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Dark-light: Model for nightblindness from the human rhodopsin Gly-90 → Asp mutation

(genetic disease/neurodegeneration/retina/signal transduction/electrophysiology)

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ABSTRACT **A human rhodopsin mutation, Gly-90 → Asp (Gly90Asp), cosegregated with an unusual trait of congenital nightblindness in 22 at-risk members of a large autosomal dominant kindred. Although rhodopsin mutations typically are associated with retinal degeneration, Gly90Asp-affected subjects up to age 33 did not show clinical retinal changes. Absolute threshold for visual perception was elevated nearly 3 logarithmic units in 7 individuals tested (ages 11–64), indicating greatly compromised rod threshold signaling. However, *in vivo* rhodopsin density was normal. Although the 38-year-old proband could not perceive dim lights, his rod increment threshold function was normal on brighter backgrounds. The impaired rod vision for dim but not bright backgrounds is consistent with a mechanism of increased basal "dark-light" from thermal isomerization equivalent to an increase of $>10^4$ over that of wild-type rhodopsin. The Gly90Asp mutation on the second transmembrane helix places an extra negative charge in the opsin pocket; this could contribute to partial deprotonation of the retinal Schiff base and thereby increase photoreceptor noise. *In vitro* evidence had suggested that transducin is activated by the Gly90Asp mutation in the absence of both the retinal chromophore and light, termed "constitutive activity." The apparent preservation of functioning rods despite extensive and lifelong nightblindness in this kindred is inconsistent with one current hypothesis that chronic rod activation from constitutively active mutant rhodopsin necessarily contributes significantly to photoreceptor demise in human retinal dystrophies.**

Vision normally is initiated when photons isomerize rhodopsin in rod photoreceptors. Rods are remarkably sensitive to dim light and can respond to single quanta in total darkness (1). Rod sensitivity is limited by the stability of rhodopsin, since occasional thermal activation will falsely signal visual sensation even in the absence of light. Such "dark-light," termed "Augenschwartz" by Fechner in 1860 (2), is indistinguishable from a true light response and will impair rod perception in dim but not brighter light. With one exception (3), previously reported rhodopsin mutations are associated with autosomal dominant retinitis pigmentosa (adRP) (4) that initially causes partial nightblindness from rod dysfunction but that ultimately progresses towards total blindness as rods (and subsequently cones) die. The mechanisms of nightblindness and retinal degeneration in rhodopsin adRP are not well understood (5). The phenotype resulting from the Gly-90 → Asp (Gly90Asp) mutation in human rhodopsin is unusual for causing extensive nightblindness from earliest childhood (presumably congenital) but without apparent retinal degeneration until a much later age. In this kindred the mechanism of nightblindness is consistent with increased dark-light from spontaneous Gly90Asp signaling.

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MATERIALS AND METHODS

Electroretinography. Corneal electroretinograms (ERGs) were elicited by full-field 10-μs xenon photostrobe flashes and recorded at 0.1–1000 Hz by using methods and control values presented previously (6).

Molecular Biology. Genomic DNA was isolated from peripheral blood samples. The Gly90Asp mutation was found by sequencing the entire rhodopsin coding region, with target DNA generated by PCR amplification (7) of genomic DNA during 40 rounds of thermal cycling [94°C for 90 s, 54°C for 60 s, and 72°C for 180 s] followed by 72°C for 10 min. Exon 1 primers were 5′-AGCTCAGGCCTTCGCAGCAT-3′ and 5′-GAGGGCTTTGGATAACATTG-3′. The codon 90 region was sequenced by the Sanger dideoxynucleotide chain-termination method (8) with primer 5′-ACGCAGCCCCTTC-GAGTAC-3′. The presence of the Gly90Asp mutation was assayed in a population of normal subjects by allele-specific oligonucleotide hybridization with primers 5′-TGGT-GAAGCCACCTAGGAC-3′ for Gly-90 and 5′-TGGTGA-AGTCACCTAGGAC-3′ for Asp-90.

Densitometry. The 530-nm test and 880-nm reference beams of a modified Florida retinal densitometer (9) were reflected from the human retina *in vivo* onto a photomultiplier tube. The 7° test field was centered on the 9.1° bleaching field. Measurements were made at 17.8° in the temporal retina of the left eye of the 38-year-old proband. A bleaching beam was either "white" or "monochromatic" from 10-nm interference filters (Baird-Atomic type B-1). Dark-corrected photon counts of test and reference beams were counted separately in 2-s intervals, and the ratio was averaged to give T, with T_0 at the end of a long full bleach and T_d after full dark recovery.

$$(T_0 - T_d)/T_0 = (1 - s)[1 - e^{-2\beta(\lambda_m)}], \quad [1]$$

in which s is the fraction of dark-corrected test counts evoked by photons that have not passed twice through rhodopsin (i.e., stray light) and β is rhodopsin density ($\log_e$) expressed as $\alpha(\lambda_m)cl$ where $\alpha(\lambda_m)$ is the molecular extinction coefficient at the test wavelength λ_m, c is the concentration, and l is the optical path length through rhodopsin in the rod outer segment. Since s is not easily measured, it is common to assume that $s = 0$ and then to solve Eq. **1** for 2β. Converting to $\log_{10}$ gives the "two-way density" Δ, which is the frequently used parameter of retinal densitometry. At equilibrium, the kinetic equation of rhodopsin bleaching (10) is:

Abbreviations: adRP, autosomal dominant retinitis pigmentosa; ERG, electroretinogram; sc-td, scotopic trolands; tvi, threshold versus retinal illuminance curve; Gly90Asp, Gly-90 → Asp.

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$$\alpha(\lambda_b)\gamma = \beta(\lambda_b)(1 - P)/\{[t_o I(\lambda_b)]\tau(\lambda_b)[1 - e^{-\beta(\lambda_b)P}]\}, \quad [2]$$

where α is the extinction coefficient at the bleaching wavelength λ_b; γ is the quantum efficiency of rhodopsin bleaching; P is the fraction of rhodopsin at equilibrium; t_o is the time constant of rhodopsin bleaching and regeneration *in vivo; I* is the bleaching irradiance; and $\tau(\lambda)$ is eye medium transmissivity. For $\lambda_b \neq \lambda_{max}$, $\alpha(\lambda_b) < \alpha(\lambda_{max})$, particularly at the long wavelength extreme, and the higher order terms in the series expansion of $[1 - \exp^{-\beta(\lambda)P}]$ in Eq. **2** become negligibly small, and Eq. **2** reduces to a simpler form:

$$P = I_o/(I_o + I), \quad [2a]$$

in which $I_o = 1/(\alpha_\lambda \gamma t_o \tau_\lambda)$ is the irradiance that bleaches 50% of rhodopsin at equilibrium. In the bleaching action spectrum, we constrain the analysis to light incident at the cornea, so that $\tau(\lambda) = \tau = 1.0$. Eq. **2a** was used to calculate the irradiance (I_1) that would bleach 10% of rhodopsin of normal subjects at equilibrium [$p(t) = P = 0.9$) at various test wavelengths (11). These calculated I_1 values were then used in measuring the rhodopsin fraction actually present (P_1) at equilibrium. From the amount actually bleached for every λ_b, we used Eq. **2a** as a template to estimate I_{10}, which is the irradiance actually required for 10% equilibrium bleaching in the proband's retina, as follows: we plotted P_1 vs. $\log_{10} I_1$ and adjusted I_o by sliding the template curve (Eq. **2a**) horizontally until it intersected P_1. The ordinate $P = 0.9$ for this template position then gives $I_{10\%}$ on the abscissa. The error in using Eq. **2a** rather than Eq. **2** to estimate I_{10} is <2.8%, which is less than the precision of irradiance measurements of our lights.

Psychophysics. The Maxwellian view optical system provided a test flash centered on a steady "red" background (Wratten no. 29: λ_{max} = 632 nm). The test spectrum was set by 10-nm interference filters; intensity was measured with a calibrated Pin-10 diode (UDT, Santa Monica, CA), and the quantum distribution was calculated (12). Two-color increment thresholds were determined by the method of adjustment under rod isolating conditions (11) at 10° location in the temporal retina of the proband's left eye: a 530-nm, 200-ms, 7.5°-diameter circular test flash (every 3 s) entered the dilated pupil 2.5 mm nasal to its center, and a second beam provided a 632-nm, 21°-diameter, concentric, steady background that entered through the pupil center. M-cone sensitivity was studied uncontaminated by rods with this test-background combination during the 4- to 9-min interval after terminating a desensitizing 3-min white bleach of 5.3 logarithmic scotopic trolands (sc-td). In this dark interval, M-cone sensitivity levels off at the lower limit of its maximum, while rod sensitivity is substantially less. Action spectra were evaluated by using test parameters and pupil entry position as given above for increment thresholds. Quantized V'_λ curves (C.I.E. scotopic luminous efficiency function; ref. 13) were normalized to agree with absolute thresholds at 510 nm for the proband and a normal individual (see Fig. 4). The remaining spectral curves were fixed as follows: the S-cone curve of Stiles π_1 mechanism (14) was set by the upper horizontal asymptote of responses to the 440-nm test on the 632-nm background. M-cones and L-cones, represented by π_4', and π_5, respectively, were shifted vertically as a unit until π_4' matched the proband's 530-nm threshold, which is the M-cone absolute threshold for both the normal and the proband (see Fig. 5).

RESULTS

All affected members of kindred UM:V490 (Fig. 1) reported extensive nightblindness from their youngest years. This did not worsen with age. None complained of persistent after images, phosphenes, or other visual disturbances in the dark. Seven affected members, ages 11–64, were evaluated clinically, and all had normal 6/6 acuity and normal color discrimination (Farnsworth Panel D-15 panel). All had dark-adapted thresholds elevated by ≈3 logarithmic units (Goldmann–Weekers Darkadaptometer) with a blue (440-nm, 70-nm half bandwidth) stimulus of 5.7° visual angle presented repetitively for 800 msec to central and peripheral retinal positions. This is known as "complete" nightblindness. Subject III-10 showed no change of threshold after 12 hr in darkness. The four affected 11- to 33-year-old individuals (IV-1, III-12, 10, and 9) had clinically normal retinae and did not show outer retinal atrophy, "bone-spicule" intraretinal pigment, "waxy" optic nervehead pallor, or attenuated arterioles that typically occur in adRP. These clinical changes were present only minimally in the 63-year-old (II-3). Visual fields (Goldmann perimetry, I4e white 0.25-mm^2 target) were full (>130° horizontal diameter) for the four 11- to 33-year-old individuals, and fields of the 63-year-old (II-3) were minimally constricted (110° diameter). However, the 38-year-old proband (III-6) and his 64-year-old uncle (II-2) showed peripheral retinal pigmentary changes and had I4e fields constricted to 80° and 25° diameters, respectively.

The complete nightblindness cosegregated as an autosomal dominant trait with a nonconservative rhodopsin Gly90Asp mutation in 12 affected members tested by direct DNA sequencing. No other mutations were found in the >99% of the rhodopsin coding region that was sequenced (1043 securely scorable bands out of 1047 base-pair total). This mutation was absent from 10 at-risk but unaffected relatives and from 5 normal spouses, and it is not a normal variant in a population of 97 unrelated normals. These genetic data in 22 members of the kindred localize the etiologic factor to the immediate chromosomal vicinity of the rhodopsin gene and strongly implicate the Gly90Asp mutation as the cause of the nightblindness.

Electroretinography. These seven affected individuals, including the 11-year-old (IV-1), all showed marked loss of rod ERG responses tested by dim blue flashes in the dark-adapted state (Fig. 2). Cone responses to 30-Hz flicker had normal amplitude in all except the 38-year-old (III-6) and 64-year-old (II-1). Cone responses to single flashes, light-adapted, were

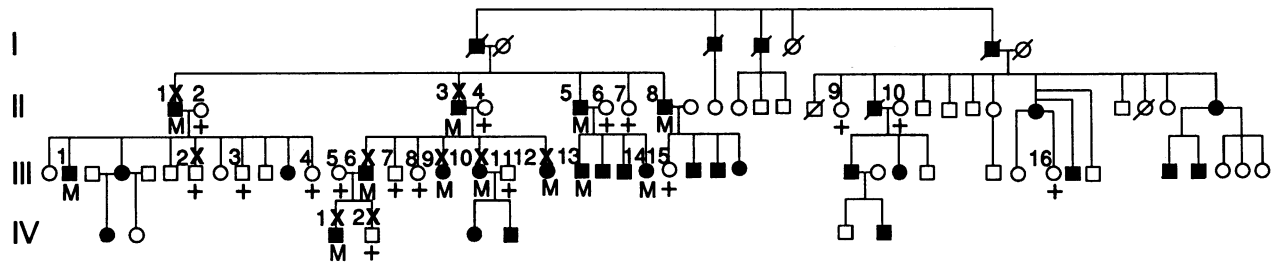

FIG. 1. adRP kindred UM-V490. The congenital nightblindness phenotype cosegregates with the mutation of codon 90 encoding glycine (GGC) to one encoding aspartate (GAC) in rhodopsin exon 1 (squares, male; circles, female; solid symbols, nightblind; open symbol, unaffected; **X**, examined at University of Michigan; +, homozygous for normal rhodopsin; M, heterozygous for normal and Gly90Asp mutant rhodopsins.

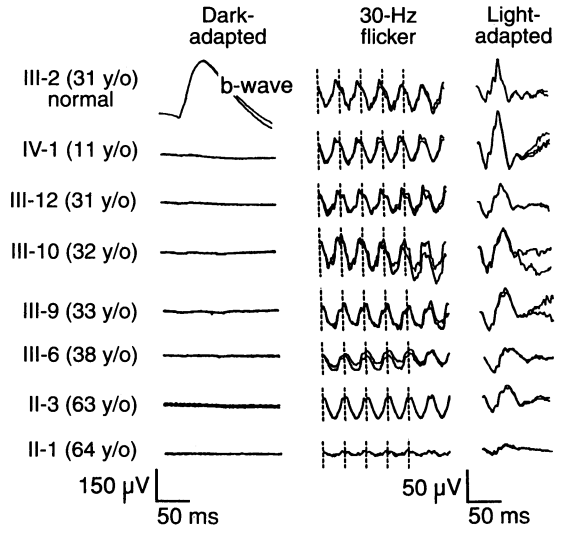

FIG. 2. ERGs in kindred UM-V490. Responses at top are from the unaffected 31-year-old (III-7), and responses below are from Gly90Asp-affected members. Vertical dashed lines for 30-Hz flicker are flash markers at 33.3-ms intervals. Stimulus conditions were as follows. "Dark-adapted": single dim blue (440-nm peak) flash (−1.86 log candela·s/m^2) after 1 hr of dark adaptation; "30-Hz flicker": "white" 30-Hz flashes (0.62 log candela·s/m^2); and "Light-adapted": single "white" flash (1.0 log candela·s/m^2) after 4 min of light adaptation with 3.3 log sc-td. ERG control values from 50 normal subjects 10–60 years old (y/o): dark-adapted b-wave, >210 μV; 30-Hz flicker, >55 μV, with implicit times < 32 ms; and light-adapted b-wave, >50 μV.

normal in all except the 64-year-old, and even his remaining small responses had normal waveform and timing, as though his cones were fewer in number but still functioned well. The apparent lack of rod signaling raised the possibility that the mutant opsin might have prevented the production of rhodopsin or rendered it unbleachable. Retinal densitometry was used to address these possibilities.

***In Vivo* Rhodopsin Densitometry.** Measurements were made at λ_m = 589.6 nm rather than the more usual 530 nm to minimize distortion from short-wavelength-absorbing bleach photoproducts, particularly metarhodopsins I, II, and III. Setting $s = 0$ in Eq. **1** gave $[(T_0 - T_d)/T_0]_{589.6} = (1 - e^{-2\beta_{589.6}}) = 0.069$ and $\beta_{589.6} = 0.0358$. The value for human wild-type rhodopsin at a λ_{max} of 493 nm was obtained from the Dartnall nomogram: $(\alpha_{589.6}/\alpha_{493}) = 0.03767 = (\beta_{589.6}/\beta_{493})$. This yields $\beta_{493} = 0.950$ or, in $\log_{10}$ units, $\Delta_{493} = 0.413$, which is within normal limits (0.444 − 0.284) of four subjects (10). This is a minimum estimate, since it is unlikely that $s = 0$ is strictly true. When measured with λ_m = 530 nm, our estimates of β_{493} with Eq. **1** fall in the range 0.24–0.49 on four other normal subjects. This difference in β_{493} values obtained with λ_m = 530 vs. 589.6 nm is attributed to contamination by metarhodopsin I, II, and III when measured with λ_{530}. Thus, estimates of β_{493} on the proband are within normal limits when measured with $\lambda_{589.6}$, whereas estimates of β_{493} when using λ_{530} were 0.16 ± 0.04 (n = 3) and fall below our comparable normals (although within normal estimates found in the literature) (15, 16), suggesting possibly increased blue-absorbing products for Gly90Asp.

Rhodopsin regeneration was studied by following the fraction $p(t)$ as a function of time in the dark after a full bleach (Fig. 3), where $p(t) = [T_0 - T(t)]/(T_0 - T_d)$. This gave a single exponential regeneration time constant of 4.8 min, which is in the 4- to 10-min range for normal human rhodopsin found in the literature (16, 17) but is somewhat faster than the 6- to 7.5-min range that we usually find.

Further evidence of the presence of rhodopsin came from quantification of the monochromatic irradiance for a 10%

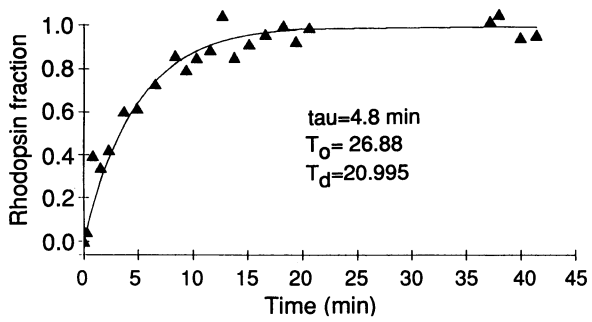

FIG. 3. Rhodopsin regeneration in the dark after >10-min full bleach of 6.4 $\log_{10}$ sc-td (from a xenon arc spectrally limited by a Wratten no. 15 filter) in the left retina of the affected proband (III-6). Regeneration follows exponential kinetics with a time constant of 4.8 min, with T_0 = 26.88 and T_d = 20.995.

equilibrium bleach (■ in Fig. 4), which is well described by $1/V'_\lambda$. Fitting $\log 1/V'_\lambda$ gave the irradiance of 11.80 $\log h\nu_{493}$ s^{-1}·cm^{-2} for λ_{max} = 493 nm of wild-type human rhodopsin. Photosensitivity $\alpha(\lambda_{max})\gamma$ was calculated after estimating ocular prereceptor light loss as $\tau_{493} = 0.469$ from a 42-year-old subject of approximately the proband's age (18). Using these in Eq. **2** along with λ_b = 493 nm, $\beta = 0.95$, t_o = 288 s, and $P = 0.9$ yielded an *in vivo* estimate of the proband's rhodopsin molecular cross-sectional area of 1.94 × 10^{-15} cm^2. This is 4.8–7.9 times larger than for three healthy young adults (2.45–4.07 × 10^{-16} cm^2) estimated by a slightly different method (10).

Psychophysical Measurements. To determine whether the proband detected signals received by this bleachable rhodopsin, we tested his vision by spectral sensitivity and probed the adaptive properties of the rods. Threshold versus retinal illuminance curve (tvi) showed that normal rods (△) detected the 530-nm test at −3.5 log sc-td on the red background and followed the Weber–Fechner curve until saturation began near the 2 log sc-td background (Fig. 5). However, the proband (▲) detected the 530-nm test at −0.494 log sc-td and was unaffected by backgrounds below 0 log sc-td but followed the normal's rod Weber–Fechner line for higher intensity backgrounds. The proband showed no evidence of incipient rod saturation at a 3-log sc-td background because of (*i*) individual differences in the background levels at which rods begin to saturate and (*ii*) probability summation with rods and S-cones,

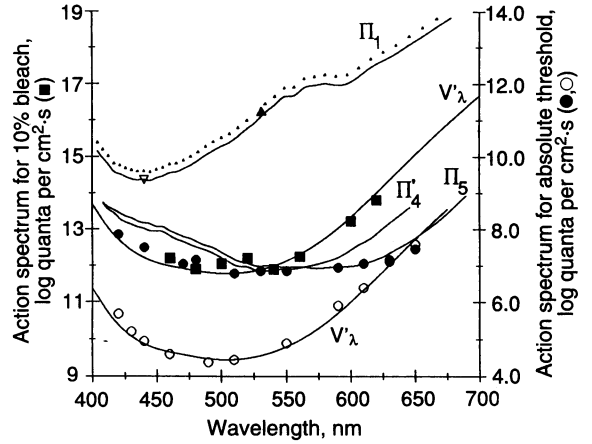

FIG. 4. Psychophysical and densitometric action spectra. Absolute threshold (right scale) for proband (●) and normal (○) with test parameters and pupil entry positions that isolate rod responses (11). ▲, Proband's sensitivity to 530-nm test on 10^3 sc-td 632-nm field; ▽, normal's sensitivity to 440-nm test on $10^{1.6}$ sc-td 632-nm field; ■, densitometric action spectrum for 10% equilibrium bleach of the proband's rhodopsin.

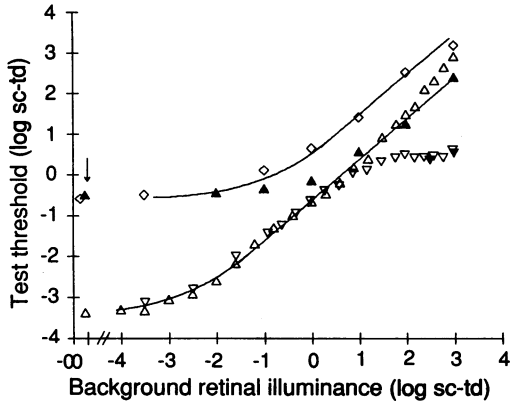

FIG. 5. Psychophysical tvi results from normal (△) and proband (▲) for rod-isolating conditions of 530-nm test on 632-nm background (11). Arrow indicates total darkness. After bleach cessation (◇), the 530-nm test isolates M-cones for normal. tvi curve of 440-nm test on 632-nm background, both entering through pupil center, for normal (▽) and proband (▼), shows "a change in law" near 1 log sc-td background as thresholds switch from being rod-mediated to being mediated by the S-cone π_1 mechanism.

which have sensitivities depressed to approximately the same level by the 3 log sc-td background [note the position of the proband's datum point (▲) in Fig. 4 relative to the dotted π_1 action spectrum described below]. For backgrounds of −1 to 1 log sc-td, the proband's rod-isolated tvi curve was more sensitive than that of the normal's M-cones or S-cones or the proband's S-cones. L-cones are desensitized by the red background and cannot account for his rod-isolated tvi function. The proband reported the 530-nm test as "green" at threshold, where his rod sensitivity was essentially the same as that of the normal's M-cones.

The psychophysical action spectrum of the absolute threshold of a normal (○ in Fig. 4) was well described by the standard V'_λ function. V'_λ also fits the proband's data (●) for shorter wavelengths, but it deviates substantially at longer wavelengths as the cones intervene, even though the proband's densitometric action spectrum for 10% rhodopsin bleach (■) continued to follow the rhodopsin V'_λ curve even for $\lambda > 530$ nm. The proband reported the 480-nm test as "blue" at threshold rather than colorless; we attribute this to the desaturated blue sensation evoked by rods under conditions in which rods and M-cones can interact (19, 20). This "blue" sensation cannot be from S-cones, since these exhibited a sensitivity only 0.01 of that required (see Fig. 5, at 3 log sc-td). In Fig. 4, the top-most solid spectrum is $\pi_1(\mu)$, and the dotted spectrum is shifted up by 0.247 logarithmic unit to reflect the amount that $\pi_1(\mu)$ is desensitized by the 3 log sc-td 632-nm background (▲). From these results it appeared that the proband's rods mediated the elevated psychophysical threshold for $\lambda < 510$ nm even though cones set the threshold for longer wavelengths.

DISCUSSION

This Gly90Asp mutation is associated with the unusual phenotype of congenital and complete nightblindness. Since the proband's rhodopsin density was normal *in vivo*, the etiology of the nightblindness must differ from those of adRP patients whose dark sensitivity loss depends primarily on the extent of rhodopsin lost (15, 21) or from those opsin mutants that exhibit low yields or a failure of trafficking (22). The normal density found for the proband suggests that mutant Gly90Asp rhodopsin remains bleachable *in vivo* and is not primarily in the free all-trans 380 nm-absorbing state as was suggested by *in vitro* experiments (23).

How then might mutant Gly90Asp rhodopsin interfere with normal signaling by the wild-type rhodopsin in dim but not bright light conditions? Light signals from both mutant and wild-type rhodopsin converge at the rod membrane, and increased thermal activation of the Gly90Asp variant would act as though a background light were always present that impaired threshold sensitivity (24). The extra negative charge from the Gly90Asp change across the opsin pocket from the Lys-296 retinylidine Schiff base (25, 26) could partially deprotonate the Schiff base and lead to increased thermal isomerization even in the absence of light (27). An estimate of equivalent *in vivo* dark-light from the mutant Gly90Asp rhodopsin can be derived from the background intensity that elevates the normal's rod increment threshold to that of the proband (Fig. 5). This was about 0.2 log sc-td, or ≈10 photoisomerizations per rod per s, which is $>10^4$ times greater than normal physiological dark-light (28), when equal expression of mutant and wild-type opsin is assumed. *In vitro* data have suggested that the mutant Gly90Asp opsin without the chromophore can activate transducin in the absence of light (29).

After this work was completed (30), we learned that the Gly90Asp rhodopsin mutant *in vitro* exhibits a hypsochromic absorption shift compared to wild-type rhodopsin (23, 29). We cannot verify this in the proband's action spectra, since (*i*) the dominant trait assures that his rod visual pigment is some unknowable mixture of wild and mutant rhodopsin, (*ii*) we have been unable to measure the spectral absorbance in the proband's eye medium that this verification would require, and (*iii*) the action spectrum measurements for the proband are insufficiently precise to resolve the small <10-nm difference predicted.

This kindred could possibly be considered to have adRP showing minimal disease with variable expression. However, an adRP designation would overlook the remarkable clinical features and would obscure the unusual pathophysiological mechanism suggested by these data—i.e., that reduced rod sensitivity in a rhodopsin mutation kindred is caused by anything other than decreased probability of light absorption from reduced levels of rhodopsin (31). The minimal retinal degeneration in Gly90Asp individuals up to 33 years of age was remarkable, given the extensive and lifelong nightblindness. adRP patients with rhodopsin mutations who have the same or less degree of nightblindness usually manifest considerably more retinal degeneration than that observed in the Gly90Asp kindred.

Although the nightblindness in our Gly90Asp kindred was constant across the 53 year span from 11- to 64-year-old affected subjects, we hesitate to apply the term "stationary" (i.e., nonprogressive with aging) because peripheral degeneration was observed in the 38- and 64-year old affected individuals. A 34-year-old patient with a Ala-292 → Glu rhodopsin mutation that shows constitutive activity *in vitro* (3) was reported as having "congenital stationary nightblindness," but examinations at later ages are not available in that genetically isolated case. tvi results of our Gly90Asp subjects are similar to those reported for Shubert–Bornshein-type congenital stationary nightblindness (32). However, Shubert–Bornshein subjects show ERG a-waves from rod photoreceptors, and, consequently, the absence of b-waves implicates a synaptic transmission defect in such subjects (33). This is unlike our Gly90Asp kindred, none of whom exhibit rod a-waves even with higher intensity white flashes of 0.62 log candela·s/m^2, dark-adapted (not shown).

Increased dark-light has been postulated to occur in some cases of retinitis pigmentosa (34), and, among other mechanisms, inappropriate signal transduction had been suggested to contribute to photoreceptor demise (35). In the case of Gly90Asp, the preservation of rods into middle and later age and the considerable disparity of visual field loss between the

63-year-old and 64-year-old affected brothers suggest that greater complexity is involved in the retinal degeneration than simply chronic rod activation by dark-light. Since the night-blindness phenotype in this kindred shows considerable physiologic rod dysfunction early but without significant rod loss until later, the Gly90Asp mutation may provide an important and simplified system for studying the complex processes of cell death in rhodopsin adRP.

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