Estimates of L:M cone ratio from ERG flicker photometry and genetics

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Estimates of L:M cone ratio for males with normal color vision were derived using the flicker-photometric electroretinogram (ERG). These were obtained by best fitting ERG spectral sensitivity functions to a weighted sum of long (L)- and middle (M)-wavelength-sensitive cone spectral absorption curves. Using the ERG, measurements can be made with extremely high precision, which leaves variation in the wavelength of maximal sensitivity (λ_{max}) of the cone photopigments as the major remaining source of inaccuracy in determining the ratio of cone contributions. Here that source of inaccuracy was largely eliminated through the use of individualized L-cone spectral absorption curves deduced from L-pigment gene sequences. The method was used on 62 normal males as part of an effort to obtain a true picture of how normal variations in L:M cone ratio are distributed. The percentage of L cones in the average eye was 65%L [where %L = 100 X L / (L+M)]. There were huge individual differences ranging from 28%–93%L, corresponding to more than a 30-fold range in L:M ratio (0.4–13). However, the most extreme values were relatively rare; 80% of the subjects fell within ±15 %L of the mean, corresponding to a 4-fold range in L:M ratio (1–4). The method remedies major weaknesses inherent in earlier applications of flicker photometry to estimate cone ratio; however, it continues to depend on the assumption that the average L cone produces a response with an identical amplitude to that of the average M cone. A comparison of the ERG results with the distribution of cone ratios estimated from cone pigment messenger RNA in cadaver eyes indicates that the assumption generally holds true. However, there may be a small number of exceptions in which individuals have normally occurring (but relatively rare) amino acid substitutions in one of their pigments that significantly affect the physiology of the cone class containing that pigment, so as to reduce the amplitude of its contribution to the ERG. Consistent with this possibility, extreme cone contribution ratios were found to be associated with atypical L-pigment amino acid combinations.

Keywords: color vision, L:M cone ratio, cone photopigments, electroretinogram, ERG, opsin genes

Introduction

An electroretinogram (ERG) adaptation of a standard psychophysical method, flicker-photometry, has been used previously to measure spectral sensitivity (Jacobs, Neitz, & Krogh, 1996). This technique has been used extensively to assess the photopigment complements in a wide variety of animals (Jacobs, 1993; Jacobs, Deegan, Neitz, Murphy, Miller, & Marchinton, 1994; Jacobs, Deegan, & Neitz, 1998; Carroll, Murphy, Neitz, VerHoeve, & Neitz, 2001; Jacobs, Deegan, Tan, & Li, 2002). From ERG spectral sensitivity functions, under the appropriate chromatic adaptation conditions, it has been possible to determine the number of different cone types in an individual (e.g., Jacobs, Neitz, Deegan, & Neitz, 1996), and in monochromatic and dichromatic individuals, the values of the spectral peaks of the photopigments can be determined (e.g., Carroll, McMahon, Neitz, & Neitz, 2000). Two of the greatest advantages of the technique are that it is extremely efficient, making it possible to make a large number of measurements in relatively little time, and the individual measurements are highly precise. Both of these merits make the technique attractive for obtaining spectral sensitivity measurements from trichromatic eyes for use in estimating the ratio of long (L)- to middle (M)-wavelength-sensitive cones. Estimates of cone contribution ratio made by obtaining the best fit of a weighted sum of L- and M-cone absorption curves require extremely precise measurements, because very small errors in spectral sensitivity lead to large errors in the cone ratio estimate. For example, a change in L:M ratio from 2:1 to 3:1 predicts an increase in relative sensitivity to long wavelengths of no more than 0.05 log units (Carroll et al., 2000).

Previously, the flicker ERG and psychophysical flicker photometry have been shown to give similar estimates of

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L:M cone ratio (Chang, Burns, & Kreitz, 1993). One goal of experiments in which ERG flicker-photometric measurements were used to make inferences about L:M cone ratio was to investigate differences in cone ratio between humans (Jacobs & Neitz, 1993) and other primate species (Jacobs, Deegan, & Moran, 1996; Jacobs & Deegan, 1997). However, the full potential of the ERG as a method for examining individual differences in cone ratio has not been exploited. One reason is that even though ERG flicker photometric measurements can be made with extremely high precision, in the case of humans, large individual differences in the λ_{max} of the L-cone photopigment remain as a significant source of inaccuracy in determining cone ratio from spectral sensitivity measurements for individual subjects (Bieber, Kraft, & Werner, 1998). To remedy this problem, we recently developed a method intended to remove this source of error using individualized photopigment spectra in the analysis of each subject's ERG-derived spectral sensitivity data (Carroll et al., 2000).

Previously, direct imaging of the cone mosaic using adaptive optics demonstrated that there can be large differences in L:M cone ratio among males with normal color vision (Roorda & Williams, 1999; Brainard et al., 2000). However, with so few subjects tested, there is little indication of how the variation is distributed in the population. Here we combine the techniques of ERG and genetics to obtain estimates of L:M cone ratio in 62 color normal males. From earlier psychophysical studies (DeVries, 1946; DeVries, 1948; Rushton & Baker, 1964; Cicerone & Nerger, 1989; Vimal, Pokorny, Smith, & Shevell, 1989; Pokorny, Smith, & Wesner, 1991), there has been disagreement about the range of variation. Estimates of the range of L:M ratio in the population vary from about 1.3 – 2.5 (Cicerone, 1990; Otake & Cicerone, 2000) to about 0.5 – 10.0 (Vimal et al., 1989; Kremers, Scholl, Knau, Berendschot, Usui, & Sharpe, 2000). Here we have measured a large sample using a method designed to have low error; thus, it should give a good representation of the true range and distribution of cone ratios in the population. In cadaver eyes, it is possible to efficiently estimate cone ratio through the analysis of messenger RNA levels (Yamaguchi, Motulsky, & Deeb, 1997; Hagstrom, Neitz, & Neitz, 1998), and this also has been used on large samples. Both methods are indirect but they rely on different assumptions. Thus, an indication of the validity of the assumptions can be gained by comparing the distributions of variation obtained using the two methods.

One more issue that has received little attention in previous studies is that variation in cone ratio arises from two different sources: (1) variability in the mechanism that determines which gene, M or L, from the X-chromosome pigment gene array is expressed in a particular cone and (2) variability that arises from the presence of heterozygous female carriers of color vision defects in the sample. We believe that the study of the first type of variation may be a key to ultimately understanding the mechanisms that determine the identity of a cone as M versus L. By restricting the sample to males, we have assured that the cone ratio variation observed in these studies reflects only genetic variation in the mechanism that determines the probability that an L versus M gene from one array will be expressed in a particular cone cell.

Finally, as a future application, with an accurate and efficient method for assessing individual differences in cone ratio, we hope to further examine the consequences for vision associated with individual variations in cone ratio. In the initial color vision assessments used in this study, all subjects performed normally, regardless of their ERG-derived cone ratio. However, it seems likely that other aspects of our vision, which are not accessed in routine color vision tests, might be influenced by variation in cone ratio. For example, a correlation between chromatic contrast sensitivity and cone ratio has been demonstrated (Gunther & Dobkins, 2002). In the future, the ERG method used here will allow us to test subjects with a wide range of cone ratios and examine potential correlations with a variety of visual capacities.

Methods

Subjects

Subjects were males recruited mainly from the Medical College of Wisconsin and local universities. The men either had normal color vision (n=62) or dichromatic color vision (n=38). Color vision was classified based on color matching performance on a Nagel anomaloscope (Strutt, 1881). Subjects classified as dichromats accepted the entire range of red-green mixtures as matching the monochromatic test light, whereas normal observers made a match over a small range of red-green mixtures and had normal match midpoints. Each dichromat was confirmed as being either a protanope or a deuteranope based on genetic analysis (see below). The color vision behavior of both the dichromats and normal males was also characterized on standard color vision tests, including the AO-HRR, the Ishihara (38 plates), the Dvorine, the D-15 (both saturated and desaturated), and the Neitz Test of Color Vision (Neitz & Neitz, 2001). The deuteranopes in this study were selected from a larger population of deuteranopes and were chosen because they had a single L-photopigment gene variant on the X-chromosome. The subjects' ages ranged from 8 to 55 years, with a mean of 27 years for the normals and 25 years for the dichromats. All research on human subjects followed the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board at the Medical College of Wisconsin. Informed consent was obtained after explanation of the nature and possible consequences of the study.

Genetic Analysis

DNA was extracted from whole blood obtained from each subject (Neitz, Neitz, & Grishok, 1995), and used in a previously described real-time quantitative polymerase chain reaction (PCR) assay to estimate the number of L and M genes in the X-chromosome visual pigment gene array (Neitz & Neitz, 2001). The L and M genes were selectively amplified by long-distance PCR, and the product obtained was subsequently used to amplify separately exons 2, 3, and 4 of L and of M genes for direct DNA sequence analysis. The primers and thermal cycling parameters for all amplifications were reported previously (Carroll et al., 2000). The resultant PCR products were directly sequenced with the AmpliTaq FS sequencing kit from ABI, and sequencing analysis was done with the ABI 310 genetic analyzer.

ERG Flicker-Photometry

The procedure and apparatus used to record the flicker photometric ERG are described elsewhere (Neitz & Jacobs, 1984; Jacobs et al., 1996; Carroll et al., 2000). Briefly, the subject's pupil was dilated with tropicamide 0.5%. Two beams (identified here as a reference and a test beam) of a three-channel Maxwellian-view optical system were superimposed to illuminate a circular portion of the retina subtending approximately 70°. High-speed electromagnetic shutters (Uniblitz; Vincent Associates, Rochester, NY) were used to alternately present the reference and test lights at 31.25 Hz, with a neutral density wedge used to control the intensity of the test light. A Varispec liquid-crystal electronically tunable filter (Cambridge Research & Instrumentation, Boston, MA) was used to control the wavelength of the test light. These conditions were designed to eliminate rod and S-cone contributions. The electrode configuration used to record the ERG signal was as follows: a ground electrocardiogram conductive electrode above the right eye, a similar electrode below the right eye to serve as the reference electrode, and a third electrode, made from fiber from the DTL Plus™ electrode, was used as the active corneal electrode. Spectral sensitivity was determined by adjusting the intensity of the test light until the ERG signal it produced exactly matched that produced by the fixed intensity reference light. For all subjects, this null point was determined at 10-nm increments over a range of 480–670 nm. For most normal males and all deuteranopes, readings at 680 nm were also obtained. For the protanopes, an additional reading at 470 nm was acquired. In addition, for all dichromats, four recordings at 5-nm increments were taken near the expected peak (530 nm for protanopes and 560 nm for deuteranopes). The average of two complete runs through each wavelength was used to determine a subject's spectral sensitivity function. Final spectral sensitivity values are reported as quantal intensities, and they were corrected for lens absorption with an age-dependent lens correction (Pokorny, Smith & Lutze, 1987).

Results and Discussion

Spectral Sensitivities of Dichromats

Spectral sensitivity functions from dichromats were used to derive cone spectra that were then used to analyze the normal trichromats (see below). For each dichromat, a flicker-photometric ERG spectral sensitivity function was measured, the number of L and M genes was estimated, and the genes were sequenced. The individual protanope spectral sensitivities were best fit to a photopigment absorption template expression (Carroll et al., 2000), allowing both λ_{max} and optical density (OD) to vary. The values from all protanopes (n=24) were averaged to yield an M-cone spectral sensitivity function with an estimated λ_{max} of 530 nm and OD of 0.22. We did not limit the analysis to protanopes who had a single X-encoded M photopigment gene. The variations in spectral sensitivity that result from amino acid polymorphisms in the M-pigment gene have been shown to have a minimal impact on the L:M ratio estimates in normal trichromats (Bieber et al., 1998; Carroll et al., 2000). We used the average M-cone spectral sensitivity function derived from the 24 protanopes to represent the M-cone spectral sensitivity of the normal males.

All of the deuteranopes (n=14) in this study had a single L-pigment gene variant on the X-chromosome. Template curve fits to the deuteranope data provided a set of L-cone spectral sensitivity functions to be used in the analysis of the normal trichromats. For the deuteranope curve fits, we used a fixed OD of 0.35, which was chosen because it is close to the average OD value obtained when fitting the deuteranope data and allowing *both* OD and λ_{max} to vary. Using a fixed OD value allowed us to characterize the different L-cone pigments using λ_{max} as the only parameter (Carroll, Neitz, & Neitz, 2001). It was shown previously that spectral sensitivity functions obtained with the ERG from single gene dichromats are highly reliable. Repeated measurements obtained from the same subject showed an average absolute deviation in λ_{max} of less than 1 nm (Carroll, Neitz, & Neitz, 2001). Among the L pigments, variations have been observed at 11 amino acid positions, 3 encoded by exon 2, 5 by exon 3, and 3 by exon 4 (Neitz, Neitz, & Kainz, 1996). These are indicated by the single letter code in Table 1 and in Figure 1. There were 6 L-pigment sequence variants found among 14 deuteranopes. Shown in Figure 1 are the λ_{max} values derived from ERG flicker photometric measurements and the amino acid identities

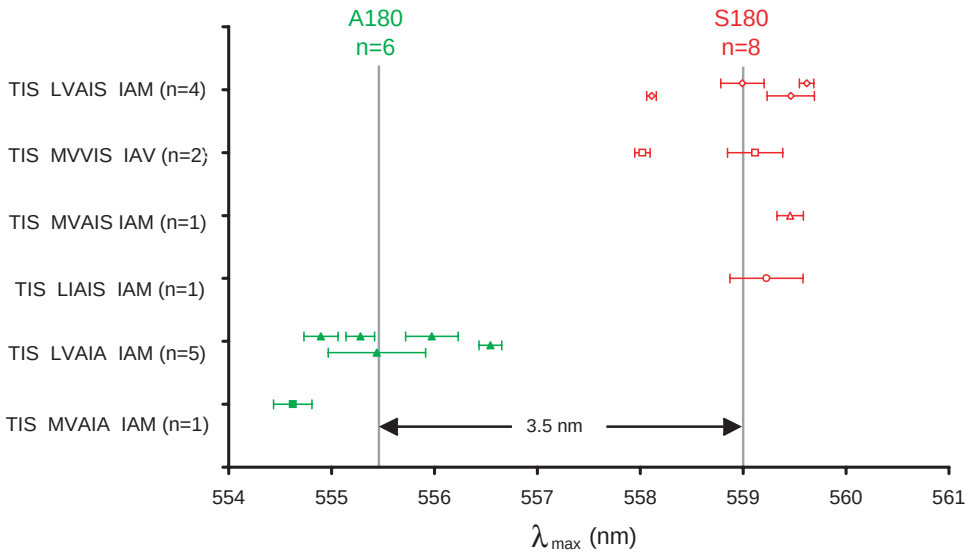

Figure 1. Individual deuteranope λ_{max} values and sequences. The 14 deuteranopes were divided into two classes according to whether their L-pigment gene specified alanine or serine at amino acid position 180. Mean values from these classes were used to derive L-cone spectra for estimating L:M cone ratio in normal trichromats. The λ_{max} values represent the average best fit of the template curve for each subject's spectral sensitivity data when a fixed OD of 0.35 was used. Horizontal error bars represent ± 1 SEM for the individual λ_{max} estimates. Single letter amino acid code is T=threonine, I=isoleucine, S=serine, L=leucine, V=valine, M=methionine, and A=alanine.

at the 11 variant positions for the deuteranopes. One of the amino acid substitutions observed among the deuteranopes, serine versus alanine at position 180, affects the spectral peak of the encoded photopigment (Neitz, Neitz, & Jacobs, 1991; Merbs & Nathans, 1992; Asenjo, Rim, & Oprian, 1994; Sharpe et al., 1998). Of the 14 deuteranopes, 8 had S180, and 6 had A180. The 8 with S180 had a mean λ_{max} of 559 nm; the 6 with A180 had a mean λ_{max} of 555.5 nm. The λ_{max} values for the pigments within the S180 group and within the A180 groups have a total range of less than ± 1 nm from the mean. The S180 and A180 groups do not overlap, and the tails of the two distributions are separated by a gap of nearly 1.5 nm.

There are no reports that any of the other 4 amino acid dimorphisms encoded by exon 3, besides 180, have an affect on λ_{max}; however, this has not been investigated thoroughly. The experiments here (Figure 1) demonstrate that if the other amino acid differences do have an effect on λ_{max} it must be quite small. In addition, the 3.5-nm shift attributed to the A180S polymorphism by these ERG measurements is in reasonable agreement with other estimates (Neitz et al., 1991; Merbs & Nathans, 1992; Neitz, Neitz, & Jacobs, 1993; Kraft, Neitz, & Neitz, 1998; Sharpe et al., 1998).

The overall range in λ_{max} is in agreement with previously reported values for deuteranopes obtained using a similar ERG method (Jacobs & Calderone, 1997). Previous examination of the spectral sensitivities of deuteranopes where the genetic identity of the L pigment was not known showed a bimodal distribution of λ_{max} values. The separation of the 2 distributions was 3.5 nm, identical to that observed here (where we compared A180 with S180 L pigments).

L:M Ratios of Normal Trichromats

We estimated the L:M cone ratio for each of the 62 normal males by determining the weighted sum of an L- and an M-cone spectral sensitivity function required to best fit the spectral sensitivity data. Figure 2 illustrates the fitting technique. Shown are five independent data sets for subject 043 obtained over a 2-month testing period. There was relatively little variability among the %L estimates, with the average deviation of any one trial from the average (59.05 %L) being 2.2 %L. Repeated measurements obtained from 3 other subjects showed similar variation among their respective %L estimates (data not shown), indicating that the ERG method employed here gives extremely reliable estimates of the relative contributions of the L and M cones. Moreover, the reproducibility of the %L measurement does not change as a function of L:M ratio. For instance, the 2 subjects with the highest estimated %L values (91.3 %L and 92.5 %L) showed average variations of only 1.2 and 2.2 %L, respectively, when each was measured on 5 separate occasions. The estimated L:M ratios (in terms of %L) for all 62 subjects are given in Figure 3A and Table 1. For each normal male, an L-cone spectral sensitivity function was assigned based on the sequence of his L pigment (Carroll et al., 2000). If the deduced sequence of the normal male's L pigment exactly

matched one of the deuteranope pigments of Figure 1, then we used either the average λ_{max} value of 555.5 nm if the pigment had A180, or the average value of 559 nm if the pigment had S180.

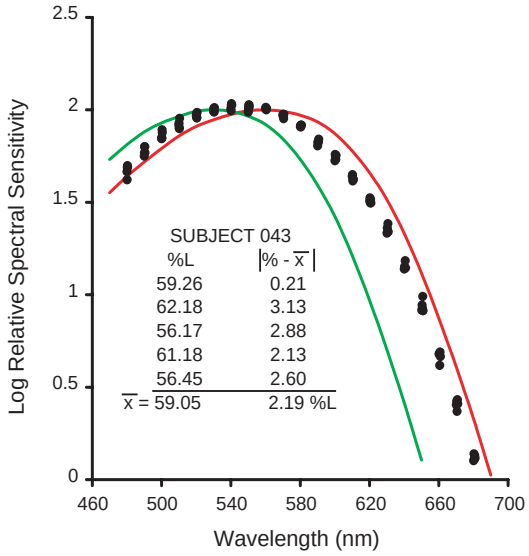

Figure 2. Estimating L:M cone ratio from ERG spectral sensitivity functions. To determine the %L for each subject, the weighted sum of an L- and an M-cone absorption spectrum was best fit to the subjects' ERG spectral sensitivity data. Each male was assigned a λ_{max} for his L cone based on the sequence of his L-pigment gene (see text). Shown above are five independent measurements of spectral sensitivity on one color normal trichromat (filled circles) and the cone spectral sensitivities used to estimate %L (red line, L-cone spectrum, λ_{max} = 559 nm, OD = 0.35; green line, M-cone spectrum, λ_{max} = 530 nm, OD = 0.22). %L estimates were obtained for each of the five trials and the average was calculated to be 59.05 %L. The average deviation of any individual trial from the average was 2.2 %L.

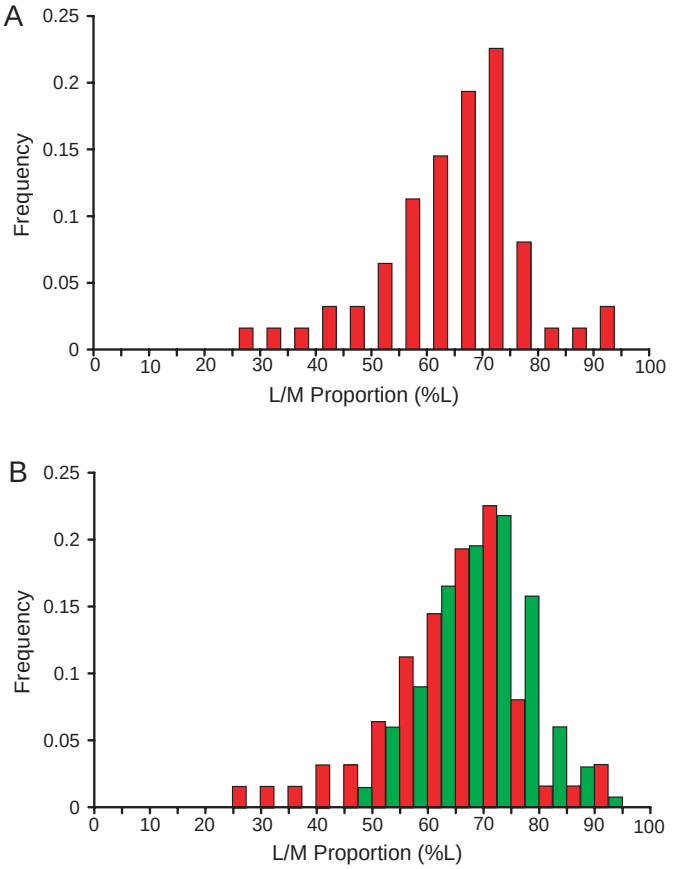

Figure 3. Normal variation in L:M cone ratio. A. Distribution of ERG-derived L:M cone ratios for 62 males with normal color vision. The relative L:M contributions are represented in terms of %L, in linear combination with M required to best fit each subject's flicker-photometric ERG spectral sensitivity function. The mean value was 65%L. B. Distribution of L:M mRNA from the retinas of 133 eye donors (M.N., unpublished data, 1998) (green bars), represented in terms of %L, compared to that of the ERG-derived ratios (red bars).

About 20% (12 of 62) of the subjects had L-pigment gene sequences for which there was not a match among the deuteranopes. Six of these subjects had L-pigment gene sequences that specified amino acid polymorphisms thought not to influence the λ_{max} of the photopigment. For these subjects, we used the average L-cone spectral sensitivity from either the A180 or S180 group of deuteranopes (depending on the identity of position 180 in the subject's L pigment). The other 6 had L-pigment gene sequences specifying amino acid polymorphisms known to significantly shift the photopigment spectrum. For these subjects, we estimated L-cone spectral sensitivity functions by extrapolating from the λ_{max} of the appropriate genotype class (A180 or S180) using previous estimates of the spectral tuning effects of the other polymorphisms in the L pigment (Neitz et al., 1991; Merbs & Nathans, 1992; Asenjo et al., 1994; Sharpe et al., 1998).

In addition to the ERG-derived %L, the L-cone spectral sensitivities used to analyze each normal male (along with the corresponding sequence for the L pigment from which the λ_{max} of his L-cone spectral sensitivity was derived) are given in Table 1. All subjects had a single L-pigment gene sequence, but about 5% had multiple copies of the L gene. This is consistent with previous data indicating that color normal individuals can have gene arrays with multiple copies of the L-pigment gene (Neitz et al., 1995; Hayashi, Motulsky, & Deeb, 1999). Two normal male subjects with multiple L-pigment subtypes were observed, but we do not know which of the L genes are expressed or in what ratio. To avoid this as a source of error, these individuals were excluded from the present study.

Table 1. L-Pigment Sequences Deduced from Gene Sequences, λ_{max}, and ERG-derived %L Estimates for 62 Males with Normal Color Vision

#	Exon 2	Exon 3	Exon 4	λ_{max}	%L	Rayleigh Match[a]	#	Exon 2	Exon 3	Exon 4	λ_{max}	%L	Rayleigh Match[a]
003	TIS	MVAIA	IAM	555.5	65.76	40-42	087	TIS	MVAIS	IAM	559	67.98	39-41
004	TIS	LVAIA	IAM	555.5	58.77	41-42	091	TIS	LVAIA	IAM	555.5	55.31	39.5-44
005	TIS	MVVIS	IAV	559	70.70	37.5-38.5	092	TIS	LVAIS	IAM	559	54.59	38-43
013	TIS	LVAIS	IAM	559	66.72	38-43.5	096	TIS	LVAIA	IAM	555.5	74.80	43-45
014	TIS	MVVIS	IAV	559	69.60	39-41	097	TIS	LVAIS	IAM	559	80.70	39-41
015	TIS	MVVIS	IAV	559	92.55	35-39	098	TIS	MVAIS	IAM	559	73.38	37-43
023	TIS	MVAIA	IAM	555.5	43.41	41-44	100	TIS	MVVIA	TSV	551.5[b]	39.40	40-46
030	TIS	LVAIS	IAM	559	79.53	37-40	103	TIS	LVAIS	IAM	559	57.44	38-41
043	TIS	LVAIS	IAM	559	59.05	35-42	104	TIS	LIAIS	IAM	559	48.94	38-40
056	TIS	LVAIA	IAM	555.5	61.16	42-43	106	TIS	MVAIA	IAM	555.5	61.86	39-42.5
057	TVY	MVAIA	IAM	554[c]	86.78	38-45	108	TIS	MVAIS	IAM	559	70.53	38-42
058	TIS	LVAIS	IAM	559	74.31	38-41	111	TIS	LVAIA	IAM	555.5	69.35	41-44
059	TVY	LVAIS	IAM	557.5[c]	66.21	39-42	112	TIS	MVAIS	IAV	559[d]	79.73	37-43
065	TIS	MVAIA	IAV	555.5[d]	71.47	40-45	113	TIS	MVAIS	IAM	559	76.18	35.5-40
067	TIS	LVAIS	TSV	555[b]	53.93	39-41.5	114	TIS	LVVVA	IAM	555.5[d]	49.99	40-43
068	TIS	LVAIS	IAM	559	72.36	39-40	116	TIS	MVAIA	IAM	555.5	66.78	40-43.5
070	TIS	LVAIS	IAM	559	74.41	36-40	117	TIS	LVAIA	IAM	555.5	61.75	39-44
071	TIS	LVAIS	IAM	559	66.19	38.5-42	119	TIS	LVAIA	IAM	555.5	32.94	38.5-41.5
072	TIS	LVAIS	IAM	559	91.26	39.5-44	120	TIS	MVAIS	IAM	559	74.72	39-42.5
075	TIS	LVAIA	IAM	555.5	73.47	41-43	123	TIS	MVAIA	TSV	551.5[b]	60.49	40-47
076	TIS	MVVIS	IAM	559[d]	28.14	39-42	129	TIS	LVAIS	IAM	559	78.76	38-40
077	TIS	LVAIS	IAM	559	69.39	39-40	157	TIS	LVAIA	IAM	555.5	67.79	42-44
078	TIS	LVAIS	IAM	559	50.71	40-42	160	TIS	MVAIA	IAM	555.5	74.41	42-43
079	TIS	LVAIA	IAM	555.5	51.95	40.5-42	163	TIS	LVAIS	IAM	559	64.48	37.5-44.5
080	TIS	LVAIS	IAM	559	57.78	39-42	182	TIS	LVAIS	IAM	559	67.97	37-40
081	TIS	MVAIA	IAV	555.5[d]	65.72	42.5-44	183	TIS	LVAIS	IAM	559	78.15	38-41
082	TIS	MVAIA	IAM	555.5	73.40	41-43.5	200	TIS	LVAIS	IAM	559	56.95	39-41.5
083	TIS	LVAIS	IAM	559	60.89	35-37	203	TIS	MVAIA	IAM	555.5	60.19	41-43
084	TIS	LVAIS	IAM	559	64.46	35-42	204	TIS	LVAIS	IAM	559	70.54	39-41
085	TIS	LVAIA	IAM	555.5	71.96	41-44	234	TIS	LVVVA	IAM	555.5[d]	44.82	41-44
086	TVY	LVAIA	IAM	554[c]	60.67	41-43.5	235	TIS	LVAIA	IAM	555.5	59.55	42-45

Each subject's L-cone spectral sensitivity function was based on the sequence of his L pigment (amino acid positions encoded by: exon 2 – 65, 111, 116; exon 3 – 153, 171, 174, 178, 180; exon 4 – 230, 233, 236). Some subjects had gene variants not seen in any of our deuteranopes; their L-pigment λ_{max} values were estimated using known spectral tuning differences (see text). a. Color matching range from Nagel anomaloscope. b. Estimated peak based on the spectral tuning effect of exon 4 specified substitutions TSV. c. Estimated peak based on the spectral tuning effect of exon 2 specified substitutions VY. d. Estimated peak based on polymorphisms with no known influence on the photopigment spectrum. Single letter amino acid code is T=threonine, I=isoleucine, S=serine, L=leucine, V=valine, M=methionine, A=alanine, and Y=tyrosine.

mRNA Results From Donor Retinae

The ERG technique is an indirect method for estimating the ratio of L:M cones in the retina. It relies on two assumptions: (1) the average L cone and the average M cone contribute equally to the generators of the ERG signal, and (2) under these recording conditions, the ERG taps signals that represent the linear sum of L and M cone contributions. Recently, L:M cone ratios were estimated from L:M mRNA ratios measured from excised patches of central retina from cadaver eyes (Hagstrom et al., 1998; Sjoberg, 1998; Hagstrom, Neitz, & Neitz, 2000). As with the ERG method, the mRNA analysis is also indirect, but the underlying assumptions are different. The mRNA analysis has the disadvantage that it cannot be used on living humans, but it is similar to the ERG in that it can be used to examine a large number of individuals. The analysis assumes that the average L cone has the same amount of mRNA as the average M cone. However, the linear additivity of the

amounts of L- and M-pigment mRNAs is not an assumption; this linearity has been demonstrated through measurements of known mixtures of L and M mRNAs. Figure 3B compares the results obtained using the ERG with those obtained previously from mRNA analysis on 133 donor retinas (M.N., unpublished data, 1998). The mRNA data are from retinal punches centered on the fovea, with radii that subtend ~10° of visual angle. Even though the ERG stimulus covers a much larger area of retina, the L:M mRNA levels remain relatively flat over this area (Hagstrom et al., 1998). If the assumptions of both techniques are valid, then the distribution obtained from mRNA analysis should match that obtained from the present study. The mean L:M ratios from the two studies are nearly identical (2:1, or ~ 66 %L) and the distributions are fairly similar. This is qualitative evidence that assumptions for both methods are reasonably valid. One curious difference between the two distributions is that with the mRNA method, almost all retinas had greater than 50%L, whereas with the ERG method, a significant number of individuals had L-cone contributions of less than 50%L. This suggests the possibility that although the assumption of equal amplitude ERG responses from L and M cones holds true for the majority of subjects, some may violate it. We examine this possibility further in the next section.

Relationship Between L-Pigment Subtype and L:M Ratio

This study makes it dramatically clear (Table 1) that there is not one "normal" L pigment. In our sample of 62 males with normal color vision, we identified 16 different normal L-pigment variants. The effect of various, normally occurring amino acid substitutions on the λ_{max} of the L-cone pigment has been studied (Neitz et al., 1991; Merbs & Nathans, 1992; Asenjo et al., 1994; Sharpe et al, 1998). However, it is not known how the variant amino acids might alter other aspects of cone photoreceptor function. There is evidence that some amino acid combinations that do not affect λ_{max} may influence other aspects of cone function, such as the efficiency with which the photoreceptor absorbs light (Neitz, Neitz, He, & Shevell, 1999). The fact that some outliers were observed in the ERG-derived distribution (which measures electrical signal) but not in the mRNA results (which may be a more direct measure of cone ratio) suggests that certain individuals may have ERG response ratios that do not reflect their actual cone ratio. It may be that amino acid combinations in some of the rare photopigment variants have detrimental effects; they may reduce the efficiency of the pigment in capturing photons or in activating the phototransduction cascade. For example, if someone had a normal percentage of L cones, say 50%, but he had an L-pigment variant that was only 50% as efficient at absorbing photons or transducing photon capture as normal, then from the ERG, his relative cone contribution would be only 33%L, a value observed in the ERG results but not in mRNA results. If true, this must be relatively rare or overall there would be a large disparity between the ERG and mRNA results. Thus, the hypothesis that there are specific L-pigment variants that are responsible for abnormal L-cone responses predicts that there should be an association between unusual ERG cone contribution ratios and unusual L-pigment variants. To test this, we obtained an estimate of the frequency of each of the L-pigment variants in the population by looking at the distribution of pigment variants in a larger sample that combined the 62 gene sequences here with those obtained previously from 87 males with normal color vision (Sjoberg, 1998). Each subject could then be characterized in terms of the frequency of his particular L-pigment variant and in terms of relative frequency of his cone contribution ratio. For example, subject 182 had the most commonly occurring L-pigment variant (TIS LVAIS IAM), which is observed in about 30% of males, and he had 68%L from the ERG analysis, which fell into one of the highest frequency bins, containing about 25% of the males. Similar frequency pairs were determined for each of the males and results for all 62 are plotted in Figure 4. A log scale is used in order to emphasize the low frequencies along each dimension.

In order to analyze the results statistically, we divided the subjects to separate out those falling in the lower 25th percentile – both in frequency of L:M ratio and in frequency of L-pigment gene sequence. In Figure 4, the upper right quadrant represents those subjects with both the most typical L-pigment gene sequences and the most typical L:M ratios. The lower left quadrant corresponds to those people who had both an unusual (falling at or below the 25th percentile) L:M contribution to the ERG and an unusual L-pigment gene sequence. The likelihood of these two low-probability events occurring together by chance is very low (0.25 x 0.25), whereas the odds of a subject being atypical in just one of these two dimensions are much higher (0.25 x 0.75). Thus, chance would predict that nearly 38% of our subjects would fall into the upper left or lower right quadrants. That is, if there was no relationship between gene sequence and cone ratio, there should be three times as many subjects in each the upper left and lower right than there are in the lower left. Instead, nearly equal numbers are observed in each of the three quadrants. The probability of getting this result by chance is low (p = .03; Fisher exact test). This association between the unusual gene sequences and the unusual ERG results is consistent with the hypothesis that the amino acid combinations that occur in some specific L pigments have detrimental effects on the flicker ERG response for that cone class. The results also suggest that variants that produce large detrimental effects must be relatively rare, so this will not generally affect the ability to assess cone ratio using the ERG for most subjects. However, it needs to be kept in mind when interpreting results from ERG-flicker photometry.

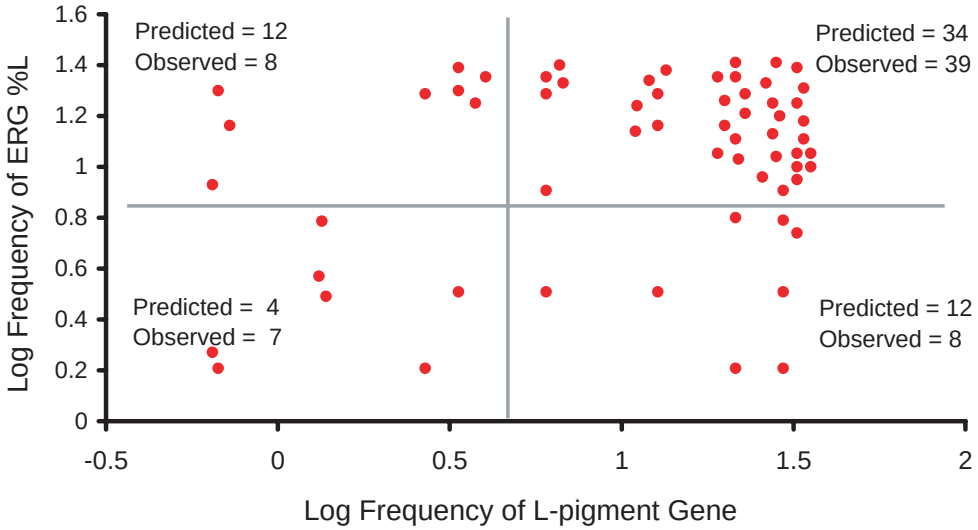

Figure 4. Correlation of L-cone contribution to the ERG with sequence variations at the L-pigment gene locus. For each subject, the frequency of his L-cone contribution to the ERG was plotted and compared to the frequency of his particular L-pigment sequence. The solid vertical line indicates the 25th percentile for the frequency of L pigment – all subjects falling to the left of the line are in the lower 25th percentile. Similarly, all subjects falling below the horizontal line have L:M contributions to the ERG found in the lower 25th percentile of the distribution. Included is the predicted number of subjects that should fall within each quadrant (as predicted by chance), along with the observed number. The association between L-pigment gene sequence and L:M cone contribution to the ERG was higher than would be predicted by chance (p=.03; Fisher exact test). Many subjects had overlapping frequencies, and these data points were displaced so that all 62 are visible in the graph. This did not change which quadrant a given data point fell into, but simply allowed each data point to be seen.

Because it is based on a statistical argument, this experiment does not identify specific unusual amino acid combinations as candidates for having detrimental effects on the ERG response. However, this is the second study (the first was Neitz et al.,1999) to suggest that normally occurring amino acid substitutions can have effects on cone function besides altering the λ_{max} of the pigments. So far, efforts to understand the implications of amino acid differences among L and M pigments have focused on λ_{max}. Future studies focused on other functional differences among the photopigment variants will be extremely important and interesting.

Effectiveness of Our Method

We have used the fitting of ERG-derived spectral sensitivity functions to individualized L-cone spectral absorption curves as a method for removing variation in the λ_{max} of the underlying photopigments as a source of error. To test the effectiveness of this method in removing the error, we compared the individual cone ratio estimates to Rayleigh matches on the Nagel anomaloscope. The Rayleigh match midpoint is linearly related to the spectral sensitivities of the underlying cone pigments, and thus, the match midpoint is highly correlated to variation in λ_{max} among the normal pigments (Neitz & Jacobs, 1986). If the variability in λ_{max} introduces error into the estimates of cone proportion derived from ERG spectral sensitivity, then the estimates of L:M proportion should also be significantly correlated with the Rayleigh match midpoints because both measures will share a strong component from variability in λ_{max}. Figure 5A shows the results for the 62 males prior to being corrected by using the individualized L-cone spectral sensitivities. To obtain the results for Figure 5A, a single L-cone fundamental was used for all subjects. As expected from the fact that the variability in the cone pigments is normally a strong source of error in the cone contribution estimates obtained from spectral sensitivity, there is a strong correlation between the two measures that is highly statistically significant (r^2 = 0.30, p < .0001). However, in Figure 5B, when we use corrected L-cone spectral sensitivity functions, based on the deduced amino acid sequence for each subject's L pigment, the correlation is reduced to near zero and the extremely low residual correlation is not statistically significant (r^2 = 0.04, p = .11). Thus, at least from this analysis, there is no measurable error associated with variations in λ_{max} remaining after corrections have been made using the genetic data.

In the sample used here, 35 subjects had S at position 180, whereas the remaining 27 had A180. In the absence of any other amino acid substitution, the S180 subjects have a pigment with a λ_{max} of 559 nm. However, 2 of the S180 subjects had polymorphisms in exon 4, which are known to shift the spectral sensitivity of the pigment toward the shorter wavelengths (Neitz et al., 1991; Merbs & Nathans, 1992; Asenjo et al., 1994; Sharpe et al., 1998). Thus, nearly one half of the subjects (35 – 2 = 33) had 559-nm L pigments, whereas the other half had

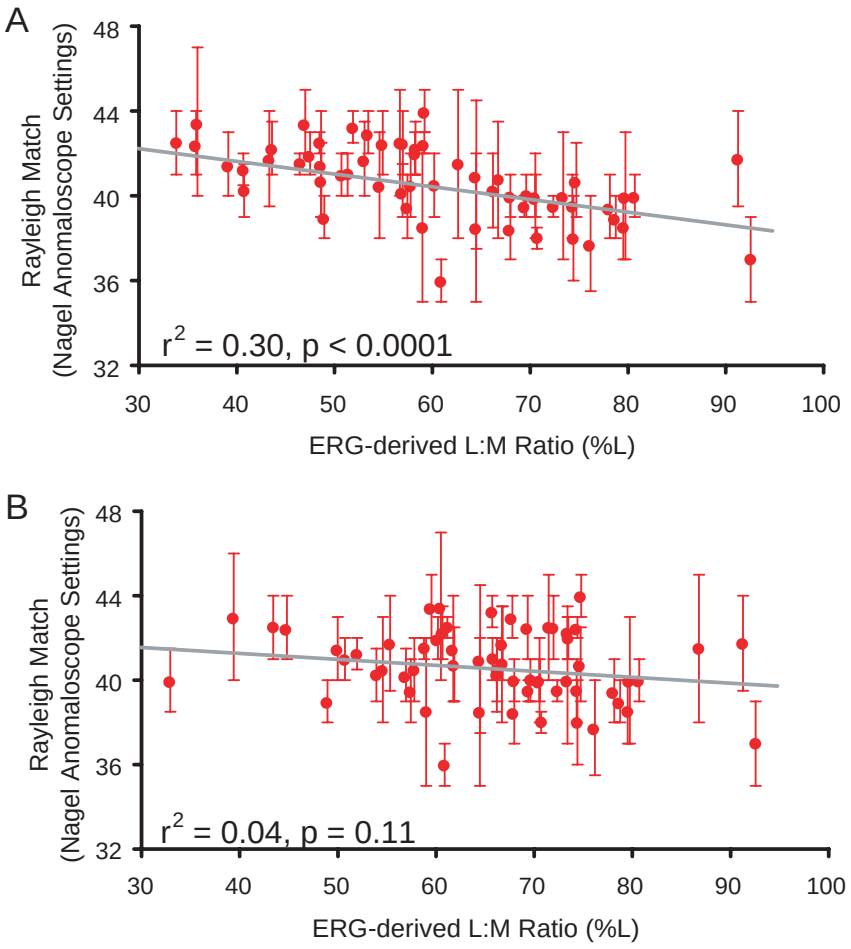

Figure 5. Estimated L:M ratios (expressed as %L cone contribution to the ERG) compared with Nagel anomaloscope Rayleigh match settings for 62 men with normal color vision. A. %L-cone contribution calculated with a single L-cone spectral sensitivity function versus anomaloscope setting. Vertical error bars represent the matching range for each subject; the data points are the midpoints. B. Same as A, except that the estimated %L contribution was corrected by using an individualized L-cone spectral sensitivity for each subject (see text).

pigments with λ_{max} values 3.5 nm shorter or more. This means that in a sample where no genetic information was known and an average L-cone spectral sensitivity was used for all subjects, all estimates would be subject to error from the λ_{max} variation. Alternatively, if an L pigment with a 559-nm spectral peak was used, nearly one half of the subjects would have accurate L:M estimates, and the other half would show L:M estimates that were incorrect. To determine how inaccurate these estimates would be, we derived a %L estimate using a 559-nm L pigment for all 29 of the subjects who had either A180 or some other polymorphism that shifted the spectrum of their L pigment. For one subject, the %L estimate was 25%L lower with the 559-nm curve than his individualized spectrum with a λ_{max} of 554 nm. This is the equivalent to misidentifying a person with a 3:1 L:M ratio as having only a 1:1 ratio. The average error was about 15%L, roughly the difference between a 1:1 and a 2:1 L:M ratio.

Conclusions

The combination ERG and genetics used here proves to be an efficient technique for obtaining measures of the L:M cone ratio. Measurements made on 62 males with normal color vision provide an estimate of how variation in L:M cone ratio is distributed in the population. There is remarkable variation among males with normal color vision that persists even though a large amount of measurement error has been removed. We assume that this widespread variability is the result of individual differences in the genetic mechanisms that determine the identity of a cone as being L versus M.

The method described here will be valuable in addressing questions about the biological mechanisms responsible for color vision. Of particular significance is understanding the basis for the large individual differences in cone ratio. We have begun to examine this

by characterizing the inheritance patterns of L:M ratio in normal men. In addition, by being able to rapidly and reliably identify subjects with unusual L:M ratios, we can investigate the consequences for vision that might come from having an abnormally skewed ratio.

The estimates of cone ratio that are derived with the methods used here appear to be highly accurate for the vast majority of individuals. However, about 10% of the ERG subjects had an L-cone contribution that was lower than the M-cone contribution, a situation that was not observed in the mRNA results. These subjects with abnormally low L-cone contributions were significantly more likely to have atypical L-pigment gene sequences. This suggests that in a small subset of individuals, the percent of L cones is underestimated because of abnormally low amplitude responses that are the result of expression of atypical L-pigment sequence variants. Preliminary results show that L:M ratios obtained from direct imaging with adaptive-optics closely agree with those obtained from an ERG method such as ours (Brainard et al., 2000), and this is being pursued further. Individuals in whom a low ratio is coupled with an atypical L gene are candidates for having L pigments with impaired function. An interesting test would be to obtain adaptive-optics retinal images from such individuals and see if the L-cone contribution to the ERG is significantly lower than predicted from the images. These sorts of experiments are also underway. This strategy might open the door for other, more comprehensive analyses of the functional differences among pigment variants.

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