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The sensory demands of photoentrainment have imposed a unique set of selection pressures, which have led to the evolution of specialised photoreceptor systems. Our work studies on retinally degenerate mammals have shown that visual blindness need not mean circadian blindness, and that two functionally distinct systems for processing light information must exits within the mammal eye. An image-forming system, which constructs an representation of the environment, and a non-image-forming photoreceptor system, which deduces gross changes in the overall amount of light at different times of day. specialisations of the mammalian photoentrainment pathway include a distinct set of retinal ganglion cells that project exclusively to the circadian centres within the brain, and the possible utilisation novel ocular photo-receptors. The features of the light environment that mediate entrainment have yet to be fully defined. Environmental irradiance appears to be a critical influence, but spectral changes and/or the position of the sun could theoretically provide useful information about the phase of twilight. Finally, the extent to which expressed circadian rhythms arise directly from a clock, or are the product of an interaction between a clock and the entrainment pathway, remains unclear in the vertebrates. In mammals at least, major lesions to the retina, at a time when both the retina and SCN are developmentally plastic, appear to markedly influence some aspects of the circadian phenotype.

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2. Objectives: It is the central aim of this proposal was to determine the functional organization of the photic system mediating biological rhythms in mammals. Light provides the primary environmental synchronizing (entraining) agent for most circadian systems, and so an understanding of entrainment mechanisms is central to understanding circadian organization. In addition, light can be considered to be the most reliable "drug" currently available to regulate circadian time in our own species. The manipulation of circadian phase has enormous potential in medicine, agriculture and industry, areas in which an understanding of basic mechanisms is required in order to develop treatment paradigms (Wetterberg, 1993).

Our goal is to determine whether an identifiable chain of receptor cells, intra-retinal neurons, and their target retinal ganglion cells forms an exclusive channel conveying photic information for synchronization of the biological clock. A major problem in the study of the entrainment pathway is that it is obscured by a mass of visual receptors and neurons devoted to the processing of visual information. By using mammals which lack specific retinal elements, and determining the effect of these defects on circadian responses to light, we have been able to identify components of the entrainment pathway. We have use mice with naturally occurring retinal disorders (*rd* & *rds*), transgenic mice which lack specific retinal photoreceptors or retinal opsins and a subterranean mammal with rudimentary subcutaneous eyes, the blind mole rat (*Spalax ehrenbergi*).

3. Status of effort: In mammals the primary circadian clock is located within the suprachiasmatic nuclei (SCN) (Ralph et al., 1990) and is regulated by photoreceptors within the eye. Unlike the other vertebrate classes, eye loss in the mammals blocks all circadian (e.g. (Foster et al., 1991; Nelson and Zucker, 1981). It should be stressed that there is no physiological or biochemical evidence for extraocular photoreception in adult mammals. In mammals light information from the eye reaches the SCN via a specific retino-hypothalamic tract (RHT). In the mouse retina as few as 0.001% of retinal ganglion cells (RGCs) project exclusively to the SCN to form this tract (Provencio et al., 1997). Although the RGCs have been defined, the photoreceptors and inner retinal neurones that are connect to these cells remain unidentified. Part of the problem is that the photoentrainment pathway is obscured by the large number of photoreceptors and inner retinal neurones devoted to image formation. Disentangling which retinal cells mediate photoentrainment from the mass of neurones dedicated to image detection has been a major problem.

In recent years, my laboratory has pioneered the use of retinally degenerate models as reduced preparations, correlating the loss of specific retinal elements with an animal's ability to entrain and phase shift in response to different light stimuli. Our first experiments in this area used mice homozygous for *retinal degeneration* (*rd/rd*). These mice experience a massive degeneration of the rods and cones. By 60 days of age all rod cells have degenerated, and between 90 and 150 days of age even the crudest electrophysiological and behavioural responses to bright light have disappeared (Provencio et al., 1994). Although all rods are lost in the *rd/rd* retina a few cone cells survive. These cones lack outer segments and constitute only 2-5% of the cone cells

found within the normal (wild-type, +/+) retina. Despite this massive loss of photoreceptors, *rd/rd* mice show circadian responses to light that are indistinguishable from those of mice with phenotypically normal retinas (*rd/+*, +/+). The irradiance (light intensity) required to produce both saturating and half-saturating responses was found to be the same for all groups. It is important to stress that not only does some photosensitivity remain in mice with degenerate retinas, but the circadian photosensitivity shown by these animals is not different from wild-type mice. In addition, unattenuated sensitivities are maintained in *rd/rd* mice greater than two years of age. These data demonstrate that the sensitivity of the circadian system to light does not parallel loss of either rod or cone photoreceptors in the *rd/rd* genotype (Provencio, et al., 1994). It should be stressed that the site of circadian photoreception must reside within the eye of *rd/rd* mice because bilateral enucleation of these animals abolishes all circadian responses to light (Foster, et al., 1991). Note that although cone opsins and cell bodies remain in the *rd/rd* retina (Argamaso et al., 1995), most of the cones have been lost and the remaining cones lack outer segments.

4. Accomplishments/New findings:

a) The photoreceptors that mediate entrainment: The results from *rd/rd* mice show that rods are not required for photoentrainment. However, the data do not exclude the possibility that a few cone cell bodies are sufficient to maintain normal entrainment. The requirement for cone photoreceptors in photoentrainment has been explicitly examined in very recent experiments that have utilised transgenic mice which contain an integrated fusion gene consisting of a portion of the human red cone opsin promoter (Wang et al., 1992) linked to an attenuated diphtheria toxin gene. Analysis of the retinae of these mice shows that the rod photoreceptors remain intact, UV cones are reduced, but green cones are eliminated (Freedman et al., 1997). Despite the loss of green cones, which are maximally sensitive around 510 nm (Jacobs et al., 1991), the circadian system of these mice responds robustly to monochromatic green light (λmax 515 nm) (Freedman, Soni, Foster - unpublished). This light stimulus would be capable of stimulating rod photoreceptors (maximally sensitive around 500 nm) but not UV cones (maximally sensitive around 360 nm) (Jacobs, et al., 1991). As a result, these data are consistent with the hypothesis that there is complete redundancy of the photoreceptor inputs to the clock. In the absence of the cones, rods mediate photoentrainment while in the absence of the rods, cones regulate the clock. However, the possibility that neither the rods nor cones are required for photoentrainment cannot be excluded. In an attempt to further clarify the role of retinal rods and cones, rodless transgenic mice *rdta* mice (see below) and the green coneless transgenic mice were bred and the progeny of these genetic crosses examined. Preliminary results suggest that low levels of green monochromatic light (λmax 515 nm) are still capable of phase shifting circadian behaviour in these mice (Freedman, Soni, Foster - unpublished). As both green cones (λmax 510 nm) and rods (λmax 500 nm) have been eliminated, the data suggest that the mammalian eye may contain an unrecognised/novel photopigment sensitive to photons around 500 nm.

The possibility that the mammalian eye contains novel photopigments is rendered a little less remote by our recent discovery of novel opsins within the eyes of another class of vertebrates. An opsin gene has been isolated from the eyes of Atlantic salmon, and other fish species, which has the key features of an opsin but is not a member of any of the known rod or cone opsin families. Several domains associated with phototransduction appear unique, and phylogenetic analysis suggests that this opsin diverged from a common ancestor before all of the other opsins (Soni and Foster, 1997). For this reason, a new family has been created and called the vertebrate ancient (VA) opsins. Whether homologues of VA opsin exist within the other vertebrate classes is currently under investigation, but it is hoped that the discovery of the VA opsin family, or perhaps other novel opsins, may ultimately help to resolve how the vertebrate eye mediates entrainment.

The most striking observation to emerge from the studies outlined above is that mice which lack classical visual responses are still capable of normal photoentrainment. This observation, however, is not restricted to mice. The blind mole rat (*Spalax ehrenbergi*) provides another example of a "blind" mammal which shows photoentrainment. The minute eyes (600 μm in diameter) of this subterranean mammal, are buried beneath the skin and brain structures involved in visual analysis are severely reduced or absent. By contrast, the SCN is well developed and receives a bilateral projection from the retina (Cooper et al., 1993). Our behavioural studies show that *Spalax* lacks any image forming ability but will entrain circadian rhythms of activity to the external light cycle, and significantly, removal of the eyes blocks all photoentrainment. Finally, several studies have now demonstrated that circadian rhythms can be regulated by light in a sub-set of humans that have their eyes but remain "blind" to visual stimuli (Czeisler et al., 1995).

Our work on retinally degenerate mice and mole rats and work by other researchers on blind humans, have shown that two functionally distinct systems must exist for processing light information in the mammal eye (and perhaps the eye of other vertebrates). Visual blindness need not mean circadian blindness. The eye contains two parallel light detecting systems. An "image-forming" system, which constructs an representation of the environment, and a "non-image-forming" photoreceptor system, which deduces gross changes in the overall amount of light at different times of day.

b) The sensory ecology of photoentrainment: In plants, and especially in animals, there has been little consideration of the features of the light environment at dawn and dusk that may be important for circadian regulation. In recent years, circadian biologists have come to realise that an understanding of the sensory ecology of the photoentrainment is essential if the cellular and molecular components of this pathway, either identified or sought, are to be placed into a functional context. During twilight the light environment changes in three important respects: 1) the amount of light, 2) the spectral composition of light, 3) and the direction of light (position of the sun in the sky) (Lythgoe, 1979). These features have been implicated in photoentrainment with varying degrees of confidence.

Deducing the amount of light at twilight. The image-forming visual system

needs a measure of brightness in a particular region of space (radiance). Indeed, the visual system exhibits extreme topographic order. Light from a region in space is focused onto a point on the retina, and then mapped precisely to different regions of the visual brain. By contrast, the circadian system needs a measure of the overall amount of light (irradiance) in the sky in order to make a reliable judgement about the phase of twilight. In the non-mammalian vertebrates, photoreceptors located beneath the skull or in the brain are unable to extract any image information. The overlying tissues act like an integrating sphere, scattering light to such an extent that all directionality is lost. By their very nature the pineal and deep brain photoreceptors are excellent irradiance detectors. But how do the mammals, deficient in extraretinal photoreceptors, extract irradiance information using their eyes? The architecture of the eye appears to make it an obligate radiance detector. Of course the eye of the blind mole rat is an obvious exception to most mammals. Its subcutaneous location, and lack of a lens (Sanyal et al., 1990), transforms this organ into an irradiance detector. However, in the rest of the mammals the mechanisms by which the eye can act as an ocular irradiance detector are less obvious.

A partial solution to the problem of extracting irradiance information using the eye, is the utilisation of a specific sub-set of retinal ganglion cells (RGCs). My laboratory has shown that the RGCs which project to the SCN in mice are relatively scarce, are spread evenly across the retina, and have relatively large dendritic fields (Provencio, et al., 1997). This effectively reduces spatial resolution and increases sampling area of the light collected by these cells. In addition, the RHT lacks any topographic order. RGCs project randomly to the retinorecipient areas of the SCN. These combined effects will successfully blur any image and provide the SCN with irradiance information.

Another striking feature of photoentrainment in all vertebrates is that the thresholds for phase shifting circadian rhythms are significantly higher than the light levels required for visual responses. In the hamster, for example, animals can recognise simple images at light levels 200 times lower than the levels necessary to induce phase shifts in locomotor rhythms (Emerson, 1980). This relative insensitivity of the circadian system may be of considerable importance. It will effectively filter out light stimuli that could not provide reliable temporal information (Nelson and Takahashi, 1991). For example, the irradiance of starlight (around 9.3×10^8 photons.cm^{-2}.s^{-1} 400-700 nm) and full moonlight (around 3×10^{10} photons.cm^{-2}.s^{-1} 400-700 nm) (Munz and McFarland, 1977) are both below the threshold for photoentrainment of both the hamster (Nelson and Takahashi, 1991) and mouse (Foster, et al., 1991).

A reliable measure of environmental light, and hence time of day, will also need to compensate for local fluctuations in the light environment. This is a particular problem for mobile organisms which may experience marked changes in light exposure as a result of shading by plants or other structures (Lythgoe, 1979). A consistent feature of the vertebrate circadian system is that it is insensitive to light stimuli of a short duration, but can integrate light information over long periods of time. For example, the circadian system of the hamster is relatively insensitive to light stimuli shorter than 30 seconds, but can integrate/add photic stimuli over periods as long as 45 min.

(Nelson and Takahashi, 1991). By contrast, integration times for image-forming visual responses are in the order of seconds. These features of the circadian system will again act to smooth out any local fluctuations in the light environment to provide a measure of irradiance.

Deducing the spectral composition of light. At twilight there are very precise spectral changes in the light environment. At the horizon there is a relative enrichment in long wavelength/red photons, but across the dome of the sky, and hence the major source of light in the environment at twilight, there is an enrichment of the shorter wavelengths (< 500 nm) relative to the mid-long wavelengths (500 - 650 nm) (Lythgoe, 1979). If the circadian system were capable of some form of spectral discrimination, then it could determine the phase of twilight very accurately. There is strong evidence that the green alga *Chlamydomonas* (Kondo et al., 1991), and the dinoflagellate *Gonyaulax polyedra* (Roenneberg and Deng, 1997), utilise the spectral changes at dawn and dusk to regulate their circadian clocks. However, there is little evidence that the vertebrates use spectral information for photoentrainment. Our work has shown that the circadian system of mice (Provencio and Foster, 1995) and of the golden hamster (Schantz von et al., 1997) is sensitive to both green light (Å 500 nm) and near-UV irradiation (Jacobs, et al., 1991). We also know that multiple photopigments, with different spectral maxima, exist in many of the extraocular photoreceptors (for review see (Meissl and Yanez, 1994). However, we do not know how the signals from these different spectral channels are utilised by the circadian system.

Deducing the position of the sun in the sky. The position of the sun relative to the horizon is a reliable marker of the time of day and is utilised by many different groups of animals for time compensated sun-compass orientation (for review see (Wallraff, 1981). To date no studies have investigated whether the position of the sun is used as a stimulus to entrain the circadian system. The sensory task of plotting the position of the sun would best be achieved by a photoreceptor organ with some form of lens and topographic mapping of light information to the brain. For this task the classical image-forming visual system would be ideally suited. In some species one could envisage the utilisation of both the non-image and image forming photoreceptor systems to bring about entrainment.

c) The role of the input pathway in defining circadian behaviour:

The tendency of animal circadian biologists to compartmentalise the circadian system into: input > clock > output, has led to the assumption that organisms exhibiting abnormal circadian rhythms, altered in their characteristic period, amplitude or phase, will have specific clock gene defects, and that genetic characterisation of these mutants will identify novel clock genes (Hall, 1995). This approach rests on the assumption that the circadian phenotype arises directly from the circadian oscillator and is not the product of an interaction between the clock and its regulatory inputs. Recent results, however, show that it is becoming increasingly difficult in some groups of organisms to distinguish between a "clock molecule" and "input molecule". Indeed, what we mean by a circadian clock is becoming rather blurred. For example, in the plant *Arabidopsis*, phytochrome and the blue light-responsive photoreceptor pathways also profoundly

influence the period of the circadian clock (Millar et al., 1995). In the fungus *Neurospora*, the gene *white collar*-2, regulates several light responses including photoentrainment, and is also an essential components of the circadian oscillator (Crosthwaite et al., 1997).

The results in *Arabidopsis* and *Neurospora* have suggested that photopigments and their transduction elements do not merely regulate the clock but may also play a critical role in the generation of the circadian rhythm. These results encourage a broader interpretation of recent results in the vertebrates. For example, a screen of mice treated with the mutagen N-ethyl-N-nitrosourea identified a long-period circadian phenotype which was called "*clock*". In constant darkness homozygous *clock* mice show a period between 27 - 28 hours for several cycles before becoming arrhythmic (Vitaterna et al., 1994). Now although both the persistence and period of the circadian rhythm have been affected by *Clock*, and the gene has been partially characterised (Antoch et al., 1997; King et al., 1997), no assumptions can yet be made about the function of this gene. For example, the circadian period of most organisms can be substantially altered upon exposure to constant light "Aschoff's Rule", and normal mice exposed to constant bright light will increase their circadian period by 1-3 h. In addition, circadian behaviour in some vertebrates will become arrhythmic after a period of constant light exposure (Aschoff, 1981). These data could be used to support the hypothesis that a period or arrhythmic mutant may result from a defect in the photoentrainment pathway. In the case of *clock*, it is possible that the gene defect mimics the effects of constant light on the circadian system. *Clock* need not be a central component of the oscillator. Further analysis will undoubtedly clarify the function of this fascinating gene.

The extent to which the photoentrainment pathway of mammals can influence the circadian phenotype remains largely unexplored. In rodents, the *rd* mutation (Foster, et al., 1991), or adult enucleation of normal mice (unpublished observation), has no effect upon circadian period. By contrast, a study in blind humans suggested that ocular damage can causes changes in the period (Lockley et al., 1995) and the phase (Lockley, et al., 1995) of circadian rhythms, and these effects cannot be duplicated in sighted individuals maintained under constant lighting conditions (constant dim light or darkness). Our recent studies in mice have looked at the transgenic ablation of retinal photoreceptors during early development, and in these animals there does appear to be a marked effect upon the circadian phenotype.

Rodless transgenic mice (*rdta*) were constructed using a fusion gene consisting of a 1 kb fragment of the human rhodopsin promoter linked to an attenuated diphtheria toxin gene. Morphological, physiological and molecular analysis of *rdta* mice, suggests that diphtheria toxin is expressed exclusively within the rod photoreceptors, and these cells begin to degenerate shortly after the onset of rhodopsin expression (around postnatal day 6). Rod loss appears complete shortly after postnatal day 28 (McCall et al., 1996). There is a secondary loss of cone photoreceptors, but a significant number of cone cell bodies (lacking outer segments) persist in the aged retina. The *rdta* phenotype resembles that of the *rd/rd* mutation; the only obvious difference being that rods degenerate earlier in development than in the *rdta* mice. In *rdta* mice several circadian parameters appear to have been affected. The period of locomotor rhythms has been

significantly reduced by approximately 15 min., and the total duration of activity (*alpha*) has increased by approximately 50 min.. The most striking difference, however, is a large increase in the amplitude of circadian responses to light in both the delay and advance portion of the phase response curve. At irradiances that produce saturating phase shifts in wild-type mice, congenic *rdta* mice showed shifts approximately 2.5 fold greater. A comparison of the irradiance response curves for *rdta* and wild-type controls shows that the threshold for inducing phase shifts is the same in both genotypes. This argues that it is the amplitude of clock responses that has been affected in *rdta* mice and not the sensitivity of the clock to light. (Lupi, McCall & Foster - in preparation). The retinae of *rdta* and *rd/rd* mice appear very similar, both lack rods and have a reduced number of cones which lack outer segments. As a result the differences in circadian behaviour between *rdta* and *rd/rd* mice are difficult to understand. One obvious difference between these two genotypes is that the onset of rod ablation in *rdta* mice occurs approximately one week earlier than in *rd/rd* mice (McCall, et al., 1996). It is possible that the earlier loss of rods in the *rdta* retina may occur when the retina and/or its central projections are sufficiently plastic to allow significant reorganisation during development. An altered signal to the developing SCN may permanently alter the expressed phenotype of the circadian system. What ever the physiological basis for the results, the data do suggest that the circadian phenotype of mammals is not dictated by the SCN developing in isolation from the rest of the circadian system.

Conclusions: The sensory demands of photoentrainment have imposed a unique set of selection pressures, which have led to the evolution of specialised photoreceptor systems. Our work studies on retinally degenerate mammals have shown that visual blindness need not mean circadian blindness, and that two functionally distinct systems for processing light information must exist within the mammal eye. An image-forming system, which constructs an representation of the environment, and a non-image-forming photoreceptor system, which deduces gross changes in the overall amount of light at different times of day. Specialisations of the mammalian photoentrainment pathway include a distinct set of retinal ganglion cells that project exclusively to the circadian centres within the brain, and the possible utilisation novel ocular photoreceptors. The features of the light environment that mediate entrainment have yet to be fully defined. Environmental irradiance appears to be a critical influence, but spectral changes and/or the position of the sun could theoretically provide useful information about the phase of twilight. Finally, the extent to which expressed circadian rhythms arise directly from a clock, or are the product of an interaction between a clock and the entrainment pathway, remains unclear in the vertebrates. In mammals at least, major lesions to the retina, at a time when both the retina and SCN are developmentally plastic, appear to markedly influence some aspects of the circadian phenotype.

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6. Publications (arising from current AFOSR grant):

Garcia-Fernandez, J.M., Jimenez, A.J. and **Foster, R.G.**, The persistence of cone photoreceptors within the dorsal retina of aged retinally degenerate mice (rd/rd): implications for circadian organization, *Neuroscience Letters*, 187 (1995) 33-36.

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7. Interactions/Transitions:

Presentations:

1995

Institute of Ophthalmology, Moorfields Eye Hospital, London (January)
State-of-the-art-in-Biology, Georgia Center for Continuing Educ., U. Georgia (January)
University of Virginia, Medical School, Pathology Department (January)
University of Virginia, National Science Foundation Workshop (February)
Biological Rhythms & Body Functions, Nara University, Japan (March)
University of Wisconsin, Neuroscience Lecture Series (April)
Gordon Research Conference on Chronobiology, Italy (May)
ARVO, Fort Lauderdale, Florida (May)
American Physiological Society, Understanding the Biol. Clock, Dartmouth, USA (July)
World Congress on Chronobiology, Italy (September)
Light Symposium Foundation, Atlanta, Georgia (October)
University of Toronto, Dept. Biology, Canada (October)
University of Edinburgh, Dept. Biological Sciences (November)
University of Nijmegen, Biochemistry Department (December)
Imperial College, Biological Society (December)
University of Surrey, Dept. of Biological Sciences (December)

1996

Charing Cross & Westminster Medical School, Department of Anatomy (February)
Imperial College, Biology 2000 (March)
Imperial College, "What's New in Biology" (April)
Society for Research in Biological Rhythms, Florida (May)
French Chronobiology Society (May)
Kristineberg Marine Research Station, Oscillatory Behav. in the CNS, Sweden (June)
Imperial College, What's New in Biology (July)
Imperial College, Vision Group (July)
Behavioural and Cognitive Neurosci. Summer School, University of Groningen (July)
Erasmus Summer School, Max-Planck-Institute for Behavioural Physiology (July)
Nordic Eye Research Group, Lingatan, Sweden (September)
University of Leicester, Dept. Genetics (December)

1997 (until April 1997)

The Colour Group, Institute of Ophthalmology (January)
Harvard Medical School, Boston, USA (January)
University of Virginia, USA (January)
4th International Symposium on Bioscience and Human-Technology, Japan (February)
Kings College London, Anatomy Department (February)
University of Bristol, Biology Department (March)
Change in the visual system: From molecules to disease, Imperial College (March)
Society for Experimental Biology, University of Kent (April)

8. New discoveries, inventions, or patent disclosures:

None

9. Honors/Awards:

1995: Research Professor, Department of Biology, University of Virginia.
1995: Senior Fellow, The NSF Center for Biological Timing.
1997: Honma Prize in Biological Rhythms Research. "An international award given to an outstanding scientist in the field of biological rhythms research. This awarded is given every two years and recipients are usually under 40 years".