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Dark-adapted light response in mice is regulated by a circadian clock located in rod photoreceptors

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Highlights

- In mice, amplitudes of a- and b-waves of dark-adapted ERG are significantly increased at subjective night.
- Dark-adapted ERG responses are regulated by a clock contained within rods.
- Disruption of the rod circadian clock does not affect overall oscillating capacity of the retina.

Abstract

The retinal circadian system consists of a network of clocks located virtually in every retinal cell-type. Although it is established that the circadian clock regulates many rhythmic processes in the retina, the links between retinal cell-specific clocks and visual function remain to be elucidated. *Bmal1* is a principal, non-redundant component of the circadian clock in mammals and is required to keep 24 h rhythms in the retinal transcriptome and in visual processing under photopic light condition. In the current study, we investigated the retinal function in mice with a rod-specific knockout of *Bmal1*. For this purpose, we measured whole retina PER2::Luciferase bioluminescence and the dark-adapted electroretinogram (ERG). We observed circadian day-night differences in ERG a- and b-waves in control mice carrying one allele of *Bmal1* in rods, with higher amplitudes during the subjective night. These differences were abolished in rod-specific *Bmal1* knock out mice, whose ERG light-responses remained constitutively low (day-like). Overall, PER2::Luciferase rhythmicity in whole retinas was not defective in these mice but was characterized by longer period and higher rhythmic power compared to retinas with wild type *Bmal1* gene. Taken together, these data suggest that a circadian clock located in rods regulates visual processing in a cell autonomous manner.

Keywords: *Bmal1*, rods, scotopic, circadian rhythms, retina, electroretinogram

The mammalian retina displays various daily rhythms in biochemical and cellular processes that allow visual function to adapt to the light/dark (LD) cycle (reviewed in McMahan *et al.*, 2014; Felder-Schmittbuhl *et al.*, 2018). These rhythmic processes include mRNA expression of photopigments and phototransduction-related genes in rods and cones (Brann & Cohen, 1987; von Schantz *et al.*, 1999; Sakamoto *et al.*, 2006; Bobu *et al.*, 2013; Kunst *et al.*, 2013) and visual sensitivity (Bassi & Powers, 1986). Several studies provided evidence that 24 h rhythms in retinal functions are controlled by an autonomous circadian clock present within the retina (Terman *et al.*, 1993; Tosini & Menaker, 1996). In addition, expression of clock genes has been described in all investigated retinal cell types (Gustincich *et al.*, 2004; Ruan *et al.*, 2006; Liu *et al.*, 2012). The presence of autonomous, coupled circadian clocks was evidenced in retinal outer nuclear layer, inner nuclear layer and ganglion cell layer (Ruan *et al.*, 2008; Dkhissi-Benyahya *et al.*, 2013; Jaeger *et al.*, 2015). At the molecular level, *Bmal1* is an essential and non-redundant component of the circadian pacemaker in mammals (Bunger *et al.*, 2000). Indeed, knockout (KO) of *Bmal1*

abolishes most daily rhythms in gene expression in the eye, including the retina (Storch *et al.*, 2007). In addition, retina-specific KO of *Bmal1*, similar to the whole organism KO, induces a decrease in the amplitude of the b-wave of both scotopic and photopic electroretinograms (ERGs). Thus, *Bmal1* disruption most likely affects the transmission of light information from both rods and cones to bipolar cells and/or its treatment in the inner retina (Storch *et al.*, 2007; Sawant *et al.*, 2017; Baba *et al.*, 2018b). Storch *et al.* more specifically demonstrated the importance of *Bmal1* in the light-adapted, photopic ERG whose b-wave has higher amplitude during the subjective day than the subjective night. In contrast, it is stuck at the lower night-time level in the retina-specific KO (Storch *et al.*, 2007). Similarly, this rhythm was attenuated in both *Cry1*^{-/-} and *Cry2*^{-/-} mice (Wong *et al.*, 2018) and totally blunted in the double *Cry1/Cry2* KO (Cameron *et al.*, 2008), further confirming the involvement of the circadian clock in this process. Additional recent studies showed that the loss of retinal *Bmal1* compromises retinal development and photoreceptor cell viability, and contributes to visual function decline during aging (Baba *et al.*, 2018b; Baba *et al.*, 2018a; Sawant *et al.*, 2019). Together, these data demonstrated the fundamental role of retinal clocks in the structural integrity and functional performance of retinal tissue. However, the molecular pathways that link cell-specific clocks to retinal health and function remain to be elucidated.

Here, we investigated the consequences of rod-specific circadian clock disruption for rhythms in retinal physiology. In control mice, we observed circadian rhythms in the amplitudes of dark-adapted ERG a- and b-waves, with higher amplitudes during the subjective night. In contrast, in the rod-specific *Bmal1* KO mice, these parameters were constitutively low (day-like). Our results suggest that a circadian clock located in rods regulates visual processing in a cell-autonomous manner.

All animal procedures were carried out according to the European Parliament and Council Directive (2010/63/EU) and authorized by the French Ministry for Higher Education, Research and Innovation (APAFIS#10213-2017060920001367-v3). *Rho-iCre*, *Bmal1*^{fl/fl} and *mPer2*^{Luc} mice were purchased from Jackson laboratories (Bar Harbor, ME, USA). All mice (males and females) had C57BL/6J background, the absence of the *rd8* mutation being previously validated. Animals were bred in the Chronobiotron animal facility (UMS 3415) on a 12h/12h light/dark (LD) cycle (ZT0 – light on, ZT12 – light off; 300 lx during the light phase, dim red light < 5 lx during the dark phase) in an ambient temperature of 22 ± 1°C, with free access to food and water.

The study was performed on five genotype groups obtained by breeding mice carrying the floxed *Bmal1* allele (*Bmal1*^{fl/fl}) (Storch *et al.*, 2007) with the Rhodopsin-iCre (*Rho-iCre*+) transgenic mouse line (Li *et al.*, 2005). All mice were also bred on the *mPer2*^{Luc} clock reporter background (Yoo *et al.*, 2004). We

generated 4 groups of control mice with distinct genotypes: *Bmal1^{fl/+};Rho-iCre/+* (named rod-*Bmal1HT*; HT - heterozygous) containing one functional *Bmal1* allele in rods, and *Bmal1^{fl/+}* or *Bmal1^{fl/fl}* or *Rho-iCre/+* (named control) whose both *Bmal1* alleles code for the wild type (WT) BMAL1 protein (Storch *et al.*, 2007). The fifth group, *Bmal1^{fl/fl};Rho-iCre/+* (named rod-*Bmal1KO*) displayed rod-specific deletion of *Bmal1*. Mice were genotyped by PCR on tail genomic DNA as described for *Bmal1* and *Per2* (Yoo *et al.*, 2004; Storch *et al.*, 2007). The primers for *Rho-iCre* transgene detection were: 5'-AGCAGCCTTGGTCTCTGTCTAC-3' and 5'GATTCTCCTCATCACCAGGGAC-3' (PCR product 500 bp).

Relative gene expression was quantified by qRT-PCR in whole retinas sampled at ZT9. 150 ng total RNA isolated by using the RNeasy Micro kit (Qiagen, Germany) were submitted to cDNA synthesis (iScript Advanced cDNA synthesis Kit, Biorad, USA) and qPCR by the TaqMan technology (Thermo Fisher, France), as described (Bagchi *et al.*, 2020). mRNA levels (*Bmal1*, Mm00500226_m1; *Cry1*, Mm00514392_m1; *Nr1d2*, Mm00441730_m1; rhodopsin, Mm01184405_m1) were normalized using *Gapdh* (Mm99999915_g1), *B2m* (Mm00437762_m1) and *Tbp* (Mm00446971_m1) whose expression did not vary between genotypes.

Immunohistochemical staining was performed on 10 μ m eye sections with primary antibodies for BMAL1 (NB100-2288 Novus) and synaptophysin (SVP38, sc12737 Santa Cruz) as previously described (Saidi *et al.*, 2011; Baba *et al.*, 2018b) (n = 3/genotype, 4 month-old mice). Secondary antibody incubation was performed at room temperature for 2 h with, respectively, anti-rabbit and anti-mouse goat IgG- Alexa 488 conjugated antibodies and nuclei were stained with DAPI (Molecular Probes). Slides were scanned with a NanoZoomer S60 (Hamamatsu) at 40x and images exported using NDP.view2 software.

ERG recordings and analysis were performed on 55-80 day-old rod-*Bmal1KO* mice and control rod-*Bmal1HT* animals (Bunger *et al.*, 2000) according to previously described procedures (Cameron *et al.*, 2008; Tanimoto *et al.*, 2009; Ait-Hmyed Hakkari *et al.*, 2016). Mice (n = 4/genotype/time-point) previously raised in LD cycle were transferred to constant dark (DD) starting at ZT12 and tested the following day at the circadian time (CT) CT6 (middle of subjective day) or CT18 (middle of subjective night) (Fig.1A). Scotopic ERG was recorded from both eyes using corneal/active electrodes (thin gold-wire with a 2-mm ring end). Ocrygel eye drop (Virbac, Carros, France) was applied to ensure good electrical contact and to keep the cornea hydrated during the entire procedure. Flash white light intensities were: 3×10^{-4} , 10^{-3} , 3×10^{-3} , 10^2 , 3×10^2 , 10^{-1} , 3×10^{-1} , 1, 3, and 10 cd.s/m².

PER2::Luciferase bioluminescence recordings were carried out on whole retina explants prepared from 1.5 month-old mice (homozygous for the *Per2^{Luc}* allele) as previously described (Jaeger *et al.*, 2015)

and analysed using the Lumicycle Analysis Program (Actimetrics, USA). Raw data were baseline (24h running average) subtracted and fitted to LM Fit (damped sine) wave to determine the period of oscillations, and analysed with Periodogram function to calculate the relative rhythmic power. Goodness-of-fit >95% and relative amplitude >100 counts/sec were set as cut-off values to define the samples to be analysed (55-75% of recorded samples in each genotype). The first oscillation was excluded from rhythm analysis which was performed on the following 3 complete cycles. Parameters of bioluminescence rhythms showed no difference between the *Bmal1^{fl/fl}* or *Rho-iCre/+* genotypes (expressing both WT alleles of *Bmal1*) and which were hence used together as a control group. To assess rhythms in the central clock in the suprachiasmatic nucleus (SCN), coronal sections (500µm) in the SCN region were made using a mouse brain matrix in ice-cold 1X HBSS (Sigma-Aldrich, Steinheim, Germany). A 1 mm² section containing the SCN was isolated with a scalpel and cultured as described (Salaberry *et al.*, 2017). Rhythm parameters were determined with the Periodogram function of Lumicycle Analysis.

Results are presented as means ± SEM. Statistical analysis (Sigma Plot 13, Systat Software, San Jose, CA, USA) was performed with t-test for qRT-PCR data, 2-way repeated measures ANOVA for ERG, 1-way ANOVA and t-test for retina and SCN bioluminescence, respectively. When no equality of variance, analysis was performed using ANOVA on ranks. ANOVAs were followed by post hoc Holm-Sidak or Dunn's multiple comparisons. The significance level was set at $P < 0.05$.

In the present study we questioned whether rods are the locus of circadian regulations that might fine-tune vision along with the 24 h cycle. To that end, we used a rhodopsin promoter-driven CRE recombinase to delete floxed sequences from *Bmal1^{fl/fl}* and generate a rod-specific KO of *Bmal1*. CRE-mediated excision of *Bmal1* was validated by PCR of whole retina genomic DNA (Fig.1B). We also confirmed that this deletion significantly impacts *Bmal1* expression by quantifying its mRNA levels in whole retinas from 120 day-old mice. We observed a significant (around 30%) reduction of *Bmal1* mRNA expression levels in rod-*Bmal1KO* (n=4) compared with *Bmal1^{fl/+}* (n=3) ($P = 0.028$) (Fig.1C). However, the (15%) difference in mRNA levels between the rod-*Bmal1HT* (n=4) and both rod-*Bmal1KO* and *Bmal1^{fl/+}* retinas did not reach statistical significance ($P = 0.286$ and 0.363 respectively). A similar gradual decrease of *Bmal1* expression from *Bmal1^{fl/+}* to rod-*Bmal1HT* and to rod-*Bmal1KO* was reflected upon BMAL1 immunostaining (Fig.1D). Furthermore, analysis of mRNA levels for distinct clock genes from the main and secondary regulatory loops, confirmed that the molecular clockwork was distinctly altered in the rod-*Bmal1KO* with respect to the rod-*Bmal1HT* and *Bmal1^{fl/+}* retinas. Indeed, the levels of *Cry1* and *Nr1d2* transcripts were significantly increased ($P < 0.001$) and decreased ($P < 0.001$), respectively, in rod-*Bmal1KO* compared with

either *Bmal1^{f/+}* or rod-*Bmal1HT* retinas (Fig.1E, F). Finally, we observed no difference in *rhodopsin* mRNA expression levels between the 3 genotype groups, suggesting that there is no major alteration of rods following KO of *Bmal1* in this cell type (Fig.1G).

We next carried out dark-adapted ERG recordings in rod-*Bmal1HT* and rod-*Bmal1KO* mice, at two time-points of the circadian cycle, CT6 and CT18 (n = 4/genotype/time-point). At both times of day, we recorded a typical scotopic ERG waveform from rod-*Bmal1HT* (Fig.1H) and rod-*Bmal1KO* (Fig.1L) mice. Rod-*Bmal1HT* mice showed a significant difference between time-points for both a- and b-wave amplitudes of the dark-adapted ERG ($P = 0.008$ a-wave, $P = 0.006$ b-wave) (Fig.1I, J). More precisely, the b-wave amplitude was distinctly higher at CT18 than CT6, with significant differences at both dimmer (-3.5 to -1.5 log cd.s/m², $P = 0.002$) and higher irradiances (>-1.5 log cd.s/m², $P = 0.019$) (Fig. 1J). Importantly, at light intensities where the a-wave amplitude could be reliably measured (>-1 log cd.s/m²) it also proved significantly higher at CT18 than at CT6 ($P = 0.013$). No difference between time points was detected in a- and b-wave implicit times ($P = 0.677$ a-wave, $P = 0.563$ b-wave) and oscillatory potential (OP) amplitude ($P = 0.318$) (Fig. 1P,R).

Thus, our data suggest that the retinal response to scotopic (rod pathway) and mesopic (cone and rod pathways) stimuli varies significantly between circadian midday and midnight. However, no significant difference was observed between CT6 and CT18 when considering the b-wave/a-wave amplitude ratios (>-1 log cd.s/m², $P = 0.332$), suggesting that the difference between CT6 and CT18 visual responses essentially originates from photoreceptors (Fig. 1K).

Unlike rod-*Bmal1HT* mice, rod-*Bmal1KO* mice displayed no difference between time-points in either of the a- and b-wave amplitudes of dark-adapted ERG ($P = 0.729$ a-wave, $P = 0.276$ b-wave) (Fig. 1M, N). They also did not show any difference in implicit times ($P = 0.483$ a-wave, $P = 0.102$ b-wave) and OPs ($P = 0.977$) (Fig.1Q, R). KO retinas also did not differ from control retinas at the photoreceptor pre-synaptic terminals as assessed by anti-synaptophysin staining (Fig. 1S). These results indicate that the daily modulation of visual sensitivity is clock-regulated and specifically relies on *Bmal1* function in rods.

We next compared a- and b-wave amplitudes between genotypes. We found no statistically significant difference for b-wave amplitudes at both time-points ($P = 0.377$ at CT6 and $P = 0.127$ at CT18). Neither did a-wave amplitudes differ between genotypes at CT6 (>-1 log cd.s/m², $P = 0.570$). By contrast, a-wave amplitudes were significantly different at CT18 (>-1 log cd.s/m², $P = 0.026$). Taken together, these data show that the dark-adapted ERG differs significantly between circadian day and night in both scotopic and mesopic conditions and that these differences depend on the integrity of *Bmal1* in rods.

Based on the ERG results described above, we further investigated whether clock disruption in rods also impacted the global oscillating capacity of the retina. We compared PER2::Luciferase bioluminescence rhythms between retinas from the same genotypes as above. Retinal explants from both groups showed similar bioluminescence profiles (Fig.2B, C) without any significant difference in period (23.16 ± 0.09 h, $n = 10$ for rod-*Bmal1HT*; 23.31 ± 0.1 h, $n=9$ for rod-*Bmal1KO*; $P = 0.302$) and relative rhythmic power (2.02 ± 0.01 for rod-*Bmal1HT*; 2.03 ± 0.01 for rod-*Bmal1KO*; $P = 0.568$). This indicates that the subjective day/night difference in light processing is not directly linked to the oscillating capacity of the whole retina and that the latter is not greatly perturbed when the circadian clock in rods is impaired.

We further compared retinal clock functionality from the preceding genotype groups to controls whose rods retained both WT *Bmal1* alleles. These control explants also showed sustained oscillatory capacity (Fig.2A) but they had a significantly lower period (22.86 ± 0.07 h, $n = 14$) than rod-*Bmal1KO* and rod-*Bmal1HT* mice ($P = 0.003$ and $P = 0.036$ respectively) (Fig.2D), indicating different intrinsic properties of retinal clocks between these mice. Interestingly, the period of controls is in agreement with the one measured in identical experimental settings for retinal explants from *mPer2^{Luc}* mice without any other genetic perturbation (Jaeger *et al.*, 2015). This supports the idea that the global retinal clock is altered in the rod-*Bmal1HT* and rod-*Bmal1KO*. Accordingly, control retinas also displayed a reduced relative rhythmic power (1.94 ± 0.02) that proved significantly different from the other genotype groups ($P = 0.003$ and $P = 0.016$ with respect to rod-*Bmal1KO* and rod-*Bmal1HT* respectively) (Fig.2E). We also determined whether alteration of light processing and clock properties in the retina could affect rhythms in the central clock. Therefore, we analysed PER2::Luciferase profiles in SCN explants from rod-*Bmal1KO* mice ($n = 3$), as compared to controls ($n = 4$). Circadian rhythms in bioluminescence were retained in both groups (Fig.2F) but, unlike in retinas, no alteration was observed in any of the parameters (Fig.2G, H, I for period; $P = 0.569$, relative rhythmic power; $P = 0.753$ and phase; $P = 0.314$).

In the present study we found that light processing, as measured by dark-adapted ERG in mice, showed a circadian component. We observed higher response amplitudes at mid-subjective night than mid-subjective day. Similar day/night difference in dark-adapted ERG was observed in wild type mice (data not shown). We also found that this difference was totally abolished upon rod photoreceptor-specific deletion of the *Bmal1* clock gene. However, the same mutation did not compromise *in vitro* oscillating capacity of whole retinas, as assessed by PER2::Luciferase bioluminescence recording. Overall, our results suggest that rods do contain a circadian clockwork that regulates visual response at the level of photoreceptors in dark-adapted condition.

Circadian tuning of retinal cone light responses has been demonstrated in several studies (Barnard *et al.*, 2006; Storch *et al.*, 2007; Cameron *et al.*, 2008; Wong *et al.*, 2018) but has been barely evaluated for rods. Early studies in rats suggested that light sensitivity at very low light intensities is regulated according to time of day (Rosenwasser *et al.*, 1979; Sandberg *et al.*, 1986). However, this daytime effect has remained controversial based on more recent investigations using scotopic ERG (Cameron *et al.*, 2008; Sengupta *et al.*, 2011; Di *et al.*, 2019). In the present study we found that control mice showed significantly larger responses to scotopic light stimulations at CT18 than at CT6, as assessed by the amplitude of the (measurable) b-wave, indicating that rod response to light is also clock-controlled. Notably, this day/night difference was totally lost in mice carrying a rod-specific deletion of *Bmal1*, further confirming it is clock regulated.

Circadian clocks are present in virtually every cell of the retina and robustness of rhythms depends on coupling between these oscillators (Jaeger *et al.*, 2015). Localisation of clocks in photoreceptor cells or layer was previously established by several studies including ours (Tosini *et al.*, 2007; Schneider *et al.*, 2010; Sandu *et al.*, 2011; Dkhissi-Benyahya *et al.*, 2013; Jaeger *et al.*, 2015). We and others suggested that these clocks are involved in the regulation of visual function (Felder-Schmittbuhl *et al.*, 2017 for review). However, due to the low level of clock gene expression in rods, the existence of a circadian clock in these cells has remained under debate (Ruan *et al.*, 2006; Liu *et al.*, 2012; Baba *et al.*, 2018b). Yet, several rhythmic functions in rods are under clock control, such as phagocytosis of their outer segments by the underlying pigmented epithelium (Bobu & Hicks, 2009) or expression of rhodopsin visual pigment (von Schantz *et al.*, 1999; Bobu *et al.*, 2013). This current study provides the first functional evidence that rods indeed carry a circadian clock depending on the integrity of *Bmal1*. This statement is corroborated by immunostaining results that showed expression of the BMAL1 protein in the ONL of control mice and almost complete absence in the ONL of conditional rod-*Bmal1KO*.

Our data show that the dark-adapted ERG varies in control mice between CT6 and CT18 also in the mesopic range of light stimulation. Circadian rhythmicity and day/night differences in the a- and b-wave amplitudes have already been reported in mesopic conditions from C57BL/6 mice (melatonin deficient) (Cameron *et al.*, 2008) and C3H/*f^{+/+}* mice (melatonin proficient) (Baba *et al.*, 2009; Baba *et al.*, 2012). Although both cone and rod pathways are involved in the response to mesopic light, at least three lines of evidence support the idea that the circadian regulation found in our study is generated primarily in rods: (1) we found a marked effect of circadian time on the a-wave amplitude; (2) no circadian time effect on

the b/a amplitude ratio; (3) the circadian time effect on the a-wave amplitude is absent in rod-*Bmal1KO* animals.

The mechanism responsible for increasing the light-response triggered by rods during subjective night is presently unknown. The a-wave is determined notably by the magnitude of phototransduction (Li *et al.*, 2018) and ionic currents at the level of photoreceptors (Robson & Frishman, 2014). We previously reported that the circadian clock regulates genes related to neurotransmission, ion channel activity and visual perception in mouse photoreceptors (Felder-Schmittbuhl *et al.*, 2017; Bagchi *et al.*, 2020; Milicevic *et al.*, 2021). For instance, mRNA expression of several genes of the phototransduction cascade in rods (*Pde6b*, *Cnga1*, *Sag*, *Rcvrn* encoding subunits of the phosphodiesterase and of the cGMP-gated cation channel, the arrestin and recoverin) as well as of a number of ion channels vary along with the 24 h cycle in isolated photoreceptors (Milicevic *et al.*, 2021). It might be possible that the rhythmic expression of some of these genes is involved in the observed cyclic light response. Increased response in the rod pathway during subjective night may also involve the gap junction-mediated coupling between rods and cones, previously shown to be increased during the subjective night (Ribelayga *et al.*, 2008). Thus it could be that this increased rod-cone coupling promotes the second order rod pathway in nocturnal condition (Tsukamoto *et al.*, 2001; Völgyi *et al.*, 2004). Our present results and previous transcriptomics data were obtained in mouse lines which do not display a night-time peak in melatonin release, in the pineal gland and in the retina. The same holds true for the rhythm in scotopic light response reported in C57BL/6J mice (Di *et al.*, 2019). This raises the question of the signalling pathway linking the circadian clock to the daily regulation of light-response. It was shown in melatonin-proficient mice that this is mediated by a melatonin-PKC-zeta pathway (Baba *et al.*, 2009; Piano *et al.*, 2018). We do not know by which mechanism similar regulations take place in melatonin-deficient mice but, based on the numerous relevant genes whose expression varies over 24 h in (rod) photoreceptors, we hypothesize that at least some of them might be directly controlled by the molecular clock. In this respect it is worth mentioning that in the rod-*Bmal1KO* the a- and b-wave amplitudes appear at their lower, daytime values, in subjective day and night, whereas in the *Cry1/Cry2* double KO the mesopic response was found increased during day time with respect to WT (Cameron *et al.*, 2008). This suggests opposite effects of the *Cry1/Cry2* clock factors with respect to *Bmal1* [as was proposed for the control of the photopic visual response (Cameron *et al.*, 2008; Wong *et al.*, 2018)]. This difference between the two genotypes has been proposed to reflect the opposite roles of *Bmal1* and cryptochromes in the molecular oscillator. BMAL1, as a transcriptional activator, is part of the positive arm of the molecular clock, trans-activating clock genes and clock-controlled genes by binding to E-box sequences. Thus, the observed increase in dark-adapted light response during the subjective night might be a direct

clock/Ebox effect involving some of the genes mentioned above, which remains to be investigated in future studies. Importantly, photoreceptor response has also been reported to be modulated daily by post-translational processes such as translocation of phototransduction cascade-elements [reviewed in (Piano *et al.*, 2018)] or changes in the affinity of cGMP-gated channels (Ko *et al.*, 2001). The importance of these processes in the current phenotype should be considered as well in future investigations.

Although *Bmal1* appears to play a central regulatory role in the visual response of rods, our PER2::Luciferase bioluminescence data show that global retinal rhythmicity persists when *Bmal1* is deleted from these cells. Furthermore, circadian period and rhythmic power were slightly, but significantly, increased in rod-*Bmal1*KO mice compared to controls. Also, we found pronounced changes in mRNA levels of two genes of the molecular clockwork between these genotypes. Whereas these results confirm the existence of a rod-specific clockwork, they also underline the fact that this clock contributes moderately to the overall retinal clock network. This was also suggested previously by the fact that rhythmic PER2::Luciferase bioluminescence signals mainly originated in the inner nuclear layer of the retina (Ruan *et al.*, 2008). Nevertheless, the precise link with the aforementioned increased period and robustness remains to be elucidated. Unexpectedly, deletion of either a single or both alleles of *Bmal1* in rods increases the period and strengthens PER2::Luciferase rhythms, as measured at the level of whole retina. Similar effect was described in another clock mutant, the *Cry2* KO, that displayed increased period and robustness in PER2::Luciferase rhythms in SCN and lung tissues (Liu *et al.*, 2007). We speculate that by weakening rhythms moderately, (rod-specific) *Bmal1* might increase responsiveness of the retinal clock to yet unknown physiological signals. It might also be possible that *Bmal1* gene dosage critically affects the expression levels of molecules regulating the coupling between oscillators within the retina. Such gene dosage was shown for the regulation by *Bmal1* of sensitivity to oxidative damage (Musiek *et al.*, 2013). Expression levels would be reduced in the heterozygote and KO situations, and decrease coupling, as suggested by the lengthening of whole retina period (Jaeger *et al.*, 2015).

The results of the ERG and bioluminescence studies appear contradictory since the heterozygous and homozygous mutants of *Bmal1* in rods display distinct phenotypes in the former and are similar in the latter. We propose that this is linked to the fact that we study a cell-autonomous process in one case (ERG) vs. a network effect in the other (bioluminescence). Indeed, the heterozygous expression of *Bmal1* in rods might allow the generation of rhythms in these cells. By contrast, as suggested above, *Bmal1* gene dosage

might play a role in the expression of regulatory factors affecting the connectivity within the global retinal clock network.

Finally, unlike retinas, SCN sampled from rod-*Bmal1KO* and control mice displayed similar PER2::Luciferase bioluminescence rhythms. This result is consistent with the conclusions from Storch et al. (2007) who reported that absence of *Bmal1* from the retina did not impact the SCN timekeeping. Thus, our data combined with those from the literature, suggest that neither retina-specific nor retinal cell-specific clocks are required for molecular circadian rhythms in the SCN.

In conclusion, our study provides evidence that light processing under scotopic and mesopic conditions in mice is regulated by the circadian clock. They support the view that rods do contain a circadian clock required for optimal performance of the visual system, at least in this nocturnal animal. They also suggest that the regulation and function of rod-specific clock genes in retinal health and disease warrant further investigation.

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Author Contributions

S.G. designed, performed experiments, analyzed data, wrote the draft; C.S. and J.M. performed experiments, analysed data and reviewed the manuscript A.B. and M.P.F.S. obtained funding, designed experiments and reviewed the manuscript.

Competing interest statement

The authors declare no potential competing interests with respect to the research, authorship, and/or publication of this article.

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Figure legends

Figure 1: Subjective day/night difference in dark-adapted ERG is lost in rod-*Bmal1KO* mice. (A) Graphical representation of lighting regimes for animal housing (12 h light (white bar):12 h dark (black bar)) and ERG recording (constant dark). Upward arrows at subjective midday (CT6) and subjective midnight (CT18) indicate the approximate times (± 1 hr) of ERG recording. (B) Detection of Cre-mediated deletion of *Bmal1* in the rod-*Bmal1KO*. Conventional PCR was performed on genomic DNA from whole retinas of following genotypes (n=3/genotype): *Bmal1^{fl/+}*, rod-*Bmal1HT*, rod-*Bmal1KO*, and *Bmal1^{fl/fl}*. M, marker; bp, base-pair. Relative mRNA expression levels of *Bmal1* (C), *Cry1* (E), *Nr1d2* (F) and *Rhodopsin* (G), in *Bmal1^{fl/+}*, rod-*Bmal1HT*, and rod-*Bmal1KO* retinas (n=3-4/genotype). (D) In addition to the inner retina (INL and GCL), BMAL1 immunostaining (green) is detected in the outer (ONL) retina of *Bmal1^{fl/+}* and (to a lesser extent) rod-*Bmal1HT* mice. Retinas from rod-*Bmal1KO* show expression of BMAL1 in the inner retina and cone somas but not in rods. Scale bar, 50 μ m. (H and L) Representative ERG traces in response to a 10 cd.s/m² flash of light in rod-*Bmal1HT* (H) and rod-*Bmal1KO* (L) mice at CT6 (blue line) and CT18 (black line), two opposite time-points of the circadian cycle in DD. (I, J) rod-*Bmal1HT* mice exhibit significantly larger a-wave (I) and b-wave (J) amplitudes at CT18 vs CT6. (M, N) rod-*Bmal1KO* mice display no difference in scotopic ERG a-wave (M) and b-wave (N) amplitudes between CT18 and CT6. (K, O) b-wave/a-wave amplitude ratio (b/a ratio) comparison between CT18 vs CT6 in rod-*Bmal1HT* (K) and rod-*Bmal1KO* (O) mice. (P, Q) a-wave and b-wave implicit times were not significantly different between CT6 and CT18 in rod-*Bmal1HT* (P) and rod-*Bmal1KO* (Q) mice. (R) Oscillatory potentials amplitude did not show significant difference between CT6 and CT18 in both genotypes. (S) Control *Bmal1^{fl/+}* and rod-*Bmal1KO* retinal sections immuno-labelled for synaptophysin (SVP38, green). SVP38 staining at photoreceptor pre-synaptic terminals did not differ between genotypes. Scale bar, 25 μ m. Comparisons between time-points was performed by 2-way repeated measures ANOVA (*P* values are indicated in the provided graphs and significant differences between CT6 vs. 18 at specific light intensities upon post hoc analysis are indicated; n = 4 /genotype/time-point). Statistical evaluation of qPCR data was performed by t-test. Data are presented as means \pm SEM. * *P* < 0.05, ** *P* < 0.01, ****P* < 0.001, ns not significant. ONL, Outer Nuclear Layer; INL, Inner Nuclear Layer, GCL, Ganglion Cell Layer; OPL, Outer Plexiform Layer; IPL, Inner Plexiform Layer.

Figure 2: *Bmal1* in rods modulates period and rhythmic power of PER2::Luciferase rhythms in whole retinas. (A-C) Representative bioluminescence traces of whole retina explants prepared from control (A), rod-*Bmal1HT* (B), and rod-*Bmal1KO* (C) mice. PER2::Luciferase oscillations appeared more persistent over multiple cycles in retinal explants from rod-*Bmal1HT* (B) and rod-*Bmal1KO* (C) mice. (D, E) Genotype group

data (n = 9-14 retinas/group) for retinal circadian period (D) and relative rhythmic power (E). Deletion of either one (rod-*Bmal1HT*) or both WT *Bmal1* alleles (rod-*Bmal1KO*) in rods significantly increased whole retina period and rhythmic power, compared to controls. Comparisons between genotypes were performed by One-way ANOVA followed by Holm-Sidak or Dunn's multiple comparisons; **P* < 0.05, ***P* < 0.01, ****P* < 0.001. (F) Representative bioluminescence traces of SCN explants from controls (black) and rod-*Bmal1KO* (green). (G-I) No significant difference between genotypes was observed for circadian period (G), relative rhythmic power (H) and phase (I). (n = 4 for controls, n = 3 for rod-*Bmal1KO*; t-test). cps, counts per second. Data are presented as means ± SEM. Individual data points are shown as dots in G, H, I.



