X-Linked Retinoschisis: *RS1* Mutation Severity and Age Affect the ERG Phenotype in a Cohort of 68 Affected Male Subjects

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PURPOSE. To assess the effect of age and *RS1* mutation on the phenotype of X-linked retinoschisis (XLRs) subjects using the clinical electroretinogram (ERG) in a cross-sectional analysis.

METHODS. Sixty-eight XLRs males 4.5 to 55 years of age underwent genotyping, and the retinoschisis (*RS1*) mutations were classified as less severe (27 subjects) or more severe (41 subjects) based on the putative impact on the protein. ERG parameters of retinal function were analyzed by putative mutation severity with age as a continuous variable.

RESULTS. The a-wave amplitude remained greater than the lower limit of normal (mean, -2 SD) for 72% of XLRs males and correlated with neither age nor mutation class. However, b-wave and b/a-ratio amplitudes were significantly lower in the more severe than in the less severe mutation groups and in older than in younger subjects. Subjects up to 10 years of age with more severe *RS1* mutations had significantly greater b-wave amplitudes and faster a-wave trough implicit times than older subjects in this group.

CONCLUSIONS. *RS1* mutation putative severity and age both had significant effects on retinal function in XLRs only in the severe mutation group, as judged by ERG analysis of the b-wave amplitude and the b/a-ratio, whereas the a-wave amplitude remained normal in most. A new observation was that increasing age (limited to those aged 55 and younger) caused a significant delay in XLRs b-wave onset (i.e., a-wave implicit time), even for those who retained considerable b-wave amplitudes. The delayed b-wave onset suggested that dysfunction of the photoreceptor synapse or of bipolar cells increases with age of XLRs subjects. (*Invest Ophthalmol Vis Sci.* 2011;52:9250–9256) DOI:10.1167/iovs.11-8115

A monogenic condition, X-linked retinoschisis (XLRs) is a leading genetic cause of macular degeneration in young males and affects 1:5000 to 1:25,000.¹ It is caused by mutations in the retinoschisin gene (*RS1*) at Xp22.² Nearly 150 disease-

causing *RS1* mutations have been identified.³ *RS1* encodes a 24-kDa protein, retinoschisin (RS), which contains a discoidin domain and is found in the retina and pineal gland.⁴ Retinoschisin is thought to be important for cell adhesion and cell signaling because the protein has been demonstrated in the photoreceptor synapse onto bipolar cells.⁵ RS is associated with the Na⁺/K⁺ ATPase in the photoreceptor inner segment membrane.⁶ Atomic force microscopy implicates a structural role for retinoschisin in organizing lipid membranes, which may help to stabilize retinal cell integrity.⁷

XLRs subjects exhibit considerable heterogeneity on clinical examination and functional vision testing.⁸ Although most XLRs-affected males first come to attention for reduced acuity during school screening, some XLRs experience retinal detachments in infancy. In addition, our clinical cohort suggests that XLRs subjects rarely retain sufficient visual function to obtain an unrestricted driver's license by the fourth and fifth decades of life.

The consequence of abnormal retinoschisin protein or complete absence of RS on retinal function can be evaluated clinically using the full-field electroretinogram (ERG). The characteristic dark-adapted bright-flash ERG response in XLRs is an "electronegative waveform" in which the b-wave amplitude is disproportionately smaller than the normal or near-normal a-wave. ERG a-wave modeling has shown that phototransduction remains normal in some XLRs subjects despite reduced b-waves,⁹ indicating that one site of dysfunction lies beyond the photoreceptors. Abnormal timing of the light-adapted 30-Hz cone flicker ERG response is also reported, further suggesting dysfunction of the inner retina when associated with normal a-wave responses.¹⁰

In this genotype-phenotype study, we used the full-field ERG to evaluate retinal function of 68 XLRs subjects who were examined and genotyped at the National Eye Institute (NEI). The *RS1* gene contains six exons that encode for a signal sequence (exons 1 and 2), a retinoschisin domain (exon 3), and the discoidin domain (exons 4–6).² Most *RS1* mutations analyzed to date have implicated primarily a loss function rather than an abnormal function from the mutant protein.⁷ Signal sequence domain mutations impair protein transport to the endoplasmic reticulum and consequently yield a functional null-protein effect. Some discoidin domain mutations allow the production of retinoschisin mutant protein, but it is rapidly degraded and lost.¹¹ The diversity and complexity of *RS1* mutations precluded our incorporating all molecular nuances in a phenotype analysis. Instead, we simplified the approach by segregating the *RS1* mutations into two groups based on the putative severity of effect on protein function. We included molecular modeling¹² and, in some cases, biochemical analysis¹¹ in this approach to classify our subjects as having either a less or a more severe mutation and a putative effect on the resultant protein. We then examined for an association of disease phenotype (ERG measurements) with genotype severity.

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SUBJECTS AND METHODS

Subjects

We performed a retrospective study of 68 XLRs subjects who were examined at the NEI between 2004 and 2011 and for whom we identified *RS1* mutations. Subjects were self-referred or referred by a physician because of a clinical XLRs presentation. We limited the analysis to subjects age 55 years and younger because the ERG is known to decline in older age.^{13,14} The research was authorized by the National Institutes of Health Neuroscience Institutional Review Board and conformed to the Declaration of Helsinki. Subjects gave written informed consent before undergoing complete ocular examination that included full-field ERG, and all had blood drawn for genetic testing. Both eyes were tested by ERG in all but three cases: one child underwent testing of only one eye for compliance with the ERG routine; in two other subjects, medical reasons precluded bilateral ERG testing, one for keratoconus and a second for a glaucoma filtering bleb. An additional subject did not complete the 30-Hz flicker recording. In all, 133 eyes of 68 subjects 4.5 to 55 years of age were evaluated by ERG.

Electroretinogram Recordings

ERGs were recorded according to ISCEV standards¹⁵ with 2.4 cd · s/m² flashes to elicit the dark-adapted combined response and the photopic 30-Hz flicker against a 34 cd/m² background using a visual diagnostic system (UTAS 2000 or Sunburst Ganzfeld Visual Testing System; LKC Technologies Inc, Gaithersburg, MD). Pupils were dilated with topical phenylephrine and tropicamide. Subjects were dark adapted for 30 minutes before ERG recording began. Burian-Allen electrodes (Hansen Ophthalmic Laboratories, Iowa City, IA) were inserted with the help of artificial tears (Refresh Celluvisc; Allergan, Irvine, CA) for conductivity and subject comfort. Dark-adapted ERG responses were recorded first, followed by 10 minutes of light adaptation at 34 cd/m² before photopic testing. The lower limit of normal for all ERG parameters was considered to be 2 SD below the mean response calculated from 96 subjects with normal vision recorded on our ERG systems.

RS1 Mutation Analysis

DNA was extracted from peripheral blood leukocytes, and all six *RS1* exons were amplified and sequenced using intron/exon boundary PCR primers previously described² (ABI PRISM 310 Genetic Analyzer; Applied Biosystem, Foster City, CA). The putative impact on retinoschisin protein structure by particular mutations was assessed, and subjects were grouped into two categories by mutation severity. Twenty-seven subjects had conservative *RS1* missense mutations which retain the

same residue charge and similar size and are likely to alter protein structure minimally; these were binned in the genetically “less severe” category. Other mutations were classified as putatively “more severe” (41 subjects) because they cause major structural change or eliminate the production of retinoschisin protein. Eighteen of the 41 subjects had the addition or subtraction of a cysteine residue that would disrupt a disulfide bond important for tertiary folding structure; seven subjects had frameshift mutations that would alter all downstream amino acid residues; two subjects had splice site alterations that would disrupt coding at the intron-exon junction; one subject had misspelling of the start codon that would prevent transcription; eight subjects had non-sense mutations and five subjects had large DNA deletions, both likely to cause premature protein truncation.

The less severe and more severe mutation groups were of comparable age: 27 ± 12 years and 26 ± 17 years, respectively. The more severe group had a larger percentage of subjects younger than 10 years and older than 45 years (27% and 17%, respectively) than the less severe group (7% and 11%, respectively).

All less severe mutations occurred in exons 4 to 6, and most of the more severe mutations involved exons 1 to 3. However, in the 18 subjects with an altered cysteine residue, 16 of the alterations occurred in exons 4 to 6, and only two occurred in exons 1 to 3. One XLRs family had a severe mutation in exon 5 from a 1-base pair deletion replaced by an 18-base pair insertion, which caused rapid degradation of retinoschisin protein shown by biochemical analysis.¹¹ Although both the less and the more severe genetic groups likely encompass a spectrum of actual effect on the protein, this binning approach provides a starting point from which to explore possible genotype-phenotype correlations in this data set.

ERG Data Analysis

Dark-adapted combined-response ERG amplitudes, implicit times, and photopic 30-Hz flicker responses were measured according to ISCEV standards.¹⁵ The b-wave amplitude can sometimes be difficult to measure in XLRs because the position of the peak may become ambiguous if the response is greatly reduced. XLRs subjects have a wide range of ERG dark-adapted responses (Fig. 1) ranging from an overall positive b-wave but with generally subnormal amplitude to what is termed an electronegative waveform in which the b-wave does not return even to the prestimulus baseline. We selected the first main positive peak within 70 ms of the flash stimulus for XLRs subjects because this interval includes the expected time of the b-wave for subjects with normal vision, which occurs 50 to 60 ms after the stimulus flash.

FIGURE 1. Representative ERG dark-adapted combined responses for a healthy subject (A) and for XLRs subjects (B, C). XLRs waveforms typically are electronegative (B), with the b-wave remaining below the pre-a-wave baseline (81 of 133 eyes [61%]) in this cohort). However, a substantial number of XLRs subjects (52 of 133 eyes [39%]) in this cohort (C) have b-wave amplitudes that are reduced but not electronegative. ERG stimulus flash occurred at 0 ms.

FIGURE 2. a-Wave amplitude versus age for subjects with less severe mutations (green circles) and more severe mutations (red circles). Solid line: regression of a-wave amplitude versus age for the entire cohort ($-0.86 \mu\text{V/y}$, $P = 0.14$, 133 eyes). Dashed line: NEI clinical normal lower bound of $188 \mu\text{V}$ (mean, -2 SD, 96 subjects).

Statistical Analysis

Statistical analysis was performed using analysis software (Proc GENMOD, version 9.2; SAS Institute, Cary, NC). Analyses included both eyes of a subject, adjusted for intrapersonal correlation (Generalized Estimating Equation Model). Continuous linear regression and correlation coefficients were computed across all ages of a given group. In a population of healthy eyes, b-wave amplitudes are not normally distributed^{13,14} and often are transformed to meet the assumptions of most statistical analyses. We performed a log transform on the a-wave and b-wave amplitudes before statistical analysis.

RESULTS

a-Wave Amplitude

For all 68 XLRs subjects, a-wave amplitudes generally remained healthy across all ages (mean, $208 \pm 52 \mu\text{V}$), and only 39 eyes (28%) had a-wave amplitudes below the clinical lower normal bound of $188 \mu\text{V}$ (Fig. 2). Linear regression for the entire cohort showed that a-wave amplitude trended downward minimally with age ($-0.86 \mu\text{V/y}$; $P = 0.14$; 133 eyes). No statistical difference in a-wave amplitude was found between the less severe (56 eyes) and the more severe (77 eyes) mutation groups (difference, $6.21 \mu\text{V}$; $P = 0.37$). Several younger subjects with severe mutations were among those with the largest a-waves.

b-Wave Amplitude

Even though the combined XLRs population of 68 subjects had a large spread of individual b-wave amplitudes across all ages, a strong effect of age was present ($-2.36 \mu\text{V/y}$; $P = 0.0005$; 133 eyes) (Fig. 3). This age-dependent decrease of b-wave amplitude occurred at a younger age for this XLRs cohort than is found in normal aging studies.^{13,14} Subjects 10 years of age and younger had b-wave amplitudes substantially larger than those who were older for the entire 68 subjects ($250 \pm 71 \mu\text{V}$ for 27 eyes in subjects 10 years and younger vs. $172 \pm 89 \mu\text{V}$ for 106 eyes in subjects 11–55 years; $P < 0.0001$).

Grouping subjects by mutation severity showed that subjects with more severe mutations (77 eyes; Fig. 3, top) had smaller b-wave amplitudes than those with less severe mutations (56 eyes, Fig. 3, bottom) (difference, $48.5 \mu\text{V}$; $P = 0.006$). Further, across all ages, 23 eyes in the more severe mutation group had b-wave amplitudes $<100 \mu\text{V}$ compared with only four eyes in the less severe group.

The age effect on the b-wave was concentrated in the more severe mutation cohort. Although subjects with less severe

mutations (56 eyes; Fig. 3, bottom) showed no age effect (slope = $-0.7 \mu\text{V/y}$; $P = 0.33$), those with more severe mutations (77 eyes; Fig. 3, top) had a considerable effect of age (slope = $-2.95 \mu\text{V/y}$; $P < 0.0001$). The data for the more severe mutation group had greater complexity than is captured by a simple line fit across all ages, however, because there was rapid fall-off of amplitude after a young age. For those 11 to 55 years of age (54 eyes), b-wave amplitude essentially did not change with age across the 45-year span (slope = $-0.06 \mu\text{V/y}$; $P = 0.07$). Although some younger subjects with more severe mutations had near-normal b-wave amplitudes, we do not expect this to persist with age because several had older affected male relatives with markedly electronegative responses.¹¹

b-/a-Wave Ratio

As the b-wave originates from activity postsynaptic to photoreceptors, the ratio of b-wave to a-wave amplitudes (b/a-ratio)

FIGURE 3. b-Wave amplitude versus age for XLRs eyes with more severe mutations (red circles) or less severe mutations (green circles). Solid lines: regression of b-wave amplitude versus age for each cohort. Subjects with more severe mutations showed decreasing b-wave amplitude with age, whereas those with less severe mutations did not. Dashed line: NEI clinical normal lower bound of $188 \mu\text{V}$ (mean, -2 SD, $n = 96$ subjects).

FIGURE 4. ERG amplitude ratio of b/a-wave versus age in XLRS subjects. *Solid lines:* regression of b/a-ratio versus age for each cohort. More severe mutations (*top, red circles*): slope = $-0.01/y$, $P = 0.0001$, 77 eyes. Less severe mutations (*bottom, green circles*): slope = $-0.0034/y$, $P = 0.56$, 56 eyes. A b- or an a-wave ratio < 1.0 indicates an electronegative waveform, with the b-wave amplitude smaller than the a-wave amplitude. *Dashed line* at 1.2 shows the lower limit of the normal b/a-ratio (mean, -2 SD; mean = 1.92, SD = 0.36; 96 subjects).

reflects the integrity of photoreceptor synaptic transmission and of bipolar activity, normalized by photoreceptor function that drives these events.¹⁶ For the entire XLRS cohort, the b/a-ratio mirrored the b-wave and decreased significantly with age ($-0.0084/y$; $P = 0.0007$, 133 eyes) (Fig. 4). For the whole cohort, subjects aged 10 years and younger had larger b/a-ratios than those who were older (≤ 10 years: mean = 1.15 ± 0.3 , 27 eyes; 11–55 years: mean = 0.83 ± 0.3 , 106 eyes; $P = 0.0001$). This effect appeared to be driven by the subjects with genetically more severe mutations (Fig. 4, top), who showed a significant decrease of b/a-ratio with age (slope = $-0.01/y$; $P = 0.0001$, $n = 77$ eyes), whereas the subjects with less severe mutations (Fig. 4, bottom) showed no effect (slope = $-0.0034/y$; $P = 0.56$, 56 eyes). Subjects with more severe mutations (56 eyes) had lower b/a-ratios averaged across all

ages than did those with less severe mutations (77 eyes) (difference = 0.21, $P = 0.0072$). Among XLRS subjects aged 11 to 55 years, the waveform was “electronegative” for 80% of those with more severe mutations (44 of 56 eyes) compared with only 51% of those with less severe mutations (27 of 52 eyes; $P = 0.018$).

a-Wave Implicit Time

The ERG a-wave trough marks the transition from the negative-going photoreceptor a-wave to the positive-going b-wave and, hence, nominally represents the onset of depolarizing bipolar cell activity. We evaluated timing of the a-wave trough to learn whether bipolar cell activation might be impaired in XLRS (Fig. 5). For the entire set of 68 XLRS subjects, the average a-wave implicit time was approximately 21 ms (range, 15–40 ms). Older subjects (limited to those 55 and younger in this study) had slower a-wave implicit times than younger subjects (-0.13 ms/y; $P < 0.0001$, 133 eyes). Again, this change was driven by subjects with more severe mutations who had faster a-wave implicit times at ages 10 years and younger (25 eyes) compared with those at ages 11 to 55 years (difference = -3.58 ms; $P < 0.0001$, 52 eyes), whereas subjects with less severe mutations showed no age effect on prolongation of the a-wave implicit time (difference = -0.27 ms; $P = 0.49$). However, a-wave implicit time did not differ by mutation severity group (difference = -0.56 ms; $P = 0.41$, 56 eyes with less severe mutation, 77 eyes with more severe mutation), indicating a stronger aging effect than mutation.

Given that the a-wave trough nominally represents the onset of the positive-going b-wave, we wondered whether b-wave amplitude might affect a-wave implicit time because larger b-wave amplitudes would rise more quickly on the negative-going a-wave and thereby reduce the a-wave implicit time. In the more severe mutation cohort, b-wave onset (i.e., a-wave implicit time) was significantly negatively correlated with b-wave amplitude (Fig. 6A; 77 eyes, $r = -0.72$; $P < 0.0001$). This was confounded by age, however, because implicit time for younger subjects was considerably faster than for older subjects (Figs. 5, 6). No correlation was found for less severe mutations (Fig. 6; 56 eyes; $P = 0.89$), and several subjects in this group had substantial reductions in b-wave amplitude loss despite almost normal b-wave onset.

FIGURE 5. a-Wave implicit time for subjects with more severe mutations (*red circles*) and less severe mutations (*green circles*). *Solid line:* regression of implicit time versus age for the entire cohort (slope = 0.13 ms/y, $P = 0.001$, 133 eyes). *Dashed line* at 24 ms shows upper bound of normal timing determined at NEI from 106 subjects up to 55 years of age.

FIGURE 6. ERG a-wave implicit time (i.e., b-wave onset) versus b-wave amplitude for XLRs subjects with more severe (*top*, red circles) or less severe (*bottom*, green circles) mutations. Smaller circles: younger subjects; larger circles: older subjects. b-Wave onset correlated inversely with b-wave amplitude for the more severe mutation cohort (77 eyes, $r = -0.72$, $P < 0.0001$) but not for the less severe cohort (56 eyes, $r = 0.04$, $P = 0.80$). Dashed line at 24 ms shows upper bound of normal timing determined at NEI from 106 subjects up to 55 years of age.

30-Hz Flicker Responses

Cone-driven ERG activity was also abnormal in this XLRs cohort, as judged by 30-Hz flicker responses. Most XLRs subjects exhibited decreased and delayed photopic 30-Hz flicker ERG responses (mean amplitude = $55.8 \pm 24 \mu\text{V}$; mean time = $35 \pm 3.3 \text{ ms}$; 133 eyes). For the group as a whole, although 30-Hz flicker amplitude did not vary with age (129 eyes; $P = 0.17$), prolonged flicker times correlated significantly with age (slope = 0.11 ms/y ; $P < 0.0001$, 129 eyes). Neither amplitude nor timing of 30-Hz flicker responses showed an effect of mutation (53 eyes less severe mutation group, 77 eyes more severe mutation group; amplitude difference = $5.02 \mu\text{V}$, $P = 0.38$; timing difference = 0.07 ms , $P = 0.91$).

Summary of Findings

The 68 XLRs subjects generally had normal a-wave amplitudes through age 55, whereas b-wave amplitudes declined with age. When subjects were binned by mutation severity, those with genetically more severe mutations had lower b-wave amplitudes and b/a-ratios than subjects with less severe mutations (b-wave difference = $48.5 \mu\text{V}$, $P = 0.006$; b/a-ratio difference = 0.21 , $P = 0.002$). In addition, subjects 11 to 55 years of age (52 eyes) who had more severe mutations had smaller b-wave amplitudes and b/a-ratios than those who were younger (≤ 10 years, 25 eyes; difference = $0.39 \mu\text{V}$, $P = 0.0001$). The a-wave implicit time (i.e., nominally b-wave onset) of the older subjects (11–55 years, 52 eyes) with more severe mutations was delayed compared with younger subjects (≤ 10 years, 25 eyes; difference = -3.58 ms , $P < 0.0001$). This delay correlated with b-wave amplitude in subjects with more severe mutations but not for those with less severe mutations. The less severe mutation group showed no effect of aging in b-wave amplitude, b/a-ratio, or a-wave implicit time.

DISCUSSION

This study found a correlation between the ERG phenotype abnormality of XLRs subjects and the expected impact of the *RS1* mutation on the RS protein. As a group, mutations that highly perturb or even eliminate the protein affected b-wave amplitude and the b/a-ratio to greater degree than conservative changes in the RS protein. In addition, b-wave amplitude and b/a-ratio both declined with age in subjects with more severe mutations in this population of XLRs subjects. However, neither mutation severity nor age systematically affected a-wave amplitude. A finding not previously noted for XLRs was that a-wave implicit time was prolonged with age. Implicit time is an attractive ERG feature to track because it can be judged without ambiguity.

XLRs characteristically causes b-wave reduction disproportionate to a-wave change, which reduces the b/a-ratio to 1.0 or less and gives a waveform described as “electronegative.” However, the b/a-ratio remained greater than 1.0 for 39% of eyes of our XLRs cohort, and three XLRs subjects (ages 6, 14, and 48) had clinically normal b-wave amplitudes ($\geq 374 \mu\text{V}$), whereas four others (ages 14, 25, 28, and 31) had b-wave amplitudes $>300 \mu\text{V}$ in at least one eye. These examples illustrate that synaptic retinal function can be well preserved in some *RS1* mutation subjects, including 2 of 34 eyes of subjects older than 30 years in the severe mutation group, and indicate that careful clinical examination, including OCT, retinal imaging, and *RS1* mutation screening, remain essential for proper diagnosis.

There may be a subtle bias between the two mutation groups in our population because proportionally more younger subjects were examined with putatively more severe mutations, and fewer subjects between ages 20 to 40 with severe mutations were included. More families in the more severe mutation group presented to our clinic with two generations affected: a young proband and the affected maternal grandfather, whereas subjects with putatively less severe mutations tended to present as single individuals or as an affected sibling. An intriguing possibility is that more severe phenotypes reflect underlying more severe genotypes.

This study indicates that molecular consideration of *RS1* mutations is useful in identifying XLRs patients expected to have more severe retinal ERG functional changes, as a group. However, the phenotype-genotype correlations still fall short for individual subjects because the two mutation groups show considerable overlap. Studies of multigenerational families have noted a considerable range of XLRs disease even for subjects with the same mutation or mutation type.^{17–19} Con-

sequently, XLRs genotype should not be understood as phenotype destiny. For instance, severe XLRs disease, including considerable acuity loss, vitreous hemorrhage, and varying extent of peripheral schisis, was reported within a Chinese family with a conservative missense mutation.¹⁸ Others reported well-preserved ERGs and mild clinical disease in three adult XLRs subjects with *RS1* mutations that cause addition or loss of a cysteine residue,¹⁷ which thereby fits our criterion of a "more severe mutation." Because XLRs disease is highly pleomorphic, it is unlikely that a more detailed specificity of mutation genotype would narrow the phenotype range. Readers interested in a different approach to considering *RS1* mutation effects may consult Sergeev et al.,¹² who performed computational molecular modeling of the mutant retinoschisin atomic structure and evaluation of protein stabilization energy to calculate a graded impact index.¹²

Questions remain as to whether photoreceptors are much affected in XLRs.¹⁶ Retinoschisin protein is heavily expressed in photoreceptor inner segments and is present in the photoreceptor-bipolar cell synapse.^{20,21} Molday et al.²² demonstrated that retinoschisin protein helps anchor the Na/K ATPase, which, if altered, might be expected to affect rod function. However, a number of clinical reports, including this present study, find generally normal a-wave amplitudes in XLRs despite reduced b-wave amplitudes.^{1,23,24} Khan et al.⁹ evaluated photoreceptor function in XLRs subjects by ERG a-wave modeling and found the majority had normal rod-photoreceptor function. This finding is consistent with results of the present study, in which 71% of our XLRs subjects maintained normal a-wave amplitudes, indicating normal rod photoreceptor circulating dark current. Our result also concurs with that of Bradshaw et al.,¹⁶ who reported decreased a-wave amplitudes in only one-third of their XLRs males. In aggregate, these studies indicate that static evaluation of a-wave amplitude is unlikely to identify functional deficits of photoreceptors in XLRs disease.

Our study gives reasonable evidence of progressive global loss of retinal function during the early decades of life in subjects with molecularly more severe mutations, for both b-wave amplitude and reduced b/a-ratio. Kjellstrom et al.¹⁰ performed a small XLRs longitudinal study but did not show any particular progressive ERG changes in longer term follow-up. However, two XLRs subjects in that study had exon 3 truncating mutations likely to prevent the production of any RS protein, and (as our present study would predict) the b/a-ratios were progressively diminished over time (subject 4: b/a-ratio = 1.20 on first examination but b/a-ratio = 0.88 on second examination 12 years later; subject 6: b/a-ratio = 1.10 on first examination but b/a-ratio = 0.77 on second examination 21 years later). This raises the question of whether the lack of RS protein may cause progressive structural change over time.¹¹

We noted that a-wave implicit time was prolonged for XLRs subjects, particularly by middle age. The a-wave trough represents the transition to dominance of postsynaptic bipolar-cell activity in the ERG. In the presence of a normal photoreceptor response, prolonged a-wave implicit time likely represents a delay in b-wave onset, consistent with previous indications that retinoschisin is expressed in the photoreceptor-bipolar cell synapse⁵ and that the natural history of XLRs pathology progressively alters synaptic function in the XLRs mouse.²⁵

Precise determinants of a-wave implicit time are not understood. Two conditions, central retinal vein occlusion (CRVO) and complete X-linked congenital stationary night-blindness (CSNB), give examples, respectively, of abnormal versus normal a-wave implicit times, both with electronegative b-waves. Human CRVO has a delayed a-wave implicit time compared with the unaffected fellow eye, which was attributed to photoreceptor hypoxia.^{26,27} CSNB shows selective reduction or

loss of ON-bipolar cell activity, and the b-wave amplitude is reduced while the a-wave amplitude and implicit time remain normal, which again implies that a-wave timing is determined primarily by photoreceptor activity.²⁸

However, in the case of XLRs, current evidence points away from the photoreceptors as causing the delayed a-wave trough because a-wave modeling had indicated normal phototransduction sensitivity (S) and velocity (K_{max}) in subjects with the *RS1* genotype.⁹ Consequently, we believe the delay stems from impaired synaptic structure, which may, for instance, affect the rate of glutamate release/uptake and thereby retard neural signal transfer to bipolar cells. Glutamate clearance from the synaptic cleft depends on diffusion from the extracellular space (and, hence, on the structural configuration of that synaptic space) and also on uptake capacity, and both parameters are malleable, at least during development.²⁹ XLRs may cause a failure of presynaptic machinery to properly modulate glutamate release or, more likely, from impaired function of the postsynaptic elements in which retinoschisin protein is normally located and which is diminished in photoreceptor synapses of the *RS1*-knockout mouse.⁵ Synaptic markers PSD95 (presynaptic) and mGluR6 (postsynaptic) both decline with age in the XLRs mouse, in parallel to the decline of b-wave rather than a-wave amplitude.⁵ A hypothesis involving the synapse in XLRs disease ties retinoschisin to the maintenance of synaptic structure and function in the outer retina.

Finally, the cone system also exhibited ERG pathology in XLRs, in addition to the rod system, because photopic 30-Hz flicker response amplitude was diminished, and time-to-peak was delayed in >70% of our XLRs cohort. This is consistent with a generalized photoreceptor synaptic defect because the great majority of the photopic 30-Hz response develops postsynaptically to the cones.³⁰ Additional study of the temporal properties of retinal signaling in XLRs disease may shed light on the cellular role of retinoschisin protein.

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References

- George ND, Yates JR, Moore AT. X linked retinoschisis. *Br J Ophthalmol*. 1995;79:697-702.
- Sauer CG, Gehrig A, Warneke-Wittstock R, et al. Positional cloning of the gene associated with X-linked juvenile retinoschisis. *Nat Genet*. 1997;17:164-170.
- Functional implications of the spectrum of mutations found in 234 cases with X-linked juvenile retinoschisis: the Retinoschisis Consortium. *Hum Mol Genet*. 1998;7:1185-1192.
- Takada Y, Fariss RN, Muller M, Bush RA, Rushing EJ, Sieving PA. Retinoschisin expression and localization in rodent and human pineal and consequences of mouse *RS1* gene knockout. *Mol Vis*. 2006;12:1108-1116.
- Takada Y, Vijayasarathy C, Zeng Y, Kjellstrom S, Bush RA, Sieving PA. Synaptic pathology in retinoschisis knockout (*Rs1-y*) mouse retina and modification by rAAV-*Rs1* gene delivery. *Invest Ophthalmol Vis Sci*. 2008;49:3677-3686.
- Molday RS, Warren R, Kim TS. Purification and biochemical analysis of cGMP-gated channel and Na⁺/Ca²⁺-K⁺ exchanger of rod photoreceptors. *Methods Enzymol*. 2000;315:831-847.
- Molday RS. Focus on molecules: retinoschisin (*RS1*). *Exp Eye Res*. 2007;84:227-228.
- Nakamura M, Ito S, Terasaki H, Miyake Y. Japanese X-linked juvenile retinoschisis: conflict of phenotype and genotype with novel mutations in the *XLRs1* gene. *Arch Ophthalmol*. 2001;119:1553-1554.

9. Khan NW, Jamison JA, Kemp JA, Sieving PA. Analysis of photoreceptor function and inner retinal activity in juvenile X-linked retinoschisis. *Vision Res.* 2001;41:3931-3942.
10. Kjellstrom S, Vijayasarathy C, Ponjavic V, Sieving PA, Andreasson S. Long-term 12 year follow-up of X-linked congenital retinoschisis. *Ophthalmic Genet.* 2010;31:114-125.
11. Vijayasarathy C, Ziccardi L, Zeng Y, Smaoui N, Caruso RC, Sieving PA. Null retinoschisin-protein expression from an RS1 c354del1-ins18 mutation causing progressive and severe XLRS in a cross-sectional family study. *Invest Ophthalmol Vis Sci.* 2009;50:5375-5383.
12. Sergeev YV, Caruso RC, Meltzer MR, Smaoui N, MacDonald IM, Sieving PA. Molecular modeling of retinoschisin with functional analysis of pathogenic mutations from human X-linked retinoschisis. *Hum Mol Genet.* 2010;19:1302-1313.
13. Birch DG, Anderson JL. Standardized full-field electroretinography: normal values and their variation with age. *Arch Ophthalmol.* 1992;110:1571-1576.
14. Weleber RG. The effect of age on human cone and rod Ganzfeld electroretinograms. *Invest Ophthalmol Vis Sci.* 1981;20:392-399.
15. Marmor MF, Fulton AB, Holder GE, Miyake Y, Brigell M, Bach M. ISCEV standard for full-field clinical electroretinography (2008 update). *Doc Ophthalmol.* 2009;118:69-77.
16. Bradshaw K, George N, Moore A, Trump D. Mutations of the XLR1 gene cause abnormalities of photoreceptor as well as inner retinal responses of the ERG. *Doc Ophthalmol.* 1999;98:153-173.
17. Li X, Ma X, Tao Y. Clinical features of X linked juvenile retinoschisis in Chinese families associated with novel mutations in the RS1 gene. *Mol Vis.* 2007;13:804-812.
18. Xu J, Gu H, Ma K, et al. R213W mutation in the retinoschisin 1 gene causes X-linked juvenile retinoschisis in a large Chinese family. *Mol Vis.* 2010;16:1593-1600.
19. Suganthalakshmi B, Shukla D, Rajendran A, Kim R, Nallathambi J, Sundaresan P. Genetic variations in the hotspot region of RS1 gene in Indian patients with juvenile X-linked retinoschisis. *Mol Vis.* 2007;13:611-617.
20. Molday LL, Hicks D, Sauer CG, Weber BH, Molday RS. Expression of X-linked retinoschisis protein RS1 in photoreceptor and bipolar cells. *Invest Ophthalmol Vis Sci.* 2001;42:816-825.
21. Reid SN, Akhmedov NB, Piriev NI, Kozak CA, Danciger M, Farber DB. The mouse X-linked juvenile retinoschisis cDNA: expression in photoreceptors. *Gene.* 1999;227:257-266.
22. Molday LL, Wu WW, Molday RS. Retinoschisin (RS1), the protein encoded by the X-linked retinoschisis gene, is anchored to the surface of retinal photoreceptor and bipolar cells through its interactions with a Na/K ATPase-SARM1 complex. *J Biol Chem.* 2007;282:32792-32801.
23. Eksandh LC, Ponjavic V, Ayyagari R, et al. Phenotypic expression of juvenile X-linked retinoschisis in Swedish families with different mutations in the XLR1 gene. *Arch Ophthalmol.* 2000;118:1098-1104.
24. Lesch B, Szabo V, Kanya M, et al. Clinical and genetic findings in Hungarian patients with X-linked juvenile retinoschisis. *Mol Vis.* 2008;14:2321-2332.
25. Park TK, Wu Z, Kjellstrom S, et al. Intravitreal delivery of AAV8 retinoschisin results in cell type-specific gene expression and retinal rescue in the Rs1-KO mouse. *Gene Ther.* 2009;16:916-926.
26. Johnson MA, Hood DC. Rod photoreceptor transduction is affected in central retinal vein occlusion associated with iris neovascularization. *J Opt Soc Am A Opt Image Sci Vis.* 1996;13:572-576.
27. Kaye SB, Harding SP. Early electroretinography in unilateral central retinal vein occlusion as a predictor of rubeosis iridis. *Arch Ophthalmol.* 1988;106:353-356.
28. Miyake Y, Yagasaki K, Horiguchi M, Kawase Y, Kanda T. Congenital stationary night blindness with negative electroretinogram: a new classification. *Arch Ophthalmol.* 1986;104:1013-1020.
29. Thomas CG, Tian H, Diamond JS. The relative roles of diffusion and uptake in clearing synaptically released glutamate change during early postnatal development. *J Neurosci.* 2011;31:4743-4750.
30. Kondo M, Sieving PA. Primate photopic sine-wave flicker ERG: vector modeling analysis of component origins using glutamate analogs. *Invest Ophthalmol Vis Sci.* 2001;42:305-312.