Spectral tuning of a circadian photopigment in a subterranean 'blind' mammal (*Spalax ehrenbergi*)

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Abstract The atrophied subcutaneous eyes of *Spalax ehrenbergi* (the blind mole rat) express a long wavelength sensitive (LWS) cone opsin. Our data provide strong evidence that this photopigment is spectrally tuned to enhance photon capture in the red light environment of the eye. Furthermore, novel mechanisms appear partially responsible for this sensory fine-tuning. These data support the hypothesis that the LWS opsin of *Spalax* acts as a functional photopigment and that it is not a 'residue' of the pre-subterranean visual system. As the eye of *Spalax* has only one known function, the entrainment of circadian rhythms to environmental light, the LWS photopigment is implicated in this task. These results, together with our recent findings that rod and cone photopigments are not required for murine photoentrainment, suggest that multiple photopigments (classical and novel) mediate the effects of light on the mammalian circadian system.

Key words: Circadian photopigment; Spectral tuning; LWS photopigment; Blind mammal; *Spalax ehrenbergi*

1. Introduction

Spalax ehrenbergi (the blind mole rat) is a subterranean fossorial rodent [1]. *Spalax* has subcutaneous atrophied eyes [2] and shows a dramatic reduction (87–93%) of those regions of the brain associated with the image-forming visual system [3]. Although visually blind [4,5], the minute eyes ($\approx$600 μm in diameter) can perceive light and are used to entrain circadian rhythms of locomotor behavior [6–9]. In the wild, photoentrainment is thought to occur when *Spalax* removes debris from their tunnel complexes, during which they are exposed to a brief period of natural light [10]. Although exposed to direct sunlight during this event, the light reaching the eye has to pass through several tissue layers, which will both reduce and spectrally modify the light available for photoreception.

Recently, we isolated a long wavelength sensitive (LWS) opsin cDNA from the eye of *Spalax* and demonstrated that this opsin is capable of forming a photopigment in vitro. Because *Spalax* lacks any capacity to generate a visual image, we speculated that this photopigment is used to entrain circadian rhythms of locomotor behaviour [6]. A weakness of this hypothesis is that apparently functional opsins are expressed in other species which *never* see natural light. For example, the blind cave fish [11] and blind crayfish [12] are found in submerged cave systems which have been isolated from the outside, and hence from any sunlight, for at least 1 000 000 years. Despite this isolation, and the possession of atrophied eyes, these animals have opsins which seem capable of forming fully functional photopigments. In view of these results we cannot exclude the possibility that the LWS opsin in *Spalax* represents some non-functional 'residue' of the pre-subterranean visual system. This dilemma forms the central focus of the current study, and is addressed by using approaches developed by visual ecologists [13].

In the aquatic environment, the light available for vision may be confined to relatively narrow spectral ranges (e.g. deep oceanic waters [14,15]), and correlations between photopigment maxima and the spectral quality of light in order to enhance visual sensitivity are well documented [16,17]. Unlike the aquatic environment, light in the terrestrial realm is commonly more abundant and is of a broad spectrum [18,19]. As a result, the photopigment maxima of terrestrial animals appear fine-tuned to enhance specific tasks of visual contrast (an object against its background) rather than for absolute sensitivity [20]. *Spalax* is unique in having the most regressed subcutaneous eyes of any known terrestrial mammal [21]. The light reaching these eyes will therefore be both attenuated and spectrally modified, and in this regard *Spalax* is faced with a photosensory problem analogous to that of certain aquatic species.

If the LWS photopigment of *Spalax* is used as a functional photopigment, then we would predict that this opsin will be fine-tuned to enhance sensitivity in its subcutaneous light environment. Furthermore, the unique evolutionary pressures imposed by this light environment might be expected to give rise to novel spectral-tuning mechanisms [22]. To examine these possibilities we have: (1) determined the spectral quality of light reaching the subcutaneous eye and compared this to the absorbance maxima of the LWS photopigment; (2) determined the amino acids at the five sites known to be primarily involved in spectral tuning of the mammalian photopigments; and (3) compared the predicted and deduced λ_{max} of the LWS photopigment to determine whether novel mechanisms might be involved in the spectral tuning of this photopigment.

2. Materials and methods

2.1. Isolation

2.1.1. cDNA synthesis. Total RNA was isolated from ocular tissue

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of adult blind mole rats ($2n = 60$ chromosomal species, [1]). Ocular cDNA was synthesized from 500 ng of total RNA using reverse transcriptase (Superscript II) and oligo dT primers (Life Technologies).

2.1.2. PCR methodology and primer sequences. Opsin cDNA was amplified using mammalian cone opsin primers MG1(sense) and MG2(antisense).

- MG1: 5′-AATGTGAGATTTGATGCTAAG-3′
- MG2: 5′-TGCAGGTGACACTGAAGAGA-3′

The following thermal profile was performed using a 'hot start' protocol; 94°C for 3 min, 94°C for 1 min, 55°C for 1.5 min, 72°C for 2 min for 30 cycles. The remaining 3′ and 5′ coding sequences were amplified using RACE-PCR (Gibco BRL) with nested gene specific primers:

- ZDG1 (3′ RACE PCR) 5′-TGAACCGGCAGTTTCAAAACTGC-3′
- ZDG2 (3′ RACE nested PCR) 5′-CCCGTCGACGATAGCTCT-GAACTTTCCAGCA-3′
- ZDG8 (5′ RACE cDNA synthesis) 5′-AGACCCATGCGAAGGT-3′
- ZDG9 (5′ RACE PCR) 5′-TGCAGACTACCATCCACCTC-3′
- ZDG10 (5′ RACE nested PCR) 5′-CCCGAATTCTGTGATGC-CACACAGGGAC-3′

2.1.3. Isolation, cloning and sequencing of full length LWS cone opsin cDNA. Full length cDNA was amplified using species-specific primers directed against the 5′ and 3′ untranslated regions and *Pfu* DNA polymerase (Stratagene).

All PCR products were cloned into pUC 18 vector (Pharmacia) (3′ RACE) and pUC 19 vector (MBI Fermentas) (5′RACE and full length clone) and sequenced on both DNA strands using the Sequenase kit (Amersham Life Sciences).

2.2. Measurement of spectral transmission through the skin

The skin of the mole rat was carefully removed and flattened on a 3-mm thick glass plate. Using a 100-W tungsten-halogen light source and a 1-mm diameter fiber optic, light was directed through the skin. A sensitive photometer-photomultiplier (International light IL 600-PM200C) equipped with narrow band interference filters (Schott, FWHM ± 10 nm, 350–700 nm) was used to measure transmitted light through specific regions of the skin.

3. Results and discussion

3.1. Structure of the LWS photopigment of Spalax

The deduced amino acid sequence of the full coding region of a LWS photopigment cDNA (GenBank accession number AF139726) from *Spalax* ocular mRNA shares a high similarity with both mouse (91%) and human LWS (red/green) (87%) photopigments. The opsin cDNA encodes a 364-amino acid protein that exhibits all the conserved structural features required for photopigment function (Fig. 1): A conserved lysine within Helix VII (K-312) required to bind retinal chromophore via a Schiff base linkage [23], a glutamate counterion within Helix III (E-129) which is necessary to stabilize the

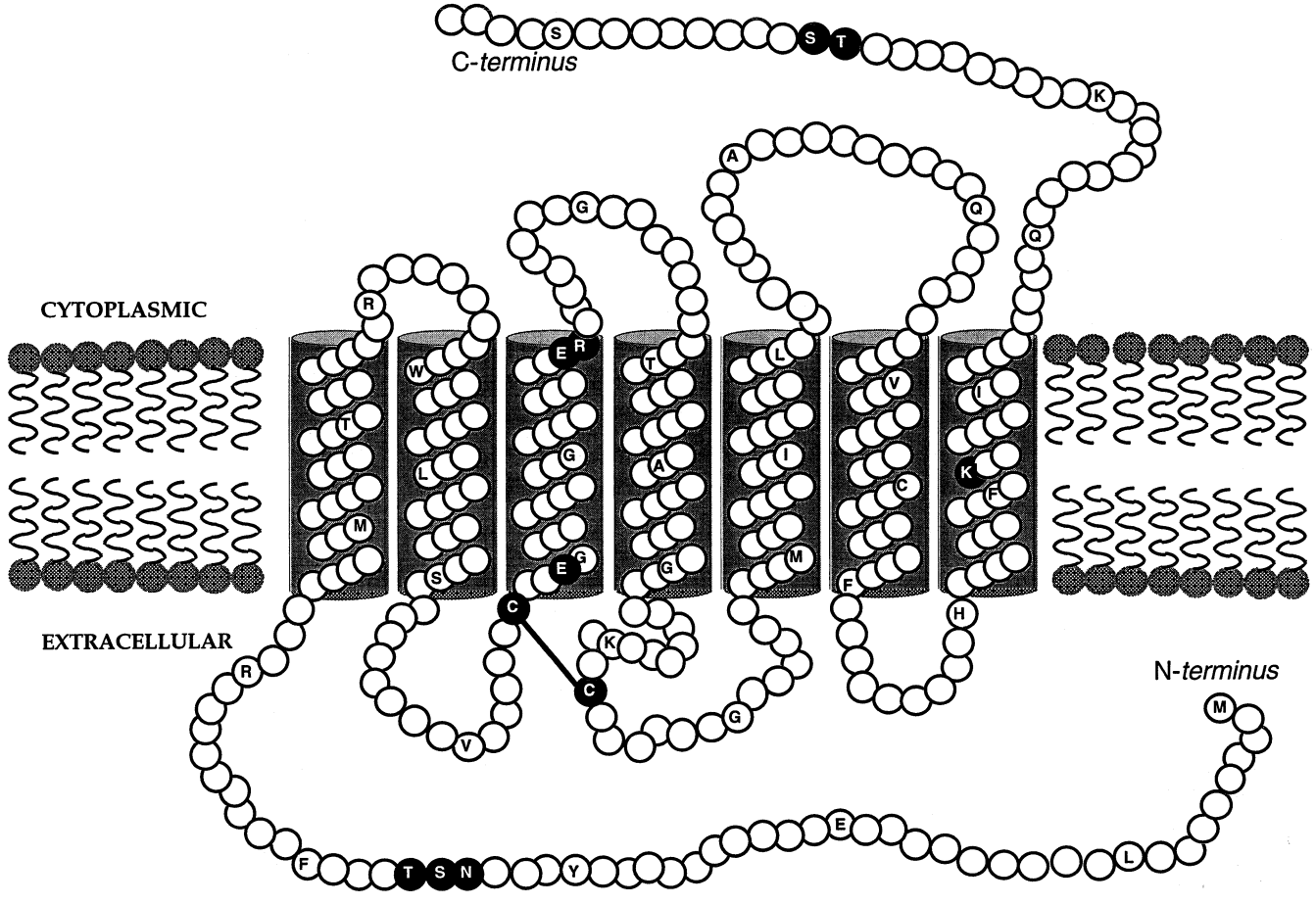

Fig. 1. Topological model of *Spalax* LWS cone opsin. The structural features known to be required for photopigment function are highlighted (black circles). Abbreviations: K: Lysine at position 312, E: Glutamate at position 129 and 150, C: Cysteine at position 126 and 203, R: Arginine at position 151, N: Aspargine at position 34, S: Serine at position 35 and 351, T: Threonine at position 36 and 350. Every 10th amino acid (white circles) is highlighted for reference (see text for details).

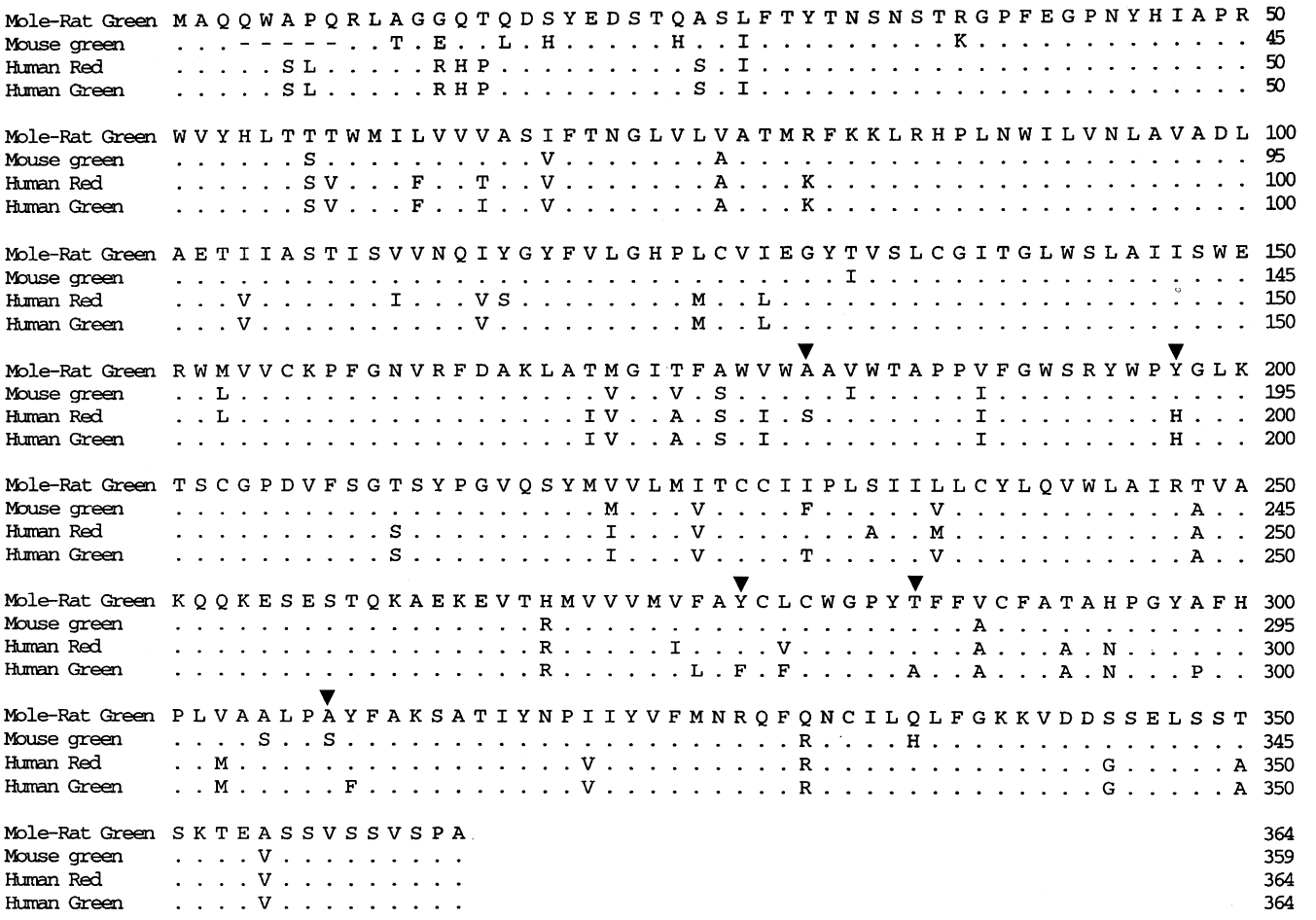

Fig. 2. Multiple sequence alignment of mole-rat, mouse and human green and human red LWS photopigments. *Spalax* shares 91% similarity with the mouse green and 87% with both human green and red (see text for details). The five amino acid sites known to be primarily involved in spectral tuning in human and rodent LWS photopigments (positions 180, 197, 277, 285 and 308) are indicated by arrowheads. Conserved amino acids are indicated by dots.

Schiff base linkage [24,25], two conserved cysteines (C-126 and C-203) which are necessary for the formation of a disulfide bond essential in correct opsin folding, a conserved ER motif at the junction of helix III and the second cytoplasmic loop required for G-protein binding [26], a single putative *N*-linked glycosylation site (N-34 S-35 T-36) and serine and threonine residues in the carboxyl-terminal tail which serve as potential phosphorylation sites [27]. Furthermore, in vitro expression studies have confirmed this LWS opsin is capable of forming a fully functional photopigment [6].

3.2. Mechanisms of spectral tuning in mammalian LWS photopigments

Molecular characterisation and site directed mutagenesis studies of the human red and human green LWS photopigments has shown that the 30-nm difference observed in their λ_{max} can be explained primarily by amino acid substitutions at three critical sites, S180A (i.e. replacement of serine for alanine at position 180), Y277F and T285A (using the amino acid numbering system of the human red cone photopigment). Replacement of hydroxyl-bearing with non-polar amino acids at these sites in the human red cone photopigment reduces the λ_{max} in an additive manner by approximately 7, 10 and 16 nm, respectively [28,29]. Both mouse and rat LWS photopigments are identical to human red at these three critical positions (Fig. 2) despite the fact that the expressed photopigment of mouse and rat have λ_{max} that are dramatically blue shifted (508 ± 2 nm) [30,31]. Recent work on the spectral tuning mechanisms responsible for the 508-nm λ_{max} in the mouse LWS photopigment showed that amino acid substitutions in the human red photopigment, H197Y and A308S, produced a functional photopigment with λ_{max} identical to that observed in mouse (508 nm). These two substitutions resulted in a 28-nm and 18-nm blue shift, respectively [30]. Therefore, amino acid differences at these sites in the mouse LWS photopigment fully account in an additive manner for the 44-nm blue shift relative to the human red LWS photopigment to which it shares greatest similarity. Thus, five critical sites appear to account for all the spectral shifts associated with these mammalian photopigments.

3.3. Mechanisms of spectral tuning in Spalax

Spalax shares identical residues with the mouse at four of the five critical sites (A180, Y197, Y277 and T285), differing only at position 308 where *Spalax* has an alanine and the mouse has a serine. In mice, the replacement of an alanine for a serine was reported to be responsible for 18 nm of the 44-nm blue shift (relative to the human red LWS photopigment). Hence, using the amino acid composition at the five sites shown to be important in spectral tuning in other mam-

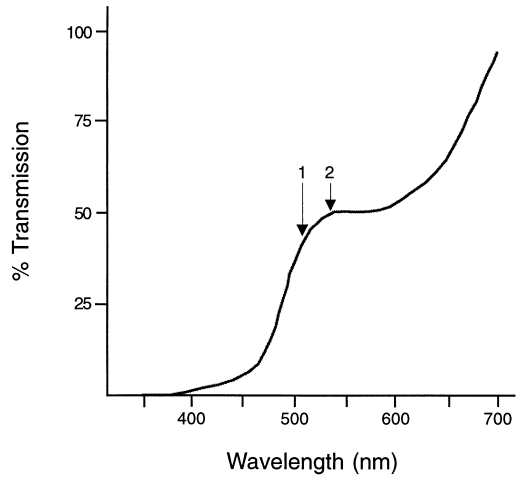

Fig. 3. Transmission spectra of light passing through the fur and skin overlaying the eyes of *Spalax*. The light available for photoreception by the eye is enriched for wavelengths greater than 500 nm. The 508-nm λ_{max} of mouse and rat LWS photopigments are indicated by arrow 1; The red-shifted 534-nm λ_{max} of *Spalax* LWS photopigment is indicated by arrow 2. The λ_{max} of this photopigment falls on that part of the transmission spectrum that confers maximum sensitivity but results in minimum thermal isomerisation.

mals we tentatively predicted a λ_{max} of 526 nm for the LWS photopigment of *Spalax*. However, and as described elsewhere [6], our in vitro expression studies showed that the LWS photopigment of *Spalax* has a λ_{max} of 534 nm, which is 8 nm longer than the calculated λ_{max} for this pigment (526 nm). This suggests that novel spectral tuning sites are involved in determining the λ_{max} of this photopigment. However, reported differences of this sort are not unique and could result from several different causes. For example, unexplained differences of approximately 10 nm between the observed λ_{max}, based on ERG recordings, and the predicted λ_{max} value have been recorded in the white tailed deer, gray squirrel and guinea pig [31–33]. The basis of these differences remain unclear but methodological problems associated with ERG recordings may provide a partial answer. For example, a recent report on the rabbit documented differences between the λ_{max} obtained by ERG recordings and that obtained by functional expression [31]. Functional expression of the LWS photopigments in these species will help to resolve this ambiguity.

Methodological problems cannot, however, account for all the inconsistencies noted. For example, the dolphin LWS photopigment shows differences between the λ_{max} values predicted by the amino acid composition at the five critical spectral tuning sites and that observed from in vitro expression. In this instance, site directed mutagenesis studies have identified a serine at position 308 as responsible for causing a blue shift of 28 nm [22]. Similar studies on mouse LWS photopigment found that a serine in this position is responsible for a blue shift of only 18 nm relative to the human red photopigment. Thus, in the dolphin, the discrepancy between the observed and predicted λ_{max}, can be attributed to an apparent species specific difference in the spectral shift associated with a particular amino acid substitution. It appears therefore, that interactions between as yet unidentified amino acids present in the opsin molecule of the dolphin enhance the blue shifting effect of the serine substitution at position 308, relative to the situation found in mouse.

In *Spalax* we are currently unable to account for the 8-nm discrepancy between the deduced and predicted in λ_{max} of the LWS photopigment. Because this difference is based upon functional expression, rather than on ERG recordings, we attribute this difference to additional novel spectral tuning sites within the LWS photopigment. Amino acids shown to be primarily important in spectral tuning are typically located within the transmembrane domains facing either towards the chromophore or an adjacent helix [34]. Additionally, further residues located within the extracellular loops have also been shown to contribute to the spectral tuning of mammalian photopigments [28,35], suggesting that sites not in the immediate environment of the chromophore can have indirect effects on λ_{max}. Therefore any additional novel tuning sites in the *Spalax* LWS photopigment may in part be due to changes in opsin structure external to the transmembrane domains which form the chromophore binding pocket. To address this issue we are currently undertaking site directed mutagenesis on the LWS photopigment of *Spalax*.

3.4. Spectral transmission in light limiting environments

Both the dolphin LWS and rod photopigments are blue shifted relative to terrestrial mammals [17] a situation also reported in deep sea and abyssal fish [15,16,36,37] and attributed to selective pressures associated with the aquatic light environment of these species. The selective filtering and scatter of light as it passes through the water column profoundly restricts the spectral range of photons available for vision. Thus, the absorbance properties of photopigments in different species vary in relation to the spectral environment. In *Spalax*, the spectral transmission of light through the skin and tissues overlying the subcutaneous eyes, reveal that the light available for photoreception is significantly enriched for wavelengths greater than 500 nm (Fig. 3).

The long-wavelength tail of all opsin based-photopigments absorbs at low levels into the far-red and thermal part of the electromagnetic spectrum. Thus in any population of photopigments there will be a low level of thermal isomerizations and hence 'thermal noise'. The rate of thermal isomerizations increases as photopigment λ_{max} are shifted to longer wavelengths [13]. For normal daylight vision this is not a problem as the abundance of photons enables light signals to be easily distinguished from background thermal noise. However, in low light environments, photopigment λ_{max} have to be positioned so that they maximize photon capture but minimize the effects of heat [16]. This problem is particularly acute for the photoreceptors of *Spalax*, which encounter few, and primarily long wavelength, photons (Fig. 3).

The λ_{max} of the LWS photopigment of *Spalax* is uniquely red-shifted to the precise wavelength of light that would maximize sensitivity but minimize thermal noise (Fig. 3). We propose that the subcutaneous location, and hence the hemoglobin-dominated light environment of the eye, has exerted strong positive selective pressures on the LWS photopigment. Thus, in this terrestrial species we see the same principle of ecological adaptation as that seen in several aquatic animals, fine tuning of the sensory organ to its photic environment.

3.5. Cone photoreceptors contribute to photoentrainment

We have strong evidence that the LWS photopigment of *Spalax* is fine-tuned to match the light environment of the subcutaneous eye, and that novel mechanisms are partially

responsible for this match. These results argue that the LWS opsin functions as a photopigment and is not merely a 'residue' of the pre-subterranean visual system. The eye of the blind mole rat has only one known function, the entrainment of circadian rhythms to the environmental light:dark cycle [4,6–9]. Collectively these results provide circumstantial evidence that the LWS cone photopigment contributes to photoentrainment in this species, and by implication, other mammals. This conclusion appears to contradict our recent findings that the loss of both rod and cone photoreceptors has no effect on rodent photoentrainment and that the retina contains novel 'circadian photoreceptors' within the inner retina [38,39]. These results appear less contradictory when pineal photoreceptors are examined. In all non-mammalian vertebrates multiple photopigments are associated with the regulation of rhythmic physiology. Classical rod- and cone-like opsins, as well as novel opsins, co-exist within the same organ [40]. But why should the non-mammalian pineal possess multiple photopigments? It is possible that multiple photopigments might be used to gather information about spectral changes within the environment. For example, at twilight the overall spectral quality of light changes in a precise manner. By monitoring this spectral change, the phase of twilight can be accurately determined. Alternatively, multiple photopigments, with differing spectral sensitivities, could be used to provide measures of irradiance over a broad spectral range [41].

In many respects the eye of the blind mole rat is analogous to the non-mammalian pineal. Both organs are screened by overlaying tissues and lack optical structures for image detection, and both act as obligate irradiance detectors. This analogy between the eyes of *Spalax* and the non-mammalian pineal, and our finding that novel retinal photopigments regulate the circadian system of mice [38] suggests that in this species, and perhaps all mammals, multiple photopigments (classical and novel) mediate the effects of light on the circadian system.

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References

[1] Nevo, E. (1991) Evolutionary biology 25, 1–125.
[2] Sanyal, S., Jansen, H.G., de Grip, W.J., Nevo, E. and de Jong, W.W. (1990) Invest. Ophthalmol. Vis. Sci. 31, 1398–1404.
[3] Cooper, H.M., Herbin, M. and Nevo, E. (1993) Nature 361, 156–159.
[4] Haim, A., Heth, G., Pratt, H. and Nevo, E. (1983) J. Exp. Biol. 107, 59–64.
[5] Rado, R. (1992) Isr. J. Zool. 38, 323–331.
[6] David-Gray, Z.K., Janssen, J.W., DeGrip, W.J., Nevo, E. and Foster, R.G. (1998) Nat. Neurosci. 1, 655–656.
[7] Tobler, I., Herrmann, M., Cooper, H.M., Negroni, J., Nevo, E. and Achermann, P. (1998) Behav. Brain Res. 96, 173–183.
[8] Goldman, B.D., Goldman, S.L., Ricco, A.P. and Terkel, J. (1997) J. Biol. Rhythms 12, 348–361.
[9] Rado, R., Gev, H., Goldman, B.D. and Terkel, J. (1991) in: Photobiology (Riklis, E., Ed.), pp. 581–587, Plenum Press, New York.
[10] Rado, R., and Terkel, J. (1989) in: Environmental quality and ecosystem stability (Spanier, E., Steinberger, Y. and Luria, M., Eds.), Vol. IV-B, pp. 391–400, ISEEQS, Jerusalem.
[11] Yokoyama, S., Meany, A., Wilkens, H. and Yokoyama, R. (1995) Mol. Biol. Evol. 12, 527–532.
[12] Crandall, K.A. and Hills, D.M. (1997) Nature 387, 667–668.
[13] Archer, S. (1999) in: Adaptive mechanisms in the Ecology of Vision (Archer, S.N., Djamgoz, M.B.A., Loew, E.R., Partridge, J.C. and Vallerga, S., Eds.), Kluwer Academic Publishers, Dordrecht.
[14] Lythgoe, J.N. (1979) Clarendon Press, Oxford.
[15] Hope, A.J., Partridge, J.C., Dulai, K.S. and Hunt, D.M. (1997) Proc. R. Soc. Lond. B Biol. Sci. 264, 155–163.
[16] Bowmaker, J.K., Govardovskii, V.I., Shukolyukov, S.A., Zueva, L.V., Hunt, D.M., Sideleva, V.G. and Smirnova, O.G. (1994) Vis. Res. 34, 591–605.
[17] Fasick, J.I., Cronin, T.W., Hunt, D.M. and Robinson, P.R. (1998) Vis. Neurosci. 15, 643–651.
[18] McFarland, W.N. and Munz, F.W. (1975) Vis. Res. 15, 1063–1070.
[19] McFarland, W.N. and Munz, F.W. (1975) Vis. Res. 15, 1071–1080.
[20] Mollon, J.D. (1991) in: Vision and Visual Dysfunction (Gregory, R.L. and Gronly-Dillion, J.R., Eds.), 2: Evolution of the Eye and Visual System, pp. 306–319, Macmillan, London.
[21] Cooper, H.M., Herbin, M. and Nevo, E. (1993) J. Comp. Neurol. 328, 313–350.
[22] Fasick, J.I. and Robinson, P.R. (1998) Biochemistry 37, 433–438.
[23] Findlay, J.B. and Pappin, D.J. (1986) Biochem. J. 238, 625–642.
[24] Sakmar, T.P., Franke, R.R. and Khorana, H.G. (1989) Proc. Natl. Acad. Sci. USA 86, 8309–8313.
[25] Zhukovsky, E.A., Robinson, P.R. and Oprian, D.D. (1991) Science 246, 928–930.
[26] Franke, R.R., Konig, B., Sakmar, T.P., Khorana, H.G. and Hofmann, K.P. (1990) Science 250, 123–125.
[27] Thompson, P. and Finlay, J.B. (1984) Biochem. J. 220, 773–780.
[28] Asenjo, A., Rim, J. and Oprian, D. (1994) Neuron 12, 1131–1138.
[29] Neitz, M., Neitz, J. and Gerald, J. (1991) Science 252, 972–974.
[30] Sun, H., Macke, J.P. and Nathans, J. (1997) Proc. Natl. Acad. Sci. USA 94, 8860–8865.
[31] Radlwimmer, F.B. and Yokoyama, S. (1998) Gene 218, 103–109.
[32] Guenther, E. and Zrenner, E. (1993) J. Neurosci. 13, 1543–1550.
[33] Jacobs, G.H. (1993) Biol. Rev. Camb. Philos. Soc. 68, 413–471.
[34] Bowmaker, J.K. and Hunt, D.M. (1999) in: Adaptive Mechanisms in the Ecology of Vision (Archer, S.N., Djamgoz, M.B.A., Lowe, E.R., Partridge, J.C. and Vallerga, S., Eds.), Kluwer Academic Publishers, Dordrecht.
[35] Wang, Z., Asenjo, A.B. and Oprian, D.D. (1993) Biochemistry 32, 2125–2130.
[36] Archer, S., Hope, A. and Partridge, J.C. (1995) Proc. R. Soc. B Lond. 262, 289–295.
[37] Hunt, D.M., Fitzgibbon, J., Slobodyanyuk, S.J. and Bowmaker, J.K. (1996) Vis. Res. 36, 1217–1224.
[38] Freedman, M.S., Lucas, R.J., Soni, B., von Schantz, M., Munoz, M., David-Gray, Z. and Foster, R. (1999) Science 284, 502–504.
[39] Lucas, R.J., Freedman, M.S., Munoz, M., Garcia-Fernandez, J.M. and Foster, R.G. (1999) Science 284, 505–507.
[40] Shand, J. and Foster, R.G. (1999) in: Adaptive Mechanisms in the Ecology of Vision (Archer, S.N., Djamgoz, M.B.A., Lowe, E.R., Partridge, J.C. and Vallerga, S., Eds.), pp. 197–222, Kluwer Academic Publishers, Dordrecht.
[41] Roenneberg, T. and Foster, R.G. (1997) Photochem. Photobiol. 66, 549–561.