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Variations in cone populations for red–green color vision examined by analysis of mRNA

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In the central human retina, there are estimated to be nearly two L cone photoreceptors for each M cone. The extent to which this value varies across individuals is unclear and little is known about how the M:L cone ratio might change with retinal location. To address these questions, the ratio of M:L cone pigment mRNA was examined at different locations. For patches of central retina, the average M:L ratio was about 2:3 which decreased to about 1:3 for patches 40° eccentric. There were also large individual differences among the 23 eyes examined. The extremes differed in central M:L mRNA ratio by a factor of > 3. The measured differences in mRNA ratio are proposed to reflect differences in photoreceptor ratio. Such variations provide unique opportunities for understanding how the neural circuitry for color vision is affected by changes in cone ratio. *NeuroReport* 9: 1963–1967 © 1998 Rapid Science Ltd.

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Introduction

Normal human color vision is based on three classes of cone photoreceptor, long (L), middle (M), and short wavelength sensitive (S). Knowing the relative numbers, ratios, and distribution of the different cone types is fundamental to understanding the biological basis of color vision.

About 7% of the cones are S cones. Their density changes with eccentricity, the foveal center lacks S cones and there is a small area of maximum density at ~1° eccentricity.[1–4] Less is known about the numbers, ratios, and topographical layout of the M and L cones. It has not been possible to distinguish L from M cone types by immunological methods. However, microspectrophotometry has been used to examine patches of the cone mosaic in a monkey.[5] Results suggest a random placement of the L and M cones. Psychophysical methods have been used to address questions about the L and M cone populations in humans. In the central retina, it is estimated that, on average, there are 1.5–2.0 L cones for every M cone.[6–8] However, there is some evidence to suggest large differences across individuals. The range of variation has been estimated to be as large as from 3:1 to 1:3.[9] Such variation has long been suggested as an explanation for large individual differences in relative sensitivity to red and green flickering lights that occur among people with normal color vision.[10]

There are also changes in peripheral color vision that might be related to changes in the distribution of cones found within an eye at different retinal locations. Results from color naming,[11,12] colour matching[13] and color discrimination perimetry[14] characterize vision in the peripheral retina as resembling a deutan color vision defect in which M cone contribution is abnormal or lacking. This is consistent with results from psychophysical hue cancellation studies that show the red response system increases in dominance relative to the green response system in the peripheral retina.[15]

In summary, on average, L cones have been estimated to outnumber the M cones in the central retina by a factor of 1.5–2.0, but there may be significant individual differences. Studies of color vision would be consistent with a change in cone ratio in the far peripheral retina toward higher L cone dominance. We have investigated these issues by estimating the M:L cone pigment mRNA ratio at different eccentricities and locations in 23 individual human retinas.

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Materials and Methods

Human eyes (obtained through the Wisconsin Lions Eye Bank) were enucleated within 3 h of death, placed on ice and retinas were dissected within a few hours. A trephine was used to punch 13 patches of retina, 6 mm in diameter, from each eye as shown in Fig. 1A. Patch 1 was centered approximately on the fovea. The middle six patches surrounding the central patch were centered ~6 mm or 21° eccentric from the fovea. The outer six patches were centered approximately 12 mm or 41° eccentric from the fovea. Nucleic acids were isolated from retinal tissue patches as described previously.[16]

Retinal RNA was reverse transcribed (RT), and the visual pigment cDNA amplified in the polymerase chain reaction (PCR) using the RNA-PCR kit from Perkin Elmer. Exon 5, which encodes the two amino acid positions that determine whether the specified pigment will be L or M,[17] was amplified with primers that give PCR products for genomic DNA and cDNA that differ in size. The forward primer was 5′-CCGAGCGGTGGCAAAGCAG-3′ and the reverse primer 5′-TGGCAGCAGCAAAGCATGC-3′. Amplification conditions were 1 cycle at 94° for 5 min, followed by 40 cycles of 94°C for 45 s, 57°C for 45 s and 72°C for 45 s, and 1 cycle of 72°C for 10 min. PCR products were gel purified and an aliquot of amplified cDNA was used again in PCR exactly as above. The final product was analysed in an *Rsa*I restriction digestion assay and quantitated by PhosphorImage analysis as described previously.[18] *Rsa*I cleaves within exon 5 of L but not M genes.

Results

Initially, we examined four eyes from two male donors. For each eye, 13 patches of retina were dissected as shown in Fig. 1A. The proportion of M:L mRNA was estimated from a quantitative *Rsa*I restriction digestion assay analysing exon 5 of the M and L pigment cDNAs. A standard curve confirming the validity of the method is shown in Fig. 1B. Fig. 1C shows examples of results from retinal samples. The estimated proportions of M pigment mRNA (calculated as described in Fig. 1C) from the individual patches are summarized in Table 1. Measurements were performed in triplicate for subject 1 and in duplicate for subject 2. The values are the average of replicate experiments (± s.e.m.) for each patch. We were interested in learning if there were differences in the M:L mRNA ratio over large topographic retinal regions: (1) superior *vs* inferior, (2) nasal *vs* temporal, and (3) central *vs* far-peripheral. Results from the 13 patches were pooled in various combinations to represent these different retinal regions. Patches 2–7 comprised the nasal retina, and patches 8–13 made up the temporal retina. In Table 1, the top panel of results represents tissue patches of the nasal retina, and the middle panel of results represents the temporal retina. The first column in the bottom panel, displaying the central patch, numbered 1, completes the results from all individual patches. The remaining columns in the bottom panel are the results from specified patches recombined to yield averages representing superior, inferior, and far-peripheral retina. There was no significant difference in the average proportion of M to L mRNA for the nasal *vs* temporal retina (0.33 and 0.34, respectively). Similarly, no significant difference was seen in the average proportion for superior *vs* inferior retina (0.27 and 0.29, respectively). However, the comparison of the central retina to the far-peripheral retina suggested a significant difference in the proportion of M to L mRNA with retinal eccentricity.

There was a significant difference between the central regions of the two eyes in subject 1, a 77-year-old, even though all values outside this area were similar between his two eyes. We can only speculate about the reason for the difference between eyes, but the fact that the large difference was seen only in the macular region might suggest that one retina was in the early stages of developing an age-related macular degeneration. If so, it is possible that one cone type is more susceptible to the disease thereby producing the difference in cone proportion. If the results from the aberrant eye are excluded, the change in proportion is even more dramatic for the remaining three eyes. The average percentage of M pigment mRNA drops from 41% in the central retina to 26% in the far-periphery. If expressed as ratios rather than proportions, the corresponding M divided by L values are 0.69 for the central retinal and 0.35 for the far-periphery, a difference of about a factor of 2. By comparison, the nasal/temporal and superior/inferior differences were small. These findings motivated us to further explore the differences in M to L mRNA proportion as a function of retinal eccentricity.

Individual retinas from 23 male donors ranging in age from 5 to 96 years were examined. Patch 1, centered approximately on the fovea, represents the central retina. Six patches: 2, 4, 6, 8, 10, and 12 were combined to provide a mid-peripheral sample whose center corresponds to a region in the living eye ~21° of visual angle eccentric from the fovea. Patches 3, 5, 7, 9, 11, and 13 were combined to provide a far-peripheral sample whose center corresponds to the region ~41° of visual angle eccentric from the fovea. Procedures were similar to those for the

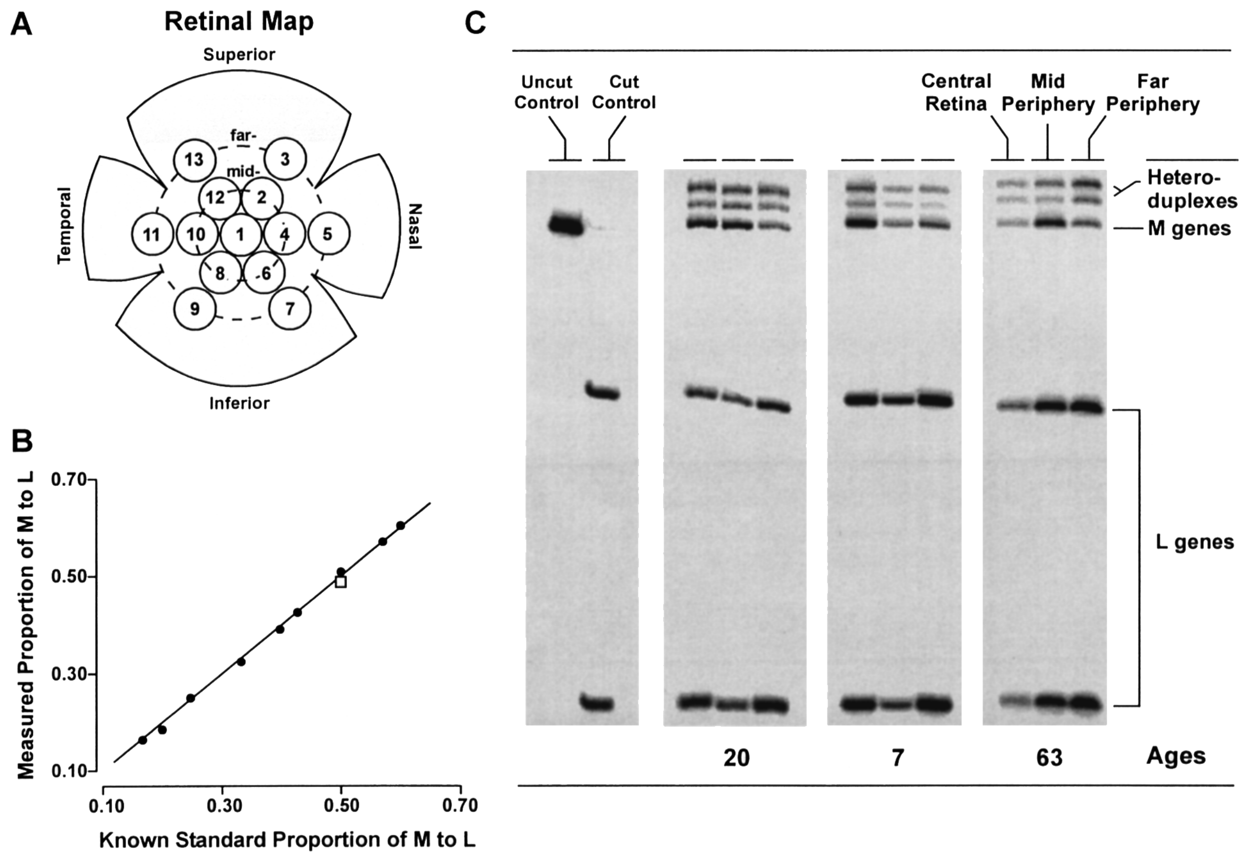

FIG. 1. (**A**) Location of retinal patches. The center of patch 1 is at 0° eccentricity. The center of each patch in the middle ring of patches represented by the small dashed circle is at 21° eccentricity. The center of each patch in the outer ring of patches represented by the large dashed circle is at 41° eccentricity. (**B**) The validity of the method used to estimate M:L cDNA proportions was demonstrated by measuring the proportions of mixtures of L and M cDNA clones. The clones were mixed in known proportions. Filled circles plot the known proportions *vs* those measured with this method. The open square symbol is the measured proportion of M:L genomic genes in a male known to have one L and one M gene[18] and serves as an additional reference point for a 1:1 mixture. (**C**) An example of the results from three individuals for three retinal eccentricities. Two bands represent L cDNA fragments produced by *Rsa*I cleavage, and one band represents M cDNA fragments that are not cut by *Rsa*I. Two heteroduplex bands are present when both the L and M genes are expressed. The proportion of M to L cDNA was estimated as described previously.[16] One strand of each heteroduplex is from L and the other is from M cDNA. Thus, half the counts in the heteroduplexes (H/2) were added to the counts in the uncut band (U), and the other half were added to the sum of the counts in the two cut bands (C). The sum (U + H/2) yields an estimate of the amount of M cDNA and (C + H/2) the amount of L cDNA in the sample. These were used to calculate proportions (M/(M + L)). It is evident that the proportion decreases with increasing eccentricity and that there is variability across individuals.

previous four eyes except the patches were combined to produce a single mid-peripheral and a single far-peripheral tissue sample before the nucleic acid isolation stage.

Figure 1C shows the results from three eyes drawn from three different age groups for the three eccentricities. By inspection, the tendency for the proportion of M pigment mRNA to decrease with increasing eccentricity can be seen. The estimated proportions of M to L mRNA for the 23 eyes are summarized in Table 2. All measurements were made in triplicate and values reported are the mean (± s.e.m.) for the replicate experiments. The individuals are categorized by age. The first group of eyes were from donors under the age of 10. The second group combined adults between the ages of 20 and 60 years. The final group consists of retinas over the age of 60. The means for each age group are also shown.

Data were grouped in this way to allow the detection of age-related differences in M:L mRNA ratio. We wanted to be alert to this idea for two reasons. First, it is uncertain if cone cell development, migration, and distribution is complete in young retinas.[19–21] One report predicts that human foveal cone density increases until 8 years of age.[19] Second, in older adults, age-related diseases that lead to retinal degeneration are common. Disorders of the macula are responsible for the majority of cases of blindness affecting 1 in 25 people over the age of 65.[22] As mentioned previously, subject 1 in Table 1 was a 77-year-old who had very different values in the M to L mRNA proportion in the central retina of his two eyes. It is possible that other older eyes in our sample might have similarly low M cone proportions.

In the 20–60 year age category, there was only

Table 1. Proportion of M to L mRNA for 13 individual retinal patches

		M/M + L mRNA (mean ± s.e.m.)					
		Nasal patches					
Subject[a]	Eye	2	3	4	5	6	7
1	O.D.	0.35 ± 0.02	0.32 ± 0.03	0.24 ± 0.02	0.16 ± 0.01	0.26 ± 0.04	0.20 ± 0.01
1	O.S.	0.18 ± 0.04	0.26 ± 0.00	0.26 ± 0.05	0.16 ± 0.02	0.35 ± 0.10	0.38 ± 0.08
2	O.D.	0.27 ± 0.05	0.95 ± 0.80[b]	0.34 ± 0.06	0.28 ± 0.05	0.45 ± 0.26	0.27 ± 0.05
2	O.S.	0.34 ± 0.01	0.23 ± 0.02	0.31 ± 0.05	0.28 ± 0.01	0.27 ± 0.04	0.16 ± 0.05
mean	0.33						
		Temporal patches					
Subject	Eye	8	9	10	11	12	13
1	O.D.	0.43 ± 0.23	0.16 ± 0.01	0.37 ± 0.02	0.30 ± 0.06	0.33 ± 0.02	0.30 ± 0.03
1	O.S.	0.24 ± 0.04	0.24[c]	0.20 ± 0.02	0.20 ± 0.02	0.27 ± 0.03	0.20 ± 0.04
2	O.D.	0.39 ± 0.23	0.29 ± 0.02	0.24 ± 0.02	0.33 ± 0.01	0.32 ± 0.08	0.28 ± 0.02
2	O.S.	0.28 ± 0.04	0.28 ± 0.11	0.32 ± 0.00	0.41 ± 0.33	0.25 ± 0.01	0.13 ± 0.03
mean	0.34						

Subject	Eye	Central patch 1	Far-peri patches 3,5,7,9,11,13	Superior patches 2,3,12,13	Inferior patches 6,7,8,9
1	O.D.	0.40 ± 0.04	0.24 ± 0.03	0.33 ± 0.03	0.26 ± 0.07
1	O.S.	0.22 ± 0.01	0.24 ± 0.03	0.23 ± 0.03	0.30 ± 0.07
2	O.D.	0.36 ± 0.03	0.29 ± 0.03	0.29 ± 0.05	0.35 ± 0.14
2	O.S.	0.46 ± 0.23	0.25 ± 0.09	0.24 ± 0.02	0.25 ± 0.06
mean		0.36	0.26	0.27	0.29

[a]Subject 1 was a male age 77 and subject 2 was a male age 41.
[b]Value not included in total mean of regions which include this patch.
[c]One experiment performed on this patch.

a slight difference in the overall mean M to L mRNA proportion between the central retina and mid-peripheral retina, 0.40 and 0.37 respectively. However, there was a large decrease in M pigment mRNA proportion between the mid- and far-periphery, from 37% down to 25%. As seen in the preliminary sample, there was a decrease in ratio from the central patch to the most eccentric ring by a factor of about 2. At most, the M to L mRNA proportion decreased very gradually between the central retina and the mid-periphery, but there was a dramatic decrease between the mid-periphery and the far-periphery.

Results also show considerable differences across individuals, which were most dramatic in the central retina. The range for the 20–60 year group was 30–55% M pigment mRNA. Individual differences were generally much smaller for the far-peripheral region. There was, however, an exception. One subject had a very high M pigment mRNA proportion in the far-periphery. As seen from the s.e.m. for that measurement, it was reliably different from the results for the other subjects.

Results for both the younger and older retinas were similar to those of the 20–60 year retinas with subtle differences. The older retinas had a slightly lower proportion of M pigment mRNA in the central patch which was not statistically significant. The young group had lower proportions of M pigment mRNA at all three eccentricities. Interestingly, the difference between the young group and the 20–60 year group was marginally statistically significant ($p = 0.04$) for the central patch. These features of the results can be best appreciated from Fig. 2, which represents the data in Table 1 in graphical form.

Discussion

On average, there was a significant drop in the proportion of M pigment mRNA in the far-peripheral retina. On average the central retina contained about 40% M pigment mRNA and the far-periphery only 25%. There were also differences between individuals, particularly in the central retina. The range was between about 25% and 50% M pigment mRNA. We propose that the M and L cones produce about equal amounts of mRNA and thus, the measured mRNA ratios reflect the cone ratios. This is supported by the fact that our measure of the average proportion of M to L mRNA in the central retina is identical to the cone proportions proposed for the human fovea on the basis of a variety of other measurements.[7,23–25] However, our results provide new information about the range of variation. When translated to cone ratios, our results would suggest that individuals at one extreme have about one L cone

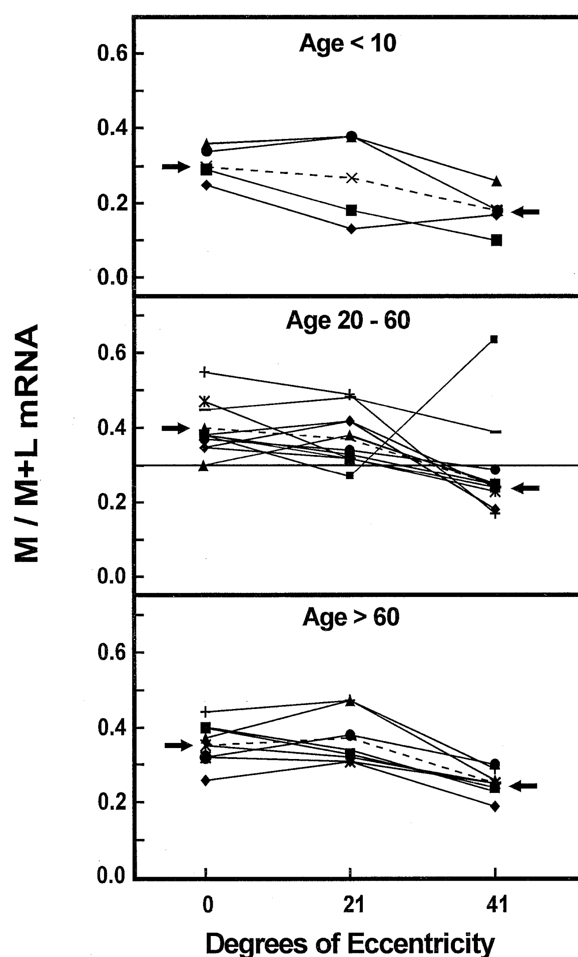

FIG. 2. A decrease in the M to L cDNA proportion with increasing eccentricity. Three separate age categories are each shown at three retinal eccentricities. Each data point is the mean M to L cDNA proportion for experiments done on one eye at the plotted retinal regions. Each eye is represented by a separate symbol and can be traced across the three eccentricity points by a solid line. The mean of each age category is represented as a dashed line and highlighted by small arrows.

for every M cone and at the other extreme they have three L cones for every M cone.

A loss of M cone response in the periphery has been reported frequently. Here are two examples. Stabell and Stabell measured chromaticities of spectral colors at 17°, 25°, 40° and 60°.[13] The wavelength, 570 nm, which evoked a green-yellow color at the fovea was perceived as orange in the far-periphery. This is consistent with a loss in strength of M cone signal in the periphery. Hibino used a hue cancellation technique to measure the spectral sensitivities of red-green and yellow-blue opponent color responses at 0°, 3.3°, 10°, 21° and 31°.[15] The red-green functions at 0° through 21° were similar to one another but the 31° red-green function decreased in the green response. The biological basis for these losses of M cone response in the periphery has not been known. The present study suggests that the perceptual change observed may be correlated with relatively fewer M cones in the periphery. It is notable that in the psychophysical studies the green response system is fairly constant up to about 20° from the fovea but the red system becomes relatively more dominant at greater eccentricities. This is parallel to the present study in which the retinal samples centered on the fovea and at 21° are similar in mRNA ratio but a change in ratio occurs between 21° and 41°.

Conclusion

The M and L cone populations are intermixed in the retina. The two cone classes seem to share the same environment and the same complement of genomic DNA, yet one population expresses L pigment and the other expresses M pigment. Here we report evidence that there are large differences in the ratio of these cone populations across people and within a person at different retinal locations. The characterization of these differences is an essential step toward understanding the biological mechanisms that determine whether an individual cone cell will express an M or an L cone pigment. Furthermore, these significant variations in cone ratio may be very useful in experiments designed to understand how the nervous system can be wired to extract color signals in the face of large variations in cone populations.

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