Visual Pigment Coexpression in Guinea Pig Cones: A Microspectrophotometric Study

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PURPOSE. To determine the visual pigment content of the rods and cones of the guinea pig (Cavia porcellus) and to quantify the level of coexpression of pigments within individual cones.

METHODS. Microspectrophotometry was used to measure the absorbance spectrum of visual pigments in individual rods and cones from three retinal regions: dorsal, ventral, and a subequatorial transition zone. Partial bleaching was used to establish whether two spectrally distinct visual pigments were present within a single cone.

RESULTS. Rods possessed a pigment with a wavelength of maximum absorbance (λmax) close to 500 nm. A population of middle-wave-sensitive cones (M cones) contained a pigment with λmax at approximately 530 nm, and a short-wave-sensitive cone population (S cones) contained a pigment with λmax close to 400 nm. The majority of cones in all regions were M cones. Approximately 10% of cones in the transition region were found to coexpress the M and S cone pigments in a ratio of approximately 4:1. Coexpression was not detected in S cones.

CONCLUSIONS. In C. porcellus, coexpression of cone pigments occurs in a small number of cells but is biased in favor of the M pigment. Given the relatively low level of coexpression, detectable in only approximately 10% of the cones in the transition region, it is unlikely to cause any significant detriment to dichromatic color vision. (Invest Ophthalmol Vis Sci. 2002;43:1662–1665)

Color vision requires at least two spectrally distinct populations of cones, and it has been assumed that a sole cone opsin is expressed in each population. However, coexpression of cone opsins has been reported in a number of species, most notably in small nocturnal mammals.1–4 Coexpression can occur either temporally during development or simultaneously in the mature adult. Temporal coexpression may be a relatively common phenomenon among lower vertebrates, notably tei- leosts,5,6 and evidence is accumulating for serial expression of opsins across a number of higher vertebrates, including humans.7 Most studied, however, are various small nocturnal mammals, particularly the rat,4 where, during development, the short-wave (S) opsin appears to be the default pathway, occurring in all cones, followed by a switch in the majority of cones to middle-wave (M) opsin expression, with a transient phase of coexpression.

Simultaneous opsin coexpression has also been reported in a number of lower vertebrates, including guppies (Poecilia reticulata) in which L cones may express two separate opsins,8 cichlids from Lake Malawi, which may coexpress different levels of two S opsins,9 and salamander (Ambystoma tigrinum), in which cones, maximally sensitive to UV, also express low levels of two longer-wave cone opsins.10 However, it is within small nocturnal mammals, primarily rodents that simultaneous coexpression is most marked. A number of these species exhibit a spatial differentiation of their two cone classes in which the superior (dorsal) retina is M-cone rich and the inferior (ventral) retina is S-cone rich.1–3 Coexpression may occur in a transition zone between the two segregated regions. Nevertheless, at least in some of these species, dichromatic color vision has been demonstrated.11,12 The majority of the coexpression data were obtained using antibody labeling of intact cones, which is basically a qualitative technique, although semiquantitative data can be obtained for variations across a retina. In the mouse, where the majority of cones are suggested to coexpress, Northern blot analysis of retinal mRNA suggests three times more S-mRNA than M-mRNA,3 but this technique cannot determine the posttranslational levels of opsin. An electroretinographic study, also in mice,13 of the spectral sensitivities of the M and S cones inferred that S cones express a maximum of 3% M pigment.

The guinea pig, Cavia porcellus, is one of the species in which simultaneous coexpression has been shown by antibody labeling.1 The dorsal retina is reported to be dominated by M cones, whereas the ventral retina is dominated by S cones, although, in this region, the S cones are never completely devoid of M pigment.1 Between these two zones there is a substantial transition region in which coexpression occurs. Despite the regional segregation and coexpression of the two cone pigments, guinea pigs have been shown to be behavioral dichromats.12 Because antibody labeling can only give qualitative indications of the degree of coexpression, we have taken advantage of the relatively high percentage of cones in the guinea pig retina14 to use microspectrophotometry to obtain a direct, quantitative measure of coexpression in individual cones. Our data support the reported predominance of M cones in the dorsal retina and the presence of a population of cones in the transition region that coexpress, with M cones containing approximately 20% S pigment. A small number of S cones that did not show any evidence of coexpression were also identified.

METHODS

Guinea pigs (C. porcellus), both pigmented and albino, were dark adapted overnight and killed by lethal injection of pentobarbitone sodium, and their eyes were enucleated under dim red light (Safelight No. 2; Eastman Kodak, Rochester, NY). All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Eyes were hemisected, and the eyecup lightly fixed in 2% glutaraldehyde for 10 to 15 seconds to extend the resilience of the photoreceptors.15 Retinal samples were taken from the dorsal M-cone rich, ventral S-cone rich, and central transition zones, according to the scheme of Röhlich et al.1 Samples were
teased apart with razor blades and flattened between two coverslips before mounting on the microscope stage of the microspectrophotometer.

Absorbance spectra of individual photoreceptors were measured with a modified dual-beam Liebman microspectrophotometer under computer control. With the help of an infrared converter, the measuring beam (normally a 2-μm² cross section) was aligned to pass transversely through a rod or cone outer segment, while the reference beam passed through a clear space adjacent to the photoreceptor. Spectra were scanned from 750 to 350 nm in 2-nm steps and back from 351 to 749 nm at the interleaved wavelengths. The outward and return scans were averaged. A baseline spectrum was measured for each cell, with both beams in an unoccupied area close to the cell, and this was subtracted from the intracellular scan to derive the final spectrum. Two baseline scans were recorded for each cell and averaged. All cones were routinely bleached with a single exposure to light, but in the case of cones that gave indications of higher short-wave absorbance, the cells were partially bleached. In this instance, the cells were bleached first with a 2-minute exposure to 550-nm light, to remove any M pigment, followed by a 10-minute white-light bleaching to remove any remaining S pigment. As a control, a similar number of cones that gave no indication of more than one pigment were also partially bleached.

Absorbance and difference spectra were analyzed as described previously. After a preliminary analysis, to obtain mean absorbance data, selection criteria were applied to records from rods and M cones (that gave no indication of coexpression), to exclude "poor" records (for the rationale, see Mollon et al.18). In the case of S cones and coexpressing cones, where the number of cells was limited, all the records were included in further analysis. It was found that the initial exposure to 550-nm light bleached approximately 90% of the M pigment, and the residual pigment was bleached by the subsequent exposure to white light. In the case of cones that coexpressed both the M and S pigments, the S pigment was isolated from the white-light exposure. The absorbance difference spectra were then derived by subtracting the baseline spectrum from the intracellular scan.
bleach difference spectrum by subtracting a scaled pure M-cone difference spectrum to remove the contribution from the remaining M pigment.

RESULTS

The majority of retinal samples were taken from the transition region (as defined by Röhlisch et al.1), but tissue was also examined from the dorsal and ventral zones. Rods dominated in all regions and had a wavelength of maximum absorbance (\(\lambda_{\text{max}}\)) close to 500 nm (Fig. 1A; Table 1). In the dorsal and ventral regions, we recorded only M cones with \(\lambda_{\text{max}}\) of 524 to 532 nm (Table 1), although cones in the ventral retina were scarce. In the transition zone, three classes of cone were recognized. The majority contained the M pigment with \(\lambda_{\text{max}}\) of approximately 530 nm (Fig. 1C; Table 1). A second, rare population of cones contained only an S pigment with \(\lambda_{\text{max}}\) close to 400 nm (Fig. 1B; Table 1). However, approximately 10% of cones in the transition region showed increased short-wave absorbance when compared with the majority of M cones (Figs. 1C, 1D). The disparity between these cones is more clearly seen in the difference spectrum derived from a complete bleaching (550-nm light followed by white light) in which there was clear evidence of an increased loss of pigment at short wavelengths (Figs. 1C, 1D).

Partial bleaching of M cones and all the S cones revealed only a single photopigment in each class. However, in the M cones with high short-wave absorbance, partial bleaching exposed two pigments. The initial 550-nm bleach isolated a pigment with \(\lambda_{\text{max}}\) identical with that of normal M cones (Fig. 2A), although there was also evidence of a small increased loss at short wavelengths. The subsequent white light exposure bleached the residual M pigment, but revealed clear evidence of a short-wave pigment with \(\lambda_{\text{max}}\) close to 400 nm, spectrally similar to that of the S cones (Fig. 2B).

Further evidence for the S pigment’s contribution to the coexpressing cones can be derived from a comparison of the difference spectra of the pure M cones with the coexpressing cones (Fig. 2C). After normalizing the spectra to the 530-nm peak, the difference between the two spectra revealed an

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<th>Mean of Individual Spectra</th>
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* Data are the mean ± SD.

**FIGURE 2.** Difference spectra from partial and complete bleaching, comparing pure M cones (dotted lines) and coexpressing cones (solid lines). The lines are locally weighted least squares (Lowess) fits to the data (data points have been removed for clarity). (A) After a 2-minute exposure to 550-nm light with the curves normalized to the long-wave peak. (B) After a further 10-minute exposure to white light, corrected for residual M-cone pigment (bleached by white light), showing the loss of the presumptive S-cone pigment in the coexpressing cones. (C) Normalized total-difference spectra after a complete bleach of coexpressing cones and pure M cones. (D) Relative difference between the two spectra in (C); note the similarity to the S-cone difference spectrum in Figure 1B.
absorbance that again showed a $\lambda_{\text{max}}$ close to 400 nm (Fig. 2D), similar to the S-cone–difference spectrum (Fig. 1B).

**DISCUSSION**

The $\lambda_{\text{max}}$ of the rod (500 nm) and M-cone (530 nm) pigments presented herein are in good agreement with those from previous reports. An extract of the rod pigment gave a $\lambda_{\text{max}}$ of 497 nm, and flicker ERG suggested a $\lambda_{\text{max}}$ of 494 nm for rods and 529 nm for the M cones. However, the $\lambda_{\text{max}}$ of the S-cone pigment (400–410 nm), determined here from microspectrophotometry, is significantly shorter than the value of 429 nm derived from flicker ERG. The discrepancy could be due to short-wave absorbance by the lens that, if significant at wavelengths of approximately 400 nm, would displace the effective spectral sensitivity of the cone, as measured by ERG, to longer wavelengths.

Partial bleaching of the coexpressing cells indicates that the S pigment accounts for approximately 20% of the total pigment content (the relative absorbances are approximately 0.002 for the S pigment and 0.007 for the M pigment), whereas the comparison of the pure M cones with the coexpressing cones suggests a slightly higher S pigment component of up to 30%. Because the signal-to-noise ratio of the microspectrophotometer at short wavelengths is low, it is unlikely that anything less than approximately 15% of an S pigment would be detected above the noise. Because of this, we cannot rule out the possibility that a further population or indeed all the remaining M cones coexpress at a low level. In the case of S cones, the situation is somewhat different. At longer wavelengths of approximately 530 nm, the signal-to-noise ratio of the microspectrophotometer is much higher, so that a smaller component of an M pigment in an S cone would be detected. From this, we estimate that if there were coexpression in the S cones, the percentage of M pigment would be less than approximately 10%.

The distribution of cones across the guinea retina, as determined from antibody labeling of cone opsins, is highly asymmetric, with the dorsal retina dominated by M cones with approximately 5% S cones, whereas in the ventral region all the cones label strongly for the S pigment but also show positive for the M pigment. Slightly ventral of the equatorial region is a broad transition zone in which the majority of cones exhibit coexpression. The microspectrophotometric data presented herein are somewhat contradictory. Our limited data from the dorsal region support the findings of Rölich et al. In the transition region, we recorded primarily M cones, with approximately 10% showing clear signs of coexpression, as well as a small population of S cones. These data are in general agreement with the findings from antibody labeling, although the percentage of cones showing coexpression is considerably less. This discrepancy may imply that the levels of coexpression can vary and may reflect the difficulty of microspectrophotometry in detecting low levels of coexpression. We recorded an unexpectedly sparse population of M cones in the ventral retina, a region purported to contain cones dominated by the S pigment.

The benefits of coexpression to a species are not immediately obvious, although one possible advantage is that coexpression broadens the spectral range over which a cone can operate. However, this would affect only S cones in which addition of a longer-wave-sensitive pigment increases the range of longer wavelengths that can be detected. In the situation reported in this study in the guinea pig, the coexpression of a relatively small amount of S pigment in an M cone did not increase the spectral range of the cone, but only lowered the threshold for detection of short wavelengths. In the transition region short-wave sensitivity will be increased without the incorporation of additional S cones, but with some detrimental effect on chromatic discrimination. However, the presence of coexpressing cones in the transition region may simply be a consequence of the asymmetry of cone distribution and pigment expression across the retina, although our data imply that the cone asymmetry is less extreme than suggested by antibody labeling techniques. Given the relatively low level of coexpression, detectable in only approximately 10% of the cones in the transition region, it is unlikely to cause any significant detriment to color vision, so that it is perhaps not surprising that dichromatic color vision can be demonstrated behaviorally in guinea pigs.

**References**