Recovery of Rod Photoresponses in ABCR-Deficient Mice

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Purpose. The ABCR protein of the rod outer segment is thought to facilitate movement of the all-*trans* retinal photoproduct of rhodopsin bleaching out of the disc lumen. This study was undertaken to investigate the extent to which ABCR deficiency affects the post-bleach recovery of the rod photoresponse in ABCR-deficient ($abcr^{-/-}$) mice.

Methods. Electroretinographic (ERG) a-wave responses were recorded from $abcr^{-/-}$ mice and two control strains. A bright probe flash was used to examine the course of rod recovery after fractional rhodopsin bleaches of $\sim 10^{-6}$, $\sim 3 \times 10^{-5}$, $\sim$0.03, and $\sim$0.30 to $\sim$0.40.

Results. Dark-adapted $abcr^{-/-}$ mice and control animals exhibited similar normalized near-peak amplitudes of the paired-flash-ERG-derived, weak-flash response. Response recovery after $\sim 10^{-6}$ bleaching exhibited an average exponential time constant of 319, 171, and 213 ms, respectively, in the $abcr^{-/-}$ and the two control strains. Recovery time constants determined for $\sim 3 \times 10^{-5}$ bleaching did not differ significantly among strains. However, those determined for the $\sim$0.03 bleach indicated significantly faster recovery in $abcr^{-/-}$ mice (2.34 $\pm$ 0.74 minutes) than in the controls (5.36 $\pm$ 2.20 and 5.92 $\pm$ 2.44 minutes). After $\sim$0.30 to $\sim$0.40 bleaching, the initial recovery in the $abcr^{-/-}$ mice was, on average, faster than in control mice.

Conclusions. By comparison with control animals, $abcr^{-/-}$ mice exhibit faster rod recovery after a bleach of $\sim$0.03. The data suggest that ABCR in normal rods may directly or indirectly prolong all-*trans* retinal clearance from the disc lumen over a significant bleaching range, and that the essential function of ABCR may be to promote the clearance of residual amounts of all-*trans* retinal that remain in the discs long after bleaching. (*Invest Ophthalmol Vis Sci.* 2008;49:2743–2755) DOI:10.1167/iovs.07-1499

The ATP-binding cassette transporter protein known as ABCR, or ABCA4, facilitates processing of the all-*trans* retinal photoproduct of rhodopsin bleaching in rod photoreceptors.[1–7] In an ATP-dependent reaction, ABCR moves *N*-retinylidene-phosphatidylethanolamine (*N*-ret-PE), a complex formed between all-*trans* retinal and phosphatidylethanolamine, from the luminal to cytosolic side of the rod disc membrane. The all-*trans* retinal in the rod cytosol is then enzymatically reduced to all-*trans* retinol for further processing in the retinoid visual cycle that enables rhodopsin regeneration in bleached rods.[8–11] As all-*trans* retinal can interact with opsin to form a metarhodopsin II (MII)-like signaling state,[12–15] the clearance of all-*trans* retinal out of the disc (i.e., from the vicinity of opsin) promotes the shut-off of the phototransduction cascade.

The impairment of ABCR activity leads to the build-up of *N*-ret-PE in the rods and to the resulting accumulation, in the retinal pigment epithelium (RPE), of retinoid-based components of lipofuscin that produce atrophy of the RPE.[16–18] Furthermore, mutations in ABCR are associated with Stargardt disease and other retinal degenerations.[19–26] However, relatively little information is available as to how ABCR deficiency affects recovery of the rod electrophysiological response after rhodopsin-bleaching illumination (i.e., after the generation of all-*trans* retinal). In electroretinographic (ERG) experiments on 16- to 20-week-old $abcr^{-/-}$ mice, Weng et al.[1] investigated recovery of the ERG a-wave after illumination that bleached approximately 45% of the rhodopsin. These investigators found that a-wave recovery in $abcr^{-/-}$ mice was substantially slower than that exhibited by wild-type mice of similar age, consistent with a delayed clearance of all-*trans* retinal from the disc lumen in mice lacking ABCR. For example, a-wave recovery at 30 minutes after the bleach amounted to approximately 75% in wild-type mice but only approximately 50% in $abcr^{-/-}$ mice.

The signaling activity of the MII-like complex formed by all-*trans* retinal and opsin far exceeds that due to free opsin (i.e., opsin devoid of chromophore) and may be as great as $\sim$10% of that of MII generated in the phototransduction process.[13,14,27,28] Furthermore, studies of both single rod photocurrents and the in vivo massed rod response show that in dark-adapted mouse rods, photoactivation of as little as $\sim$100 rhodopsins per rod (i.e., a fractional bleach of $\sim 10^{-6}$; Ref. 29), produces a rod photocurrent response of near-saturating peak amplitude (see, for example, Ref. 30). On the basis of these findings, and of previous ERG data obtained with $\sim$45% bleaching,[1] we reasoned that $abcr^{-/-}$ mice might exhibit a detectable delay in rod recovery at fractional bleaches well below $\sim$45%. The present study was undertaken to test this possibility. Preliminary results have been reported (Pawar AS, et al. *IOVS* 2006;47:ARVO E-Abstract 4736 and Pawar AS, et al. *IOVS* 2007;48:ARVO E-Abstract 608).

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Materials and Methods

All procedures conformed to the principles embodied in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Experiments were conducted on mice of ages 3 weeks to 3 to 4 months that were maintained on a light/dark cycle (12-hour light/12-hour dark or 14-hour light/10-hour dark) at an ambient illumination of ~2 to ~19 lux. The *abcr*$^{-/-}$ mice were derived from breeding pairs that were generously provided by Gabriel H. Travis (University of California at Los Angeles). Two wild-type (i.e., *abcr*$^{+/+}$) strains were used as control animals. The first of these was a C57-derived strain that, like *abcr*$^{-/-}$, possesses the leucine variant at amino acid position 450 of the retinal pigment epithelium (RPE) protein RPE65 (Travis GH and Radu R, personal communication, August 2006, and Ref. 31). Breeding pairs of these mice (N10, C57BL/6J) were generously provided by Michael Danciger (Loyola Marymount University). The second was strain C57BL/6J (Jackson Laboratories, Bar Harbor, ME), which is known to possess the methionine variant at amino acid position 450 of RPE65.[32] As noted in the Discussion section, the investigation of control mice possessing both the leucine 450 and methionine 450 variants was of importance to the present study in light of RPE65's role in the retinoid visual cycle.[33–35] These control strains possessing the leucine and methionine RPE65 variants will henceforth be termed C57-leu450 and C57-met450, respectively.[31]

All mice were dark-adapted overnight before the experiment. Equipment and procedures used for single- and paired-flash ERG recordings were similar to those described.[36–38] Briefly, under dim red light, the mouse was anesthetized with an intraperitoneal injection of ketamine and xylazine [0.15 and 0.01 mg (g body wt)$^{-1}$, respectively]. Pupil dilation was performed with 2.5% phenylephrine HCl and 1% tropicamide, and the cornea was anesthetized with proparacaine HCl (0.5%). Drops of a moistening/lubricating agent (Tears Naturale Forte; Alcon, Fort Worth, TX) were periodically applied to the corneal surface. The mouse's body temperature was maintained in the range of 37.5°C to 38.5°C with use of a heating pad positioned beneath the animal. Boosts of anesthetic (approximately one sixth of the initial dose) were delivered subcutaneously at ~20-minute intervals beginning ~40 minutes after the initial dose. Responses to full-field test flashes (green light of ~20-μs duration) and to probe flashes (white light of ~1.7 ms duration) were obtained with the use of a stainless-steel recording electrode positioned on the cornea, a stainless-steel reference electrode placed in the mouth, and a platinum subdermal needle ground electrode positioned in the nape of the neck. Responses were amplified (band-pass: 0.3−3000 Hz), sampled at 100 kHz, stored in a computer, and subsequently analyzed (MatLab software; MathWorks, Natick, MA). Paired-flash ERG determinations of the rod response to a given test stimulus (i.e., in the present experiments, to a bleaching illumination of defined strength) used the methodology described previously.[36–39] In the paired-flash method, a bright (i.e., rod-saturating) probe flash is presented at a defined time after the test stimulus. The bright probe flash, which rapidly drives the rods to saturation, produces an a-wave response that essentially titrates the prevailing level of rod circulating current. As referenced to the "probe-alone" response obtained from the fully dark-adapted eye (i.e., in the absence of a recent presentation of the test stimulus), the probe response obtained in the paired-flash trial yields the prevailing "derived" amplitude of the rod response to the test stimulus (see, for example, pp. 519–520 in Ref. 39).

ERG experiments investigating rod recovery were conducted on 18 *abcr*$^{-/-}$, 17 C57-leu450, and 20 C57-met450 mice, the age ranges of which were 23 to 116, 23 to 95, and 23 to 86 days, respectively. The initial phase of each experiment consisted of a series of single- and paired-flash measurements on the dark-adapted mouse. In the single-flash trials we recorded the a-wave response to a bright probe flash of fixed strength (773 scotopic candela seconds per square meter [sc cd-s · m^{-2}]), to determine the peak amplitude (A_{peak}) and time to peak (t_{peak}) of this response. The derived rod response to a weak test flash (0.3 sc cd-s · m^{-2}) at a fixed post-test-flash time (t = 86 ms) was determined in paired-flash trials, by using a test-probe interval (t_{probe}) of 80 ms, a probe flash of 773 sc cd-s · m^{-2}, and a determination time (t_{det}) of 6 ms.[36] Consecutive experimental runs were separated by a dark-adaptation period of ≥2 minutes. The normalized amplitude, at t = 86 ms, of the dark-adapted derived response to the weak test flash was obtained from the relation.[36]

$$\text{Normalized response} = A(t)/A_{moD} = 1 - [A_m(t)/A_{moD}] \quad (1a)$$

$$t = t_{probe} + t_{det}, \quad (1b)$$

where $A(t)$ is the derived response amplitude, A_{moD} is the amplitude (at 6 ms) of the response to the probe flash in a probe-alone trial under dark-adapted conditions, and $A_m(t)$ is the amplitude of the probe response in the paired-flash trial.

After completion of the dark-adapted characterization, a bleaching stimulus was delivered, and the time course of recovery of the derived response $A(t)$ to this bleaching light was investigated through analysis of the response to the 773 sc cd-s · m^{-2} probe flash. The present study employed four different bleaching stimuli: (1) the weak test flash (0.3 sc cd-s · m^{-2}) used in the dark-adapted characterization; (2) a single bright flash of strength sufficient to produce rod saturation (7.9 sc cd-s · m^{-2})[36]; (3) a series of 20 flashes (each of 477 sc cd-s · m^{-2}) delivered over a period of approximately 100 seconds [cumulative bleaching energy: (20) (477 sc cd-s · m^{-2}) = 9.5 × 10^3 sc cd-s · m^{-2}]; and (4) a 2-minute exposure to intense full-field green light from a microscope illuminator positioned above the eye under investigation.[40] The recording electrode was withdrawn during the 2-minute illumination period. In experiments of types 1 and 2, the bleaching light was presented in each experimental run,[36] and the experiment typically involved two determinations at a given t_{probe}. Some experiments involved the investigation of recovery from both the subsaturating (type 1) and saturating (type 2) bleaching flashes. Unless otherwise indicated, values of $A(t)$ determined after the bleaching illumination were normalized to the dark-adapted probe-alone amplitude A_{moD} determined early in the experiment. For recoveries from the 20-flash and 2-minute bleaching stimuli, the 6-ms determination time t_{det} was negligible and was ignored. The conclusion of the bleaching illumination defined time 0 in each experiment or (in type 1 and type 2 experiments) each experimental run. For the 20-flash and 2-minute bleaching illuminations, the bleaching light was presented only once in the experiment, and the recovery time course was determined by presentations of the probe flash alone at various times. In all four types of experiment, probe responses obtained after the bleaching illumination were analyzed to yield $A_m(t)$, and the derived response $A(t)$ was obtained through equation 1. In experiments of types 3 and 4, both the post-bleach times of measurement and the overall post-bleach period of investigation differed somewhat among experiments. To permit ANOVA of individual sets of recovery data in the type 3 experiments, consecutive values of the determined, normalized derived response $A(t)/A_{moD}$ were linearly interpolated to yield minute-by-minute values of $A(t)/A_{moD}$. For sets of recovery data in the type 4 experiments, determined values of $A(t)/A_{moD}$ were grouped within 3-minute bins to permit ANOVA (see the Results section).

For low extents of rhodopsin bleaching (i.e., those relevant to the present experiments of types 1–3), the fractional bleach B produced by a bleaching stimulus of strength L (in sc cd-s · m^{-2}) is approximately given by $B = aL/R_o$, where R_o is the population of rhodopsin molecules in the dark-adapted (i.e., unbleached) rod, and a is the number of photoisomerizations (R*) produced by a flash of unit strength. Previous estimates of a (based on different experimental approaches) have ranged from 100 R* (sc cd-s · m^{-2})$^{-1}$ (Ref. 36) to 490–580 R* (sc cd-s · m^{-2})$^{-1}$ (Ref. 29). If one assumes that $R_o = 7 \times 10^7$ (Ref. 29) and $a \sim 250$ R*, the fractional bleaches corresponding with the bleaching illuminations in the present type 1 to 3 experiments (0.3, 7.9, and 9.5 × 10^3 sc cd-s · m^{-2}, respectively) are ~10^{-6}, ~3 × 10^{-5}, and ~0.03, respectively. Procedures used to determine the extent of

rhodopsin bleaching by the 2-minute illumination (type 4 experiment) followed those described.[37,40] The anesthetized mouse was killed by cervical dislocation immediately after the bleach, and the retinas and RPEs were isolated. The retina and RPE of a given eye were extracted by using formaldehyde (for analysis of retinaldehydes) and isopropanol/hexane (for analysis of all-*trans* retinol and retinyl ester) extraction procedures. The extracts were analyzed for molar amounts of 11-*cis* retinal, all-*trans* retinal, all-*trans* retinol, and retinyl ester by using normal-phase high-performance liquid chromatography and standard curves. The difference in molar percents of 11-*cis* retinal measured for the illuminated versus unilluminated eyes was used to determine the percentage of rhodopsin bleached.[37,40] The data yielded bleaching extents of 40% ± 11% in the $abcr^{-/-}$ mice ($n = 3$; ages of 73, 78, and 78 days); 32% ± 8% in the C57-leu450 mice ($n = 4$; ages of 73, 73, 69, and 69 days); and 31% ± 5% in the C57-met450 mice ($n = 3$; ages of 63, 58, and 63 days). Based on their average values, the following text quotes the extent of bleaching as ~30% to ~40%.

Results

Dark-Adapted Characterization

Waveforms labeled PA in Figure 1A–C show, respectively, dark-adapted responses recorded from $abcr^{-/-}$, C57-leu450, and C57-met450 mice in probe-alone trials (i.e., on presentation of only the bright probe flash: 773 sc cd-s · m^{-2}). These probe-alone responses were analyzed for the peak amplitude (A_{peak}) and time-to-peak (t_{peak}). Table 1A–C shows, for each of the three investigated strains, the number of experiments and ages of the mice for which determinations of A_{peak} (column 1) and t_{peak} (column 2) were made. Columns 1 and 2 in Table 1D–F show data (means ± SD) obtained for A_{peak} and t_{peak}, respectively, in the $abcr^{-/-}$, C57-leu450, and C57-met450 mice. Peak amplitude of the probe-alone response ranged, on average, from 315 to 369 μV. Between-groups ANOVA showed a significant difference among the three investigated strains for A_{peak} ($F_{2,52} = 3.183$; $P = 0.050$); in addition, values of t_{peak} among the strains differed significantly ($F_{2,52} = 6.974$; $P = 0.002$. Post-hoc comparisons of A_{peak} and t_{peak} in the $abcr^{-/-}$ and C57-leu450 mice indicated a significant difference only for t_{peak} ($P = 0.001$). In the $abcr^{-/-}$ versus C57-met450 mice, there were significant differences in both A_{peak} and t_{peak} ($P = 0.015$ and $P = 0.008$, respectively). Between the C57-leu450 and C57-met450 mice, A_{peak} and t_{peak} did not differ significantly. Average values of t_{peak} ranged from 7.10 to 7.64 ms among strains.

The dark-adapted characterization conducted in the initial phase of each experiment typically also included paired-flash determinations of the normalized derived response to a weak test flash (0.3 sc cd-s · m^{-2}). Waveforms labeled 80 in Figure 1 show probe responses obtained in paired-flash trials with the use of an 80-ms test-probe interval. As indicated in column 3 of Table 1D–F, the average, normalized derived response amplitudes determined for the $abcr^{-/-}$, C57-leu450, and C57-met450 mice were within a narrow range (0.65–0.67). Between-groups ANOVA showed no significant difference among strains for the dark-adapted, normalized derived response ($F_{2,46} = 0.387$; $P = 0.681$). Post-hoc pair-wise comparisons showed no significant difference between values for the $abcr^{-/-}$ versus C57-leu450 mice ($P = 0.472$). In addition, there was no significant difference between the $abcr^{-/-}$ and C57-met450 mice ($P = 0.972$) or between the C57-leu450 and C57-met450 mice ($P = 0.436$).

~10^{-6} and ~3 × 10^{-5} Fractional Bleaches

Figure 2A shows the recovery time course of the derived response to a brief flash (0.3 sc cd-s · m^{-2}) estimated to produce a fractional bleach of ~10^{-6}. The strength of this flash

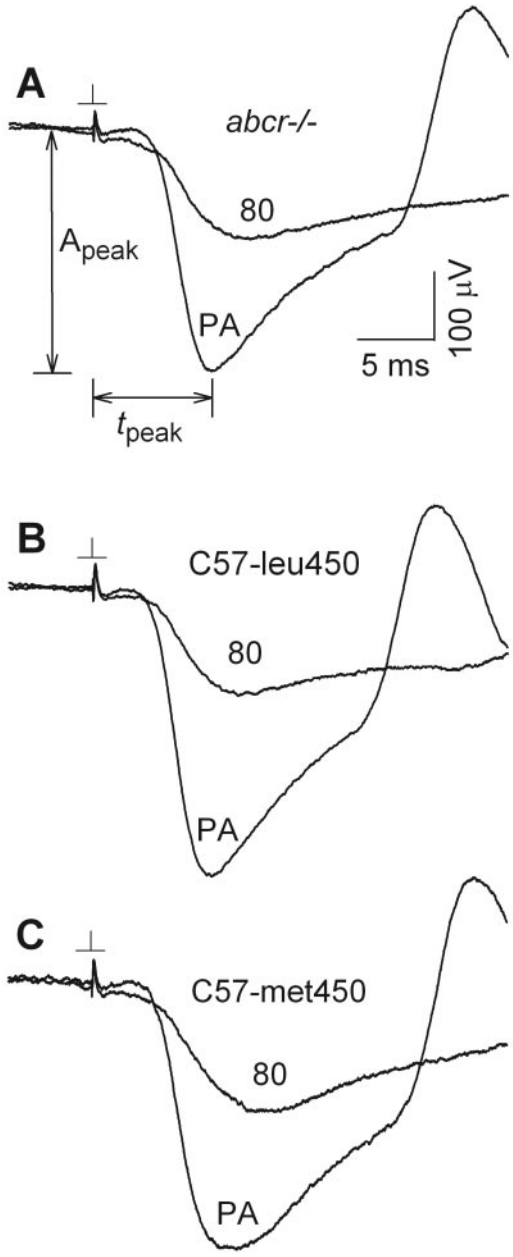


Figure 1. Dark-adapted responses recorded from $abcr^{-/-}$ (**A**), C57-leu450 (**B**), and C57-met450 (**C**) mice in single-flash and paired-flash trials. Each illustrated waveform is a single response. Traces labeled PA: responses to the 773 sc cd-s · m^{-2} probe flash presented alone. The A_{peak} and t_{peak} of the probe response were determined as shown in (**A**). Traces labeled 80: probe responses recorded in paired-flash trials with an 80-ms test probe interval.

was identical with that used to measure weak-flash sensitivity under dark-adapted conditions (described above). Waveforms at the left in Figure 2A show probe responses obtained in paired-flash experiments on the $abcr^{-/-}$, C57-leu450, and C57-met450 mice. To quantify the time course of recovery of the paired-flash-derived response, we analyzed determinations of the normalized response $A(t)/A_{moD}$ beginning at $t_{probe} = 200$ ms (i.e., $t = 206$ ms) in relation to the exponential decay function (curves in Fig. 2A)

$$A(t)/A_{moD} = \eta \exp[-(t - 206)/\tau_r], \quad (2)$$

TABLE 1. Dark-Adapted Characterization and Recovery Parameters

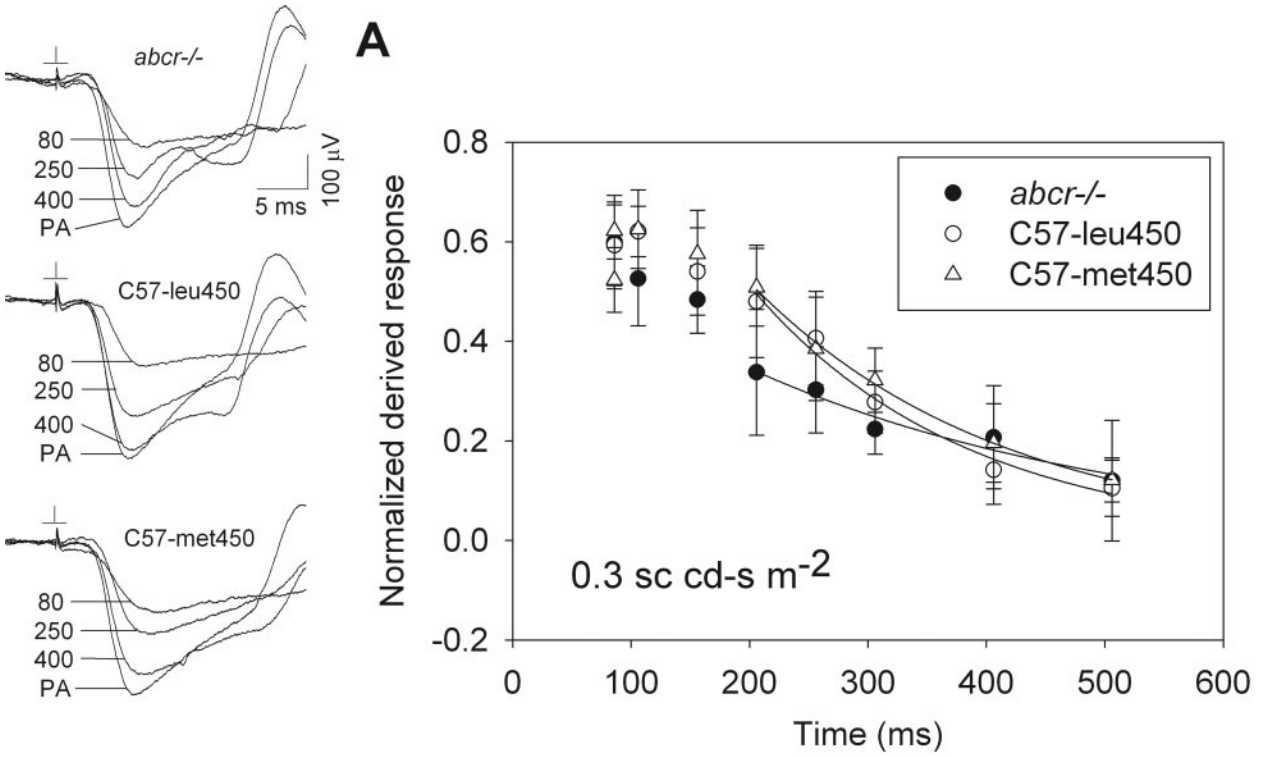
						Approximate Bleach †			
			1	2	3	4	5	6	7
Type of measurement			Dark-Adapted Characteristics *			~10^{-6}	~3 x 10^{-5}	~3%	~30% to ~40%
			A_{peak}	t_{peak}	Norm DR	τ_r	τ_ω	τ	Slope (σ)
A	*abcr* $^{-/-}$	No. of mice	18	18	15	3	5	8	4
		Age (days)	55 ± 30	55 ± 30	57 ± 32	40 ± 6	43 ± 6	63 ± 40	58 ± 27
B	C57-leu450	No. of mice	17	17	17	4	3	8	4
		Age (days)	58 ± 26	58 ± 26	58 ± 26	53 ± 26	43 ± 24	61 ± 24	63 ± 36
C	C57-met450	No. of mice	20	20	17	3	5	9	5
		Age (days)	56 ± 19	56 ± 19	58 ± 19	62 ± 3	51 ± 13	50 ± 21	72 ± 14

Determined parameters		Dark-Adapted Characteristics *			~10^{-6}	~3 x 10^{-5}	~3%	~30% to ~40%
		A_{peak} (μV)	t_{peak} (ms)	Norm DR	τ_r (ms)	τ_ω (ms)	τ (min)	σ (min^{-1}) ‡
D	*abcr* $^{-/-}$	315 ± 60	7.10 ± 0.37	0.65 ± 0.07	319 ± 24 320 (R^2 = 0.95)	321 ± 39 315 (R^2 = 0.99)	2.34 ± 0.74 2.45 (R^2 = 0.96)	–0.0281 ± 0.0079 –0.0280 (R^2 = 0.95)
E	C57-leu450	340 ± 56	7.64 ± 0.36	0.67 ± 0.04	171 ± 26 181 (R^2 = 0.98)	362 ± 29 362 (R^2 = 0.98)	5.36 ± 2.20 4.95 (R^2 = 0.99)	–0.0099 ± 0.0068 –0.0079 (R^2 = 0.87)
F	C57-met450	369 ± 79	7.50 ± 0.56	0.65 ± 0.08	213 ± 41 211 (R^2 = 0.99)	395 ± 39 392 (R^2 = 0.96)	5.92 ± 2.44 4.93 (R^2 = 0.97)	–0.0200 ± 0.0127 –0.0225 (R^2 = 0.98)

* A_{peak} and t_{peak}: peak amplitude and time to peak, respectively, of the dark-adapted response to the 773-sc cd-s · m^{-2} probe flash; Norm DR: normalized derived response to a 0.3-sc cd-s · m^{-2} test flash at t = 86 ms. Determinations of A_{peak} and t_{peak} within a given experiment are based on data obtained in three presentations of the probe flash. Determinations of Norm DR within a given experiment are based on data obtained in three paired-flash trials.

† Columns 4–7 indicate, for the four investigated bleaching conditions, the numbers of mice investigated (A–C) and the determinations of recovery parameters (D–F). In D–F, recovery kinetics determined with fractional bleaches of ~10^{-6}, ~3×10^{-5}, and ~0.03 are described in relation to the time constants τ_r, τ_ω, and τ, respectively. These time constants are defined by equations 2, 3, and 4. Recovery kinetics determined with the ~30% to ~40% bleach are described in relation to the slope σ, as defined by equation 5. Values of R^2 in sections D–F denote the goodness of fit of equations 2, 3, 4, or 5 to the aggregate data.

‡ Determinations of slope σ within a given experiment are based on fitting a straight line to ≥3 data points obtained within ~12 to ~20 minutes after bleaching.



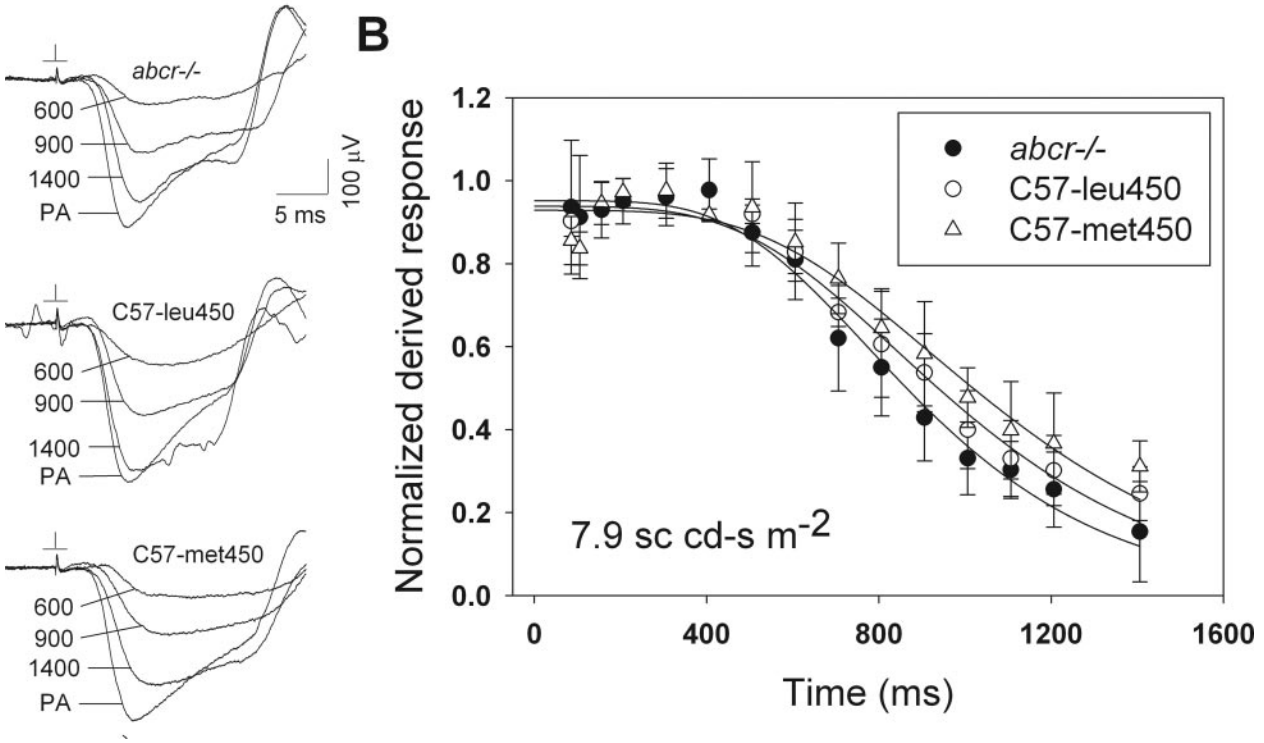


FIGURE 2. Recovery after relatively weak bleaching illumination. (**A**) Bleaching flash of 0.3 sc cd-s · m^{-2} ($\sim 10^{-6}$ fractional bleach). *Left*: paired-flash data obtained in single representative experiments. Labels indicate values of t_{probe}. *Right*: aggregate results obtained from three *abcr*$^{-/-}$, four C57-leu450, and three C57-met450 mice. The curves illustrate the fitting of equation 2 to data obtained with $t_{probe} \geq 200$ ms. (**B**) Bleaching flash of 7.9 sc cd-s · m^{-2} ($\sim 3 \times 10^{-5}$ fractional bleach): *Left:* data obtained in single representative experiments. *Right:* aggregate results obtained from five *abcr*$^{-/-}$, three C57-leu450, and five C57-met450 mice. The curves illustrate the fitting of equation 3 to the data.

where the dimensionless parameter η and the exponential time constant τ_r are free parameters. Determinations of the recovery time constant τ_r in these $\sim 10^{-6}$ bleach experiments (numbers of experiments and animal ages shown in column 4 of Table 1A–C) are summarized in column 4 of Table 1D–F. These data are organized to indicate results obtained from the fitting of equation 2, both to data from individual experiments and to the aggregate data set obtained from a given strain. Thus, for example, the column 4 data in Table 1D indicate, for the *abcr*$^{-/-}$ mice, the mean ± SD of τ_r values obtained in individual experiments (upper entry) and the single τ_r value determined from equation 2 fitting to the aggregate data set (lower entry). Accompanying the aggregate best-fit value is the corresponding goodness of fit (R^2). Among the investigated strains, the average τ_r determined from the individual fits ranged from 171 to 319 ms and corresponded closely with the aggregate fitted values of τ_r. Between-samples ANOVA of τ_r indicated a significant difference ($F_{2,7} = 20.517$; $P = 0.001$). Post-hoc pair-wise comparisons of the data showed that τ_r for the *abcr*$^{-/-}$ mice significantly exceeded those for both the C57-leu450 ($P < 0.001$) and C57-met450 ($P = 0.004$) mice. There was no significant difference in τ_r between the C57-leu450 and C57-met450 mice. For the three investigated strains, η (equation 2) was 0.34 ± 0.07 (*abcr*$^{-/-}$), 0.51 ± 0.07 (C57-leu450), and 0.50 ± 0.04 (C57-met450).

Figure 2B shows recovery results obtained with the 7.9 sc cd-s · m^{-2} flash. This flash, of strength sufficient to saturate the rod response,[36] produced a fractional bleach of $\sim 3 \times 10^{-5}$. Recovery data obtained in these experiments were analyzed in relation to a nested exponential function similar to those used previously[36,37]:

$$A(t)/A_{moD} = \theta_1\{1 - \exp[-\theta_2 \exp(-t/\tau_\omega)]\}, \quad (3)$$

where the dimensionless parameters $\theta_1$and θ_2 and the recovery time constant τ_ω are free parameters. Column 5 of Table 1D–F summarizes the results obtained. The upper entry in each row is the mean ± SD for the recovery time constant τ_ω based on the fitting of equation 3 to individual data sets; the lower entry indicates the value of τ_ω obtained by fitting equation 3 to the aggregate data set for a given strain. As illustrated in Figure 2B, aggregate recovery data obtained from the three investigated strains exhibited a generally similar pattern, although recovery in the C57-met450 mice was, on average, somewhat slower than that in the *abcr*$^{-/-}$ and C57-leu450 mice. ANOVA of the values of τ_ω (Table 1D–F, column 5) indicated a significant difference ($F_{2,10} = 4.808$; $P = 0.034$). Post-hoc pair-wise comparisons showed a significant difference between the *abcr*$^{-/-}$ and C57-met450 mice ($P = 0.011$), no significant difference between the *abcr*$^{-/-}$ and C57-leu450 mice, and no significant difference between the C57-leu450 and C57-met450 mice. θ_1 and θ_2 (equation 3) for the three strains were, respectively, 0.96 ± 0.03 and 11.76 ± 4.28 (*abcr*$^{-/-}$), 0.93 ± 0.02 and 10.67 ± 0.51 (C57-leu450), and 0.92 ± 0.02 and 11.07 ± 3.38 (C57-met450).

~3% Bleach

Figures 3A, 3C, and 3E show results from single representative experiments on *abcr*$^{-/-}$, C57-leu450, and C57-met450 mice, respectively, that involved a cumulative luminance of 9.5×10^3 sc cd-s · m^{-2} (series of bright flashes delivered over an approximately 100-second period; see the Methods section) and produced a fractional bleach of ~0.03. Figures 3B, 3D, and 3F show aggregate results obtained in eight experiments on the *abcr*$^{-/-}$ mice, eight on the C57-leu450 mice, and nine on the C57-met450 mice (Table 1A–C, column 6). In these experiments, the normalized derived response determined after the bleaching illumination declined toward the prebleach baseline and in a number of cases exhibited an overshoot (i.e., an amplitude that exceeded the dark-adapted amplitude, A_{moD}). A similar overshoot in the post-bleach recovery of wild-type rods has been reported[41] (also see Ref. 42). To quantify the results obtained, data collected in each experiment (which typically differed in post-bleach times of determination of the derived response) were linearly interpolated to yield values of $A(t)/A_{moD}$ at post-bleach times of 2, 3, . . . , 30 minutes and (for a subset of the data set) at times 31, 32, . . . , 60 minutes. Repeated-measures ANOVA demonstrated a significant interaction between strains as a function of time for the interval of 2–30 minutes ($F_{56,616} = 3.934$; $P < 0.001$). Post-hoc tests showed a significant difference between the *abcr*$^{-/-}$ and C57-leu450 mice and between the *abcr*$^{-/-}$ and C57-met450 mice, but no significant difference between the C57-leu450 and C57-met450 mice. Unlike the case of the 2- to 30-minute interval, repeated-measures ANOVA indicated no significant difference among the investigated strains for the 31- to 60-minute interval.

The recovery time course in these ~3% bleach experiments was further characterized by determinations of a characteristic time constant, τ. In the first of two methods used, data obtained in a single given experiment were analyzed by fitting a simple exponential function,

$$A(t)/A_{moD} = \alpha + \beta \exp(-t/\tau), \quad (4)$$

where α, β, and τ are free parameters, to recovery data obtained between time 0 (i.e., the time of conclusion of the bleaching illumination) and post-bleach time T. The value of T was chosen based on visual inspection of the data, and corresponded with the time of completion of a visually apparent plateau in the derived response amplitude. Among experiments, values of the selected period T were, respectively, 29.75 ± 10.28 (*abcr*$^{-/-}$), 42.50 ± 8.86 (C57-leu450), and 40.78 ± 14.65 (C57-met450) minutes. Overall results obtained for the recovery time constant τ are shown in the upper entries in column 6 of Table 1D–F. The average τ determined for the *abcr*$^{-/-}$ mice (2.34 minutes) was substantially less than that determined for the C57-leu450 and C57-met450 mice (5.36 and 5.92 minutes, respectively). ANOVA of these determinations of τ indicated a significant difference between strains ($F_{2,22} = 7.144$; $P = 0.004$). Furthermore, post-hoc comparisons between the strains indicated a significant difference between the *abcr*$^{-/-}$ and C57-leu450 mice ($P = 0.008$) and between the *abcr*$^{-/-}$ and C57-met450 mice ($P = 0.002$). There was no significant difference between the C57-leu450 and C57-met450 mice. The second of the two analysis methods involved the fitting of equation 4 to aggregate data obtained from a given strain (Figs. 3B, 3D, 3F). Vertical arrows in Figures 3B, 3D, and 3F indicate the conclusions of the post-bleach periods used for this analysis of aggregate data, and lower entries in column 6 of Table 1D–F show the values of τ obtained. These aggregate-fit values of τ corresponded closely with the average values determined by fitting to the individual data sets.

Aggregate data obtained from all three strains (Figs. 3B, 3D, 3F) exhibited an upward trend of the derived response in the later phase of the experiments (i.e., a positive-directed trend that opposed the downward-directed recovery process). This upward-directed process, if of sufficient magnitude, might be anticipated to skew determination of (i.e., lead to underestimation of) the recovery time constant τ. However, ANOVA of the values of the excursion β of the simple exponential function fitted to individual data sets (equation 4; values of β: 0.68 ± 0.26 for *abcr*$^{-/-}$; 0.74 ± 0.17 for C57-leu450; and 0.77 ± 0.27 for C57-met450) showed no significant differences

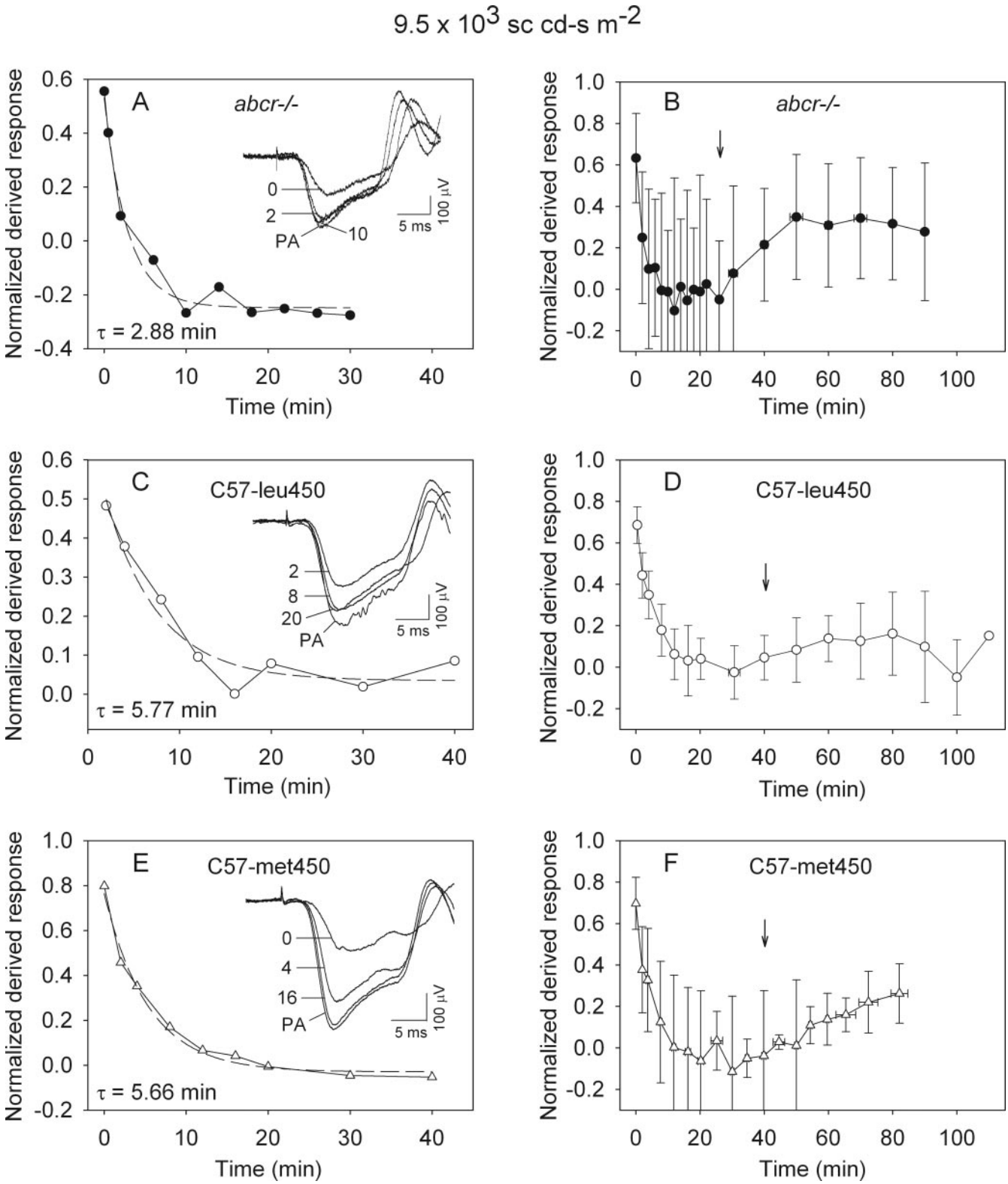


FIGURE 3. Recovery after the ~3% bleach. (**A**, **C**, **E**) Recovery data obtained in single experiments on the *abcr*$^{-/-}$, C57-leu450, and C57-met450 mice, respectively. *Insets:* representative responses to the probe flash. *Dashed curve*: simple exponential function fitted to the data obtained from the time of conclusion of the bleaching light (time 0) to the apparent plateau of recovery. (**B**, **D**, **F**) Aggregate data obtained in groups of experiments on *abcr*$^{-/-}$, C57-leu450, and C57-met450 mice, respectively. In cases for which data obtained at different values of t_{probe} were binned, the abscissa value and horizontal error bar of the illustrated data point represent the mean ± SD of the binned t_{probe} values. *Vertical arrows*: conclusion of the period over which data were analyzed in relation to the exponential function.

($F_{2,22} = 0.252$; $P = 0.779$), and post-hoc pair-wise comparisons indicated no significant differences. In addition, aggregate data for the *y*-intercept of this fitted exponential function [i.e., for the time 0 value, given by the sum ($\alpha + \beta$)] were similar among strains (0.66 ± 0.22 for *abcr*$^{-/-}$; 0.74 ± 0.10 for C57-leu450; and 0.68 ± 0.12 for C57-met450). Thus, although the basis of the opposing process remains unclear, the similarities of the excursion β and of the *y*-intercept ($\alpha + \beta$) among strains suggest that this process operates in similar fashion among strains and does not account for the relatively fast recovery determined for the *abcr*$^{-/-}$.

~30% to ~40% Bleach

Figure 4 shows overall results obtained in experiments with the 2-minute illumination that bleached ~30% to ~40% of the rhodopsin. Figure 4A shows aggregate results obtained from the investigated strains over periods that ranged up to 100–110 minutes after bleaching. As illustrated by Figure 4A, recovery in *abcr*$^{-/-}$ mice was, on average, faster than those in the C57-leu450 and C57-met450 mice. The initial ~12 to ~20 minutes of recovery determined in the investigated strains was further analyzed by determining the slope of a straight line

$$A(t)/A_{moD} = \psi + \sigma t, \quad (5)$$

where the slope σ (in units of inverse time) and the dimensionless intercept ψ are free parameters fitted to the data obtained over the initial ~12- to ~20-minute post-bleach period. Figures 4B–G illustrate results obtained in representative single experiments on *abcr*$^{-/-}$ (Figs. 4B, 4C), C57-leu450 (Figs. 4D, 4E), and C57-met450 (Figs. 4F, 4G) mice, and data for the determinations of slope are summarized in column 7 of Table 1D–F. ANOVA of the values of the slope σ indicated no significant differences among the investigated strains ($F_{2,10} = 3.420$; $P = 0.074$). However, post-hoc pair-wise comparisons showed that slopes determined for the *abcr*$^{-/-}$ mice differed significantly from those of the C57-leu450 mice ($P = 0.026$). There was no significant difference between the *abcr*$^{-/-}$ and C57-met450 mice or between the C57-leu450 and C57-met450 mice. For the three investigated strains, values of the *y*-intercept ψ of the fitted linear functions were 0.88 ± 0.12 (*abcr*$^{-/-}$), 0.92 ± 0.08 (C57-leu450), and 0.97 ± 0.04 (C57-met450). ANOVA of the values of ψ yielded no significant differences among strains ($F_{2,10} = 1.516$; $P = 0.266$), and post-hoc pair-wise comparisons also indicated no significant differences between strains.

Age Dependence

The preceding sections have considered rod response properties of *abcr*$^{-/-}$, C57-leu450, and C57-met450 mice, independent of the age of the animals. To investigate the possibility of a difference in properties exhibited by older versus younger mice, we separately grouped and analyzed data obtained from mice <2 months of age (1–59 days) and ≥2 months (60 days and greater) of age. The histograms of Figures 5A, 5C, 5E, and 5G summarize data for these two subgroups with respect to A_{peak} (Fig. 5A) and t_{peak} (Fig. 5C) of the dark-adapted response to the probe flash; to the normalized weak-flash response at t = 86 ms (Fig. 5E); and to values of the recovery time parameter τ determined with ~3% bleaching (Fig. 5G). The triplets of histograms within each of these panels describe results obtained from the *abcr*$^{-/-}$, C57-leu450, and C57-met450 mice. The filled bar within each triplet indicates the overall (i.e., age-independent) result obtained and is identical with that described in the corresponding column of Table 1D–F. The shaded and open bars of each triplet indicate results for mice of age <2 months and ≥2 months, respectively. Beneath each histogram is the number of mice from which the data were obtained. The scatterplots of Figures 5B, 5D, 5F, and 5H provide further description of the data sets summarized in the histograms. These scatterplots show, as a function of age, and for each of the investigated mice, the value of the parameter considered in the accompanying left-hand histogram. Two-way ANOVA with age (<2 months versus ≥2 months) and strain as between-sample factors indicated no significant differences for either A_{peak} or t_{peak}. There was a significant difference for the normalized derived response ($F_{2,49} = 3.446$; $P = 0.041$). Among the *abcr*$^{-/-}$ mice, ANOVA for <2-month versus ≥2-month animals yielded a significant difference only for the normalized derived response ($F_{1,13} = 5.841$; $P = 0.031$) and for τ ($F_{1,6} = 18.273$; $P = 0.005$). In the C57-leu450 mice, there was no significant effect of age on any of the parameters described in Figure 5. In the C57-met450 mice, there was a significant effect of age only for A_{peak} ($F_{1,18} = 5.340$; $P = 0.033$). Across strains, ANOVA of data obtained from a given age group yielded, in the <2-month mice, a significant difference only for A_{peak} ($F_{2,28} = 7.399$; $P = 0.003$) and t_{peak} ($F_{2,28} = 8.236$; $P = 0.002$). In the ≥2-month mice, there was no significant effect for any of the investigated parameters. Post-hoc pair-wise comparisons indicated, in the <2-month *abcr*$^{-/-}$ versus the C57-leu450 mice, a significant difference in t_{peak} ($P = 0.001$) and a marginal effect with respect to the normalized dark-adapted derived response ($P = 0.051$). In the ≥2-month *abcr*$^{-/-}$ versus the C57-leu450 mice, there were no significant differences. In the <2-month *abcr*$^{-/-}$ versus the C57-met450 mice, there was a significant effect only with respect to the A_{peak} ($P = 0.001$) and t_{peak} ($P = 0.016$). In the ≥2-month *abcr*$^{-/-}$ versus the C57-met450 mice, there were no significant differences. In the <2-month C57-leu450 versus the C57-met450 mice, the only significant difference was with respect to the normalized derived response ($P = 0.050$). In the ≥2-month C57-leu450 versus the C57-met450 mice, there were no significant differences.

Discussion

In the present study, we addressed the kinetics of rod recovery in *abcr*$^{-/-}$ versus control mice after bleaching stimuli that correspond with fractional rhodopsin bleaches of ~10^{-6} to ~30%–~40%. The most striking difference in recovery kinetics concerns the exponential time constant that describes recovery after ~3% bleaching. As shown by Table 1 and Figure 3, the exponential time constant that describes recovery in *abcr*$^{-/-}$ rods under this condition is about half that exhibited by the C57-leu450 and C57-met450 control mice. In addition, the rate of initial recovery in *abcr*$^{-/-}$ mice after ~30% to ~40% bleaching significantly exceeds that in C57-leu450 mice and is on average faster than that in C57-met450 mice (Table 1; and Fig. 4 and accompanying text). The relatively fast recovery time course in *abcr*$^{-/-}$ mice cannot be attributed to differences among the investigated strains in pupil size, other preretinal factors, or absorptivity (i.e., amount) of rhodopsin in the rods. That is, such differences would be expected to have produced differences in the dark-adapted, derived rod response to a weak test flash, yet these determinations of weak-flash sensitivity were similar among the *abcr*$^{-/-}$ mice and controls (Table 1). Accordingly, we interpret the significantly faster recovery kinetics observed in *abcr*$^{-/-}$ mice under the above-summarized conditions to reflect an intrinsically rapid process of post-bleach recovery in *abcr*$^{-/-}$ rods.

The relationship observed between the *abcr*$^{-/-}$ mice and wild-type control animals after significant bleaching differs

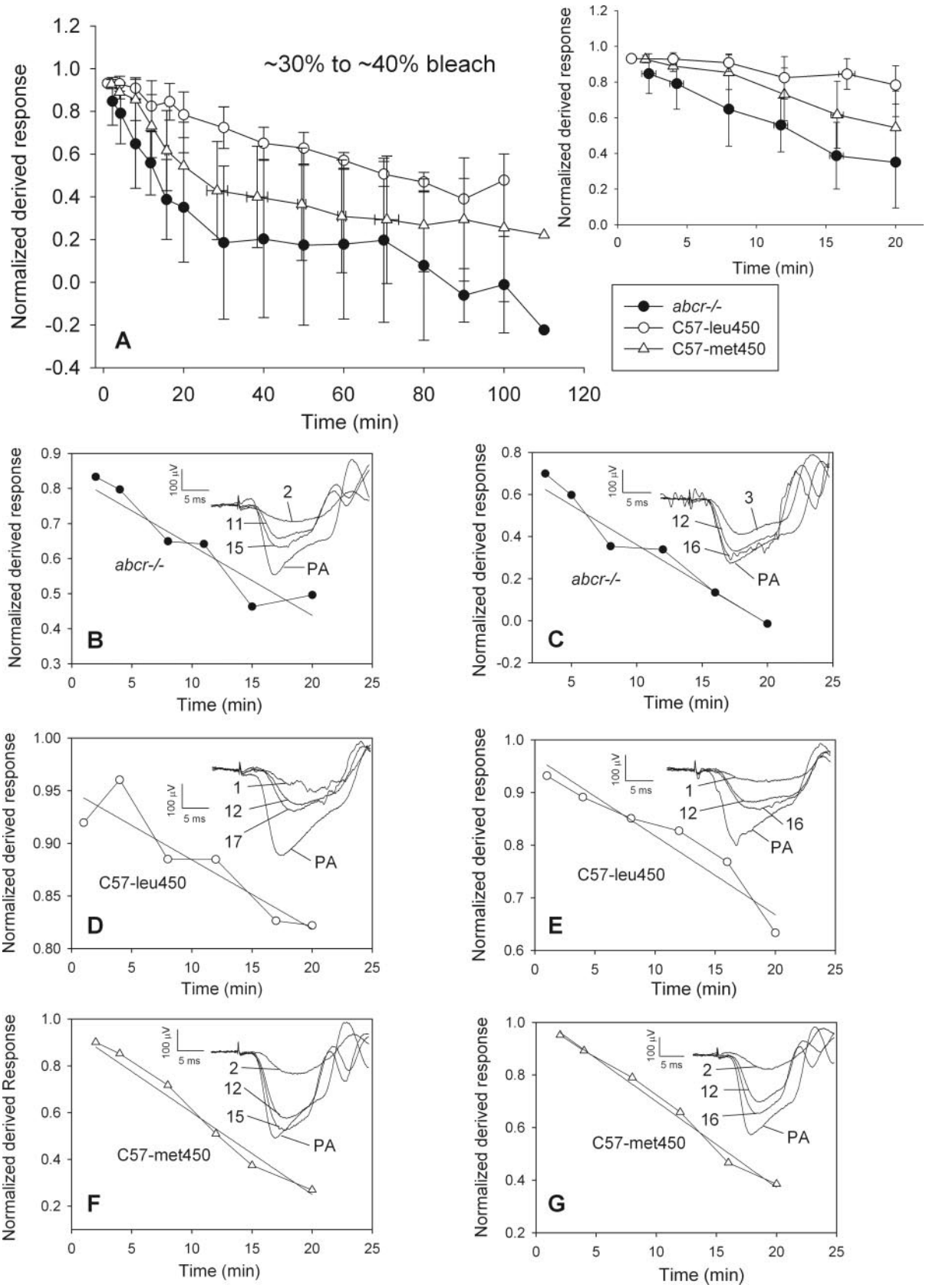


FIGURE 4. Recovery after the ~30% to ~40% bleach. (**A**) Aggregate data obtained from four *abcr*$^{-/-}$, four C57-leu450, and five C57-met450 mice. *Inset:* data illustrated on a faster time scale. (**B**–**G**) Data obtained in representative single experiments on *abcr*$^{-/-}$ (**B**, **C**), C57-leu450 (**D**, **E**), and C57-met450 (**F**, **G**) mice over the first ~12 to ~20 minutes after the conclusion of the bleaching illumination. Each panel illustrates the fitting of a straight line to the data. *Insets*: paired-flash data obtained in representative single experiments. Labels indicate the post-bleach time, in minutes.

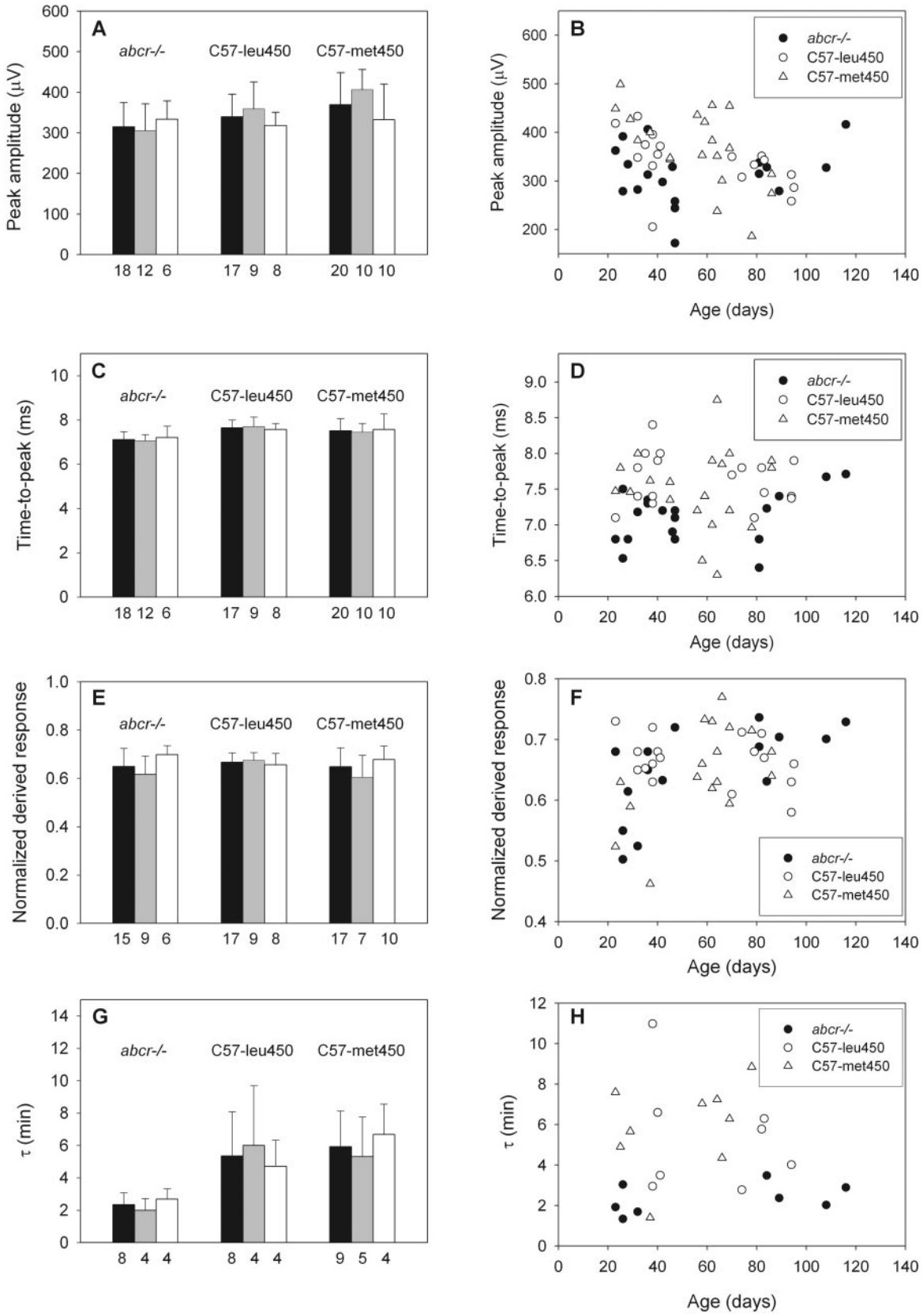


FIGURE 5. Analysis of data in relation to age. Parameters described are the A_{peak} of the dark-adapted response to the 773 sc cd-s · m^{-2} probe flash (**A**, **B**); the t_{peak} of this response (**C**, **D**); the normalized derived response to a 0.3 sc cd-s · m^{-2} test flash at $t = 86$ ms (**E**, **F**); and the recovery time constant τ determined with ~3% bleaching (**G**, **H**). *Filled bars* (**A**, **C**, **E**, **G**) replicate aggregate data reported in Table 1. *Shaded bars* and *open bars* represent results obtained from animals <2 months and ≥2 months of age, respectively. The number beneath each histogram indicates the number of mice from which the data were collected.

from that reported by Weng et al.[1] That is, the absolute time course of recovery reported by Weng et al. for *abcr*$^{-/-}$ mice after a 45% bleach is comparable with that reported here after a roughly similar (~30% to ~40%) bleach. However, by contrast with the Weng et al. study, we find post-bleach rod recovery in the wild-type strains to be (in the C57-met450) on average slower than, or (in the C57-leu450) significantly slower than, that in the *abcr*$^{-/-}$ mice after ~30% to ~40% bleaching (Fig. 4). In addition, the presently observed rod recovery in both wild-type strains was substantially slower than that in the *abcr*$^{-/-}$ mice after ~3% bleaching. Conceivably, differences in the specific wild-type strains used, and perhaps also the ages of the investigated mice, could be the bases of the contrasting findings of the present study and that by Weng et al.

The wild-type mice used in this study were pigmented C57-derived lines possessing the leu450 and met450 variants of RPE65, the isomerohydrolase of the retinoid visual cycle that promotes the conversion of all-*trans* retinyl ester to 11-*cis* retinol in the RPE.[34,35] The leu450 and met450 variants of RPE65 mice are known to differ with respect to the rate of isomerohydrolase activity.[33,43,44] Specifically, leu450, the RPE65 variant expressed by the presently studied *abcr*$^{-/-}$ mice, has greater catalytic activity than does the met450 variant. In light of the evidence that the isomerohydrolase reaction is a critical step in the retinoid visual cycle[34,35] and that rhodopsin regeneration is critical for full dark adaptation of the rods,[45,46] the comparison of rod recovery in the *abcr*$^{-/-}$ mice with that in control animals possessing both the leu450 and met450 variants of RPE65 was important in the present study. Interestingly, these two wild-type strains exhibited remarkably similar rod recovery properties under the present experimental conditions. In particular, there were no significant differences among data obtained from the C57-leu450 and the C57-met450 after ~3% bleaching or during the initial ~12- to ~20-minute period after ~30% to ~40% bleaching. These findings imply that occurrence of the leu450 variant of RPE65 in *abcr*$^{-/-}$ mice does not underlie the observed relatively rapid recovery of the *abcr*$^{-/-}$ rod response after the more extensive bleaches used here. Significant differences in rod recovery kinetics in albino mice possessing the leu450 versus met450 variant of RPE65 have been reported by Nusinowitz et al.[32] Specifically, these investigators compared ERG a-wave recovery in BALB/c mice, which possess the leu450 variant, with that in c2J mice, which possess the met450 variant. For bleaching illuminations of 3.61 and 3.97 log sc td-s, the exponential time constant describing recovery in the BALB/c was, respectively, approximately 48% and 21% faster than in c2J mice. Furthermore, after ~80% to ~90% bleaching, recovery of the ERG b-wave proceeded more rapidly in the BALB/c mice.

The observation of relatively fast post-bleach rod recovery in the *abcr*$^{-/-}$ mice raises several interesting issues relevant to the processing of all-*trans* retinal in *abcr*$^{-/-}$ mice and to the activity of ABCR in wild-type mice. Previous biochemical experiments indicate that ABCR's facilitation of all-*trans* retinal movement across the disc membrane contributes to the post-bleach processing of all-*trans* retinal in the visual cycle. For example, Weng et al.[1] found that the amount of all-*trans* retinal per eye in *abcr*$^{-/-}$ mice at up to 1 hour after a 45% bleach significantly exceeded that in wild-type controls. However, the magnitude of this increase, on average up to ~30 picomoles per eye (Fig. 3C in Ref. 1) represents only a small fraction of the decrease in rhodopsin produced by the 45% bleach (Figs. 3A, 3B in Ref. 1). Thus, ABCR deficiency under the conditions investigated by Weng et al. corresponds with a relatively small, albeit significant, prolongation of all-*trans* retinal clearance. Interestingly, the rod outer segments of *abcr*$^{-/-}$ mice contain an abnormally high level of phosphatidylethanolamine (PE), the lipid that combines with all-*trans* retinal to form *N*-ret-PE. A high PE level in (presumably, the disc membranes of) *abcr*$^{-/-}$ rods conceivably could underlie the present observation of relatively fast recovery kinetics in *abcr*$^{-/-}$ mice. For example, the abnormally large amount of PE in *abcr*$^{-/-}$ disc membranes (the luminal surface, the cytosolic surface, or both; see also Ref. 47) might promote, by mass action, the sequestering of all-*trans* retinal as *N*-ret-PE at a rate considerably exceeding that in wild-type rods, thereby accelerating response recovery relative to the wild-type. Alternatively, the high level of PE in the *abcr*$^{-/-}$ rod, by binding 11-*cis* retinal arriving from the RPE through operation of the visual cycle, might localize 11-*cis* retinal to the vicinity of opsin and thereby promote rhodopsin regeneration.[48] A further possibility derives from the finding that ABCR itself can bind 11-*cis* retinal.[48] That is, the ABCR of wild-type rods, a major protein of the disc rims, might delay recovery relative to *abcr*$^{-/-}$ by *competing* with opsin for 11-*cis* retinal and thereby slowing rhodopsin regeneration.

In summary, the present results suggest that ABCR in normally functioning rods directly or indirectly prolongs, rather than accelerates, the post-bleach recovery of the rod photoresponse over much of its excursion after substantial bleaching. This notion might seem puzzling in view of the likely disadvantage, in photoreceptor evolution, of processes that slow dark adaptation. The seeming paradox is resolved, however, if the primary physiological role of the ABCR-mediated reaction is to promote clearance, from the disc, of *minute, residual amounts* of all-*trans* retinal that other mechanisms, such as thermal diffusion across the disc membrane, cannot achieve. That is, the ABCR-mediated reaction may have little if any accelerating effect on removal of the major portion of bleach-generated all-*trans* retinal and, thus, on the bulk removal of MII-like complexes of all-*trans* retinal and opsin. Rather, ABCR's essential function may be to eliminate trace amounts of all-*trans* retinal from the disc and thereby oppose the buildup, over the lifetime of the rod disc,[49] of retinoid-based compounds that otherwise would be transferred to the RPE and there accumulate as A2E and other retinoid-based components of lipofuscin.[18] On this hypothesis, the slower rod recovery observed in normal rods upon the bleach-induced elevation of all-*trans* retinal in the disc lumen represents a cost, or tradeoff, associated with the presence of a system (ABCR) that can clear tiny remaining amounts of all-*trans* retinal. Beyond its consistency with the observed modest difference in post-bleach all-*trans* retinal levels in ABCR-deficient versus wild-type rods,[1] this hypothesis is consistent with the finding that abnormal A2E build-up in *abcr*$^{-/-}$ mice amounts, on average, to only several tens of picomoles per eye (~21 picomoles per eye over 4 to 5 months[1]; ~30 picomoles per eye over 8 to 9 months[43]), a molar quantity small by comparison with, for example, the amount of 11-*cis* retinal present as rhodopsin chromophore in fully dark-adapted rods.[40,50] The hypothesis is also consistent with the near-normal course of rod recovery frequently observed in human subjects with Stargardt disease and an ABCA4 (i.e., ABCR) mutation,[25,26] and with the prolongation, in these subjects, of primarily the final, tail phase of psychophysically measured dark adaptation after major bleaching of rhodopsin (Fig. 9 in Ref. 25).

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