

Research Note

More than Three Different Cone Pigments Among People with Normal Color Vision

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A fundamental feature of normal color vision is that red and green lights can be mixed to appear identical with a monochromatic yellow light. Another characteristic of normal color vision is that people often disagree on the amounts of red and green needed in the mixture to exactly match the yellow. Comparison of such color vision differences with photopigment gene differences reveals that a serine/alanine polymorphism at amino acid position 180 of X-encoded pigments can account for this type of color vision variation. This amino acid change shifts the spectrum of the pigment produced by about 6 nm, a value that would predict a larger minimum color vision difference between individuals than is actually observed. This discrepancy can be explained if, counter to the Young–Helmholtz theory as the explanation of trichromacy, many people with normal color vision have more than three spectrally different cone pigments.

Photopigments Cones Genetics Color matching Color anomaly

INTRODUCTION

It is a long favored view that three types of visual pigments underlie human color vision and that they are the same for all who enjoy normal color vision. Contrary to this conventional wisdom, people with normal color vision do not all have the same cone pigments. Individual differences in the spectral positions of normal pigments are manifest as differences in color vision. When asked to mix red and green primary lights to match the appearance of a standard yellow light, people who differ in middle wavelength sensitive (M) or long wavelength sensitive (L) cone pigments disagree in the proportion of the mixture primaries required (Neitz & Jacobs, 1986, 1990; Shevell & He, 1992; Lutze, Cox, Smith & Pokorny, 1990; Lindsey, Winderickx, Sanocki, Teller, Deeb & Motulsky, 1992; Elsner & Burns, 1987; Eisner & MacLeod, 1981; Waaler, 1967). The exact nature of the cone pigment differences that yield these individual differences in normal color vision and their underlying cause, have been matters of debate (Alpern & Moeller, 1977; Jordan & Mollon, 1988; Webster & MacLeod, 1988; *Frontiers of visual science: Proceedings of the 1985 symposium*, 1987).

Results from comparative studies of cone pigment genes identify molecular differences in the X-encoded opsins that affect photopigment spectral sensitivities. These suggest that amino acid differences at just three positions in the opsins can account for spectral differences among the middle-to-long wavelength pigments (Neitz, Neitz & Jacobs, 1991a). Of the three substitutions, the one (Ser¹⁸⁰ for Ala¹⁸⁰) that produces the smallest spectral shift (5–7 nm) may occur among normal pigments. Nathans, Thomas and Hogness (1986) determined the nucleotide sequences of a normal L pigment gene isolated from genomic DNA and two L pigment genes from a cDNA library representing several dozen human eyes. The L pigment encoded by the genomic gene differed from the two L pigments encoded by the cDNAs at the site specifying position 180.

We have now tested the hypothesis that spectral variations in both the M and L pigments produced by serine/alanine substitutions at position 180 can explain normal color vision variations. Specific nucleotide differences in the X-linked pigment genes of males with normal color vision were examined and compared with individual differences in Rayleigh color matches. For each subject we obtained estimates of the relative proportion of genes that encode Ala¹⁸⁰ vs Ser¹⁸⁰ among all his X-linked pigment genes. This allowed an estimate of how much of the color matching variation among these subjects with normal color vision might be apportioned to polymorphism at this single amino acid position in the M and L pigments.

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METHODS

Subjects

Observers were sixteen young males. All but two of the observers that served in this study were participants in one of two earlier studies of Rayleigh match variation in color normal observers (Neitz & Jacobs, 1986, 1990).

Rayleigh matches

The Rayleigh match results of all but two observers were reported earlier (Neitz & Jacobs, 1986, 1990). A three-channel Maxwellian view optical system was used. The subjects viewed an annular stimulus field with an inner diameter of 3° and an outer diameter of 11°. The mixture of red (690 nm) primary and green (546 nm) primary lights was viewed in alternation with a 600 nm comparison light. The experimenter controlled the proportion of red to green light in the mixture and the subject controlled the intensity of the comparison light. A series of red/green settings were presented. On each trial, once the observer adjusted the intensity of the comparison to achieve the closest possible match he made one of three choices: the mixture and comparison lights were indistinguishable, the mixture appeared as if too much red had been added, or too much green had been added. The settings judged "redder" and "greener" on all trials defined the limits of the matching range. For each subject, the midpoint of that range was used in the comparisons presented here. The matches obtained with this procedure are highly reliable. Earlier, a group of eighteen subjects were tested on two occasions, separated by 5 months (Neitz & Jacobs, 1990). The average absolute difference between test and retest was 0.0066 R/R + G (test/retest $r = 0.99$).

DNA isolation, amplification and sequencing

Genomic DNA was isolated from peripheral leukocytes by the method of Poncz, Solowiejczyk, Harpel, Mory, Schwartz and Surrey (1983) and used in the polymerase chain reaction (PCR) to amplify exon 3 of the X-linked visual pigment genes. All PCR reactions

were carried out in duplicate for each subject. PCR primers hybridized to both M and L pigment genes. The 5' primer is specific to the 3' end of intron 2 and its sequence is 5' TTCCCCTTTGCTTTGGCTCAAAGC 3'. The 3' primer is homologous to the 5' end of intron 3 and is 5' GACCCTGCCCACTCCATCTTGC 3'. The intron sequences were determined by J. Nathans (personal communication) from genomic clones of L and M pigment genes. The sequences corresponding to the primers begin 38 nucleotides from the 3' end and 48 nucleotides from the 5' end of exon 3 respectively. 1 μ g of genomic DNA was used in each PCR reaction and the reactions contained: 50 mM KCl; 10 mM Tris-HCl, pH 8.3; 200 μ M of each of the deoxynucleotide triphosphates; 1 μ M of each primer; and 1.5 mM MgCl₂. The thermal cycling parameters were one cycle of 95°C for 5 min followed by 30 cycles, each of 94°C for 30 sec, 67°C for 30 sec and a final cycle of 67°C for 5 min. PCR reactions were subject to gel electrophoresis through 4% seaplaque GTG low melting temperature agarose and, for each subject, the band corresponding to amplified visual pigment gene segments was excised from the gel. Approximately 1 ng of amplified DNA in the gel slice was then used in asymmetric PCR and nucleotide sequence analysis was performed on the resulting single strand DNA. Asymmetric PCR thermal cycling parameters were essentially the same as those described above except that 40 cycles, each of 94°C for 30 sec and 67°C for 30 sec were used. The 5' primer (described above) was present in limiting concentrations (0.02 μ M) in the asymmetric PCR reactions and was used as the primer in the sequencing reactions. DNA sequencing reactions were carried out using the Sequenase Version 2.0 kit (United States Biochemical) and deoxyadenosine 5'(α -thio) triphosphate [³⁵S]. Each series of sequencing reactions was run on duplicate sequencing gels. DNA sequencing gels were exposed to X-ray film for 16–24 hr and the X-ray films were scanned with the Biorad 620 video densitometer. The scans were analyzed with the Biorad 1D analyst software.

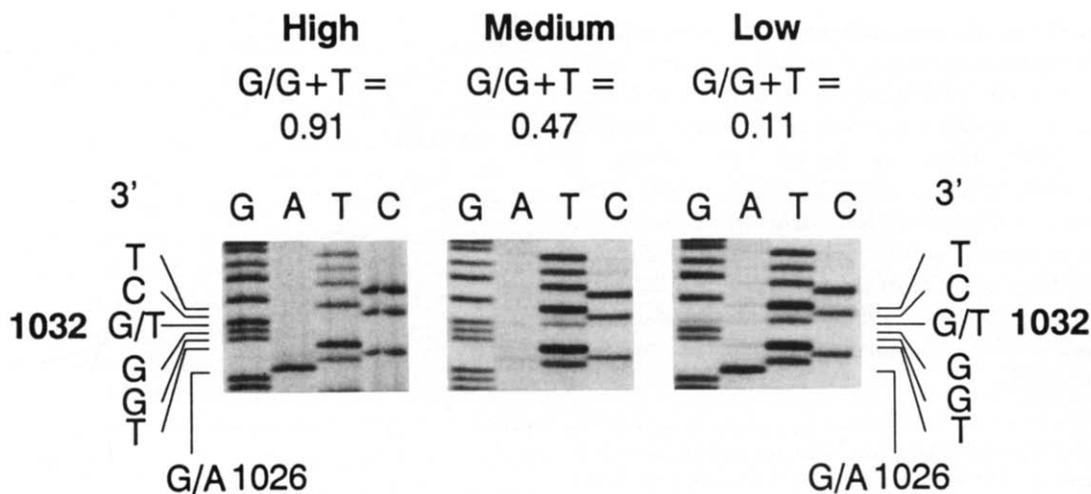


FIGURE 1. Nucleotide sequences from three representative color normal males who differ in the proportion of pigment genes with G at position 1032 (numbering convention from Nathans *et al.*, 1986). The proportion G/G + T was determined from densitometer scans of the sequence ladder autoradiograms.

RESULTS

The substitution of Ser¹⁸⁰ for Ala¹⁸⁰ in the X-encoded cone opsins corresponds to a base change of thymine (T) for guanine (G) at nucleotide position 1032 in the genes. DNA sequence analysis spanning a region that includes nucleotide 1032 of the X-linked pigment genes is shown for three subjects in Fig. 1. There are widespread differences among subjects at position 1032. These are exemplified by the sequences in Fig. 1. The sequence ladders (Fig. 1) correspond to residues 1024–1043 of the sense strands of the X-linked pigment genes. For example, the sequence ladder of the subject, labeled "High" G/G + T, reads from bottom to top (1024–1043): GG ATC TGG GCT GCT GTG TGG (the italic G is position 1032). In each autoradiogram, the sequences of all of the particular subject's X-linked pigment genes are represented. At each nucleotide position that is identical among all a subject's X-linked pigment genes, a single band appears in the autoradiogram. Nucleotide differences among a subject's X-linked pigment genes are evident in the autoradiogram as bands in multiple lanes at one nucleotide position. Most of the sixteen subjects had bands corresponding to both G and T at position 1032, i.e. most have some X-linked pigment genes that encode alanine at amino acid position 180 and others that encode serine at 180. The two examples (labeled "medium" and "low") in Fig. 1 have bands in both the G and T lanes at position 1032.

For the sixteen subjects, autoradiograms were scanned with a densitometer and the relative optical densities in the G and T lanes at position 1032 were determined. The band intensity ratio (G^{1032}/T^{1032}) was taken as an indicator of the relative ratio of the number of X-linked pigment genes with G¹⁰³² to the number with T¹⁰³². These were converted to the relative proportions of genes with G at 1032, G/G + T. The G/G + T values ranged from 0.08 to 0.95 across subjects. Two separate genomic DNA samples from each subject were analyzed. The measured proportions, G/G + T, obtained from the different samples were highly reliable within subjects. The average absolute difference between the two measurements for each individual was 0.03. To derive an objective measure for comparison across all subjects, we scanned the area of the autoradiogram that corresponded to position 1032 in both the G and T lanes even though a few subjects had no apparent band in the T lane at position 1032. For those few subjects, the noise in the T lane was enough above background that this procedure yielded G/G + T values of slightly < 1.0 even though they probably have no genes with T at 1032. An example of this is shown in Fig. 1. The person in Fig. 1 with the highest proportion of genes with G (labeled "High") has a calculated G/G + T value of < 1.0 even though all of his X-linked pigment genes probably have G at 1032. Conversely, like the subject with the low G/G + T shown in Fig. 1, none of the sixteen subjects examined appeared (by inspection) to be completely lacking genes with G at 1032, i.e. all had a clearly defined band in the G lane.

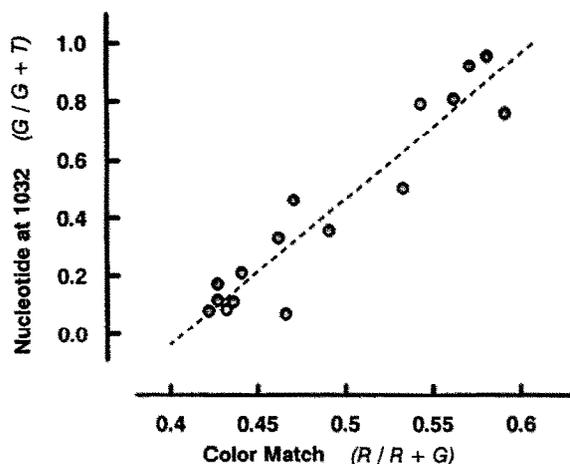


FIGURE 2. Comparison of individual differences in color vision with differences at nucleotide 1032 of the pigment genes. The proportion of red light (690 nm) in a mixture with green light (546 nm) that each subject required to match the appearance of a standard light (600 nm) is plotted vs the relative proportion of X-chromosome pigment genes that have G at position 1032 (as measured from band intensities in autoradiograms of sequencing gels).

The proportions, G/G + T, determined from sequence analysis were compared to the proportions of red light in the mixture required to match the comparison light in Rayleigh color matches. The result of this comparison between individual differences at position 1032 of the genes and the Rayleigh match differences is illustrated in Fig. 2. There is a high correspondence between the two measures ($R^2 = 0.90$).

There is another highly polymorphic site (position 1026) very near position 1032; in different genes either a guanine (G) or an adenine (A) base occupies position 1026. The polymorphisms at 1032 and 1026 did not correlate well. This is illustrated in Fig. 1. Two subjects (labeled "High" and "Low") with quite disparate proportions of genes with G at position 1032 are identical at position 1026, i.e. both subjects appear to have an A at position 1026 of all their X-linked pigment genes. The middle sequence of Fig. 1 shows the opposite extreme for position 1026; all of that subject's genes have a G at 1026. The independence of these two polymorphisms supports the conclusion that the behavioral differences are caused by the one at 1032 and not by other polymorphic sites.

DISCUSSION

It was shown earlier that two L pigments with about a 6 nm spectral separation had a serine/alanine substitution at position 180 as the only non-homologous difference (Neitz *et al.*, 1991a). More recently, two M pigments from human protanopes that differ at position 180 were found to have a spectral separation of about 7 nm (Neitz, Neitz & Jacobs, 1991b). Thus, a substitution of Ser¹⁸⁰ for Ala¹⁸⁰ produces a redward spectral shift of about 6 nm, in either an M or L pigment. The polymorphism at this site, therefore, could produce two types of L pigments and two types of M pigments in the color normal population. These subgroups of each the

M and L pigments were apparent earlier in microspectrophotometric results (Dartnall, Bowmaker & Mollon, 1983).

A person whose M and L pigments all have Ala¹⁸⁰ would be expected to require a high proportion of red light in the Rayleigh mixture to compensate for the lower sensitivity of both pigment types to the red primary. A 6 nm redward shift of the L pigment, or a 6 nm redward shift of the M pigment are predicted to have very similar effects on this color match (Neitz & Jacobs, 1990). A person whose L pigments have serine and M pigments have alanine would make a similar match to one whose L pigments have alanine and M pigments have serine—both would require less red light in the match than if all pigments had Ala¹⁸⁰. A person whose M and L pigments all have Ser¹⁸⁰ would require less red light in the Rayleigh match than any of the three examples above. The proportion of pigments (no matter M or L) that have Ala¹⁸⁰ should, thus, be related to the proportion of red light in the Rayleigh match, just as we found.

Lindsey *et al.* (1992) also recently found a relationship between Ser¹⁸⁰/Ala¹⁸⁰ individual differences and Rayleigh color match variation. They used PCR conditions that were intended to selectively amplify only L pigment genes. The amino acid encoded at position 180 was compared with differences in Rayleigh matching. On average, those subjects with Ala¹⁸⁰ encoded by the selectively amplified gene required more red light in the Rayleigh mixture than those with Ser¹⁸⁰. They concluded that spectral shifts produced by a Ser¹⁸⁰/Ala¹⁸⁰ substitution in the L pigment influences the Rayleigh matches of color normal males.

Our ideas of which cone mechanisms vary in normal color vision have changed over the years. From our earliest analysis of color match variation, we suggested that among observers with normal color vision there is variability in the L cone mechanism but there is no corresponding variability in the spectral position of the M cone mechanism (Neitz & Jacobs, 1986). That analysis relied on the assumptions that there were only two types of males with regard to photopigment complement and that only one cone mechanism varied. Based on those assumptions, a calculation that used the average red/green ratios and the average intensities of the comparison light for the two main groups of males suggested that it was the L cone mechanism that varied. Later results indicated that males whose matches fell outside of the two main groups had different pigment complements than those who fell within them. We concluded that the assumptions of only two types of men and that only one cone mechanism varied were incorrect and that particularly to account for the matches of color normal males who fall outside the main groups, both M and L pigments must vary in spectral position (Neitz & Jacobs, 1990). Webster and MacLeod (1988) also concluded that both M and L cone pigments vary among observers with normal color vision. The present molecular genetic results do not distinguish nucleotide differences among the M pigment genes from those among the L pigment genes. However, given that both pigments vary, the high

correspondence between the genetic and behavioral results over the full range of normal color matches shown here indicates that, among color normal males, both the M and L pigment spectral variation results from polymorphism of the respective genes at the site specifying amino acid 180.

Genetic changes that shift the pigment spectra are not expected to completely account for color match variation in this sample. Other factors, e.g. differences in ocular pigmentation and in visual pigment optical density also influence the Rayleigh match. These must be relatively minor contributors to the Rayleigh match variation in our sample of young male observers because, despite them, there is a high correspondence between the genes and behavior (Fig. 2). Individual differences in the proportion of X-linked pigment genes that encode alanine vs serine at position 180 are, alone, sufficient to predict 89% of the variance in the observed color matches. This further suggests that, among these observers, this polymorphism is likely to be the only source of between-subject differences in the spectral positions of the M and L cone pigments.

Implications for theories to explain human trichromacy

Trichromacy is one of the earliest-established facts of human color vision (for a discussion see Brindley, 1970). The celebrated theory proposed by Young (1802) that there exist three types of light sensitive “particles” (visual pigment molecules in modern terms) has long been used as explanation of why human vision is trichromatic. However, the findings here suggest that in addition to one S pigment, the polymorphism at position 180 produces four different middle-to-long wavelength cone pigments in the normal population: a longer M and a shorter M pigment, 5–7 nm separated in peak and two similarly separated, longer and shorter, L pigments. If more than three of these occur in normal eyes it would invalidate the three pigment hypothesis as the explanation of trichromacy.

If only three different cone pigments occur in one normal eye (one S, one M and one L), then combinations of the pigments described above would lead to three easily distinguishable groups of color normal males in the Rayleigh match: (1) those with both the longer L and M pigments, (2) those with either the longer L or the longer M pigment and (3) those with both the shorter L and M pigments. The difference in the color match between the most extreme groups would be that predicted by a cumulative shift in pigments of about 12 nm. The difference between the intermediate group and either extreme would be that predicted by a pigment shift of about 6 nm. This is illustrated in the lower half of Fig. 3.

Under stimulus conditions designed to favor the detection of variation in the spectral positions of M and L cone pigments we find that the Rayleigh color matches of normal males are distributed into groups (Fig. 3, top). The mean differences between groups, however, are not those predicted by pigment shifts of 6 nm; instead, the pigment shift required to produce the difference between adjacent groups is slightly < 3 nm (Neitz & Jacobs,

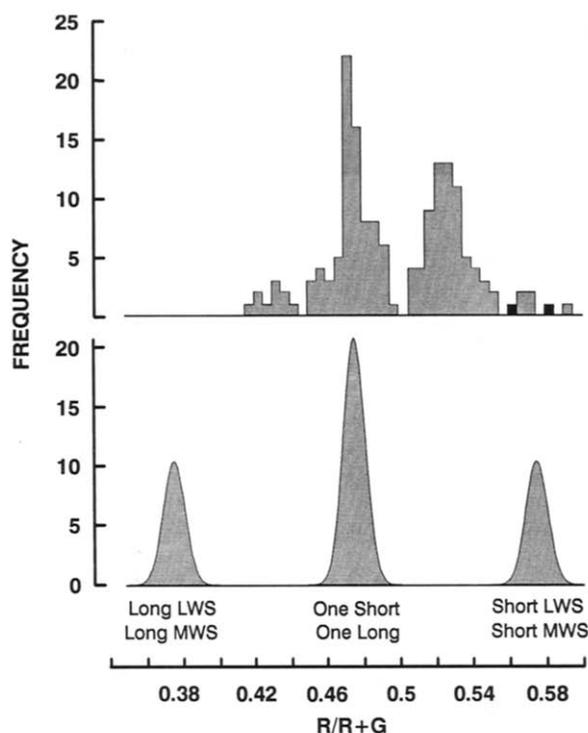


FIGURE 3. (Top) Distribution of Rayleigh color matches for 162 males with normal color vision. These were compiled from Neitz and Jacobs (1986, 1990); subjects of Fig. 2 were from one of those studies except for the two shown in black. (Bottom) Distribution of color matches predicted by the hypothesis that each person has only three cone pigments—one S pigment, one of two M pigments (different by 6 nm), and one of two L pigments (different by 6 nm). Some normally distributed variation around the prediction for each group has been added to stimulate individual differences in preretinal filtering, photopigment optical density, and measurement error.

1990). These intermediate color matches can be explained if some males with normal color vision express a third X-encoded pigment, i.e. they express both of the different M pigment types (or both L types). If such pigment pairs act together as a single mechanism in color matching then, for example, people with both M types would produce Rayleigh matches that are intermediate to the three predicted groups shown in the lower half of Fig. 3, just as are observed (Fig. 3, top).

We note that the present results confirm the earlier findings that serine/alanine substitutions at position 180 produce spectral shifts with magnitudes of 5–7 nm in both M and L pigments. The disparities between the observed color matches of the most extreme subjects are those predicted by cumulative L plus M spectral shifts of nearly 10 nm. The polymorphism at position 180 accounts for these large color vision differences well. If a serine/alanine substitution at position 180 produced a spectral shift much smaller than 5–7 nm in either the M or L pigment it could not account for these extremes. Also, if the serine/alanine substitution produced a spectral shift in the M pigment that was much different in magnitude than the one it produces in the L pigment, then our genetic measure (that does not differentiate M from L pigment genes) would not correlate well with the behavioral differences. The largest differences between color matches observed are not quite as large as the

prediction shown in the lower half of Fig. 3 where the cumulative L plus M shift between the two most extreme groups equals 12 nm. Our data would predict this. If people usually express all of their X-linked pigment genes then the only way that two people could differ by the full 12 nm is if one had all genes encoding Ala¹⁸⁰ and the other had all genes encoding Ser¹⁸⁰. Some subjects do indeed appear to have all genes encoding Ala¹⁸⁰ but we found none who appear to have all genes encoding Ser¹⁸⁰. If no one completely lacks genes that encode Ala¹⁸⁰ then no one with a match like the left-most prediction in Fig. 3 (lower distribution) is expected.

The pigment genes of people with normal color vision are polymorphic with regard to the amino acid encoded at position 180, the site responsible for a 5–7 nm spectral shift. Also, most people have more than two X-linked pigment genes (Nathans *et al.*, 1986). These facts alone suggest that some people are likely to have X-linked genes encoding more than two spectrally distinct pigments. The validity of the three pigment hypothesis thus hinges on the question: are more than two middle-to-long wavelength pigments expressed in one eye? The differences between the distribution of color matches predicted by the theory that only two X-linked pigment genes are expressed and the observed distribution (in Fig. 3) indicates that more than two spectrally different X-linked pigment genes are expressed in many people.

A consideration of the degree of correspondence between the genetic and behavioral results further supports the conclusion that more than two X-linked pigment genes are expressed. If each person expressed only one M and one L gene, any unexpressed pigment genes would contribute to the measured G/G + T proportion but not to the measured behavior. The result would be an inability to predict precisely color matching behavior from the genes. To give a simple illustration, among people with three X-linked pigment genes, for the four possible G/G + T proportions (3/3, 2/3, 1/3, 0/3) two of them (3/3 and 0/3) would produce predictable behavior, but the other two (2/3 and 1/3) would produce unpredictable behavior depending on which two of the three genes were expressed. If the unexpressed gene is equally likely to have G or T at position 1032 and its identity is independent of the expressed genes, the average difference between the observed behavior and that predicted by the genes would be one-sixth of the total range of observed behaviors. The genes predict behavior better than that. The average difference between predicted and observed color matches is 0.097 (SD = 0.0709) times the total range of observed color matches.

The high correspondence between predicted and observed matches suggests that the three pigment hypothesis is not the explanation for human trichromacy—more than one M and one L pigment gene do contribute to behavior in many people. (Interestingly, however, the “extra” pigments do not appear to confer vision that extends beyond trichromacy.) Another recent result supports a conclusion that males with normal color vision have more than three spectrally different cone types. A fundamental property of color vision based on three

cone types is that color matches made with an unadapted eye will persist after selective color adaptation (Brindley, 1970; Jameson & Hurvich, 1972). To the contrary, some color normal males change their color matches after adaptation to red light, as predicted, if they have more than three spectrally different cone receptor types (Neitz, Neitz & Jacobs, 1991c).

For decades models of the genetic basis for normal and defective color vision were devised under the assumption that there are two X-chromosome pigment gene loci. That assumption has proved invalid. Molecular genetic results reveal that most people have more than two pigment genes on the X-chromosome (Nathans *et al.*, 1986; Drummond-Borg, Deeb & Motulsky, 1989). Now evidence is accumulating to suggest that the idea, that individuals with normal color vision have only three spectrally different cone photoreceptors, is also invalid.

The subgroups of L and M pigments that occur in normal color vision are spectrally separated by about 5–7 nm. 5–7 nm may seem a small translation in pigment spectra. However, the sensitivity ratio of two M pigments, so separated, changes by more than a factor of 2 between wavelengths near 530 and those longer than 620 nm. If two such pigments were in separate cones, wired appropriately, this is enough to produce an ample color signal. For example, the ratio of L-to-M pigment sensitivity differs by about a factor of 2 for monochromatic lights of 580 and 605 nm. In people with normal color vision, the color difference that corresponds to this change in sensitivity ratio is dramatic—from yellow to red-orange. There is a second example. The two X-encoded pigments of anomalous trichromats have a spectral separation of about 5–7 nm (Pokorny, Smith & Katz, 1973; Nagy, Purl & Houston, 1985) and most of them have sufficient color discrimination ability to reject easily the color matches of normal trichromats (Pokorny, Smith, Verriest & Pinckers, 1979). Normal human color vision is uniformly trichromatic [even female heterozygous carriers of color anomaly, who have been long believed to have more than three cone types, appear trichromatic; Nagy, MacLeod, Heyneman and Eisner (1981)]. The extra pigment types in people with normal color vision are sufficiently different to support tetra- or even pentachromacy. The fact that they don't indicates that the trichromacy of normal vision has its origin at a level of the visual pathway beyond that of the cone pigments, likely beyond the receptors.

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