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Science **315**, 1723 (2007);
DOI: 10.1126/science.1138838

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Emergence of Novel Color Vision in Mice Engineered to Express a Human Cone Photopigment

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Changes in the genes encoding sensory receptor proteins are an essential step in the evolution of new sensory capacities. In primates, trichromatic color vision evolved after changes in X chromosome–linked photopigment genes. To model this process, we studied knock-in mice that expressed a human long-wavelength–sensitive (L) cone photopigment in the form of an X-linked polymorphism. Behavioral tests demonstrated that heterozygous females, whose retinas contained both native mouse pigments and human L pigment, showed enhanced long-wavelength sensitivity and acquired a new capacity for chromatic discrimination. An inherent plasticity in the mammalian visual system thus permits the emergence of a new dimension of sensory experience based solely on gene-driven changes in receptor organization.

The retinas of most nonprimate mammals contain two classes of cone photopigments that support dichromatic color vision (1). A cone pigment most sensitive to short (S) wavelengths is encoded on an autosome, and a second cone pigment most sensitive to middle (M) or long (L) wavelengths is encoded on the X chromosome. Many primate lineages have added an additional type of M or L pigment, permitting the evolution of trichromacy (2). In Old World primates, the third cone pigment arose by duplication of the ancestral X-chromosomal cone pigment gene (3). By contrast, in most New World monkeys, cone pigment diversity derives from polymorphic variation in a single X-linked gene (4, 5). In heterozygous females, X-chromosome inactivation produces a mosaic of spectrally distinct cone types, which provides the foundation for trichromacy. The polymorphic X-linked pigment genes in New World monkeys may represent the ancestral arrangement for primates, as judged by the observation that the same three amino acid substitutions confer the spectral difference between M and L pigments in all present-day primates (3). Color vision requires both multiple photopigments and appropriate neural wiring, and it has been argued that the organization of the primate retina, and in particular the low-convergence midgen bipolar and ganglion cell system, is such that the addition of a new class of cone photoreceptors may be all that is required for comparing M versus L cone signals (6–8).

Our experiments used genetically engineered mice to model the first step in the evolution of primate trichromacy. We asked whether the sud-

den acquisition of an additional and spectrally distinct pigment and its production in a subset of cones suffice to permit a new dimension of chromatic discrimination that would imply that (i) the mammalian brain is sufficiently plastic that it can extract and compare a new dimension of sensory input and (ii) the heterozygous female primate that first inherited an additional X-chromosome allele would have immediately enjoyed a selective advantage with respect to chromatic discrimination. We produced a line of mice in which most of the coding sequences of the normal mouse X-chromosomal M pigment gene [specifying a pigment with wavelength of maximum absorption λ_{\max} ~510 nm, hereafter “M”] was replaced with a human L pigment cDNA (specifying a hybrid pigment with a λ_{\max} of ~556 nm, hereafter “L”) (9). A similar knock-in mouse has recently been produced (10). As in New World monkeys, breeding yields mice with three distinct complements of M/L pigments: Males and homozygous females have either M or L pigments, and heterozygous females have a mixture of the two pigments. Earlier we observed that cones containing the L pigment could transduce light signals with an efficiency roughly equal to that of native M cones, as judged by the electroretinogram (ERG), and that the responses of individual retinal ganglion cells (RGCs) in M/L heterozygotes showed substantial cell-to-cell variability in chromatic sensitivity despite the large number of cones that contribute to the receptive field center of a typical mouse RGC. This functional heterogeneity likely reflects the spatial graininess of M versus L cone territories created by X-chromosome inactivation, which is consistent with the ~50- μ m average diameters for X chromosome–inactivation patches in the retina (9).

We first performed an analysis of cone-based spectral sensitivity using ERG flicker photometry (11) [see also supporting online material (SOM)]. The mouse S cone pigment is maximally sensitive in the ultraviolet range (12, 13); we minimized its activation by using lights of relatively long wavelength. Spectral sensitivity functions, obtained from mice whose retinas contained either the M

pigment ($n = 12$ mice) or the L pigment ($n = 17$ mice), were fitted with photopigment absorption functions (Fig. 1, A and B). The spectral maxima (512 and 556 nm) are close to those determined by earlier measurements of these pigments (9, 12, 13). Spectral sensitivity functions, similarly obtained from 86 M/L heterozygotes (Fig. 1C), were best fit by linearly summing the spectra from mice with either M or L pigments. On average, there is a twofold larger contribution from the M pigment than from the L pigment (presumably reflecting a bias in the M:L cone ratio), and there is substantial individual variability in this relative weighting (Fig. 1D). The latter likely reflects stochastic variation in X-chromosome inactivation (14).

Although heterozygotes have, on average, greater M than L sensitivity, this is not because the L pigment compromises cone viability or function. First, immunolabeling with antibodies to M/L opsin demonstrated nearly identical densities of M or L cones in retinas from animals expressing only one or the other of these pigments [mean M cone densities \pm SD = 8129 ± 995 per mm^2 and mean L cone densities \pm SD = 8288 ± 952 per mm^2 ($n = 2$ mice for each genotype)]. Second, ERG voltage versus intensity (V -log I) functions recorded from M or L retinas are indistinguishable when the intensity of the stimulating light is specified according to its calculated effectiveness for each pigment (Fig. 1E), implying that signals from the L and M pigments activate the ERG with the same efficiency.

To examine whether vision is altered by the added photopigment, we tested mice in a behavioral three-alternative forced-choice discrimination task. In this task, the animal was required to identify which one of the three test panels was illuminated differently from the other two, with the location of the correct choice varying randomly between trials (15). The L pigment has greater sensitivity to long wavelengths than does the M pigment, so one might expect enhanced behavioral sensitivity to such lights in mice whose retinas contain the L pigment. Increment thresholds were determined for 11 mice (four having only M pigment and seven M/L heterozygotes) by briefly adding either of two monochromatic test lights (500 and 600 nm) to one of the three stimulus panels, all of which were continuously illuminated with an achromatic light. The threshold differences between the 500- and 600-nm test lights were significantly smaller for M/L heterozygotes (Fig. 1F), indicating that these mice can extract visual information from L cones.

Color vision implies the ability to discriminate variations in spectral composition irrespective of variations in intensity. To assess this possibility, we derived brightness matches between a monochromatic test light and a series of standard lights of different wavelengths (see SOM) and then asked whether mice could discriminate between a 500-nm test light and two identical standard lights of 600 nm. At each trial, the location of the test light varied randomly among the three panels, and its intensity varied randomly in steps of 0.1 log

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units to encompass a range of ± 0.5 log units around the brightness match. As a control, we first tested a mouse whose retina contained M but not L pigment. As expected, despite extensive testing (totaling $\sim 15,600$ trials), no significant discrimination was achieved (open circle in Fig. 2A). With the use of the same procedures, two M/L heterozygotes also failed to show evidence of color discrimination (open square and triangle in Fig. 2A; $\sim 16,000$ trials per subject).

The two M/L heterozygotes that failed in the wavelength discrimination had relatively skewed M:L ratios (78:22 and 65:35). Unbalanced representation of M and L cones has little impact on human color vision (16, 17), but, with smaller numbers of cones, we thought that this might not be true for mice. After extensive training ($\sim 17,000$ trials), an M/L heterozygote with a more balanced M:L ratio (44:56) successfully discriminated 500-nm from 600-nm lights. The averaged asymptotic performance across the full range of brightness variation at this test wavelength is indicated by the vertical bar and arrow in Fig. 2A. The test wavelength was then progressively displaced in steps of 5 or 10 nm toward 600 nm; the mouse succeeded at each of these discriminations until the test and standard lights were separated by ~ 10 to 15 nm, at which point discrimination failed. This process was repeated for six other standard lights covering the range from 570 to 630 nm, yielding in each case a qualitatively similar result. As an internal control in these experiments, trials in which the test and standard lights were set to the same wavelength were randomly interspersed. The cumulative results of this control procedure (Fig. 2B) show that (i) discrimination failed when both chromatic and brightness cues were eliminated and (ii) the derived brightness matches accurately predict the points of discrimination failure. Two additional M/L heterozygotes also succeeded at the color discrimination task (Fig. 2A).

For the M/L heterozygotes, wavelength discrimination varied across the range tested, being most acute at 590 to 600 nm (Fig. 2C). The U-shaped wavelength discrimination function is qualitatively like those for other dichromatic visual systems (1); in particular, it is similar to wavelength discrimination functions for human tritanopes (18), a dichromatic subtype in which color vision is based only on M and L pigments with peaks at ~ 530 and ~ 560 nm, respectively. At present, we cannot fully explain the variation among M/L heterozygotes in the color discrimination task, but it presumably reflects some combination of individual variation in M:L cone ratio, vagaries in the spatial distribution of cone types vis-à-vis RGC receptive fields, or central factors related to memory, intelligence, or motivation.

To assess dichromatic color matches, we used a test light that was composed of an additive mixture of two monochromatic lights. Using the same brightness control measures as before, we progressively added 620-nm light to 530-nm light to determine what proportions could be discriminated from two monochromatic standard lights

(600 and 580 nm, in successive experiments). Results obtained from the M/L heterozygote with an M:L ratio of 44:56 (solid circles in Fig. 2A) are summarized in Fig. 2, D and E. In both cases, this animal successfully discriminated the combination of test lights when it was composed largely of the 530-nm component. However, as more of the 620-nm component was added, discrimination became more difficult, eventually dropping to chance performance, thereby defining a color match. Dichromatic color matches can be predicted by calculating the proportions of each test light at which the net quantal absorption in the two pigments is equal to the absorption from the standard light. These predicted matches (downward-pointing blue arrows in Fig. 2, D and E) are close to the actual location of discrimination failure, as is also seen for a human equivalently tested (red square and red arrow in Fig. 2D). These results

imply that color vision in this M/L heterozygous mouse is based on a comparison of quantal catches between the M and L pigments.

In primates, midget bipolar and ganglion cells mediate the chromatically opponent M versus L color vision pathway (19, 20). Like many non-primate mammals, the mouse lacks a midget system (21), and thus the color vision documented in M/L heterozygotes must be subserved by other means. Most mouse RGCs have a receptive field center with an antagonistic surround (22), albeit a weak one, and chromatic information could be extracted based on differences in M versus L input to these two regions. In a variation on this idea, chromatic information could also be extracted simply based on variation among RGCs in the total M versus L weightings.

In M/L heterozygous mice and in heterozygous New World monkeys, the stochastic process

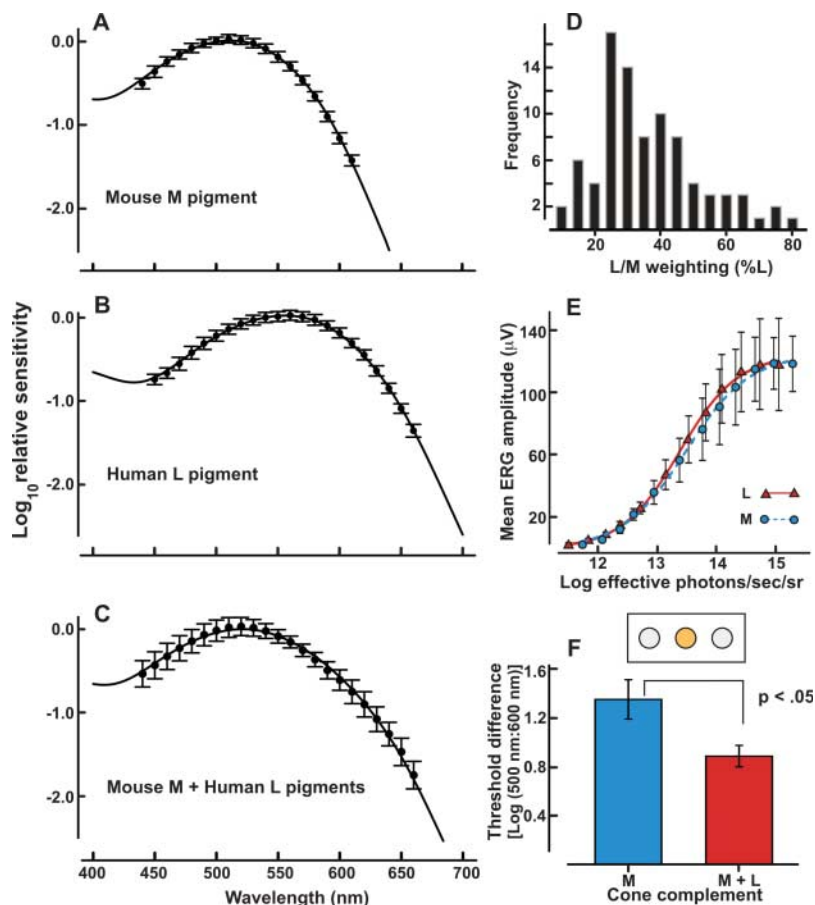


Fig. 1. (A) ERG spectral sensitivity for mice expressing the M pigment. Data points are mean values for 12 animals. The curve is that for the best-fitting photopigment absorption function. (B) Mean spectral sensitivity function for 18 mice expressing the human L pigment. (C) Spectral sensitivity for 82 heterozygous mice. The curve is the best-fit linear summation of curves derived from those in (A) and (B). (D) Distribution of the L:M cone weightings required to best fit each of the heterozygous mice represented in (C). (E) Mean V -log I functions obtained from activation of either mouse M or human L pigments. Light intensity has been specified according to its calculated effectiveness on each of these pigments. sec, seconds; sr, steradians. For derivation of the fitted functions, see SOM. (F) Increment-threshold measurements. The inset schematizes the discrimination context in which, on each trial, a monochromatic test light was added to any one of the three panels, all of which were steadily illuminated with identical achromatic light. The colored bars depict the difference in the thresholds obtained for 500- and 600-nm test lights for mice whose retinas contained either mouse M ($n = 4$ mice) or both M and L ($n = 7$ mice) pigments. Error bars in [(A) to (C)] and [(E) and (F)] indicate 2 SDs.

(X-chromosome inactivation) by which M versus L cone territories are generated and the presumed lack of any molecular distinction between M and L cones other than pigment content have parallels in a recent model for M versus L cone development in Old World primates, in which M versus L pigment gene expression is hypothesized to occur in a stochastic and independent manner in each cell, unaccompanied by any other differences in gene expression (23). The successful acquisition of chromatic discrimination based on X-chromosome

inactivation lends indirect support to this Old World primate model by demonstrating the possibility of behavioral trichromacy in the context of a stochastic process for receptor determination.

These results have general implications for the evolution of sensory systems. In the visual, olfactory, and gustatory systems, genetic manipulations have either added a new receptor protein to the existing complement of receptors or replaced an endogenous receptor protein with a new one (24–27). In each case, the behavioral or electro-

physiological responses show the predicted expansion or modification of sensitivity. However, these earlier experiments were not designed to create an additional class of sensory neurons or to facilitate the emergence of neural circuitry for comparing new and existing sensory responses. In the present experiments, X-chromosome inactivation has provided the means to both express a non-native receptor protein and to localize it to a distinct class of primary sensory neurons. Our observation that the mouse brain can use this information to make spectral discriminations implies that alterations in receptor genes might be of immediate selective value not only because they expand the range or types of stimuli that can be detected but also because they permit a plastic nervous system to discriminate between new and existing stimuli. Additional genetic changes that refine the downstream neural circuitry to more efficiently extract sensory information could then follow over many generations.

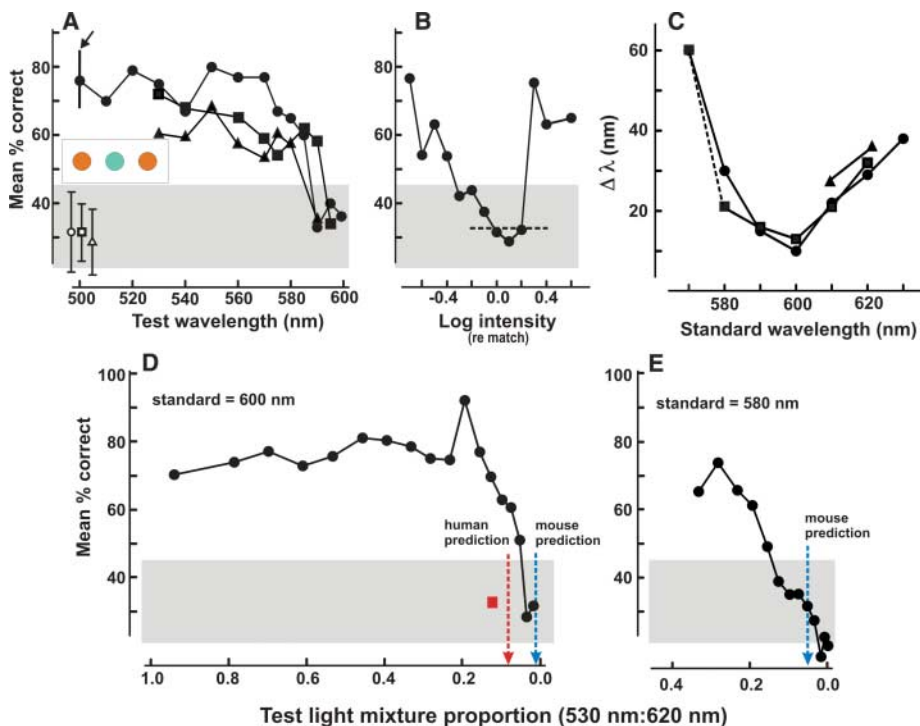


Fig. 2. Tests of color vision in mice. **(A)** Wavelength discrimination. The inset symbolizes the context in which the mouse was required to detect which of three stimulus panels was distinctively illuminated; its luminance and position relative to that of the standard wavelength (600 nm) were randomized from trial to trial. Lower left symbols show asymptotic performance levels (for the final 100 trials \pm 1 SD) for three animals at the calculated brightness match (see SOM) and for values \pm 0.1 log units from that point (the open circle indicates the mouse with M pigment; the open square and triangle indicate M:L heterozygotes with M:L ratios of 78:22 and 65:35, respectively). The solid circles connected by lines are the asymptotic performance levels achieved at the calculated brightness match by the M:L heterozygote with an M:L ratio of 44:56. Averaged performance over the full range of luminance variation for a test wavelength of 500 nm (brightness match \pm 0.5 log units) is indicated by the vertical line (arrow). The solid triangles and solid squares show results from two other heterozygous animals (M:L ratios of 46:54 and 53:47, respectively). For the latter animal, the standard wavelength was 610 nm. **(B)** Results from the embedded brightness control test. The circles are asymptotic performance levels for cases where the test and comparison lights were set to the same wavelength and the test light was systematically varied in luminance relative (re) to the value required for a brightness match. The results were cumulated across seven standard wavelengths. The horizontal dashed line indicates chance performance. **(C)** Wavelength discrimination functions for heterozygous knock-in mice. Each point plots the wavelength difference ($\Delta\lambda$) between the test and standard wavelengths that is required for successful discrimination (at the 95% confidence level). Symbols for individual animals are the same as those in (A). The dashed line connecting standard wavelengths of 580 and 570 nm for one female indicates that it failed to successfully discriminate a test light of 510 nm from a standard of 570 nm, and thus $\Delta\lambda$ for that standard must exceed 60 nm. **(D)** Color-matching data. Plotted are asymptotic levels of performance achieved by an M:L heterozygous mouse (44:56 M:L ratio) at the brightness match where the test light was a variable mixture of 530- and 620-nm lights, and the standard light was 600 nm. The location of the predicted match based on the M and L photopigments is indicated by the blue arrow. The match made by a human trichromat (red square) is shown along with the predicted human match (red arrow). **(E)** Color-matching data. Details are the same as for (D) except that the standard light was 580 nm. The shaded area in [(A) and (B)] and [(D) and (E)] indicates chance performance (at the 95% confidence level).

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- We thank J. Fenwick for help with animal testing. This work was supported by grant EY002052 from the National Eye Institute (G.H.J.), the Howard Hughes Medical Institute (J.N.), and the Visual Neurosciences Training Program of the National Eye Institute (H.C.).

Supporting Online Material

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Materials and Methods
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14 December 2006; accepted 21 February 2007
10.1126/science.1138838