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Topography of long- and middle-wavelength sensitive cone opsin gene expression in human and Old World monkey retina

MAUREEN NEITZ, SHAWN D. BALDING, CARRIE MCMAHON, STACY A. SJOBERG, AND JAY NEITZ

Department of Ophthalmology, and Department of Cell Biology, Neurobiology & Anatomy, Medical College of Wisconsin, Milwaukee, Wisconsin

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Abstract

The topographical distributions of the relative ratio of long- (L) and middle- (M) wavelength sensitive cone opsin messenger RNA (mRNA) in human and baboon retinas were mapped using real-time polymerase chain reaction. The L:M mRNA ratio increased in a central-to-peripheral gradient in both species, being quite pronounced for humans.

Keywords: molecular genetics of color vision; cone photopigments, cone topography, cone photopigment gene expression, real time quantitative PCR

Introduction

Information about the topographical arrangement of short-, middle-, and long-wavelength sensitive cones (abbreviated S, M, and L respectively) in retinas from humans and Old World monkeys is critical for understanding the mechanisms that govern L and M opsin gene expression, and for understanding how the circuits for color vision arise. The mosaic of S *versus* L/M cones has been examined using antibody staining and *in situ* hybridization. These methods can distinguish S from M/L cones, but not M from L cones (Ahnelt et al., 1987; Wikler & Rakic, 1990; Curcio et al., 1991; Bumsted & Hendrickson, 1999; Martin & Grunert, 1999; Ahnelt et al., 2000; Martin et al., 2000). Microspectrophotometry and adaptive optics coupled with retinal densitometry have allowed examination of all three classes of cone in the central retina (Mollon & Bowmaker, 1992; Roorda & Williams, 1999; Roorda et al., 2001; Bowmaker et al., 2003; Hofer et al., 2005). Statistical analyses of the data have revealed that the distributions of M and L cones are generally not different from random, although there is a slight tendency for clumping of L and M cones in humans (Hofer et al., 2005).

An unresolved question is whether circuits for red-green color vision are established by random wiring or by cone-selective wiring (Martin et al., 2001; Diller et al., 2004; Solomon et al., 2005). The degree to which peripheral midget ganglion cells exhibit red-green opponency has been offered as a way to distinguish between these two competing hypotheses (Lennie et al., 1991; Martin et al., 2001; Diller et al., 2004; Solomon et al., 2005). For a cell to exhibit red-green opponency, the receptive field center must receive pure or nearly pure L or M cone input, but input to the surround can be mixed (Lennie et al., 1991; Reid & Shapely, 1992; Lee, 2004). The probability that the input to a peripheral ganglion cell's receptive field center will be purely L or purely M will depend on the relative representation and "clumpiness" of L and M cones in the periphery. Thus, the knowledge of the topography of L and M cones is important for distinguishing between the random *versus* selective connections hypotheses.

The absence of distinguishing molecular markers other than the opsin expressed, and the high degree of similarity of the opsins has prevented separate mapping of L and M cones using antibody staining or *in situ* hybridization. Adaptive optics have not yet allowed high resolution imaging of peripheral retina, and with microspectrophotometry (MSP) it is difficult to obtain data from a sufficiently large number of cones to quantify their distributions. However, previous studies have demonstrated that the analysis of L *versus* M opsin messenger RNA (mRNA) using reverse transcriptase and the polymerase chain reaction (PCR) is an effective method for extrapolating the relative L:M cone ratio at specific retinal locations (Hagstrom et al., 1998; Deeb et al., 2000; Hagstrom et al., 2000; Bollinger et al., 2004). In humans and other Old World primates the relative ratio of L *versus* M opsin mRNA has been observed to vary as a function of retinal location (Hagstrom et al., 1998; Deeb et al., 2000; Hagstrom et al., 2000), and it has been reported that in humans there is a pronounced dominance of L opsin mRNA at eccentricities greater than about 40° (Hagstrom et al., 1998; Hagstrom et al., 2000). These findings have been further supported by MSP in the peripheral human retina (Bowmaker et al., 2003). Here we investigated the topography of the L:M mRNA ratio in a larger group of human male eye donors and in greater detail than previously studied. We also examined the

Address correspondence and reprint requests to: Maureen Neitz, Department of Ophthalmology, The Eye Institute, 925 North 87th Street, Milwaukee, WI 53226. E-mail: mneitz@mcw.edu

topography of L:M mRNA in baboon retinas; both species showed a dramatic central-to-peripheral gradient of increasing L mRNA, but only in humans did L mRNA overwhelmingly dominate the retinal periphery.

Materials and methods

Tissue preparation and nucleic acid extraction

Human eyes were obtained through the Wisconsin Lions Eye Bank. Eyes were enucleated and refrigerated within 5.5 hours of death. Male donors ranged in age from 3 to 91 years, with an average age of 66 years, a median age of 71, and a modal age of 77. Female donors ranged in age from 62 to 96 years, with an average age of 80, and a modal age of 75. A 6-mm trephine was used to take a punch of retina centered on the fovea from 107 male and 33 female eyes, and 10 male eyes were flat mounted onto a nylon filter printed with a 3-mm^2 grid and frozen at -80°C. Retinas from four male baboons (*Papio anubis*) were obtained through the Tissue Distribution Program of the Regional Primate Center at the University of Washington. Their ages ranged from 1.96 to 2.32 years. Baboon retinas were flat mounted onto nylon filters printed with a 3-mm^2 grid and frozen at -80°C. Flat mounted retinas were cut along the grid lines while frozen. Nucleic acids were extracted from each square and from each trephine punch as described previously (Neitz et al., 1998), and resuspended in 50 μl of 10-mM Tris, pH 8.0, 1-mM EDTA pH 8.0.

Determination of the ratio of L:M mRNA by reverse transcription and real-time quantitative polymerase chain reaction

The relative ratio of L:M mRNA was determined using *Taq*Man assays (Applied Biosystems, Foster City, CA), as described previously (Neitz & Neitz, 2001). The primers and probes for these assays are described in Table 1. The primer pairs do not amplify genomic DNA because one primer in each pair spans two adjacent exons, and thus corresponds to a sequence found in cDNA but not in genomic DNA.

Reverse transcription and PCR were carried out sequentially in the same tube using the TaqMan RT-PCR Kit (Applied Biosystems, Foster City, CA). For human samples, each 25 μl reaction contained a final concentration of 5 mM $MgCl_2$, 0.3 mM each dATP, dUTP, dCTP, and dGTP, 0.2 units/μl MuLV reverse transcriptase, 0.24 units/μl RNase Inhibitor, 150 nM L-probe, 250 nM M-probe, and 900 nM each of the forward and reverse primers. For baboon samples, each 25 μl reaction contained a final concentration of 7.5 mM $MgCl_2$, 0.3 mM each dATP, dUTP, dCTP, and dGTP, 0.2 units/μl MuLV reverse transcriptase, 0.24 units/μl RNase Inhibitor, 200 nM L-probe, 200 nM M-probe, and 300 nM each of the forward and reverse primers.

L- and M-specific TaqMan probes were labeled on the 3′ end with a quencher dye (Tamara) and on the 5′ end with different reporter dyes (6'Fam, Joe, or Vic, see Table 1). The single nucleotide difference between the human L and M gene probes corresponds to the second nucleotide of codon 309, which has been observed to always distinguish L from M genes in humans (Bollinger et al., 2001; Neitz & Neitz, 2001), and to be the only polymorphic nucleotide in the probe region. The difference in sequence between monkey L and M probes corresponds to regions in exon 5 that distinguish L from M genes for all monkeys used in this study. Real-time quantitative PCR was performed in an ABI Sequence Detection System model 7700. Reactions were first incubated at 48°C for 30 minutes, then at 95°C for 10 minutes to allow the reverse transcriptase reaction to be completed. The reactions were then subjected to 3 cycles of 95°C for 30 seconds and 67°C for 1 minute, then 37 cycles of 95°C for 15 seconds and 67°C for 1 minute. Sufficient template was added to each reaction so that the fluorescence intensity from the reporter dyes exceeded background fluorescence between cycle 16 and 24. For each sample analyzed, results from triplicate or quadruplicate reactions were averaged to estimate the relative amount of L:M mRNA.

Standard curves and reproducibility of the measurements

Separate standard curves were generated for humans and monkeys. L and M opsin cDNAs were cloned from retinal RNA isolated from normal human, baboon, and macaque retinas using standard methods, and the clones were sequenced. For the monkeys, sequences for exons 4 and 5 of the cloned L and M opsin cDNAs were compared to the corresponding regions in the genome to verify that the two matched. Monkey gene sequences were obtained by PCR amplification and direct sequencing of individual exons from genomic DNA. There were no nucleotide differences across the monkey species (*Papio anubis, Macaca nemestrina, Macaca fascicularis*) within the region of the L and M cDNAs used in the assay, so the same primers and probes were used for all three monkey species. We generated the standard curves shown in Fig. 1 by using real-time quantitative PCR to estimate the L:M ratios in mixtures of cloned L and M opsin cDNAs. The reproducibility and reliability of the real-time PCR assays were determined by repeatedly measuring the L:M RNA ratios for retinas from 2 humans and 1 macaque monkey over a period of several months (Neitz & Neitz, 2001). The average of quadruplicate measurements provides

Table 1. *Primers and probes used in real-time quantitative PCR*

Species	Primers/Probes	Location
Baboon	5′CCGAGCGGTGGCAAAG	Exon 5, L&M
Baboon	5′GCAGCAAAGCATGCGAAGA	Exon 5/6 junction
Baboon	5′6-FAM-AGATCATCACCATCACCATGCGCGT-TAMARA	L-Exon 5
Baboon	5′VIC-TGATGTTCCTGGCGTTCTGCTTCTGC-TAMARA	M-EXON 5
Baboon	5′TTTGCTGCTGCCAACCCT	Exon 4/5 junction
Human	5′TCGAAACTGCCGGTTCATAA	Exon 5
Human	5′6-FAM-CCTGCCGGCCTACTTTGCCAAA-TAMARA	L-EXON 5
Human	5′JOE-CCTGCCGGCCTTCTTTGCCAAA-TAMARA	M-EXON 5

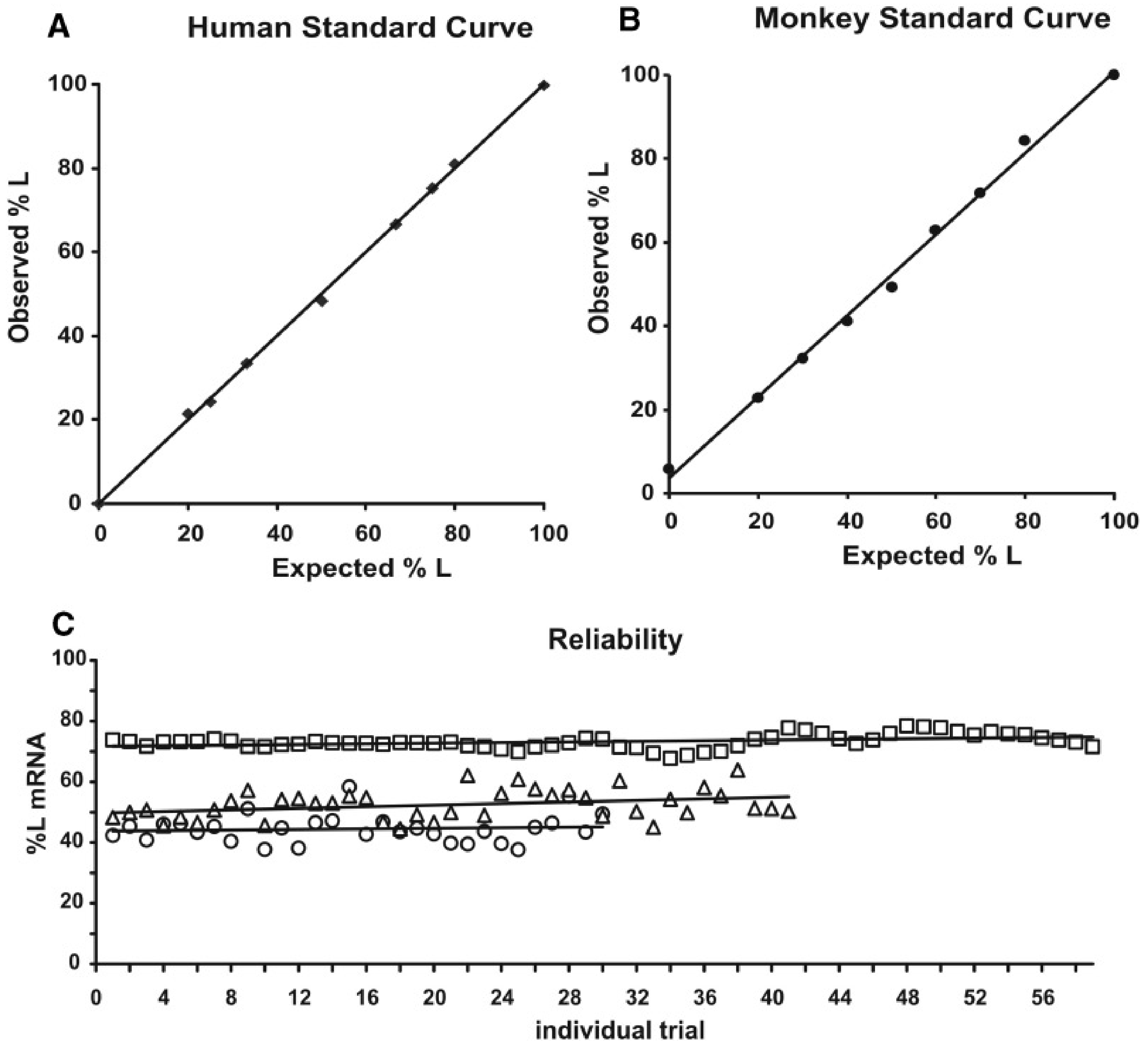

Fig. 1. Standard curves and reliability of real-time quantitative PCR estimates of L:M cDNA ratios. (A) Human or (B) monkey L and M opsin cDNA clones were mixed in known ratios, and the ratios were measured in quadruplicate with real-time quantitative PCR. The average of the quadruplicate measurements for each ratio are plotted against the input ratios. (C) Averages of quadruplicate measurements of the relative L:M mRNA ratios in retinal samples from 2 human male eye donors (squares and triangles), and one monkey (circles) retina were made repeatedly over many days to illustrate the reliability of the method over time.

a reliable estimate of the L:M mRNA ratio with 95% confidence limits of ±4%.

Results

Shown in Fig. 2 is the distribution of the percentage of the L plus M opsin mRNA that was L (%L mRNA) in a 6-mm circular punch of retina centered on the fovea for 107 male and 33 female human retinas. Each donor retina expressed L and M opsin genes. The range of variation was from 41% to 97% L, with an average of 66% L for the males, and from 53% to 84% L, with an average of 68% L for the females.

The %L mRNA across the entire retina was evaluated by estimating the L:M mRNA ratio for every grid square of one flat mounted human retina. The results, shown in Fig. 3, demonstrate a clear trend in the pattern of variation: the highest levels of M opsin mRNA were present in the central retina near the fovea and optic disk, the lowest levels were in the far peripheral retina near the ora serrata, and there was a central-to-peripheral gradient of increasing L mRNA. In order to determine whether this pattern of variation is common for humans, we measured the L:M mRNA ratio in grid squares along the horizontal and vertical meridia for 11 retinas from 10 donors. The results are shown in Fig. 4 where the same general trend observed in Fig. 3 was also evident along the horizontal and vertical meridia for all 11 donor retinas. At the most peripheral retinal locations, near the ora serrata, the proportion of M opsin mRNA was quite low, representing 20% or less of the total L plus M opsin mRNA, and in some cases M opsin mRNA

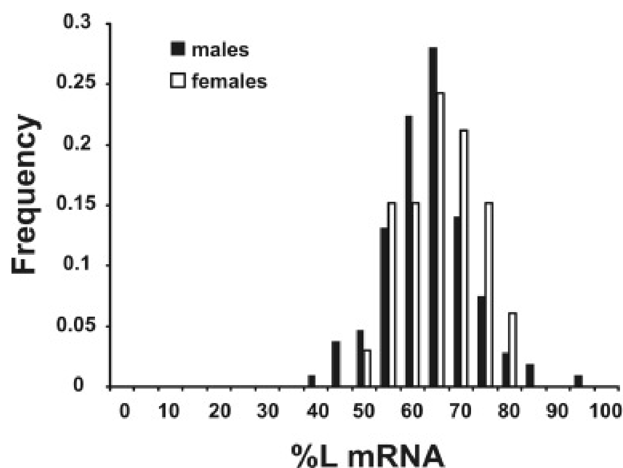

Fig. 2. Distribution of percent L opsin mRNA in central retina from 140 eye donors. The L:M ratio was determined independently by 2 methods: real-time quantitative PCR as described in Materials and Methods, and by quantitative restriction digestion analysis of PCR products, as described previously (Hagstrom et al., 1998). There was good correspondence between the values obtained by the two methods. Plotted is the average percentage L mRNA estimates from the two methods. Black bars are males, and white bars are females

was not detected in the most peripheral locations (e.g., retina 980237 in Fig. 4). The average for the foveas from the 11 retinas was 64% L, which corresponds well to the average cone ratio of 66% L estimated using flicker photometric ERG (Carroll et al., 2002) and 66% L opsin mRNA for the 140 eye donors in Fig. 2.

The topographical expression pattern of L and M opsin genes in baboons compared to humans was evaluated. The percentage of the L plus M opsin mRNA was measured for two columns of grid squares extending from the center to the superior ora seratta for five baboon retinas. The baboon retinas showed a similar trend to humans with an increase in the relative amount of L opsin mRNA in a central-to-peripheral gradient (Fig. 5). The average for the baboon retinas was 42% L opsin mRNA overall, with 32% L in the fovea, which is considerably lower than for humans and corresponds well to previously published estimates of 38% of the L plus M cones being L (Marc & Sperling, 1977). Interestingly, we have also examined the L:M mRNA ratio along the horizontal meridia of three macaque retinas and did not observe a well-defined central-to-peripheral gradient of L:M mRNA (data not shown, McMahon et al., in preparation). The results for macaque monkeys confirm previously reported findings that showed variability in the L:M mRNA ratio within macaque retina, but no gradient (Deeb et al., 2000).

Discussion

In the developing primate retina, S and L/M cones differ in morphology, in the timing of the onset of opsin expression, and in the types of second order neurons with which they form synaptic connections (Bumsted et al., 1997; Bumsted & Hendrickson, 1999; Xiao & Hendrickson, 2000). These major differences indicate that S cones are indeed a different cell type than are L/M cones. However, other than the photopigment, there are no known characteristics that distinguish L from M cones, suggesting that they represent a single cell type, and that the final identity as an L versus M cone is simply determined by the opsin gene that is

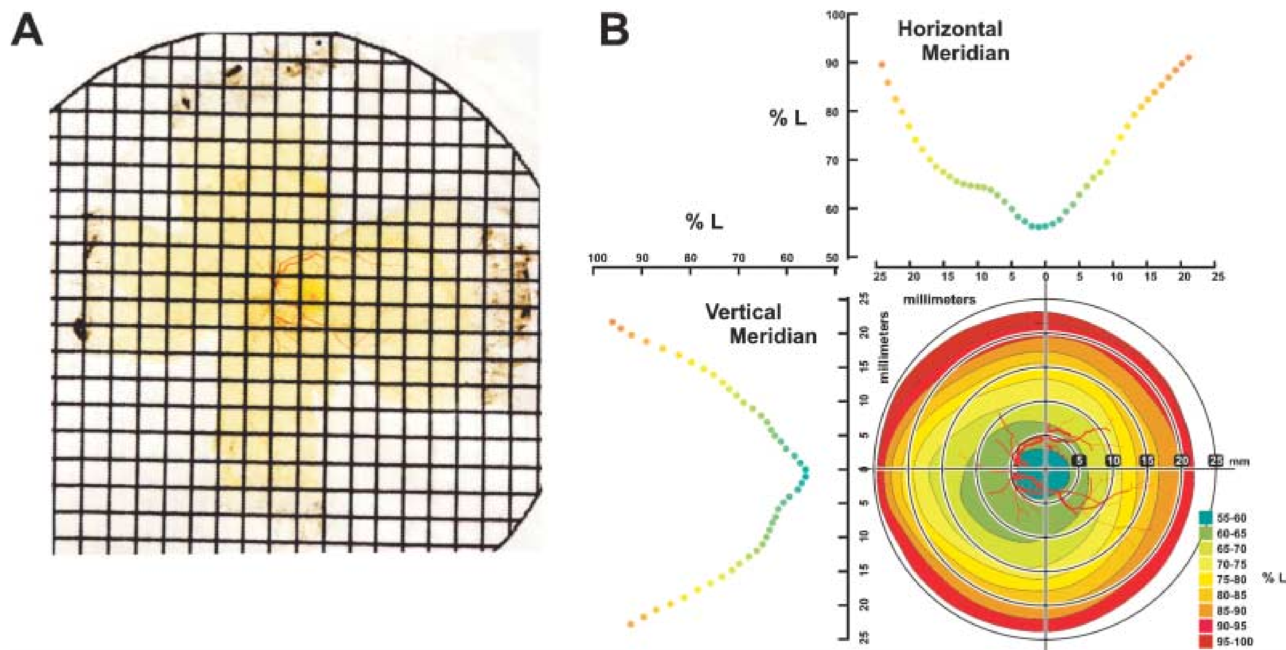

Fig. 3. Topographical map of the percent L opsin mRNA in a complete male retina. (A) Human retina flat mounted on nylon filter printed with a 3 mm^2 grid. (B) Recreated image of the flat mounted retina in A is color coded to indicate the %L mRNA in each square (lower right). The average of quadruplicate estimates of the % L mRNA for each grid square along the horizontal (upper right) and vertical (lower left) meridia from real-time quantitative reverse transcriptase PCR were used to create the color coded dotted lines shown by extrapolating between the measured data points.

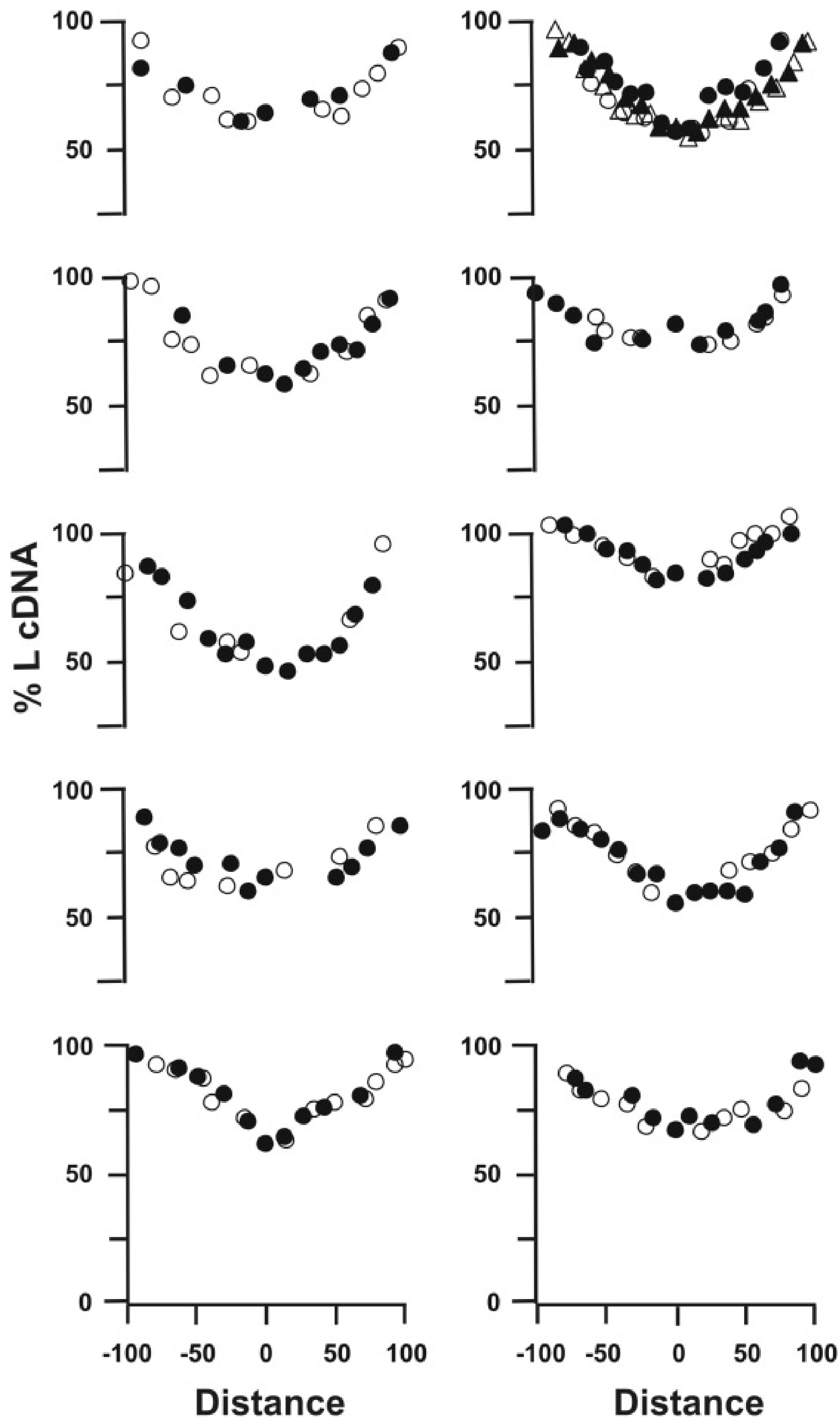

Fig. 4. Real time quantitative PCR estimates of the %L mRNA along the horizontal and vertical meridians for 10 male eye donors. On the abscissa numbers indicate the percentage of the distance between the fovea (0) and ora serrata (±100). Negative numbers are used for inferior and nasal, and positive numbers for temporal and superior. From top to bottom on the left are donors 980375, age 72 years, left eye; 971165, age 66, right eye; 971110, age 63, left eye; 980262, age 36, right eye; donor 921057, age 12, right eye. On the right, from top to bottom are donors 971155, age 71 years, right and left eyes; 000224, 76 yrs, right eye; 000237, age 48; right eye; 980440, age 67, right eye; 980476, age 57, right eye. For donors 000224 and 000237, each data point is the average of quadruplicate real-time quantitative RT-PCR measurements, for all others each data point is the average of triplicate measurements. For all donors, open symbols are data from the horizontal meridian, closed circles are from the vertical meridian. For donor 971155, circles are for the right eye, triangles are for the left eye.

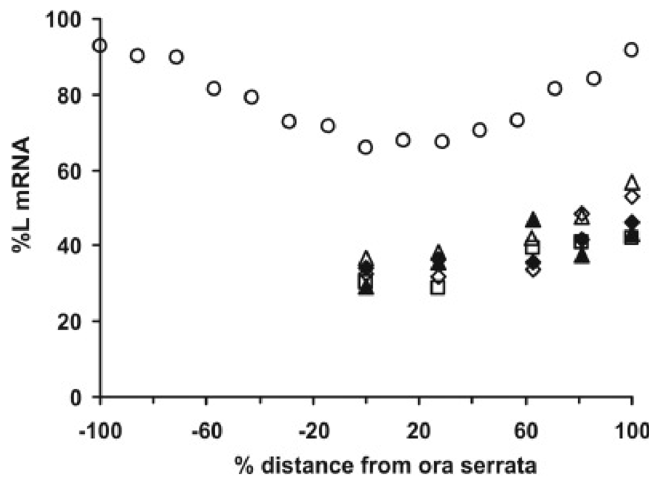

Fig. 5. Topographical map of %L mRNA in baboon retinas compared to humans. Open circles are the normalized average % L mRNA for the 11 human retinas in figure 4 plotted as a function of the percentage of the distance between the fovea and the ora serrata. Each of the remaining data points is the average of quadruplicate real time quantitative PCR estimates of the %L mRNA in grid squares from five flat-mounted baboon retinas from four animals.

expressed (Nathans, 1999; Wang et al., 1999; Smallwood et al., 2002). An enhancer that has been termed the locus control region (LCR) is shared by the tandemly arrayed L and M genes in Old World primates and is required for normal opsin gene expression (Nathans et al., 1989; Wang et al., 1992). Nathans and colleagues have proposed that the LCR stochastically binds to the promoter of an X-chromosome visual opsin gene in a one-time irreversible event that simultaneously determines the L or M fate of the photoreceptor, ensures long-term mutually exclusive expression of L or M opsin, and sets the L:M cone ratio (Smallwood et al., 2002). In humans, the L gene is normally positioned first in the array, followed by one or more M genes, so the L gene is the one most proximal to the LCR. The idea that linear proximity favors the L gene for expression has been hypothesized to account for the average 2:1 L:M cone ratio typical of humans (Winderickx et al., 1992; Wang et al., 1999; Smallwood et al., 2002). The order of the L and M opsin genes on the X-chromosome is conserved across humans, macaques, and baboons (McMahon et al., in preparation), yet the ratios of L:M cones (Marc & Sperling, 1977) and mRNA are reversed in the baboons. Thus, linear proximity alone cannot account for the fact that the average human has twice as many L as M cones. Instead, these results provide evidence that other factors, such as DNA polymorphisms that contribute to the success of the L *versus* M gene promoters in associating with the LCR, are instrumental in determining the L:M ratio.

The process by which the LCR binds to an L or M gene promoter is envisioned to be entirely random; however, the observed foveal-to-peripheral gradient of increasing L:M mRNA ratio represents a non-random feature of opsin gene expression. The gradient of opsin gene expression parallels that previously described for other developmental processes (LaVail et al., 1991; Bumsted et al., 1997; Martin et al., 2000; Xiao & Hendrickson, 2000), suggesting that it may be a consequence of the sequence of events that occur in the developing retina. The temporal and spatial patterns of L/M opsin gene expression during development are similar for humans and macaques (Xiao & Hendrickson, 2000). Chromatin remodeling is associated with differentiation and controls activation and silencing of gene expression in an ongoing, dynamic process that occurs before and after cells undergo their final cell division (Ringrose & Paro, 2004). Accessibility of transcriptional complexes to genes is improved or restricted by chromatin modifications that are transmitted through mitosis from mother to daughter as a record of the cell's transcriptional history. Daughter cells can change the epigenetic chromatin marks; however, this capacity diminishes as development progresses, until ultimately each cell is locked into a specific pattern of gene expression that is characteristic of its differentiated state. Cells at the retinal periphery are born late relative to cells in the fovea (LaVail et al., 1991), and therefore at the time of their birth and later when opsin gene expression is initiated, the peripheral cells have accumulated a much longer transcriptional history than foveal cells. The gradient of increasing L:M mRNA with distance from the fovea may be a consequence of developmental events such as chromatin remodeling that diminish the capacity of peripheral cells to express M opsin. The relative length of gestation and the relative size of eyes from largest to smallest is humans $\gg$ baboons $>$ macaque. Thus, the time lapse between events occurring in central *versus* peripheral retina may be significantly longer in humans, and may produce the pronounced foveal-to-peripheral gradient in L:M mRNA ratio and an L dominance of the peripheral human retina.

Whether or not the circuitry for color vision is wired by random connections is a controversial issue that has been difficult to resolve. For example, conflicting results have been reported (Martin et al., 2001; Diller et al., 2004), but whether the inconsistencies are real or caused by experimental conditions remains unclear (Solomon et al., 2005). Nevertheless, the probability that random wiring could produce red-green opponent peripheral midget ganglion cells must be compared to the observed frequency of such opponent cells to evaluate the merits of the random wiring hypothesis, and this will require knowledge of the topography of L and M cones in the peripheral primate retina. In a retina with an extremely biased L:M cone ratio, as in the peripheral human retina, a random wiring mechanism could easily produce peripheral midget ganglion cells that have receptive field centers with a sufficiently pure L cone input to be spectrally opponent. In contrast, macaques and baboons have nearly equal numbers of L and M cones in the peripheral retina, so that in the absence of significant local areas of clumping, a random wiring mechanism is unlikely to produce red-green opponent peripheral midget ganglion cells. However, if cones of the same class occur in clumps in the peripheral retina, this would greatly increase the probability that random wiring would produce red-green opponent cells. During the time period from midgestation to birth there is a tremendous expansion in the area of the retina (LaVail et al., 1991; Bumsted et al., 1997) and this process intermixes cells, which to some extent disrupts clumps of clonally related cells (Reese & Tan, 1998; Reese et al., 1999). Non-random topological arrangements of L and M cones are expected to be observed to the extent that clonally related cells remain in proximity. Cellular migration associated with formation of the fovea is much greater than the lateral spread of cells associated with expansion of retinal area (LaVail et al., 1991), yet even at one degree eccentric from the fovea, a tendency for local "clumpiness" of L and M cones has been observed (Hofer et al., 2005). Thus, in peripheral retina, larger clumps of clonally related cells may be found. Resolution of the controversy between the random and selective connections hypotheses must await future experimentation; however, the results reported here underscore the

dramatic species differences in the topography of L and M cones in the peripheral primate retina that will have a bearing on the interpretation of the experimental data.

Acknowledgments

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