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Journal

Investigative Ophthalmology & Visual Science, 53(10)

ISSN

0146-0404

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Publication Date

2012-09-14

DOI

10.1167/iovs.12-9569

Peer reviewed

Discordant Anatomical, Electrophysiological, and Visual Behavioral Profiles of Retinal Degeneration in Rat Models of Retinal Degenerative Disease

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Purpose. To assess structural, functional, and visual behavioral relationships in mutant rhodopsin transgenic (Tg) rats and to determine whether early optokinetic tracking (OKT) visual experience, known to permanently elevate visual thresholds in normal rats, can enhance vision in rats with photoreceptor degeneration.

Methods. Eight lines of pigmented Tg rats and RCS rats were used in this study. OKT thresholds were tested at single ages (1, 2, 3, 4, and 6 months) in naïve groups of rats, or daily in groups that began at eye-opening (P15) or 10 days later (P25). Electroretinogram (ERG) response amplitudes were recorded after OKT testing, and outer nuclear layer (ONL) thickness measurements were then obtained.

Results. OKT thresholds, when measured at a single time point in naïve Tg lines beginning at P30, did not decline until months after significant photoreceptor loss. Daily testing of Tg lines resulted mostly with OKT thresholds inversely related to photoreceptor degeneration, with rapid degenerations resulting in sustained OKT thresholds for long periods despite the rapid photoreceptor loss. Slower degenerations resulted in rapid decline of thresholds, long before the loss of most photoreceptors, which was even more pronounced when daily testing began at eye opening. This amplified loss of function was not a result of testing-induced damage to the rod or cone photoreceptors, as ERG amplitudes and ONL thicknesses were the same as untested controls.

Conclusions. The unexpected lack of correlation of OKT testing with photoreceptor degeneration in the Tg rats emphasizes the need in behavioral therapeutic studies for careful analysis of visual thresholds of experimental animals prior to therapeutic intervention. (*Invest Ophthalmol Vis Sci.* 2012;53:6232–6244) DOI:10.1167/iovs.12-9569

Retinal degenerative diseases (RDDs) affect millions of people and are a leading cause of vision loss and blindness worldwide. Although effective treatments for most of these diseases are limited, numerous animal models are available for study. The most widely used model of RDD is the Royal College of Surgeons (RCS) rat. The RCS rat has a mutation in the *Mertk* gene[1] expressed in RPE cells[2,3] that prevents them from phagocytizing photoreceptor outer segments.[4] This dysfunction results in progressive photoreceptor cell death[5,6] and loss of electrophysiological responses such as the electroretinogram (ERG).[5] Other widely used rat models of human RDDs are mutant rhodopsin transgenic (Tg) rats, 3 lines of P23H and 5 lines of S334ter rhodopsin, with different rates of degeneration (in the public domain, http://www.ucsfeye.net/mlavailRDratmodels.shtml). Rhodopsin mutations represent a much larger human population than the *Mertk* mutation. These rhodopsin mutations also cause progressive photoreceptor degeneration with mostly concomitant decline in retinal function as measured with the ERG[7,8] and visual activity in the superior colliculus.[9] Both the RCS and mutant rhodopsin models are currently being used for preventative therapeutic approaches such as neuroprotection,[10–14] cell transplantation and cell-based therapy,[15–18] and gene therapy.[8,19–21]

The ultimate measure of successful treatment in human patients is the prevention of vision loss or restoration of vision. Thus, a number of recent studies have now focused on analysis of vision as a consequence of progressive photoreceptor degeneration.[22,23] In several cases, however, behavioral tests of vision have not shown a direct correlation with loss of photoreceptors and ERG function. For example, in pigmented RCS rats at 4 months of age, the outer nuclear layer (ONL) consists of approximately one row of photoreceptor nuclei (approximately 10% of normal) and ERG b-waves can no longer be elicited[24]; yet visual acuity measured in a perceptual based task remains at 35% of normal,[22] and spatial frequency thresholds of the optokinetic tracking (OKT) response are ~60% of normal.[23] Somewhat later, at 6 to 7 months of age, photoreceptor cell survival is minimal, and luminance thresholds recorded from the superior colliculus in response to small spots are no longer measurable (Girman S, et al. *IOVS*

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Supported by an FFB-Canada graduate student scholarship (TJM); Knight's Templar Eye Foundation-Pediatric Ophthalmology Grant (TJM); and NIH Grants EY001919, EY006842, EY002162 (MML), and K08 EY00415 (JLD); The Foundation Fighting Blindness and an Unrestricted Award to University of California San Francisco (UCSF) from the Research to Prevent Blindness (MML and JLD); and The Bernard A. Newcomb Macular Degeneration Fund (JLD).

Submitted for publication January 24, 2012; revised June 11, 2012; accepted August 13, 2012.

Disclosure: **T.J. McGill**, None; **G.T. Prusky**, None; **R.M. Douglas**, None; **D. Yasumura**, None; **M.T. Matthes**, None; **R.J. Lowe**, None; **J.L. Duncan**, None; **H. Yang**, None; **K. Ahern**, None; **K.M. Daniello**, None; **B. Silver**, None; **M.M. LaVail**, None

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2003;44:ARVO E-Abstract 482), yet visual acuity remains at ~30% of normal and OKT thresholds are ~40% of normal.[22,23] Visual acuity in the RCS rat remains quantifiable until ~11 months of age, albeit at low levels, and OKT thresholds can be elicited until ~10 months of age.[22,23]

Studies using rapidly degenerating S334ter-3 mutant rhodopsin rats have also shown similar discordance between anatomical and electrophysiological measurement compared with measurements of vision. For example, Sagdullaev et al.[9] reported that by postnatal day (P) 28, photoreceptor cell death is near complete in the central retina, and multiunit recordings from a corresponding area of the superior colliculus (SC) are nonresponsive. At P63 and later, the entire SC was nonresponsive.[9] However, Thomas et al.[25,26] reported quantifiable optokinetic thresholds up to 6 to 7 months of age using the same S334ter-3 rat line.

Congruency between measures of the visual system has previously been reported in normal (nondystrophic) rodents, such as the rise of OKT thresholds coinciding with maturation of photoreceptors in the developing mouse retina.[27] However, in a recent study that examined the potentially toxic effect of high concentrations of CNTF injected into the normal rat eye, visual thresholds were impaired significantly longer than were ERG responses or photoreceptor outer segments.[28] Whereas the authors discussed multiple possible explanations for this effect, plasticity within the visual system could also account for the incongruent visual system measurements in retinal degeneration models. Experience-dependent plasticity in the mammalian visual system is evident in early,[29–31] juvenile,[32–35] and adult phases of life.[36–39] A number of studies have examined neuronal plasticity in animal models with RDD, focusing particularly on secondary changes following the death of photoreceptors.[40–48] Although much of the evidence of visual system plasticity has come from loss-of-function studies in normal animals, a few studies have examined visual experience to stimulate a form of plasticity that can lead to gain of function. One such study[49] showed that providing a specific visual stimulus early in life can permanently enhance visual thresholds in normal rats. These findings are particularly relevant to RDD therapy, as they suggest that vision could potentially be rescued before, or perhaps even after, significant retinal degeneration has occurred by engaging gain-of-function plasticity.

Therefore, the goals of this study were to generate a comprehensive evaluation of visual function in 8 lines of mutant rhodopsin Tg rats and compare this with previously reported morphological and electrophysiological degenerative changes; and to examine the effect of early visual experience, that can enhance vision in normal rats,[49] on visual deterioration in the Tg and RCS rat models of human RDD.

Methods

Animals

This study was conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of the Institutional Animal Care and Use Committees (IACUC) at the University of Lethbridge and the University of California, San Francisco (UCSF). Development of the eight lines of Tg rats is described elsewhere (in the public domain, http://www.ucsfeye.net/mlavailRDratmodels.shtml).[50] The Tg rats, with different rates of retinal degeneration, were three lines of P23H rhodopsin (a mouse opsin gene with a single amino acid substitution at codon 23) and five lines of S334ter rhodopsin (a mouse opsin gene bearing a termination codon at residue 334, which results in a C-terminal truncated opsin protein lacking the last 15 amino acid residues and, thus, all of the phosphorylation sites of the molecule). Pigmented Tg rats, optimal for OKT analysis, were produced by crossing homozygous albino Tg rats (carrying two copies of the transgene) with normal, wild-type pigmented Long-Evans (LE) rats. All offspring were pigmented, each carrying a single copy of the transgene (in the public domain, http://www.ucsfeye.net/mlavailRDratmodels.shtml).

Behavioral Measurements of Vision

Measurement and analysis of OKT thresholds followed the same procedures and used the same equipment as described elsewhere (OptoMotry; CerebralMechanics, Lethbridge, Alberta, Canada).[23,27,51] Whenever possible, experimenters were blind to previously recorded results, and thresholds were regularly confirmed by more than one observer. In addition, thresholds were measured from multiple litters of rats at both UCSF and University of Lethbridge with multiple observers. OKT thresholds measured in the two locations were slightly different in both mutant and wild-type rats. The normal OKT thresholds were consistently approximately 0.427 cyc/deg at UCSF and 0.528 cyc/deg at the University of Lethbridge. The thresholds reported here, except where indicated, have been normalized between testing locations to the 0.427 cyc/deg level.

Electroretinography

Rats were dark-adapted overnight and were anesthetized with an intramuscular mixture of ketamine (13 mg/kg) and xylazine (87 mg/kg) while in dim red light. Corneas were anesthetized with 0.5% proparacaine, and pupils were dilated with 2.5% phenylephrine hydrochloride followed by 1.0% atropine. Bilateral, simultaneous full-field scotopic ERGs were elicited with 10-μs flashes of white light, and responses were recorded using contact lens electrodes[52] with a visual electrodiagnostic system (UTAS-E 3000; LKC Technologies, Inc., Gaithersburg, MD) as previously described[28] to determine the dark adapted (scotopic) a- and b-wave response amplitudes and light adapted (photopic) b-wave responses. In addition, we carried out flicker analysis of cone function after measuring light adapted b-wave responses using a standard 20-Hz flicker stimulus at intensities of 0.4 and 0.9 log cd s/m^2, averaging 50 responses.

Histological and Morphological Analysis

At the University of Lethbridge, animals were euthanized via lethal dose of euthansol (sodium pentobarbital; 0.3 mL/kg), and those sacrificed at UCSF were killed with an overdose of carbon dioxide. The rats were then immediately perfused with a fixative of 2% paraformaldehyde and 2.5% glutaraldehyde in PBS, and the eyes were dissected out later. Eyes were dissected from the orbit following marking for orientation and fixed by immersion in the same fixative. The eyes were then bisected along the vertical meridian and embedded in an Epon-Araldite mixture, with sections cut at 1-μm thickness as described.[6] Measurements of the ONL thickness were taken as an index of the stage of retinal degeneration,[53] and were obtained from 54 locations around the retina as described.[54]

Experimental Design

Evaluation of visual function was performed using multiple groups (n = 3–6) of Tg rats from each line and a single litter (n = 6) of RCS rats. The first functional evaluation (naïve) involved a single behavioral and ERG evaluation prior to sacrifice in Tg rats beginning at P30 and then at monthly intervals up to 4 months, and at 6 months (i.e., group 1 was tested only at P30 then sacrificed, group 2 was tested only at P60 then sacrificed, etc.). The second evaluation with different groups of animals consisted of daily evaluation that began at P25 and ended when the animals no longer generated an optomotor response to the stimuli, or when OKT thresholds persisted for a significant period after

considerable or complete photoreceptor cell death. Finally, to examine the effect of early exposure to the testing stimulus,[49] Tg and RCS rats were tested daily beginning at eye-opening (P15). To confirm effects seen as a result of early (P15) daily testing were attributable to the rotating stimulus, a yoke-control was also used. The yoke-control consisted of a control group of S334ter–line-9 animals in which half of the group received normal testing from P15 onward, and the second half received the identical stimuli as the first half minus movement of the gratings. On P25, all animals in this control group were tested using normal procedures.[49]

Results

Structural and Functional Degenerative Changes in Tg Rats

A summary of the rates of photoreceptor degeneration as seen structurally in the pigmented mutant rhodopsin Tg rats is shown in Figure 1. Light microscopic examples of each of the lines at P30 are shown in Figure 2. The loss of photoreceptors is extremely slow in three of the lines, with the ONL thickness almost indistinguishable from that of wild-type LE controls through the age of P120 in P23H-2 and P23H-3 rats (Fig. 1A) and through the age of P180 in S334ter-9 rats (Fig. 1B). By contrast, two of the lines, S334ter-3 and S334ter-7, show very rapid degeneration and have already lost most of their photoreceptors by the age of P30 (Figs. 1B, 2I, 2J). The other lines (P23H-1, S334ter-4, and S334ter-5) show intermediate rates of degeneration (Figs. 1, 2D, 2F–H). By comparison, the widely studied pigmented RCS rat[6] has a rate of degeneration most closely resembling that of the S334ter-5 line. After each of the behavioral studies (described below), the eyes were examined histologically, and in every case, the stage of retinal degeneration as measured by the thickness of the ONL matched that shown in Figure 1. Thus, in all cases, the testing light exposure produced no negative structural changes as viewed in plastic-embedded sections.

A number of structural-functional studies have shown a relatively close concordance of declining ERG response amplitudes with the decline in photoreceptor numbers with advancing age in rhodopsin Tg[7,8] and RCS rats.[5] Our studies confirm this for all lines, except for the very slowly degenerating S334ter-9 line, in which the ERG response amplitudes decline somewhat more rapidly than the loss of photoreceptors, but not as rapidly as those of the S334ter-4 line (to be described elsewhere). An example of the concordant structural-functional relationships in one of the slowest (P23H-2) and fastest (S334ter-7) degenerating lines are illustrated in Figures 6A–C and 6F. In this case at P90, in the slowly degenerating P23H-2 line, almost the full complement of photoreceptors is still present, and the scotopic and photopic ERG response amplitudes are almost normal. By contrast, in the rapidly degenerating S334ter-7 line, almost all photoreceptors have degenerated and disappeared, and the ERG responses are almost nonexistent (Figs. 6A–C, 6F).

Delayed Visual Degeneration following Single Time Point Assessment (Naïve Testing)

To explore the structural-functional-visual behavioral relationships in mutant rhodopsin Tg rats, we examined OKT thresholds from each line measured only once at a single time point prior to sacrifice (e.g., for each line, group 1 was tested at P30 then sacrificed, group 2 at P60, and so on). Despite strikingly different rates of photoreceptor degeneration (Fig. 1), OKT measurements recorded from each of the three P23H lines resulted in OKT thresholds of ~0.400 to 0.450 cyc/deg

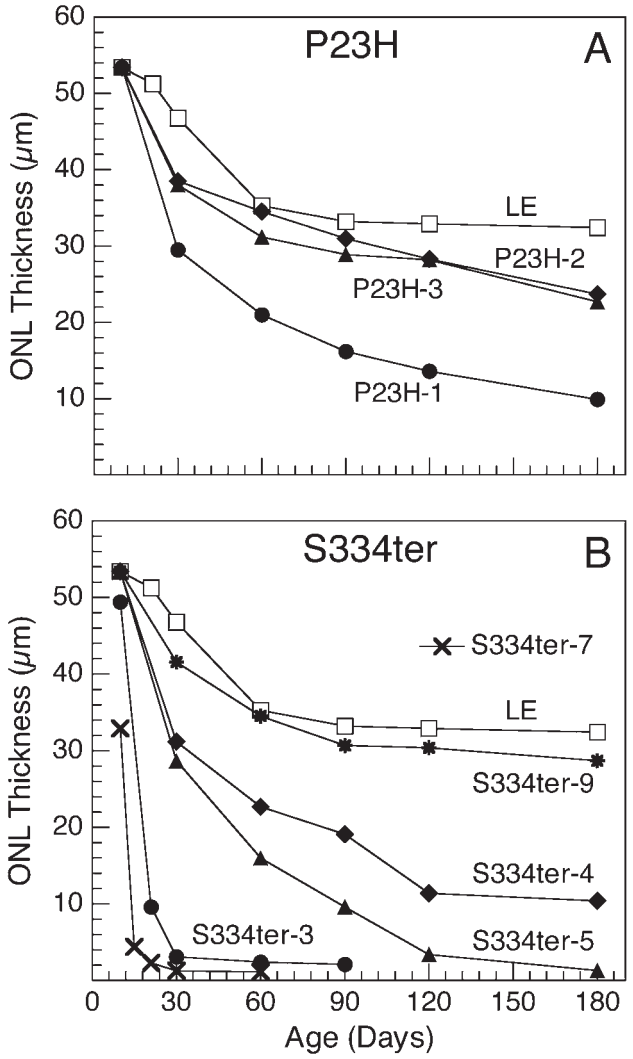

Figure 1. Rates of degeneration in mutant rhodopsin Tg rats. ONL thickness, which is proportional to the number of photoreceptor nuclei, is shown in wild-type pigmented Long-Evans rats (LE) and different lines of pigmented Tg rats. (**A**) LE rats and pigmented Tg rats carrying the P23H transgenes. (**B**) LE rats and pigmented Tg rats carrying the S334ter transgenes. The data are modified from those presented at (in the public domain, http://www.ucsfeye.net/mlavailRDratmodels.shtml) and are the means of 3 to 4 rats at each age. For reference to light micrographs of plastic-embedded sections (Fig. 2), each photoreceptor nucleus is approximately 4.5 mm in diameter.

up to P180, which were virtually identical to those of LE controls (Fig. 3A), despite the loss of 25% to 30% of photoreceptors in P23H-2 and P23H-3 and approximately 70% in P23H-1 Tg rats at this age (Fig. 1A). Thresholds maintained near LE control values were also observed in two lines with the S334ter mutation, lines S334ter-4 and S334ter-9 (Fig. 3B). Most surprisingly, the three fastest degenerating S334ter lines (and the three fastest degenerations of all the lines) also showed near control values through P90 (lines S334ter-3 and S334ter-7) or P120 (line S334ter-5; Fig. 3C), after which they declined. However, they still had measurable thresholds through P180. Remarkably, the OKT thresholds in the most rapidly degenerating lines, S334ter-3 and S334ter-7, were virtually identical with those of controls through at least P90 (Fig. 3C), yet almost all photoreceptors had been lost much earlier, by P30 (Figs. 1B, 2I–J). Thus, none of the lines

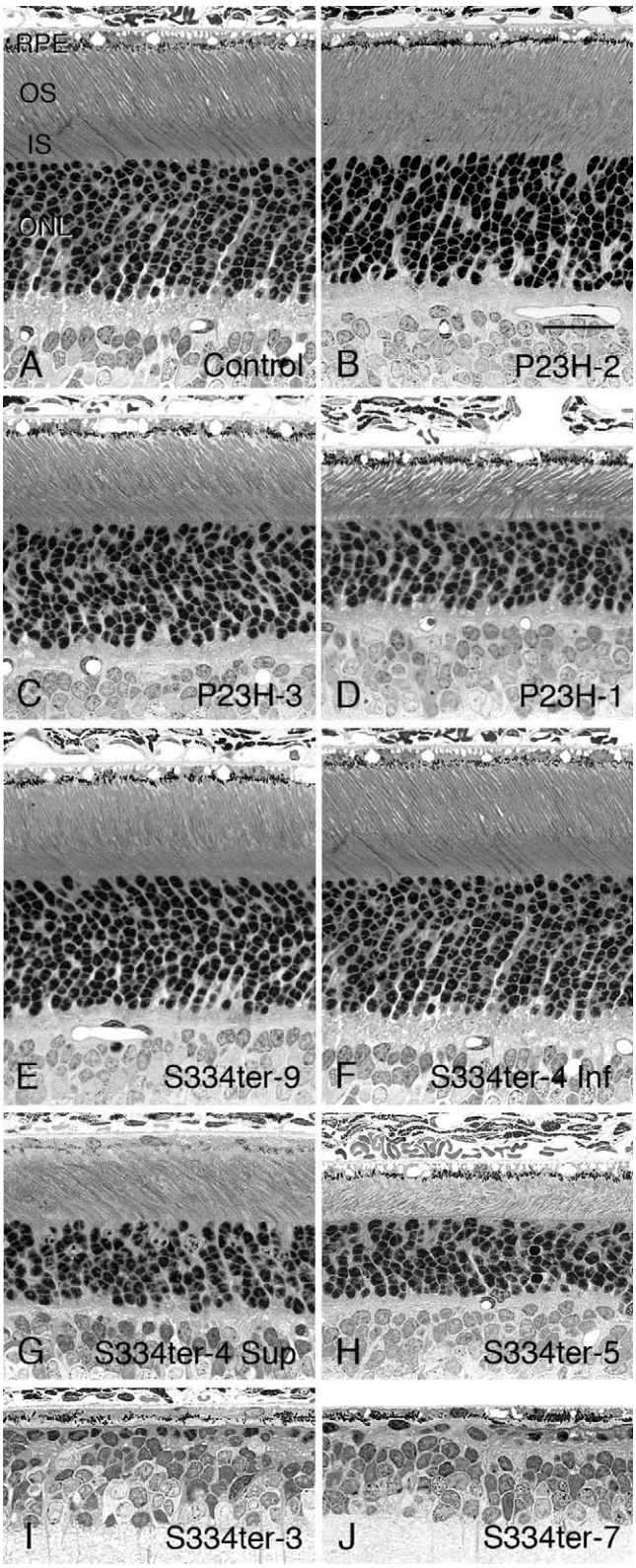

Figure 2. Light micrographs from the posterior retinas. (**A**) LE control rats at P30. (**B–D**) P23H rats at P30. (**E–J**) S334ter rats at P30. Within each of the mutant rhodopsin types, the retinas are shown in the order of the slowest to more rapid rate of retinal degeneration. In the P23H rats (**B–D**), the P23H-2 retinas (**B**) are almost normal, as are the P23H-3 retinas (**C**); although the rod outer segments (OS) have shortened slightly by this stage of degeneration in the P23H-3 retinas (**C**). The P23H-1 retinas (**D**) show a greater loss of photoreceptor nuclei and even shorter rod OS. In the S334ter retinas, the S334ter-9 retinas (**E**) are indistinguishable from controls (**A**). All of the Tg lines have a slightly greater degree of degeneration at most ages in the superior hemisphere than in the inferior hemisphere, and this is most evident in the S334ter-4 line, where the inferior hemisphere (**F**) is almost indistinguishable from the controls, but the superior hemisphere (in the same eye) shows significantly more degeneration (**G**). Line S334ter-5 at P30 (**H**) is considerably more degenerated, with a slightly thinner ONL than the superior hemisphere of the S334ter-4 line (**G**), but the rod inner segments and outer segments are much shorter and more disorganized. The S334ter-3 (**I**) and S334ter-7 (**J**) retinas at P30 have already lost all but one incomplete row of photoreceptor nuclei. *Scale bar* = 25 μm.

had a decline in OKT threshold that matched the loss of photoreceptors through P180.

Role of Cone Photoreceptors in OKT Performance

In rhodopsin mutations and others that are expressed in rod photoreceptors only, cones usually survive longer than rods in these degenerations.[12,55–57] It has recently been shown that cones in P23H-3 rats survive for an extraordinarily long time.[58,59] This, and the fact that the light levels in the OKT testing apparatus used in the present study are within the photopic range, led us to investigate whether surviving cone photoreceptors specifically could be mediating the normal OKT thresholds found in naïve testing (Fig. 3) despite the loss of most photoreceptors. We analyzed cone function with photopic flicker ERGs in all lines of Tg rats. These rats had not been tested for OKT thresholds, and thus were comparable with the rats that had naïve OKT testing (Fig. 3). The results show that the two fastest degenerations (S334ter-3 and S334ter-7) had maximum cone responses of only 15% to 20% of control at P30 and P60, which progressively declined thereafter and were effectively nonmeasureable by P120 (Fig. 4B). The slowest degenerations (P23H-2, S334ter-9, and P23H-3) had wave amplitudes near LE control values at P30, which declined to 50% to 75% of control by 180 days of age (Figs. 4A, 4B). Photoreceptor degenerations of intermediate rate (S334ter-4, P23H-1, and S334ter-5) had flicker amplitudes 75% to 90% of normal at P30, which progressively declined to 20% to 35% of normal by P180 (Figs. 4A, 4B). The flicker ERG profiles were therefore similar to ONL thickness degeneration profiles (Fig. 1) and those of photopic and scotopic ERG responses (Figs. 6, 8). Thus, the flicker analysis of cone function did not match the naïve OKT thresholds (Fig. 3). This strongly suggests that residual cone function does not mediate the OKT response in naïve mutant rhodopsin Tg rats.

Effects on Visual Thresholds of Daily Testing Beginning on P25

Prusky et al.[49] reported that OKT thresholds could be enhanced in normal pigmented Long-Evans rats if repeated daily testing began on P15 and carried past P25. If testing was not begun until P25 or after, there was no observed enhancement effect. Given the results of the Prusky et al. study,[49] we explored whether the presumed loss of OKT thresholds in degenerating Tg rat retinas could be enhanced. As a first step, we began our evaluations at P25 using the same daily testing protocol described in Prusky et al.[49]

Daily testing starting on P25 surprisingly resulted in a nearly inverse relationship between the rate of photoreceptor degeneration and the rate of decline of OKT thresholds (Fig. 5). For example, the fastest ONL degenerating line (S334ter-7), where the ONL has declined to less than 10 μm and lost most photoreceptors before P30, maintained OKT thresholds close to normal (0.42–0.48 cyc/deg) up to at least 90 days of age (Fig.

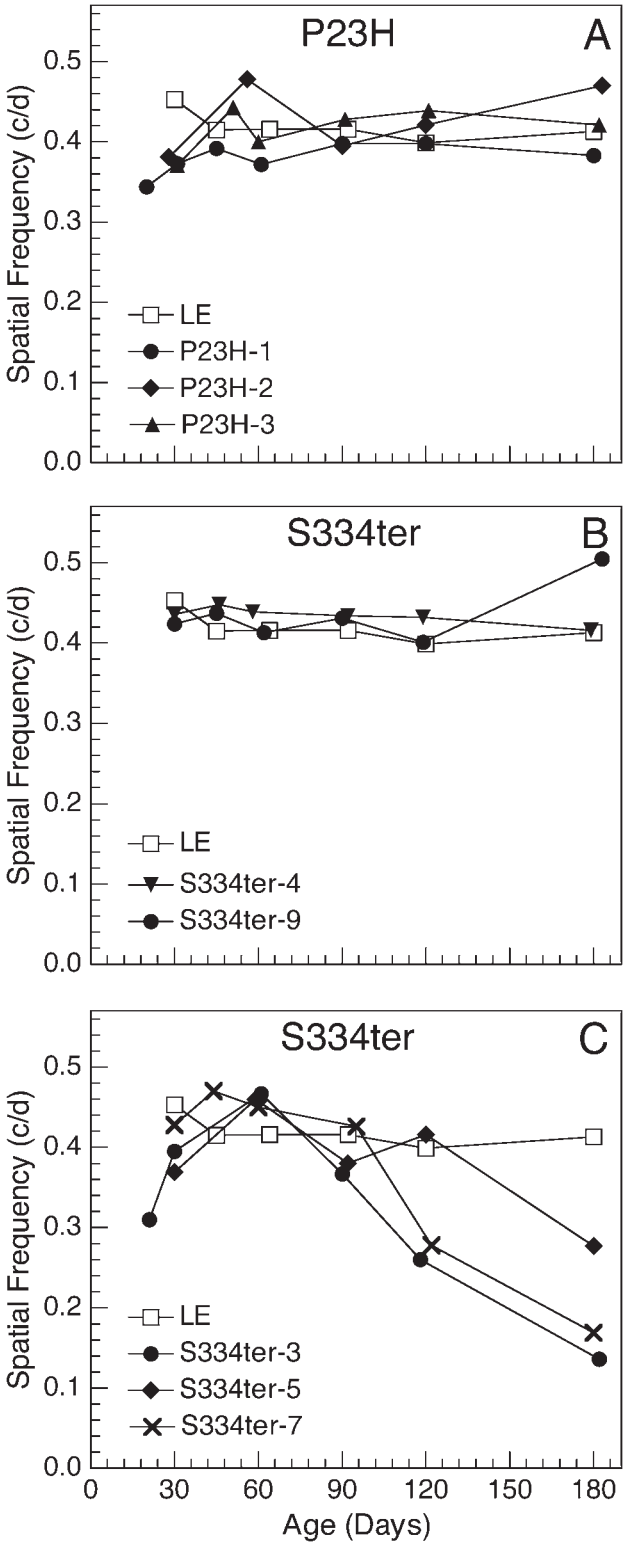

Figure 3. Changes in spatial frequency thresholds of the OKT in different Tg rat lines compared with that of wild-type Long-Evans (LE) controls using single time point assessment (naïve testing). Each data point is the mean of 3 to 8 rats, each of which had only one session of OKT threshold measurements (i.e., a different group of rats was used for each age). The measurements within a group were so consistent that the SEM fell within the size of the symbol in each case and, thus, are not shown. In the P23H lines (**A**) and the S334ter lines with very slow photoreceptor degeneration (S334ter-9) and intermediate rate (S334ter-4) (**B**), the OKT thresholds are very close to those of LE controls; they either are not significantly different at each time point, or do not show any data point at any age with a reduction from the LE control value by more than 10%. In the fastest degenerating lines (S334ter-3, S334ter-5, and S334ter-7), the OKT thresholds did not differ by more than 12% from those of LE controls up to P90 for lines S334ter-3 and S334ter-7 (**C**), and up to P120 for line S334ter-5 (**C**). Thereafter, the S334ter-3 and S334ter-7 lines showed a decline of 30% to 35% from LE control value at P120 and 60% to 65% decline by P180. The slightly more slowly degenerating line S334ter-5 showed a decline of approximately 33% at P180 (**C**).

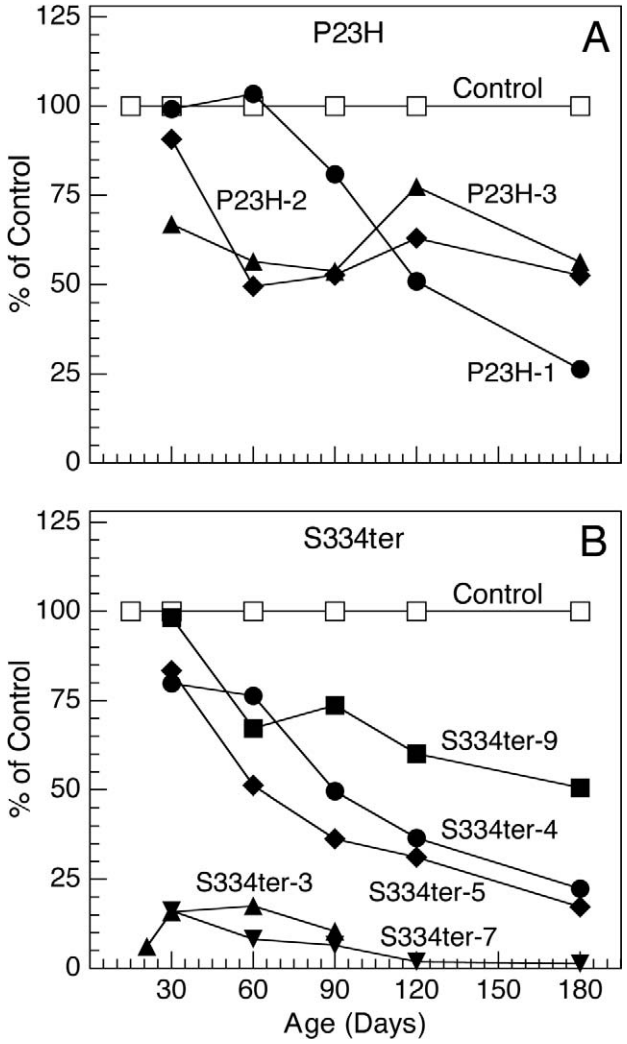

Figure 4. Flicker photopic ERG analysis. (**A**) P23H mutant rhodopsin Tg rats. (**B**) S334ter mutant rhodopsin Tg rats. The results are expressed as percent of LE control at each age. The data curves are similar to those of the conventional photopic b-wave measurements of the full-field ERG (Figs. 6, 8) and are shown from a standard 20-Hz flicker stimulus at an intensity of 0.4 log cd s/m^2, averaging 50 responses. The waveforms were nearly identical with stimulus intensity of 0.9 log cd s/m^2.

5A). (Note that Fig. 5 shows an x-axis to only P90 for clarity of differences among the lines, but some descriptions here include OKT thresholds at older ages.) Similarly, line S-3 also undergoes rapid photoreceptor degeneration at nearly the same rate as S334ter-7, yet OKT thresholds were measured at 0.4 cyc/deg at P30 and 0.26 cyc/deg at P90, or 80% and 50% of normal at P30 and P90, respectively (Fig. 5A), with slow decline thereafter. Remarkably, in S334ter-3, the OKT threshold

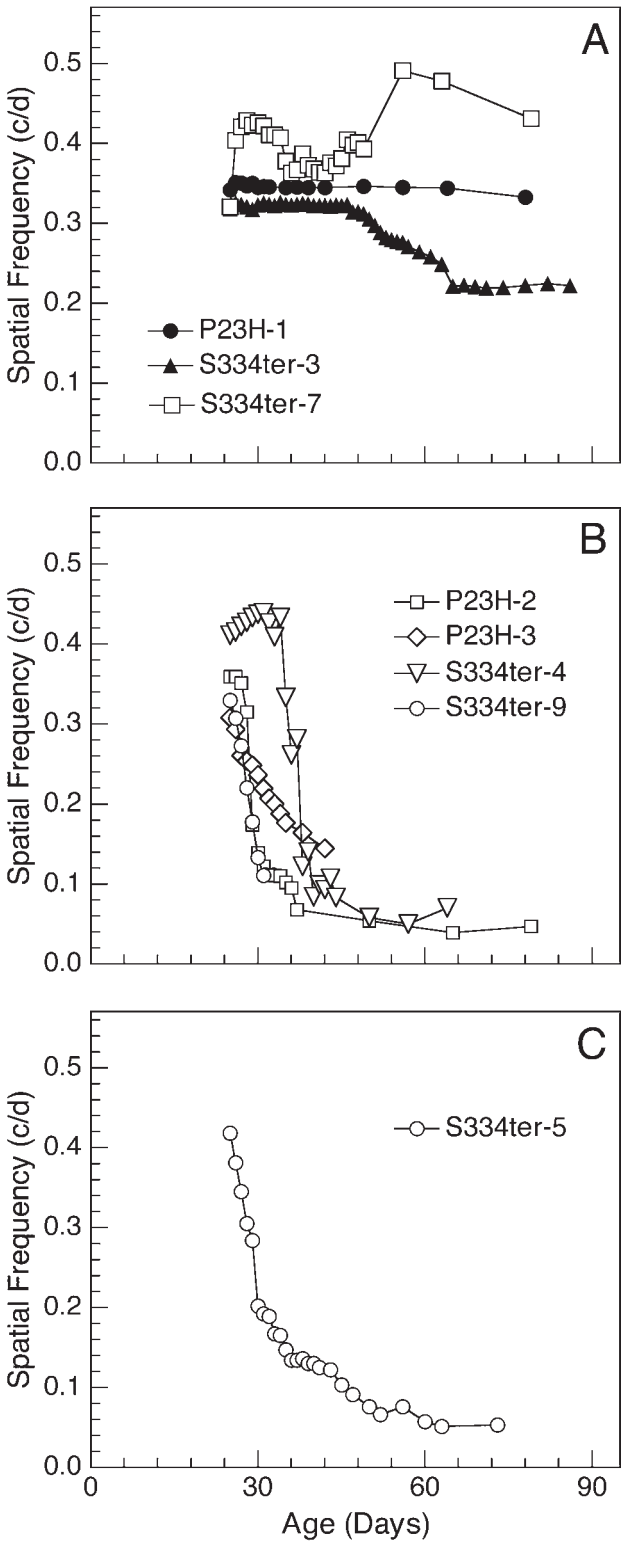

Figure 5. Changes in spatial frequency thresholds of the OKT in different ages when tested daily beginning at P25. Each data point is the mean of 3 to 6 rats, and the measurements within a group were so consistent that the SEM fell within the size of the symbol in each case and, thus, are not shown. Daily testing starting on P25 resulted in a nearly inverse relationship between the rate of photoreceptor degeneration and the rate of decline of OKT thresholds. (**A**) The fastest degenerating lines S334ter-3 and S334ter-7, as well as line P23H-1 with an intermediate rate of degeneration, all show sustained OKT thresholds, with the S334ter-3 line being recordable up to at least P170. (**B**) The slowest degenerating lines (P23H-2, P23H-3, and S334ter-9) and the one with an intermediate rate of degeneration (S334ter-4) all show an early and rapid decline in OKT threshold, reaching almost nonrecordable levels by P60. (**C**) The relatively rapid photoreceptor degeneration in the S334ter-5 line is fairly closely matched by the rate of decline in OKT threshold. The OKT threshold for LE wild-type controls, as shown in Figure 3, is 0.427 cyc/deg. Note that the x-axes are shown to P90 for clarity of the early changes in OKT thresholds. As described in the text, some of the lines were measured beyond P90, including P23H-1 (to P150) and S334ter-3 (to P170).

was still measurable at P170 (not shown), even though most photoreceptors had degenerated and disappeared by P30 (Figs. 1B, 2I). Photoreceptor degeneration in P23H-1 is faster than in other P23H lines and has progressed to ~20% remaining cells by P90, yet OKT thresholds remain at ~80% of normal until P120 and only decline to ~70% of LE values by P150 (not shown).

Conversely, lines that exhibit very slow (P23H-2, P23H-3, S334ter-9) or intermediate (S334ter-4) rates of degeneration exhibited rapid decline in OKT thresholds, with each line being nonresponsive to the OKT stimuli by P60 (Fig. 5B), despite the photoreceptor complement being almost normal at this age (Figs. 1A, 1B). Only line S334ter-5 appeared to have a decline in OKT threshold that was similar to the rate of photoreceptor cell loss (Fig. 1B).

To highlight the two extremes of discordance between ONL thickness and OKT thresholds, we compared the fastest degenerating (S334ter-7) and one of the slowest (P23H-2) lines in Figure 6. In line S334ter-7, photoreceptors are lost rapidly very early in development (Fig. 6A), and almost all are missing by P30 (Fig. 2J) and thereafter (Fig. 6F, right panel). This results in the near absence of ERG responses, with only minimal scotopic b-wave (Fig. 6B) and photopic b-wave (Fig. 6C) responses, both of which fall within the noise level of the ERG. However, naïve (Fig. 6D) and daily (Fig. 6E) testing resulted in persistent OKT thresholds at or very near normal levels up to P90, despite having virtually no surviving photoreceptors (Figs. 6A, 6F, right panel).

In the slow degenerating P23H-2 line, the ONL (Figs. 6A, 6F) and ERG responses of all waveforms (Figs. 6B, 6C) were very close to those of LE control rats. In this line, naïve testing also resulted in persistent OKT thresholds (Fig. 6D). However, daily testing resulted in a rapid decline from 0.35 cyc/deg at P25 to 0.10 by P30, and thresholds were nearly nonmeasurable by P60 (Fig. 6E), despite a normal ONL thickness (Figs. 1A, 6A, 6F, center panel).

Effect of Early Exposure to OKT Stimulus (P15)

To determine if early daily exposure beginning at P15 would enhance OKT thresholds over those beginning at P25 in the Tg rats (Figs. 5B, 5C), as shown in normal LE rats,[49] we retested all the lines with different groups of animals beginning at P15. We also tested RCS rats daily from P15 and compared these data with previously reported thresholds tested from P25 onward.[23] When tested from P15 onward, the OKT thresholds of Tg lines that exhibit the fastest photoreceptor degeneration (S334ter-3 and S334ter-7) increased from ~0.2 cyc/deg up to 0.4 to 0.5 cyc/deg by P25, virtually the same values as the OKT thresholds when begun at P25 (Figs. 7G, 7H). Thereafter, they followed the same or similar profiles as animals tested from P25 onward, staying at a relatively high level throughout the testing period (Figs. 7G, 7H). The slowly degenerating P23H-3 line (Fig. 7B) and the faster degenerating P23H-1 line (Fig. 7C) achieved the same levels as the series beginning at P25 and also showed no enhancement of OKT thresholds.

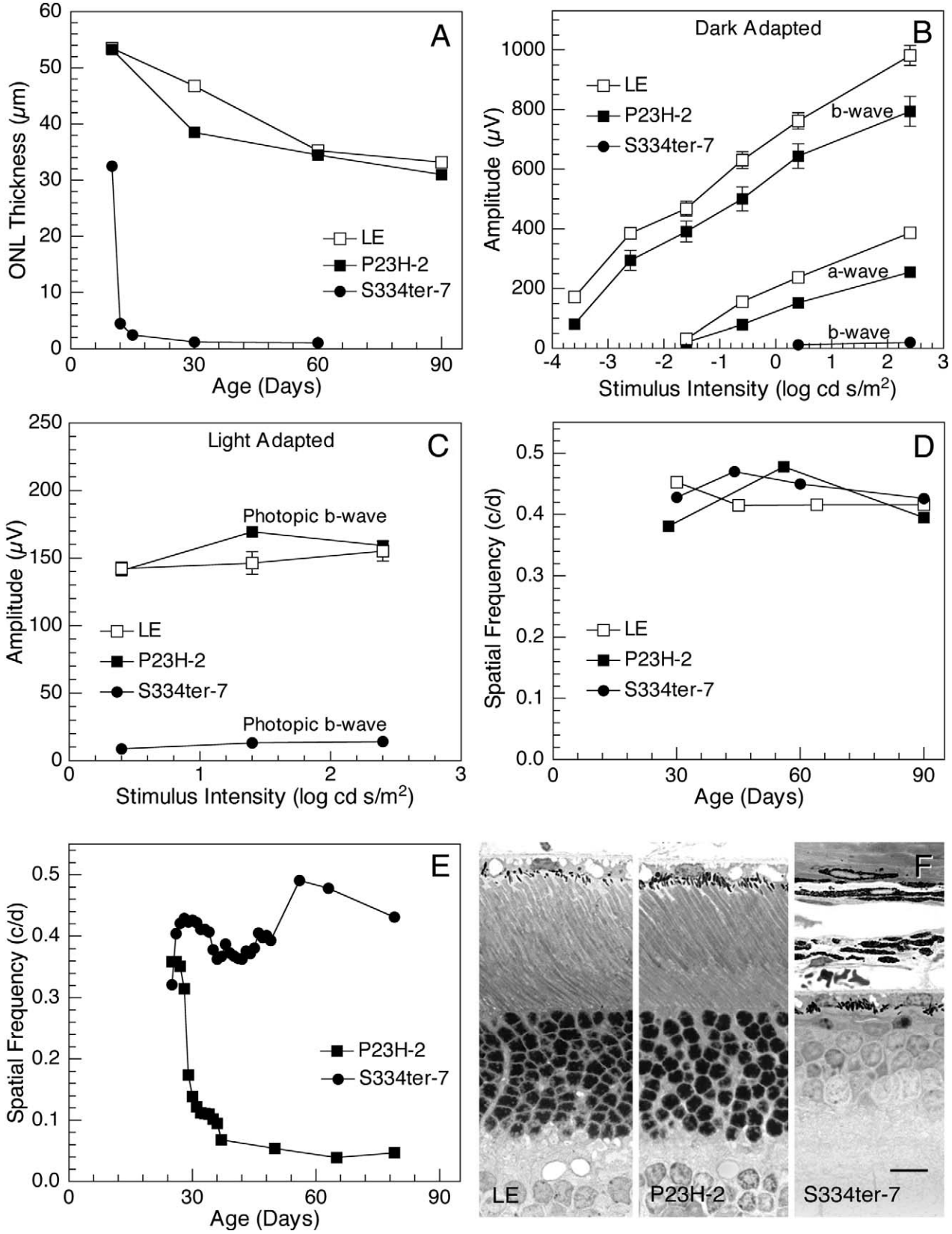

Figure 6. Comparison of fastest degeneration (line S334ter-7) and one of the slowest degenerations (line P23H-2). (**A**) ONL thickness. (**B**) Dark-adapted (scotopic) ERG responses. (**C**) Light-adapted (photopic) ERG responses. (**D**) Naïve OKT testing. (**E**) Daily OKT testing. (**F**) Light micrographs of these retinas at P90. *Left panel*: LE wild-type control. *Middle panel*: P23H-2. *Right panel*: S334ter-7. Both OKT behavioral tests show extreme discordance with the structure and function of the retina in one or both lines of Tg rats. In the naïve (single time point) testing (**D**), the OKT thresholds are virtually normal with both lines, expected in the nearly normal P23H-2 line (**A–C, F**) but unexpected in the fast degenerating S334ter-7 line, where the photoreceptors and most retinal function are lost by P30 and thereafter. In the daily testing (**E**), the OKT thresholds are

reversed from the expected, with those of the fast degenerating S334ter-7 line sustained at essentially normal levels, but those of the slow degenerating P23H-2 line declining precipitously and virtually abolished by P60, despite a nearly normal complement of photoreceptors and ERG responses. *Scale bar* = 10 μm.

Other lines that show rapid visual degeneration when tested beginning on P25 (P23H-2, S334ter-4, and S334ter-9; Fig. 5B), and when tested beginning at P15, exhibited an exacerbated visual degeneration that resulted in slightly lower maximal thresholds (Figs. 7A, 7D, 7E). We cannot explain the difference in pattern between lines P23H-2 (Fig. 7A) and P23H-3 (Fig. 7B), since they not only have a near-identical timing of loss of photoreceptors (Fig. 1A), but also are indistinguishable

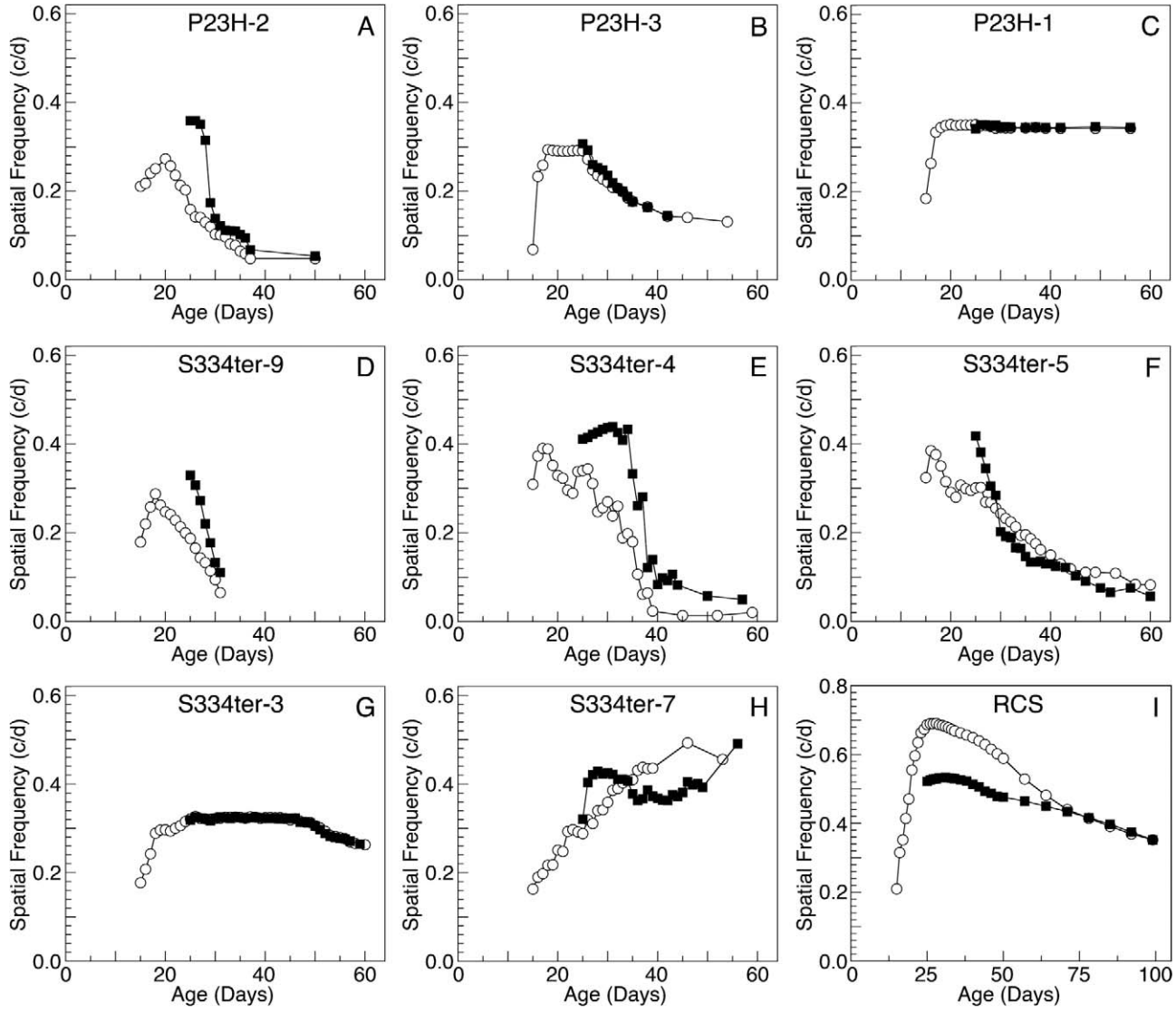

Figure 7. Changes in spatial frequency thresholds of the OKT at different ages when tested daily beginning at P15 (*open symbols*) to determine the effect of early exposure to OKT stimuli compared to that beginning at P25 (*solid symbols*). Each data point is the mean of 3 to 6 rats, and the measurements within a group were so consistent that the SEM fell within the size of the symbol in each case and, thus, are not shown. The data plotted beginning at P25 are taken from Figure 5 and are given here for comparison. The early testing beginning at P15 shown in *open symbols* did not result in an elevation of OKT threshold over that of daily exposure beginning at P25 in the Tg rat lines (**A–H**); but enhancement did occur in RCS rats (**I**), albeit temporarily. The slowest degenerating lines, P23H-2 (**A**) and S334ter-9 (**D**), as well as S334ter-4 (**E**) with an intermediate rate of degeneration, showed lower maximal thresholds and more rapid and earlier decline when testing began at P15 than at P25. In one slow degenerating line, P23H-3 (**B**); two lines with intermediate rates of degeneration, P23H-1 (**C**) and S334ter-5 (**F**); and the most rapid photoreceptor degenerations, S334ter-3 (**G**) and S334ter-7 (**H**), the early OKT thresholds increased to the levels at or near those tested beginning at P25, and thereafter followed a relatively similar profile to those tested from P25 onward, each with somewhat different patterns (see text). Note that the x-axes (to P60) are expanded from those in Figure 5 (to P90) to show the early differences in the two groups. When testing began at P15 and continued past P60, OKT thresholds were maintained at a level almost identical to those of the group where testing began at P25, which included RCS (to P175); P23H-2 (to P80); P23H-1 (to P150); S334ter-4 (to P70); S334ter-5 (to P75); S334ter-3 (to P165); and S334ter-7 (to P90). Also, data for RCS rats (**I**) are not normalized, and they show at least transient elevated visual thresholds with early OKT visual experience, unlike the Tg rats which do not. Data for the RCS rats are shown to P99, an age where almost all photoreceptors have degenerated and disappeared in pigmented RCS rats.[6]

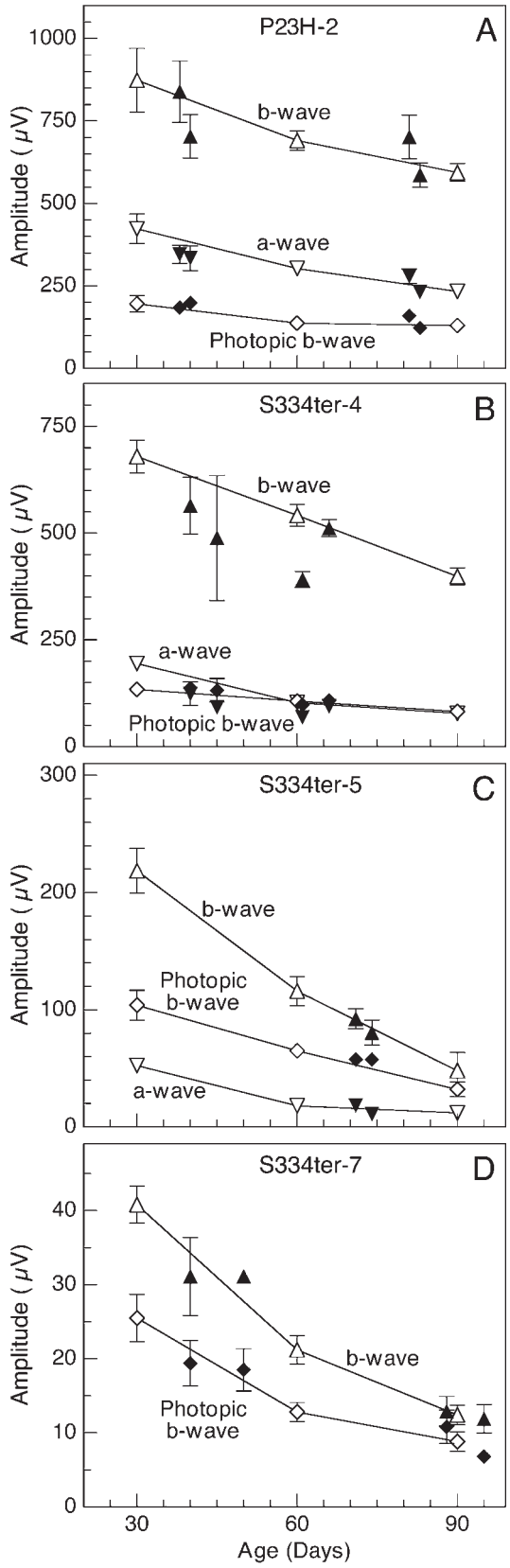

Figure 8. ERG response amplitudes of scotopic a- and b-waves and photopic b-waves of Tg rats following daily OKT testing (*solid symbols*) at different ages compared with those of untested Tg rats of the same lines at P30, P60, and P90 (*open symbols*). (**A**) Line P23H-2. (**B**) Line S334ter-4. (**C**) Line S334ter-5 (**D**) Line S334ter-7. In the OKT-tested rats, the first pairs of *solid symbols* are those where testing began at P15 and second, those that began at P25. Where the *symbols* are immediately adjacent to one another, they were tested at the same age, but the *symbols* have been shifted by a day for clarity. Each *symbol* represents the mean of 2 to 6 rats (mostly 4–6) ± SEM. Where error bars are not shown, the SEM fell within the *symbol*. Since the ages of the tested animals often fell at an age between those of the Tg controls, statistical analysis (unpaired Student's *t*-test) was carried out between the tested group and its nearest control group. As shown in Figures 8A–D, most of the ERG response amplitudes of OKT-tested rats appeared to lie along the normal response curves. All values showed no statistical difference from controls except the scotopic a- and b-waves at P61 in the S334ter-4 rats (**B**), both $P < 0.005$. So overall, there appeared to be no effect on the ERG response amplitudes due to the OKT testing. The ERG responses plotted were from stimulus intensities of 0.4 log cd s/m^2 for scotopic b-waves and 2.4 log cd s/m^2 for scotopic a-waves and (following exposure to 1.5 log cd/m^2 rod-desensitizing adapting background light) photopic b-waves.

histologically at every age through P180 (data not shown). The other relatively fast degenerating line, S334ter-5, had an OKT profile beginning at P15 that peaked early, approximately P16 to P20, before falling and joining the declining threshold pattern of those beginning at P25 (Fig. 7F). As noted earlier, this is the only line in which the decline in OKT threshold with daily measurement was similar to the loss of photoreceptors, albeit slightly faster (Figs. 1B, 7F).

Interestingly, when RCS rats were tested daily from P15, their OKT thresholds began at ~0.18 cyc/deg and increased to ~0.7 cyc/deg by P25 (normalized to 0.566 cyc/deg; Fig. 7I), which subsequently declined to match P25 tested animals by 65 to 70 days of age. Of all the models tested for the effect of early exposure to OKT stimuli, the RCS rat is the only one to exhibit an enhancement of function, albeit a temporary one, unlike the permanent one in normal LE rats.[49] Yoke control tested animals (S334ter-9) resulted in identical visual thresholds to those reported in Figure 7D, confirming that the effects of testing reported here are attributable to generating the OKT response and not any other testing parameter (not shown).

Repeated OKT Testing is Not Harmful to Photoreceptors

To examine whether testing OKT thresholds is harmful to photoreceptors or retinal function in Tg rats, we analyzed the ERG of untested Tg rats and those that had undergone OKT testing beginning at P15 and P25 in each of the P23H-2, S334ter-4, S334ter-5, and S334ter-7 lines. Scotopic a- and b-wave and photopic b-wave response amplitudes in P15 and P25 OKT-tested groups were very similar to Tg rats that had not undergone OKT testing at all ages tested (Fig. 8). In addition, there was almost no difference in ERG response amplitudes measured between P15 and P25 groups in each of the lines (Fig. 8). ONL thickness measurements taken following the final ERG revealed no significant histological difference between animals tested beginning on P15 and P25 within each line, and no difference between the experimental animals and age-matched Tg controls (data not shown). Thus, although repeated OKT testing results in accelerated visual (OKT) decline in the slow degenerating lines (Fig. 5B), there does not appear to be a detrimental effect of OKT testing on photoreceptor or retinal structure or function.

Discussion

Photoreceptor degeneration ultimately results in progressive vision loss. Numerous studies using various animal models of RDD have shown that abnormalities in visual indices including

visual acuity tests, optomotor thresholds, ERG response amplitudes, and luminance thresholds recorded from the superior colliculus result from progressive photoreceptor death. In the present study, we investigated the effects of photoreceptor degeneration on visual function in Tg rat models of human RDD. In these models, while electrophysiological and histological indices of retinal degeneration were usually similar, behavioral measurements of visual degeneration based on OKT thresholds were often contradictory to the other measures. When OKT thresholds are measured at a single time point (naïve experiments), they do not decline until very late in the photoreceptor degeneration profile, long after significant photoreceptor degeneration has occurred. Daily testing of Tg lines with fast degenerations resulted in sustained OKT thresholds for long periods despite the rapid loss of photoreceptors. Conversely, daily testing of OKT thresholds in slow degenerations resulted in a rapid decline of thresholds, which was even more pronounced when testing began earlier, at eye opening. The cause of the amplified loss of function was not a result of testing-induced damage to the rod or cone photoreceptors, as ERG response amplitudes and ONL thickness measurements (pre- and post-OKT testing) reflected expected values congruent with age-matched Tg controls. Finally, early enhancement of OKT thresholds, similar to that seen in normal rats, was not achieved in Tg rats but was obtained temporarily in RCS rats.

Visual Receptors for the OKT

The present findings raise the question of what role photoreceptors play in mediating the OKT in Tg rats. In several of the Tg rat lines, the OKT thresholds are indistinguishable from those of wild-type controls as late as P180, yet most of their photoreceptors had degenerated and disappeared by P30 to P60. We also found that cone ERG responses disappeared concomitant with the loss of photoreceptors, in general; so the long-term survival of some cones in most of the lines of Tg rats could not be responsible for the OKT signaling, as suggested in the more slowly degenerating P23H-3 rats.[58,59] Thus, normal OKT thresholds are achievable in mutant rhodopsin Tg rats without a full complement of rod and cone photoreceptors, and it appears that rods and cones may not be necessary for a normal OKT in these retinal degenerations.

By contrast, studies using knockout mice in which there is loss of both rod and cone phototransduction, the animals failed to track the drifting grating of the same apparatus (CerebralMechanics) used in the present studies.[60] This is consistent with the rods and cones being essential for the OKT to occur. In the study by Ecker et al.,[60] the additional knockout of melanopsin expression also resulted in a non-recordable OKT, suggesting that melanopsin-containing intrinsically photosensitive retinal ganglion cells are not the sensors in the eye for the OKT.[60] A similar study using photoreceptor knockouts[61] showed that under photopic conditions, cone-only mice have an inverted-U shaped contrast sensitivity (CS) curve that matched that of normal sighted controls, and rod vision animals were nonresponsive. Conversely, under scotopic conditions, cone vision animals were nonresponsive, and rod vision animals matched control values closely. Therefore, in normal mice, where photoreceptors are not degenerating, both rods and cones presumably have specific roles in producing the OKT tailored specifically to that cell-type's luminance sensitivity range.[62] In the present study, all OKT thresholds were examined under photopic conditions, and although we did not examine rod photoreceptor OKT function under scotopic conditions, rod photoreceptors are unlikely to account for the results reported here as most Tg lines tested had significant reductions in scotopic ERG wave amplitudes and near complete rod photoreceptor death prior to, or during, the sustained OKT thresholds. Thus, there are clearly differences in species, age of onset of the loss of rod and cone function and/or other factors that lead to these apparent contradictory findings on the role of rods and cones in the OKT.

Role of the Visual Cortex in Naïve and Daily OKT Testing

The loss or absence of photoreceptors in the fast degenerating lines, in some way, prevents the testing-induced reduction in OKT thresholds. This suggests that the action is either on the photoreceptors themselves or on some aspect of visual function that depends upon or results from photoreceptor presence or activity. The fact that throughout the various components of this study, ERG amplitudes measured from each line at various ages resulted in amplitudes that closely resemble the degree of ONL thinning, suggests that the action is not on the photoreceptors themselves, but rather on a different aspect of visual system activity. A previous study[49] reported that early (P15) and repeated exposure of LE rats to the OKT stimuli could result in a cortically mediated enhancement of visual thresholds long-term. However, animals tested from P25 onward did not exhibit this same visual cortex–based enhancement as seen in wild-type rats. It was also shown that if enhanced LE rats had bilateral primary visual cortex lesions, the enhancement effect disappeared and thresholds fell to naïve levels. Furthermore, LE rats that underwent primary visual cortex ablation on P14 did not exhibit the enhancement effect when tested from P15 onward, confirming that the visual cortex must be intact for enhancement to occur.[49] In the present study, all animals tested had intact visual cortices, yet there was no enhancement effect of the early exposure in any of the Tg lines, even though the maximum thresholds reached near nondystrophic rat levels[23,51] in some lines. In slow degenerations, however, we found that rather than enhancing the visual thresholds, the early exposure resulted in accelerated visual decline. Although we cannot discount the role of the visual cortex in this phenomenon, it is not likely to be involved given that the normal output of this optokinetic response is mediated through subcortical circuitry[49] and that thresholds in each of the Tg lines never exceeded the naïve LE rats' maximal threshold (~0.530 cyc/deg, normalized to 0.427), above which the cortex becomes actively involved.

OKT-Dependent Retinal Reorganization

The variability between measures is not likely due to normal inner retinal changes that are evident in many models of RDD, including the mutant rhodopsin Tg rats used here.[40,41,45] Although it has been shown that before significant retinal degeneration, processes of bipolar and horizontal cells show abnormalities[63] and these retinal circuitry changes occur concurrently with impaired ERG amplitudes in P23H-1 rats.[64] Changes seen in OKT thresholds in the current study occurred independent of the progressive decline in ERG wave amplitudes. In addition, the majority of inner retinal changes that occur in rodent models of retinal disease do not occur until very late in the degeneration profile, yet some of the behavioral changes seen in this study occur within the first few weeks of life. It is possible that driving the OKT could increase the rate of occurrence of these changes, but whether these potential changes are sufficient to cause such drastic outcomes has yet to be examined.

The OKT in Tg Rats as a Measure of Visual Rescue in Therapeutic and Other Retinal Studies

Because vision loss is the fundamental clinical feature of RDD, and because vision rescue is the goal of any successful therapy, it was important to extend the previous anatomical and electrophysiological studies into thorough evaluations of behaviorally measured visual thresholds as a comprehensive background for potential therapeutic studies. The surprising findings in both the naïve and daily OKT testing in the Tg rats have several ramifications for the use of these animals with OKT testing, and the OKT for testing vision, in general. While the Tg rats have and continue to be valuable animal models for several areas of therapeutic studies, as noted above,[8,10–13,15–21,65] the use of the OKT is limited as a therapeutic outcome with these animals. In most therapeutic studies, an agent/gene is administered before or as photoreceptor degeneration has begun, and then the outcome measurements are made at a later time. This has been most frequently and effectively used with structural and functional (ERG) measures.[8,66–69] This paradigm is equivalent to the naïve experiments in the present study, so at the end of the post-treatment period, the OKT thresholds will not have been reduced from normal and there will be no "headroom" to detect a change due to the therapeutic measure.

In the RCS rat,[70–74] however, OKT have largely been tested once per month (sometimes only two tests total; P60 and P90) and report reduced thresholds in unoperated RCS rats by P60 and further decline at P90 unlike the naïve group in this study, suggesting that naïve testing in the RCS rat would not result in sustained OKT thresholds as seen in the Tg rats. If other mutation types or other species are studied using the OKT as a measure of vision in therapeutic studies, it will be important to define the naïve OKT pattern in the untreated animals before proceeding.

When measured daily, the different lines of Tg rats showed OKT threshold patterns with age that were mostly reciprocal to the rate of retinal degeneration, and therefore it is not clear what would be measured using the OKT as an outcome measure. When daily OKT thresholds are tested in the RCS rat, however, OKT thresholds measured from eye opening (P15) increased to enhanced levels before beginning to decline, and thresholds tested beginning at P25 were equal to LE controls for a short period of time before declining.[23,28] Despite daily testing, we did not observe an amplified degeneration of OKT thresholds in either group (testing beginning at P15 or P25) of Tg rats, although OKT thresholds do persist in RCS rats longer than ERG wave amplitudes,[75–77] SC luminance sensitivity,[78,79] and rod photoreceptor survival.[6] Thus, early OKT testing does not result in amplified degeneration of OKT thresholds in all models of RDD.

OKT is a measure of visual performance based on a reflexive behavior that attempts to stabilize a moving stimulus on the retina. Strictly speaking, OKT is not a true measure of visual perception. Visual perception such as acuity measured using the visual water task (CerebralMechanics)[80] has been reported in the RCS rat.[22] In the McGill et al. study,[22] visual acuity at P30 was ~0.82 cyc/deg, slightly lower than LE controls at 1.0 cyc/deg. The acuity of RCS rats progressively declined toward the inability to discriminate between a black and a white computer screen by 11 months of age. The authors discuss the possibility of thresholds above 0.6 cyc/deg being cortically dependent, while thresholds below 0.6 cyc/deg being dependent upon subcortical circuitry, which is consistent with current OKT findings.[49] In addition, the visual acuity in both enhanced and nonenhanced LE rats using moving gratings in the VWT are nearly indistinguishable from those measured by OKT.[49] Collectively, these findings suggest that although the OKT may not be true visual perception, it is a very accurate predictor of visual acuity measured in a perceptual task.

The study reported here is the first to examine the relationship between behaviorally measured visual thresholds and electrophysiological and morphological indices of vision in mutant rhodopsin Tg rats, other than the several reports of OKT measures in the S334ter-3 line noted above,[25,26,65] which are consistent with the results found here for this line.

The remarkable and consistent differences in the OKT patterns following daily testing in the Tg rats with different rates of degeneration make these animals useful for exploring the cellular and neural mechanisms that regulate OKT in rats.

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