Impaired Retinal Differentiation and Maintenance in Zebrafish Laminin Mutants

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METHODS. Mutant larvae were observed for defects in visual behavior by testing their optokinetic response (OKR). In addition, electroretinograms (ERG) were measured and retinal morphology was examined by standard histology, immunocytochemistry, TUNEL assay, and electron microscopy.

RESULTS. Both, gup and sly showed no OKR at any light intensity tested, whereas bal embryos showed some remaining OKR behavior at more than 40% of contrast. Consistent with the OKR result, gup and sly did not show an ERG response at any light intensity tested, whereas bal mutants exhibited small a- and b-waves at high light intensities. All three laminin mutants showed altered ganglion cell layers, optic nerve fasciculations, and lens defects. Again, bal showed the least severe morphologic phenotype with no additional defects. In contrast, both, gup and sly, showed severe photoreceptor outer segment shortening and synapse alteration (floating ribbons) as well as increased cell death.

CONCLUSIONS. Lamb1 and laml1 chains play an important role in the morphogenesis of photoreceptors and their synapses. In contrast, lam1 is not involved in outer retina development.

laminins are heterotrimERIC glycoproteins that are composed of α, β, and γ chains. So far, 15 laminin isoforms have been identified in different tissues and at different developmental stages.1,2 They can bind to integrins, dystroglycan, syndecans, and other cell surface molecules that act as their cellular receptors. Laminin proteins are crucial elements of the extracellular matrix and are known to be involved in central nervous system (CNS) development, in particular in forming and maintaining neuronal contacts, both during development and in the adult. This is also the case in the retina, where laminins are present in many different compartments—namely, Bruch’s membrane, the interphotoreceptor matrix, the external limiting membrane (ELM), the outer plexiform layer (OPL), inner plexiform layer (IPL), and inner limiting membrane (ILM).3 Because of the presence in these retinal compartments, laminins are thought to play a central role in development and maintenance of photoreceptor structure and synaptic function.3 In addition to their effects on the neural retina, it has been shown recently that laminins are crucial for lens development.4–6

In humans, mutations in laminin-encoding genes lead to severe diseases such as junctional epidermolysis bullosa,7 congenital muscular dystrophy,8 and Pierson syndrome.9,10 a disease causing a severe nephrotic syndrome followed by early-onset end-stage renal disease accompanied by ocular impairments such as extreme nonreactive narrowing of the pupils and complex maldevelopment of the eyes, including lens abnormalities, atrophy of the ciliary muscle, corneal changes, and retinal alterations.

In the zebrafish, three laminin mutants, named bashful (bal; laml1), grumpy (gup; laml1), and sleepy (sly; laml1) have been identified due to their reduced body length and defects in notocord differentiation.11–13 Anterior–posterior axon trajectory defects in the retinotectal projection are apparent in gup and sly mutant larvae after Dil and DiO labeling.14 These two mutants were also identified in a screen for defects in visual behavior with defective optokinetic response (OKR), ERG, and retinal morphology.15

We describe the morphologic, behavioral, and electrophysiological alterations in the retinas of these mutants.

METHODS
All experiments were conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Fish Maintenance and Breeding
Fish were maintained and bred as described elsewhere.16 We used the zebrafish strains Tubingen (TÜ) and Tup Long Fin (TL),17 grumpy (gupts210), sleepy (slypl265), and bashful (balpo62) larvae were obtained by mating of identified heterozygous carriers, and the embryos were sorted according to their small body and eye size. A comparison of the strength of these alleles with the published data12,14,16,19 suggests that the alleles used are functional null alleles. 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 post-fertilization (dpf).

Retinal Histology
Larvae were anesthetized at 4°C on ice before fixation. For light microscopy, they were then immediately fixed in 4% paraformaldehyde in 0.2 M phosphate buffer (PB; pH 7.4) for 1 hour (4°C).
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 resin (Technovit 7100; Kulzer, Wehrheim, Germany). Microtome sections (3 μm) were prepared and mounted on slides (SuperFrost Plus; Menzel-Gläser, Braunschweig, Germany). They were then air dried at 60°C, stained with Richardson solution (1% azure, 1% methylene blue, and 1% borax in deionized water), and coverslipped with mounting medium (Entellan; Merck, Darmstadt, Germany).

Quantifications

Photoreceptor Layer: Thickness Quantification. Toluidine blue-stained resin-embedded sections with a thickness of 5 μm were evaluated by using bright-field optics. In each larva, we measured the photoreceptor layer thickness as the distance, from the inner end of the photoreceptor soma to Bruch’s membrane. In each laminin mutant, three sections from two to three different fish were quantified. In each section, 10 loci in proximity to the optic nerve were analyzed. Thus, 20 to 30 loci were analyzed per replicate. The bar graphs were designed and the statistic tests performed in commercial software (Prism 4.03; GraphPad, San Diego, CA). Significance between bal, gup, and sfy retinas was determined by one-way ANOVA and the Tukey post hoc test (Prism; GraphPad, San Diego, CA).

Lens Phenotype Quantification in the bal Mutant. Toluidine blue-stained, resin-embedded sections and unstained cryostat sections visualized by differential interference contrast (DIC) optics were screened for lens phenotypes in 35 eyes of 19 different bal larvae.

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. After they were rinsed in 0.1 M PB, the specimens were dehydrated in a graded series of ethanol-water mixtures up to 100% ethanol. After preinfiltration in 1:1 100% ethanol/embedding resin (Fluka, Buchs, Switzerland), larvae were infiltrated in pure embedding resin overnight. Larvae were then positioned in embedding capsules (Beem caps; Canemco & Marivac, Canton de Gore, Quebec, Canada) with fresh resin and polymerized at 60°C for approximately 16 hours. Ultrathin (60 nm) transverse sections were prepared, contrasted with lead citrate, examined, and photographed with a transmission electron microscope (model EM 900; Carl Zeiss Meditec, Oberkochen, Germany).

Cell Death Detection

Fixed larvae were cryoprotected in 30% sucrose for at least 4 hours. Entire larvae were then embedded in tissue-freezing medium (Cryomatrix; Reichert-Jung, Vienna, Austria) and rapidly frozen in liquid N2. Cryosections (25 μm thick) were cut at −20°C, mounted on slides (Super Frost Plus; Menzel-Gläser, Braunschweig, Germany), air dried at 37°C for at least 2 hours, and stored at −20°C until further use. For cell death detection, the slides were thawed, washed three times in PBS (50 mM, pH 7.4), and incubated in blocking solution (BS; 10% NGS and 1% BSA in 0.3% PBS/Triton X-100) for 1 hour. Sections were then incubated overnight in primary antibody in BS at 4°C. The immunolabeling was visualized by using Alexa488-conjugated anti-mouse IgG or anti-rabbit IgG (1:1000; Invitrogen-Molecular Probes, Basel, Switzerland) as a secondary antibody. The following primary antibodies were used for immunostaining: monoclonal mouse anti-zipr1 (1:20; University of Oregon Stock Center), monoclonal mouse anti-glutamine synthetase (GS; 1:700; Chemicon, Harrow, UK), and polyclonal rabbit anti-syntaxin3 (Synt3; 1:400; Alamine Laboratories, Jerusalem, Israel).

After the immunostaining, the slides were coverslipped and analyzed under a fluorescence microscope (Axioskop; Carl Zeiss Meditec, with AxioVision 4 as the imaging software).

OKR Measurements

Visual performance was assessed by measuring the OKR as previously described.22 To measure eye velocity, single larvae were placed dorsal-side-up in the center of a Petri dish (35 mm diameter) containing 3% prewarmed (28°C) methylcellulose. Moving sine-wave gratings were projected by a linearized HP cp6111 projector onto a screen within the visual field of the larva. The apparent distance from the larva’s right eye to the screen was 4.65 cm, and the projection size on the screen was 8 × 6 cm, subtending a visual angle of 65.6° horizontally and 55.1° vertically. Eye movements were triggered by the visual stimulus and recorded by an infrared-sensitive CCD camera. Custom-written software (based on LabView (MAQ ver. 5.1; National Instruments, Austin, TX) was used to control the stimulation and the camera and to analyze the resulting images. Contrast sensitivity functions for wild-type (wt) and laminin mutant larvae were measured as eye velocity as a function of the spatial frequency of the moving grating with alternating movement direction (0.53 Hz). The average eye velocity for each spatial frequency was calculated by integration of eye-velocity traces. Graphs were designed and the statistical tests performed with commercial software (Prism 4.03; GraphPad). Significant differences between bal (n = 5), gup (n = 5), sfy (n = 5), and their respective siblings (n = 5) were determined by a two-way ANOVA and Bonferroni post hoc test.

Electroretinographic Recordings

Electroretinograms (ERGs) were recorded from 5 dpf larvae as previously described.23 All specimens were dark-adapted for 30 minutes before positioning them in the recording chamber. Each larva was placed on the surface of a moist sponge with E3 medium and paralyzed by directly applying a droplet of the muscle relaxant (Esmeron; 0.8 mg/mL in larval medium; Organon Teknika, Eppelheim, Germany). Light flashes of 200 ms duration were separated by 7-second intervals. Unattenuated irradiation at the position of the subject as measured by a photometer with photopic sensitivity profile was 5.7 mW/cm2. A virtual instrument (VI) (NI LabVIEW ver. 5.1; National Instruments) was used to control the stimulation and to record ERG traces on computer. Sampling was performed in buffered acquisition mode with a sampling rate of 1000 Hz, and responses were averaged between five acquisitions.

RESULTS

Laminin Mutations Lead to Alterations in Retinal Morphology

In previous studies, it has been shown that laminin mutations encoding for laminin α1, β1, and γ1 are important both for notochord differentiation and for proper intersegmental blood vessel formation.11–15,18 In addition, defects in vision have been reported for β1, and γ1 laminin mutant larvae.15 To assess the general retinal morphology of these laminin deficient fish, we analyzed laminin mutant and wt retinas with standard histologic techniques (Figs. 1A–D) at 5 dpf. All three laminin mutants showed similar retinal alterations: They pos-
Retinal Alterations in Zebrafish Laminin Mutants

We wanted to know whether the different laminin mutations would affect the expression of specific synaptic markers of the OPL. Therefore, we labeled ribbon synapses by using a Syntaxin 3 antibody. In this staining, ribbon synapses were labeled to the same extent in wt and all three laminin mutants (Fig. 2E–H), thus indicating that the remaining synapses between photoreceptors and second order neurons still possess normal ribbon synapses.

Ultrastructure of Photoreceptors and Their Synapses in Laminin Mutants

Knowing that photoreceptors are affected by the laminin mutations in gup and sly, we examined the ultrastructure of the outer retina by using electron microscopy to gain further insight. Again, the situation in the bal mutant outer retina at 5 dpf (Fig. 3B) resembled very much the situation in wt larvae (Fig. 3A). Bal mutants showed normally elongated cone outer segments and healthy RPE cells. As expected from the immunohistochemical data, outer segments in gup (Fig. 3C) and sly (Fig. 3D) are strongly shortened. In addition to this photoreceptor defect, gup larvae express an altered RPE with fewer melanin granules and abundant vacuoles in the RPE (Fig. 3D). However, Bruch’s membrane, a structure where laminin-1 is deposited is unaffected by the mutations (Figs. 3E–H).

Next, we analyzed the photoreceptor ribbon synapse at the ultrastructural level. We identified classic cone pedicles including triads of invaginating horizontal (HC) and bipolar (BC) cell dendrites adjacent to respective ribbon synapses in wt (Fig. 3E) and bal larvae. However, invaginating dendrites were reduced and instead of classical “triade associated” ribbons, floating ribbons were frequently observed in gup larvae (Fig. 3G). The photoreceptor synapse of sly mutant larvae is even more strongly affected with only very few or no ribbons and hardly any invaginating dendrites formed (Fig. 3H). Moreover, the classic bell-shaped structure of cone pedicles was lost in the sly mutant.

Retinal Cell Death in Laminin Mutants

We next asked whether the retinal defects in these mutants are associated with an increase in apoptotic cell death by applying the TUNEL assay. The number of TUNEL-positive cells detected in 5 dpf wt (Fig. 4A) and bal (Fig. 4B) mutant larvae was...
very low with no more than one to three labeled cells per section and thus corresponded closely to the rate reported from zebrafish normal development. In contrast, the amount of cell death observed in gup (Fig. 4C) and sly (Fig. 4D) was very high, showing numerous dying cells all over the retina. In the sly mutant, most of the apoptotic cells were labeled in the outer nuclear layer (ONL), thus suggesting severe photoreceptor degeneration (Fig. 4D).

Cells of the Inner Retina in Laminin Mutant Zebrafish

To examine whether the alterations induced by the laminin defect also affect neurons of the inner retina, we labeled several cell populations by using specific antibodies: 5e11 to label amacrine cells, TH to label dopaminergic amacrine cells, cPKC to label mixed rod cone bipolar cells, and GS to label Müller cells (MCs). All these cell types were clearly identified by the respective antibody in the mutant retinas (data not shown) and the labeling did not show any altered morphology concerning the intensity and localization of the immunolabeling in the respective individual cells. However, GS staining was altered in all three mutant retinas with regard to the Müller cell end feet (Fig. 5). Despite a normal morphology with clearly labeled somata and radial processes spanning from the OLM to the ILM, the staining pattern was clearly altered in all three mutants at the level of the ILM, where the mutants (Figs. 5B–D) did not form a regularly shaped margin between the vitreous and the GCL.

**Figure 2.** Double cone labeling (zp1; A–D) and ribbon synapse labeling (syntaxin 3; E–H) in transverse sections of wt and laminin mutant larvae at 5 dpf. Entire double cones, including the inner and outer segments, were clearly labeled in wt (A) and bal (B) larvae. In contrast, zp1 labeling was restricted to the inner segments and somata in gup (C) and sly (D) larvae. Ribbon type synapses were labeled to the same extend in wt (E) as well as in all mutant retinas (F–H). Scale bar, 50 μm.

**Figure 3.** Transmission electron microscopy of transverse sections of the photoreceptor layer (A–D) and OPL (E–H) in wt and laminin mutant larvae at 5 dpf. Photoreceptor layer: (A) In the 5-dpf wt, photoreceptor outer segments (OS) were elongated and melanin-filled granules were clearly visible in the RPE. (B) In the 5-dpf bal mutant, OS were normally elongated in bal and RPE pigmentation was normal. Both (C) gup and (D) sly showed strongly reduced OS length and melanin granules with fainter pigmentation. Bruch’s membrane was identifiable in wt and in all three laminin mutants (A–D, arrows). Synaptic layer: wt (E) and bal (F) showed classic cone pedicles with triads built by invaginating horizontal and bipolar cell dendrites and ribbon type synapses (R). (G) gup did not show normal triads: dendritic invagination was reduced and ribbons (R) were floating. (H) sly showed very few to no ribbons and very few invaginating dendrites. In addition, the classic bell-shaped morphology of cone pedicles was lost. Scale bars: (A–D) 5 μm; (E–H) 1 μm.
as was seen in their wt siblings (Fig. 5A). In contrast, close examination of individual Müller cells clearly showed that Müller end feet of all three laminin mutants terminated irregularly at different depths of the inner retina (Fig. 5B–H11032–D/H11032) thereby not building any separating membrane between the neuronal and the vitreal part of the retina. Taking into account that the ILM is composed by Müller glia end feet and ECM components it is very much likely that the altered composition of the ECM leads to the breakup of the regular Müller end feet pattern.

Contrast Sensitivity and Retinal Signal Transmission in Laminin Mutants

To assess the impact of the described morphologic changes on vision, we measured contrast sensitivity of the OKR, a behavioral assay for visual performance22 and ERG, an electrophysiological assessment of outer retinal function.23

Contrast sensitivity of all three laminin mutant larvae was severely reduced compared with that in respective wt siblings (Fig. 6; bootstrap resampling test, \( P < 0.05 \)). However, the bal mutant (Fig. 6A) showed some residual OKR at light intensities of 50%, 70%, and 100%, thus suggesting that bal mutants are still able to see at higher light intensities.

To obtain insight in the overall physiology of the mutated eyes, we examined the ERGs of 5 dpf laminin mutant larvae and their wt siblings (Fig. 7). In accordance with the above described morphologic findings, bal mutants showed reduced but still clearly measurable ERG-signals (Fig. 7A). In contrast, both, gup and sly mutants did not show any remaining signal at any light intensity measured (Figs. 7C, 7E). These data are consistent with the behavioral analysis, indicating that bal mutants are able to see, whereas gup and sly are completely blind.

Given the nearly normal ERG in bal larvae, we hypothesized that the poor visual performance in the behavioral assay is due to defects in the retina and the lens. A small or degenerated lens should have a severe impact on visual behavior but should compromise the ERG only slightly.

DISCUSSION

In this study, we describe the retinal phenotype of three different laminin mutants, bal (lama1), gup (lamb1), and sly (lamc1) that had been previously described to have defects in notochord differentiation and intersegmental blood vessel formation.11–13,18 All three laminin mutants showed an altered GCL and optic nerve and lens defects. The bal mutant did not show any other morphologic alterations, whereas gup and sly showed severe degeneration at the level of photoreceptors and synapses. In addition, no OKR and no ERG were detectable in gup and sly larvae, whereas bal homozygous showed some remaining OKR and a clear ERG at high contrast levels.

According to our results, a mutation in the lama1 gene encoding for the \( \alpha_1 \) laminin chain (bal) leads to defective laminin111 (formerly known as laminin 1; see Ref. 26) and defective laminin121 but leaves all other 13 known laminins unaffected. In contrast, mutations in lamb1 and lamc1 lead to
Studies of the role of laminins in the zebrafish eye have shown that a defect in laminin expression leads to alterations in lens morphology. Semina et al. recently showed that lam1 mutants have severe lens defects, varying from small lenses to no lenses. This deficiency is likely to be due to a compromise in lens capsule integrity. However, because we still found lenses in some of the mutant eyes we suggest that the lens capsule defect leads to defective lens formation during early development, and later on, to a complete clearing of the lens from the laminin mutant eye.

In conclusion, we found that lam1, lam1, and lamc1 mutations lead to lens defects and morphologic alterations of the GCL and the optic nerve, whereas only lam1 and lamc1 mutations lead to severe photoreceptor dysfunction.
The affected retinal tissues are located in the retinal compartments known to contain laminin proteins in rodents, such as Bruch’s membrane, the interphotoreceptor matrix, and the ELM, OPL, IPL, and ILM. The lack or mutation of one chain of a trimeric laminin protein leads to developmental malformation and/or degeneration in the respective retinal compartments such as the photoreceptors, the OPL, or the GCL. As the mutation in the lama1 chain affects only the lens and the GCL and leaves other layers normal, we favor the idea that lama1 chains are not involved in outer retina development and maintenance or may be replaced by lama5 or other lama chains to compensate for the absent lama1 chain. The recent study by Semina et al. furthers this hypothesis by showing that other alleles of lama1 mutation show similar phenotypes, affecting the GCL and lens, such as baltp82. Thus, all three laminin chains examined in this study are crucial in the formation of a proper ILM and a normal GCL, but only lamb1 and lamc1 are vital for photoreceptor development and maintenance. Finally, the outcome of this hypothesis is that the location of the respective laminin chains throughout the zebrafish retina correlates nicely with the laminin chain distribution suggested by Libby et al. in the rat retina.

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References


Figure 7. ERG recordings from dark-adapted laminin mutants and their wt siblings at different light intensities (OD 1–5) at 5 dpf. (A) The bal mutant showed a classic ERG at higher light intensity. The gup (C) and sly (E) mutants are completely blind, as they showed no ERG signal.


