Chapter 7
Retinal Circadian Rhythms in Mammals Revealed Using Electroretinography

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Abstract Light levels can change by up to ten orders of magnitude between midday and midnight. As a result, the visual system is faced with a large diurnal variation in functional demands. Two mechanisms exist to allow the retina to function under such varied conditions: adaptation and circadian rhythmicity. Adaptation occurs in response to the presenting light conditions and circadian rhythmicity allows the tissue to anticipate those light conditions. Circadian rhythmicity has been described at many points along the visual projection from its photoreceptive origins to the highest levels of visual processing. Electroretinography has proved a very useful tool in the assessment of retinal rhythms. It offers a noninvasive and quantitative assessment of the activity of first- and second-order cells in the retina and has been used by a number of researchers to describe diurnal and/or circadian rhythms and probe their mechanistic origins in several mammalian species. Here we review the various attempts to investigate these retinal rhythms, predominately by use of the electroretinogram, in several mammalian species.

Keywords Electroretinogram • Circadian • Retina • Mammalian • Adaptation

Light levels can change by up to ten orders of magnitude between midday and midnight. As a result, the visual system is faced with a diurnal variation in functional demands at least as great as any other physiological system. It is perhaps unsurprising then that daily variations in structure, biochemistry, and function have been described at many points along the visual projection from its photoreceptive

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G. Tosini et al. (eds.), *The Retina and Circadian Rhythms*, Springer Series in Vision Research 1, DOI 10.1007/978-1-4614-9613-7_7,
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origins to the highest levels of visual processing [1–5]. These diurnal rhythms reflect, in part, direct responses to the changing environmental illuminant (light adaptation), but are also influenced by endogenous circadian clocks. The inclusion of such circadian control has an important functional benefit—by providing a sense of time it allows elements of the visual system to change in advance of predicted alterations in illuminance.

Other chapters in this volume address rhythms in retinal anatomy, biochemistry, and physiology in a variety of vertebrate species. Here we concentrate upon systems level investigations in mammals, with a primary focus on data obtained using the electroretinogram (ERG). Electroretinography has proved a very useful tool in the assessment of retinal rhythms. It offers a noninvasive and quantitative assessment of the activity of first- and second-order cells in the retina and has been used by a number of researchers to describe diurnal and/or circadian rhythms and probe their mechanistic origins in several mammalian species. The ERG, however, cannot reveal the ultimate output of the retina—the pattern of action potentials produced by retinal ganglion cells (RGCs) and sent to the brain. We aim also to review the rather few attempts that have been made to describe rhythmicity in this visual code by recording the activity of either RGCs or retinorecipient nuclei in the brain.

7.1 Electroretinography

The ERG is a graphical representation of field potential changes across the eye elicited by a light stimulus. It is often used clinically as a diagnostic tool to assess the physiological integrity of the retina. However, it is also widely used for research purposes in both human and animal subjects. The process of recording an ERG is relatively straightforward and noninvasive, making it an attractive research tool for recording from laboratory animals. An active electrode is placed on, or near, the cornea and a reference electrode is applied elsewhere on the body. Field potential changes are then recorded that are associated with extracellular currents in the retina elicited by a visual stimulus. The visual stimuli used can vary depending on the response required, and will be discussed later. The most commonly used technique is the full-field ERG, where a brief flash (usually <10 ms) is presented to the retina and the combined response of all cells recorded. However, the development of the multifocal ERG by Erich Sutter 20 years ago allowed for assessment of ERG activity in small, focal, areas of retina in response to the same visual stimuli [6]. This technique was invaluable for detecting local areas of dysfunction in human retina such as macular degeneration or small scotomas. For most assessments of circadian rhythmicity, however, the full-field ERG has been utilized, as circadian regulation is assumed to impact all areas of the retina.

The full-field ERG response to a brief flash of light gives a trace similar to that shown in Fig. 7.1. This trace is a composite waveform to which several defined, and likely some undefined, physiological events contribute [7]. Nevertheless, it is commonly described in terms of three major components, each of which is attributed to a specific event in the transfer of visual information through the retina. These can have one of two notations. Ragnar Granit (1933) designated the different

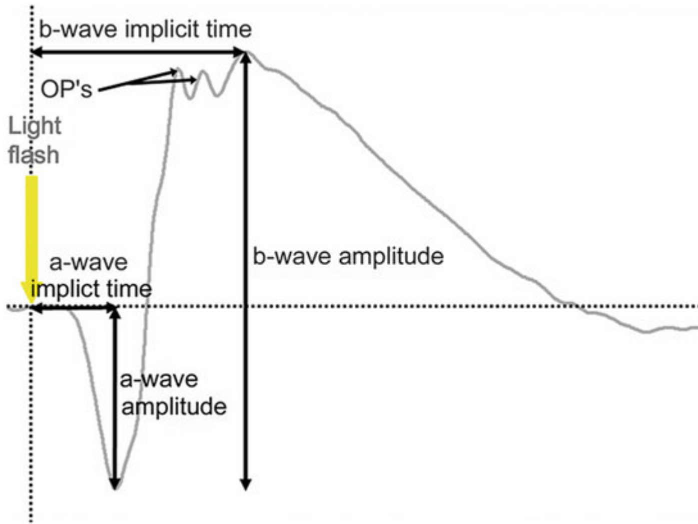

Fig. 7.1 Schematic representation of a flash ERG and the retinal events contributing to it. A representative ERG trace is shown in *gray* (*x*-axis: time; *y*-axis: voltage; arbitrary units), and *yellow arrow* shows time of flash. The earliest element of the ERG is a negative deflection (the a-wave) 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 (OPs; high frequency wavelets superimposed on the b-wave)

components in the sequence of their disappearance following anesthesia as PI, PII, and PIII. Those experiments emphasized that the ERG waveform is in fact the sum of several separable physiological events. The relative amplitude and latency of these components determines qualitative and quantitative aspects of the ERG waveform. Although the PI–PIII notations are still used today, the more common component names are described below, along with their relationship to Granit's notations.

7.1.1 a-Wave

The earliest component (and most resistant to anesthesia) is a negative deflection (corneal negative potential) termed the a-wave. The a-wave reflects the first physiological event in visual transduction, activation of rod and cone photoreceptors by light, and is a reflection of Granit's PIII component [8]. When assessing the a-wave empirically, the convention is to measure the amplitude from the baseline (pre-stimulus) to the trough of the transient a-wave (see Fig. 7.1). Additionally, the latency of this wave, the so-called implicit time, can be measured as the time from the onset of the light flash to the trough of the a-wave. These measurements can then

be compared over the course of the circadian day. The a-wave therefore gives the potential to reveal circadian rhythmicity in the earliest steps of vision.

7.1.2 b-Wave

The a-wave is curtailed by appearance of a corneal positive deflection (Granit's PII component) which is apparent in the integrated waveform as the b-wave. The b-wave is thought to originate with the activity of second-order neurons, specifically ON bipolar cells [9]. Both rod and cone activation can give rise to the b-wave as all rod bipolar cells and several cone bipolar cells are ON-type [10]. Hyperpolarizing (OFF) bipolar cells and horizontal cells also contribute to shape of this wave by causing repolarization of the ERG response after the b-wave peak [11]. The b-wave can be measured in a similar way to the a-wave: amplitude is measured from the trough of the a-wave, to the positive peak of the b-wave; and the implicit time from the flash onset, to the peak of the b-wave (see Fig. 7.1). There is a strong correlation between the latency and amplitude of a- and b-waves and the magnitude of the light stimulus and, consequently, the visual response. Dim flashes are associated with small amplitudes and long implicit times, and increasing the flash intensity correlates with increasing amplitude and decreasing implicit times. Additionally, light adaptation state of the retina can play a role in the magnitude of these components with adaptation to a background light correlated with an increase in the amplitude and decrease in the implicit time of a- and b-waves [12].

7.1.3 Oscillatory Potentials

High frequency wavelets, termed oscillatory potentials, frequently appear superimposed on the b-wave of the ERG (shown in Fig. 7.1). Although their cellular origin remains controversial, it is generally accepted that they reflect third-order neural events (activation of amacrine and/or ganglion cells; [13, 14]).

7.1.4 Other Components

Other components of the ERG (that, for simplicity, have not been annotated in Fig. 7.1) may appear depending on the stimulus and recording conditions such as a slow corneal positive "c-wave" (corresponding to Granit's PI), and a "d-wave" that is sometimes observed at the cessation of the stimulus. 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 [15]. Multiple cellular events are thought to define the d-wave, but the largest contribution is from OFF bipolar cells [9, 16–18].

7.1.5 Separating Rod and Cone Responses

Given the shift in reliance from rod- to cone-based vision and back again over the natural diurnal cycle, it is important that assessments of retinal rhythmicity target visual events from both outer-retinal photoreceptor types. As discussed above, different recording conditions can bias the ERG to reveal responses originating from either rod or cone photoreceptors. However, it is important to remember that rod and cone signals are mixed at all steps in information transfer [19], including at the level of the photoreceptors themselves. As a result, even if an ERG waveform is elicited by selective activation of cones, its characteristics will be impacted also by the physiological state of rods and the retinal pathways downstream of them (and vice versa).

The species under investigation and the type of recording condition can alter the balance of rod and cone input to the a-wave. Nocturnal species, e.g., mice, contain so few cones that only a minimal cone a-wave can be recorded, while species with more cone-rich retinae (including humans) display large cone a-waves. Mammalian rod-dominated retinas usually all show large rod-driven a-wave responses. The easiest way to separate these responses is to take advantage of the fact that, under dark-adapted conditions, rods are roughly 1,000 times more sensitive than cones [20]. Consequently, using dim stimuli, it is possible to isolate rod-dependent (scotopic) responses. At absolute threshold for the ERG response, the waveform can take on a different shape comprising simply a small corneal negative potential (scotopic threshold response), the cellular origin of which is not entirely clear [21, 22]. However, at slightly higher flash intensities a classical rod-dependent b-wave appears. The b-wave can be recorded at lower intensities than the a-wave, likely reflecting substantial amplification of the initial response when passed to the second-order cells.

The difference in absolute sensitivity between rod and cone photoreceptors unfortunately cannot be easily used to isolate cone pathways as brighter flash stimuli under dark-adapted conditions activate both cones and rods. Strategies to isolate a purely cone-dependent (photopic) ERG usually involve eliminating rod influence by saturating them with bright light. A cone ERG may therefore be elicited by a very bright flash presented against a constant background light of sufficient intensity to saturate the rod light response. The rod influence can also be eliminated using the “paired-flash” protocol where a cone ERG can be recorded by a probe flash presented shortly after a very bright flash that saturates rods [23]. Temporal response differences between rods and cones can also be exploited, as cones possess superior temporal resolution compared to rods. 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, using a flicker that is above the rod critical flicker

fusion can isolate cone responses [24]. Finally, in some species there is a clear difference in spectral sensitivity of rod and cone photoreceptors, allowing judicious use of different wavelengths to bias stimuli towards targeting one or other receptor class. While all these cone-isolating techniques exclude the rod response to the presenting flash stimuli, it must be noted that rods are of course "activated" in all these protocols. Assessing the activity of cones without activating rods is thus only possible using transgenic models that permanently remove or inactivate rod responses [25, 26].

7.2 Rhythmicity of the ERG

There are many advantages of using a technique such as the ERG to examine retinal rhythms. The noninvasive nature of the technique allows many different species to be assessed (including humans), with relative ease and speed. It is also possible to record responses over the course of the circadian day from the same animal, instead of using cross-sectional experimental design. The ERG has therefore been used extensively to study circadian control of the retina at a systems level.

The difference between diurnal and circadian rhythms has likely been extensively reviewed in previous chapters, but we would like to emphasize this distinction before reviewing the ERG literature, particularly because light adaptation is such a ubiquitous feature of visual processes. Even in the absence of endogenous circadian clocks therefore, strong 24 h rhythms in retinal function would be expected under the natural light:dark cycle. In order to determine the extent to which diurnal rhythms reflect circadian control, as opposed to such direct effects of light exposure, experiments should include an assessment under constant lighting conditions (usually constant darkness (DD)).

7.2.1 Local Versus Central Clocks

A second important variable to consider when assessing retinal rhythms, diurnal or circadian, is the influence of the central circadian pacemaker, the suprachiasmatic nucleus (SCN) of the hypothalamus, versus endogenous circadian clocks in the retina itself. The existence of local clocks within the retina was first revealed by Besharse and Iuvone [27] in the retina of the *Xenopus laevis* 30 years ago [27]. They reported a circadian rhythm in activity of the enzyme serotonin *N*-acetyltransferase in isolated eye-cups in vitro and this rhythm was able to be entrained to exactly 24 h by administration of a 12:12 h light/dark (LD) cycle. Since then, autonomous local retinal circadian clocks have been reported in many other vertebrates, including mammals [28]. This provides clear evidence that the retina contains autonomous circadian clocks, but, in vivo, it does receive innervation and hormonal inputs from the brain, and SCN itself, providing an opportunity for central clocks to exert an

influence [29–32]. The question of which exerts the predominate influence was elegantly addressed by White and Hock in 1992, who examined a daily suppression in the dark-adapted (scotopic) ERG around the time of rod outer segment disk shedding [33]. Rabbits were entrained to a high amplitude 12:12 h LD cycle for 3 weeks and then one eye was sutured shut and patched to occlude light input. Scotopic ERGs were measured after 12 h in the dark, and the time at which a drop in the amplitude of the b-wave was observed was noted. For the unpatched eye, this always occurred ~30 min after subjective dawn, but for the patched eye the drop in b-wave amplitude occurred earlier as the days progressed. After 4 days of continuous DD for the patched eye, the b-wave reduction, and histological disk shedding correlates, occurred 10 min prior to subjective dawn, ~40 min earlier than the unpatched eye. This corresponded to a "free-running" circadian rhythm of ~23 h 50 min, and provides support for the notion that, even in an intact in vivo system, retinal clocks are the predominate timekeeper in the mammalian retina. Further evidence for this was reported recently in the mouse retina. Storch et al. [34] used a constitutive retinal knockout of *Bmal1* (an essential component of the molecular circadian clock) to display loss of circadian rhythmicity of the light-adapted (photopic) ERG in animals possessing a functional SCN [34]. It is therefore clear that circadian rhythmicity of the retina likely depends largely on intrinsic clocks located within this tissue. This raises a number of supplementary questions including how these local clocks regulate retinal physiology and how they retain synchrony with external time—are they reset by local photoreceptors or do they rely upon a synchronizing signal from the central hypothalamic oscillator (as is the case with other non-SCN clocks)?

7.2.2 ERG Rhythms in Human and Mouse

As previously stated, the noninvasive nature of the ERG technique means it can easily be used to assess rhythms in human retinal function. However, due to the somewhat tedious and impractical nature of circadian experiments that must be conducted in constant conditions (usually DD), most human studies have concentrated on diurnal retinal rhythms. Several studies correlated a change in the scotopic ERG b-wave amplitude (usually a drop) shortly after dawn [35–37], with rod disk shedding and subsequent phagocytosis thought to happen around this time. In fact, disk shedding, and scotopic b-wave amplitude and threshold sensitivity reduction, is highly correlated in both rabbit and rats [33, 38, 39]. However, this reduction in sensitivity could not be entirely accounted for by the shortening of rod outer segments, suggesting the influence of other circadian processes [39]. Studies on photopic ERGs in humans have generally reported alteration of the implicit time of the b-wave, and additionally the d-wave, with longer implicit times exhibited at night [40–42].

More recently, this analysis has been extended to mice, due to the vast opportunity for genetic manipulation in these animals. In C57BL/6 mice we have previously reported a marked rhythm in the amplitude and implicit time of the cone-isolated photopic ERG recorded against a rod saturating background light,

with smaller, longer latency responses recorded during the subjective night [43]. Dark-adapted ERGs also showed circadian rhythmicity but only at flash intensities high enough to reflect mixed activation of both rods and cones. This observation was also repeated in sighted $C3H^{f+/+}$ mice that can synthesize the circadian "neuro-hormone" melatonin, indicating that in wild-type mice, it is primarily retinal responses to bright stimuli that are subject to circadian regulation [44]. Some aspects of these findings may reflect the nocturnal temporal niche, and underground burrowing system utilized by rodents. Under these conditions, rod vision could be useful at all diurnal phases and therefore should be maintained across the circadian cycle. On the other hand, irrespective of such considerations, cone vision should be a feature only of "daytime," and therefore could benefit from being under circadian control.

7.3 Mechanisms Underlying ERG Rhythmicity

A variety of pharmacological and genetic approaches have been used to probe the mechanisms responsible for the ERG rhythmicity.

7.3.1 Rod/Cone Pathway Balance

As rod and cone pathways are intrinsically shared in the mammalian retina [19], the segregation of these two pathways, possibly though modulation of gap-junction coupling, appeared an attractive candidate to explain the enhancement of photopic b-wave amplitude and implicit time in the subjective day. We employed the *Gnat1*$^{-/-}$ mouse that lacks normal rod phototransduction (although morphologically intact rods remain [25]) to assess the influence of rod phototransduction on cone ERG rhythmicity [45]. However, these animals retained large amplitude circadian rhythms in the ERG b-wave, and instead showed specific deficits in light adaptation [45]. Nonetheless, as rods are morphologically still intact in these animals, it is possible that circadian rhythmicity of gap-junctions connecting rod and cone pathways remains intact.

7.3.2 Melanopsin-Containing RGCs

The recently discovered melanopsin-containing retinal ganglion cells (mRGCs) are generally known to be responsible for non-image forming vision, providing light information to the SCN for the entrainment of circadian rhythms, the olivary pretectal nuclei (OPN) for the pupil light reflex (PLR), and several other higher brain areas [46–49]. In addition to these higher inputs we have shown that mRGCs perform a

local modulatory function within the retina. Although, mRGCs probably do not make a direct contribution to the ERG waveform, ERG studies in humans and mice have revealed the influence of these photoreceptors on ERG rhythmicity. In the case of humans, mRGCs appear to be responsible for some aspects of long-term light adaptation. The nocturnal increase in cone b-wave implicit time (discussed above) can be reversed by acute bright light stimulation. The action spectrum for this effect (λ_{max} = 483 nm) matches the reported spectral sensitivity of mRGCs [50]. The role of mRGCs in the diurnal and circadian modulation of the ERG was further investigated in melanopsin-deficient mice (*Opn4*$^{-/-}$), which lack mRGC photosensitivity [51]. Circadian rhythmicity of the cone-isolated photopic ERG (described above) was lost in these animals with ERGs resembling a middle ground between "day-time"- and "night-time"-like [52]. This suggests that mRGCs are required to maintain ERG rhythmicity under circadian conditions, perhaps by retaining the synchrony of local clocks.

7.3.3 Melatonin

The so-called circadian neurohormone melatonin plays a widespread role in the temporal regulation of many aspects of physiology [53]. Its influence is no less important in the retina, with several studies assessing the impact of systemic and/or local release of melatonin on retinal function [53]. Retinal melatonin is synthesized in photoreceptors under the direct control of a circadian clock [54, 55], where levels are high during the night and low during the day. Similar to centrally released melatonin from the pineal gland, the synthesis of melatonin can also be suppressed by light stimulation [56]. For this reason, it is thought to provide a night-time cue to influence retinal physiology by playing a role in both diurnal and circadian modulation. Exogenous melatonin administration appears to have a differential effect in humans and rodents, possibly reflecting the difference in ecological niche. In humans, high salivary melatonin was correlated with the low photopic ERG b-wave amplitudes under diurnal conditions [57] and oral dosing with melatonin caused a reduction in the amplitude of the b-wave under both scotopic and photopic conditions [58]. However, other studies in humans have produced varied results [59–61].

In mice, studies involving melatonin have been limited, due to the rarity of mouse strains available that are capable of synthesizing the hormone [62, 63]. The C3H mouse does produce melatonin, but is often coupled with the *rd1* mutation that causes rapid photoreceptor degeneration in these animals. Baba et al. (2009) recently crossed C3Hf$^{+/+}$ mice lacking the *rd1* mutation with mice containing a targeted deletion of the melatonin 1 receptor (MT1$^{-/-}$) to allow further insight into the function of melatonin in the mouse retina [64]. The MT1 receptor is localized on photoreceptors, inner retinal neurons, and ganglion cells providing several loci for melatonin to exert an influence. C3Hf$^{+/+}$MT1$^{-/-}$ mice lack both diurnal and circadian rhythmicity of the photopic ERG with overall suppressed responses, showing a similar

phenotype to the *Opn4*$^{-/-}$ animals [44, 64]. Injection of melatonin in the day in C3Hf$^{+/+}$ decreased the scotopic response threshold and increased a- and b-wave amplitudes, but not in C3Hf$^{+/+}$MT1$^{-/-}$ animals. Interestingly, circadian dopamine metabolism, thought to exert a strong influence on ERG rhythmicity (see below), was normal in the retinae of C3Hf$^{+/+}$MT1$^{-/-}$ animals, indicating that the circadian rhythm in dopamine release is not sufficient to drive circadian rhythmicity of the photopic ERG [44].

7.3.4 Dopamine

Dopamine plays an important role in the nervous system where it functions both as a neurotransmitter and neuromodulator acting on two subfamilies of dopamine receptor. D1-like receptors (D1 and D5) couple positively to adenylyl cyclase and D2-like (D2–D4) negatively to produce opposing responses based on cellular receptor expression. Dopamine release in the retina is strongly light-dependent [65–68] and modulated by a circadian clock [69] with greater release in the subjective day. Many aspects of retinal physiology are thought to be affected by light and/or circadian-induced changes in dopamine release, including gap-junction coupling, melatonin synthesis, disk shedding, and growth and development [70]. It is therefore unsurprising that dopamine plays a role in the rhythmicity of the ERG. Dopamine is solely produced in dopaminergic (DA) amacrine cells within the retina that are identifiable due to high expression levels of the rate-limiting enzyme in dopamine metabolism, tyrosine hydroxylase (TH). Jackson et al. have recently published an extensive report on the influence of dopamine on the mouse ERG, using retina-specific dopamine-deficient mice (rTHKO) and selective dopamine receptor subtype knockouts (D1RKO, D4RKO, D5RKO) [71]. In agreement with previous work, a circadian rhythm in the mouse scotopic ERG was not observed; therefore, the authors focused their efforts on the photopic light-adapted ERG using the rod saturating background protocol developed in our laboratory [43]. Mice lacking dopamine in the retina showed abolishment of photopic ERG rhythmicity after 2 days in DD with reduced cone ERG amplitudes in comparison to wild-types. In contrast to the *Gnat1*$^{-/-}$ mice, surprisingly, adaptation (growth of the b-wave in response to background illumination) was unaffected in these animals. Furthermore, circadian rhythmicity of *Per2* (an integral clock gene) was maintained, suggesting the retinal clock remains fully functional in these animals. As these animals display a similar phenotype to the *Opn4*$^{-/-}$ it could be postulated that the loss of melanopsin abolishes the rhythmic release of dopamine in the retina, possibly through a desynchrony of the DA amacrines. We have shown that mRGCs are not sufficient to elicit a global change in dopamine [72], but an excitatory input from mRGCs to DA amacrines has been identified [73] which may play a large role in synchronizing the circadian release of dopamine from these cells.

Jackson et al. expanded their study by examining dopamine receptor-specific knockouts. Their main findings implicated D1 and D4 receptors in the regulation of the ERG (and also higher visual function). D4RKOs displayed similar responses to the rTHKO with the abolishment of circadian rhythmicity after 2 days in DD suggesting this receptor may influence circadian rhythmicity through rhythmical dopamine release. D1RKOs, interestingly, showed deficits in light adaptation of the ERG but intact circadian rhythms. The mechanism of action for this result is unclear, especially due to the lack of adaptation phenotype in the rTHKO animals. However, it could indicate that rapid changes in D1-receptor expression levels underlie the adaptation seen in the wild-type ERG, rather than in dopamine release per se.

7.3.4.1 Importance of Retinal Clocks for General Retinal Function

When considering the prevalence of physiological processes within the retina that are affected by the circadian clock, it has been hypothesized that this circadian modulation is vital to retinal function [5]. This hypothesis has been addressed by studying ERGs in animals lacking integral components of the molecular circadian clock, *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice [43]. These animals lack behavioral circadian rhythms, showing highly disrupted wheel-running rhythms in constant conditions. They do, however, show "normal" diurnal wheel-running behavior when exposed to 12:12 LD cycles, active in the dark and inactive in the light [74]. Whether this reflects actual diurnal rhythmicity or not is still a matter for discussion, but does indicate that some manner of visual function in these animals remains intact. When analyzing the ERG, we observed that, as may have been expected, circadian rhythmicity was abolished in these animals. However, surprisingly, ERG b-wave amplitudes were constitutively enhanced under both scotopic and photopic conditions indicating that clocks are certainly dispensable for general retinal health, at least under laboratory conditions. In another study, Storch et al. [34] also examined ERGs in animals bearing either a retina-specific or whole animal lesion of the circadian clockwork, namely the core clock gene *Bmal1* [34]. Again, loss of circadian rhythmicity of the photopic ERG was apparent. However, in contrast to our data from the *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice, modest decreases in b-wave amplitude of cone and mixed rod + cone ERGs were observed. When this data is taken together with our *Cry1*$^{-/-}$*Cry2*$^{-/-}$ findings, it appears likely that this reduction in ERG amplitude is attributed to the loss of circadian regulation of retinal pathways rather than any generalized pathology. Indeed, 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, *Bmal1* is a transcriptional promoter and *Cry1/2* are transcriptional repressors, these two lesions stop the clock in a different state. This could explain why *Bmal1*$^{-/-}$ ERGs are constitutively 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).

7.4 Ganglion Cell Rhythmicity

All light signals elicited in the retina, if they are to be passed to higher visual areas in the brain, must pass through RGCs. As the ERG is generally used to reflect the function of photoreceptors and bipolar cells in the retina, it is important to discuss circadian rhythmicity in the cells that actually carry the ultimate output signals from the retina. Of course, any rhythmicity arising in these cells could come from presynaptic origins so it is difficult to assign the loci of circadian regulation by recording from these cells alone. However, as previously discussed, mRGCs act as photoreceptors and it is possible to identify intrinsic circadian rhythmicity in these cells using the relevant light stimulation techniques. Often referred to as the "circadian photoreceptor," relating to their light input into the central circadian pacemaker, could circadian control mechanisms intrinsic to these cells contribute to the time-of-day dependence observed in the SCN light response [75]?

Core molecular clock components have been localized to the majority of cell types in the mammalian retina [76]; however, a large heterogeneity exists between cell type in the level and rhythmical occurrence of clock protein expression [77]. mRGCs themselves exhibit all six of the "core" clock proteins, making it likely that these cells are capable of autonomous circadian rhythm generation [77]. Certainly, a circadian variation in melanopsin mRNA levels has been observed in these cells [78, 79]; however, this rhythm is abolished after degeneration of rods and cones [80]. Weng et al. [81] addressed circadian rhythmicity of mRGCs using multielectrode array recordings of rat mRGCs pharmacologically isolated from synaptic inputs. They found that although mRGCs did exhibit an increase in response gain of mRGC phototransduction (increased firing rate) in the subjective night, no significant circadian rhythmicity in light sensitivity was observed [81]. In some respects, these results make sense for an input to the circadian clock that must retain sustained irradiance-coding for accurate relay of the presenting light conditions to the central circadian clock.

The circadian rhythmicity of mRGCs could contribute to time-of-day dependence in central light responses. The SCN circadian clock itself shows a strong rhythmicity in photosensitivity, with light adjusting clock phase only when presented during the subjective night [82]. Many individual SCN neurons remain photoresponsive during the day, indicating that retinal afferents are active at all circadian phases. However, electrophysiological recordings indicate that SCN light responses are qualitatively and quantitatively different across the circadian cycle [75, 83], raising the possibility that some of the variation in clock photosensitivity could originate in the retina. Another mRGC-driven response to show strong circadian variation is the PLR. The PLR, although driven by all three types of photoreceptor [84], relies solely on the output of mRGCs [85]. Zele et al. analyzed the post-illumination pupil light response (PIPR), sustained pupilloconstriction after light cessation that is thought to represent activation of melanopsin specifically [86]. They found that the PIPR did display a circadian rhythm with a minimum post-illumination pupil response occurring in the subjective evening ~1.31 h after melatonin onset [87].

This circadian change was effected via altered redilation kinetics observed at this time of day. It is certainly possible that these results could reflect central circadian modulation at the level of the OPN where the PLR is controlled, but also could suggest that the mRGC input could vary over the course of the circadian day.

7.5 Conclusions

The ERG represents an attractive technique for studying circadian control of the retina. It can be applied to both laboratory animals and human subjects, using comparable protocols, facilitating translation of basic physiological insights into an understanding of human biology and pathophysiology. Moreover, because the ERG waveform reflects the integrated response of the retina to a visual stimulus, it reveals circadian regulation at the systems level, in a way that targeted recordings of individual retinal cells cannot. Finally, repeated recordings can be taken from the same individual, raising the possibility of tracking rhythmic changes within a single subject.

The ERG has therefore proved a very good method of describing retinal circadian rhythmicity. Its capacity to determine the physiological origins of such rhythmicity has been less widely exploited. Because the ERG waveform is a composite of multiple physiological events, it is often not possible to assign an alteration in a given ERG parameter to a particular change in the behavior of the retinal circuitry. Thus, for example, reduced a-wave could reflect changes in photoreceptor physiology (e.g., rod:cone coupling), or a reduction in b-wave latency indicative of alterations in ON bipolar cell activity. Combining ERG recordings with intravitreal injections of pharmacological agents can be a good way of bridging this gap, as can the use of genetically modified organisms. Viral gene targeting methods and RNA silencing methods are also increasingly applied to the retina and will surely be incorporated into circadian ERG studies in future.

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