

We measured the locus of gnn at a distance of 11.4 cM from the simple sequence length polymorphism (SSLP) marker Z6984 and 4.2 cM from marker Z15453 on LG 16 on the Tübingen map of the zebrafish genome (http://wwwmap.tuebingen.mpg.de/). Because the distance between these two markers amounts to approximately 8 cM, it is likely that gnn maps 4.3 cM distal to Z15453. Thus, we localized the gnn mutation between Z15453 and Z9559 between 60.1 and 78.9 cM from the top of LG 16.
**DISCUSSION**

In our screening for mutations in the visual system, we isolated the recessive mutant zebrafish gantenbein. In early embryonic stages, (i.e., up to 3 dpf), the retinal development of mutant larvae was identical with that of their WT siblings. Beyond that stage, cone photoreceptors became dystrophic, and the RPE began to degenerate, whereas the rods remained unaffected in all the developmental stages examined. Cone dystrophy started at 5 dpf in the red cones, which were affected sooner and more severely by the mutation than the other cone types. Electron-dense debris was restricted to the outer segments of the red-green DC subpopulation. With progressing degeneration (after 6 dpf), all other cone types started to degenerate as well. RPE degeneration led to an altered melanin granule shape and melanin accumulation in RPE cells, to cell swelling, abnormal microvilli elongation, and degenerative protrusions reaching far into the ONL.

**Altered CMS**

Because PNA labeling failed to stain the gnn cone matrix in any of the stages examined, we suggest that the gnn mutation may affect the composition of the CMS. Thus, the gnn mutation exhibits morphology similar to those of other animal models of retinal dystrophies. For example, the structural integrity of the CMS, when labeled with PNA, weakens concomitantly with degeneration of the cone photoreceptor in the rd mouse and is diffuse in the rds mouse.** Similar results were obtained in two canine models (rcd, rcd1) for retinal degeneration.**

PNA labeling of the CMS and associated cone structures was present throughout the progressing degeneration in all these animal models. In light of these studies, we suggest that the absence of the CMS labeling in the gnn mutant may be due, at least partially in regard to missing photoreceptor labeling, to an altered release of extracellular matrix molecules from the dystrophic cones.

**Cone Dystrophy and Degeneration**

In the gnn mutant, cone development was initially normal. The gnn retinas showed the same ultrastructural morphology as WT retinas, including differentiated cones with elongating outer segments and increasing pigmentation in the RPE at 3 dpf.** This argues against cone aplasia, the congenital absence of cones in the gnn mutant. Severe morphologically observable dystrophy became visible at 5 dpf. Our results show that the four different cone types were not affected equally, but that, initially at least, the DCs were selectively affected by the mutation. We therefore assume a special susceptibility of the DCs to the biochemical changes induced by the gnn mutation, leaving the SCs primarily unaffected. This may be partially because red cones are the first to express opsin in the zebrafish and the goldfish.** Thus, the degeneration may be related to the timing of opsin expression. However, with deteriorating dystrophy of the DCs, the SCs start to degenerate as well, thus leading to generalized cone degeneration.

**Interdependence between Cones and RPE**

Apparently as a consequence of the dystrophic cones, gnn retinas exhibit progressive degeneration in the RPE—namely, reduction of RPE pigmentation because of altered melanin granule morphology, protrusion of RPE cells into the inner retina, and, in the later stages, melanin-filled vacuoles. All these alterations show unequivocal similarities to retinal dystrophies in other animal models (RCS rat,** and** rds mice,** and** prcd** and** rcd1 dogs**) and in human RP.**

The fact that cones and RPE cells degenerated parallel to each other (i.e., the morphology and availability and, in consequence, the usefulness of both cell types lessened) raises the question of whether the gnn mutation is cell autonomous to the cones or to the RPE. Because RPE and photoreceptors are mutually dependent, both scenarios are plausible. As we know from the RCS rat, a malfunctioning RPE may be unable to shed photoreceptor discs and may therefore lead to photoreceptor degeneration, as is known to be the case in the RCS rat.** On the other hand, mutated proteins in the phototransduction cascade may cause cone dysfunction and concomitant loss of normally derived metabolic byproducts, which then lack the RPE cells, thus leading to their consecutive degeneration.** Because the alteration of CMS in the gnn retinas may be due to metabolism defects in the dystrophic cones, the second hypothesis may hold for the mutant retina and the RPE degeneration may also be caused by cone dysfunction in the gnn.
retina. However, because all the cones lack the PNA labeling, not just the red cones, and the RPE demonstrates overgrowth of the lens, a primary defect in the RPE may be equally likely. Future experiments including blastula cell transplantation and subsequent characterization of the resultant chimeric retinas may elucidate the question of whether the primary defect is in the cone or in the RPE.

**Second-Order Neurons**

Milam et al.\(^3\) stated that photoreceptor loss in human RP need not be accompanied by subsequent degeneration of INL cells, even in the presence of severe outer retinal degeneration. We confirmed these results for one distinct retinal cell population, the Mb-ON BCs in the *gun* mutant. These cells expressed morphology and had a number of cells identical with those of WT retinas, thus suggesting normal wiring from the first synapse in the OPL through the Mb-BCs toward the ganglion cells. However, reduced immunoreactivity for AMPA-type glutamate receptors on MC radial processes and end feet indicated altered receptor distribution on *gun* MCs. We therefore suggest a change in MC physiology as a reaction to the degenerating photoreceptors. Thus far, we have not been able to determine...
clearly whether the number of MCs is reduced in the gnn retina. Should this be the case, the decrease in MCs could contribute to a loss in the total number of cells in the mutant INL. In contrast, an increase in the number of MCs may indicate a reactive gliosis in the degenerating gnn retina, a phenomenon currently observed in patients with RP.40 In contrast to these similarities between gnn and RP morphology, we did not observe any degenerative reduction in the number of ganglion cells (a common feature in RP).45

**Similarities to Other Zebrafish Mutants**

Several screenings for visually impaired zebrafish8,11 have led to the identification of several recessive mutations affecting many parts of the visual system and a few dominant mutations.12,41 Identified by OKR screening, most of the phenotypes have been superficially analyzed by standard histology, and eventually by additional measuring of OMR and ERG.8 So far, the retinal morphologies of a few mutants have been studied in detail (partial optokinetic response b (pob),14 shrunken head (shr),42 night blindness b (nbb),41 young (yng),43 no optokinetic response c (arc),16,44 lakritz (lak),17 mikre oko,45 perplexed (plx), confused (cfs),46 and nagie oko (nok)).47 Two of these mutants, pob and nbb, express cone photoreceptor-specific degenerations. Although the disruption of the olfactoretinal centrifugal pathway is supposed to cause rod malfunction in nbb,41 the mutant gene responsible for red-blindness in pob14 is thought to be a novel, red-cone-specific gene, because the mutation does not cosegregate with the red opsin locus. Therefore, in both pob and gnn mutants, DCs are affected by the mutations. However, red cones were exclusively affected in the pob mutant, but other cone types and the RPE appear to remain unaffected by this mutation. Linkage analyses of the pob mutation revealed no linkage to the red opsin, and we therefore suspect a novel red-cone-specific gene to be affected by the pob mutation. In contrast to pob, gnn exhibits DC dystrophy and consecutive

![Figure 7: Electron micrograph of the outer retina in transverse sections of gnn larvae at 6 dpf. The gnn retina showed severe RPE degeneration: RPE cells, densely filled with melanin granules (✱), protruded into the photoreceptor layer. OS did not elongate any more but became shorter because of ongoing degeneration. Normal pigmentation in the RPE was almost completely lost. Arrow: Bruch’s membrane. Scale bar, 2 μm.](http://www.iovs.org/doi/abs/10.1167/iovs.03-0704)

![Figure 8: (A, B) AMPA-type glutamate receptor subunit (GluR4); (C, D) PKCβ1, and (E, F) GS labeling in transverse sections of WT and gnn larvae at 5 dpf. (A) GluR4 immunoreactivity was present on MC end feet (arrowheads) and in the OPL (arrows) of WT. In the gnn mutant retina (B), the OPL labeling (arrow) was identical with the WT, whereas the MC labeling (arrowheads) was weaker. PKCβ1-immunoreactive BCs showed identical labeling in (C) WT and (D) gnn. BC somata (✱), BC processes in the OPL (arrow), and BC terminals (arrowheads) in the IPL were labeled with the same intensity in the WT and the gnn mutant retinas. GS-immunoreactive MCs showed identical labeling in WT (E) and gnn (F). MC somata (✱), MC processes in the OPL (arrow), and MC end feet (arrowheads) in the GCL were labeled with the same intensity in the WT and the gnn mutant retinas; however, fewer MC somata were labeled in gnn. Scale bar: (A–D) 10 μm; (E–F) 25 μm.](http://www.iovs.org/doi/abs/10.1167/iovs.03-0704)
SC and RPE degeneration. Another very recently published mutant, nrc, showed no OKR and a highly unusual ERG. The malfunction of the nrc retina may be attributable to abnormal, immature-appearing photoreceptor terminals, characterized by “floating” synaptic ribbons that remain unassociated with postsynaptic processes or arciform densities. In contrast to nrc, gnn showed normal ribbon synapses, and horizontal and bipolar cell dendrites invaginated normally into the cone pedicles. Furthermore, AMPA-type glutamate receptors were identically distributed throughout the bipolar cell dendrites of gnn mutants and their WT siblings, thus giving evidence for a morphologically normal synapse in the gnn retina.

Similarities to Other Animal Models and to Human Hereditary Eye Diseases

The gnn mutant zebrafish shows several ultrastructural analogies to other animal models of retinal degeneration and also to human hereditary retinal diseases. The degenerative shortening of photoreceptor outer segments in gnn is a typical feature of retinal degeneration, commonly observed not only in zebrafish retinal degeneration mutations, but also in most other animal models and in retinal diseases such as RP or AMD.

The deposits in the DC-OS observed in the gnn mutant present yet another parallel to other animal models such as the rds mouse, because inner segments of photoreceptors undergoing lysis in rds retinas contain similar electron-dense inclusions, as observed in gnn DC-OS. However, these inclusions must not be observed in all degenerating photoreceptors and, so far, the biochemical and molecular composition of these deposits in the rds mouse retina remains unclear.

RPE degeneration, as observed in gnn, is a common morphologic characteristic in RP. The RPE defect in RP is assumed to be a secondary-effect mutationally induced by the loss of the photoreceptors. Although the genetic origin of the degenerations may vary, the phenotypic analogies concerning the RPE between gnn retinas and RP are striking. Late stages of the degenerated gnn retina showed RPE cells densely filled with melanin granules, a phenomenon equally observed in human retinas in advanced RP. Furthermore, many of the RPE cells in gnn retinas at 6 dpf protruded into the photoreceptor layer. In analogy to this process, RPE cells in patients with RP show similar protrusions into the photoreceptor layer. With further progress of the degeneration in RP, numerous RPE cells start migrating through the remaining outer retina to perivascular spaces in the inner retina, producing bone spicule pigment.

Unfortunately, we were not able to find this degenerative RPE migration in the gnn retina, probably because of the high mortality rate of the gnn larva at approximately 9 dpf. Nevertheless, in both RP and gnn retinas, the absence of metabolic byproducts normally derived from the photoreceptors may be the reason for RPE degeneration and subsequent RPE cell relocation in late stages of the degeneration.

Acknowledgments

The authors thank Dagmar Ripper for excellent technical assistance.

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