REVIEW ARTICLE

The electroretinogram as a method for studying circadian rhythms in the mammalian retina

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Abstract

Circadian clocks are thought to regulate retinal physiology in anticipation of the large variation in environmental irradiance associated with the earth's rotation upon its axis. In this review we discuss some of the rhythmic events that occur in the mammalian retina, and their consequences for retinal physiology. We also review methods of tracing retinal rhythmicity *in vivo* and highlight the electroretinogram (ERG) as a useful technique in this field. Principally, we discuss how this technique can be used as a quick and noninvasive way of assessing physiological changes that occur in the retina over the course of the day. We highlight some important recent findings facilitated by this approach and discuss its strengths and limitations.

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Environmental light intensity can change by up to 10 orders of magnitude between midday and midnight. This represents a huge variation in functional demands for the visual system. However, no rhythm in the physical environment is more predictable than this light:dark cycle and, as a result, there is great scope for endogenous circadian clocks to adjust visual physiology in preparation for changes in illumination. One might therefore expect circadian control to be an important aspect of the visual system and there is abundant evidence that this is the case.

Circadian and diurnal variations have been described at the highest levels of visual processing by psycho–physical assessments of visual sensitivity in humans and rodents (Terman and Terman 1985; Bassi and Powers 1986; O'Keefe P. and Baker 1987), and by using electroencephalography to describe rhythms in visual evoked potentials (Stolz *et al*. 1988). However, the most extensive evidence for circadian control concerns the earliest events in vision, those occurring in the retina.

Retinal rhythms

Rhythms in retinal anatomy, biochemistry and physiology have been widely reported in nonmammalian vertebrates (Lythgoe and Shand 1983; Kolbinger *et al*. 1996; Manglapus *et al*. 1998; Green 2003; Green and Besharse 2004). In this review however, we concentrate on the situation in mammals. There is a growing literature on this topic, with reports of rhythms in electrotonic coupling between the rod and cone photoreceptors (Ribelayga *et al*. 2008); retinal pH (Dmitriev and Mangel 2001); phosducin phosphorlylation (Pozdeyev *et al*. 2008) and extracellular adenosine (Ribelayga and Mangel 2005). However, to date the most extensively studied rhythms are those in neuromodulator (melatonin and dopamine) activity, photoreceptor outer segment disc shedding, gene expression and aspects of the electroretinogram (ERG).

Rhythms in neuromodulatory substances

High amplitude circadian rhythms in melatonin production and dopamine release have been identified in the mammalian retina (Nir *et al*. 2000; Tosini 2000; Doyle *et al*. 2002; Sakamoto *et al*. 2004; Tosini *et al* 2007). These have been mainly studied in postmortem tissue by radio-immunoassay or HPLC, although there have also been reports of rhythmic melatonin production from cultured hamster and mouse retinae *in vitro* (Tosini and Menaker 1996, 1998). Broadly, rhythms in these two neuromodulators are in anti-phase, with

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dopamine being produced during the day and melatonin at night (Tosini *et al*. 2008). The precise site of melatonin production in the mammalian retina is a little controversial, with evidence for a photoreceptor origin (Niki *et al*. 1998; Tosini *et al* 2007) and for inner retina and ganglion cell layer contributions (Garbarino-Pico *et al*. 2004; Liu *et al*. 2004). Retinal dopamine arises from a subset of amacrine cells and is released upon light exposure and, under conditions of constant darkness, by a circadian clock (Dubocovich 1983; Witkovsky 2004). The anti-phase nature of melatonin and dopamine rhythms is supported by an interaction between these two neuromodulatory substances. Thus, melatonin inhibits the release of dopamine (Dubocovich 1983) and, conversely, dopamine inhibits melatonin production (Nguyen-Legros *et al*. 1996; Tosini and Dirden 2000). Indeed, it has been suggested that rhythms of dopamine release are absent in mice strains lacking melatonin (Doyle *et al*. 2002).

Both melatonin and dopamine are capable of modulating multiple aspects of retinal function. Melatonin receptors have been localized in mammals to photoreceptors and horizontal, amacrine and ganglion cells in the retina (Wiechmann and Summers 2008). Diverse physiological responses to melatonin have been reported including a decrease in photoreceptor membrane conductance (Cosci *et al*. 1997), an increase in the degree of light-induced photoreceptor cell death (Wiechmann and Osteen 1992), and release of the inhibitory neurotransmitter GABA from amacrine cells (Fujieda *et al*. 1999). Dopamine receptors (both D_1-like and D_2-like) are also widely expressed within the retina, supporting the notion that dopamine plays a role as an important effector of light adaptation, driving profound alterations in cell–cell electrical coupling (Hampson *et al*. 1992; He *et al*. 2000; Xia and Mills 2004; Mills *et al*. 2007).

Photoreceptor disc shedding

The rod and cone opsins that form the origin of the visual system are membrane-associated proteins. To maximize the likelihood of photon capture, rods and cones have a unique morphology in which extensive ciliation gives rise to banks of membranous 'discs' packed full of opsin protein. These discs form at the photoreceptor inner segments (adjacent to the cell body) and are shed at the apical surface. Shed discs are engulfed in phagosomes in the retinal pigment epithelium (RPE) (Young and Bok 1969). As such, the processes of disc shedding and phagocytosis are critical events in retinal maintenance. In several mammalian species disc shedding has been reported to occur in a burst shortly after dawn as demonstrated by an upregulation in phagosome numbers in the RPE at this time of day (Nguyen-Legros and Hicks 2000). This process has also been shown to occur in a circadian manner when the animal is kept in constant darkness (LaVail 1980), and even following SCN lesion (Terman *et al*. 1993), suggesting that it is driven by a local circadian oscillator (but see Teirstein *et al*. 1980). The rhythm in disc shedding appears to be defined by melatonin and dopamine signalling (Nguyen-Legros and Hicks 2000) although disc shedding rhythms have been observed in mice genetically deficient in melatonin synthesis (Grace *et al*. 1999).

Gene expression

A recent microarray study reported circadian rhythms in expression of over 250 genes in the mouse retina (Storch *et al*. 2007). The most widely studied of these are genes expressing elements of phototransduction cascades. Transcripts for both rod and cone opsins have been reported to display a circadian rhythm peaking around dusk (von Schantz 1999), while diurnal rhythms in other elements of the phototransduction cascade peak around dawn (Brann and Cohen 1987; Bowes *et al*. 1988). A diurnal rhythm in expression of melanopsin (the photopigment of newly discovered ganglion cell photoreceptors) has also been reported (Hannibal *et al*. 2005). Additionally, rod alpha-transducin shows diurnal translocation, reportedly localised to the rod outer segments during the night, and the inner segments during the day (Brann and Cohen 1987).

Electroretinography

Perhaps the most accessible method by which to explore rhythmicity in retinal function is electroretinography. The ERG is a graphical representation of field potential changes across the eye elicited by a light stimulus. In the clinic it is a powerful diagnostic tool employed to assess the physiological integrity of the retina. It is also widely used for research purposes in human subjects and across the animal kingdom.

Recording ERGs is noninvasive and relatively straightforward. Briefly, an active electrode is placed on, or near, the cornea and a reference electrode is applied elsewhere on the body. These record field potential changes associated with extracellular currents in the retina elicited by a visual stimulus (usually a flash of light).

The nature of the ERG waveform depends upon the recording conditions. The most commonly used is the flash ERG, in which the response to a discrete flash of light is recorded. The ERG reflects the integrated activity of all cells in the retina and defining the precise cellular origins of individual aspects of the waveform is an area of study in itself (reviewed by Heckenlively *et al*. 2006). However, the flash ERG is commonly described in terms of several discrete components (figure 1) each of which is attributed, at least to a first approximation, to a specific event in the transfer of visual information through the retina.

a-wave

The earliest noticeable component is a negative deflection (reflecting a corneal negative potential) termed the a-wave. The a-wave reflects the first event in visual transduction: activation of rod and cone photoreceptors by light (Brown 1968). The degree to which rod and cone photoreceptors contribute

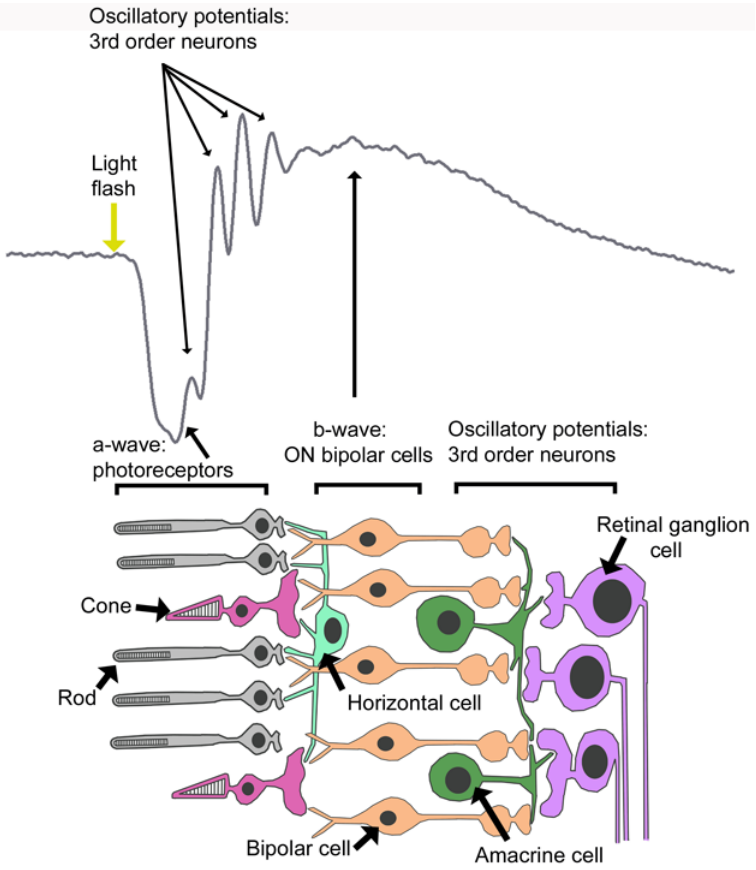

Figure 1. Schematic representation of a flash ERG and the retinal events contributing to it. A representative ERG trace is shown in grey (x-axis, time; y-axis, voltage; arbitrary units), yellow arrow shows time of flash. The earliest element of the ERG is a negative deflection (the a-wave) originating from photoreceptor activation. The next step in signal transfer is activation of ON bipolar cells (the origin of the b-wave, the large slow positive deflection). Subsequently, bipolar cells activate retinal ganglion cells which pass the light signal down the optic nerve. Horizontal and amacrine cells modulate the signal flow through the retina and it is thought that the amacrine cells and/or the retinal ganglion cells contribute to oscillatory potentials (high frequency wavelets superimposed on the b-wave).

to the a-wave is dependent on recording conditions (see below) and also on the species under investigation. The retina of nocturnal species e.g. mice contains so few cones that they generally are incapable of driving a measurable a-wave, while species (including humans) with more cone-rich retinas can have a large cone a-wave.

b-wave

The next and most noticeable component of the flash ERG is the b-wave, a corneal positive deflection thought to reflect the activity of second-order neurons, specifically ON-bipolar cells (Stockton and Slaughter 1989). All rod bipolar cells and at least three of the nine morphological cone bipolar cells are ON-type, therefore the b-wave can be elicited following photoreception by either rods or cones. Hyperpolarising (OFF) bipolar cells and horizontal cells subsequently contribute to repolarisation of the ERG response after the b-wave peak (Bush and Sieving 1994).

Oscillatory potentials

High frequency wavelets frequently appear superimposed on the b-wave of the ERG (shown in figure 1). These are termed oscillatory potentials. Their cellular origin remains controversial but it is generally accepted that they reflect third-order neural events (activation of amacrine and/or ganglion cells; Heynen *et al*. 1985; Yu and Peachey 2007).

Other ERG components

The a-waves and b-waves and oscillatory potentials represent the most easily measureable elements of the flash ERG. However, other components may appear depending on the stimulus and recording conditions. For simplicity these are not represented in figure 1. A corneal positive c-wave is

sometimes observed following the b-wave, and can be accentuated by using an extended duration flash stimulus and/or pharmacological inhibition of the b-wave. The c-wave is thought to result from an increase in the transepithelial potential of the retinal pigment epithelium caused by electrical separation of the basal and apical membranes by tight junctions (Brindley and Hamasaki 1963). A d-wave reflecting the response of the retina to cessation of the stimulus is also sometimes observed. This can be either corneal positive or negative, depending on the stimulus conditions and/or species studied. In a flash ERG, the b-waves and d-waves are almost simultaneous, so an extended flash of > 100 ms is required to separate them. Multiple cellular events define the d-wave, but at least in humans, the largest contribution is from OFF bipolar cells (Stockton and Slaughter 1989; Gurevich and Slaughter 1993; Sieving *et al*. 1994; Szikra and Witkovsky 2001).

Isolating rods and cones

Many modifications of the basic flash ERG protocol have been introduced in order to enhance elements of the waveform that reflect particular aspects of the retinal light response. Perhaps the most fundamental of these are those designed to separate rod-based from cone-based vision. The easiest approach to this problem is to take advantage of the fact that, under dark adapted conditions, rods are roughly 1000 times more sensitive than cones (Fu and Yau 2007). Consequently, it is possible to isolate rod-dependent (scotopic) responses using dim stimuli. At absolute threshold (about 1 log unit brighter than the psycho–physical threshold) the ERG waveform can take on a different shape comprising simply a small corneal negative potential (the so-called scotopic threshold response), the cellular origin of which is unclear (Saszik *et al*. 2002; Bui and Fortune 2004). However, at slightly higher flash intensities a classical rod-dependent b-wave appears.

The difference in absolute sensitivity between rod and cone photoreceptors cannot be easily used to isolate cone-pathways. Although brighter flash stimuli under dark adapted conditions activate cones, rods continue to contribute under these conditions. Nevertheless, it is possible to isolate a cone-dependent (photopic) ERG. One way to achieve this is to rely upon the fact that rods can be saturated ('bleached') by bright light. Thus, a cone ERG may be elicited by a very bright flash presented against a constant background light of sufficient intensity to saturate the rod light response. Similarly, cone ERG can be recorded by a probe flash presented shortly after a very bright flash that saturates rods (Verdon *et al*. 2003). Another approach to isolating cone-dependent responses is to take advantage of their superior temporal resolution compared to rods. Due to the difference in kinetics of these photoreceptors the frequency at which a flickering light stimulus is perceived as a constant light (critical fusion frequency) is much higher for cones than rods. Therefore, the response of cones to a flickering light can be isolated using a flicker that is above the rod critical flicker fusion (Peachey *et al*. 1992). A limitation of this latter approach is that the flicker ERG has a different waveform from the flash ERG and it is not possible to identify components relating to specific elements of retinal pathways.

Factors affecting the flash ERG waveform

The fundamental waveform of the flash ERG is retained under most recording conditions. However, the nature of the stimulus and the adaptation state of the retina can have a large effect on the timing and magnitude of individual elements. There is a strong correlation between the latency (time to peak or implicit time) and amplitude of a-waves and b-waves and the magnitude of the light stimulus and, consequently, the visual response. Thus, dim flashes are associated with small amplitudes and long implicit times (indeed, under these circumstances the a-wave is often undetectable), while increasing flash intensity correlates with increasing amplitude and decreasing implicit time. Similarly, the improvement in contrast sensitivity during adaptation to background light correlates with an increase in the amplitude and decrease in latency of a-waves and b-waves elicited by a flash superimposed on this background (Alexander *et al*. 2006).

ERG rhythmicity

Electroretinography has many advantages as a method of studying retinal rhythmicity. Firstly, because it reflects the integrated response of the retina to a light stimulus it provides a systems level view of circadian control in this tissue. Secondly, it is noninvasive, allowing it to be applied to both human and animal subjects. Finally, multiple recordings can be taken from the same individual, allowing circadian time-courses to be described by longitudinal sampling (as opposed to the cross-sectional experimental design required to measure other retinal rhythms).

Attempts to use the ERG to study retinal rhythms in humans have concentrated primarily on diurnal variations in the retina under a constrained light:dark cycle. All of these studies report time-of-day dependence to the b-wave, but not the a-wave. While this indicates that circadian modulation is more apparent in the inner retinal response, it does not exclude a clock influence on photoreceptor activity (see below). Diurnal modulation of the dark-adapted human ERG comprises a transient suppression in amplitude shortly after dawn and an associated increase in scotopic threshold (Nozaki *et al*. 1983; Birch *et al*. 1984). Studies on the cone-isolated ERG have rather reported alterations in response latency, with b-wave and d-wave implicit times increased at night-time (Hankins *et al*. 1998, 2001).

A reduction in the sensitivity of the dark-adapted ERG b-wave around subjective dawn has also been reported in rats and rabbits. This persists in constant conditions and has been correlated with the increase in phagosome numbers in the RPE at the phase of maximal disc shedding (Sandberg *et al*.

1986; White and Hock 1992). A nocturnal increase in sensitivity and rate of dark adaptation has also been reported for the dark adapted ERG (and VEP) in rabbits (Brandenburg *et al*. 1983).

We have recently explored rhythmicity in the flash ERG of C57BL/6 mice free running in constant darkness (Cameron *et al*. 2008a). We found a marked rhythm in the amplitude and implicit time of the cone-isolated flash ERG recorded against a rod saturating background light. Thus, cone b-wave amplitude, speed and oscillatory potentials were all reduced at subjective night. Dark-adapted ERGs also showed circadian variability, but only at high flash intensities at which both rods and cones contribute to the ERG response. There was no evidence of a circadian rhythm in the response to very dim (rod-isolating) stimuli. These findings suggest that, at least in C57BL/6 mice, it is primarily retinal responses to bright light stimuli that are subject to circadian regulation (Cameron *et al*. 2008a).

Applying the ERG to explore mechanisms of retinal rhythmicity

The application of ERG methodology to retinal rhythmicity has enabled a number of mechanistic insights regarding the circadian/diurnal control of this tissue.

The significance of local versus central clocks: The existence of local circadian clocks was first revealed in *Xenopus* over 20 years ago with Besharse and Iuvone (1983) reporting a circadian rhythm in the activity of the enzyme serotonin N-acetytransferase in isolated eye-cups *in vitro*. Since then, autonomous retinal clocks have been identified in many other vertebrates including mammals (Tosini *et al*. 2008). Thus, the most direct route for exerting circadian control of retinal function is via local oscillators. However, the retina receives innervation, and hormonal inputs from the brain, including the SCN itself, providing an opportunity for central clocks to support rhythmicity (Smeraski *et al*. 2004; Gastinger *et al*. 2005, 2006; Korf and von Gall 2006). What then is the balance of central versus local clocks in providing temporal control of this tissue? An early attempt to address this question showed that the rhythms in scotopic ERG of the two eyes in rabbits could be dissociated by patching one eye, allowing local clocks to free-run (White and Hock 1992). The conclusion that retinal rhythms rely predominantly on local oscillators has more recently been supported by retina-specific knockout of the molecular circadian clock. Storch *et al*. (2007) used a conditional knockout of the *Bmal1* gene (a core clock component indispensable for rhythm generation) to selectively ablate retinal clocks. They showed that this treatment ablated the wild type rhythm in the photopic ERG, indicating that the SCN clock can not compensate for the loss of local retinal oscillators in driving this response (Storch *et al*. 2007).

Importance of rhythmicity for general retinal function: In view of the ubiquity of circadian influence on the basic physiological processes of the retina, it has been hypothesised that this circadian modulation is vital to retinal function (Green and Besharse 2004). In their study of *Bmal1*$^{-/-}$ mice, Storch *et al*. (2007) addressed this question by studying ERGs in animals bearing either a retina-specific or whole animal lesion of the circadian clockwork. In both cases they reported modest decreases in the amplitude of cone and mixed rod+cone ERGs. The authors attributed these findings to the loss of circadian regulation of retinal pathways rather than any generalized pathology (Storch *et al*. 2007). Recent data from our lab supports this conclusion (Cameron *et al*. 2008a). We assessed retinal function in *Cry1*$^{-/-}$Cry2$^{-/-}$ mice, which also lack behavioural circadian rhythms. We found that ERG rhythmicity was abolished in these animals but, surprisingly, that this was associated with constitutively enhanced ERG amplitude. This confirms that clock-loss need not necessarily induce any decrement in the retinal-light response and indicates that clocks are dispensable for general retinal health, at least under laboratory conditions. The difference between the ERG phenotype of *Bmal1*$^{-/-}$ and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice is intriguing. One possible explanation is that because *Bmal1* and *Cry1/2* are active at different phases of the molecular oscillation, these two lesions stop the clock at a different state. That could explain why *Bmal1*$^{-/-}$ ERGs are consititutively suppressed (equivalent to the wild-type response at night) and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ ERGs are constitutively enhanced (equivalent to the wild-type response during the day).

Role of ipRGCs in regulating the local retinal environment: The recent discovery of melanopsin-expressing intrinsically photoresponsive retinal ganglion cells (ipRGCs) provides an independent mechanism by which light could influence retinal physiology. Although ipRGCs do not make a direct contribution to the ERG waveform, ERG studies in humans and mice have revealed a role for these novel photoreceptors in modulating the local retinal environment. In the case of humans, they appear responsible for some aspects of long-term light adaptation. Thus, the nocturnal increase in cone b-wave implicit time (discussed above) can be reversed by acute bright light stimulation, and the action spectrum for this effect (λ_{max} = 483 nm) matches the reported spectral sensitivity of ipRGCs (Hankins and Lucas 2002). To further identify the role of ipRGCs in diurnal and circadian rhythms of the mammalian retina, we recently assessed cone ERGs in melanopsin knock-out mice (*Opn4*$^{-/-}$), which lack ipRGC photosensitivity (Lucas *et al*. 2003). While all aspects of light adaptation in the cone ERG appeared normal in these animals, we found that circadian rhythmicity was lost (Barnard *et al*. 2006). This suggests that ipRGCs are required to maintain ERG rhythmicity under circadian conditions, perhaps by retaining synchrony of local clocks. It has recently been reported that ipRGCs regulate the activity of a subset of dopaminergic DA amacrine cells in the murine retina (Zhang *et al*. 2008). This provides a potential route by which the ipRGC influence on diurnal/circadian control

of retinal physiology could be transduced. Experiments from our laboratory indicate that this ipRGC input is insufficient to influence global dopamine levels in the retina, but it certainly could influence their activity in other ways, including, perhaps, entrainment or synchronisation of the circadian clocks in these cells (Cameron *et al*. 2008b, in press, unpublished data).

Challenges and limitations

The ERG has proved very useful in describing general properties of circadian rhythms in retinal function. However, its ability to identify the specific physiological events that are under circadian control in this tissue has not yet been fully exploited. In general terms the amplitude and implicit time of the a-waves and b-waves correlate with the magnitude of the visual response. However, despite this relationship and the known dependence of individual components of the ERG on the activity of specific retinal cell types (see above) it is difficult to assign alterations in a given ERG parameter to a change in a specific aspect of the retinal light response. This is because the ERG is a composite waveform in which all components are to some extent overlapping. Thus, for example, a reduction in b-wave latency would result in an earlier truncation of the a-wave and could, in theory, result in a decrease in a-wave amplitude. Relationships such as these make it difficult to ascribe an alteration in waveform to a particular event with certainty. In future, however, it should be possible to get around this problem by using either intravitreal injections of agonists/antagonists or suitable transgenic models to fully isolate different aspects of the light response.

Another challenge in employing ERGs to trace retinal rhythmicity is the fundamental plasticity of the retinal light response. For this reason, retinal rhythms can easily be masked by experimental conditions. Great care, in particular, must be taken to control adaptation state and prior light exposure (over short-terms, medium-terms and even surprisingly long-terms (Hankins and Lucas 2002)). As a particular example of this issue, we have found that recording ERG rhythms from mice free-running in LL is prone to artefact due to a progressive decrease in ERG amplitude under these conditions. Interindividual variation is also a concern, making a within subjects experimental design desirable. In rodents, there are large age and, in some cases, strain-specific effects on ERG magnitude that need to be controlled for. A final issue to consider in experimental design that has arisen from our own work in mice is the need for pretreatment with a stable, high-amplitude light:dark cycle. For reasons that remain unknown we have found it difficult to record ERG rhythms in mice previously housed under dim or erratic lighting regimes.

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