n-3 Fatty Acid Deficiency Alters Recovery of the Rod Photoresponse in Rhesus Monkeys

Brett G. Jeffrey,1,2 Drake C. Mitchell,3 Robert A. Gibson,4 and Martha Neuringer5,6

PURPOSE. Docosahexaenoic acid (DHA) is the major polyunsaturated fatty acid within rod outer segments. Varying the dietary content of DHA or its precursor, α-linolenic acid (ALA), can alter retinal DHA levels. The purpose of the present study was to assess rod phototransduction and recovery in rhesus monkeys raised on diets with different DHA and ALA content.

METHODS. Adult rhesus monkeys had consumed from birth a diet low in ALA (0.3%) and known to induce an 80% reduction in retinal DHA. They were compared with groups receiving 8% ALA or 0.6% DHA, both of which support normal retinal DHA levels. Rod recovery was assessed with a double-flash protocol using a-wave saturating test flashes. The recovery of rod-isolated ERG a-wave amplitude was analyzed to determine $T_c$, the time to initiation of rod recovery, and $T_{50}$, the time from initiation to 50% of full recovery. Phototransduction was assessed from the fit of a quantitative model to the leading edges of rod-isolated ERG a-waves. ERG a- and b-wave amplitudes and implicit times were also measured.

RESULTS. Rod recovery ($T_{50}$) was delayed by 30% and ERG implicit times by 5% in monkeys in the low ALA group compared with the other groups. There was no significant effect of diet on ERG amplitudes, the time to initiation of rod recovery, or the parameters describing phototransduction.

CONCLUSIONS. The results indicate that mechanisms involved in deactivation and rod recovery are selectively altered in monkeys raised on a low-ALA diet whereas, at the flash intensities used, the mechanisms underlying phototransduction remain unaffected. (Invest Ophthalmol Vis Sci. 2002;43:2806–2814)

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Docosahexaenoic acid (DHA) or 22:6n-3, a 22-carbon n-3 fatty acid with six double bonds, is the major polyunsaturated fatty acid within the mammalian retina. DHA is particularly concentrated within the disc membranes of rod outer segments, where it accounts for up to 30% of total phospholipid fatty acid acyl chains and up to 60% of the acyl chains of phosphatidylinethanolamine, one of the major membrane phospholipids. Several lines of evidence suggest that DHA serves a number of important roles within the retina. The retina possesses a uniquely efficient conservation and recycling mechanism that helps preserve retinal DHA concentrations, even during periods of low n-3 fatty acid supply. Highly polyunsaturated phospholipid acyl chains such as DHA are important in providing a number of biophysical membrane properties that produce the optimal environment for the metarhodopsin I to metarhodopsin II transition and for the efficient and rapid coupling of metarhodopsin II with transducin. DHA also plays an important role in the binding and release of the retinoids, 11-cis-retinal and all-trans-retinol to the interphotoreceptor retinoid-binding protein, which transports these retinoids between the outer segments and the retinal pigment epithelium.

DHA may be obtained directly from the diet or can be synthesized from shorter-chain n-3 fatty acids such as α-linolenic acid (ALA). The synthesis of DHA depends on both the concentration of the shorter-chain n-3 fatty acid precursors and on the relative concentration of their n-6 homologues, which compete for the same desaturation and elongation enzymes and for incorporation into tissue membranes. Feeding animals an n-3-deficient diet during development induces a significant decrease in retinal DHA concentration that is compensated for by an increase in 22:5n-6, the n-6 homologue of DHA, and to a lesser extent by increases in 22:4n-6 and 20:4n-6. DHA appears to have a functional role in the retina that cannot be filled by 22:5n-6, as indicated by alteration of the electroretinogram (ERG) in animals reared on n-3-deficient diets. Reduced ERG amplitudes have been reported in monkeys, rats, and guinea pigs raised on n-3-deficient diets low in ALA compared with control animals fed high ALA, n-3 sufficient diets. In addition, rhesus monkeys raised on n-3-deficient diets show delayed ERG b-wave implicit times and delayed recovery of ERG b-wave amplitude after a b-wave saturating flash. ERG alterations also have been reported in both preterm and term human infants fed formula milk containing low levels of ALA compared with infants fed a DHA-supplemented formula.

The mechanisms by which reductions in retinal DHA concentrations affect the ERG are not yet fully understood. The macrostructure of the retina appears unaffected in n-3-deficient rats and monkeys, although a reduction in the number of phagosomes within the retinal pigment epithelium has been reported in rats. The rate of rhodopsin regeneration after 1 hour of bleaching is significantly slowed in n-3-deficient rats but slow rhodopsin regeneration is unlikely to have affected the ERG results in the studies cited, in that all with one exception, allowed sufficient time to achieve full dark adaptation. Given the lack of gross morphologic changes in the retina in n-3-deficient animals, it is necessary to consider
changes in intracellular or synaptic mechanisms as possible causes for the ERG alterations. The ERG methods used in the majority of dietary n-3 studies to date are ill suited for identifying particular retinal mechanisms underlying the reported changes in ERG a- and b-waves, due to the nonspecificity of the cellular origin of these ERG components when generated by low-to-moderate flash intensities.22 Thus, the changes reported in the ERG b-wave could reflect alteration in any one of the numerous processes from photon capture up to generation of the ERG b-wave by the bipolar cells.23–25

The uniquely high concentration of DHA within the rod outer segments suggests the need for a specific measure of the rod photoreponse as a function of retinal DHA content. A quantitative model has been reported that describes the G-protein phototransduction cascade in single photoreceptors.26 The same quantitative model, or slight variants of it, have been used to fit the leading edge of the ERG a-wave recorded to a high-intensity flash,27,28 thereby providing an in vivo method of quantifying the phototransduction process. Weisinger et al.14 applied this method to the study of n-3-deficient guinea pigs and found up to a 0.7-log-unit reduction in phototransduction sensitivity and a 0.24-log-unit reduction in the maximal rod response, when compared with guinea pigs fed a high-ALA diet. The first purpose of the present study was to assess whether rod phototransduction was similarly altered in rhesus monkeys raised on a diet with low n-3 fatty acid content.

One of the earliest ERG findings reported for n-3-deficient monkeys was a delay in the recovery of b-wave amplitude after a b-wave saturating flash,13 a result confirmed in a subsequent cohort of monkeys.24 The second purpose of the present study was to determine whether this delay could be attributed to a delay in recovery of the rod photoreponse itself. The time course of recovery of the rod photoreponse may be assessed by using a double-flash protocol with high-intensity flashes.29,30 The first high-intensity flash sends the rods into saturation. The delay before recovery begins indicates the period of photocrystall腘.31 Once recovery starts, the amplitude of the rod-isolated ERG a-wave to the second saturating flash is proportional to the amount of recovery since the initial saturating flash.

There have been no studies in humans or nonhuman pri-mates that have investigated the long-term effect of altering dietary n-3 fatty acid content on retinal function into adulthood. Human studies have only examined infants up to 4 months of age, and previous studies in the rhesus monkey included subjects up to 2 years of age, when the animals are still juveniles. The final purpose of the study was to assess retinal function in adult rhesus monkeys fed lifelong diets differing in n-3 fatty acid content.

**Materials and Methods**

**Animals and Diets**

All experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the Oregon National Primate Research Center and were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Twenty-one rhesus monkeys (Macaca mulatta) were fed one of three semipurified diets (n = 7 per group) that were identical except for the source of dietary fat. The composition of the diets has been described elsewhere.13 The fatty acid compositions of the three diets are listed in Table 1. The SOY diet (high ALA) contained 8% ALA (weight percentage of total fatty acids), with soybean oil as the sole dietary fat. The SAF diet (low ALA) contained less than 0.3% ALA; a 1:1 mixture of safflower and peanut oils was used to match the 54% linoleic acid (18:2n-6) content of the SOY diet. The dietary fat of the DHA diet consisted of a mixture of plant, animal, and fish oils that provided 0.6% DHA, 0.2% eicosapentaenoic acid (EPA, 20:5n-3), 0.2% arachidonic acid (AA, 20:4n-6) and 1.4% ALA. This diet was designed to match the fatty acid composition of rhesus milk.

The monkeys were fed a formula version of their assigned diet from birth until 18 months of age and thereafter were maintained on a solid version of the diet. Their mothers had been fed similar diets from 2 months before conception and throughout gestation (average length, 165 days) to initiate nutritional manipulation early in development. At the time of the ERG studies reported herein, the ages of the groups (mean ± SD) were 4.5 ± 0.9, 4.3 ± 0.5, and 4.6 ± 0.4 years in the SAF, SOY, and DHA groups, respectively.

**Fatty Acid Analysis**

The red blood cell fatty acid analysis obtained most recently before ERG testing was used to compare fatty acid profiles between the dietary groups. The most recent analyses for the SAF, SOY, and DHA groups occurred at 3.7 ± 1.3, 3.8 ± 0.6, and 4.1 ± 0.4 years of age, respectively (mean ± SD). The method of fatty acid analysis has been described in detail elsewhere.37

**Animal Preparation**

Monkeys were anesthetized with an intramuscular injection of ketamine (8–10 mg/kg and then 4–5 mg/kg at 40 to 60 minute intervals, as required), xylazine (0.8–1.0 mg/kg and then 0.4–0.5 mg/kg at the same intervals), and atropine sulfate (0.04 mg/kg for each dose). Lidocaine (1.5 mg/kg) was administered intravenously after anesthetic induction to prevent the cardiac arrhythmias sometimes induced by xylazine. Rectal temperature was maintained between 36.5°C and 39.5°C with water-circulating heating pads placed beneath and on top of the monkey. Respiratory function was monitored with pulse oximetry, and heart rate was monitored by electrocardiogram. Hydration was maintained during the experiment with lactated Ringer’s solution delivered intravenously (10 mL/kg per hour).

Pupils were dilated to approximately 8 mm with phenylephrine (10%) and tropicamide (1%), and the monkey was dark adapted for 30 minutes before ERG recording. The ERG response was recorded from the right eye, with an adult monkey bipolar Burian-Allen contact lens electrode (Hansen Ophthalmic Development Laboratory, Iowa City, IA). Due to large photovoltaic artifacts produced by the high-intensity test flashes, the corneal active electrode was referenced to a subdermal needle electrode at the right outer canthus (monopolar configuration). A subdermal needle electrode in the midline of the back served as ground. Although the speculum was not used as the reference, large photovoltaic artifacts were still present unless the speculum was

**Table 1. Fatty Acid Composition of Diets**

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>SAF*</th>
<th>SOY‡</th>
<th>DHA‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total saturated</td>
<td>13.7</td>
<td>14.9</td>
<td>35.6</td>
</tr>
<tr>
<td>Total monounsaturated</td>
<td>31.1</td>
<td>23.1</td>
<td>38.0</td>
</tr>
<tr>
<td>Polysaturated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2 n-6</td>
<td>54.0</td>
<td>54.0</td>
<td>20.2</td>
</tr>
<tr>
<td>20:4 n-6 (AA)</td>
<td>—</td>
<td>—</td>
<td>0.2</td>
</tr>
<tr>
<td>Total n-6</td>
<td>54.0</td>
<td>54.0</td>
<td>20.8</td>
</tr>
<tr>
<td>18:3 n-3 (ALA)</td>
<td>0.3</td>
<td>8.0</td>
<td>1.4</td>
</tr>
<tr>
<td>20:5 n-3 (EPA)</td>
<td>—</td>
<td>—</td>
<td>0.2</td>
</tr>
<tr>
<td>22:6 n-3 (DHA)</td>
<td>—</td>
<td>—</td>
<td>0.6</td>
</tr>
<tr>
<td>Total n-3§</td>
<td>0.3</td>
<td>8.0</td>
<td>2.5</td>
</tr>
<tr>
<td>Ratio n-6/n-3</td>
<td>180</td>
<td>6.8</td>
<td>9.0</td>
</tr>
</tbody>
</table>

Data are expressed as weight percentage of total fatty acids.
* Fat source was 50% high-linoleic safflower oil and 50% peanut oil.
† Fat source was soybean oil.
‡ Fat source was a blend of lard, coconut, palm, corn, soybean and tuna oils.
§ Total n-6 and n-3 fatty acids include several fatty acids present at 0.2% or less.

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22:6 n-3 (DHA) | — | — | 0.6 |
| Total n-3§ | 0.3 | 8.0 | 2.5 |
| Ratio n-6/n-3 | 180 | 6.8 | 9.0 |
Light Stimulus

Flash stimulation was provided by two high-intensity photoflash units (model 283; Vivitar, Newbury Park, CA) mounted at the north pole of a 40-cm Ganzfeld (LKC Technologies, Gaithersburg, MD). All flash intensities were measured using a photometer with attached photometric filter (350 Linear/Log spectrometer Graseby Optronics, Orlando, FL) set to integration mode for measurement of brief flashes. Retinal illumination in photopic trolands was calculated from flash energy (in candela-seconds per square meter) and measured pupil size.38 The scotopic efficiency of achromatic flashes was estimated to be 0.4 log units higher than the measured photopic troland value, based on the color temperature (7000°K) of the photoflash flashes.58 A filter (λmax = 449 nm, half-bandwidth = 47 nm; 47B Wratten filter; Eastman Kodak, Rochester, NY) was used to produce short-wavelength flashes for rod isolation. In humans, the scotopic troland value of the blue filter is estimated to be 1.35 log units higher than its photopic troland value.52 This value was used for the present experiment, because both the scotopic and photopic spectral sensitivity curves in the short-wavelength region are similar between the rhesus monkey and the human.59,60 For the average age at ERG testing (4.5 years), mean eye length of the monkey was estimated to be 19.2 mm,41 slightly smaller than the mean adult human eye length of 23 mm. The number of photoisomerizations/rod/second per troland of light is assumed to be proportionally higher in the monkey than in the adult human by a factor equal to the inverse square of the difference in average eye lengths.53 Therefore, 1 scot td was estimated to produce 12.7 photoisomerizations/rod/sec in the monkeys in the present experiment, a value 0.169 log units higher than that estimated in the adult human.53

Rod-Isolated ERG a-Wave Recovery Functions

A double-flash technique was used to investigate the recovery of rod-isolated ERG a-wave amplitudes after a saturating flash.55,56,57 The first, or test, flash was achromatic and of sufficient intensity to induce a maximal ERG a-wave response. Four test flash intensities were used to produce retinal illuminances of 4.4, 4.8, 5.2, and 5.6 log scot td-sec based on an 8-mm pupil. The second or probe flash was a short-wavelength flash (4.0 log scot td-sec for an 8-mm pupil; 47B Wratten filter; Eastman Kodak). The interstimulus interval (ISI) between the test and probe flashes varied between 1 and 180 seconds. Four rod recovery functions were measured, one for each test flash intensity. Each recovery function consisted of a sequence of 9 or 10 double-flash pairs presented in order of ascending ISI. Figure 1A shows examples of rod-isolated ERG a-waves (solid lines) recorded at different ISIs after a 5.2 log scot td-sec test flash. At the end of each recovery function, an ERG was recorded to a single probe flash presented without a preceding test flash (probe only).

Rod Isolation

For each recovery function, rod-isolated ERGs were obtained by subtracting a cone-isolated ERG from each probe flash response. The cone-isolated ERG was obtained by presenting a probe flash at a short ISI after a test flash, before the onset of rod recovery.53 Measurements from monkeys in a preliminary study indicated that cone-isolated ERGs could be obtained by presenting the probe flash 1 second after the 4.4 and 4.8 log scot td-sec test flashes and 3 seconds after the 5.2 and 5.6 log scot td-sec test flashes.

Rod-Isolated ERG a-Wave Recovery Analysis

To assess recovery of the rod response, the following P3 model55 was fitted to the leading edges of the rod-isolated ERG a-waves in each of the four recovery sequences.
where $P_3$ is the leading edge of the a-wave at time ($t_f$) seconds, in response to a flash with a retinal illumination of $i$ scotopic troland-second. The parameters derived to fit the model were: $S$, the sensitivity parameter that scales retinal illuminance (in scotopic troland-second); $t_d$, the delay due to the filter and finite duration of the flash (in seconds); and $R \max_{aP3}$, the maximum response (in microvolts).

Allowing all three parameters to vary, the $P_3$ model was fitted to the leading edge of the rod-isolated ERG a-wave recorded to a probe flash presented without a preceding test flash (probe only, Fig. 1A). The parameters $S$ and $t_d$ were then fixed, and only parameter $R \max_{aP3}$ was allowed to vary to fit the $P_3$ model to the rod-isolated ERG a-wave at a given ISI (Fig. 1A, dashed lines). The derived $R \max_{aP3}$ at each ISI ($R \max_{aP3}$) was normalized in relation to $R \max_{aP3}$ derived from the isolated probe presented without a preceding test flash ($R \max_{aP3}$) and plotted against ISI (Fig. 1B). The normalized recovery of ERG a-wave amplitude was analyzed in terms of a double-exponential recovery function

$$R \max_{aP3} \cdot R \max_{aP3} = d \cdot \{1 - \exp(-(t - T_c)/\tau_1)\}
+ (1 - d) \cdot \{1 - \exp(-(t - T_c)/\tau_2)\}$$  \hspace{1cm} \text{maxP3 $\leq$ $t_d$ $\leq$ 1} (2)

where $R \max_{aP3}$ is the derived maximum a-wave amplitude at ISI time ($t$); $R \max_{aP3}$ is the maximum a-wave amplitude from the isolated probe; $T_c$ is the critical delay before the initiation of recovery; and $\tau_1$ and $\tau_2$ are the time constants of the two exponentials. The parameter $d$ is a weighting factor that scales the relative contribution of the two exponentials to the recovery function. In Figure 1B, the solid curve shows the fit of equation 2 to a representative set of normalized recovery data. A further parameter, $T_{max}$ defined as the time taken from $T_c$ to reach 50% of full recovery, was derived from the fit of equation 2 (Fig. 1B, inset).

A double-exponential model was used to fit the recovery data, because a single-exponential model overestimated the extent of rod recovery at longer ISIs, particularly for the two highest test flash intensities (dashed curve in Fig. 1B). The double-exponential model is based on recent data demonstrating that the recovery of the rod photocurrent after moderately intense flashes has a time course dominated by two different time constants. When both time constants $\tau_1$ and $\tau_2$ were allowed to vary for the data presented herein, the second exponential accounted for less than 5% of the overall fit (i.e., the weighting factor $1 - d$ was < 0.05) at the two lowest test flash intensities, but greater than 20% of the fit (i.e., 1 - $d$ > 0.2) at the two highest test flash intensities. The average of $\tau_2$ across the two highest test flash intensities was 125 seconds, comparable in magnitude to the longest ISIs used (90–180 seconds). Consequently, the magnitude of $\tau_2$ could not be derived accurately and was therefore fixed at 125 seconds for all rod recovery functions.

**Phototransduction Analysis**

Before the start of the double-flash experiment, ERGs with mixed rod and cone contributions were obtained to three short-wavelength flashes ($R_{max}$ = 449 nm; 47B Wratten filter; Eastman Kodak) that produced retinal illuminances of 2.5, 3.1, and 4.0 log scot td-sec (8-mm pupil). Cone-isolated ERGs were obtained by presenting each short-wavelength flash 3 seconds after a 5.2 log scot td-sec test flash. Rod-isolated ERGs were then obtained by subtracting the cone-isolated ERGs from the original mixed rod/cone ERGs (Fig. 2).

The following $P_3$ model was used to fit the leading edges of the rod-isolated ERG a-waves

$$P_3(t, i) = \{1 - \exp(-i \cdot S \cdot (t - t_d^i))\} \cdot R \max_{aP3} \cdot t_i > t$$  \hspace{1cm} \text{maxP3 $\leq$ $t_d$ $\leq$ 1}  \hspace{1cm} \text{(3)}

where $\otimes$ represents the convolution integral.

This is the same $P_3$ model outlined in equation 1 with the addition of the capacitance term ($r$) to account for the time constant in seconds, due to membrane capacitance. Equation 3 has the advantage over equation 1 of providing a good description of both rod- and cone-isolated a-waves. The cone ERG data from the current experiment are reported elsewhere.

The parameters of the $P_3$ model were determined by fitting equation 3 simultaneously to the leading edges of the three rod-isolated ERG a-waves (ensemble analysis). All analysis was performed with the program NONLIN, which uses a modified Gauss-Newton nonlinear least-squares algorithm, with subroutines specifying the $P_3$ model written by the authors. The numerical solution of equation 5 for discrete data was used during the fitting process. The fits of equation 3 to the rod-isolated ERG a-waves recorded from a typical DHA monkey are shown by the solid curves in Figure 2.

**Other ERG Analyses**

Conventional a- and b-wave amplitudes and implicit times also were determined for the three rod-isolated ERGs used for phototransduction analysis. ERG a-wave amplitude was measured from baseline to the trough of the a-wave, and b-wave amplitude was measured from the trough of the b-wave to the peak of the b-wave. Implicit times were measured from flash onset to the trough of the a-wave and to the peak of the b-wave.

**Statistical Methods**

The first author was masked to both the diets and groupings of monkeys during the recording and analysis of ERG results. Data were checked for normality and homogeneity of variance, using either the Shapiro-Wilk test (for < 50 observations, i.e., number of monkeys x number of parameter measurements used in comparison) or the Kolmogorov-Smirnov test with a Lillifores correction (for number of observations > 50). Rod phototransduction parameters were analyzed using a one-way ANOVA for the main effect of diet. All other data were analyzed using a general linear model (GLM) for main effects of diet (SAF, SOY, and DHA) and flash intensity (2.7–4.0 log scot td-sec for phototransduction parameters or 4.4–5.6 log scot td-sec for rod recovery parameters). The effect of flash intensity in the GLM analyses was significant for all variables and therefore is not listed for each variable in the results section. For analyses that indicated a significant effect of diet, post hoc analyses were performed with the Bonferroni correction.
for multiple comparisons. For all comparisons the level of statistical significance was set at $P < 0.05$ before Bonferroni correction.

**RESULTS**

**Red Blood Cell Fatty Acids**

There was a significant effect of diet on red blood cell DHA ($P < 0.001$). As expected, DHA was greatly elevated in the DHA group ($8.8\% \pm 0.7\%$ of total fatty acids, mean $\pm$ SD), almost absent in the SAF group ($0.3\% \pm 0.1\%$), and intermediate in the SOY group ($1.8\% \pm 0.6\%$). There was no significant effect of diet on AA ($P < 0.08$). SAF monkeys had the highest AA level ($18.1\% \pm 1.5\%$), which was only slightly elevated over that in the SOY ($16.9\% \pm 1.1\%$) and DHA groups ($16.4\% \pm 1.5\%$). The red blood cell compositions in the three groups had been stable since 12 months of age (Conner WE, Neuringer M, unpublished data, 1996).

**Rod Recovery Functions**

There was no significant effect of diet ($P < 0.86$) on $T_{50}$, the time to the initiation of rod ERG a-wave recovery (Fig. 3A). However, there was a highly significant effect of diet ($P < 0.001$) on $T_{50}$, the time to reach 50% of rod ERG a-wave recovery (Fig. 3B). Post hoc analysis indicated that $T_{50}$ in SAF monkeys was significantly delayed in comparison with that in the SOY ($P < 0.001$) and DHA groups ($P < 0.004$). $T_{50}$ in SAF monkeys was delayed across the three lowest test flash intensities on average by 31% (range, 21%–36%) in comparison with that in the SOY monkeys and by 28% (19%–39%) in comparison with that in the DHA monkeys. The difference in $T_{50}$ was less consistent at the highest test flash intensity, at which it was delayed by 21% and 1% in SAF monkeys in comparison with the SOY and DHA monkeys, respectively.

There was also a significant effect of diet on $\tau_r$, the time constant of the first exponential that contributed to the rod ERG a-wave recovery function ($P < 0.001$). This effect was essentially identical with the effect on $T_{50}$ and was expected given that the first exponential function is the dominant contribution to the fit of each recovery function. With $\tau_r$ set to 125 seconds, the mean weighting factor $1 - d$ for the second exponential averaged across monkeys was 0.02, 0.06, 0.23, and 0.30 for the 4.4, 4.8, 5.2, and 5.6 log scot td-sec test flashes, respectively. There was no significant effect of diet ($P < 0.94$) for the relative contribution ($1 - d$) of the second exponential to the overall recovery function.

The mean rod ERG a-wave recovery functions in the SAF and SOY monkeys are shown at the two lowest test flash intensities in Figure 4A and at the two highest test flash intensities in Figure 4B. The similarity in the time to initiation of rod recovery between the SAF and SOY monkeys is evident across the three lowest test flash intensities. However, it is clear that once recovery started, SAF monkeys were markedly delayed in comparison with the SOY monkeys. The recovery functions of the SOY and DHA monkeys were very similar, as shown in the insets, and therefore the DHA group has been omitted from the main graph for clarity.

![Figure 3](https://example.com/fig3.png)

**Figure 3.** The means of $T_{50}$ (A) and $T_{50}$ (B) in each diet group and at each test flash intensity (4.4, 4.8, 5.2, and 5.6 log scot td-sec). The level of significance for the effect of diet in the general linear model (GLM) analysis is shown at the top of each graph. Asterisks in the post hoc comparison matrix indicate significant differences between diet groups after Bonferroni correction for multiple comparisons.

![Figure 4](https://example.com/fig4.png)

**Figure 4.** Mean normalized rod ERG a-wave recovery of the SOY and SAF diet groups at the (A) 4.4 and 4.8 log scot td-sec and (B) 5.2 and 5.6 log scot td-sec test flash intensities. Error bars, SEM. Mean fits for each recovery function, calculated from the individual fits for each monkey, are also shown for SOY (solid lines) and SAF (dashed lines) diet groups. Insets: Mean recovery functions for the SOY (solid lines) and DHA (dashed lines) diet groups.
b-waves (a-wave implicit times were significantly delayed in SAF monkeys in comparison with those in SOY and DHA monkeys, respectively). Similarly, post hoc analysis indicated that b-wave implicit times were delayed in SAF monkeys (P < 0.001 for both). When averaged across the three intensities, in SAF monkeys rod-isolated ERG a-waves were delayed on average by 5.5% and 4.8% and rod ERG b-waves by 7.5% and 8.6% in comparison with those in SOY and DHA monkeys, respectively.

### DISCUSSION

#### Retinal Function in Rhesus Monkey n-3 Nutritional Studies

The present results reported in adult monkeys confirm and extend the previous results in infant and juvenile rhesus monkeys fed the same or similar diets. In a previous study, recovery of scotopic ERG b-wave amplitude after a b-wave saturating flash was significantly delayed in n-3-deficient monkeys at 3 months to 2 years of age, compared with monkeys fed a high-ALA diet.13,18 When the monkeys from the present study were tested at 3 and 12 months of age, SAF monkeys again had delayed ERG b-wave amplitude recovery.54 By using high-intensity saturating flashes and fitting a P3 model to the recovery of the ERG a-wave, the present study has established that a delay also occurs in recovery of the rod photoreceptor after an a-wave saturating flash, suggesting that the previous findings are attributable to altered function of the photoreceptor.

In the earlier studies, scotopic and photopic ERG b-wave implicit times also were delayed at 2 years of age in the n-3-deficient group compared with monkeys fed a high-ALA diet.19 The same effect was found in the n-3-deficient monkeys in the present study. However, ERG b-wave amplitude and implicit time saturate at approximately 2.0 log scot td-sec (0.5 log below our lowest intensity), so the differences in b-wave implicit time we observed in the n-3-deficient monkeys may reflect differences in a-wave implicit time and may also be due to alterations at the level of the photoreceptor.

None of the ERG parameters measured differed significantly between the SOY monkeys and the DHA monkeys. The similarity of these two groups suggests that in adult rhesus monkeys, a diet containing a high level of DHA (0.6%) does not provide any additional benefit for retinal function over a diet containing a high level of ALA (8%) as the only n-3 fatty acid, despite the substantially higher red blood cell DHA levels induced by DHA supplementation. Because the monkeys used in this study were involved in ongoing studies, we do not have direct information on their retinal DHA levels. However, the diet and blood levels of n-3 fatty acids in both the SAF and SOY groups are essentially identical with those in previous studies, in which monkeys fed the SAF diet showed a 80% reduction in retinal DHA in the major membrane phospholipids compared with monkeys fed the SOY diet at 2 years of age (e.g., 7.3% ± 1.6% vs. 36.0% ± 2.4% of total fatty acids in phosphatidylethanolamine, mean ± SEM, respectively).17 Retinal fatty acid composition has also been measured in rhesus monkeys who were initially breast-fed (thereby receiving ≈0.6% DHA from rhesus milk) and then consumed a stock diet containing approximately 0.3% DHA. These animals, with DHA intake comparable to our DHA group, had retinal DHA levels equivalent to those in monkeys fed the SOY diet (35.1% ± 2.4%).50 The expected similarity in retinal DHA levels between the SOY and DHA groups, compared with the substantial reduction in the SAF group, is paralleled by the present results for retinal function. Thus, changes in retinal function are correlated with retinal DHA levels and not with circulating levels as reflected in red blood cells.

#### Retinal Function in n-3–Deficient Rodents

The effects of a low ALA, n-3–deficient diet on rod phototransduction in rhesus monkeys appear to be different from those observed in rats and guinea pigs. In rats and guinea pigs fed an n-3–deficient diet, S was reduced by 0.3 to 0.7 log units and maxP3 by 0.2 to 0.3 log units, compared with control animals fed a high-ALA diet,14,51 whereas neither S nor maxP3 was altered in the SAF monkeys. Differences between species are also evident with conventional ERG analysis. In rats and guinea pigs fed n-3–deficient diets, large reductions in ERG a- and b-wave amplitudes were reported, but ERG implicit times were not affected.14,16,17,19,52,53 In contrast, in the present study

### Table 2. Rod Phototransduction Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SAF</th>
<th>SOY</th>
<th>DHA</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>S (scot td-sec)−1 sec−2</td>
<td>12.8 ± 2.2</td>
<td>12.9 ± 2.6</td>
<td>13.7 ± 2.3</td>
<td>&lt;0.74</td>
</tr>
<tr>
<td>R maxP3 (µV)</td>
<td>295 ± 67</td>
<td>273 ± 51</td>
<td>313 ± 61</td>
<td>&lt;0.49</td>
</tr>
<tr>
<td>t0 (ms)</td>
<td>2.95 ± 0.18</td>
<td>2.82 ± 0.15</td>
<td>2.87 ± 0.13</td>
<td>&lt;0.34</td>
</tr>
<tr>
<td>τ (ms)</td>
<td>0.86 ± 0.31</td>
<td>0.84 ± 0.36</td>
<td>0.96 ± 0.15</td>
<td>&lt;0.72</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± S.D.  
* By ANOVA.

### Table 3. Rod ERG a-Wave and b-Wave Amplitudes

<table>
<thead>
<tr>
<th>Diet Group</th>
<th>Rod ERG a-Wave Amplitudes</th>
<th>Rod ERG b-Wave Amplitudes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.7*</td>
<td>3.3*</td>
</tr>
<tr>
<td>SAF</td>
<td>198 ± 57</td>
<td>240 ± 70</td>
</tr>
<tr>
<td>SOY</td>
<td>173 ± 46</td>
<td>208 ± 41</td>
</tr>
<tr>
<td>DHA</td>
<td>210 ± 49</td>
<td>249 ± 61</td>
</tr>
</tbody>
</table>

Data are expressed as mean microvolts ± S.D.  
* Retinal illuminance in log scot td-sec.
SAF monkeys had no alteration in ERG amplitude but had a small significant delay in ERG implicit times. A previous study in monkeys found reduced scotopic and photopic ERG a- and b-wave amplitudes at 3 to 4 months of age but not at later ages. It is not clear why this early and transient effect was not replicated in the present study.

**Visual Function in Human n-3 Nutritional Studies**

ERG alterations also have been reported in preterm human infants fed formula milk containing a low level of ALA (0.5%) compared with infants fed either human milk, which contains DHA, or a formula supplemented with 0.35% DHA from fish oil. These ERG alterations included reduced maximal amplitudes, decreased retinal sensitivity, and elevated rod threshold of the conventional ERG b-wave at 36 weeks postconceptional age. Term infants fed diets with 1.7% ALA had lower sensitivity compared with those receiving 0.35% DHA at 6 weeks of age. In both preterm and term infants, these changes were no longer present at 4 months of age (using corrected, or postterm, age for preterm infants). This finding is consistent with the transient effect on ERG amplitudes found in the earlier monkey study. However, rod-isolated responses and rod recovery have not been examined in human infants as a function of n-3 fatty acid intake. In humans, DHA is rapidly incorporated into retinal membranes during the last trimester of pregnancy and early postnatal months, during which time the outer segments continue to grow. Altered retinal function in human infants fed formula with ALA as the sole n-3 fatty acid suggests that infants cannot synthesize sufficient DHA from ALA during early life to meet the demand for normal retinal DHA accretion. Whether higher dietary levels of ALA would ameliorate the early ERG changes in human infants remains to be determined.

**Rod Recovery Models**

The double-exponential model described in equation 2 provided a better fit of rod recovery than a single-exponential model, particularly at long ISIs and higher intensities (Fig. 1B). In all monkeys, 90% of full rod recovery was achieved within 35 seconds after the two lowest test flash intensities and after 120 seconds at the highest two test flash intensities (Fig. 4). The mean value of \( \tau_2 \) was 125 seconds, which explains why the second exponential improved the fit of the recovery function to the data at longer ISIs, but only at the two highest test flash intensities. Based on our estimate for adult rhesus monkeys of 12.7 photoisomerizations/scot td-sec, it is estimated that the two brightest test flashes produced bleaches of 3.1% and 6.9% of available rhodopsin. Lamb described the time course of the recovery of rod sensitivity after a bleach as having three components, the second of which had a time constant of 100 seconds. Recent evidence suggests that this second component corresponds to the first-order decay of a photoproduct, believed to be the rhodopsin-phosphate-arrestin complex (Rh-P-Arr). Given the similarity in the magnitude of our \( \tau_2 \) to that of Lamb’s component 2, it may be that the second exponential in our equation 2 represents the decay of the Rh-P-Arr complex during rod recovery.

**Proposed Mechanisms of DHA in the Rod Photoreceptors**

The high concentrations of DHA and dipolyunsaturated phospholipids in the disc membranes are thought to impart a number of biophysical properties that result in a more loosely packed lipid bilayer. Rhodopsin is a membrane-spanning, integral membrane protein within the outer segment disc. The activation proteins, transducin and phosphodiesterase (PDE), and the deactivation proteins, rhodopsin kinase and recoverin, are bound to the disc membrane by fatty acids. A more loosely packed lipid bilayer should facilitate the lateral diffusion of these proteins through the disc membrane. This assertion is supported by recent results that establish that lateral diffusion determines the rate of rod phototransduction. The role of DHA in providing optimum membrane properties is supported by the findings that the coupling of metarhodopsin II with transducin is more efficient and rapid in recombinant model membranes containing DHA compared with model membranes containing a medium-chain monounsaturated, oleic acid (18:1). However, in the retina of n-3-deficient monkeys, DHA is largely replaced by its n-6 homologue, DPA (22:5n-6), also a 22-carbon chain fatty acid but with one less double bond. Given the small difference between these two fatty acids, it might be predicted that the packing density of the disc membrane lipid bilayer is only marginally altered. The high-intensity test flashes used in the current experiment bleached relatively large proportions of the available rhodopsin (0.5%-6.9%) and ensured that all transducin (which saturates at 0.02% bleach) and PDE (which saturates at 0.004% bleach) were activated. A reduction in the rate of diffusion of the activation proteins would be observed as a reduction in \( S \), the gain of phototransduction and/or a slowing of the photoreponse. However, SAF monkeys had very similar values of \( S \) compared with both SOY and DHA monkeys. The 5% delay in the peak of the ERG a-wave may reflect a slowing of the phototransduction cascade. However, this cannot be determined from the present data, because the location of the a-wave peak depends on the interaction of several ERG com-
ponents, including the falling phase of the ERG a-wave and the rising phase of the postreceptorial ERG b-wave. Because both activation and deactivation proteins are bound to the disc membrane, it would be predicted that both processes should be equally affected by alteration in the biophysical properties of the disc membrane. Clearly, this is not the case, because the delay in rod recovery is approximately six times larger than the maximum delay in phototransduction estimated from the delay in ERG implicit time. This differential effect suggests that a specific mechanism within the recovery process is altered by n-3 deficiency.

One possible explanation for the 30% delay in rod recovery observed in the SAF monkeys would be a transient increase in cytosolic Ca\(^{2+}\) once the cyclic guanosine monophosphate (cGMP)-gated cation channels begin to reopen after rod saturation. In Figure 4, the rising portions of the exponential recovery functions reflect the time course of the reopening of the cGMP-gated ion channels after rod saturation. The delay in recovery of the SAF monkeys occurred after rod saturation (\(T_d\)), once cGMP channels began to reopen and Ca\(^{2+}\) and Na\(^{+}\) began to flow through the reopened cGMP-gated ion channels into the photoreceptor outer segments. Ca\(^{2+}\) decreases the affinity of cGMP-gated channels for cGMP and slows both the synthesis of cGMP and the phosphorylation and deactivation of rhodopsin,\(^6\) events that would slow the rate of cGMP-gated channel reopening and therefore, rod recovery. Whether alteration of retinal fatty acid status alters the rate of Ca\(^{2+}\) and Na\(^{+}\) current flow through cGMP-gated ion channels has yet to be determined. However, investigations in rat cardiomyocytes and CA1 neurons from the hippocampus have shown that DHA as a free fatty acid suppresses Ca\(^{2+}\) and Na\(^{+}\) currents through voltage-gated \(L\)-type channels.\(^6,\)\(^65\) DHA constitutes a large proportion of the free fatty acid pool in the retina.\(^6\) We speculate that the reduced DHA status of the SAF monkeys leads to a reduction in free DHA in the retina, which may result in a larger transient Ca\(^{2+}\) influx through the cGMP-gated ion channels. Such a transient Ca\(^{2+}\) influx could explain the observed delay in rod recovery in the SAF monkeys and account for the observation that rod recovery is more affected than photocapacitance.

In summary, a diet low in n-3 fatty acids was associated with specific changes in retinal function, including increased implicit times and a substantial delay in the recovery of the rod-isolated photoreponse. These changes appear to be related to differences in retinal DHA levels that may alter biophysical properties and lipid-protein interactions in retinal membranes.

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References