Nuclear receptor NR2E3 gene mutations distort human retinal laminar architecture and cause an unusual degeneration

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Mutations in the nuclear receptor gene, NR2E3, cause a disorder of human retinal photoreceptor development characterized by hyperfunction and excess of the minority S (short wavelength or blue) cone photoreceptor type, but near absence of function of the majority rod receptor. NR2E3 disease can also progress to blindness. How the human retina accommodates mis-specified types and numbers of neurons and advances to retinal degeneration are unknown. We studied the retinal organization in vivo of patients with NR2E3 mutations. Early human NR2E3 disease with S cone hyperfunction showed thickened retinal layers within an otherwise normally structured retina. With visual loss, however, lamination was coarse and there was a strikingly thick and bulging appearance to the retina, localized to an annulus encircling the central fovea. This pattern was not found in other retinal degenerations. The abnormal laminar retinal architecture of early NR2E3 disease may be due in part to larger cells with an S cone phenotype in place of rods that failed to differentiate. The later-stage dysplastic appearance suggests a previously unrecognized proliferative response in human retinal degeneration.

INTRODUCTION

The mature human retina is a central nervous system structure with a precisely formed laminar organization. The primary light-sensing visual cells, cones and rods, form the most distal neural layer. The cones (day vision cells) are enriched in the central retina and the rods (night vision cells) are most dense in a ring about 4 mm from the center (1). Spatial topography of the retina has localized thickening and thinning with the underlying mosaics of cells of different type, size and density fitting together to achieve functional neural circuits. This complex adult retinal structure requires a precisely coordinated developmental process, which is increasingly understood at a molecular level (2,3).

The nuclear receptor gene, NR2E3, is postulated to have a role in human rod photoreceptor development (4,5). NR2E3 mutations are associated with an unique autosomal recessive retinal disease, the enhanced S cone syndrome (ESCS). ESCS was first identified when unexpected hyperfunction of the normally minor S (short wavelength sensitive, blue) cone population was found in patients with a severe deficit in rod function (6–8). Proof of greater numbers of photoreceptors with an S cone phenotype in ESCS retinas was provided nearly a decade ago by in vivo electrophysiology (9) and, more recently, by histopathology in a postmortem donor retina of a 77-year-old patient with late-stage ESCS due to NR2E3 mutation (10). Two murine models, the rd7 (Nr2e3 mutant) and the Nrl−/− (with no Nr2e3 expression) mouse,
have shown concordant results of increased cone cells and greater percentage with an S cone phenotype (11–13).

How does human retinal structure accommodate misspecified numbers and types of photoreceptors? With in vivo high-resolution microscopy and correlative measures of vision in patients with NR2E3 mutations, we sought to determine the effects of abnormal NR2E3 on human retinal organization. Architecture of the NR2E3-mutant retina differed remarkably from normal at all disease stages; there were relatively subtle increases in layer thickness as well as surprisingly coarse lamination and regional expansions of retinal tissue. The latter findings suggest a very different retinal degenerative process than previously recognized in man (14).

RESULTS

Retinal topography and lamination are abnormal in the NR2E3-mutant retina

An en face view of the transparent normal human adult retina has obvious features like the optic nerve head and the major vessels coursing along the retinal surface into and out of the nerve (Fig. 1A). En face views of NR2E3-mutant retinas show a multitude of abnormalities including clumps of pigment and atrophic lesions (Fig. 1B and C). Topographical maps of retinal thickness using high-resolution depth (cross-sectional) imaging also show differences between normal (Fig. 1D) and NR2E3-mutant retinas (Fig. 1E and F). The normal retina shows a number of distinctive features: a central depression or foveal pit; a surrounding ring of increased thickness with displaced inner retinal layers from foveal formation; a decline in thickness with distance from the fovea and a prominent crescent-shaped thickening in the nasal retina extending into the superior and inferior poles of the optic nerve, attributable to the converging axons from ganglion cells (Fig. 1D). The topography of NR2E3-mutant retinas also shows a foveal depression, but unlike normal there is a large annular increase in retinal thickness approximately at the eccentricity of the optic nerve; thickness of the annulus is notably greater in superior and inferior retina than along the horizontal meridian in the temporal retina (Fig. 1E and F).

To probe further the differences between normal and NR2E3-mutant retinal topography, we studied cross-sectional images along horizontal (Fig. 2) and vertical meridia (Fig. 3). The normal human retina in cross-section has a foveal depression and discernible laminae (Fig. 2A). A magnified view of the very central retinal region (box, right) with overlaid reflectivity profiles illustrates the laminar differences between the foveal pit and an adjacent thicker region at 1.7 mm temporal to the fovea. The profile through the pit at the level of cone photoreceptor (PR) inner segments (IS) and outer segments (OS) and retinal pigment epithelium (RPE). At 1.7 mm temporal to the fovea, where rod photoreceptors are numerous, at a density of \( \sim 100,000 \text{cells/mm}^2 \) (1), the cellular layers of low reflectivity have intervening higher reflectivity laminae that represent synaptic connections. The layer of photoreceptor nuclei [outer nuclear layer (ONL)] averages 62.9 \( \mu \text{m} \) in thickness at this location (SD, 5.1 \( \mu \text{m} \); \( n = 8 \); ages 19–28 years).

A retina affected with retinitis pigmentosa (RP), in contrast to normal, has neither rod nor S cone function; only a central island of reduced L cone function remains (Fig. 2B). The cross-sectional retinal image shows a foveal pit, but the adjacent temporal retina appears thinned compared with the normal. The magnified view (box, right) shows that the foveal reflectivity profile has a vitreo–retinal peak, but the low-reflectivity zone, representing photoreceptor nuclei, is thinned compared with normal. At the 1.7 mm temporal locus, inner retinal lamination is present, but outer retinal layers are abnormal. The low-reflectivity zone representing the ONL is thinned (11.7 \( \mu \text{m} \)) and the usual multi-peaked deep high reflectivity has become single-peaked (indicating PR IS/OS signal is reduced but RPE signal is retained) (15–17). The decline in L cone vision with distance from the fovea and the decline in outer retinal layers tend to coincide.

NR2E3-mutant retinas representing two different degrees of disease severity (assayed by amount of cone vision) are shown (Fig. 2C and D). Like the RP retina, there is no measurable rod vision. Unlike RP, NR2E3-mutant retinas (exemplified by P3) can have hyperfunction of S cones, and L cone function at the lower limit of normal. P4 has slightly reduced S cone function and greatly reduced L cone function (7). Both P3 and P4 retain normal-appearing laminar organization in the central 3 mm of retina. The magnified view for P3 (box, right) shows a normal foveal reflectivity profile. At 1.7 mm temporal, inner retinal lamination is intact, but the ONL is thicker than normal (89.7 \( \mu \text{m} \)); the PR IS/OS signal is multi-peaked and approximates the normal. Further temporal in P3, the retina does not decline gradually in thickness but becomes reduced at about 3 mm and then remains thicker than the normal. P4 also has a normal foveal reflectivity profile (magnified view not shown), and at 1.7 mm the ONL is normal in thickness (64.5 \( \mu \text{m} \)). There are, however, unexpected variations in retinal thickness in the temporal retina. The outer retinal layers taper between the fovea and about 4 mm temporal, somewhat coinciding with the tapering of L cone function. Retinal thickness then increases again (giving the appearance of bulges in the tissue) and lamination becomes indistinct in the region with retained but limited S cone vision. Analyses with high-resolution imaging at 1.7 mm were also performed in two other NR2E3-mutant retinas, and both showed ONL thickness at the upper limit of normal (72–74 \( \mu \text{m} \)).

Coarse thickening outside the central retina in NR2E3 mutations

In the vertical meridian of the normal mature human retina (Fig. 3A; about 3–6 mm from the fovea), there are not only neural and synaptic laminae but also ganglion cell axons traversing superficially to converge and exit at the optic nerve, and the retinal arteries and veins. Cross-sectional images in the superior retina show the same laminations and tapering of thickness with distance from the fovea, as along the horizontal meridian (Fig. 2A); the prominent band of high reflectivity near the retinal surface represents the nerve fiber layer (NFL), and retinal surface irregularities are from blood vessels (protrusions into the vitreous that cast ‘shadows’
Figure 1. NR2E3-mutant human retinas are abnormally thickened, most prominently in a ring at the eccentricity of the optic nerve. Infrared en face views of the central retina of a representative normal individual, age 22 (A), and two patients (B and C) with NR2E3 mutations (P1, age 32; P2, age 54). White boxes define limits of the topographical map from high-resolution depth imaging in the same individuals, shown to the right. (D) Normal average retinal thickness \( n = 5 \); overlaid retinal vessel pattern is from one normal) compared with retinal thickness maps in the NR2E3-mutant retinas (E and F). All images are depicted as right eyes. F, fovea.
through the image; Fig. 3A). An RP retina (Fig. 3B), without rod and cone vision in this region, shows dramatic thinning and indistinct laminations. Three NR2E3-mutant retinas with only S and L cone function (Fig. 3C–E), representing progressively decreasing vision, differ from both the normal and the RP retinas by having increased thickness, abnormal laminations, and irregularity of the vitreo–retinal surface.

Magnified views of a region expected to have highest rod density (4.4 mm superior to the fovea) (1) are shown with overlaid reflectivity waveforms for each of the five retinas (Fig. 3, right panels). Normal retinal laminae at this location include: the multi-peaked deep signal representing PR IS/OS, RPE and anterior choroid; the alternating cellular and synaptic layers and the NFL (17). In the thinned RP retina, laminae are not easily discernible; there is a single-peaked deep signal (presumed RPE) (15) with enhanced backscatter, a deep hyporeflective and more superficial hyperreflective layer. The NR2E3 retina of P3, with preserved S and L cone function, has a definite laminar organization and is slightly increased in total thickness, compared with the normal. Notable details include: a diminished PR IS/OS peak; normal or slightly increased ONL and INL, and a normal NFL. In addition, the intervening laminae of the depth image appear increased in thickness. NR2E3. P4 and P2 retinas with little or no function in this region have thick coarsely laminated retinas with a single-peaked deep signal (presumed RPE), deep hyporeflective and more superficial hyperreflective layers.

**NR2E3 topographical defects are not present in RP and CRX-mutant retinas**

To determine whether these observations were specific, we quantified retinal thickness in all 17 patients with NR2E3 mutations (Fig. 4A) and related these data to those of normal subjects (n = 18) and two other subgroups of patients with retinal degeneration: five patients with cone-rod dystrophy or Leber congenital amaurosis caused by CRX mutations (Fig. 4B) and 10 patients with RP of unknown genotype (Fig. 4C). NR2E3-mutant retinas (without retinoschisis) can have a foveal depression, but beyond the central retina there is abnormally increased thickness. The patients with CRX mutations, with various severities of dysfunction, all had reduced central retinal thickness, and either normal or reduced thickness beyond the central retina (Fig. 4B) (18). The RP patients showed normal or reduced thickness in the central retina and declining thickness outside the center (Fig. 4C), as anticipated from previous histopathological studies of human RP and in vivo cross-sectional imaging (14,17,18).

**DISCUSSION**

Retinal laminar defects in this human disease of a nuclear receptor transcription factor: separating early from late effects

Lamination of the vertebrate retina depends on a complex process in which birth order and cell fate are spatially and temporally coordinated by genes coding for transcription factors, cell cycle regulators and other molecules (2,3,19). The photoreceptor-specific NR2E3 gene is a member of the family of ligand-activated nuclear receptor transcription factors; it is expressed in rod photoreceptors, functioning as a transcriptional activator of rod genes in concert with NRL and CRX (5,20).

More than a decade of intense study in animals shows that mutation or misexpression of certain transcription factors can result in defects, which include: (i) failure to specify correctly one or more cell types (13,21–23); (ii) increased formation of alternative cell types (13,22,24–26); (iii) failure to complete differentiation of correctly specified cells (27); (iv) loss of retinal organization (28,29); (v) failure of complete cell cycle exit in abnormally specified cell types (22,24,26,30); and (vi) cell death as a consequence of dysplasia (12,22,30).

Within the context of a dauntingly complex but increasingly understood vertebrate retinogenesis, how do we even begin to explain the striking and different retinal architectural abnormalities in human NR2E3 mutations? On the basis of data from our cohort of patients, we propose a disease sequence with the modest first goal of distinguishing the congenital laminopathy from progressive effects of retinal remodeling and degeneration. Using severity of visual loss and associated laminar changes, we postulate five stages (Fig. 5). Data in support of the sequence were taken from outside the central retina, specifically the inferior retina at about 5 mm from the fovea, in which there is a large range of retinal thickness values among the NR2E3-mutant retinas (Fig. 4A; 219–378 μm; normal range 128–230 μm, n = 22). We suggest that stage I represents the congenital laminopathy and stages II–IV represent the progressive aspects of retinal remodeling and degeneration (Fig. 5).

Stage I (represented by P3, age 31) shows a laminated retina in which some of the layers are abnormally thick (ONL = 66 μm, normal = 53 ± 5 μm; OPL = 41 μm, normal = 22 ± 3 μm; IPL + GCL = 80 μm, normal 29 ± 4 μm), and there is supernormal S cone function and normal L cone function. Stages II and III (represented by P1, age 31 and P5, age 51) show a coarsely laminated abnormally thick retina with either supernormal or normal S but abnormally reduced L cone function (stage II), or abnormally reduced S and L cone function (stage III). Stage IV
(represented by P6, age 77), corresponds to a delaminated retina with near normal thickness, but no measurable visual function. This disease stage is probably similar to that described in the only post-mortem donor retina from a patient with NR2E3 mutation studied by histopathology (10).

Interpreting the human NR2E3 disease sequence in the context of experimental studies

Retained laminar architecture with thickened ONL in stage I NR2E3 disease, taken together with the co-localized vision results of supernormal S, normal L cone and no rod function, leads to the hypothesis that NR2E3 mutation perturbs rod development, specifically rod cell differentiation. Rather than a thinned and disrupted photoreceptor layer from rod cells not forming (23), there is normal or greater thickness of this layer, suggesting a compensatory increase in alternative cells or retained immature ‘rod’ cells expressing S-opsin. Evidence that L cone differentiation was not grossly perturbed comes from our finding of normal structure and function of the L cone-rich foveola (which lacks rod and S cone photoreceptors) (1,31) and normal L cone function at other retinal loci (6–10). The thicker ONL beginning at the foveal edge (Fig. 2) may simply be due to increased cell size. For example, replacement of rod nuclei with cone-like nuclei that are ~15% larger (32) would predict a ~50% increase in ONL layer thickness, assuming random close packing of ellipsoid-shaped nuclei and a packing density that is independent of small size changes (33). In the region of the rod-rich ring of normal retina (1), lamination was retained in stage I NR2E3 disease, but there could be increased thickness of inner as well as outer retinal layers, suggesting cell gain, both in ONL and in secondary and tertiary neurons, as predicted from earlier studies (8,34).

Loss of retinal organization with a coarsely laminated and bulging appearance was a feature of stages II and III of NR2E3 disease and was related to increased visual loss. The topography of this retinal disorganization was an annulus with eccentricity near that of the optic nerve (Fig. 1), a distribution reminiscent of the normal rod-rich ring (1), and possibly marking the location of an earlier stage with high density of cells having S cone phenotype. Interestingly, the two mouse models with Nr2e3 loss of function, rd7 and Nr1l, also show postnatal disturbance of retinal organization with variable-sized folds or whorls of S cone cells in the outer retina that precedes degeneration and dysfunction (11–13,35). We offer the following speculative explanations for the retinal disorganization. There could be continued

Figure 3. Cross-sectional images in the vertical meridian in NR2E3-mutant retinas show coarse lamination. (A) Normal cross-sections from superior retina of normal retina have identifiable laminae and there is a full complement of rod and cone function. (B) Retinitis pigmentosa patient with no measurable vision in this region has a thinned retina with poorly defined laminae. (C–E) NR2E3-mutant retinas with different degrees of S and L cone vision show thickened retina with coarse laminar structure, more pronounced in the retinas with less vision. Magnified cross-sections (white box, green coloration) with overlaid reflectivity waveforms at 4 mm in the superior retina in the five subjects. Calibration bars for both sets of images are shown.
slow proliferation of neuronal (or glial) cells in the NR2E3 deficient retina, resulting in the coarsely laminated bulging human retina and, possibly, the murine whorls. Proliferation may be associated with failure to completely exit the cell cycle (22,23). Alternatively, this is a secondary response to changes in the microenvironment from cell death. For example, release of ciliary neurotrophic factor (CNTF), which can cause proliferation in adult retinal progenitors (36,37), may cause hyperplasia and coarse lamination in regions of greater visual loss in NR2E3-mutant retinas. Photoreceptor degeneration is associated with increased retinal progenitor cell proliferation in the patched (ptc) mouse mutant (with loss of Shh receptor function) (38). The photoreceptor cells with S cone phenotype in NR2E3-mutant retinas have the unusual finding of co-expression of S and L opsins in some of these cones, a property more often seen in fetal cones than adult human cones (10), and may respond differently to neurotrophic activation than cells in other retinal degenerations (Fig. 4B and C).

Stage IV disease in NR2E3-mutant retinas was associated with thinning of the dysplastic-appearing retina and no visual function. Cell death and glial scarring, the likely basis (10), may have several explanations. This may be consistent with the failure of abnormal S cone-like cells to completely exit the cell cycle, which can be associated with apoptosis (30,39,40). In addition, continued proliferation of S cone-like cells could also be followed by a wave of (quasi-development) apoptosis, which is not a prominent feature in normal photoreceptor development although it affects other retinal cells (e.g. ganglion cells) (41). Finally, there may be increased sensitivity to cell death of the photoreceptors with S cone phenotype owing to different energy metabolism and oxygen utilization than rods (42,43).

In summary, we propose a disease sequence in which mutations in NR2E3 are associated with a primary phase comprising: (i) failure of rod photoreceptor differentiation; and (ii) a compensatory increase in functional S cone-like cells, associated with thickening of retinal layers. This is followed by a secondary phase comprising: (iii) concomitant loss of retinal lamination and S and L cone function, owing to a combination of cell death and cell proliferation. The thickened and coarsely laminated retinal regions without measurable function may thus be regions with neurons and/or glia reacting to cell death of neighboring L or S cones. This would be a
previously unrecognized type of retinal remodeling (14,44) and may be found in other human retinopathies.

Clinical implications of the laminopathies in NR2E3 disease

Are the present results helpful to form future treatment strategies for NR2E3-mutant retinas? The altered cell composition in adult retina resulting from a developmental flaw would not seem approachable, but preventing or slowing the degenerative disease component would be a worthy goal. The finding of coarsely laminated thickened retina in regions of increasing visual loss adds serious complexity. If this is a secondary proliferative process in immature cells following natural cytokine release, there should be concern about use, in these patients, of certain otherwise-promising treatments for retinal degeneration such as CNTF-releasing intraocular drug delivery system (45), now in clinical trials of RP patients. If ESCS-NR2E3 is currently not amenable to treatment, then focus should shift to avoid misdiagnosis of these patients as RP. Relatively simple diagnostic functional tests have been devised for all stages of the disease (46,47) and the ESCS phenotype has been associated with NR2E3 mutation in at least 95% of patients (4,48,49).

MATERIALS AND METHODS

Subjects

Informed consent was obtained from subjects after the nature of all studies had been explained. Research procedures were in accordance with institutional guidelines and the tenets of the Declaration of Helsinki. Molecular diagnoses have been previously published (4,10,18,49,50). Of the 32 participating patients, 17 had NR2E3 mutations (ages 11–77; patients, P, are numbered consecutively by appearance in figures), five had CRX mutations (ages 15–50) and there were 10 patients with retinitis pigmentosa of unknown genotype (ages 29–68). Normal subjects (n = 26; ages 4–58) were also included.

Optical coherence tomography: data acquisition

Cross-sectional retinal reflectivity profiles were obtained with optical coherence tomography (OCT) (Zeiss Humphrey Instruments, Dublin, CA, USA) in all 32 patients and in normals. The principles of the method (51) and our techniques (15–18) have been described. Data were acquired with one of two instruments: OCT1 with a theoretical axial resolution in retinal tissue of ~10 μm or OCT3 with a resolution of ~8 μm. Retinal cross-sections were sampled in all patients with vertical and horizontal scan groups centered on the anatomical fovea and consisting of five overlapping segments of linear scans, each 4.5 mm in length, extending 9 mm in either direction. Each segment was repeated at least twice and usually three or more times. In a subset of normals and patients, six scan groups were used to cover a 18 × 12 mm² region of the retina centered on the anatomical fovea at high lateral resolution. Each scan group covered a region of 6 × 6 mm² of retina by using 21 parallel horizontal (‘raster’) scans of 6 mm length, vertically separated by 0.3 mm. A video fundus image showing the location of the scanned segment was acquired and saved with each OCT scan by the commercial software. In addition, the fundus video visible during the complete session was recorded continuously on a video cassette recorder to allow post-acquisition digitization of fundus images when necessary.

OCT: data analysis

Post-acquisition processing of OCT data was performed with custom programs (MATLAB 6.5, MathWorks, Natick, MA, USA). Longitudinal reflectivity profiles (LRPs) making up the OCT scans were aligned using a dynamic cross-correlation algorithm (15,16) with a manual override when crossing structures (e.g. intraretinal pigment), which interrupted local lateral isotropy by shadowing deeper signals. Spatial maps of retinal thickness were derived from the groups of raster scans. First, the precise location and orientation of each scan relative to retinal features (blood vessels and optic nerve head) were determined using the video images of the fundus. LRPs were allotted to 0.3 × 0.3 mm² bins in a rectangular coordinate system centered at the fovea; the waveforms in each bin were aligned and averaged. The retinal thickness, as measured by OCT, was assumed to be the distance between previously defined features T1 (corresponding to the vitreo–retinal interface) and T4 (corresponding to the maximal slope vitread to the signal maximum) on averaged LRPs (15–17,52). Missing data were interpolated bilinearly, thickness values were mapped to a pseudo-color scale, and location of blood vessels and optic nerve head was overlaid for reference. Regions of retinoschisis were excluded manually from the analyses. Highly magnified sections of OCT scans are displayed after using a 2 × 1 moving median filter to reduce speckle noise in lateral dimension while keeping the original resolution in the longitudinal direction. All data are displayed as equivalent right eyes.

Psychophysics

Psychophysical thresholds were measured (at 2° intervals; stimulus, 1.7° diameter, 200 ms duration) in the same retinal regions as the OCT scans. Rod function was determined using two-color dark-adapted perimetry (500 and 650 nm stimuli). S cone function was measured with 440 nm stimuli on a yellow background (170 cd m⁻²). Long/middle wavelength sensitive (L) cone function was determined with 650 nm stimuli in the dark-adapted state (6) and compared with the normal data measured during the cone plateau phase of dark adaptation following a bleach. Details of the visual function techniques and analysis methods have been described (4,6–10,34,46,53).

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