

# Retinal Circadian Clocks are Major Players in the Modulation of Retinal Functions and Photoreceptor Viability

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Circadian rhythms control many biochemical and physiological functions within the body of an organism. These circadian rhythms are generated by a molecular clock that is located in almost every cell of the body. Accumulating data indicate that dysfunction of the circadian clock negatively affects the health status of the tissue in which the circadian clock has been disabled. The eye also contains a complex circadian system that regulates many important functions such as the processing of light information, the release of neurotransmitters, and phagocytic activity by the retinal pigment epithelium, to name just a few. Emerging experimental evidence indicates that dysfunction of the circadian clock within the retina has severe consequence for retinal function and photoreceptor viability. The aim of this review is to provide the reader with a summary of current knowledge about the eye circadian system and what effects emerge with a disruption of this system.

## INTRODUCTION

Circadian rhythms are present in nearly all organisms on the planet. These rhythms are generated by series of transcriptional-translational feedback loops [1]. The circadian clock plays a key role in the regulation of the metabolic processes within a cell, hence it is not surprising that dysfunction of circadian rhythms – by genetic or environmental factors – are believed to be a cofactor in the development of several pathologies such

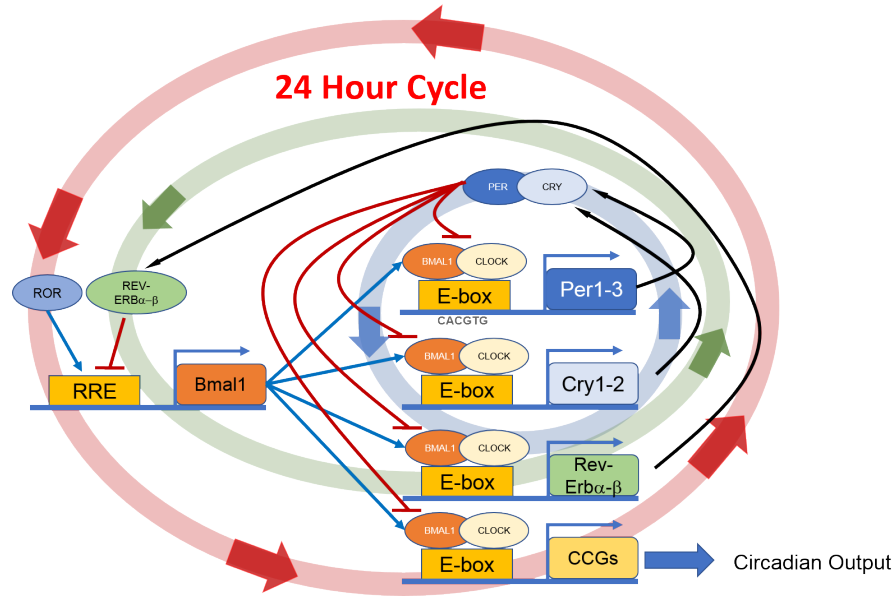
as: metabolic disorders [2], cancer [3,4], inflammation [5], and premature aging [6-8] to name just a few. Indeed, the importance of the circadian clock in regulating numerous functions within organisms has been recently underscored by awarding of the 2017 Nobel Prize in Physiology or Medicine to Drs. Jeffrey C. Hall, Michael Rosbash, and Michael W. Young for their discoveries of molecular mechanisms controlling the circadian rhythm. The aims of this review are to describe the role that circadian clocks play in the regulation of ocular functions and

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†Abbreviations: ATP, adenosine triphosphate; BMAL1, brain and muscle arnt-like 1; CCGs, clock-controlled genes; CLOCK, circadian locomotor output cycles protein kaput; Cry, cryptochrome; Dio2, type 2 iodothyronine deiodinase; ERG, electroretinogram; KO, knock-out; mRNA, messenger ribonucleic acid; NAD<sup>+</sup>, nicotinamide adenine dinucleotide; NAMPT, nicotinamide phosphoribosyltransferase; Per, period; RPE, retinal pigment epithelium; RT-PCR, reverse transcriptase-polymerase chain reaction; SIRT1, sirtuin 1.

Keywords: circadian clock, *Bmal1*, retina, RPE, cornea

Author Contributions: This work was supported by grants from the National Institutes of Health: EY026291 to GT and GM116760 to KB and by NS083932. CD, KB, and GT, wrote the paper. KB and GT designed the Figures.



**Figure 1.** Schematic illustration of the molecular circadian clock. BMAL1: CLOCK heterodimer binds to E-box present on the promoter region of *Per* and *Cry*. Then PER together with CRY inhibit their own transcript. The second feedback loop involves the transactivation of the *Rev-Erba*, *Rev-Erbβ*, and *Rora* genes by CLOCK/BMAL1. Then REV-ERB $\alpha$ , REV-ERB $\beta$  and RORA compete for binding to RRE elements in the *Bmal1* promoter, driving a daily rhythm of *Bmal1* transcription and the second feedback loop. These feedback loops generate a 24-hour rhythmic oscillation.

the consequences that circadian disruption may have on the eye of mammals.

## MOLECULAR CLOCKWORK IN MAMMALS

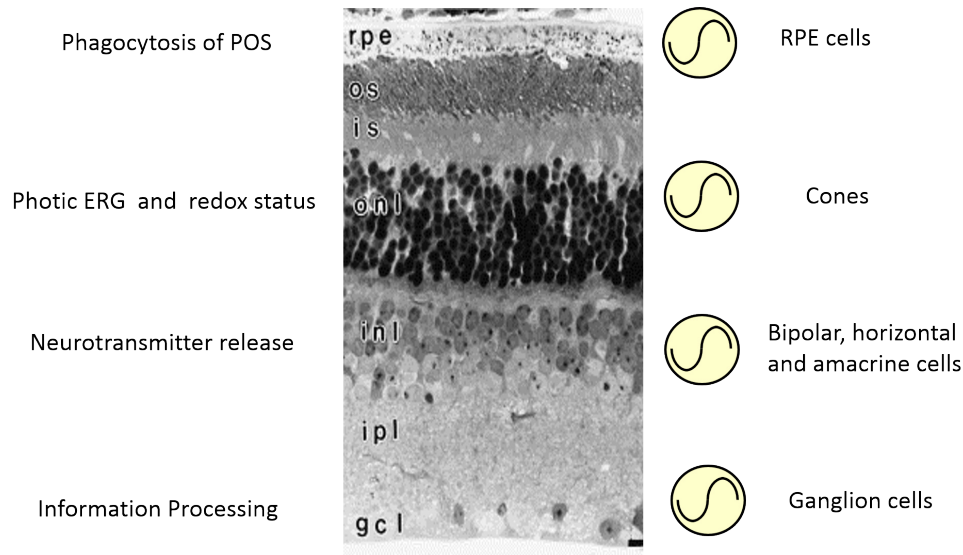
The accepted model for the molecular clockwork in mammals has two interlocking transcription-translation feedback loops involving several “clock genes” and their protein products, which ultimately regulate the transcription of other genes called clock-controlled genes (CCGs†). These feedback loops consist of positive and negative components. The positive components include the basic helix-loop-helix-PAS domain transcription factors, CLOCK and BMAL1. These transcription factors heterodimerize and bind to E-box promoter elements that enhance the transcription of genes encoding the negative components PERIOD (PER) 1, 2 and CRYPTOCHROME (CRY) 1, 2. The PER and CRY proteins feedback inhibit the transcription of the *Per* and *Cry* genes by blocking CLOCK/BMAL1-mediated trans-activation (Figure 1). The second feedback loop involves the transactivation of the *Rev-Erba*, *Rev-Erbβ*, and *Rora* genes by CLOCK/BMAL1. The protein products of these genes compete for binding to RRE elements in the *Bmal1* promoter, driving a daily rhythm of *Bmal1* transcription and closing the second feedback loop [1]. The circadian clock directly controls the rhythmic transcription of CCGs via the BMAL1/CLOCK complex that binds to DNA sequences (E-boxes)

that are present on the promoter region of these genes that ultimately regulate physiological functions.

## CIRCADIAN CLOCKS IN THE MAMMALIAN EYE

Several studies have shown that many aspects of mammalian eye physiology are under the control of retinal circadian clocks and these clocks control neurotransmitter release, processing of visual information, and cellular processes [9-11]. A few excellent reviews have recently been published on this topic, hence this review focuses on the effects of dysregulation in circadian rhythms within the eye and impact on the function and health of this organ.

Within the eye, the retina – and more specifically the photoreceptors – is the tissue that perceives light and then via neuronal pathways (*i.e.*, the optic nerve and the retinal hypothalamic tract) communicate this light information to image forming and non-image forming brain nuclei where these signals are further processed to produce vision or to entrain the body’s circadian rhythms [12-14]. The retina is a heterogenous collection of different neuronal cell types that are organized into discrete layers. These layers can be classified based on their physical characteristics with the nuclei of a cell type forming the nuclear layer and the synapses between cell types that form the plexiform layers [15]. Using genetically-mod-



**Figure 2. Schematic representation of the circadian organization in the mammalian retina.** Circadian clocks are present in many retinal cell types where they control specific cellular functions. Dysfunction of the circadian clock in retinal cells affected the functioning and viability of retinal cells.

ified animal models in which a circadian clock could be monitored in real-time [16], previous studies have shown that circadian rhythms in this tissue can be generated in the photoreceptor layers [17,18], the inner retinal layer and by the ganglion cell layer [17] (Figure 2). However, these studies did not reveal which cells within each retinal layer contains a circadian clock. This question was addressed by a seminal study using single cell RT-PCR, where Ruan *et al.* [19] reported that within the retina only horizontal, bipolar, amacrine, and ganglion neurons simultaneously expressed all the canonical clock genes, whereas no cell within the photoreceptor layer was found to contain the necessary molecular machinery to produce a circadian oscillation. This somewhat contradictory result (see [17,18]) was later explained by a study in which the distribution of clock proteins was investigated in the mouse retina [20]. The results of this study mostly confirm the previous study by Ruan *et al.* [19], but it also revealed that cones (which comprise only 2 to 3 percent of the total photoreceptors in the mouse and thus may be easily missed in the sampling by Ruan *et al.* [19]) also harbor all the molecular machinery necessary to generate a circadian oscillation [20]. The notion that cones contain a circadian clock is further supported by electroretinogram (ERGs) studies which have demonstrated that – at least in the mouse – only the photic (*i.e.*, the cone mediated response) ERG is under circadian control whereas the scotopic ERG (*i.e.*, the rod mediated response) does not show any circadian regulation [21-24]. It is also worthwhile mentioning that in addition to retinal neuronal cells, Mueller cells in the mouse retina contain functional circadian clocks [25]. Hence, it appears that within the

retina – as seen in the rest of the body – the majority of cells contain a circadian clock.

The next question that many scientists around the world started to focus on was the effect of removing clock genes (*i.e.*, disruption of circadian clockwork, Figure 1) on retinal cell function and health. The first study that tried to address this question was performed in Dr. Weitz's laboratory [22]. In this very elegant study, the authors reported that in the mouse retina many genes (more than a thousand) show a circadian rhythm and a large fraction of these genes were no longer rhythmic in the retina of mice lacking *Bmal1*. They also reported that removal of *Bmal1* abolished the circadian rhythm in the amplitude of the b-wave of the photic ERG [22]. Interestingly, they did not find any significant alteration in retinal cell morphology, at least in young mice. Since these experiments were performed on a global *Bmal1* Knock-Out (KO) mouse, the authors could not conclude whether the results obtained were due to a dysfunctional retinal clock or whether the master circadian clock (*i.e.*, superchiasmatic nucleus) located in the brain could have also contributed to the observed results. To address this important question, the authors decided to produce a mouse in which *Bmal1* was only removed from the retina (*Chx10<sup>Cre</sup>; Bmal1<sup>Fl/Fl</sup>* mice [22]). The data obtained with this retinal specific *Bmal1* KO mouse confirmed previous findings in the global *Bmal1* KO mouse, thus demonstrating that the retinal circadian clock is actually controlling the circadian rhythms in the photic ERGs [22]. The next study that tackled this important question investigated the effects of *Per1* and *Per2* removal on the health of retina [26]. This investigation indicated that deletion of

both *Per1* and *Per2* somewhat affected the organization of the retinal tissue and down-regulated the expression and the distribution of cone opsin (specifically, blue cone opsin) mRNA and protein. In an additional study, Dr. Hicks' laboratory investigated the effect of *Rev-Erba* removal on the retina, and similarly to *Per1* and *Per2* removal, their results show that a dysfunctional clock affects the processing of the visual information (where an increase in light sensitivity was observed), but there were no significant changes on the morphology of the retina [27]. Finally, the effects of *Cryptochrome 1* (*Cry1*) or 2 (*Cry2*) removal from the retina was investigated by Stuart Peirson's laboratory using global *Cryptochrome 1* and 2 knock-out mice [28]. The authors reported that contrary to previously published data [29], CRY1 immunoreactivity was observed in all retinal layers and within the photoreceptor layer only in the cone photoreceptors. When *Cry1* or *Cry2* KOs were crossed with Period 2::luciferase transgenic mice, the circadian rhythm in bioluminescence was less robust and stable in *Cry1* KO retinas, whereas in *Cry2* retinas the circadian rhythm in bioluminescence had a significantly longer period. *Cry1* KO mice did not show a circadian rhythm in the amplitude of b-wave under photopic conditions, whereas in *Cry2* KO mice they only observed a small reduction in the amplitude of the b-wave of the scotopic ERGs. The rhythms in contrast sensitivity and pupillary light response were also attenuated or even abolished in *Cry1* KO mice, but significant effects were observed in *Cry2* KO mice. Hence it appears that at least within the retinal clockwork, *Cry1* plays a key role in the circadian regulation of retinal functions, whereas the role of *Cry2* is marginal.

The observation that CRY1 is only present in the cones agrees well with those of Storch *et al.* [22] and Liu *et al.* [20] in supporting the presence of a circadian clock in cone photoreceptors. Hence, from these studies it was concluded that removal of clock genes from the retina has a modest effect and these effects seem to be concentrated on the circadian regulation of visual information processing rather than on retinal cell morphology and/or viability.

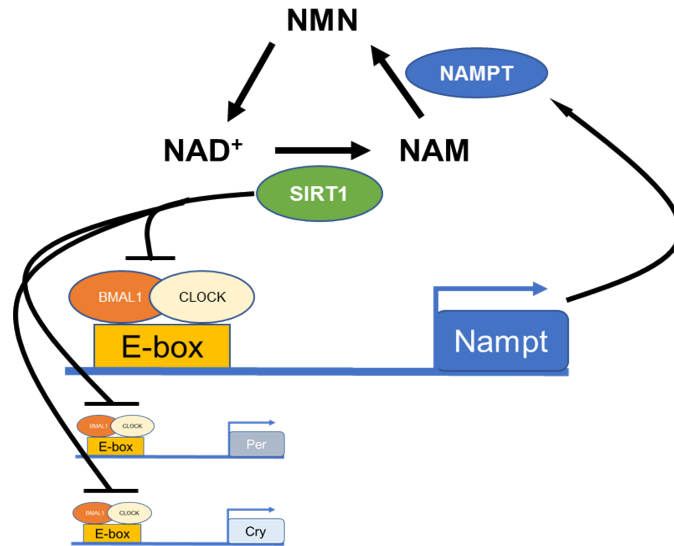
This view has been recently challenged by a series of new and exciting studies in which the role of *Bmal1* has been further investigated. In the first of these new studies, the authors used a transgenic mice line in which *Bmal1* was selectively removed only from the neural retina (*Chx10-Cre*; *Bmal1<sup>FU/FI</sup>* mice) or cone photoreceptors (*HRGP-Cre*; *Bmal1<sup>FU/FI</sup>* mice) and reported that such a removal has dramatic consequences on the spatial distribution of the cone opsin [30]. In addition, they reported that *Bmal1* regulates the expression of thyroid hormone-activating enzyme type 2 iodothyronine deiodinase (*Dio2*) and treatment with thyroid hormone partially rescued the phenotype produced by *Bmal1* removal. This result

supports the notion that *Bmal1* and *Dio2* are involved in determining cone photoreceptor identity [30]. However, apart from a small reduction in the length of the cones, the morphological analysis of photoreceptors revealed that the retina of young (2 to 3 months old) *Bmal1* KO mice was mostly unaffected compared to same age controls [30]. An additional study using 8 to 9 month old *Bmal1* KO (*i.e.*, the maximum life span for these KO mice [8]) reported a significant reduction (about 30 percent) in the thickness of the outer nuclear layer, but no significant alterations were observed in the thickness of the inner retinal layer and ganglion cells layer [31]. Furthermore, the authors also reported that removal of *Clock* and *Npas2* (*i.e.*, disabling the formation of the *Bmal1/Clock* or *Npas2* dimer, Figure 1) produced an almost identical effect of *Bmal1* removal, thus indicating that this effect is due to action of the *Bmal1/Clock* (*Npas2*) dimer and not to possible pleiotropic effects of *Bmal1* that are independent from the circadian clock.

Finally, our laboratory has further expanded our knowledge on the roles that *Bmal1* plays in the regulation of retinal functions [32] by showing that removal of *Bmal1* from the retina by using *Chx10-Cre*; *Bmal1<sup>FU/FI</sup>* mice has multiple effects on both rod pathway and cone viability. In the rod pathway, we observed that *Bmal1* removal caused stunting of rod bipolar cell dendrites and thinning of the outer plexiform layer in both young and old mice. Such an effect is likely to be due to the action of *Bmal1* during the development and/or differentiation of these cells since the dendritic abnormalities of the rod bipolar cells are already present in 28-day old mice. In addition, we also reported that the viability of cone photoreceptors during aging is greatly affected by removal of this gene since retinal specific-*Bmal1* KO mice at the age of 24 to 26 months have a dramatic reduction in the number of cones (40 to 50 percent) with respect to control mice of the same age [32]. Since loss of cones during aging is a hallmark of age-related macular degeneration, we hope that this new data will stimulate research in humans to investigate whether circadian dysfunctions (such as genetic mutations or shift-work) could be a risk factor for age-related macular degeneration.

## CIRCADIAN CLOCK ENERGY METABOLISM AND CONE VIABILITY

Accumulating evidence indicates that cellular metabolism is under direct control of the circadian clock [2]. Indeed, redox status, adenosine triphosphate (ATP) production, and gene expression in mitochondria are under control of the circadian clock [33]. In mice, clock gene deletion alters metabolism and mitochondrial energy expenditure, and increases oxidative damage in the heart and liver [34-39]. Moreover, several investigations



**Figure 3. Schematic illustration of circadian clock regulation in the NAD<sup>+</sup> salvage pathway.** BMAL1: CLOCK heterodimer binds to an E-box present on the promoter region of *Nampt* to regulate the rhythmic transcription of this gene and thus the levels of NAD<sup>+</sup> and SIRT1. SIRT1 also regulates clock gene transcript via deacetylation of histones.

have also shown that levels of the oxidoreductase factor nicotinamide adenine dinucleotide (NAD<sup>+</sup>) are under direct control of the circadian clock via the transcriptional regulation of nicotinamide phosphoribosyltransferase (NAMPT; [40,41]), the rate-limiting enzyme in NAD<sup>+</sup> biosynthesis (Figure 3). Experimental data also indicate that NAMPT modulates cellular metabolism, senescence and photoreceptor survival via the regulation of NAD<sup>+</sup>-dependent deacetylase SIRTUIN 1 (SIRT1; [42-44]). SIRT1 is expressed in the retina [45] and may be involved in the modulation of photoreceptor health and aging [46-49].

In this context is worth noting that cone photoreceptors are among the cells in the body with the highest metabolic demand [50] and they contain more mitochondria, produce more ATP, show more cytochrome c oxidase reactivity and thus more production of reactive oxygen species than any other retinal cell type [51]. Hence, even a relatively small perturbation of cellular metabolism may have significant negative effects on the cell and therefore we believe that the loss of cones observed in aged retinal specific-*Bmal1* KO mice may be a consequence of a dysfunctional circadian clock on mitochondrial functions and thus decreases the capability of cone photoreceptors to respond to oxidative stress [32].

Finally, a recent study has reported that genetic removal of *Nampt* from rod or cone photoreceptors produces degeneration of these cells and exogenous administration of nicotinamide mononucleotide (NMN, a NAD<sup>+</sup> precursor) prevents the degeneration of the pho-

toceptors in *Nampt* KO mice [52]. Thus, it is plausible to speculate that administration of exogenous NMN can prevent the loss of cones in retinal specific-*Bmal1* KO during aging [32] and thus mitigate the effects of circadian clock dysfunction on cone metabolism.

In addition to the retina, the retinal pigment epithelium (RPE) displays a circadian rhythm in the disk shedding and phagocytic activity of rod and cone photoreceptors outer segments [53-55] that is independent from the master circadian clock located in the brain [56]. However, it is still unclear whether the circadian clock controlling this rhythm is located in the RPE or in the retina. Our recent work has shown that the RPE also contains a circadian clock that is independent of the master circadian clock located in the brain [57], albeit a signal from the retina is responsible for entrainment of the RPE circadian clock [58]. No study so far has investigated whether disruption of the circadian clock has any effect on the function and health of this tissue. However, indirect evidence suggests that removing the burst of phagocytic activity after the onset of light [59] or by changing the time of the peak [60] is detrimental to this function of the RPE and its ability to support retinal photoreceptors. In addition, our study with global and retinal specific-*Bmal1* KOs suggests that the RPE clock may affect rod viability since global *Bmal1* KO show a severe phenotype with respect to photoreceptor loss compared to retinal specific-*Bmal1* KO mice [31,32].

Finally, we would like to mention that the circadian rhythm in the retina, RPE, and cornea showed a signif-

icant reduction in the amplitude in aged mice and such a reduction seems to be more pronounced in the retina than in the RPE or the cornea [61]. However, it is not known whether the reduction of these rhythms is due to a decrease in the strength of the circadian clock that may occur with aging or whether the loss of synchrony amongst the different cells is responsible for the decrease in the amplitude.

## CONCLUSIONS

Over the last twenty years, the field of circadian biology has grown tremendously and it is now clear that this system has a tremendous impact on the life of an organism. As stated previously, the importance of this biological system was recognized with awarding of the 2017 Nobel Prize in Medicine or Physiology to three circadian biologists.

The study of circadian rhythms within the eye (Figure 2) is an important topic of investigation and the data so far collected indicates that circadian dysfunctions produce significant alterations in eye function and health. We hope that awarding of the 2017 Nobel prize in Medicine or Physiology to the field of circadian biology will generate even greater emphasis on the role that this biological system plays in health and disease of an organism and how physicians and other health care providers will translate this new knowledge into new medical treatments [62].

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