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Le lien entre l'horloge circadienne déficiente et les maladies rétiniennes

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The connection between circadian clock impairment and retinal disease

Shumet Tenaw Gegnaw

The connection between circadian clock impairment and retinal disease

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The connection between circadian clock impairment and retinal disease

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General introduction and scope of the thesis

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General introduction

The Earth's rotation around its axis determines the alternation between day and night. The exposure to these variations in light and temperature from the beginning of times has had an enormous effect on the evolution of life on Earth. To adapt these daily environmental alterations, living beings utilize the internal timing system that resonates with the 24-h day (Pittendrigh, 1993). This internal timing system in all organisms has been achieved by the development of the circadian clock. This circadian clock produces circadian rhythms in physiology and behaviour that include sleep-wake, feeding behaviour, body temperature, hormone secretion, cell cycle progression, and glucose homeostasis, which persist in constant conditions with an approximate period of 24 hours. To ensure resonance with the environment, circadian clock works can adapt to entrainment cues (zeitgebers) such as light intensity, food availability, and physical activity (reviewed by Buhr and Takahashi, 2013; Hughes et al., 2015; Quante et al., 2019). Among those Zeitgebers, light is the primary signal to synchronize or entrain vertebrate circadian rhythms. The retina converts light to an electrochemical signal which is further transmitted to the principal clock located in the hypothalamus and called the suprachiasmatic nuclei (SCN). The SCN are comprised of a network of oscillating cells that are considered as the "master-pacemaker" in mammals (Buhr and Takahashi, 2013). Virtually all mammalian tissues and cells have been shown to oscillate. SCN neurons coordinate the phases of most secondary (central and peripheral) clocks distributed throughout the body, potentially via neuronal and humoral cues.

Humans in the modern lifestyle are constantly faced with drastic changes in the patterns of light exposure due to uncontrolled exposure to artificial light at night, the ability to rapidly cross several time zones when traveling, and shift work or jet lag. All these lead also to alterations in the daily rhythms in a diverse set of biological processes. These include sleep, wake, eating behaviour, which themselves have the capacity to feed back on the circadian system and hence to potentially disturb physiology or behaviour at tissue or whole organism levels. Hence, it is likely that constant misalignment of these circadian clock entrainment cues negatively modulates our health. Such effects have been observed in the extreme case of shift workers (Roenneberg and Merrow, 2016). The disruption of the normal circadian rhythm predisposes to several disease pathologies including metabolic and neurodegenerative

diseases, retinopathies, and cancer (Marcheva et al., 2010; Bhatwadekar et al., 2013; reviewed in Hastings and Goedert, 2013; Musiek et al., 2013; Sulli et al., 2019).

The molecular circadian clock

It is well known that circadian rhythmicity is generated on a molecular level within virtually all cells in the body. The molecular machinery driving these rhythms is similar in the principal SCN clock and secondary clocks, including retinal clocks. This machinery is comprised of an interlocking transcriptional and translational feedback loop (TTFL). In mammals more than a dozen genes contribute to this loop, most importantly six : *Bmal1*, *Clock*, *Per1*, *Per2*, *Cry1*, and *Cry2*. An overview of the molecular machinery of the clock is presented in Figure 1.

The molecular clockwork core loop consists of heterodimer (Buhr and Takahashi, 2013; Hughes et al., 2015; Takahashi, 2017). In turn, the heterodimers of PER and CRY proteins translocate from the cytoplasm to the nucleus and form complexes that interact with BMAL1:CLOCK complex to inhibit their transcriptional activity, and thereby repress their own transcription. Protein degradation products of PER and CRY

Response Element (RORE) where REV-ERB represses Bmal1 transcription and ROR activates it (Guillaumond et al., 2005; reviewed in Takahashi, 2017) (Figure 1). The rhythmic transcriptional activator proteins complex BMAL1:CLOCK which binds to the E-box (CACGTG) enhancer sequences in the promoters of *Per1*, *Per2*, *Per3*, *Cry1*, and *Cry2* genes to induce their transcription following post-translational modifications terminates the phase of repression thus allowing a new primary positive loop to ensue (Figure 1).

The BMAL1:CLOCK complex also connects with a second feedback loop that involves the orphan nuclear receptors REV-ERB (α and β) and ROR (α, β, and γ), whose expression is activated by BMAL1:CLOCK. In turn, the REV-ERB and ROR proteins compete for a common DNA regulatory binding site, the Retinoic acid-related Orphan receptor expression of these clock genes produces circadian clock outputs by controlling the rhythmic transcription of clock-controlled genes (CCGs) via the BMAL1:CLOCK complex that binds to enhancer sequences (E-boxes) that are present on the promoter region of these genes, via REV-ERB and ROR proteins binding to RORES, or also via other transcription factors that are themselves clock targets (reviewed by Muñoz and Baler, 2003).

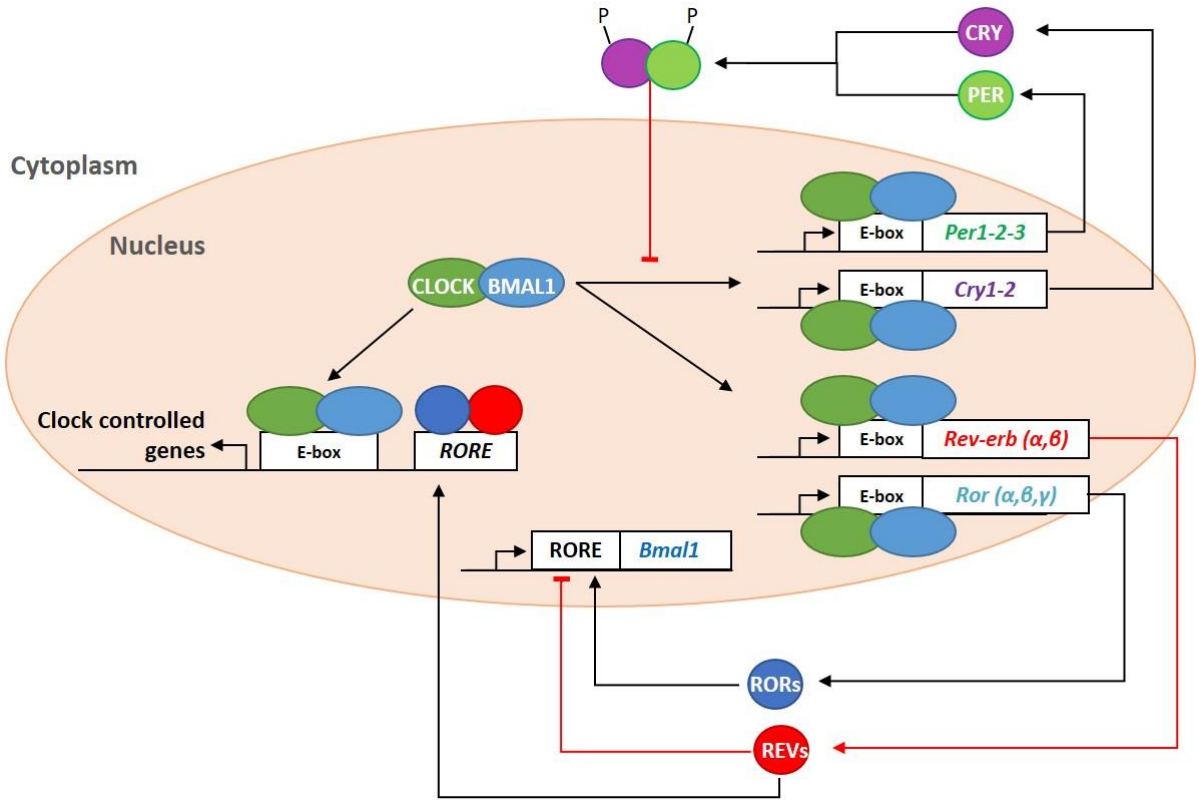

Figure 1. Schematic representation of the circadian molecular clock pathways. The core clock genes are comprised of transcriptional and translational feedback loops. BMAL1 heterodimerizes with CLOCK and activates (sharp arrow) the transcription of other clock genes such as *Per1-3*, *Cry1-2*, *Rev-Erb* (*α* & *β*), *Ror* (*α, β* & *γ*) by binding to E-boxes in their promoter region (positive loop). After transcription of these clock genes they are translationally released in the cytoplasm. The protein products PER and CRY form a heterodimer in the cytoplasm and translocate back to the nucleus to interact with the BMAL1:CLOCK complex and inhibit their own transcription, which together constitutes the core negative feedback loop of the molecular clock. Another loop, which constitutes a secondary feedback loop, involves REV-ERB and ROR factors. By competitive binding to retinoid-related orphan receptors response elements (RORE), the REVERB and ROR proteins repress (blunt arrow) and activate *Bmal1* transcription, respectively. Clock-controlled genes are the transcriptional output of this molecular network which governs rhythmic biological processes. Adapted from Felder-Schmittbuhl et al., 2017.

These feedback loops (illustrated in Figure 1) build a network of rhythmic clock genes in which expression patterns are, in addition, governed by post-translational modifications such as phosphorylation and ubiquitination, in order to set the phase coherence of cellular and tissue functions over 24h. The coordinated activity of clock genes drives the transcriptional networks of a plethora of clock-controlled genes that regulates rhythmic physiology and metabolism (reviewed in Takahashi et al., 2008). Although the same clock gene network is omnipresent in cells, the set of genes that are rhythmic varies greatly between organs or perhaps even cell types, notably due to the fact that clock control is associated with local, tissue-specific transcriptional regulations, for instance in the liver or pancreas: (Perelis et al.,

2015; Zhang et al., 2015). Thus, the circadian machinery regulates different biological processes in different organs to modulate their functions. The emergence of next-generation sequencing technologies in the circadian field offered an opportunity for a systematic analysis of temporal gene expression in a specific organ or tissue over 24h. For example, RNA sequencing and DNA array studies in 12 mouse organs have uncovered a wide array (about 43% of all protein encoding genes) of ubiquitous and tissue-specific genes under circadian control, in agreement with the need, for different organs, to fulfill distinct temporally controlled tasks (Zhang et al., 2014). A more comprehensive analysis of rhythmic transcriptional expression profiles in >60 tissues/organs from a diurnal nonhuman primate was also performed (Mure et al., 2018). This genome-wide transcriptome study uncovered a wide array (about 82%) of protein encoding, ubiquitous and tissue-specific genes undergoing rhythmic daily changes. These studies show that the circadian clock system is highly pervasive, with extensive regulation of basic biological processes such as metabolism and DNA repair but also tissue-specific functions, all intimately coordinated within the 24h period (Chaix et al., 2016). Thus, circadian rhythmicity has strong adaptive value and it is established that chronic disturbance of these timed mechanisms, as experienced in today's 24h life styles, leads to increased morbidity and reduced lifespan (West and Bechtold, 2015).

The circadian clock in the retina

The retina is the light sensitive neural tissue that lines the back surface of the eye. It comprises two main layers i.e., the neural retina layer and the retinal pigment epithelium (RPE) layer, a neural layer of pigmented epithelial cells. The neural retina is organized into three discrete cellular layers interconnected by synaptic (plexiform) layers: the outer nuclear layer (ONL), the inner nuclear layer (INL), and the ganglion cell layer (GCL). These cellular layers consist of several cell types, including the rod and cone photoreceptors (PRs) in the ONL, bipolar cells, horizontal cells, amacrine cells, Müller glial cells (MGCs) in the INL, and the ganglion cells (Figure 2) (Rodieck, 1973). The RPE lies below the photoreceptor layer.

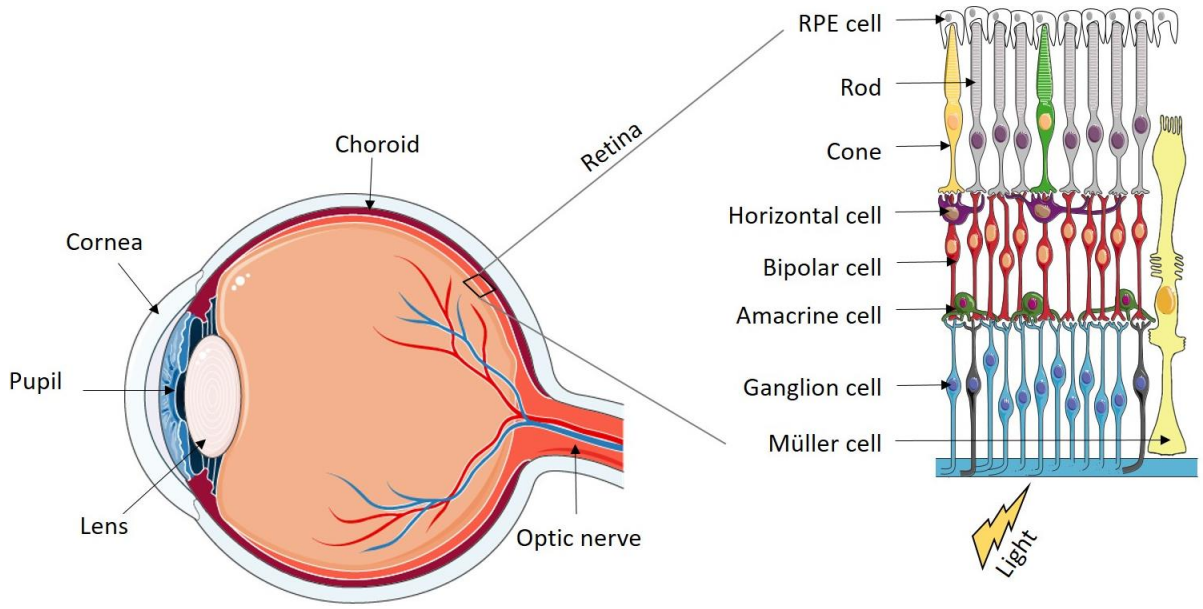

Figure 2. Schematic representation of the eye and the retinal cell layers. The retina lies on the posterior part of the eye consisting of a pigmented layer, the retinal pigment epithelium (RPE), and a multilayered neuroretina. The RPE is in close contact with the outer segments of the photosensitive rod and cone cells of the neuroretina. Figure adapted from Servier Medical Art (https://smart.servier.com/smart_image/retina/).

The retinal architecture and organization allow the visual function to adapt changes in light intensity occurring over the 24-h day, hence, virtually all investigated retinal cell types were revealed to integrate and orchestrate circadian rhythms (Figure 3). In the early 80s, the circadian clock was found in African clawed frog (Xenopus laevis) eye, presenting a daily rhythm in the activity of arylalkylamine N-acetyltransferase (AANAT: a key regulatory enzyme in the melatonin biosynthetic pathway) in isolated, cultured eye cups (Besharse and Iuvone, 1983). The AANAT rhythm occurs in retinas cultured under a daily LD cycle. This rhythm also persists for at least 3 days in constant darkness, indicative of rhythm generation by an intrinsic circadian clock, and drives the rhythm of melatonin release, the core hormone of circadian rhythms (Cahill and Besharse, 1993). The clock regulating these rhythms was later shown to be located in photoreceptor cells of the retina (Tosini et al., 2007). The existence of an independent circadian clock in the mammalian retina was suggested by studies on animals with lesions of the SCN. For example, circadian rhythms of rod outer segment disc shedding and AANAT mRNA expression persist in SCN-lesioned rats (Terman et al., 1993; Sakamoto et al., 2000). The circadian rhythm of melatonin synthesis in cultured neural retinas of the golden hamster (Mesocricetus auratus) and the functionalities of this rhythm finally established the mammalian retina as a bonafide circadian pacemaker: entrainment by light cycles and free-running in constant darkness (Tosini and Menaker, 1996).

The localization of a circadian clock in different retinal cell types within the retinal layers remains an area of ongoing research. However, it was demonstrated that the retina harbors a network of clocks localized in each retinal cell layer. Strong evidence came from the bioluminescence recordings using transgenic lines with a luciferase reporter coupled to clock (*Per1* and *Per2*) genes (Yamazaki et al., 2000; Yoo et al., 2004). The mouse whole retinal explants as well as virtually all isolated cellular retinal layers, namely ganglion cell (GCL), inner nuclear (INL), and outer nuclear (ONL) layers, exhibit autonomous PER2::LUC oscillations with unique periods (Jaeger et al., 2015). Per1-Luc bioluminescence was also shown to oscillate in ONL layers isolated from Period1-luciferase rats (Tosini et al., 2007). GCL and INL are the major sites of circadian clock gene expression rhythms in culture (Ruan et al., 2006; Ruan et al., 2008; Jaeger et al., 2015): highest levels of bioluminescence, highest rhythm amplitudes, most clock genes expressed. However, several studies reported that ONL cells or layer also express circadian clock genes and express clock gene expression rhythms in culture (Tosini et al., 2007; Schneider et al., 2010; Sandu et al., 2011; Dkhissi-Benyahya et al., 2013; Jaeger et al., 2015). It was suggested that these clocks are involved in the regulation of visual function (Felder-Schmittbuhl et al., 2017 for review). Nonetheless, due to the low level of clock gene expression in rods, the presence of a circadian clock in these cells has remained under debate (Ruan et al., 2006; Liu et al., 2012; Baba et al., 2018a). Using IHC, we provided the first functional evidence that rods indeed contain a functional clock (**Chapter 3**). Additionally, a study by Xu et al., has demonstrated that Müller cells of the mammalian retina express circadian clock genes and express circadian gene expression rhythms in cell culture (Xu et al., 2016). Taken together, these experimental evidences indicate the presence of cell-specific circadian oscillators organized in a complex cellular network in the retina.

The core clock gene components are expressed throughout the retina (reviewed in Bhoi et al., 2023). These clocks play a crucial function in adapting retinal physiology and function of the LD cycle (reviewed in McMahon et al., 2014; Felder-Schmittbuhl et al., 2018 - presented in Figure 3). They regulate these functions by controlling the mRNA expression of photopigments and phototransduction-related genes in rods and cones (Brann and Cohen, 1987; von Schantz et al., 1999; Sakamoto et al., 2006; Bobu et al., 2013; Kunst et al., 2013), visual sensitivity (Bassi and Powers, 1986), and rod-cone electrical coupling (Ribelayga et al., 2008). The mammalian retinal clock also regulates processes that are linked to retinal survival

such as nocturnal release of the cytoprotective melatonin (Tosini and Menaker, 1996), photoreceptor outer segment phagocytosis by the underlying pigmented epithelium (RPE) (LaVail, 1980; Bobu and Hicks, 2009), and vulnerability of photoreceptors to degeneration following light damage (Organisciak et al., 2000). These data suggest that in the mammalian retina the circadian clock plays a major role in photoreceptor function and survival.

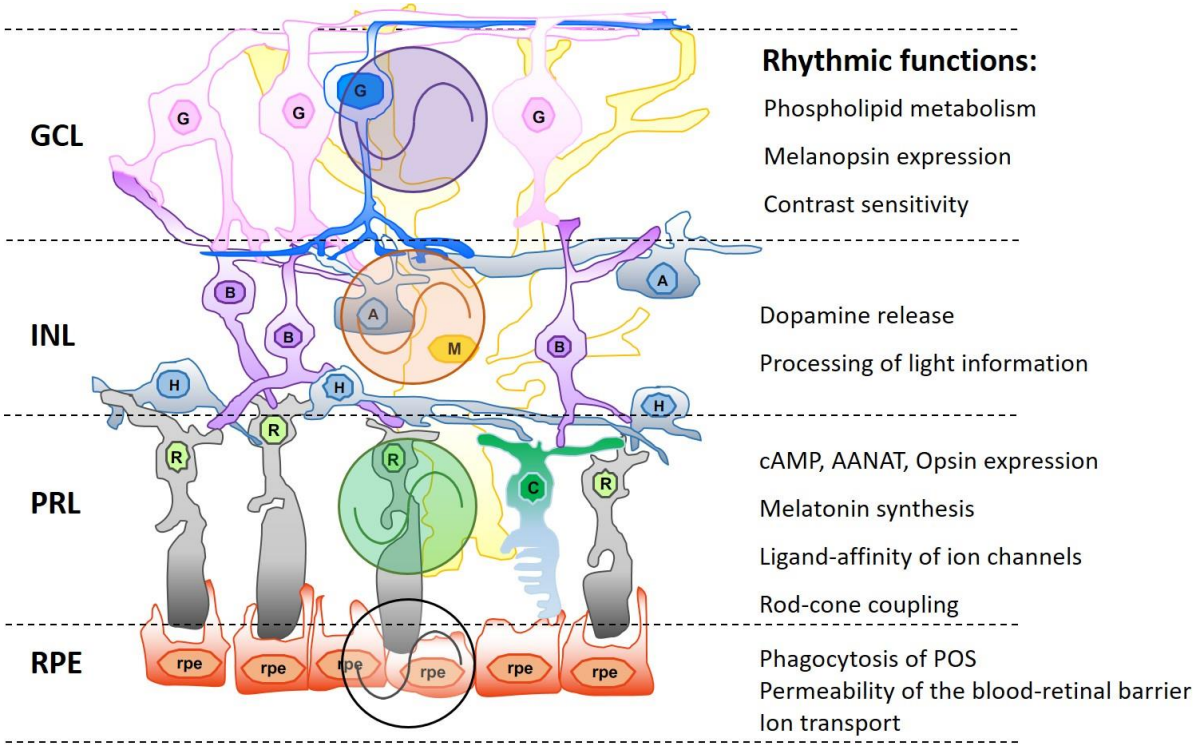

Figure 3. Rhythmic functions in the neural retina and the retinal pigment epithelium, with their schematic overview of location identified by retinal layer. A schematic transversal section through retinal layers and cell types is shown with the three main oscillators (depicted as GCL, INL, and ONL) previously identified (Jaeger et al., 2015). The RPE also contains an individual clock (Baba et al., 2010; Milićević et al., 2019). Examples of documented circadian processes are listed on the right in RGC, INL, ONL and RPE (Besharse and McMahon, 2016; Felder-Schmittbuhl et al., 2017; Felder-Schmittbuhl et al., 2018). ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer; RPE: retinal pigment epithelium; G: RGC; A: AC; B: BC; H: HC; M: MGC, R: Rods; C: Cones; POS: photoreceptor outer segments.

Additional insight into possible circadian clock-controlled functions in the retina has been inferred from the transcriptomics studies. Many genes display daily and circadian variations of their expression in the vertebrate retina, and the circadian clock exerts a great amount of control over gene expression through the role of clock proteins as transcriptional regulators. In the mouse whole eye, a genome-wide microarray study found that 3000 genes show daily rhythmic expression in normal LD conditions and 300 retinal genes show circadian rhythmic expression (Storch et al., 2007). The data show that, as is true in other peripheral oscillators such as liver (Panda et al., 2002), a large portion of the transcriptome is under circadian

control. The retinal genes with rhythmic expression were involved in basic neural and cellular functions such as synaptic transmission, photoreceptor signalling, intercellular communication, regulation of the cytoskeleton, and chromatin remodelling. Thus, the transcriptional regulation of retinal gene expression by the (retinal) clock appears to be a critical mechanism underlying (short term) control of retinal function and metabolism. Similar observations were made using a circadian proteomic study of the retina in the mouse that identified several proteins with circadian rhythmicity in their intensities (Tsuji et al., 2007; Møller et al., 2017). These include proteins involved in vesicular transport, calcium-binding, protein degradation, metabolism, RNA-binding and proteins folding. Interestingly, the retinal clock molecular mechanism also contributes to the regulation of those genes that were rhythmic only in LD cycles as ablation of the molecular clockworks by deletion of BMAL1 also disrupted rhythmic gene expression in LD cycles (Storch et al., 2007). Therefore, the synaptic and metabolic processes in the retina are most likely controlled by the circadian clock.

More recently, RNA sequencing of the diurnal transcriptome of the baboon retina identified rhythmic expression of 733 genes under normal LD cycles (Mure et al., 2018). These studies uncover how the circadian clock and LD cycles interact to regulate retinal gene expression and thereby retinal fucnctioning. Nevertheless, they are inadequate in that they do not distinguish between cell types in the retina. In a recent study, the circadian clock was specifically knocked out (KO) (conditional *Bmal1* KO) in cone photoreceptors, and its effects on the cone transcriptome was analyzed (Bhoi et al., 2021). During the day, 88 genes indicated differential expression between cone specific *Bmal1* KOs and their WT controls, uncovering genes involved in a wide range of functions (i.e., gene regulation, neuron development, protein-binding and -transport, and circadian components). At the same time, the circadian cycle related transcriptome of microdissected photoreceptor layers and retinal pigmented epithelium was investigated. This study showed that, amongst others, genes implicated in retinal metabolism, neurotransmission and disease show rhythmic expression patterns that are lost when *Per1* and *Per2* are inactivated (Milićević et al., 2021). Taken together, these data demonstrate that within the retina the cell specific circadian clocks in various cell types differentially control gene expression.

Taken together, all circadian transcriptomic and proteomic experimental evidences, suggest that disrupted transcriptional and perhaps posttranscriptional control by the retinal circadian molecular clockwork is likely to be involved in the pathogenesis of retinal genetic diseases.

Circadian clocks and retinal genetic diseases

Lighting and retinal light perception have a profound effect on daily patterns of human physiology and behaviour and on quality of life. In many studies, blindness has been considered the worst medical condition in the general population, with a higher impact on day-to-day life than other chronic conditions such as Alzheimer disease, cancer, AIDS/HIV, loss of limb, heart disease, arthritis, and deafness (Scott et al., 2016). The role of a functional circadian clock to support retinal health has been suggested by several studies (reviewed in Felder-Schmittbuhl et al., 2017). Indeed, circadian clocks modulate expression of about 43% of all (human retinal disease) protein encoding genes (Zhang et al., 2014) and most likely impact retinal homeostasis and modulate long-term health. This implies that altered circadian clock function might affect retinal disease and pathology. This idea is supported by a number of examples from the literature: dysfunction of the circadian rhythms in mice lacking *Bmal1* is accompanied by age dependent (cone) photoreceptors degeneration (Baba et al., 2018a; Baba et al., 2018b). Mice carrying adult-specific *Bmal1* deletion display premature aging symptoms and their ocular abnormalities include corneal neovascularization, keratinization and progressive inflammation (Yang et al., 2016). Also, *Clock* KO mice develop cataracts (Dubrovsky et al., 2010). A circadian retinal sensitivity to phototoxicity has been reported in rats with photoreceptor cells being more vulnerable at subjective night (Organisciak et al., 2000; Vaughan et al., 2002). This phenotype is likely linked, among others, to circadian changes in the expression of crystallins that serve a chaperone function in the photosensitive membrane system (Organisciak et al., 2011). Thus, clock dysfunction in the eye or the retina might constitute a risk factor for blinding diseases. However, besides a few examples that have been reviewed elsewhere (Felder-Schmittbuhl et al., 2017; Bery et al., 2022), the effects of circadian (long-term) influence on human ocular disease remain essentially to be explored. Below, we describe the possible relationship between clock dysfunction and ocular diseases, particularly those relating to retinal pathophysiology and fitting within the scope of this thesis (diabetic retinopathy and retinitis pigmentosa).

Clock dysfunction and diabetic retinopathy

Circadian clock disruptions have linked to diabetic retinopathy (DR), one of the major complications in patients with diabetes that leads to progressive vision loss and eventual blindness (Ola et al., 2012): Disruption of *Clock* and *Bmal1* in mice leads to diabetes and hypoinsulinaemia (Marcheva et al., 2010) and in the retina there is indirect evidence that the circadian clock disruption is associated with and contributes to DR. For example, *Per2* clock gene-mutant mice exhibit similar symptoms as diabetic damage to the retina (Bhatwadekar et al., 2013). Conversely, DR in diabetic mice and rats is associated with reduced clock gene expression, including strong downregulation of PER2 and BMAL1 (Busik et al., 2009). Furthermore, mutation of *Per2* induces DR in mice, likely through a retinal vascular phenotype similar to DR (Jadhav et al., 2016). Deleting Bmal1 specifically in endothelial cells, a key cell type that line blood vessels, results in phenotypic features like diabetic retinopathy (Bhatwadekar et al., 2017). Taken together, all studies provide evidence that the circadian function in retinal vasculature may play a critical role in maintaining the health of the retina.

Clock dysfunction and retinitis pigmentosa

Retinitis pigmentosa (RP) is a relatively common inherited form of progressive retinal degeneration, affecting 1 in 4000 people worldwide (Hartong et al., 2006). Though RP is a clinically and genetically heterogeneous disease with over 100 disease genes currently implicated (https://web.sph.uth.edu/RetNet/home.htm), all RP types follow common triphasic symptomatic course that involve night blindness, progressive concentric visual field loss resulting in tunnel vision, and eventually legal blindness (Dias et al., 2018; Verbakel et al., 2018). The progression of the disease is explained by the initial concentric loos of peripheral rod photoreceptors, which subsequently triggers the decline of more centrally located cone photoreceptors.

Many disease genes have been associated with RP, and apart from differences in the underlying genetic cause, it results in a final common cellular fate of apoptosis and death. While progress has been made in deciphering in understanding of diseases for each identified diseases, there is still much to understand regarding the nature of the preapoptotic pathways. Many reviews have examined multiple mechanisms of photoreceptor cell death (Travis, 1998; Stone et al., 1999; Pacione et al., 2003). Logic, also presented in **Figure 4**, proposes a limited

number of mechanisms responsible for the initiation of apoptosis. For example, oxidative stress, which generates toxic reactive oxygen species (ROS), is believed by many to be an initiating factor to the death of retinal cells in various ways. Another cell death mechanism is autophagy, which is normally the mechanism by which the body removes harmful reactants; however, excessive autophagy induces abnormal apoptosis and necrosis, as well as metabolic dysfunction. Moreover, degenerated retina composition analysis has revealed that massive intracellular protein aggregates are featured in photoreceptor cell death (Pfeiffer et al., 2020). More specifically, the evidence accumulating from studies into the autosomal dominant RP (adRP) mutations reveals that incorrect protein folding, leading to aggregation and stimulation of the unfolded protein response (UPR), is probably central to the death of the photoreceptors in numerious mutations (reviewed in Kennan et al., 2005). For example, dominant rhodopsin mutations are perhaps detrimental to rods because the mutant forms of rhodopsin are toxic to rods. These effects are attributable to interference with metabolism, perhaps by formation of intracellular protein aggregates (reviewed in Hartong et al., 2006).

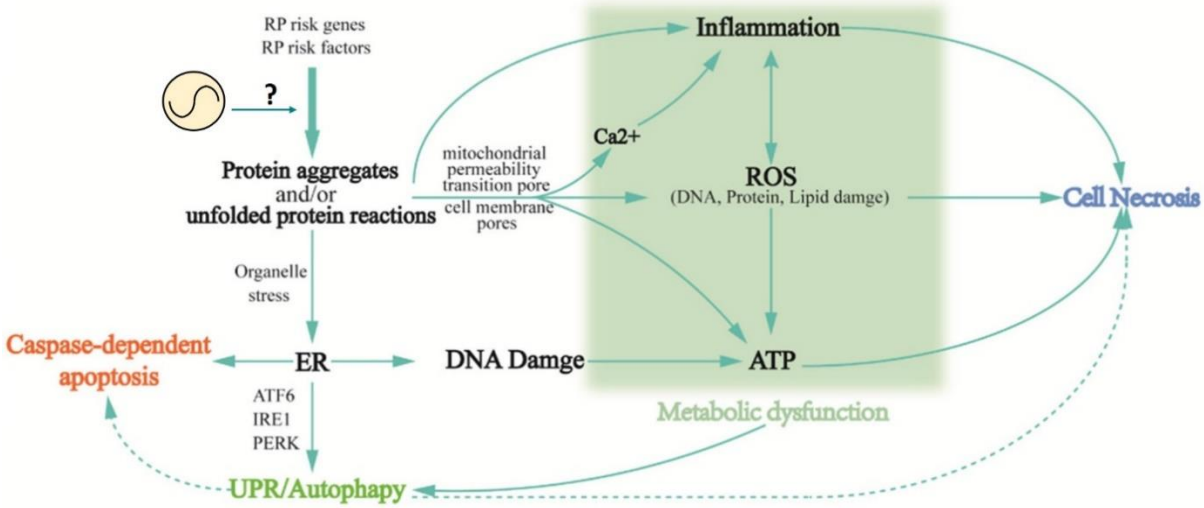

Figure 4. Retinal degeneration involves various cellular biochemical reactions that cause retinal cell death: protein aggregation, oxidative stress, the immune response, and metabolic dysfunction (Liu et al., 2022). Figure adapted from Liu et al., 2022. When gene mutations trigger intracellular protein aggregation, one causes stress in endoplasmic reticulum (ER), stimulating the UPR, but when UPR system becomes overwhelmed, cell death is induced by accumulation of excess misfolded proteins (Kaufman, 2002). Notably, the formation of misfolded proteins probably central for the death of the photoreceptors in a substantial number of adRP genes (reviewed in Kennan et al., 2005). Some of these genes normally encode proteins in the rod phototransduction biochemical reactions (van Soest et al., 1999; Hartong et al., 2006). Second, the imbalance between oxidative system and antioxidant system and aggravating oxidative stress. Third, it triggers a protective immune defense, when the active markers are immune cells to resolve inflammation. The result of these uncoordinated reactions ultimately points to the death of photoreceptor cells. Another pathway of cell death is autophagy, however excessive autophagy displays apoptosis as well

as metabolic dysfunction, which is also associated with autophagy. The link between this pathogenesis and circadian clocks might take place at the level of gene regulation, in particular of disease genes.

The role of a poorly functioning circadian clock modulating the pathogenesis of RP has been hardly investigated (Bery et al., 2022). Interestingly, individuals with similar RP disease gene mutations can have variable retinal phenotypes, even within the same family. This variability involves difference in disease onset, rates of degeneration as well as the cell types most affected, indicating an important role of environmental and dietary factors, life style, epigenetic factors/possible modifier genes (Hartong et al., 2006; Contín et al., 2016) or of light damage (Naash et al., 1996; Tam et al., 2010) in this disease, but a potential role of a partly or chronically disturbed circadian rhythm has not been excluded.

Aim and outline of this thesis

This thesis is composed of a series of experiments, and aimed to investigate the role of the circadian clocks in the retina physiology and photoreceptor-related retinal disease, that is one subtype of RP.

Part II, we investigated the effects of clock genes inactivation on retinal physiology and disease. In **chapter 2**, we explored the role of core-clock genes *Per1* and *Per2* on the developing eye physiology. By means of a functional transcriptomics approach, using mRNA abundance as a primary readout, we compared genome-wide differential gene expression in the whole eye of *Per1/Per2* double mutants relative to WT at E15, E18 and P3. In **Chapter 3**, to better define the functional circadian role of *Per1* and *Per2* genes, we performed electroretinogram (ERG) recordings in adult mice. Impairment of these genes caused loss of the day/night difference in scotopic light processing by the retina. In mutants, light response in constant darkness was constitutively high, like in the dark for wild-type mice. Additionally, in **Chapter 4**, to further decipher how/where the day/night differences in scotopic ERG response are generated, we used the rod-specific knockout of Bmal1 (rod-*Bmal1KO*) that carries an inactivated clock in rod photoreceptor cells. We conducted analyses of the light response over the circadian cycle similar to those of chapter 3. We generated mice carrying also the Per2-Luciferase reporter knock in to evaluate how Bmal1 knock out in rods could affect the global rhythmic capacity of the retina. In **chapter 5**, we investigated the regulation and function of rod-specific clock genes in the context of PR-related retinal genetic disease, i.e., retinitis pigmentosa. We used rod-*Bmal1KO*, P23H Rho mutant, and double mutants (rod-

Bmal1KO/P23H Rho (DM)) of these mice. By means of a functional transcriptomics approach, using the functional and morphological status of the retina as the primary readouts, we analysed the changes in gene expression in P23H Rho and DM retinas as compared with Ctrl, and DM retinas compared with P23H Rho retinas of 4 months-old mice.

Part III, as argued in chapter 4, rod cells contain a functional clock that regulates daily visual light processing in a cell autonomous manner. Thus in **Chapter 6**, to get further insight into the contribution of these cells to the retina clock network we examined the potential impact of these cells degeneration on the retinal clock activity using P23H Rho mutant mice. We introduced into these mice the Per2-Luc reporter knock in and recorded bioluminescence from whole retinas (WT, $Rho^{P23H/+}$, and $Rho^{P23H/P23H}$) at 36 and 70 postnatal days.

Lastly, in **Chapter 7**, I provide a reflective look at all the previous chapters, integrating them with the latest scientific findings and extrapolating the acquired knowledge to better understand the implications of the circadian system (impairment) to retinal function and health. Also, recommendations for future follow-up experiments and other potential research questions are discussed in this section. The limitations and strengths of this thesis are described as well.

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Part II

Effects of clock genes inactivation on retinal physiology and disease

Chapter 2

Core-clock genes Period 1 and 2 regulate visual cascade and cell cycle components during mouse eye development

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Core-clock genes Period 1 and 2 regulate visual cascade and cell cycle components during mouse eye development

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Abstract

The retinas from Period 1 (*Per1*) and Period 2 (*Per2*) double-mutant mice (*Per1*$^{-/-}$ *Per2*^{Brdm1}) display abnormal blue-cone distribution associated with a reduction in cone opsin mRNA and protein levels, up to 1 year of age. To reveal the molecular mechanisms by which *Per1* and *Per2* control retina development, we analyzed genome-wide gene expression differences between wild-type (WT) and *Per1*$^{-/-}$ *Per2*^{Brdm1} mice across ocular developmental stages (E15, E18 and P3). All clock genes displayed changes in transcript levels along with normal eye development. RNA-Seq data show major gene expression changes between WT and mutant eyes, with the number of differentially expressed genes (DEG) increasing with developmental age. Functional annotation of the genes showed that the most significant changes in expression levels in mutant mice involve molecular pathways relating to circadian rhythm

signaling at E15 and E18. At P3, the visual cascade and the cell cycle were respectively higher and lower expressed compared to WT eyes. Overall, our study provides new insights into signalling pathways -phototransduction and cell cycle- controlled by the circadian clock in the eye during development.

Keywords: circadian clock, eye, photoreceptor, differentiation, transcriptomics

Abbreviations: CPM, counts per million; DEGs, differentially expressed genes; FDR, false discovery rate; IPA, Ingenuity pathway analysis; LD, light-dark cycle; PCA, principal component analysis; qPCR, quantitative polymerase chain reaction; SCN, suprachiasmatic nucleus; WT, wild-type; ZT, zeitgeber time

1. Introduction

The coherent functioning of the circadian clock, driven by genetics, endogenous factors and environmental cues, is pivotal in orchestrating the rhythmicity of biological functions. The circadian system operates through a hierarchical system of oscillators. These oscillators occur in several peripheral tissues and brain areas, with the suprachiasmatic nucleus (SCN) of the hypothalamus being the primary pacemaker or the master clock (Mohawk et al., 2012). The molecular clockwork machinery comprises transcription factors encoded by ‘‘clock genes’’, which interact in interlocked transcriptional/translational regulatory loops. The key positive loop encompasses CLOCK and BMAL1 heterodimers. The negative feedback loop is formed by the Period (PER) 1–3 and Cryptochrome (CRY) 1–2 proteins which inhibit the transcriptional activity of BMAL1/CLOCK dimer. An important interconnecting secondary loop of the circadian oscillator involves both the nuclear receptor families of REV-ERBs and RORs (respectively named after the anti-sense *Rev-Erbα* in relation to the *c-ErbA-α*/*Thrα* gene; encoded by *Nr1d1–2* and Retinoic acid-related Orphan Receptors, encoded by *Rora-c*). In addition, the core clock network has many other putative regulatory genes, such as *Dbp, Hlf, Tef*, *Ciart*, *Csnk1ε*, *Csnk1δ*, *Fbxl3, Fbxl21, Nfil3, Bhlhe40, Bhlhe41,* and *Arntl2* (Takahashi, 2017).

It is well known that the peripheral tissue oscillators are synchronized by outputs from the SCN, resulting in coordinated rhythmic oscillations of peripheral organs. Without the driving force of the SCN, these organs run out of phase (Reppert and Weaver, 2002; Yoo et al., 2004). The capacity to synthetize melatonin in a rhythmic manner, in vitro, suggested the idea of an

endogenous clock in the eye, located in the retina (Tosini and Menaker, 1996; reviewed in Felder-Schmittbuhl et al., 2018). Directly sensitive to the light/dark cycle, the retina is responsible for the synchronization of circadian rhythms generated within the SCN (Yamazaki et al., 2002; Lee et al., 2003). Several functions in the mammalian eye are controlled by the retinal circadian clock (reviewed in McMahon et al., 2014; Felder-Schmittbuhl et al., 2017) extending from the expression of photopigments (von Schantz et al., 1999; Bobu et al., 2013) to visual sensitivity (Barnard et al., 2006; Storch et al., 2007; Cameron et al., 2008). Retina specific functions such as photoreceptor outer segment disk shedding and RPE mediated phagocytosis as well as susceptibility to phototoxicity have been linked to the clock (LaVail, 1980; Organisciak et al., 2000; Bobu and Hicks, 2009). In addition, the rate of mitosis measured in the cornea (Kikkawa, 1973; Xue et al., 2017) along with the daily production of aqueous humor in the ciliary body contributing to intraocular pressure (Lozano et al., 2015; Tsuchiya et al., 2017), are also well-studied rhythmic cellular processes.

The circadian system is known to time the early development of the mouse visual cortex (Kobayashi et al., 2015). It is during these developmental stages that circadian rhythms optimize growth and neurobehavioral development (Mirmiran et al., 2001; Sumova et al., 2012; Honma, 2020). Protein products of clock genes regulate cellular processes within the wild-type eye during development, at least until adulthood (Storch et al., 2007; Liu et al., 2012; Baba et al., 2018; Sawant et al., 2019). Loss of *Rev-Erbα* (*Nr1d1*) expression results not only in several defects during retinal visual processing, but also of retinal sensitivity to ambient light (Mollema et al., 2011; Ait-Hmyed Hakkari et al., 2016). We previously reported a number of developmental anatomical/histological defects related to eye physiology up to 1 year of age in the *Per1*$^{-/-}$*Per2*^{Brdm1} mutant mice (Zheng et al., 1999; Zheng et al., 2001). These defects include a reduced number of blue cone opsin (*Opn1sw*) expressing cells, decreased steady-state levels of blue cone opsin gene expression and delayed differentiation of rod inner and outer segments (Ait-Hmyed et al., 2013). Sawant and coworkers (Sawant et al., 2017) recently showed that the circadian clock gene *Bmal1* is implicated in spatial patterning of cone opsins in the retina. This corroborates the hypothesis that circadian clock genes are essential for proper cone development. Finally, in *Cry1/Cry2* double-mutant animals, cone function is also compromised (Wong et al., 2018). Nonetheless, the detailed molecular mechanisms linking clock genes and cone development remain to be elucidated.

Understanding how the circadian clock controls retinal development is essential to apprehend how its disturbance could lead to pathogenicity (Chaix et al., 2016). The recent development of whole genome-wide transcriptomic approaches allowed systematic analysis of temporal gene expression and identification of clock target genes in specific cell types/organs (Panda et al., 2002; Storch et al., 2007). It was shown for example that rhythmic regulation of gene expression programs relies on BMAL1/CLOCK binding to DNA that primes target genes for tissue specific transcription activation (Trott and Menet, 2018; Beytebiere et al., 2019). Furthermore, transcriptome characterization in 12 mouse organs over time revealed that 43% of protein-encoding genes possess a rhythmic expression profile (Zhang et al., 2014). Recently, the first transcriptome atlas of a diurnal non-human primate in more than 60 tissues/organs also unraveled a wide array of rhythmic tissue-specific genes, extending the proportion of protein encoding genes regulated by the clock in at least one tissue to about 80% (Mure and Le, 2018). To date, transcriptomic analysis in ocular tissues has provided an extensive list of cyclic processes in various compartments of the eye (Storch et al., 2007; Mure et al., 2018). Yet, the understanding of developmental signaling pathways potentially under the control of clock gene expression is still sparse. Hence, the aim of the present study is to understand transcriptional difference in the timing of developmental processes and the functional implications that arise due to clock perturbations.

We currently present the developmental expression profiles of clock transcripts in mouse WT eyes between embryonic day 13 (E13) and postnatal day 24 (P24). Furthermore, we have characterized genome wide differential gene expression in the whole eye of *Per1*$^{-/-}$*Per2*^{Brdm1} mutant versus wild-type at E15, E18 and P3 and identified functional categories and pathways. We found that the *Per1/Per2* ablation significantly affects gene expression of the secondary regulatory loop members of the circadian clockwork, of the phototransduction pathway as well as of cell cycle components during development.

2. Materials and Methods

2.1. Animal Care

Mice were handled according to the European Parliament and The Council of the European Union Directive (2010/63/EU). All experimental procedures conformed to the Association for Research in Vision and Ophthalmology Statement on Use of Animals in Ophthalmic and Vision

Research. The homozygote double mutant mice *Per1*$^{-/-}$*Per2*^{Brdm1} carrying the loss-of-function mutation of *Per1* and *Per2* genes (Zheng et al., 1999; Zheng et al., 2001) were obtained as a generous gift from Dr. U. Albrecht (University of Fribourg, Switzerland). Wild-type (WT) and mutant animals (both on mixed C57BL/6J/129Sv background) were housed and bred in the Chronobiotron animal facility (UMS 3415, CNRS-University of Strasbourg) on a light–dark (LD) cycle (12 h light/12 h dark, 300 lx during the light phase, dim red light < 5 lx during the dark period), with an ambient temperature of 22 ± 1 °C. Animals were supplied with standard chow diet and water ad libitum. Control and mutant mice were age-matched, and mostly male mice were used in the post-natal groups.

2.2. Genotyping

Genotyping was performed by PCR amplification on mouse-tail DNA separately for the WT and KO alleles. The primer sequences were as follows: *Per1* KO fwd: 5′-ACAAACTCACAGAGCCCATCC-3′ and *Per1* KO rev: 5′-ACTTCCATTTGTCACGTCCTGCAC-3′; *Per2* mutant fwd: 5′-TTT GTTCTGTGAGCTCCTGAACGC-3′ and *Per2* mutant rev: 5′-ACTTCCAT TTGTCACGTCCTGCAC-3′; (Zheng et al., 2001; Zheng et al., 1999); *Per1* WT fwd: 5′-GTCTTGGTCTCA TTCTAGGACACC-3′ and *Per1* WT rev: 5′-AACATGAGAGCTTCCAGTC CTCTC-3′; *Per2* WT fwd: 5′-AGTAGGTCGTCTTCTTTATGCCCC-3′ and *Per2* WT rev: 5′-CTCTGCTTTCAACTCCTGTGTCTG-3′. PCR conditions comprised of 35 cycles of 30 s at 94 °C, 30 s at 56 °C for Per1 (WT and KO) or 58 °C for *Per2* (WT and mutant), 1 min at 72 °C followed by a final step for 5 min at 72 °C.

2.3. Eye Sampling

Both wild-type and the mutant mice were subjected to timed breeding. After confirmation of the presence of a vaginal plug at E0, female mice were immediately transferred to a separate cage until the appropriate embryonic (E) and postnatal (P) developmental stages were reached. qRT-PCR analysis was performed for E13, E15, E18, P0, P3, P13 and P24 only in WT samples. The comparative transcriptome study was performed only on samples from E15, E18 and P3 from WT and mutant animals, based on the periods of genesis of cones and rods in mice (respectively around E13–E16 and E18–P3 (Carter-Dawson and LaVail, 1979; Cepko et al., 1996)). The E15, E18 and P3 WT samples used for RNA sequencing were distinct from those used for qPCR. For very young mice, decapitation was used. Cervical dislocation was

performed only for older animals. Eye sampling was performed at the Zeitgeber time (ZT4), 4h after lights were switched on. A Zeitgeber is any external or environmental cue that entrains/synchronizes an organism's biological rhythms (in our case it would be only light). Whole eye globes were enucleated, snap-frozen on dry-ice and stored at −80 °C until use. Histological validation was performed on whole eyes sampled on P0 and P3 animals from same breeders and showed no obvious difference between genotypes (Supplementary Fig.S1).

2.4. RNA extraction and quality control

Mouse whole eyes were individually homogenized in 500 µl TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) and incubated for 5 min at room temperature. 100 µl of chloroform were added to each lysate and after 2 min incubation at room temperature the mixture was centrifuged using phase-lock gel tubes (Heavy, 2 ml; QuantaBio, Beverly, MA, USA) at 12,000 ×g for 15 min at 4 °C. Following the phase separation, the RNA from the upper aqueous phase was precipitated with equal volume of 70% ethanol and purified using the RNeasy micro kit (Qiagen GmbH, Hilden, Germany) according to manufacturer's protocol, including the DNase digestion step. The RNA was eluted with 14 µl of RNase-free water. RNA concentration and purity were measured using NanoDrop ND-1000 V 3.5 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA; A260/A280 and A260/A230 values were between 1.8 and 2). RNA quality was evaluated with the Bioanalyzer 2100 with RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA; RNA integrity numbers were 9.7–10).

2.5. Real-time quantitative RT-PCR (qRT-PCR)

RNA to cDNA Kit (Thermofisher, Courtaboeuf, France). qPCR was performed to analyze gene expression along development at E13, E15, E18, P0, P3, P13 and P24 using the 7300 Real Time PCR system (Thermofisher) and the hydrolysed probe-based TaqMan chemistry as previously described in (Sandu et al., 2011). Serial dilutions created from the pool of all cDNA samples were used to calculate the amplification efficiency for each assay (values were between 1.8 and 2 for all assays). Each PCR reaction was done in duplicate. Nine circadian clock genes were examined: *Arntl*, *Clock*, *Per1*, *Per2*, *Per3*, *Cry1*, *Cry2*, *Nr1d1*, and *Rorb* (Supplementary Table S1). The PCR program was: 10 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing–elongation at 60 °C for 1 min. The PCR conditions were 1 × TaqMan

Universal PCR Master Mix, No AMPErase UNG (Thermofisher); 1 × Gene Expression Assays mix (containing forward and reverse primers and cognate probe; Thermofisher); and 1 µl of cDNA in a total volume of 20 µl. At the end of the elongation step, fluorescence data acquisition was performed using the 7300 System Sequence Detection Software V1.3.1 (Thermofisher) and qBase software (free v1.3.5; (Hellemans et al., 2007)) was used for data analysis. Transcript levels were normalized to *Gapdh* and *Sdha* (Adachi et al., 2015). Data were submitted to Shapiro-Wilk normality test (data for *Per1* and *Nr1d1* did not pass). Differences among the age groups were analyzed using one-way ANOVA followed by Holm-Sidak post-hoc test or (when data did not pass the normality test) a non-parametric Kruskall-Wallis test followed by a Dunn's post hoc test (Sigmaplot v12, Systat Software, Inc.). The post hoc analysis is detailed in Supplementary Table S2.

2.6. Construction of RNA-seq Libraries and RNA sequencing

RNA-Seq libraries were constructed from 500 ng of total RNA (E15, E18 and P3) using the KAPA mRNA HyperPrep Library Preparation Kit 005302-11-1 (Roche Sequencing Solutions, Pleasanton, CA USA) for Illumina Platform HiSeq 4000 as per manufacturer's instructions. RNA was treated with magnetic oligo-dT beads (Lot No: 005328-9-1) to capture the poly (A) RNA and was chemically fragmented into a desired size (around 200–300 bp) using heat in the presence of Mg2+. These RNA fragments were used for first and second strand cDNA synthesis. (A) tailing was added to the blunt ends of the dscDNA to enable adapter ligation with the (T) base overhangs. These adapter-ligated library DNA were purified and used for enrichment by using adapter-specific PCR. The libraries were amplified using a mixture of KAPA HiFi HotStart RdyMix (2×) and Lib. Amp. Primer Mix (10×) (Roche Sequencing Solutions, Pleasanton, CA USA) to produce strand-specific PCR products. The library amplification process was verified using flash gel visualization along with clean up steps. By using Agilent Bioanalyzer, quality and size distribution of the cDNA library was checked. Fragments size for the cDNA library were between 200 and 500 bp, with a peak at ~300 bp. Qubit 2.0 Fluorometer (Life Technologies, Foster City, CA, USA) was used for the quantification of libraries. The cDNA library was sequenced by single-end sequencing of 50 bases length on Illumina HiSeq 4000 sequencer (Illumina, San Diego, CA, USA).

2.7. RNA sequencing data analysis

Reads were subjected to quality control (FastQC v.0.11.5, dupRadar (Sayols et al., 2016), Picard Tools) showing low duplication- and appropriate gene detection rates, trimmed using Trimmomatic v0.32 (Bolger et al., 2014) and aligned to the reference mm10 mouse genome (Ensembl GRCm38 v87) using HISAT2 (v2.0.4) (Kim et al., 2015). Counts were obtained using HTSeq (v0.6.1) (Anders et al., 2015) with parameters "-m union -f bam -r name -s no -a 10 -t exon -i gene_id" and the mouse GTF from Ensembl (version 87).

Statistical analyses were performed using the edgeR (Robinson et al., 2010) and limma (Ritchie et al., 2015) R/Bioconductor packages using R (v3.4.3) and Bioconductor (v3.6). 22,911 genes with more than 5 counts in at least 3 of the samples were retained. Count data were transformed to log2-counts per million (logCPM), normalized by applying the trimmed mean of M-values method (Robinson and Oshlack, 2010) and precision weighted using voom (Law et al., 2014). Differential expression was assessed using an empirical Bayes moderated t-test within limma's linear model framework including the precision weights estimated by voom. Resulting p-values were corrected for multiple testing using the Benjamini-Hochberg false discovery rate. An adjusted p-value ≤ 0.05 was considered significant, and an additional fold change cut-off (≤−1.5 or ≥1.5) was applied to identify differentially expressed genes. Additional gene annotation was retrieved from Ensembl (release 91) using the biomaRt R/Bioconductor package. Principal component analysis (function plotPCA, package DESeq2) was performed on the logCPM values of the top-500 most variable genes.

Supplementary Fig. S2a shows that the relative clock gene mRNA quantities in WT eye globes between E15, E18 and P3, as assessed by qRT-PCR (data from Fig. 1), confirm the data obtained by RNA-Seq. In addition, qPCR analysis performed on whole eye globes from WT and *Per1*$^{-/-}$ *Per2*^{Brdm1} mice confirmed the gene expression changes between genotypes measured by RNA-Seq (Supplementary Fig. S2b). These analyses agree with other studies (S.M.-I Consortium, 2014; Bennis et al., 2017). The two series of quantifications (qRT-PCR/RNA-Seq) were obtained with distinct sample series, thus also providing a biological validation of our results.

Results from RNA-sequencing in *Per1*$^{-/-}$ *Per2*^{Brdm1} eye samples show the absence of reads in specific genomic regions from the *Per1* and *Per2* loci corresponding to the deletion mutations

initially introduced in these genes (Zheng et al., 1999; Zheng et al., 2001), thus validating the loss of expression of PER1 and PER2 (Supplementary Fig. S3).

2.8. Canonical Pathway Analysis

Canonical pathway enrichment analysis was performed using IPA (Ingenuity) by using both up and down regulated genes in mutant vs WT (adjusted p-value < 0.05 and fold change ≤−1.5 or ≥1.5), at each age (Ingenuity Systems, version 24718999, accessed in November 2018). Significance of enrichment was calculated using a right-tailed Fisher's Exact Test and corrected for multiple testing using the Benjamini-Hochberg false discovery rate ($p < 0.01$).

3. Results

3.1. Clock gene expression in WT control mouse eyes during development

We first performed a temporal analysis of core clock gene expression by quantitative RT-PCR (qRT-PCR) in developing whole eyes from WT mice. We chose seven age time points (E13, E15, E18, P0, P3, P13, P24) spanning photoreceptor genesis and maturation. Our data revealed distinct temporal gene expression dynamics for key core clock components (*Bmal1* or *Arntl*, *Clock*, *Per1*, *Per2*, *Per3*, *Cry1*, *Cry2*, *Nr1d1* and *Rorb*) during eye development (Fig. 1 and Supplementary Table S2).

This initial analysis revealed that all clock genes examined are already expressed at E13. Using one-way ANOVA, their abundance showed a significant age-related change (Fig. 1). The relative expression level of *Bmal1* (*Arntl*) mRNA reached a peak just after birth at P3 while maintaining moderate expression at other ages (Fig. 1a). Similarly, *Rorb* mRNA had significantly highest expression around P3 (Fig. 1i). In contrast, a steady increase in gene expression was maintained by *Clock* gene from E13 until P3 (Fig. 1b). Among Period genes, *Per1* and *Per2* expression peaked at birth (P0) whereas *Per3* expression culminated only at the postnatal ages (P13 and P24; Fig. 1c–e). *Cry2* also reached the highest expression levels close to eye opening at P13 (Fig. 1f). Finally, *Cry1* mRNA had a peak in gene expression at birth (P0) (Fig. 1e). *Nr1d1* had the most striking profile, increasing expression levels between E13 and P24 with a fold-change difference of 19, whereas differences between minimal and maximal expression did not reach 4-fold for other clock genes (Fig. 1h). This initial analysis helped us to select time points for further in-depth whole-genome RNA-Seq analysis.

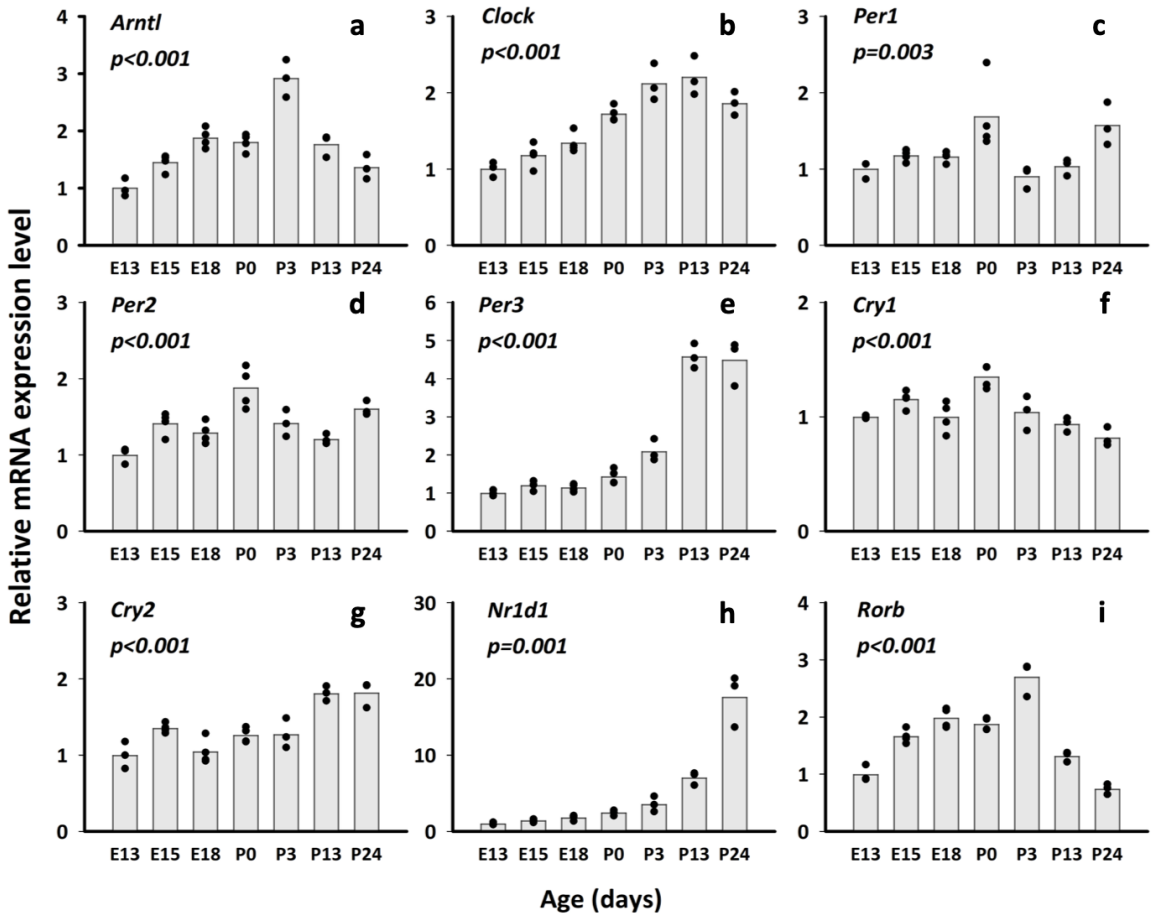

Fig. 1. Relative mRNA expression profiles of circadian clock genes in the WT whole eyes across eye developmental time points (n = 3–4/age). Bar graphs show mean ± SEM mRNA expression levels of (a) *Arntl* (*Bmal1*) (b) *Clock* (c) *Per1* (d) *Per2* (e) *Per3* (f) *Cry1* (g) *Cry2* (h) *Nr1d1* and (i) *Rorb* in the whole eyes relative to E13. *Arntl* (*Bmal1*) and *Rorb* mRNA reached a peak just after birth around P3, Clock gene expression increases steadily from E13 until P3, *Per1*, *Cry1* and *Per2* expression peaked at birth (P0), *Per3* and *Cry2* expression peaks only around P13 and P24. *Nr1d1* has an increasing expression pattern between E13 and P24. One Way ANOVA was performed and statistical significance is indicated by p-values. Values from individual samples are shown as dots. Results from post-hoc analysis are presented in the Supplementary Table S2.

3.2. RNA-Seq analysis of differentially expressed genes in *Per1*$^{-/-}$*Per2*^{Brdm1} mutants compared to WT controls

3.2.1. Whole transcriptome changes

We performed RNA sequencing to compare the whole eye gene expression profiles between *Per1*$^{-/-}$*Per2*^{Brdm1} and WT mice, as a function of time during embryonic (E15, E18) and postnatal (P3) ages. Principal component analysis clearly showed age and genotype effects in the whole eye transcriptome (Fig. 2). Age-related effects were pronounced and segregated with the first principal component (PC). This explains most of the variance in the data (PC1: 56%). Indeed,

both in mutant and WT mice, many genes (respectively 14,046 and 15,445) were significantly differentially expressed (adjusted p-value < 0.05) between different developmental stages. This possibly reflects the extensive gene expression changes occurring in eye development between E15 and P3. Genotype-related effects segregated on the second principal component, which explained only 7% of the variance in the data. Thus, gene expression profiles cluster together for mutants and WT at E15, whereas clear differences can be observed between mutant and WT expression profiles at E18 and P3.

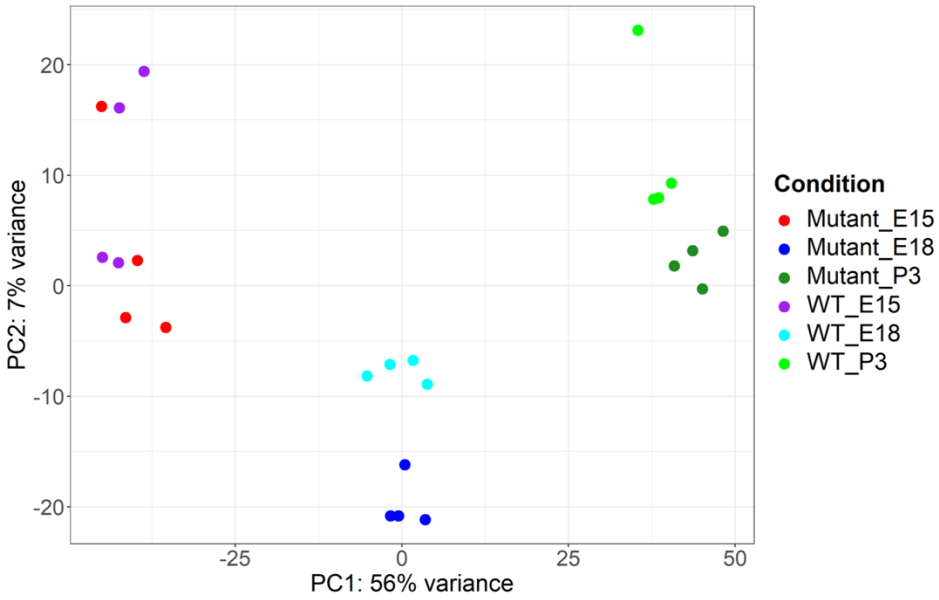

Fig. 2. Principal component analysis of eye transcriptomes in WT and mutant eyes at E15, E18 and P3. The percentage with which a principal component (PC) accounts for the variability in the data is indicated on the corresponding axis. Each circle represents an individual RNA sample from WT or mutants under LD conditions at ZT4 for three developmental time points (E15, E18 and P3) (n = 4/age/genotype).

3.2.2. Specific transcriptome changes between genotypes

Analysis of differentially expressed genes (DEG; adjusted p value < 0.05 and fold change ≤−1.5 or ≥1.5) of the *Per1*$^{-/-}$*Per2*^{Brdm1} versus WT whole eyes showed an increasing number of DEG during development. At time points E15, E18 and P3, we respectively found 36, 200 and 367 genes with significantly increased expression. At the same time points, we observed 18, 90 and 273 genes with significantly decreased expression in the mutant mice (Fig. 3a). Only limited numbers of genes (35 up and 30 down) are differentially expressed at more than one developmental stage.

Of the 23 genes most likely involved in the molecular clockwork (Takahashi, 2017), eight genes (*Per3*, *Nr1d2*, *Ciart*, *Bhlhe41*, *Rorc*, *Tef*, *Npas2* and *Dbp*) displayed statistically significant

changes between WT and mutants, in at least one age group (Fig. 3b). In contrast, expression of *Per3* and *Nr1d2* showed a large increase in the mutant eyes at all ages. *Ciart* displayed specific increase at E18 in the mutant, whereas *Bhlhe41*, *Rorc* and *Tef* mRNAs were specifically increased at P3. *Npas2* expression was markedly reduced and *Dbp* significantly upregulated in the E18 and P3 mutants.

All the differentially expressed genes at E15 within the cut-off range are presented in Fig. 4a. Selected DEGs are highlighted in the volcano plots for E18 and P3 age groups (Fig. 4b and c). Most of them relate to the eye and, more specifically, to the photoreceptor, to neuronal development and to the clock. The complete lists of DEGs sorted by statistical significance are listed in Supplementary Table S3 (E15 mutant versus WT), Table S4 (E18 mutant versus WT) and Table S5 (P3 mutant versus WT) respectively.

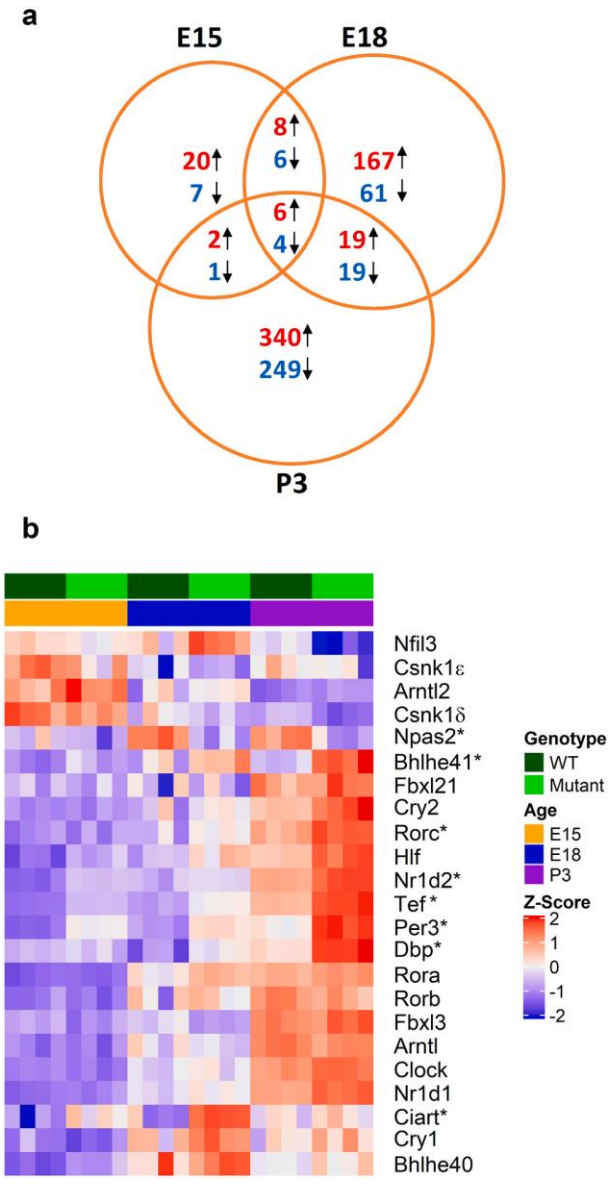

Fig. 3. (a) Venn diagram of differentially expressed genes in mutant versus WT eyes. The total numbers of differentially expressed genes (increased or decreased) in each age group and the overlaps are indicated. Upregulated (higher in mutant) genes are marked in red and downregulated (higher in WT) genes are marked in blue (adjusted p-value < 0.05 and fold change ≤−1.5 or ≥1.5). **(b)** Heat map of clock and clock-related gene expression during embryonic (E15 and E18) and postnatal (P3) ages of *Per1*$^{-/-}$*Per2*^{Brdm1} mutants versus WT. Genes were clustered using Euclidean distance and complete linkage. Color-coding corresponds to z-score of logCPM values from blue (lowest expression) to red (highest expression). *Per3*, *Nr1d2*, *Ciart*, *Bhlhe41*, *Rorc*, *Tef*, *Npas2* and *Dbp* displayed statistically significant changes between WT and mutants in at least one age group "*". logCPM, log2 counts per million.

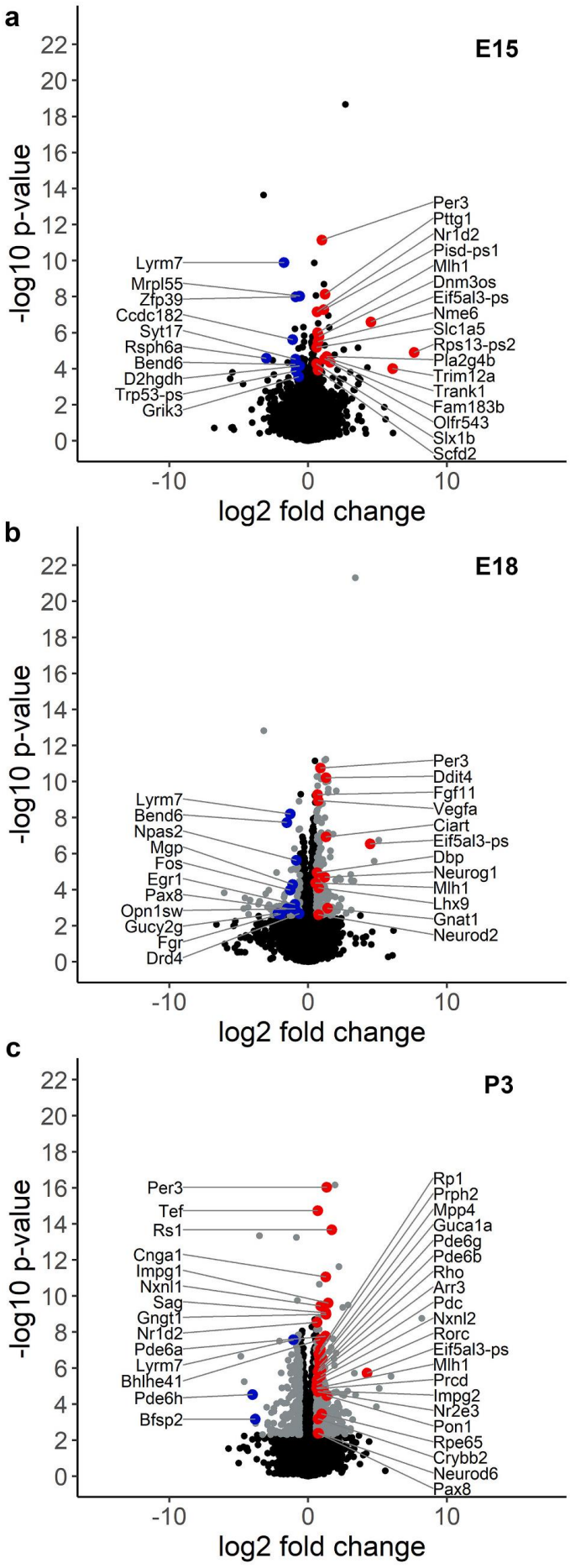

Fig. 4. Volcano plots for the genes found enriched in *Per1*$^{-/-}$*Per2*^{Brdm1} versus WT whole eyes at E15, E18 and P3. The plot shows the enrichment (x axis, log2-transformed fold change) of the genes in mutant versus WT eyes against the significance (y axis, p-value, −log10 scale). Genes with adjusted p-value < 0.05 and fold change ≤−1.5 or ≥1.5 are highlighted in grey. Selected genes are highlighted in red (upregulated) and blue (downregulated) for **(a)** E15 (complete list), **(b)** E18 and **(c)** P3 (eye and neuronal development related). Each circle represents an individual gene.

3.3. Functional differences between gene expression profiles in the *Per1*$^{-/-}$*Per2*^{Brdm1} and WT whole eyes

To evaluate gene expression profiles on the level of canonical pathways, we performed enrichment analyses on the genes differentially expressed in the different age groups (adjusted *p*-value < 0.05 and fold change ≤−1.5 or ≥1.5) using the Ingenuity Pathway Analysis (IPA) software. We focused our analysis on the presence of canonical pathways, since these are the simplest molecular representations of the relevant biological events.

Top canonical pathways associated with genes differentially expressed at E15 are related to 'circadian rhythm signaling' and the 'adipogenesis pathway' (Fig. 5a). Quite expectedly, `circadian rhythm signaling' is also the top-ranked canonical pathway at E18 followed by four other pathways suggesting enrichment in these signaling molecules (Fig. 5b).

Ten canonical pathways were significantly enriched with genes differentially expressed between *Per1*$^{-/-}$*Per2*^{Brdm1} mutants and WT at the postnatal age P3 (Fig. 5c). The topmost significant canonical pathway was related to Phototransduction, which agrees with the aberrant phenotype (alteration of photoreceptor development at early postnatal age) previously found in the mutant (Ait-Hmyed et al., 2013).

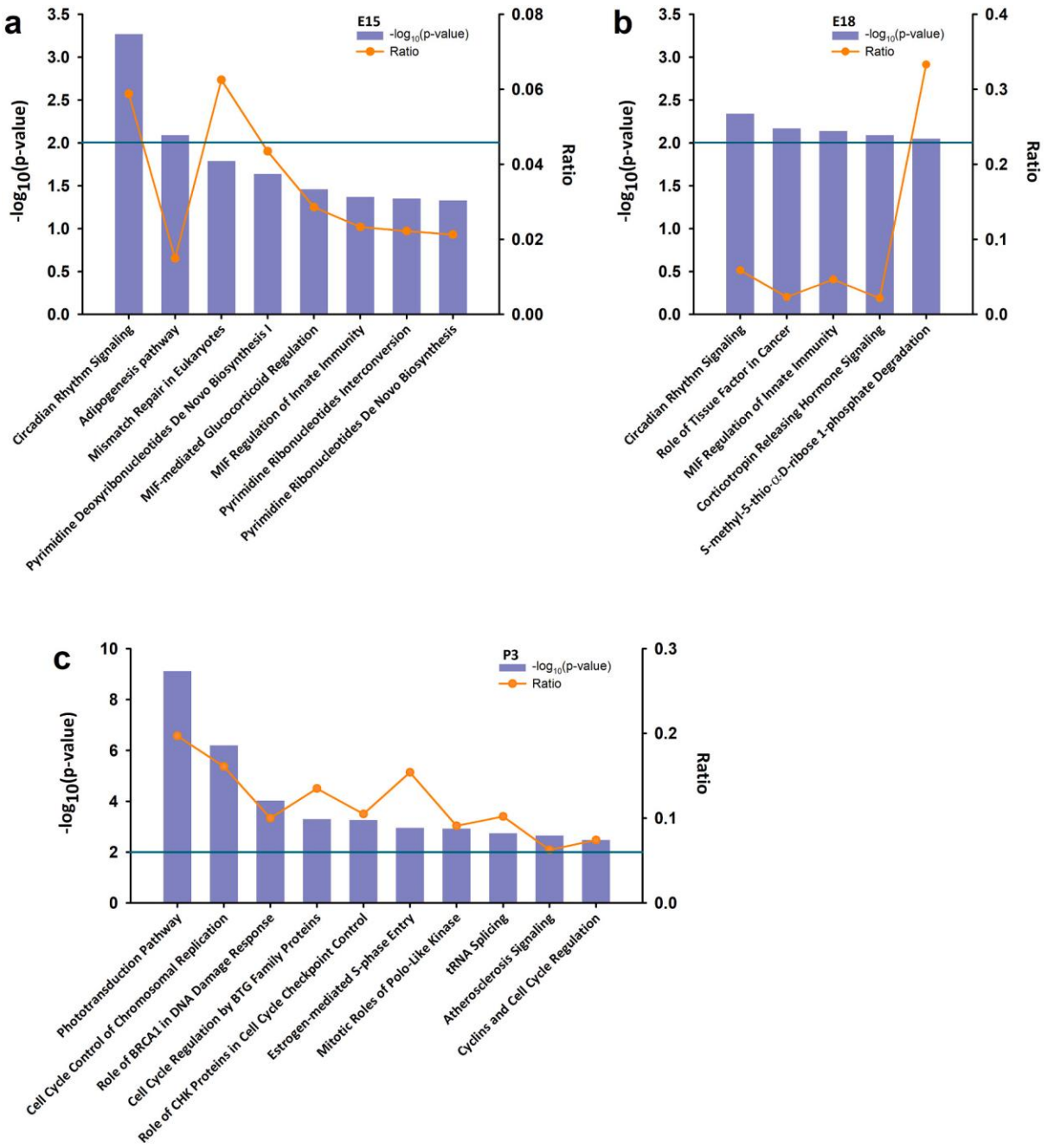

Fig. 5. (a) (b) and (c) Top-ranked canonical pathways at E15, E18 and P3 for DEG in *Per1*$^{-/-}$ *Per2*^{Brdm1} mutants versus WT. Bars represent the −log10 adjusted p value (left y-axis). Orange line represents the ratio of the number of differentially expressed genes present in the canonical pathway to the total number of genes in the pathway (right y-axis). The green line indicates the threshold at an adjusted $p < 0.01$.

The components of phototransduction cascade, which were mainly upregulated in the mutant, are highlighted in Fig. 6a,b (see also Supplementary Fig. S4 and S5a,c). We also found increased expression of photoreceptor-specific genes, like *Prph2* (peripherin 2), *Impg1* and *Impg2* (interphotoreceptor matrix proteoglycan 1 and 2), *Prcd* (photoreceptor disc component), *Rp1l1* (RP1 like 1), *Nr2e3* (Nuclear Receptor Subfamily 2 Group E Member 3),

Prom1 (prominin 1) and *Mpp4* (membrane palmitoylated protein 4) (details in Supplementary Table S5), indicating the differentiation of the photoreceptor complex.

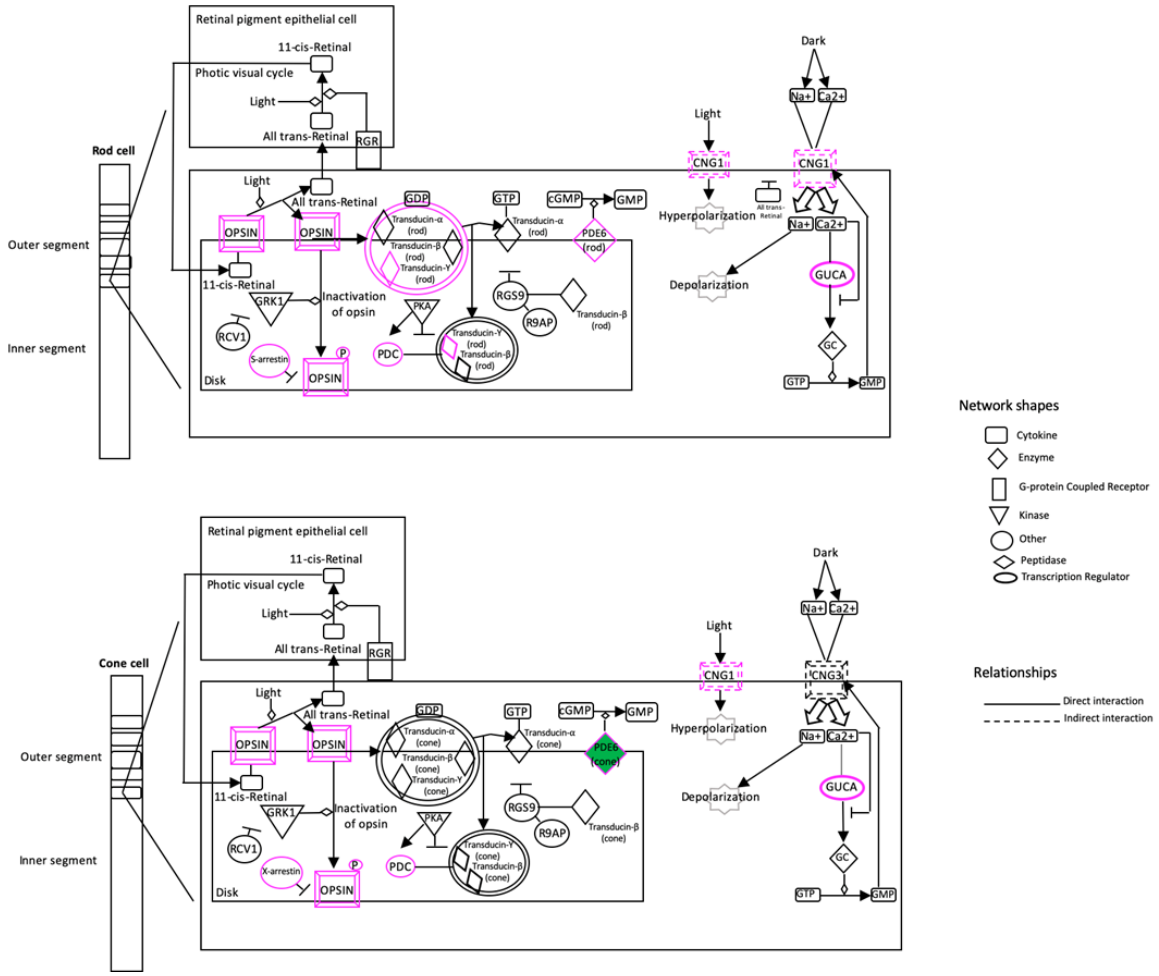

Fig. 6. Ingenuity pathway analysis identified phototransduction pathway to be associated with differentially expressed genes at P3. The phototransduction pathways of rods and cones are shown respectively in **a** and **b**. Shapes of the network elements refer to the functional category of the gene product (indicated in the legend inset), pink indicates upregulation and green indicates downregulation (IPA All rights reserved).

Interestingly, a substantial fraction of genes previously implicated in human congenital ocular pathologies were found with increased expression levels in P3 mutants: for instance *Rpe65* (retinitis pigmentosa of the RPE65 type), *Rp1* (retinitis pigmentosa 1), *Pon1* (serum paraoxonase/arylesterase 1), *Bbs7* (Bardet-Biedl syndrome 7), *Nxnl1* and *Nxnl2* (Rod-derived cone viability factors), *Ptgds* (prostaglandin D2 synthase), *Rs1* (retinoschisis (X-linked, juvenile) 1), *Samd7* (SAM domain-containing protein 7), *Tacstd2* (tumor-associated calcium signal transducer 2), *Cdhr1* (cadherin related family member 1), *Efemp1* (EGF containing fibulin extracellular matrix protein 1), *Crybb2* (crystallin, beta B2) and *Mgp* (Matrix Gla protein) (details in Supplementary Table S5).

The *Per1*$^{-/-}$*Per2*^{Brdm1} mutants at P3 also displayed a decrease in expression of cell-cycle-associated genes (Fig. 5c and 7a, b, c). There was a clear down-regulation of genes belonging to the 'Cell Cycle Control of Chromosomal Replication' pathway (Fig. 7a). DNA replication licensing factors like *Mcm2*, *Mcm3*, *Mcm6* and *Cdt1*, cell division cycle proteins like *Cdc6* and *Cdc45* along with essential enzymes like *Pole* and *Top2a* were the down-regulated components (Fig. 7a). The `Cyclins and Cell Cycle Regulation' pathway had significantly reduced expression levels of some of the major E2F family genes like *E2f2*, *E2f7* and *E2f8* along with the cyclins B1 (*Ccnb1*) and D1 (*Ccnd1*) (Fig. 7b). Within these two enriched cell-cycle-related pathways, only cell cycle genes *Cdk18* (cyclin-dependent kinase 18) and *Ppp2r2b* (protein phosphatase 2 regulatory subunit beta, a negative regulator of division) were found upregulated. It is known that there is a decrease in cell division at this period of normal eye development (Levine and Green, 2004). This is confirmed in our data by the major downregulation of genes belonging to the 'DNA replication' and 'Cell cycle' GO terms in WT eyes between E18 and P3 (Supplementary Fig. S5d). However, downregulation of these genes appears even more prominent in the mutant eyes, based on the p values of these canonical pathways (Supplementary Fig. S5d).

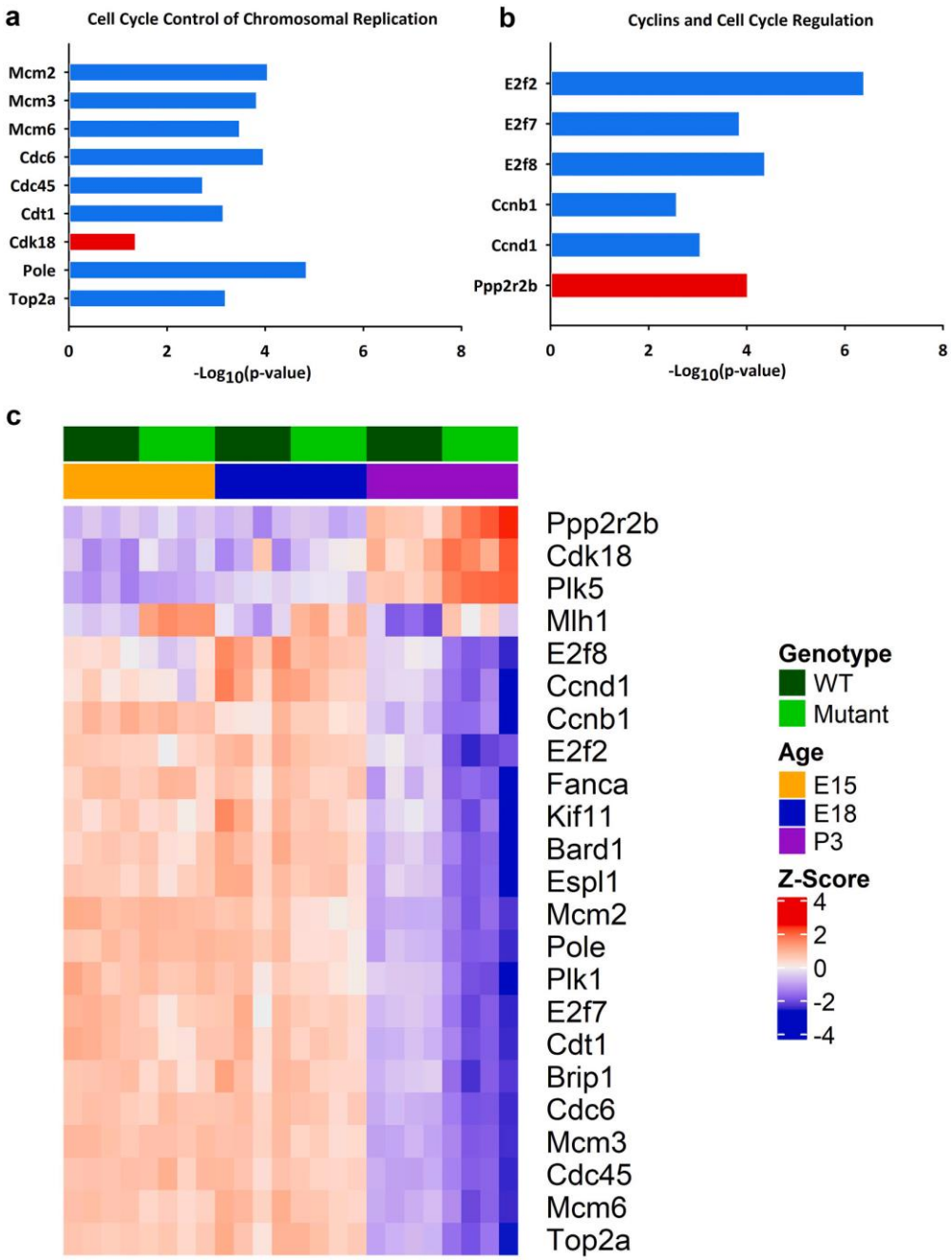

Fig. 7. IPA analysis of DEG at P3 showed enrichment in the **(a)** cell cycle control of chromosomal replication genes and **(b)** cyclins and cell cycle regulation genes pathways. Significance for the differential expression between mutant and WT eyes is shown as −log10 (Adj p-value) (x-axis). There was an overall decrease in the expression of genes of pathways **(a)** and **(b)** in the mutant eyes at P3, except *Cdk18* and *Ppp2r2b*. Up- and down-regulated genes are shown in red and blue respectively. **(c)** Heat map of the differentially expressed genes of the cell-cycle related pathways during embryonic (E15 and E18) and postnatal (P3) ages of *Per1*$^{-/-}$*Per2*^{Brdm1} mutants versus WT. Genes were clustered using Euclidean distance and complete linkage. Color-coding corresponds to z-score of logCPM values from blue (lowest expression) to red (highest expression). *E2f8, Ccnd1, Ccnb1, E2f2, Fanca, Kif11, Bard1, Espl1, Mcm2, Pole, Plk1, E2f7, Cdt1, Brip1, Cdc6, Mcm3, Cdc45, Mcm6, Top2a* displayed pronounced downregulation in the mutants at P3 while *Ppp2r2b, Cdk18, Plk5* had higher gene expression levels. *Mlh1* stands out with an unusual, increased, expression pattern throughout development in the mutants.

4. Discussion

The circadian clock is a major regulator of physiology and behaviour through the temporal control of gene expression programs on a 24 h scale. The molecular role and mechanism of action of clock genes in developmental processes have lately received increased interest (Li et al., 2007; Jensen et al., 2012; McQueen et al., 2018; Zhao et al., 2018; Zhou et al., 2018; Logan and McClung, 2019). Here we focus on the comparison between the eye transcriptome of *Per1*$^{-/-}$*Per2*^{Brdm1} and WT mice at E15, E18 and P3. In our dataset, we observed differential expression of clock genes, especially from the regulatory feedback loops. Most prominently at P3, mutant eyes display the most extensive alterations: significant upregulation of phototransduction-related genes, together with a reduction in cell cycle-related transcripts. Our work provides further evidence that clock genes play a role in eye development, likely by taking part in the signaling between differentiation programs and regulation of cell division.

Using immunohistochemistry, we previously described that retinas from *Per1*$^{-/-}$*Per2*^{Brdm1} mutant mice display altered photoreceptor differentiation with reduced blue cone numbers and opsin expression (Ait-Hmyed et al., 2013). To reveal the underlying molecular mechanisms of this observation, we here used comparative transcriptome analysis of the developing whole eye, including (premature) retinal photoreceptors, in animals from the same mutant line and WT. Upon analyzing gene expression changes in WT eyes along development, we found that 'Visual perception' and 'Sensory perception of light stimulus' are the major GO terms enriched in the genes whose expression increases from E15 to P3 (Supplementary Fig. S6, see also Fig. S5a, c). This indicates that cone and rod genes provide a substantial contribution to the characterized transcriptome and thus that our analyses are relevant for identifying related changes in the mutants.

Several groups previously reported a relationship between clock gene mutation(s) and abnormal eye/retinal development (Mollema et al., 2011; Ait-Hmyed et al., 2013; Stone et al., 2013; Ait-Hmyed Hakkari et al., 2016; Sawant et al., 2017; Baba et al., 2018; Sawant et al., 2019; Stone et al., 2019) but most frequently the underlying mechanisms have not been fully elucidated. Our data (Fig. 1) suggests that regulatory mechanisms of retinal development and differentiation driven by the clock are already at play at E13. However, it is at P3 that the gene expression changes induced by the absence of PER1 and PER2 are maximal in our transcriptome study (Fig. 3, Fig. 7c). It has been described that de-repression of cell type-

specific differentiation programs is the major epigenetic change occurring along with retinal development (Aldiri et al., 2017). In this context of global genome de-repression, and since clock genes are modulators of gene expression programs, it is not surprising that the effects of clock perturbation are more pronounced at later (P3) than earlier (E15, E18) stages. In addition, expression of Clock and Bmal1 peaks at P3 (Fig. 1). Thus, absence of repression by PER1/PER2 on BMAL1/CLOCK target genes, might have its maximal effects at P3 as well. For instance, the upregulation of *Per3* mRNA in mutant vs WT eyes is striking (FC > 2.5 at P3 but also FC > 1.9 at E15, and > 1.8 at E18). This observation could possibly also be due to a compensatory developmental effect induced by *Per1/Per2* mutation. Further studies are needed to shed light on these hypotheses.

Our results also raise a more general question whether the DEGs we identified are (in-) direct targets of the BMAL1/CLOCK complex. Therefore, we compared our dataset with a list of 2,458 binding sites/1,905 target genes for the BMAL1/CLOCK complex, previously identified in the liver by Trott and Menet (Trott and Menet, 2018). A limitation of this study is that we cannot exclude that eye-specific targets of BMAL1/CLOCK are not represented in the list of liver targets. We found that 5.7% of all our DEGs overlap with this list. However, we did not observe an enrichment for BMAL1/CLOCK targets in our DEG set (one-sided Fisher's exact test, $p = 0.74$). This indicates that the genes found differentially expressed between WT and mutant eyes contain both direct (for instance *Dbp*, *Nr1d2*) and (essentially) indirect targets of the clock.

It is presently unknown how the clock might control phototransduction gene expression in the mammalian retina. Our (Ingenuity pathway) analysis indicates that, in accordance with the literature (Corbo et al., 2010; Hao et al., 2012), NRL and CRX are the major regulators of the upregulated genes at P3. *Nr2e3*, which acts as a co-activator of rod-genes with NRL, CRX and REV-ERBα (NR1D1) (Cheng et al., 2004), has a FC > 1.6 at P3 in our $Per1^{-/-}Per2^{Brdm1}$ mutant eyes. *Nr2e3* is a photoreceptor-specific nuclear receptor (PNR) that represses cone-specific genes and activates several rod-specific genes during mammalian retinal development (Peng et al., 2005). When overexpressed in developing photoreceptors, *Nr2e3* drives their differentiation towards a rod fate at the expense of cones (Cheng et al., 2006), whereas its absence may lead to “hyperfunction” of S-cones (Haider et al., 2000; Haider et al., 2001). Thus, *Nr2e3* mutations cause enhanced S-cone syndrome among humans (Haider et al.,

2000). In the mutant eyes at P3 we find an increase in expression of rhodopsin (FC > 1.6) and strong decrease (2 fold at E18, 2.4-fold at P3) for s opsin, in agreement with the previously observed phenotype (Ait-Hmyed et al., 2013). In addition, expression of *Nr2e3* was also shown to be rhythmic in the 24 h transcriptome of mouse eye globes (Storch et al., 2007) and in zebrafish adult retinas (Laranjeiro and Whitmore, 2014), suggesting it is regulated by the clock. Combining our data with those of the literature, we speculate that NR2E3-mediated regulation might be one of the causes of the morphological/functional changes in the phototransduction machinery observed in the mutant at P3.

Another transcription factor gene displaying upregulation (FC > 1.5) in the mutant eye at P3 is *Rorc*. The *Rorc* gene codes for the ROR gamma clock factor and is itself a target of *Nr2e3* during retinal development (Haider et al., 2009; Mollema et al., 2011). It was also previously determined that loss of *Rora*, one of its paralogs, leads to defective cone differentiation and reduced expression of blue, green opsin as well as cone arrestin (Fujieda et al., 2009). In the absence of *Rorb*, complete loss of rods and over-production of blue cones occurs during retinal development (Jia et al., 2009). It will be interesting to understand if the retinal architecture is disrupted in the absence/mutation of *Rorc* as well. Obviously, further studies are needed to determine the precise mechanisms involved.

Effects of clock alteration on blue cones was also described previously (Sawant et al., 2017). More specifically, photoreceptor-specific loss of *Bmal1* induces extended distribution of the s-opsin expressing cones along the dorso-ventral axis. This was shown to involve differential thyroid hormone signaling through an effect on *Dio2* expression (Sawant et al., 2017). However, we did not detect any significant alteration of the expression of thyroid hormone pathway modulators in the $Per1^{-/-}Per2^{Brdm1}$ mutants at any of the studied ages. It might be that such alterations occur later in development. Indeed, *Dio2* expression is detectable after P5, similar to that of m-opsin (Sawant et al., 2017). In contrast, we found a decrease in the expression levels of s-opsin at E18 and P3, which is retained in the adult (Ait-Hmyed et al., 2013). This is similar to the phenotype induced by a photoreceptor-specific knockout of *Per2* (Sawant et al., 2017). Our data thus indicate that the clock effects on cone generation is two-fold: an early effect leading to reduced s-opsin expression/s-cone differentiation and a later, thyroid hormone dependent, effect on their dorso-ventral gradient.

In our study, the cell cycle appears perturbed in the eyes from *Per1*$^{-/-}$*Per2*^{Brdm1} animals at P3 (Fig. 7 and Supplementary Fig. S5d), an age at which a substantial part of precursors for late-born retinal cell types are still dividing (Cepko et al., 1996; Levine and Green, 2004). The link between circadian clock and cell cycle regulation has long been established (Masri et al., 2013). However, there are only few studies of clock regulation, cell division and cellular differentiation together. The relationship between *Bmal1* loss and cell cycle arrest, with an upregulation of p21 and a block in the G1 phase, was previously studied in the hair follicles (Lin et al., 2009). The gene expression levels of Wee1 (cell cycle checkpoint kinase) and p21 (cyclin-dependent kinase inhibitor) are also regulated by *Bmal1* in the liver (Matsuo et al., 2003). *Cry2* promotes the circadian regulation of myogenic differentiation and regeneration in the mouse (Lowe et al., 2018) whereas *Rev-Erbα* (*Nr1d1*) acts as an inhibitor of the same processes (Chatterjee et al., 2019). Also, Clock gene silencing induced spontaneous differentiation of mouse embryonic stem cells indicating an earlier exit from their pluripotent state (Lu and Yang, 2016). In general, cell-cycle specific markers have been strongly connected with retina development (Dyer and Cepko, 2001). *E2f* family transcription factors (Dagnino et al., 1997; Vuong et al., 2012) and cyclin D1 (Fantl et al., 1995; Das et al., 2009; Das et al., 2012) are well known to regulate embryonic aspects of retinal development. In the *Per1*$^{-/-}$*Per2*^{Brdm1} eyes it might be that reduced expression of genes promoting cell division triggers faster cell cycle exit, which is likely to disturb the ratio between late-born to early-born cells as shown previously regarding cyclin D1 (Das et al., 2009; Das et al., 2012): here the overproduction of rod and cone markers at the expense of blue cones. Interestingly, conditional knockout of *Bmal1* in the retina was recently shown to result in delayed cell-cycle exit and subsequent altered neurogenesis (Sawant et al., 2019). Therefore, we surmise to ensure proper generation of retinal cell types, the timing needs to be optimum and the circadian clock might be responsible for appropriate temporal expression patterns of cell cycle-related genes.

Finally, we still do not know exactly how the absence of *Per1* and *Per2* shuts down the expression of cell cycle-related genes discussed extensively above. Pathway analysis of transcriptome changes along development in WT eyes indicates a downregulation of genes belonging to the Wnt and Hippo signaling pathways between E15 and E18 (Supplementary Fig. S5b). By contrast, these pathways are absent or underrepresented in the genes turned down between E15 and E18 in mutant eyes. Also, the PI3K-Akt signaling pathway, which

includes many growth factors and associated receptors, is significant in mutants but absent in the WT (Supplementary Fig. S5b). We hypothesize that the specific combination of signals turned off between E15 and E18 in the mutant eyes induces more massive depletion of cell cycle components and hence an earlier decrease in progenitor proliferation. Future studies are needed to make sure that the downregulation of these cell cycle-related pathways occurs in the retina exclusively and not elsewhere in the eye.

5. Conclusions

This study provides new information about the role of the circadian clock in eye development. It more specifically points to signaling pathways potentially linking the clock, photoreceptor generation and control of the cell cycle in the developing retina. Moreover, our findings reveal potential mechanisms for the putative ocular effects of clock malfunction reinforcing the ubiquitous relevance of circadian clock in eye morphogenesis. More studies will be needed to determine the connection between the phenotype and retinal abnormalities.

CRediT authorship contribution statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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Table S1-References of the TaqMan probes (Thermofisher) used for real-time PCR

Gene	Taqman assay reference	RefSeq	Exon Boundary	Assay location	Amplicon length (bp)
Arntl	Mm00500226_m1	NM_001243048.1	8-9	900	87
Clock	Mm00455950_m1	NM_001289826.1	15-16	1548	81
Per1	Mm00501813_m1	NM_001159367.1	18-19	2628	106
Per2	Mm00478113_m1	NM_011066.3	19-20	3271	73
Per3	Mm00478120_m1	NM_001289877.1	4-5	1027	73
Cry1	Mm00514392_m1	NM_007771.3	1-2	740	64
Cry2	Mm00546062_m1	NM_009963.4	1-2	255	70
Nr1d1	Mm00520708_m1	NM_145434.4	1-2	664	62
Rorb	Mm00524993_m1	NM_001043354.2	2-3	730	74
Gapdh	Mm99999915_g1	NM_013556.2	2-3	276	81
Sdha	Mm01352363_m1	NM_023281.1	3-4	342	64

Table S2-Post-hoc analysis of data in Figure 1 (One-way ANOVA)

Arntl

WT	E13	E15	E18	P0	P3	P13	P24
E13		0.077	<0.001	<0.001	<0.001	0.002	0.207
E15			0.074	0.143	<0.001	0.225	0.914
E18				0.835	<0.001	0.924	0.037
P0					<0.001	0.823	0.080
P3						<0.001	<0.001
P13							0.159

Clock

WT	E13	E15	E18	P0	P3	P13	P24
E13		0.539	0.104	<0.001	<0.001	<0.001	<0.001
E15			0.462	0.003	<0.001	<0.001	<0.001
E18				0.043	<0.001	<0.001	0.008
P0					0.047	0.014	0.491
P3						0.546	0.317
P13							0.124

Per1

WT	E13	E15	E18	P0	P3	P13	P24
E13		1.000	1.000	0.082	1.000	1.000	0.197
E15			1.000	1.000	1.000	1.000	1.000
E18				1.000	1.000	1.000	1.000
P0					0.019	0.250	1.000
P3						1.000	0.056
P13							0.511

Per2

WT	E13	E15	E18	P0	P3	P13	P24
E13		0.057	0.305	<0.001	0.082	0.656	0.005
E15			0.746	0.015	0.997	0.646	0.611
E18				0.002	0.691	0.756	0.227
P0					0.027	<0.001	0.347
P3						0.675	0.613
P13							0.098

Per3

WT	E13	E15	E18	P0	P3	P13	P24
E13		0.750	0.921	0.182	0.001	<0.001	<0.001
E15			0.833	0.617	0.02	<0.001	<0.001
E18				0.337	0.003	<0.001	<0.001
P0					0.035	<0.001	<0.001
P3						<0.001	<0.001
P13							0.775

Cry1

WT	E13	E15	E18	P0	P3	P13	P24
E13		0.429	0.989	0.005	0.865	0.902	0.349
E15			0.356	0.160	0.739	0.152	0.006
E18				0.002	0.936	0.933	0.296
P0					0.012	<0.001	<0.001
P3						0.772	0.169
P13							0.714

Cry2

WT	E13	E15	E18	P0	P3	P13	P24
E13		0.046	0.966	0.213	0.211	<0.001	<0.001
E15			0.063	0.897	0.917	0.007	0.006
E18				0.274	0.261	<0.001	<0.001
P0					0.992	0.001	0.001
P3						0.003	0.003
P13							0.952

Nr1d1

WT	E13	E15	E18	P0	P3	P13	P24
E13		1.000	1.000	0.621	0.233	0.038	0.006
E15			1.000	1.000	1.000	0.229	0.04
E18				1.000	1.000	0.782	0.175
P0					1.000	1.000	1.000
P3						1.000	1.000
P13							1.000

Rorb

WT	E13	E15	E18	P0	P3	P13	P24
E13		<0.001	<0.001	<0.001	<0.001	0.099	0.175
E15			0.060	0.133	<0.001	0.052	<0.001
E18				0.359	<0.001	<0.001	<0.001
P0					<0.001	0.002	<0.001
P3						<0.001	<0.001
P13							0.002

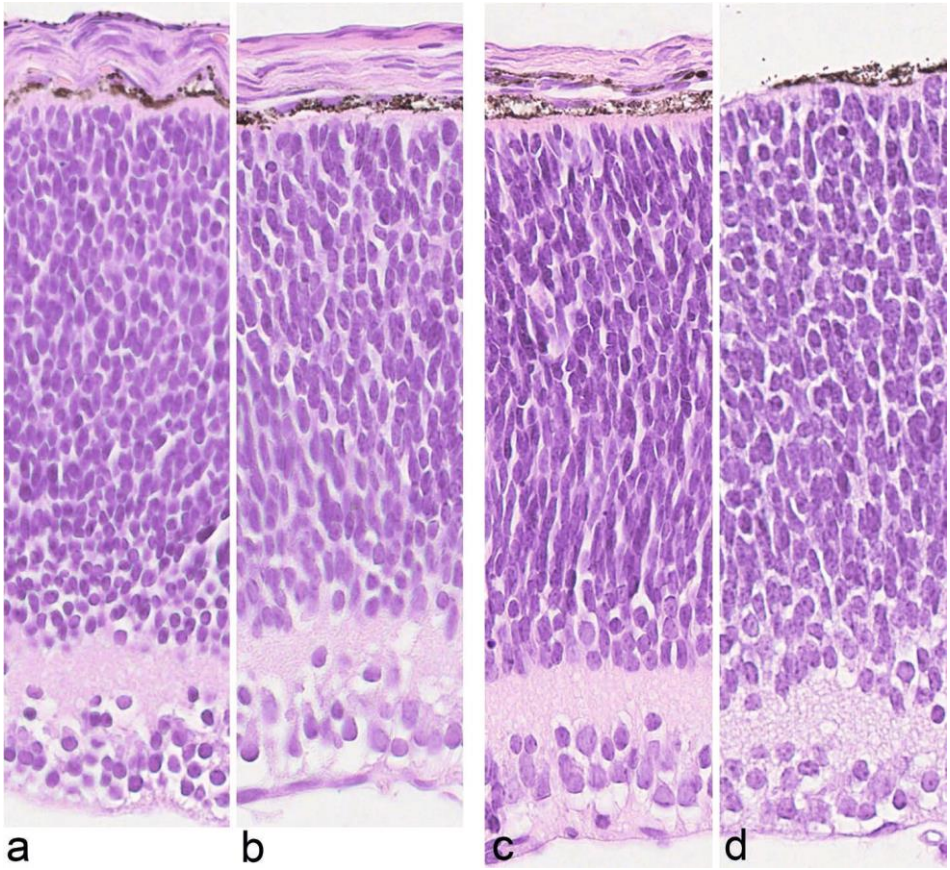


Figure S1-Photomicrographs of retinal sections from WT (a, c) and mutant (b, d) eyes at P0 (a, b) and P3 (c, d) showing no major structural differences between genotypes at these developmental ages.

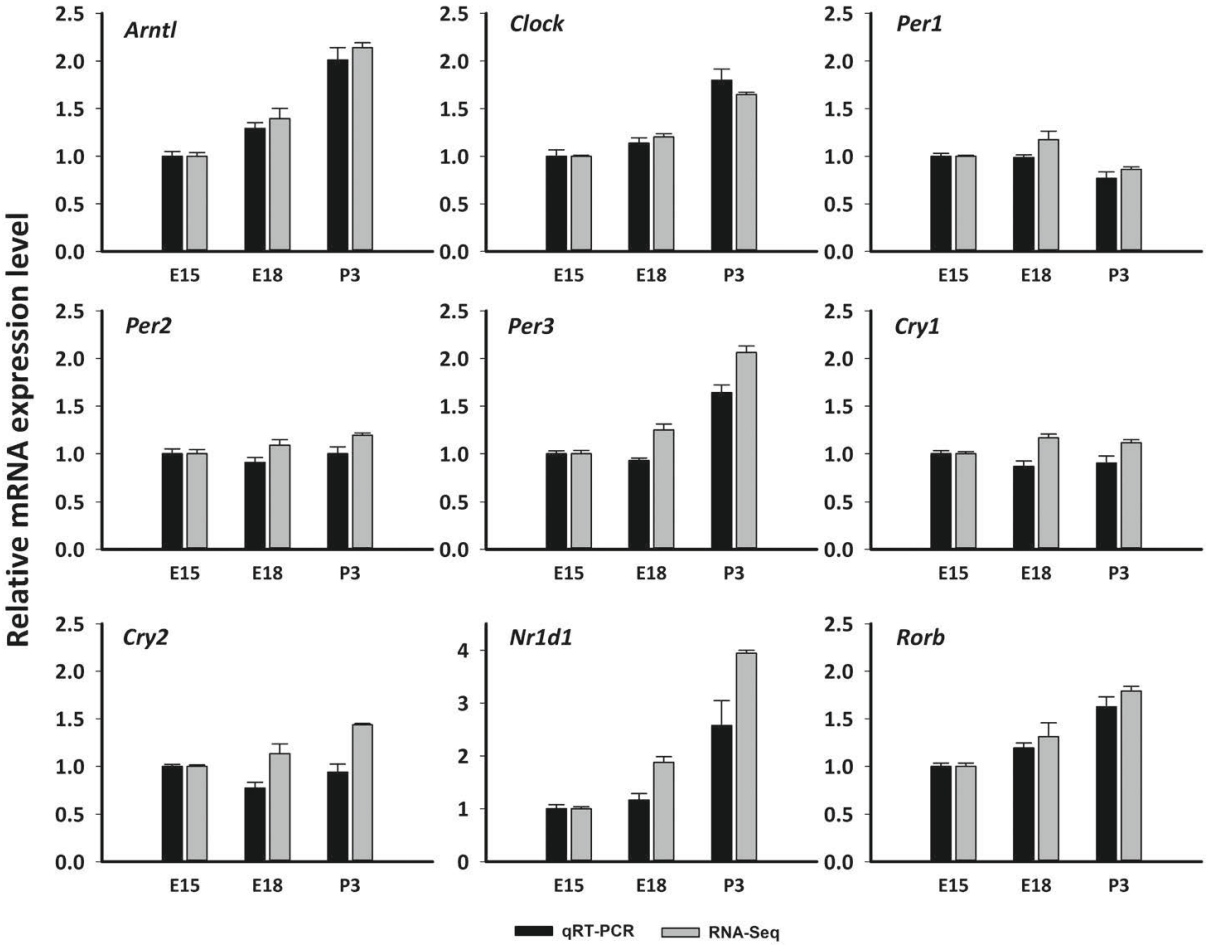
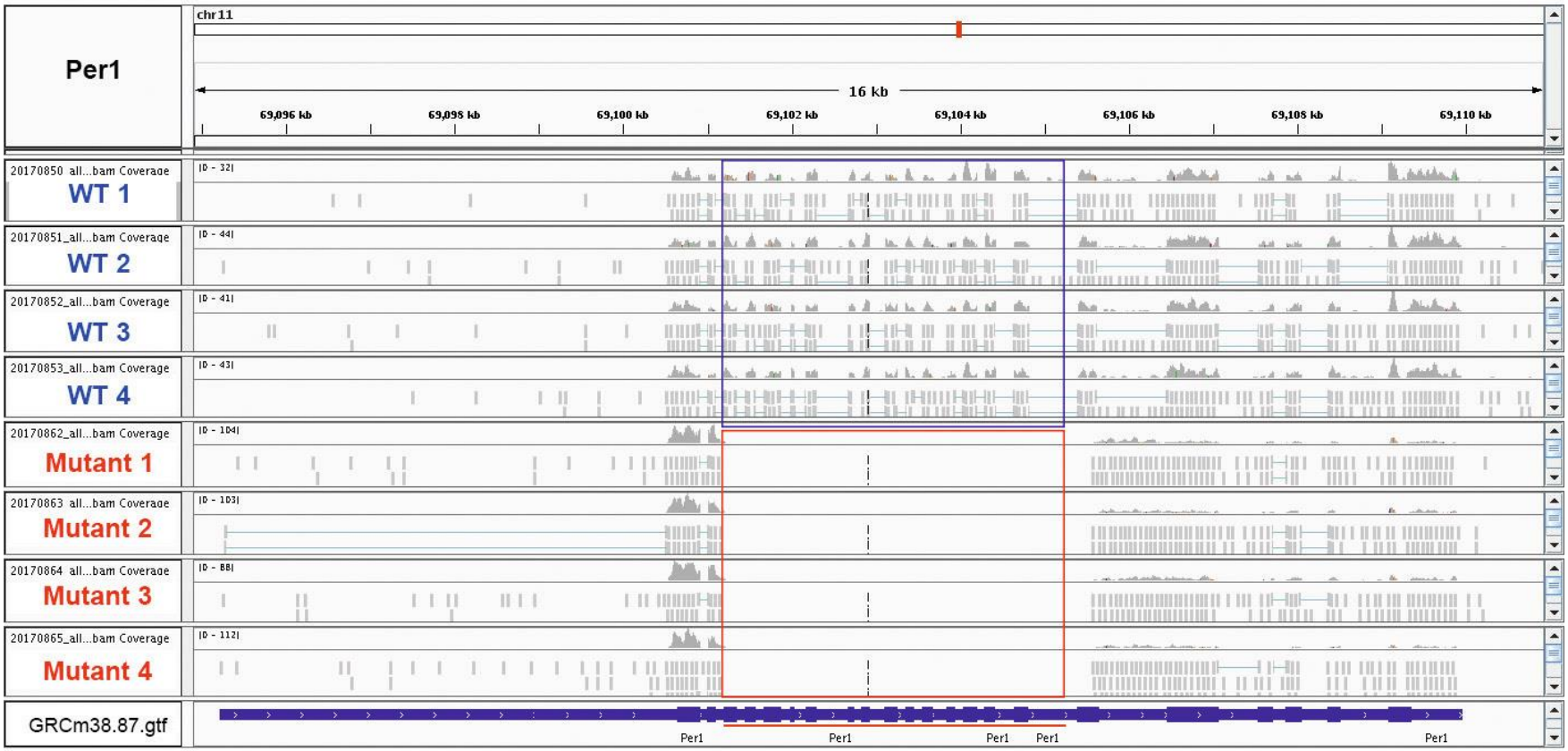


Figure S2-Comparative mRNA expression profiles of circadian clock genes in the WT whole eyes across eye developmental time points (n=3-4/age) of qRT-PCR versus RNA-seq experiments. Bar graphs show mean ±SEM mRNA expression levels of *Arntl* (*Bmal1*), *Clock*, *Per1, Per2*, *Per3*, *Cry1*, *Cry2*, *Nr1d1* and *Rorb* in the whole eyes relative to E15. The gene expression levels of all the clock genes display an identical trend of increase or decrease between the qRT-PCR and RNA-seq data.

a


Per1
chr11
16 kb
69,096 kb
69,098 kb
69,100 kb
69,102 kb
69,104 kb
69,106 kb
69,108 kb
69,110 kb
20170850_all...bam Coverage
WT 1
20170851_all...bam Coverage
WT 2
20170852_all...bam Coverage
WT 3
20170853_all...bam Coverage
WT 4
20170862_all...bam Coverage
Mutant 1
20170863_all...bam Coverage
Mutant 2
20170864_all...bam Coverage
Mutant 3
20170865_all...bam Coverage
Mutant 4
GRCm38.87.gtf
Per1
Per1
Per1
Per1
Per1

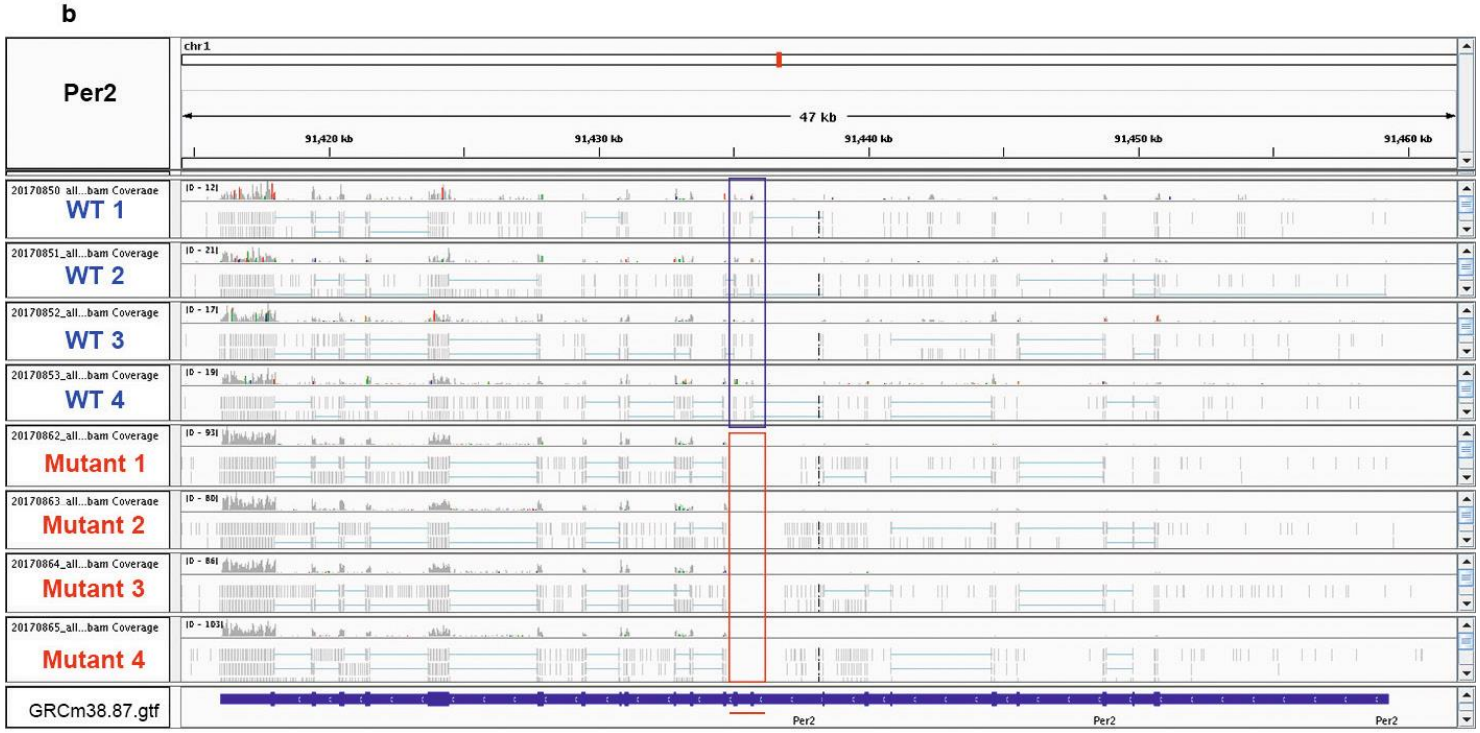


Figure S3-IGV images depicting RNA sequencing reads as obtained for wild type (WT) and mutant mice for both the (a) Per1 and (b) *Per2* gene at developmental timepoint E15. *Per* sample tracks with the read coverage (top) and the first few RNA Seq reads are shown (bottom). Each rectangle (grey by default) denotes an aligned read. The bottom track shows the gene model as present in the GRCm38 annotation (v87). Each blue rectangle represents an exon and blue connecting lines denote introns. The horizontal red line shows the exons deleted in the mutant transcripts as described by Zheng et. al. [32,33]. Blue and red boxes highlight these regions for the wild type and mutant samples, respectively.

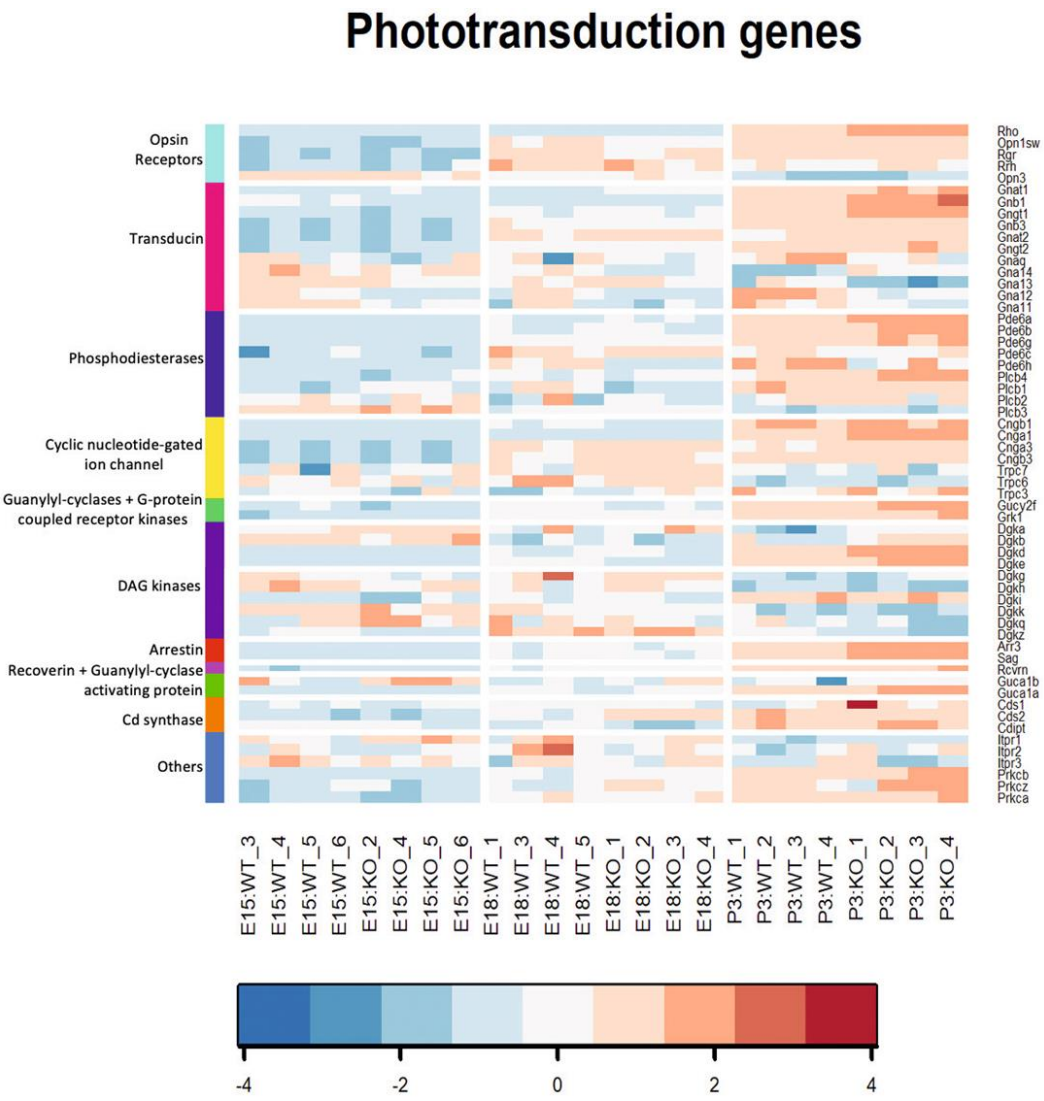


Figure S4-Heatmap of phototransduction genes during embryonic (E15 and E18) and postnatal (P3) ages of *Per1*$^{-/-}$*Per2*^{Brdm1} mutants versus WT. Genes were clustered using Euclidean distance and complete linkage. Color-coding corresponds to z-score of logCPM values from blue (lowest expression) to red (highest expression). The genes involved in phototransduction are grouped in several categories-opsin receptors, transducin, phosphodiesterases, cyclic nucleotide-gated ion channel, Guanylyl cyclases + G-protein coupled receptor kinases, DAG kinases, arrestin, Recoverin + Guanylyl-cyclase activating protein, Cd synthase, others.

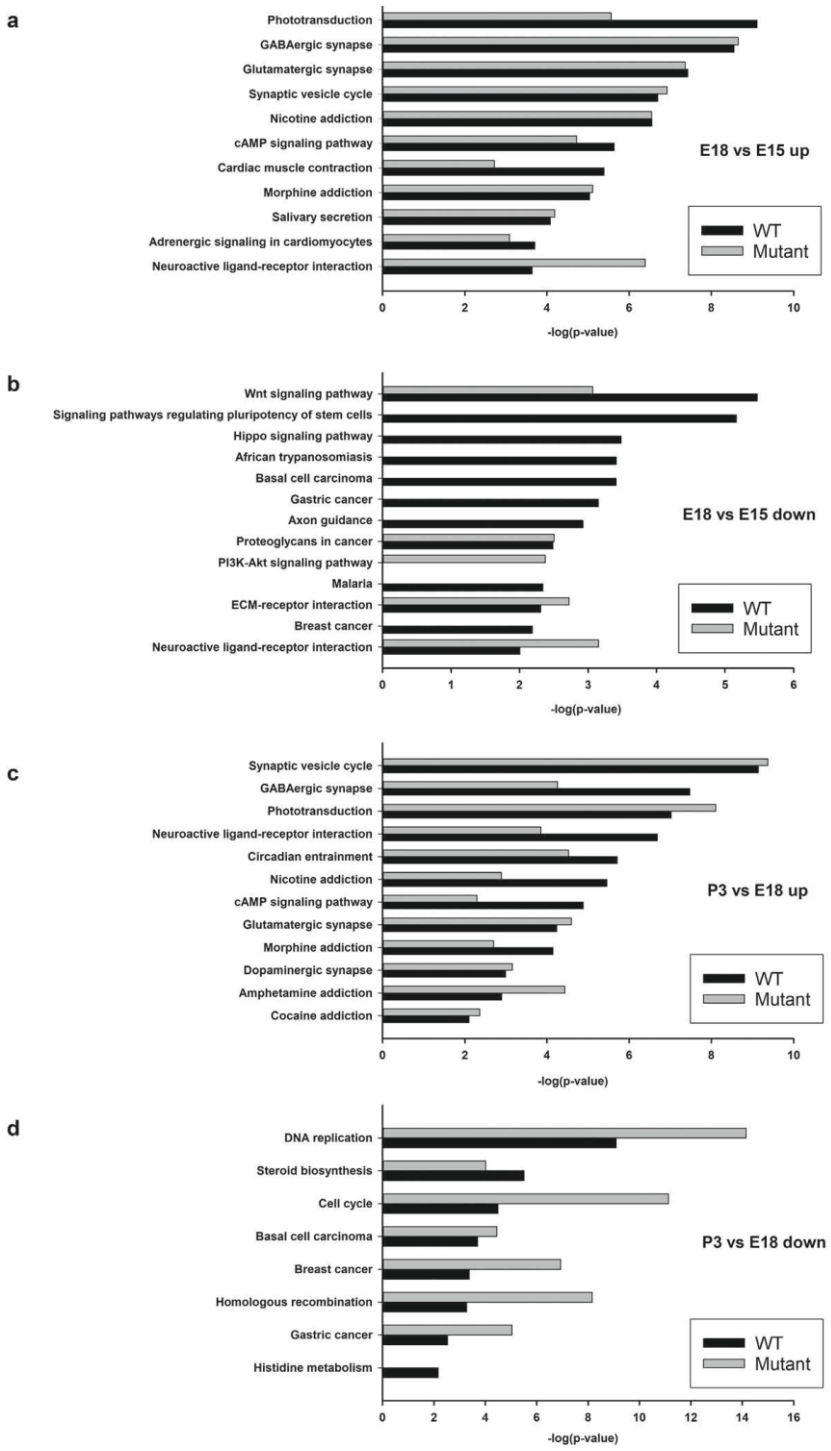


Figure S5-Canonical pathway analysis of DEG in WT and *Per1*$^{-/-}$*Per2*^{Brdm1} eyes between E15 and E18 (a and b for DEG which are respectively up or down regulated) and between E18 and P3 (c and d for DEG which are respectively up or down regulated). Bars (black for WT, grey for mutant) represent the -log10 adjusted p-value (x-axis).

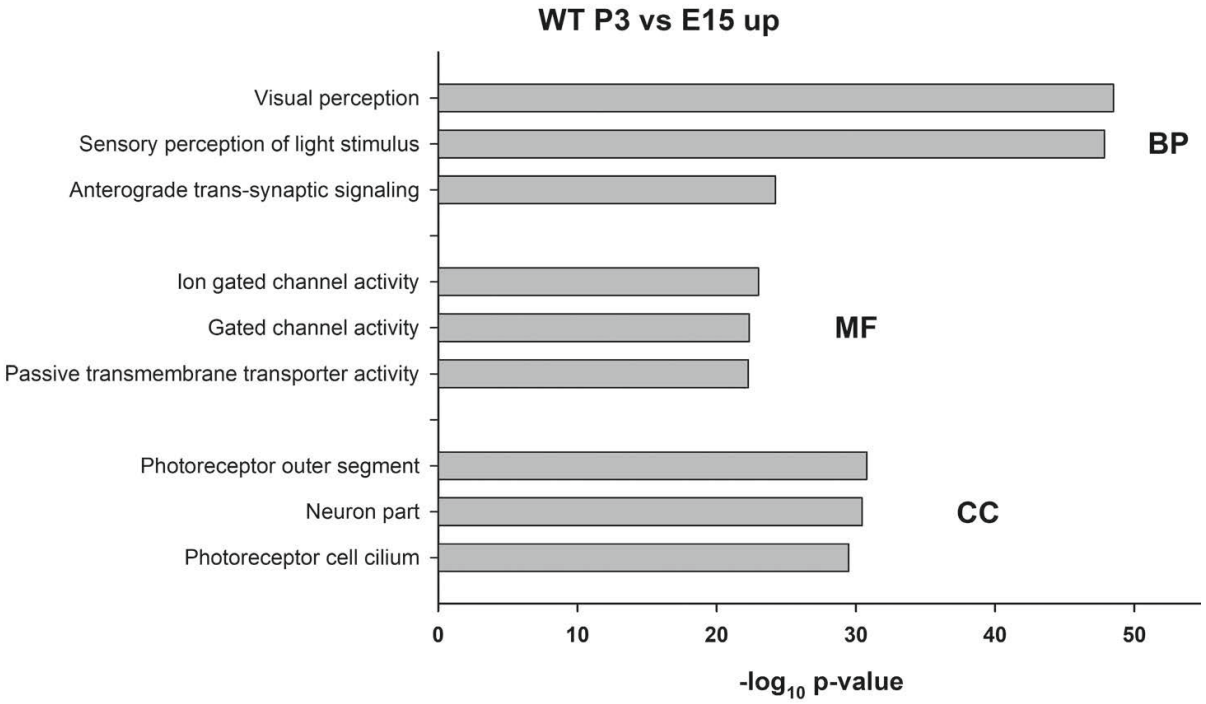


Figure S6-Canonical pathway analysis of upregulated DEGs between E15 and P3 in WT. Bars represent the -log10 adjusted p-value (x-axis). BP stands for Biological Process, MF for Molecular Function and CC for Cellular Component.

Chapter 3

Per1 and *Per2* circadian clock genes regulate daily physiological rhythms in the retina but their loss does not promote retinal degeneration upon aging

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In preparation

Per1 and _Per2_ circadian clock genes regulate daily physiological rhythms in the retina but their loss does not promote retinal degeneration upon aging

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Abstract

The mammalian retina has a localized network of clocks almost in every retinal cell-type. It is well established that plethora of rhythmic processes in the retina are regulated by the circadian clock, yet links between clock genes and retinal cell-specific clocks on visual perception is limited. Little is known regarding circadian control of retinal light responses in the *Per1*$^{-/-}$*Per2*^{Brdm1} species. Here, in the current study, we have addressed this deficit using electroretinogram (ERG) recordings and through morphometric assessment of retinal histology in *Per1*$^{-/-}$*Per2*^{Brdm1} mice versus the wild-type retina to evaluate daily rhythmicity under different light conditions and to identify clock impairment effects on the long-term survival of the retina. We observed that inactivation of *Per1* and *Per2* genes impairs the known daily modulation of visual responses as exemplified by the amplitudes of a- and b-waves in scotopic ERG conditions. In the *Per1/Per2* mutant, the ERG light-responses remained constitutively high (night-like) during 24h. Surprisingly, upon aging, we detected no marked decrease in visual sensitivity in the *Per1/Per2* mutants, despite minor histological changes. Taken together, these data suggest that the circadian clock is essential for general retinal health.

Keywords: *Per1*, *Per2*, Circadian rhythms, Retina, Electroretinogram

Introduction

Various biochemical and cellular processes occurring in the mammalian retina daily allow the adaptability of visual functions to the light/dark (LD) cycle (reviewed in McMahon et al., 2014; Felder-Schmittbuhl et al., 2018). Several studies have provided evidence that rhythmic processes in the eye include visual sensitivity (Bassi and Powers, 1986) and mRNA expression of phototransduction-related genes and photopigments in the photoreceptors (Brann and Cohen, 1987; von Schantz et al., 1999; Bobu et al., 2013; Kunst et al., 2013). In addition, it is also known that an independent circadian clock is localized within the retina and controls 24h rhythmic functions (Terman et al., 1993; Tosini and Menaker, 1996). These autonomously functioning circadian clocks are present in all the 3 key retinal layers - outer nuclear layer, inner nuclear layer, and ganglion cell layer (Tosini et al., 2007; Ruan et al., 2008; Dkhissi-Benyahya et al., 2013; Jaeger et al., 2015). So far, all investigated retinal cell types are known to express clock genes (Gustincich et al., 2004; Ruan et al., 2006; Liu et al., 2012).

Circadian rhythms in retinal function have been described in several species using ERGs, but this approach has been applied in only few clock gene knockout rodent models. Recently, in the rod specific *Bmal1* knockout mice, amplitudes of dark-adapted ERG a- and b-waves were observed to be constitutively lower (day-like) during the subjective night as compared to higher amplitudes in the control mice (Gegnaw et al., 2021). Similarly, in *Cry1*$^{-/-}$ and *Cry2*$^{-/-}$ deficient mice severely attenuated rhythms are observed in the photopic ERG (Wong et al., 2018). Moreover, these rhythms were totally blunted in the double *Cry1/Cry2* KO (Cameron et al., 2008).

The functional endpoints of circadian organization in the *Per1*$^{-/-}$*Per2*^{Brdm1} mutant have not yet been properly elucidated, although prior experiments in the same mouse line resulted in no significant differences in scotopic and photopic ERG recordings (Ait-Hmyed et al., 2013). We decided to continue exploring the consequences of circadian clock disruption in retinal rhythmicity as *Per1* and *Per2* are involved in the molecular clockwork with Bmal1, on the opposite regulatory arm, and exert contrary effects right from development to aging. For instance, in precursor proliferation (respectively, decrease vs increase) (Sawant et al., 2019; Bagchi et al., 2020); characterized by number of developmental eye defects such as global

reduction in mRNA expression of S- and M-opsin expressing cells and delayed rod IS and OS differentiation (Ait-Hmyed et al., 2013) including perturbation of cell cycle genes (Bagchi et al., 2020). Also, *Per1*$^{-/-}$*Per2*^{Brdm1} mutant mice are arrhythmic, have global clock defects (Zheng et al., 2001) and lack a daily peak in photoreceptor outer segment (POS) phagocytosis under constant darkness (Milićević et al., 2021). *Per2* mutant mice (*Per2m/m*) retinas have specifically demonstrated decrease in ERG amplitude under scotopic light coupled with increased CTGF (connective tissue growth factor) expression in retinal ganglion cells, with respect to WT littermates (Jadhav et al., 2016).

Retinal clocks are implicated in the functional performance and structural integrity of the retinal tissue. Here, we observed that invalidation of *Per1* and *Per2* impairs the daily modulation in visual responses. The visual sensitivity appears diminished in the *Per1*$^{-/-}$ *Per2*^{Brdm1} mutants but does not undergo a further decline upon aging, in line with unaltered retinal histology. This data throws some light on the link between retinal cell autonomy and the circadian visual processing in mammals.

Materials and methods

Animals

All animal experiments were authorized by the French Ministry for Higher Education, Research, and Innovation (APA- FIS#10213-2017060920001367-v3) and performed in strict accordance with the European Parliament and Council Directive (2010/63/EU). Homozygote double mutant mice on a mixed background (C57BL/6J x 129 SvEvBrd) (as a generous gift from Dr U. Albrecht, Univ. Fribourg, Switzerland) carrying the loss-of-function mutation of *mPer1* gene (*Per1*^{Brdm1}; (Zheng et al., 1999)) and mutation of the *mPer2* gene (*Per2*^{Brdm1}; (Zheng et al., 2001); later defined as *Per1*$^{-/-}$*Per2*^{Brdm1}) were used in the study. The absence of the rd8 mutation was previously validated. For the experiments, up to five generations of mutant and WT mice were bred in the Chronobiotron animal facility (UMS 3415) on a 12 h/12h LD cycle (ZT0 (Zeitgeber time 0) – 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, maintained exactly as described in (Albrecht et al., 2001) with ad libitum access to standard chow and water. Control and mutant mice were age-matched, and male and female mice were used in the study.

Genotyping

Mice were genotyped by polymerase chain reaction (PCR) amplification of tail DNA separately for the WT and KO alleles. The primer sequences were as follows: *Per1* KO fwd: 5'-ACAAACTCACAGAGCCCATCC-3' and *Per1* KO rev: 5'-ACTTCCATTTGTCACGTCCTGCAC-3'; Per2 mutant fwd: 5'-TTT GTTCTGTGAGCTCCTGAACGC-3' and *Per2* mutant rev: 5'-ACTTCCAT TTGTCACGTCCTGCAC-3'; (Zheng et al., 1999; Zheng et al., 2001); *Per1* WT fwd: 5'-GTCTTGGTCTCA TTCTAGGACACC-3' and *Per1* WT rev: 5'-AACATGAGAGCTTCCAGTC CTCTC-3'; *Per2* WT fwd: 5'-AGTAGGTCGTCTTCTTTATGCCCC-3' and *Per2* WT rev: 5'-CTCTGCTTTCAACTCCTGTGTCTG-3'. PCR conditions comprised of 35 cycles of 30 sec at 94°C ,30 sec at 56°C for Per1 (WT and KO) or 58 °C for Per2 (WT and mutant), 1 min at 72 °C followed by a final step for 5 min at 72 °C.

Electroretinography

ERG recordings and analysis were performed on aged-matched (between 2 and 18 months according to experiments) *Per1*$^{-/-}$*Per2*^{Brdm1} mice and control WT animals, according to previously described procedures (Cameron et al., 2008; Tanimoto et al., 2009; Ait-Hmyed Hakkari et al., 2016). WT and mutant mice previously raised in LD cycle were transferred to darkness starting at ZT12 and then reexposed or not to light and tested the following day according to protocole depicted on Figure 1. 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/m^2.

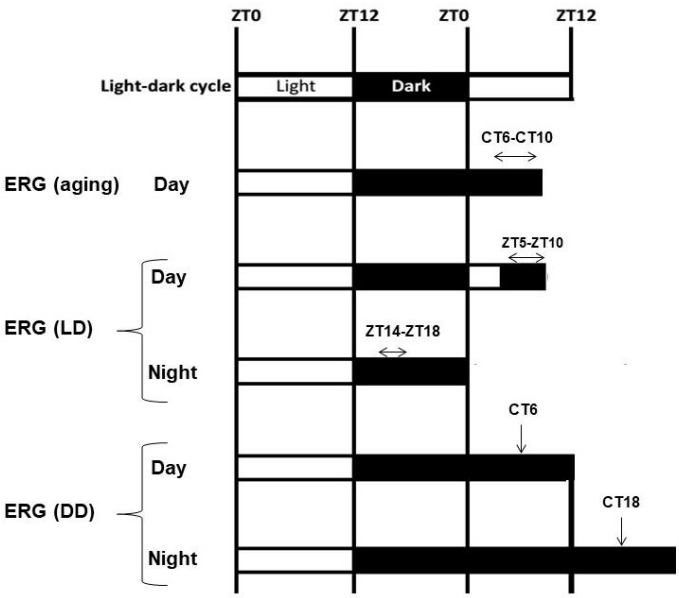

Figure 1. Protocol of light exposure during animal housing (upper line) and the distinct ERG protocols. Effect of aging (8-9, 13-14 and 18 months) was evaluated in the afternoon following overnight dark-adaptation (n = 3 to 6 per genotype). Effect of time of day in LD was analyzed during the night (ZT 14-18) after at least 2h dark-adaptation and during the day following a 3h light exposure and >2h dark adaptation (3-4 months-old mice, n = 5-6 per genotype and per time point). Circadian effect was evaluated under constant darkness (DD) at Circadian time (CT) 6 (middle of subjective day) and CT18 (middle of subjective night) (2-4 months-old mice, n = 6-7 per genotype and per time point).

Immunohistochemistry

Immunohistochemical staining was performed on 10 µm eye sections (n = 3-5/genotype, 5-, 9- and 18-months old mice, eyes collected during daytime) as previously described (Ait-Hmyed Hakkari et al., 2016). Incubation with primary antibodies was as follows: anti-PKCα (1/500); anti-GFAP (1/500). Following incubation with anti-rabbit goat IgG-Alexa 488 secondary antibody (1/500), cell nuclei were stained with 4,6-di-amino-phenylindol-amine (DAPI; Molecular Probes). Slides were mounted in PBS/glycerol (1:1) and observed using a Nikon Optiphot 2 fluorescence microscope (Nikon, Melville, NY, USA) equipped with differential interference contrast optics. Thickness of the ONL and INL was measured on (4) DAPI stained sections collected at 500 µm of the optic nerve head (n= 3-5 per genotype and per time point).

Statistics

Results are presented as means ± SEM. Statistical analysis (Sigma Plot 13, Systat Software, San Jose, CA, USA) was performed with 2-way repeated measures ANOVA for ERG and 2-way

ANOVA for layer thickness evaluation. ANOVAs were followed by post hoc Holm-Sidak's multiple comparison. The significance level was set at $P < 0.05$.

Results

Per1$^{-/-}$*Per2*^{Brdm1} mutants do not show any day-night difference in scotopic ERG

To investigate whether light response of rods was rhythmic in *Per1*$^{-/-}$*Per2*^{Brdm1} mutant mice under LD conditions, scotopic ERG was recorded in dark-adapted animals at the middle of the day (ZT5-10) and of the night (ZT14-18). At low stimulus intensities (–3.5 to -1 log cd.s/m^2), this ERG reflects the activity of rod pathways, while both rod and cone pathways contribute to responses at higher irradiances characterised by mesopic vision. In the WT control animals, the ERG was characterized by large a- and b-wave amplitudes during the night as compared to the smaller amplitudes measured during the day (P = 0.013 a-wave; P = 0.010 b-wave) (Fig. 2A and 2B). Amplitudes of b-waves were higher at night mostly throughout the intensity range tested. The day/night difference in a-wave amplitudes was mainly significant at intensity ranges where these could be reliably measured (> –1 log cd.s/m^2). By contrast, there was no significant difference in a- and b-wave amplitudes between day and night in the *Per1*$^{-/-}$ *Per2*^{Brdm1} mutants (P = 0.220 a-wave; P = 0.161 b-wave) (Fig. 2C and 2D). Day and night amplitudes in the latter showed intermediate values between day and night amplitudes of WT animals and were not distinct from WT values in the day (P = 0.095 a-wave; P = 0.272 b-wave) and in the night (P = 0.237 a-wave; P = 0.216 b-wave).

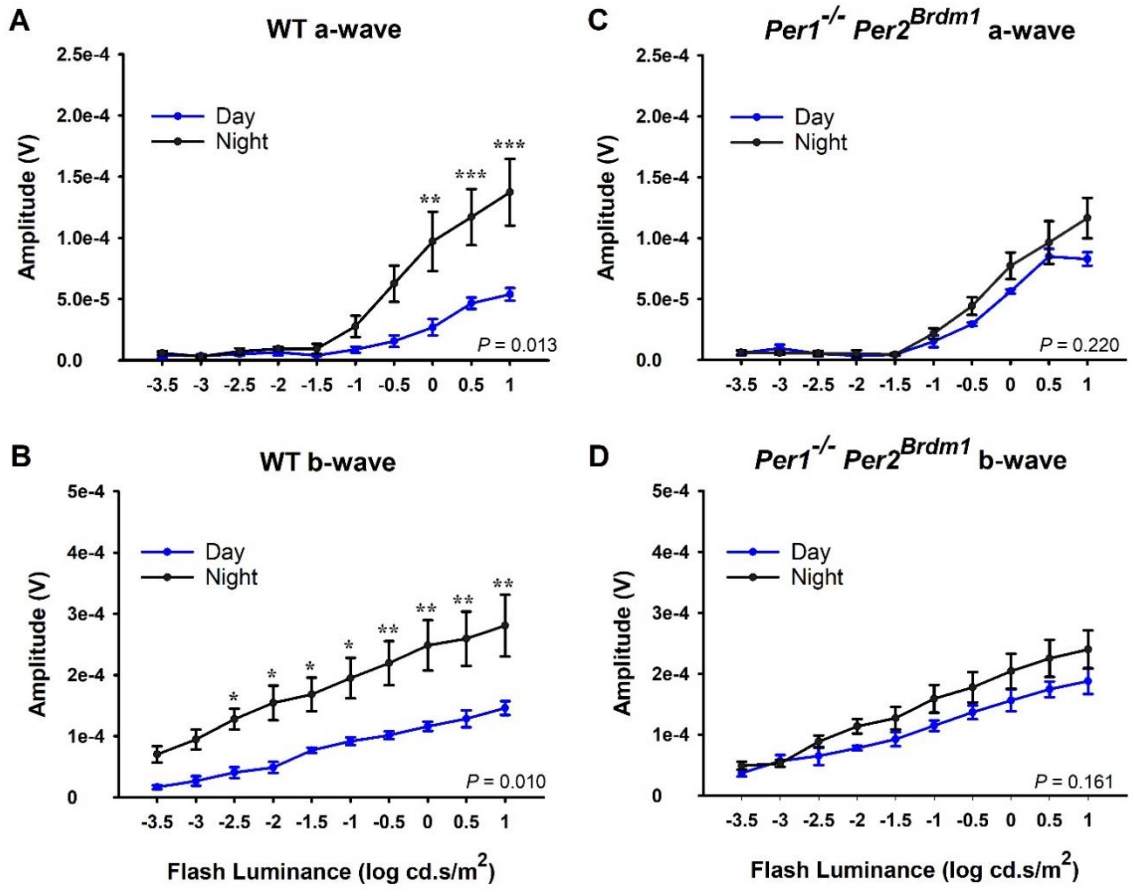

Figure 2. Scotopic ERGs in WT and *Per1*$^{-/-}$*Per2*^{Brdm1} dark-adapted mice in day and night under LD condition. In control mice, the scotopic a- and b-wave amplitudes significantly differed over the LD cycle, with values larger during the night than at day (Fig.2A, B). These day/night differences were reduced and not significant in *Per1*$^{-/-}$*Per2*^{Brdm1} mutant mice (Fig.2C, D). n = 5 per genotype in the day, n = 6 per genotype in the night. *P<0.05, **P<0.01, ***P<0.001, repeated measures 2 way ANOVA (P values for post hoc analysis are indicated on the graphs).

To evaluate whether this property of scotopic light response in dark-adapted WT animals is controlled by the circadian clock we performed a similar study with animals maintained in constant darkness (DD). Significant differences were observed when a- and b-wave amplitudes were determined at subjective day (CT6, 18h following day/night transition) and subjective night (CT18, 30h following day/night transition). Indeed, amplitudes were significantly larger during the night in control animals (P = 0.035 a-wave; P = 0.003 b-wave) (Fig. 3A and 3B). The importance of circadian clock dependence in retinal pathways was further revealed by the fact that the magnitude of the difference between scotopic responses at subjective mid-day and mid-night appeared completely blunted in the *Per1*$^{-/-}$*Per2*^{Brdm1} mutants (P = 0.805 a-wave; P = 0.975 b-wave) (Fig. 3C and 3D).

These results in WT animals in DD conditions are similar what we observed in mice carrying a unique *Bmal1* allele (Gegnaw et al., 2021), with significant increase in light response in the dark-adapted ERG, specifically during the subjective night. They confirm that light sensitivity appears higher in the night condition, as might be expected from a nocturnal animal. By contrast, and unlike what was previously reported, we did not manage to detect a day/night difference in photopic ERG amplitudes recorded on light-adapted animals (data not shown).

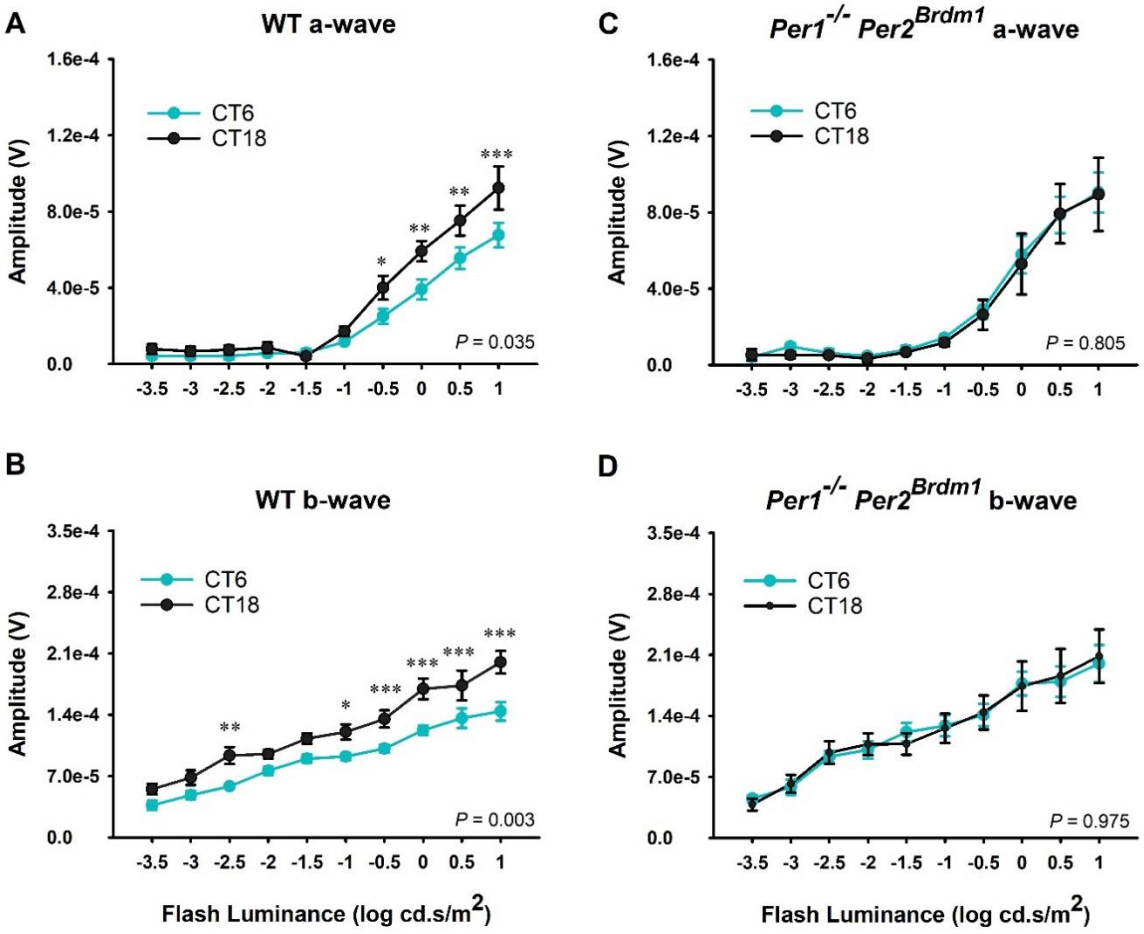

Figure 3. Scotopic ERG a- and b-wave amplitudes are controlled by the circadian clock. The WT mice exhibit significantly larger scotopic a- and b-wave amplitudes at CT18 (black) compared to CT6 (blue). The curves for both a- and b-wave amplitudes showed time of day dependence (specifically at higher luminance for a-wave and both at lower and higher light intensities for b-wave). All data points show mean ± SEM; n = 6/genotype, CT6, and n = 6-7/genotype, CT18. *P<0.05, **P<0.01, ***P<0.001, Repeated measures Two-way ANOVA.

Per1$^{-/-}$*Per2*^{Brdm1} mutants do not show overt age-dependent decline in vision

We next assessed whether aging would affect visual function more specifically in the mutant mice, as reported in the retina specific *Bmal1* knock out (Baba et al., 2018). We first performed scotopic electroretinography during day-time in dark-adapted WT and mutant animals at different ages (8-9, 13-14 and 18 months). At none of these ages did *Per1*$^{-/-}$ *Per2*^{Brdm1} mutants display any significantly different scotopic responses with respect to controls. Noteworthily at 18 months, a- and b-wave amplitudes showed a tendency for higher values in mutants, specifically at largest flash intensities (Fig. 4A, B).

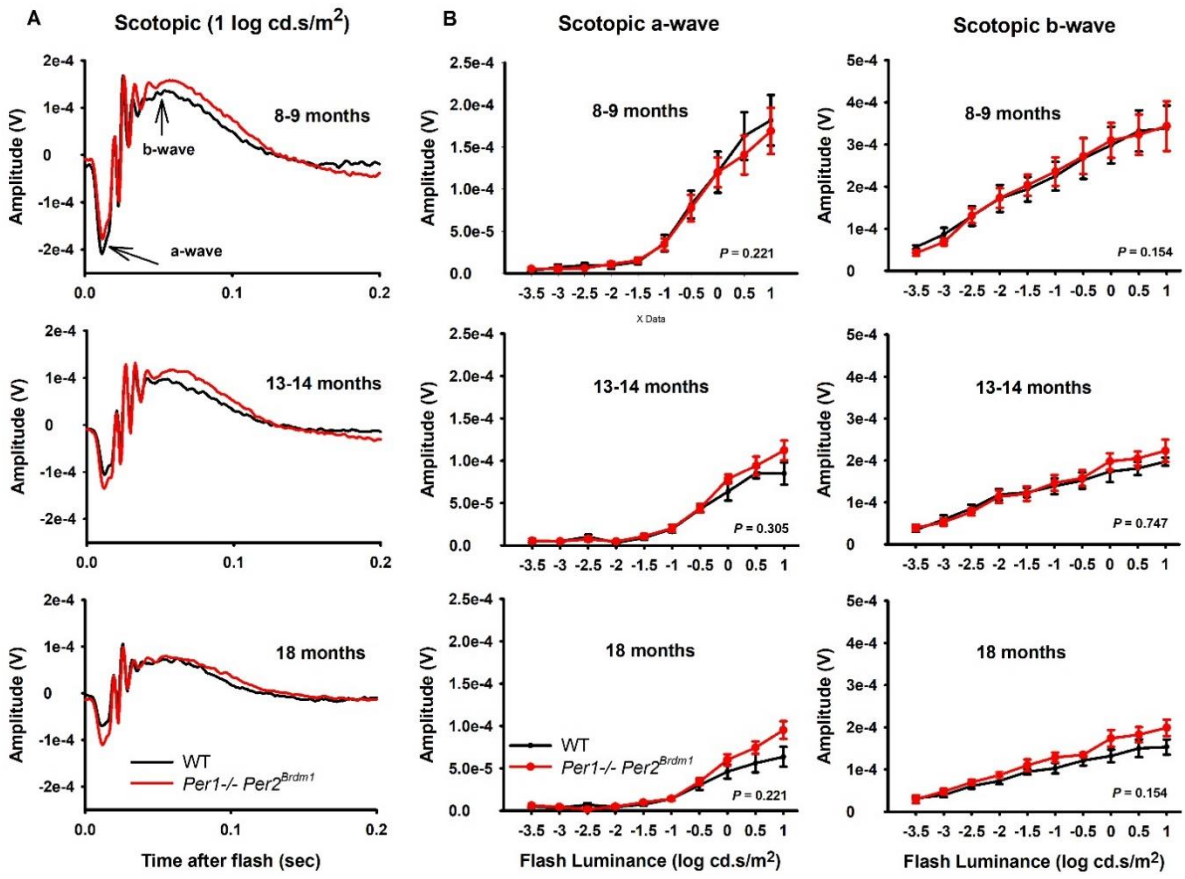

Figure 4. No major effect of aging in *Per1*$^{-/-}$*Per2*^{Brdm1} mutants in scotopic ERG responses. (A) Scotopic ERG average waveforms at 1 log cd·s/m^2 flash. These average waveforms were obtained from *Per1*$^{-/-}$*Per2*^{Brdm1} mutant mice (red lines, n= 3-6 per age) and WT (black lines, n= 5-6 per age) of different ages (8-9, 13-14 and 18 months). (B) The amplitudes of scotopic a- and b-waves in WT and *Per1*$^{-/-}$*Per2*^{Brdm1} mutant mice did not show any significant difference throughout the age study. Repeated measures two-way ANOVA (a- and b-wave amplitudes).

At all these ages we then checked if there was a difference in light response in photopic ERG but in these conditions also, mice from both genotypes behaved likewise (supplementary Figure 1A-C). We finally checked whether there was an alteration in the response to blue light

in photopic conditions in the mutant, since the latter contained a reduced population of S-opsin expressing cones (Ait-Hmyed et al., 2013) but found no difference between genotype in light responses (supplementary Figure 1D).

Histological analysis in *Per1*$^{-/-}$*Per2*^{Brdm1} and WT retinas during aging

It was previously reported that the retinal invalidation of *Bmal1* induces altered morphology of rod bipolar cells, namely stunted dendritic processes of rod-bipolar cells in the outer plexiform layer. We evaluated whether invalidation of the *Per1* and *Per2* clock genes might trigger similar abnormalities by performing anti-PKCα staining of retinal sections prepared from 9 month-old animals. However, we did not manage to detect any alteration at the levels of dendrites in the outer plexiform layer (Figure 5A). Thickness of the outer and inner nuclear layers was analysed at 9 and 18 months. No difference was detected between WT and mutant retinas (2-way ANOVA, genotype effect: P = 0.076 ONL; P = 0.644 INL; Figure 5B). We also found no difference in cell densities in these layers (data not shown). Finally, to evaluate whether the *Per1* and *Per2* invalidation induced any stress or degenerative state in the retina, we performed anti-GFAP staining at 18 months but the data looked similar between genotypes (Figure 5C).

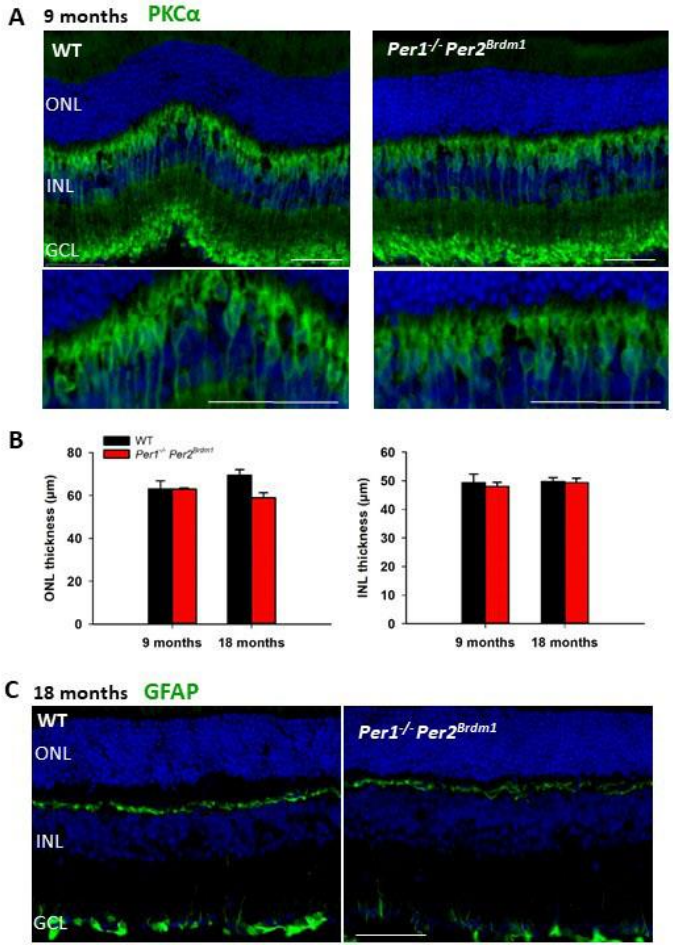

Figure 5. Immunostaining and morphological analysis of WT and mutant retinal sections. (A) Representative micrographs of retinal sections stained with anti-PKCα antibody and higher magnifications of the INL region showing the detail of dendritic processes from rod-bipolar cells in the outer plexiform layers. Sections were collected from 9 months old WT and *Per1*$^{-/-}$*Per2*^{Brdm1} mutant mice (n = 2 per genotype). (B) Morphometric quantitative analysis of retinal layer thickness in 9-10 and 18 months animals from WT (black bars) and *Per1*$^{-/-}$*Per2*^{Brdm1} mutant (red bars) mice showed no effect of genotype on the ONL (P = 0.076) and the INL (P = 0.644) (n = 4-5/genotype). Nuclear layers were stained with DAPI (blue). (C) Representative micrographs of 18 months retinal sections stained with anti-GFAP showing no sign of retinal stress or disease (n = 3 per genotype). Scale bars 50 µm.

Discussion

The present study shows that scotopic light response in dark-adapted WT animals is higher in the night than in the day, a property likely regulated by a circadian clock since this effect is retained in constant dark condition and totally absent in the *Per1*$^{-/-}$*Per2*^{Brdm1} mutant mice. We further show that, unlike mice carrying a retina-specific knock out of Bmal1 clock gene, the invalidation of *Per1* and *Per2* does not induce any accelerated aging of the retina, based on both electroretinography, immunohistochemistry and morphological analysis. These data highlight distinctive properties of the visual system either strictly dependent or not on the integrity of *Per1* and *Per2* genes.

Circadian tuning of retinal rod light responses has been barely documented; however, cone responses were evaluated in several studies (Barnard et al., 2006; Storch et al., 2007; Cameron et al., 2008; Wong et al., 2018). Time of day has been suggested to regulate light sensitivity at very low light intensities in the rats (Walker and Olton, 1979; Sandberg et al., 1986). Recent investigations have relayed disagreement over this daytime effect using scotopic ERG (Cameron et al., 2008; Sengupta et al., 2011; Di et al., 2019; Gegnaw et al., 2021). Our present data with WT mice on a mixed C57BL/6J x 129 SvEvBrd background corroborate our recent findings that there is an increase in scotopic ERG during the subjective night in C57BL/6J (Gegnaw et al., 2021), providing convincing evidence that this property mainly of rods is circadian clock regulated.

The loss of day/night differences in the *Per1*$^{-/-}$*Per2*^{Brdm1} mutant further confirm this regulation. Noteworthily, the day and night light-response in the mutant in the present study appears constitutively high (identical to the response of WT mice in the subjective night), whereas it was the opposite (constitutively low) in mice carrying a rod-specific knock out of Bmal1 (Gegnaw et al., 2021). This observation might be related to the fact that the clock factors PER1 and PER2, on one hand, and BMAL1 on the other, act on the two opposite arms of the clock molecular machinery. We previously characterized the transcriptome of WT and *Per1*$^{-/-}$ *Per2*^{Brdm1} laser-microdissected photoreceptors at CT1, CT10, CT19 and CT22. Considering that the rod light response is abnormally high in the subjective day time, we selected genes that are upregulated between CT10 and CT19 in the WT and that are turned down in the mutant vs WT at CT10. The 249 genes of this intersection display strong enrichment in GO-Terms

related to neurotransmission (data not shown), indicating that a down-regulation of synaptic function might underlie the decrease in scotopic light response.

It needs to be underlined that light responses under LD condition show a higher amplitude of day/night difference. This might be due to a direct effect of light or dark on photoreceptor response. Alternatively, it might be that the LD cycle acts on the clock amplitude, that might in turn generate increased day/night changes in gene expression as shown previously (Storch et al., 2007).

Unlike potent effect on the processing of light, the invalidation of *Per1* and *Per2* clock genes does not appear to induce any harm to the retina and vision. This stands in contrast to what was reported regarding the retina-specific Bmal1 knock out that exhibits both altered ERG responses, reduced photoreceptor layers and abnormal bipolar cell dendrites upon aging (Baba et al., 2018). One explanation would be that the clock does not play such a crucial role in the survival capacity of the retina over long term on the mixed background used in our study. Recent data with specific knock out of Bmal1 in the retinal pigmented epithelium somehow supports this hypothesis. Indeed, even if these animals have lost their daily rhythm in photoreceptor outer segment phagocytosis, they do not present premature alteration of vision or retinal structure (DeVera et al., 2022). Alternatively, it might be that *Per1* and *Per2* genes are functionally replaced here by *Per3*, as suggested earlier. Indeed, in DD condition only *Per1* and *Per2* mRNAs showed sustained rhythms in whole retinas whereas *Per3* mRNA was rhythmic in the double mutant (Milićević et al., 2021).

In conclusion, our study suggests light processing at the level of photoreceptors under scotopic and mesopic conditions in mice is mediated by circadian clockwork. It supports the view that the visual system requires rods for optimal performance, at least in nocturnal species. Our results can draw further investigation into the regulation and function of rod-specific clock genes in retinal physiology.

Author contributions

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

Declaration of competing interest

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

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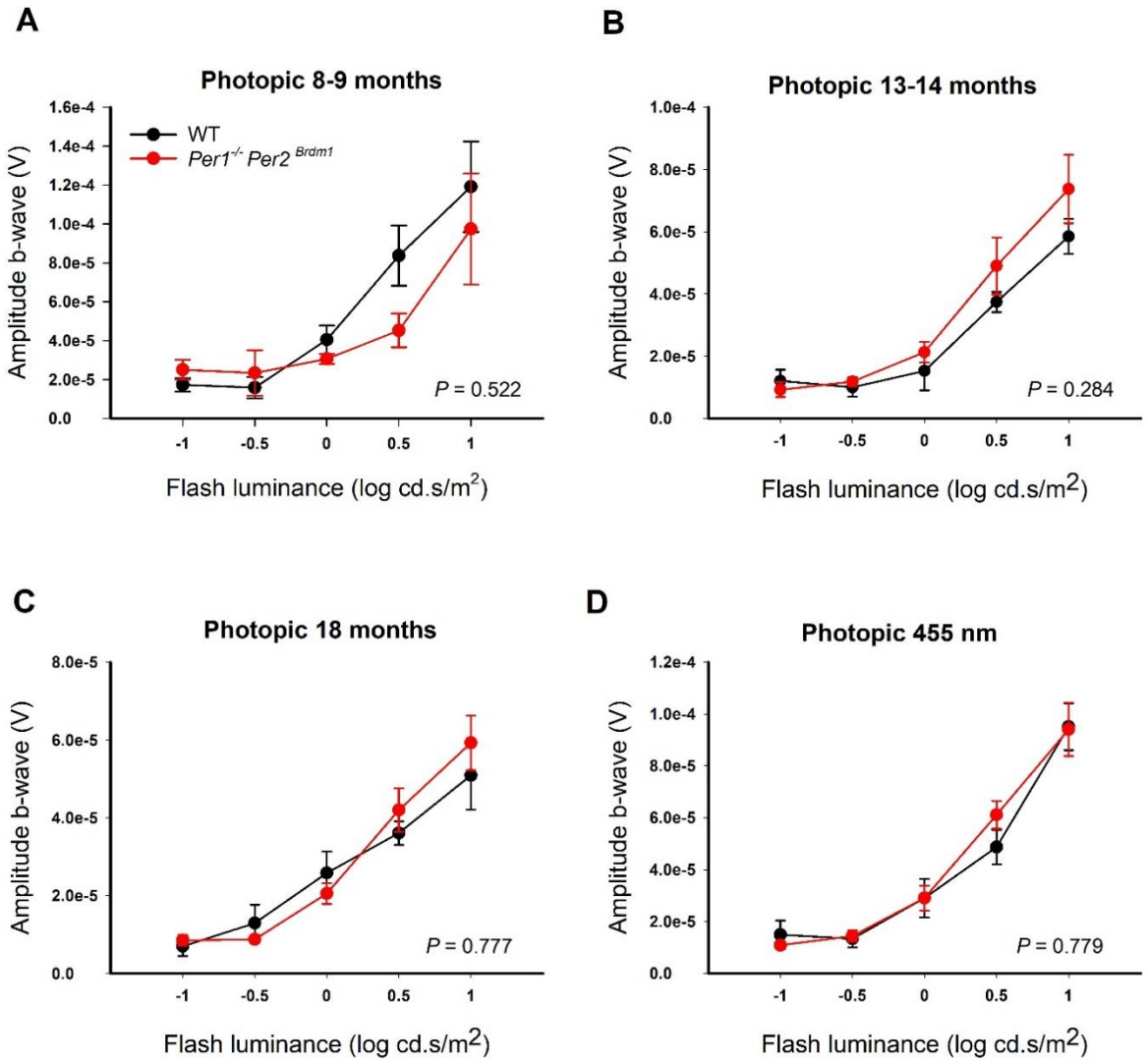
Supplementary result



Supplementary Figure 1: Photopic ERGs in WT and *Per1*$^{-/-}$*Per2*^{Brdm1} mice. (A-C) Photopic white light ERG recordings were performed in light adapted mice aged 8-9 (A), 13-14 (B) and 18 (C) months, at mid-day. No difference could be detected between genotypes (n = 3-6 per genotype, per age). (D) Photopic ERG recordings performed under 455 nm blue light on 2-3 months mice during daytime also did not show any difference between genotypes (n = 7 per genotype). Repeated measures 2-way ANOVA; P values for genotype effect indicated in the graphs.

Chapter 4

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

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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* knockout 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 McMahon 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 and Cohen, 1987; von Schantz et al., 1999; Sakamoto et al., 2006; Bobu et al., 2013; Kunst et al., 2013) and visual sensitivity (Bassi and 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 and 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., 2018a).

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., 2018a; Baba et al., 2018b; 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 mPer2Luc 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 µm 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., 2018a) (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 x 10^{-4}, 10^{-3}, 3 x 10^{-3}, 10^{-2}, 3 x 10^{-2}, 10^{-1}, 3 x 10^{-1}, 1, 3, and 10 cd.s/m^2.

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 mm^2 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*$^{fl/+}$ 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/m^2, P = 0.002) and higher irradiances (>–1.5 log cd.s/m^2, P = 0.019) (Fig. 1J). Importantly, at light intensities where the a-wave amplitude could be reliably measured (>–1 log cd.s/m^2) 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/m^2, 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/m^2, P = 0.570). By contrast, a-wave amplitudes were significantly different at CT18 (>–1 log cd.s/m^2, 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.

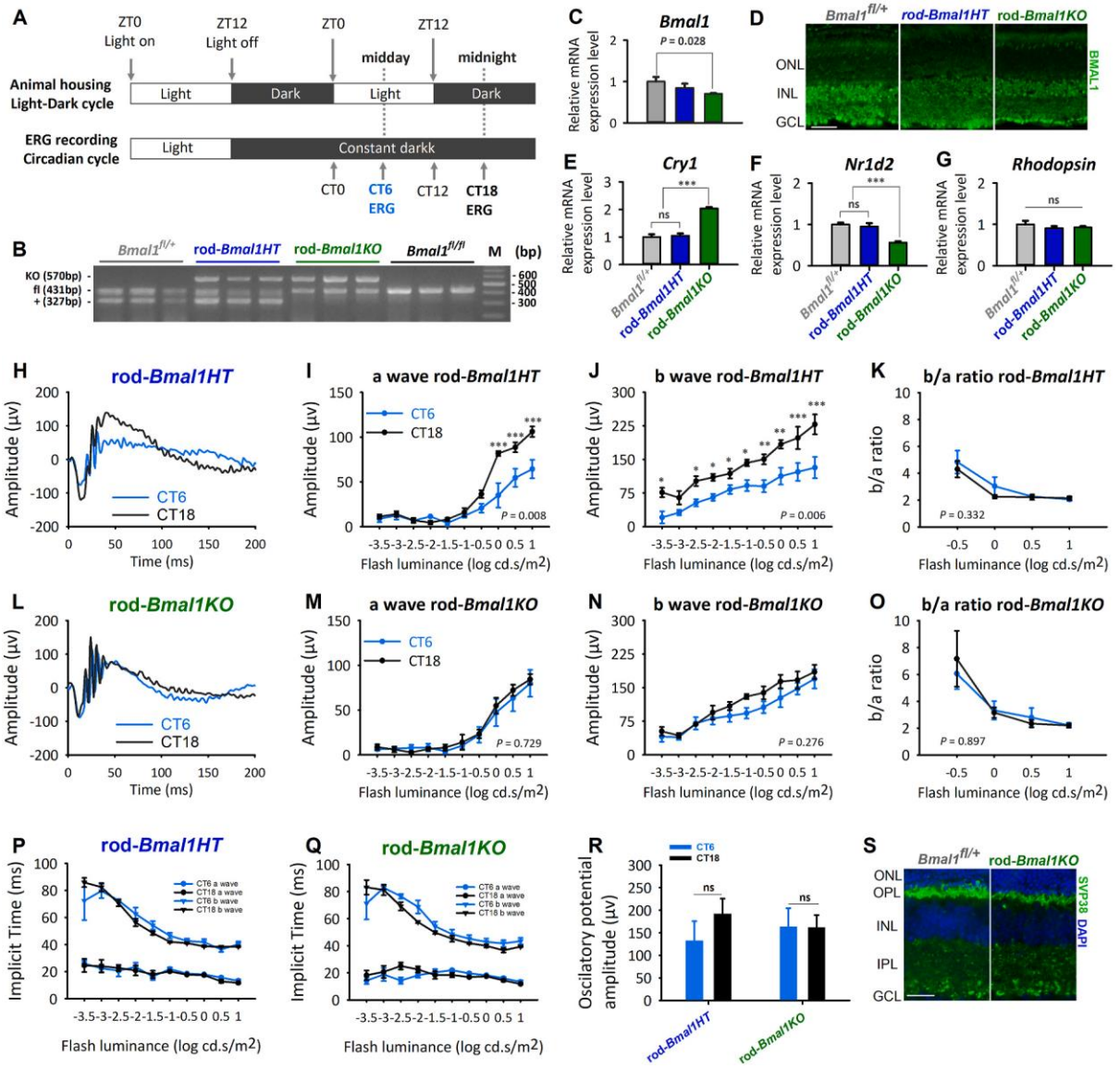

Fig. 1. Subjective day/night difference in dark-adapted ERG is lost in rod-*Bmal1KO* mice. (A) Graphical representation of lighting regimes for animal housing (12 h light (white bar):12 h dark (black bar)) and ERG recording (constant dark). Upward arrows at subjective midday (CT6) and subjective midnight (CT18) indicate the approximate times (±1hr) 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 µm. (H and L) Representative ERG traces in response to a 10 cd s/m^2 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 µm. 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 ± 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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.

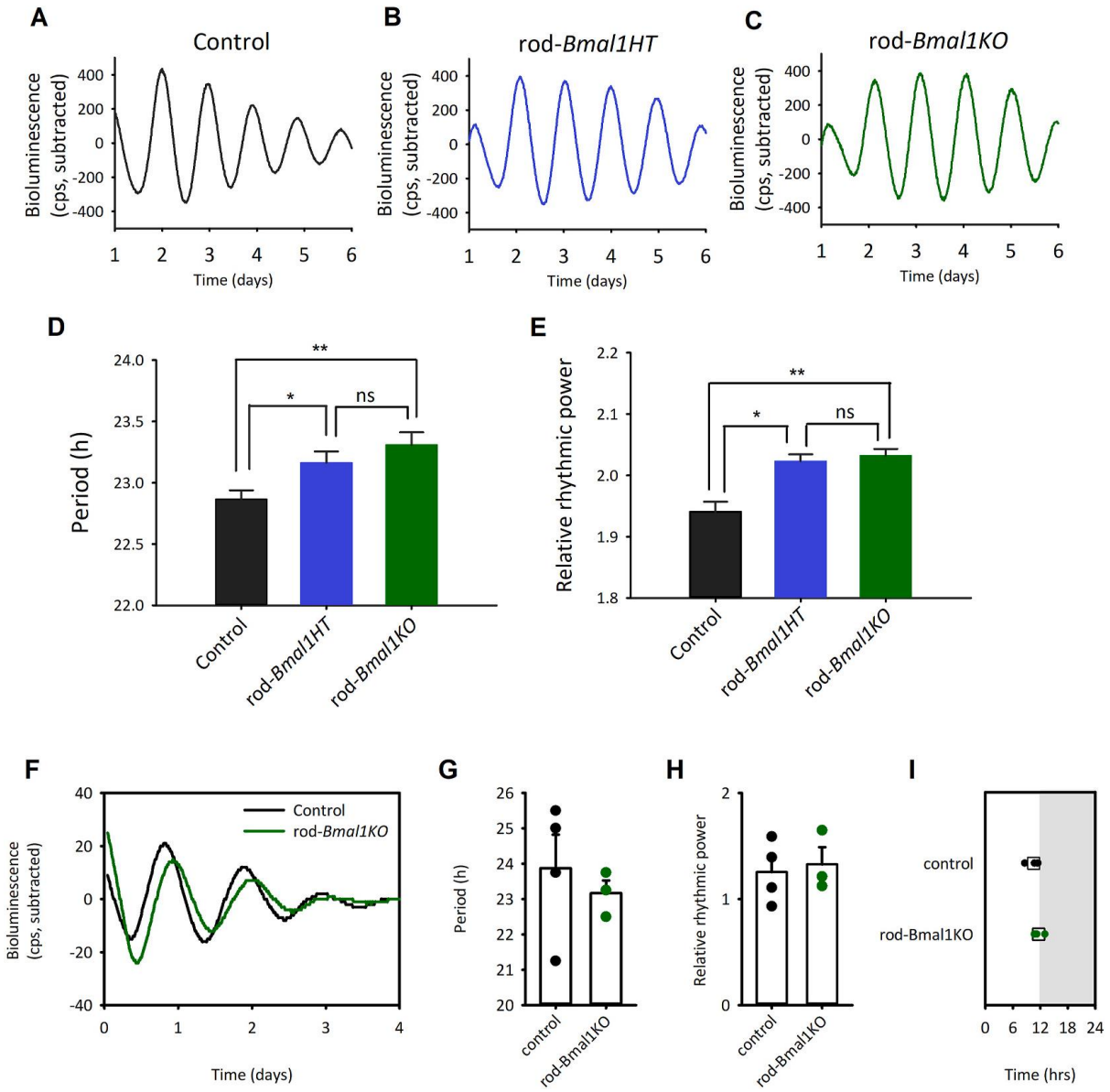

Fig. 2. *Bmal1* in rods modulates period and rhythmic power of PER2::Luciferase rhythms in whole retinas. (A–C) Representative bioluminescence traces of whole retina explants prepared from control (A), rod-*Bmal1HT* (B), and rod-*Bmal1KO* (C) mice. PER2::Luciferase oscillations appeared more persistent 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. (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. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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., 2018a). Yet, several rhythmic functions in rods are under clock control, such as phagocytosis of their outer segments by the underlying pigmented epithelium (Bobu and 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 and 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; Milićević 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 (Milićević 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/E-box 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 (Brann and Cohen, 1987; 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-*Bmal1KO* 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.

Author contributions

S.G. designed, performed experiments, analysed 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.

Declaration of competing interest

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

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Chapter 5

Circadian Clock Disruption Negatively Modulates Retinal Photoreceptor Degeneration

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In preparation

Circadian Clock Disruption Negatively Modulates Retinal Photoreceptor Degeneration

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Abstract

Daily rhythmicity is a central hallmark of vision, in particular the adaptation of retinal physiology and light information processing to the day/night cycle. These cyclic processes are regulated within the retina by a circadian clock whose precise cellular localization remains to be determined. Although hundreds of genes associated with retinal degeneration have been

identified, no direct link has been established between the clock and genetic retinal diseases. Hence, we investigated the hypothesis that a poorly functioning circadian clock modulates pathology of retinal photoreceptor disease. We performed a study in mice, using the P23H mutation of rhodopsin, one of the major causes of autosomal dominant Retinitis Pigmentosa (RP) in human, as well as a rod-specific conditional knockout of *Bmal1*, a key clock component. More specifically, we used mice either heterozygous for the rhodopsin P23H mutation or carrying the *Bmal1* (rod) deletion (rod-*Bmal1KO*) alone as well as double mutants of these strains, and WT control mice. We show by functional (electroretinography) and structural (immunohistochemistry) analyses that the introduction of the (rod-*Bmal1KO*) mutation into the P23H rhodopsin line, exacerbates the retinal RP phenotype induced by the P23H mutation alone. Indeed, we observed marked ERG amplitude reduction and loss of photoreceptor cells and gliosis in the double mutants with respect to simple P23H Rho mutants. These observations were further corroborated by RNA-Seq analysis. We found major gene expression differences between the double mutant and P23H Rho retinas. In this data, we identified unique circadian regulated gene expression sets implicating the phototransduction cascade, photoreceptor synaptic events and metabolism in response to photoreceptor degeneration. Taken together, our analyses suggest molecular mechanisms that link clock and retinal degeneration, and could perhaps inspire treatment approaches for blinding diseases.

Keywords: *Bmal1*, P23H, rod, circadian clock, Retinitis Pigmentosa, ERG, RNA Seq.

1. Introduction

Daily rhythms are a major hallmark of every cellular and physiological process throughout the body. These rhythms are programmed by molecular clocks widely distributed in mammalian cells and tissues. They are coordinated by a master clock located in the suprachiasmatic nuclei (SCN) within the hypothalamus. Circadian clocks keep time by using complex transcriptional/translational feedback loops involving transcription factors encoded by “clock genes” (mainly six core genes: *Bmal1*, *Clock*, *Per1*, *Per2*, *Cry1*, *Cry2*). They transmit their cycling patterns to their target genes, hence to cell physiology (Takahashi et al., 2008).

Genome-wide transcriptome profiling studies in mouse have uncovered a wide array (about 43%) of protein- encoding, ubiquitous and tissue-specific genes under circadian control. This seems to be in agreement with the need, for different organs, to fulfil distinct temporally

controlled tasks (Zhang et al., 2014). These studies showed that the circadian clock system is highly pervasive, with extensive regulation of basic biological processes such as metabolism and DNA repair but also tissue-specific functions, all intimately coordinated within the 24-hour period (Chaix et al., 2016; Mure et al., 2018). Thus, circadian rhythmicity has strong adaptive value. It is, moreover, established that chronic disturbance of these timed mechanisms, as experienced in today's 24h life styles, leads to increased morbidity and reduced lifespan (West and Bechtold, 2015).

In mammals, the retinal circadian clock plays a crucial function in adapting retinal physiology and function to the light/dark cycle (reviewed in McMahon et al., 2014; Felder-Schmittbuhl et al., 2018). It regulates these functions by controlling the mRNA expression of photopigment and phototransduction-related genes in rods and cones (Brann and Cohen, 1987; von Schantz et al., 1999; Sakamoto et al., 2006; Bobu et al., 2013; Kunst et al., 2013), visual sensitivity (Bassi and Powers, 1986; Barlow, 2001), and rod-cone electrical coupling (Ribelayga et al., 2008). The mammalian retinal clock also regulates processes that are linked to retinal survival such as nocturnal release of the cytoprotective melatonin (Tosini and Menaker, 1996), photoreceptor outer segment phagocytosis by the underlying pigmented epithelium (RPE) (LaVail, 1980; Bobu and Hicks, 2009), and vulnerability of photoreceptors to degeneration following light damage (Organisciak et al., 2000).

Expression of clock genes has been detected in most retinal cell types (Gustincich et al., 2004; Ruan et al., 2006; Liu et al., 2012; Milićević et al., 2021) but is still debated regarding rods. The retinal clock is currently considered as a network of layer/cell-specific oscillators (Dkhissi-Benyahya et al., 2013; Jaeger et al., 2015). In mice, loss of core clock genes negatively influenced (the combination of) retinal development, retinal structure and/or visual function (Owens et al., 2012; reviewed in Bery et al., 2022). More specifically, double mutation of *Per1* and *Per2* leads to subtle morphologic changes in the retina including a disturbed dorso-ventral distribution of cones (Ait-Hmyed et al., 2013), whereas no phenotypic alterations were observed in *Cry1/Cry2* double-knockouts (Selby et al., 2000). Invalidation of *Bmal1* or of *Clock* and *Npas2* genes in mice was reported to compromise photoreceptor viability, but only upon aging (Baba et al., 2018b). Various studies suggest that the circadian clock regulates the function of the retina in various ways. First, it regulates the light sensitivity of photoreceptors (Storch et al., 2007; Ait-Hmyed Hakkari et al., 2016). Second, the clock has been implicated in

daily variations of retinal light response (Storch et al., 2007; Cameron et al., 2008; Gegnaw et al., 2021; Allen, 2022). Finally, it drives the photoreceptor outer segment (POS) phagocytosis by the underlying pigmented epithelium (Bobu and Hicks, 2009; Krigel et al., 2010; Milićević et al., 2021), and regulates phototransduction and cell cycle pathways during eye development (Bagchi et al., 2020). Taken together, these results suggest that in the mammalian retina the circadian clock plays a major role in photoreceptor function and survival.

The requirement of a functional circadian clock to support retinal health has been suggested by several studies (reviewed in Felder-Schmittbuhl et al., 2017; Bhoi et al., 2023). Indeed, circadian clocks modulate the expression of approximately one third of all human retinal disease genes (Milićević et al., 2021). In addition, mutations in *Per2* induce diabetic retinopathy in mice, likely through retinal microvasculature pathology (Bhatwadekar et al., 2013; Jadhav et al., 2016). Similarly, Bmal1 was shown to regulate both developmental angiogenesis and neovascularization in the mouse retina (Jidigam et al., 2022). No link with the clock has been established so far in Retinitis Pigmentosa (RP), a relatively common inherited form of progressive retinal degeneration, affecting 1 in 4000 (Ferrari et al., 2011; Verbakel et al., 2018). RP is a clinically and genetically heterogeneous disorder with more than 90 implicated disease genes. The symptoms common to all RP types involve night blindness and progressive concentric visual field loss leading to tunnel vision and frequently resulting in legal blindness (Verbakel et al., 2018). The progression of the disease is explained by the initial death of rods, which subsequently triggers the degeneration of cones. Multiple disease genes and corresponding molecular pathways have been identified in RP, but exact molecular mechanisms remain partly elusive. RP disease pathways are frequently intrinsically linked and result in a final common cellular fate of apoptosis and death. Obviously, photoreceptor cell death results in vision loss. Interestingly, individuals with the similar RP disease gene mutations can have different retinal phenotypes. This variability involves the timing of disease onset, rate of progression, and the area of the retina and cell type most affected. Variability may be induced by epigenetic factors, genetic modifier loci or light damage (Ferrari et al., 2011; Contín et al., 2016). However, a potential role of temporally or chronically disturbed circadian rhythms has been hardly investigated (Bery et al., 2022).

The present study investigated the hypothesis that a poorly functioning circadian clock affects retinal function and negatively modulates the disease phenotype seen in Retinitis Pigmentonsa. Indeed, a disturbed retinal clock may alter the expression of disease genes and/or induce homeostatic imbalance. Hence, it may influence the time of onset, severity and progression of genetic retinal diseases. We tested our hypothesis by crossing the rod-specific *Bmal1* knock out (KO) (Gegnaw et al., 2021) with the *Rho*$^{P23H/+}$ mouse (Sakami et al., 2011; Sakami et al., 2014). The latter strain is a well-established model of Retinitis Pigmentosa (RP) in human, caused by a dominant P23H mutation in rhodopsin (Dryja et al., 1990). We analysed the double-mutants and controls by electroretinogram (ERG), immunohistochemistry and whole retina RNA-sequencing experiments. Experimental results support the conclusion that the conditional, rod-specific, KO of *Bmal1* exacerbates retinal RP phenotypes induced by the P23H mutation. More specifically, analysis of whole retina transcriptome in these animals pointed at clock regulated pathways, such as metabolism, that might be altered by *Bmal1* invalidation in rods and contribute to the worsened phenotype of the double mutant retinas.

2. Materials and Methods

2.1. Animals

All experimental procedures were carried out according to the European Parliament and The Council of the European Union Directive (2010/63/EU) for animal experiments, and authorized by the French Ministry for Higher Education, Research and Innovation (APAFIS#10213-2017060920001367-v3). *Rho*$^{P23H/P23H}$, *Rho-iCre* and *Bmal1*$^{fl/fl}$ 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 (Mattapallil et al., 2012). Animals were bred in the Chronobiotron animal facility (UMS 3415, CNRS-University of Strasbourg) 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 distinct genotypes generated by breeding mice carrying the P23H mutation of rhodopsin (*Rho*$^{P23H/P23H}$) (Sakami et al., 2011), the floxed Bmal1 allele (*Bmal1*$^{fl/fl}$) (Storch et al., 2007), and Rhodopsin-iCre (*Rho-iCre/+*) transgene (Li et al., 2005). We generated four main genotype groups: the double mutant carrying both the heterozygous

P23H mutation and the rod-specific deletion of *Bmal1* (DM: *Rho*$^{P23H/+}$*;Bmal1*$^{fl/fl}$*;Rho-iCre/+*), simple mutants with either only the heterozygous P23H mutation (P23HRho: *Rho*$^{P23H/+}$*;Bmal1*$^{fl/+}$*;Rho-iCre/+*) or only the rod-specific deletion of *Bmal1* (rod-*Bmal1KO*: *Bmal1*$^{fl/fl}$*;Rho-iCre/+*) and control mice with no mutation in rhodopsin but with Rhodopsin-iCre transgene and one floxed *Bmal1* allele (Ctrl: *Bmal1*$^{fl/+}$*;Rho-iCre/+*). For homogeneity, the *Rho-iCre/+* transgene was introduced into all groups which also contained at least one floxed allele of *Bmal1*. In Ctrl mice the circadian clock in rods should be unaltered (Bunger et al., 2000; Gegnaw et al., 2021). We initially confirmed that the *Rho-iCre/+* transgene did not induce any specific phenotype by comparing visual responses of the Ctrl group to mice carrying uniquely one floxed allele of *Bmal1* (Supplementary Figure 1: ERG). All mice were also bred on the *mPer2*^{Luc} clock reporter background (Yoo et al., 2004).

Mice were genotyped by PCR on tail genomic DNA. Genotyping of the *Bmal1* conditional knockout mice was performed as previously described (Storch et al., 2007). The primers to detect the *Rho-iCre* transgene were: 5'-AGCAGCCTTGGTCTCTGTCTAC-3' and 5'GATTCTCCTCATCACCAGGGAC-3'. For the detection of the P23H knock in we used: 5'-GCCTGTTTAGCTGAGAAAAC and 5'-GACCACGTAACAAACTTCTG-3'.

2.2. Electroretinography (ERG)

ERG recording was performed according to previously described procedures (Tanimoto et al., 2009; Ait-Hmyed Hakkari et al., 2016). In summary, the ERG device consisted of a Ganzfeld bowl, an amplifier, and a PC-based control and recording unit (RETI port/scan 21; Stasche & Finger GmbH, Roland Consult, Brandenburg, Germany). Mice were anesthetized with a combination of xylazine and ketamine in Ringer solution at 30 µl/g [Rompun 2% = 2.0 g/100 ml (Bayer, Puteau, France); Imalgene 1000 = 100 mg/ml (Merial, Lyon, France)] and placed on a temperature regulated heating plate to maintain a constant body temperature. Ground and reference electrodes were placed subcutaneously in the base of the tail and below ear lobes (right and left), respectively. ERG was recorded from both eyes using corneal/active electrodes (thin gold-wire with a 2-mm ring end). Ocrygel (Virbac, Carros, France) eye drop was applied to ensure good electrical contact and to keep the cornea hydrated during the entire procedure. The following single scotopic white flash ascending intensities were used: 3 x 10^{-4}, 10^{-3}, 3 x 10^{-3}, 10^{-2}, 3 x 10^{-2}, 10^{-1}, 3 x 10^{-1}, 1, 3, and 10 cd.s/m^2. Before scotopic recordings, mice were dark-adapted overnight and animal handling before recording was

performed under dim red light. ERG recordings were made in the afternoon (CT7 to CT10) and genotypes were randomly allocated, to avoid bias from circadian changes in visual responses. We chose to perform these recordings under subjective day to avoid any confounding effect from circadian time, since the response of rod-*Bmal1KO* mice stays in the daytime configuration also at night (Gegnaw et al., 2021). Prior to ERG recording we made sure that the distinct animal groups were properly synchronized to the LD cycle (Supplementary Figure 2) and that their retinas had intact rhythmicity, as assessed by recording rhythms in PER2::LUCIFERASE activity in vitro (Supplementary Figure S3). Thus subjective time for ERG recording could be defined properly.

Statistical analysis of ERG data was performed using Sigma Plot 13 (Systat Software, San Jose, CA, USA). Repeated measures two-way ANOVA was performed for ERG data. Results are presented as mean ± SEM and statistical significance level was set at $P < 0.05$.

2.3. Immunohistochemistry

Mice were euthanized by cervical dislocation. Retinas from left eyes were quickly dissected away from the eyecup in cold HBSS buffer within a petri dish, snap-frozen on dry-ice and stored at -80°C until mRNA extraction. Right eyes were quickly removed and immersion fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C, then transferred into fresh PBS (0.05% sodium azide) and stored at 4°C until needed for immunohistochemical analysis.

Immunohistochemistry analysis of retinas was performed as previously described (Ait-Hmyed et al., 2013). In summary, cryostat sections (10 µm) were permeabilized with Triton (PBS-0.1% Triton X-100, 5 min) and blocked with saturation buffer (3% bovine serum albumin, 0.05% Tween-20, and 0.1% sodium azide in PBS) for 60 min. Sections were incubated overnight with primary antibodies diluted in saturation buffer, washed 2 x 5 min in PBS, incubated with secondary antibodies (2 h at room temperature) and washed again thoroughly for 40 min. Cell nuclei were stained with 4,6-di-amino-phenylindol amine (DAPI; Molecular Probes). Slides were washed 3 x 5 min in PBS, mounted in PBS/glycerol (1:1), and scanned in a NanoZoomer at 40x and viewed using NDP.view2 software. Immunostaining was performed on four consecutive sections per eye (n=4 per genotype). All experiments gave similar results and representative images of stained sections are shown. Primary antibodies used for

immunolabeling were monoclonal anti-rhodopsin rho-4D2 (Hicks and Molday, 1986), polyclonal anti-cone arrestin (Zhu et al., 2002, a kind gift from Cheryl Craft, dilution 1:500), polyclonal anti-PKCα (ab32376, Abcam, dilution 1:500), and polyclonal anti-glial fibrillary acidic protein (GFAP). Secondary antibodies were Alexa goat anti-mouse or anti-rabbit 488 (dilution 1:500) and goat anti-rabbit 594 (dilution 1:500).

2.4. RNA extraction, RNA-seq library preparation and RNA sequencing

Sampled retinas were individually homogenized using a motorized pestle in 500 µl TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) and incubated for 5 min at room temperature. 100 µl chloroform were added to each lysate and after 2 min incubation at room temperature the mixture was centrifuged using Phase-Lock gel tubes (Heavy, 2ml; QuantaBio, Beverly, MA, USA) at 12 000 x g for 15 min at 4°C. The RNA from the upper aqueous phase was precipitated with equal volume of 70 % ethanol and extracted using the RNeasy Micro kit (Qiagen GmbH, Hilden, Germany) following manufacturer's instructions, including the DNase digestion step to remove any genomic DNA contamination. The RNA was eluted with 14 µl of RNase-free water. RNA concentration and purity were measured using NanoDrop ND-1000V 3.5 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and its quality was evaluated with a Bioanalyzer (AgilentTechnologies, Amstelveen, The Netherlands) (RNA integrity numbers were between 7.4-10).

RNA-seq library preparation was performed exactly as described previously (Bagchi et al., 2020). Briefly, libraries were prepared from 500 ng purified RNA using the KAPA mRNA HyperPrep Library Preparation Kit (Roche Sequencing Solutions, Pleasanton, CA USA) for Illumina Platform HiSeq 4000, according to manufacturer's protocol. The libraries were amplified using a mixture of KAPA HiFi HotStart RdyMix (2X) and Lib. Amp. Primer Mix (10X) (Roche Sequencing Solutions, Pleasanton, CA USA) to produce strand-specific PCR products. The cDNA library amplification process was confirmed using flash gel visualization along with cleanup steps. Quality and size distribution of the cDNA library was checked by using the Bioanalyzer. Qubit 2.0 Fluorometer (Life Technologies, Foster City, CA, USA) was used for the quantification of libraries. The cDNA library was sequenced by single-end (50bp) sequencing on Illumina HiSeq 4000 sequencer (Illumina, San Diego, CA, USA).

2.5. Bioinformatics

Reads were subjected to quality control FastQC v0.11.15, and trimmed for adapter sequences using Trimmomatic v0.32. The trimmed reads were aligned with a reference mouse genome (Ensembl GRCm38.p7) using HISAT2 (v2.1.0) (Kim et al., 2015). Counts were obtained using HTSeq (v0.6.1) (Anders et al., 2015) with parameters ''--stranded=reverse'', default parameters ''--order=name --minaqual=10 --type=exon --idattr=gene_id --mode=union'' and the mouse GTF from Ensembl GRCm38v93.

Statistical analyses were performed using the edgeR (Robinson et al., 2010) and limma (Ritchie et al., 2015) R/Bioconductor packages using R (v3.6.0) and Bioconductor (v3.9). 23,905 genes with more than 2 count-per-million reads (CPM) in at least 4 samples were retained. Count data were transformed to log2-counts per million (logCPM) using voom, normalized by applying the weights trimmed mean of M-values method (Robinson and Oshlack, 2010).

The differential expression between the different genotypes was evaluated using a moderated t-test within limma's linear model framework corrected for gender, including the precision weights estimated by voom. Resulting P-values were corrected for multiple testing using the Benjamini (Reimand et al., 2007)-Hochberg false discovery rate adjusted P value (Adj P). An adjusted P-value less than 0.05 was used as a level of statistical significance. An additional fold change (FC) cut off (≤-2 or ≥2) was applied when comparing P23H or DM to Ctrl group. Additional gene annotation was retrieved from Ensembl (v97) using the biomaRt R/Bioconductor package.

Gene ontology and pathway enrichment analyses were performed using g:Profiler (Reimand et al., 2007) by using Mus musculus genome as reference background. An adjusted $P < 0.05$ was set as threshold for significantly enriched pathways using the g:SCS method to correct for multiple testing. We investigated interaction between protein products from the list of genes up or down-regulated in the DM with respect to Ctrl by STRING analysis with threshold set at 0.7 (STRING version 11.5 (https://string-db.org/); (Szklarczyk et al., 2015).

Selection of conserved mouse/human non-coding elements (CNEs) located in introns, 5' and 3' regions up to the neighbor gene as defined in UCSC (genomes mm10 and hg19, respectively) were retrieved using the "comparative genomics" and "intersection" tools in UCSC browser and Galaxy (Karolchik et al., 2004) and https://main.g2.bx.psu.edu/. CNEs were kept for further analysis after additional selection using the following criteria: they presented either promoter or enhancer-like signature (related to H3K4me3 and H3K27ac) or CTCF-binding site ENCODE Candidate Cis-Regulatory Elements (cCREs). It is the subset of representative DNase hypersensitive sites across ENCODE samples that are supported by either histone modifications (H3K4me3 and H3K27ac) or CTCF-binding data (Consortium, 2011; 2012; Consortium et al., 2020). The CNEs were kept when the conservation was at least 80%.

Searches for enriched motifs within selected lists of CNEs were ran under the web server TFmotifView (Leporcq et al., 2020), where "enrichment" threshold is >1.5 (i.e., a given motif is present 1.5 times more in the foreground than in the background), and with a p-value set to 0.05. The "foreground" corresponded to the CNEs and the "background" corresponded to shuffled foreground sequences, based on basic permutation methods with conservation of nucleotide counts but randomized order of arrangement.

3. Results

3.1. Rod-specific knock-out of *Bmal1* exacerbates visual dysfunction induced by the P23H Rho mutation

We performed dark-adapted ERG to evaluate visual function in distinct genotype groups (DM, P23H Rho, rod-*Bmal1KO* and Ctrl) at four age time-points: postnatal day - P40, 80, 112 and 180. In contrast with the Ctrl group, significantly decreased a- and b-wave amplitudes were observed in the P23H Rho mice, already at P40 (Figure 1A, E; P = 0.001). Likewise, at P40, amplitudes in P23H Rho mice were significantly reduced compared with the rod-*Bmal1KO* group (Figure 1A, E). At the same time point (P40), also the double mutant mice rod-*Bmal1KO*/ P23H Rho (DM) showed a pronounced decline of the a- and b-wave amplitudes compared with rod-*Bmal1KO* and Ctrl mice. DM mice at P40 and P80 age time-points displayed a decline in the scotopic a-wave and b-wave amplitudes with respect to the P23H Rho single mutants, but without reaching statistical significance (Figure 1A,B,E,F; P > 0.05).

Importantly, in the older P112 DM mice, the scotopic b-wave (but not a-wave) amplitudes were significantly lower than in single P23H Rho mutants ($P < 0.05$; Figure 1G). Interestingly, there was no difference in a- and b-wave amplitudes between the rod-Bmal1KO and controls at P112, or any of the other time points measured (P40, P80, P180) (Figure 1A-D a-wave, E-H b-wave). This indicates that the decrease in ERG response observed in the DM compared with P23H Rho mutants most likely results from a synergic effect of Bmal1 disruption and P23H mutation in rod photoreceptors. Finally, at P180, b-wave amplitude in DM was not anymore significantly different from age-matched P23H Rho animals (Figure 1H; $P > 0.05$). Accordingly, a-wave amplitudes were similar between these two genotype groups and showed that light-response was mostly lost at P180 (Figure 1D). Under photopic conditions P23H mice did not significantly differ from all control groups at P80 and P112 but differed significantly at 6 months. By contrast, photopic response in the DM was already markedly reduced with respect to control groups at 4 months (Supplementary figure 4).

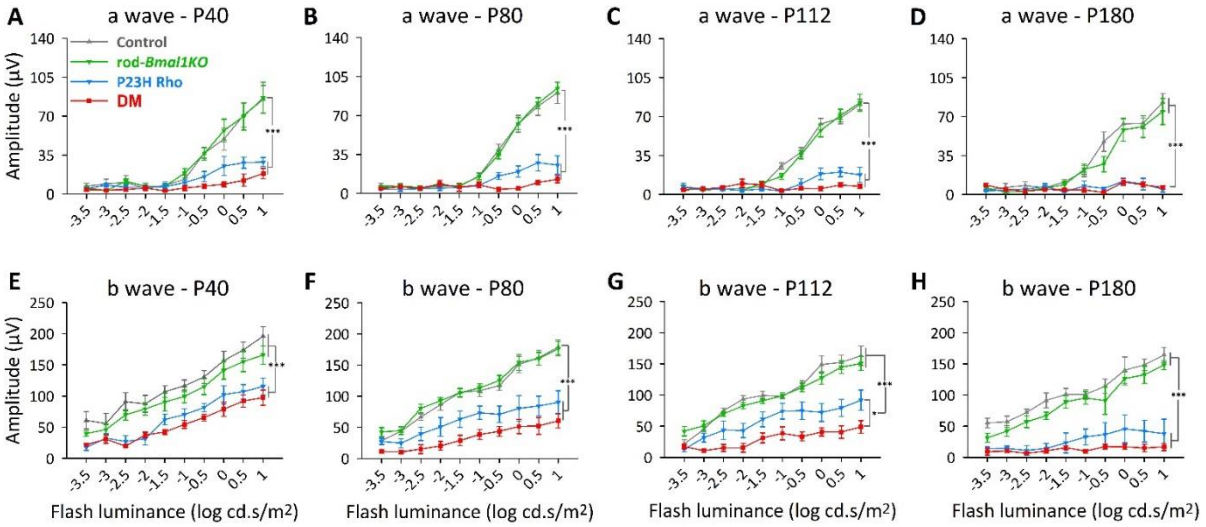


Figure 1. Dark-adapted ERG analysis reveals synergistic effect between the simple P23H mutation and the rod-specific *Bmal1* KO. Amplitudes of a- and b-waves are presented according to intensity of the light stimulus in Ctrl (gray), rod-*Bmal1KO* (green), P23H (blue), and DM (red) groups at P40 (A, E), P80 (B, F), P112 (C, G) and P180 (D, H). (A-D) a-wave in DM displayed a tendency for reduced amplitude with respect to littermate P23H Rho mice at all ages. (E-H) b-wave amplitudes in DM mice appear also reduced with respect to P23H Rho. This decrease is more marked at P80 (F) than at P40 and is significant at P112 (*$P < 0.05$, G). No more difference is detectable between DM and P23H Rho mice at P180 (H), with nearly undetectable visual responses in both genotypes. Amplitudes did not differ between rod-*Bmal1KO* and Ctrl mice throughout the study and were significantly larger than for of DM or P23H mice at all ages (***$p<0.001$). Data are means ± SEM. Comparison between genotypes was performed by 2-way repeated measures ANOVA (n = 6-10, n = 6-8, n = 5-8, and n = 5-6/genotype at P40, 80, 112, and 180, respectively).

3.2. Immunohistological examination suggests that *Bmal1* invalidation in rods promotes rod and cone loss induced by the P23H mutation

To confirm the functional ERG data, structural analysis of retinas was performed by immunohistochemistry at P120, the age at which the dark-adapted visual response was significantly reduced in DM mice compared with single P23H Rho mutants and Ctrl mice (Figure 1G).

We first performed immunostaining of retinal sections for the rhodopsin protein. Ctrl and rod-Bmal1KO mice displayed intense labelling of rhodopsin, localized to the photoreceptor outer segments (Figure 2A,B). In contrast, staining of P23H Rho retinas not only confirmed the reduced presence of rod photoreceptors but also showed that rhodopsin was mis-localized to the inner segments and cell bodies (Figure 2C). Importantly, DM retinas presented with a similar pathological picture, but more severe, with few stained inner segments and cell bodies and complete absence of outer segments (Figure 2D).

To investigate whether cones were also affected, we used anti-cone arrestin labelling. Whereas controls showed the expected labelling of cone pedicles and outer segments (Figure 2E,F), staining of the latter was mostly absent in P23H Rho sections and in DM retinas the staining was further reduced, in agreement with photopic ERG data (Figure 2G,H). GFAP immunoreactivity confirmed the diseased retina phenotype in these genotype groups, with intense staining reflecting reactive gliosis (Figure 2K,L) whereas the rod-Bmal1KO retinas stained like Ctrl (Figure 2I,J).

Finally, we investigated the structural integrity of the rod bipolar cells by immunostaining against protein kinase C α (PKCα). In the control retinas (Ctrl and rod-*Bmal1KO*), rod bipolar cells were found in the inner nuclear layer, as expected, and presented normal dendritic processes in the outer plexiform layer (Figure 2M,N). In the mutant retinas (P23H Rho and DM), bipolar cells were localized at the correct layer, but their dendritic processes in the outer plexiform layer were of reduced length or totally absent (Figure 2O,P).

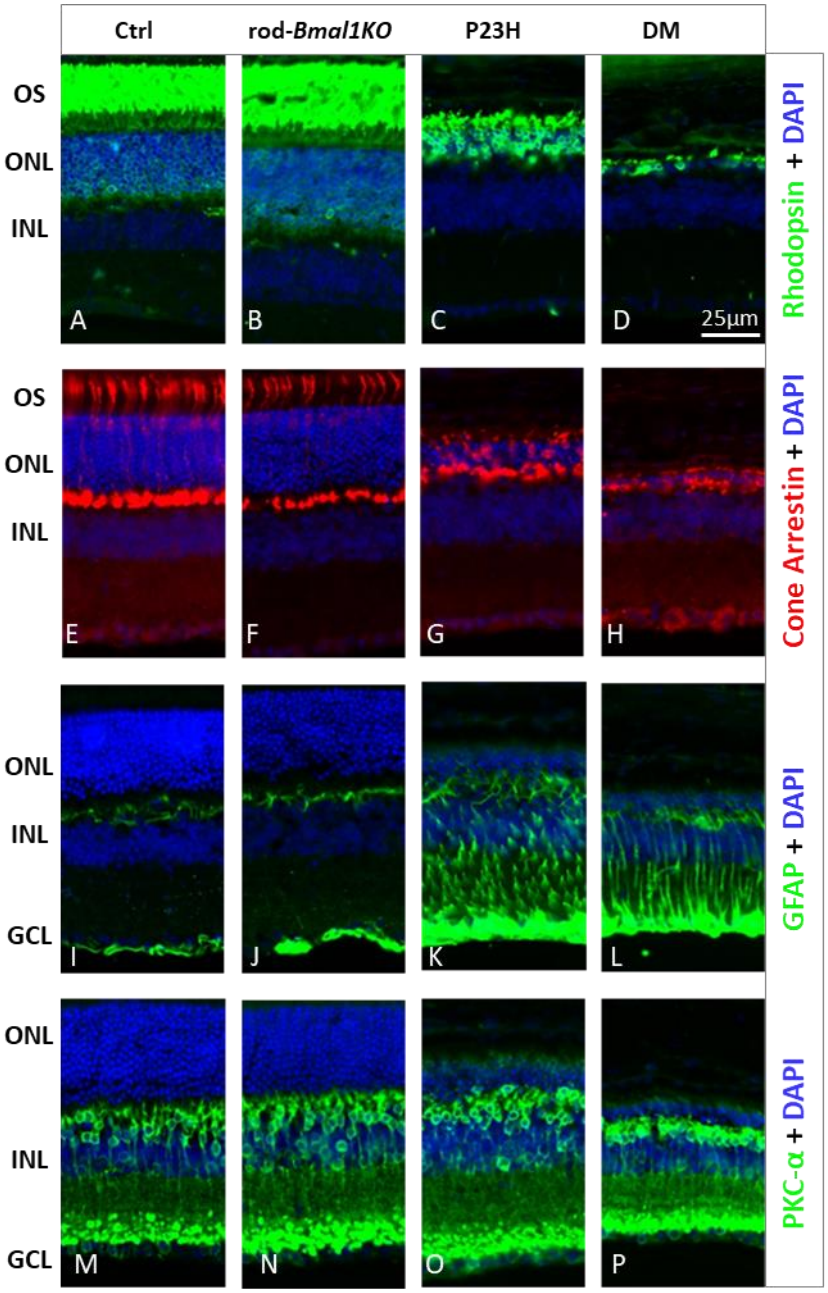


Figure 2. DM mice show severe loss of rod photoreceptor cells and general retinal disease. Representative images of immunohistochemical staining from Ctrl (A,E,I,M), rod-*Bmal1KO* control (B,F,J,N), P23H Rho (C,G,K,O), and DM (D,H,L,P) P120 retina sections stained for: Rhodopsin (A-D), cone arrestin (E-H), glial fibrillary acidic protein (GFAP) (I-L), protein kinase C α (PKCα) (M-P) (magnification = 40x) (n=4 per genotype). Rhodopsin is expressed in the photoreceptor outer segments in Ctrl (A) and rod-Bmal1KO (B) mice; rhodopsin expression is decreased in P23H Rho retina (C) and mostly disappeared in DM retina (D). The cone photoreceptor staining with cone arrestin was severely reduced in P23H Rho retina (G) and almost lost in DM retina (H), in which only few cells were labelled at synaptic terminals. P23H Rho and DM retinas showed activated Müller glial cells, detected by increased level of GFAP (K,L). The dendritic processes of ON-bipolar cells (labelled with anti-PKCα) also appeared affected in the mutant retinas (O,P). DAPI staining is shown in blue. OS, outer segment; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar: 25µm.

3.3 Retinal RNA-seq analysis confirms that *Bmal1* loss in rods enhances retinal degeneration

To further investigate the worsened retinal degeneration in DM shown by functional and structural analyses, we performed retinal RNA-sequencing at P120. We analysed and compared the retinal transcriptomes of 3 genotype groups: Ctrl, P23H Rho and DM. We found 962 differentially expressed genes (DEG) in retinas from P23H Rho relative to Ctrl group, with 625 upregulated and 337 downregulated genes; and many more (3210) genes when comparing DM to the same controls, with 1843 upregulated and 1367 downregulated genes (Adj $P < 0.05$, FC ≤ -2 or ≥ 2) (Figure 3A, Tables S1 and S2). These data confirm on a molecular level that the circadian clock dysfunction in the DM induces significantly more profound alterations than in the P23H Rho single mutant retina. Importantly, further direct comparison of retinal transcriptomes between DM and P23H Rho littermates showed 889 genes differentially expressed, with 248 upregulated and 641 downregulated (Adj $P < 0.05$) (Figure 3A and Table S3). RT-qPCR on the mRNAs extracted from the same genotype groups was performed for a few DEG and validated the RNA-seq data. In particular, rhodopsin gene expression was found drastically decreased in the simple P23H Rho mutant and even more in the DM retinas, also in agreement with immunohistochemistry data (Supplementary Figure 5).

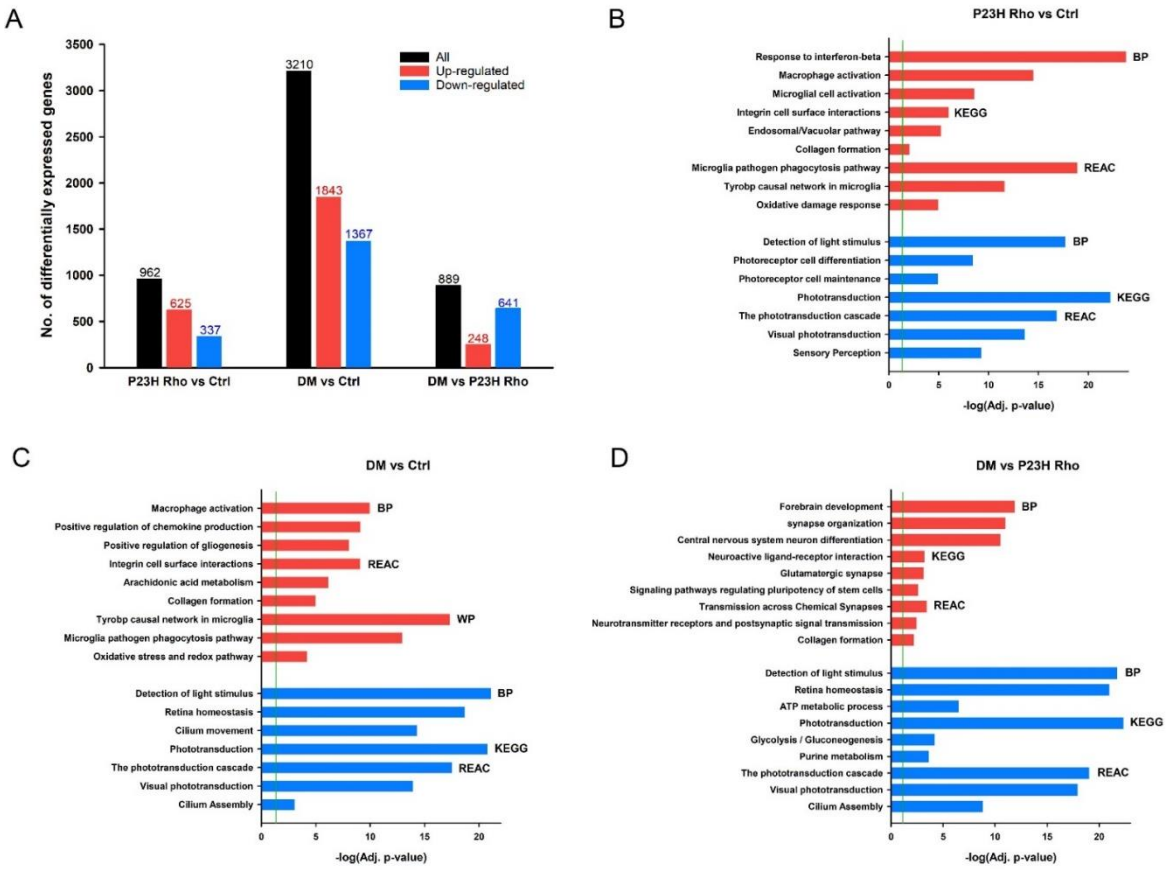


Figure 3. Transcriptional profiling of P23H Rho single mutant and DM double mutant mouse retinas with respect to controls at P120. A, Histogram shows differentially expressed genes in P23H Rho vs Ctrl (Adj P <0.05, FC ≤-2 or ≥2), DM vs Ctrl (Adj P <0.05, FC ≤-2 or ≥2), and DM vs P23H (Adj P <0.05) mouse retinas at P120 (all, up-regulated, and down-regulated; marked in black, red, and blue respectively). B, C, D Functional annotation using gProfiler of DEG (up-regulated in red, down-regulated in blue) between P23H Rho and Ctrl (A), CM and Ctrl (B) and DM and P23H Rho (D). The most relevant biological pathways from the Gene Ontology, Reactome (REAC), Wikipathways (Wiki) and KEGG databases are shown. Detailed lists of differentially expressed genes in P23H Rho vs Ctrl, DM vs Ctrl, and DM vs P23H Rho are shown in Tables S4, S5, and S6 respectively).

We used biological pathway analysis to identify significantly altered processes in the distinct transcriptome comparisons (Figure 3 and Table S1, S2, S3). This analysis showed that genes with increased expression (625 genes) in P23H Rho vs Ctrl were involved in immune system process, defence response, cell adhesion and response to oxidative damage (Figure 3B, Table S4). As expected, vision related biological processes such as detection of light stimulus and phototransduction were significantly enriched within genes whose expression was decreased (337 genes) (Figure 3B, Table S4). These results are in line with previously described data about retinas carrying the P23H Rho heterozygous mutation of rhodopsin gene (Leinonen et al., 2020). Analysis of genes found differentially expressed between DM and Ctrl groups similarly showed marked enrichment in terms related to defense response, cell adhesion and

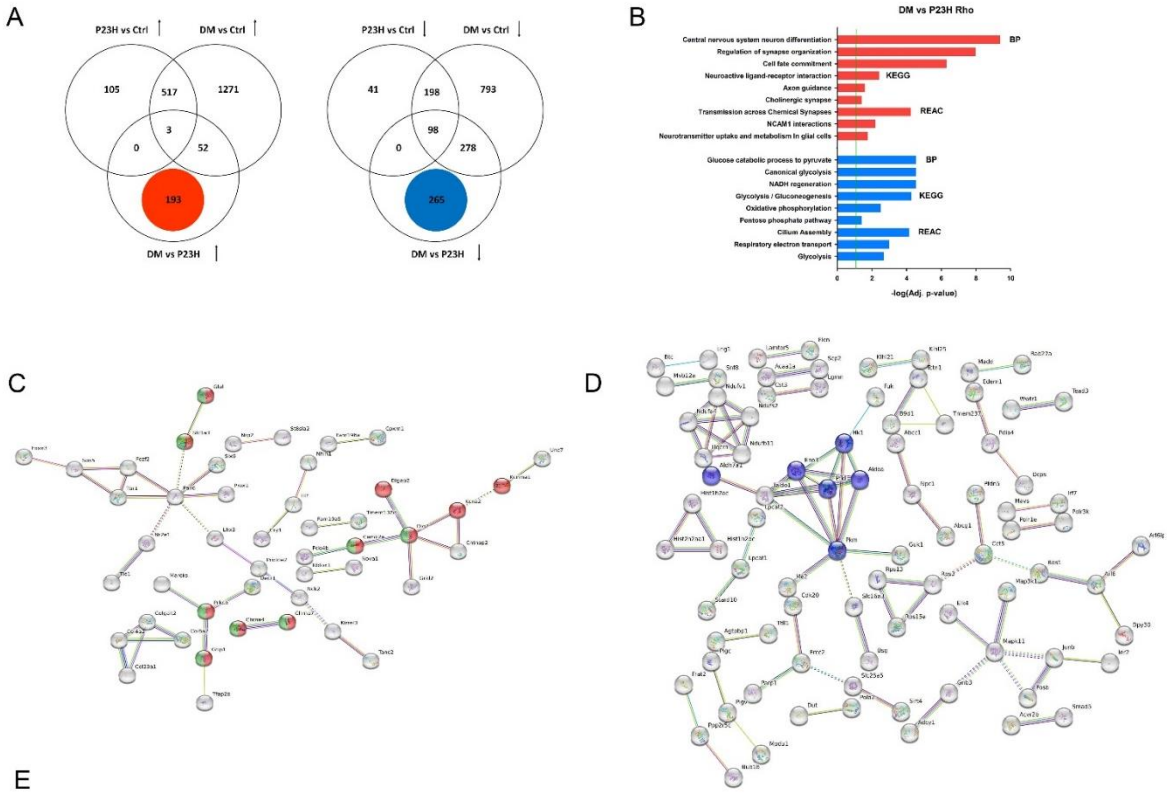
glial activation among upregulated transcripts (1,843 genes) (Figure 3C, Table S5). Genes with decreased relative expression (1,367 genes) displayed enrichment in GO terms related to vision and detection of light stimulus (Figure 3C, Table S5).

Pathway analysis of the genes differentially expressed between DM and P23H Rho groups showed that genes with increased expression (248) were involved in organ development and mainly regulation of synaptic transmission (Figure 3D, Table S6). Vision-related biological processes were significantly enriched among the (641) genes with decreased relative expression, such as visual perception, detection of light stimulus, but also metabolic processes such as glycogenesis and gluconeogenesis (Figure 3D, Table S6).

Comparison of the three sets of DEG identified above showed a number of similarities, as highlighted by the Ven diagrams of Figure 4A. However, they also pointed to some genes that are specifically up (193) or down (265) regulated in DM vs P23H Rho retinas but that were not altered in any other comparison (Figure 4A). As already seen in Figure 3D, the upregulated DEG are enriched in pathways linked to development such as Central nervous system neuron differentiation, Cell fate commitment and Axon guidance, together with synapse related processes (Figure 4B). The STRING analysis indicated that this gene set was enriched for genes involved in Neuronal system (FDR 0.001) and Transmission across chemical Synapses (FDR 0.0071) in the Reactome database, with interactions between following gene products *Camk2a, Dlg2, Dlgap2, Kcna2, Kcnma1, Slc1a3, Glul, Prkcb, Grip1, Chrna4*, and *Chrna7* (Figure 4C). These data highlight the extensive rewiring and tissue reorganisation going on in DM retinas. To further investigate which mechanisms might trigger these phenotypic changes we examined the promoter regions of the 193 DEG by concentrating on evolutionarily conserved sequences. We found highly significant enrichment in binding sites for transcription factors involved in retina development such as *Meis1, NFIA and NeuroD2*, confirming that retinal developmental mechanisms may be activated in the DM (Figure 4E).

The 265 DEG specifically downregulated in the DM vs P23H Rho comparison were enriched in pathways related to metabolism (i.e. Canonical glycolysis, Oxidative phosphorylation). Here also, STRING analysis revealed a network of 60 edges (P = 0.00889) and functional associations such as a cluster of six gene products (*Pfkl, Pkm, Eno3, Aldoa, Hk1*, and *Aldh7a1*) that are associated with the Glycolysis/Gluconeogenesis pathway. This confirms the metabolic alterations occurring in DM retinas. In line with this conclusion, the promoter analysis of the

265 genes showed marked enrichment in binding sites for GABPA a major regulator of the OXPHOS pathway (Figure 4E) (Mootha et al., 2004).



E

UP															
Transcription factor	ZFP57	MEIS1_2	NFIA	PITX3	DUX	GMEB2	PRDM4	NEUROD2	ZNF341	NR1H4	HIC1	GABPA	PKNOX1	YY2	HOXA11
Matrix ID	MA1583.1	MA1639.1	MA0670.1	MA0714.1	MA0611.1	MA0862.1	MA1647.1	MA0668.1	MA1655.1	MA1110.1	MA0739.1	MA0062.3	MA0782.2	MA0748.2	MA0911.1
Global p-value	1.20E-06	6.87E-04	7.94E-04	3.46E-03	5.02E-03	1.05E-02	1.19E-02	1.30E-02	1.72E-02	2.00E-02	2.38E-02	2.59E-02	3.04E-02	3.33E-02	3.78E-02

DOWN								
Transcription factor	GABPA	ERG	ZIC1_ZIC2	ZNF682	CREB3L4	HAND2	CEBPG	YY2	PRDM1
Matrix ID	MA0062.3	MA0474.2	MA1628.1	MA1599.1	MA1474.1	MA1638.1	MA1636.1	MA0748.2	MA0508.3
Global p-value	9.07E-05	1.05E-03	8.12E-03	1.03E-02	1.99E-02	2.26E-02	3.08E-02	3.25E-02	4.99E-02

Figure 4. Analysis of uniquely differentially expressed genes between DM and P23H Rho retinas. A) Venn diagram showing the overlap between genes that are significantly downregulated (marked in blue) or upregulated (marked in red) in P23H Rho or DM retinas as compared to Ctrl retinas, and DM retinas as compared to P23H retinas in P120 mice (n = 4/genotype). Numbers of DEG specifically between DM and P23H Rho retinas are highlighted (red, up; blue, down). B) Representative biological pathways for genes that are significantly up and downregulated in DM relative to P23H Rho retinas (red, up; blue, down). C, D) String network analysis of functional associations of protein products for gene lists highlighted in A). E) Table showing the putative transcription binding sites found enriched in the 193 DEG upregulated (top) and in the 265 DEG downregulated (bottom) specifically in the DM. The table shows the identity of transcription factors (first line), the matrix id (second line) and the global p-value for motif occurrence (third line).

4. Discussion

This study aimed at investigating the molecular and pathophysiological effects of combining circadian clock dysfunction and retinal degeneration, most importantly rhodopsin-linked RP. On molecular, morphological and functional levels our data support the hypothesis that a poorly functioning circadian clock is able to enhance degeneration in a retinal genetic disease. In summary, we found that mice with conditional, rod-specific Bmal1 knockout display retinal light responses and photoreceptor integrity similar to their Ctrl littermates. Mice (P23H Rho) with P23H-induced disease show a typical Retinitis Pigmentosa phenotype with visual function decline already at 1 month, consistent with earlier studies including ours (Sakami et al., 2011; Leinonen et al., 2020; Gegnaw et al., 2021). Most importantly, the rod-specific invalidation of Bmal1 in these mice (DM) exacerbates these phenotypes. Furthermore, transcriptome analysis shows major gene expression changes between DM and P23H Rho retinas that are linked to regulation of retina differentiation and tissue remodelling in the case of up-regulated genes, indicating a more advanced stage of degeneration. Genes whose expression is decreased, besides coding for phototransduction proteins, show marked enrichment in metabolic pathways. Together, these data suggest that the control of metabolism pathways by the circadian clock in rods is protective and supports functional adaptation in response to genetic retinal disease.

There are a number of similarities between our functional analysis and those reported in the literature. Globally, our results are in agreement with the previous description of the (heterozygous) P23H Rho model (Leinonen et al., 2020; Gegnaw et al., 2021). Indeed, we found a significant (> 50%) reduction of the scotopic a-wave amplitude in (heterozygous) P23H Rho compared with Ctrl mice at P40 (Figure 1). There are also differences between our comparative transcriptome analysis and those reported in the literature. Leinonen and co-workers described a transcriptomic fingerprint of neuronal network adaptation following early retinal degeneration already detectable at 1 month in P23H Rho retinas when compared to their age matched littermate wild types (Leinonen et al., 2020). The authors also found similar results in 3 months P23H Rho retinas. Surprisingly, we did not detect any such changes between P23H Rho and Ctrl retinas at 4 months. We found more immune response and cell stress-associated GO terms and pathways (Table S4). This might be due to the more stringent parameters chosen for our RNA-seq data (FC ≤-2 or ≥2), which yields more specific results.

Regarding clinical data, residual light response in RP patients has been proposed to involve cones (Hartong et al., 2006). Accordingly, we found that the cone function, as measured by light-adapted ERG was normal in P23H Rho mice up to 4 months age. It was however significantly decreased at 6 months when compared to the Ctrl group (Supplementary Figure S4).

Several studies reported the effects of clock disruption on retinal phenotype. In the present study, we found that the rod-mediated visual response measured during daytime did not show any major disturbances in the rod-*Bmal1KO* mice in the early months of life (Figure 1). These retinas showed neither any obvious loss of photoreceptor cells nor any stress response upon immunological staining at 4 months (Figure 4). We recently showed that these rod-*Bmal1KO* mice did not differ from controls in rhodopsin mRNA expression levels at 4 months age (Gegnaw et al., 2021). In agreement with these observations, mice with global deletion of *Bmal1* were reported to retain normal retinas up to 6 months (Storch et al., 2007; Owens et al., 2012). More generally, deletion of core clock components does not lead to drastic alterations of retinal phenotypes in young animals (Selby et al., 2000; Owens et al., 2012; Ait-Hmyed et al., 2013). However, there is evidence that photoreceptor viability decreases in *Bmal1* or *Clock/Npas2* mutant mice aged 8 months (Baba et al., 2018b). The effects of retina-specific *Bmal1* deletion indicate this is specifically affecting cones, with minor effects (stunted bipolar cell dendrites, reduced scotopic b-waves amplitude) on the rod pathway (Baba et al., 2018a). Taken together, these data indicate that clock dysfunction has mild impact on retinas, excepted during aging. By contrast in the present study, when comparing the transcriptome from DM vs P23H Rho retinas we found pathways such as Cell fate commitment or Axon guidance to be highly significant in up-regulated genes. Also, we confirmed activation of genes respectively involved in bipolar cell development and axon pathfinding, such as *Lhx3* and *Efna5* (Davenport et al., 1998; Dong et al., 2020). Thus, in case of pathological state, *Bmal1* invalidation in rods obviously impels the degenerating retina to engage into extensive reorganisation of the cellular network.

How then does clock disturbance in rods aggravate rod degeneration? Our longitudinal ERG study indicates a synergistic effect between clock disruption and retinal degeneration that becomes significant around 4 months. This is corroborated by the transcriptome analysis performed at the same age and showing more severe loss of photoreceptor and

phototransduction-related genes in DM vs P23H Rho samples. It is known that the clock regulates phototransduction genes, at least in rods (Felder-Schmittbuhl et al., 2017 for review). For instance, we observed downregulation of *Grk1*, encoding the kinase that phosphorylates rhodopsin and in turn contributes to termination of phototransduction (reviewed in Arshavsky, 2002) and subsequently to the maintenance of rod sensitivity (Cideciyan et al., 1998; Sakurai et al., 2011). Concurrent circadian oscillations of Grk1 transcripts (Kunst et al., 2013), protein phosphorylation (Horner et al., 2005; Osawa et al., 2011) and abundance of the *Grk1* inhibitor recoverin (Zernii et al., 2011; Grigoriev et al., 2012) is consistent with a key role of *Grk1* in regulating the daily adjustment of light processing and sensitivity (reviewed in Barlow, 2001). Deficiency of *Grk1* causes light-dependent retinal degeneration (Chen et al., 1999) and is responsible for the Oguchi form of congenital stationary night blindness (Hayashi et al., 2007; Oishi et al., 2007). Hence, the dramatic decrease in photoreceptor genes such as Grk1 could be involved in the worsened retinal phenotype of the DM. However, downregulation might also be the consequence of the rod degeneration.

An alternative hypothesis is that *Bmal1* might act through the loss of homeostatic mechanisms such as regulation of metabolism and of oxidative stress that play crucial roles in rods (Newton and Megaw, 2020). Data from the literature showed the circadian clock plays a broad role in maintaining health during aging. Indeed, mice deficient in *Bmal1* display reduced lifespan and symptoms of premature aging, including cataracts and neurodegeneration (Kondratov et al., 2006; Musiek et al., 2013). Similarly, circadian disruption induced by exposure to chronic jet lag reduces lifespan and alters brain activity in animal models (Davidson et al., 2006; Gao et al., 2020). In human, circadian misalignment or clock (BMAL1) gene mutation has been associated with occurrence of neurodegenerative diseases such as Alzheimer's and Parkinson's disease (Chen et al., 2015; Gu et al., 2015; Bokenberger et al., 2018). The link between clock gene mutation and neuronal pathology in mice involves impairment of redox homeostasis (Musiek et al., 2013). Oxidative and reductive cycles synchronize to 24h rhythms through tight regulation of metabolism by circadian clocks, at least in the periphery, but this relationship has not been clearly demonstrated in the central nervous system (reviewed by Smith and Musiek, 2020). In peripheral cells, a strong reciprocal link exists between clock factors and metabolic processes, at several levels. Among these,

NAD(P)(H) is at the interphase between metabolism, circadian rhythms and aging (Levine et al., 2022). Also, mitochondria metabolic activity and nutrient use are under circadian control (Jacobi et al., 2015; Neufeld-Cohen et al., 2016). Our analysis of DM vs P23H Rho downregulated genes showed highly significant enrichment in metabolic pathways such as glycolysis, oxidative phosphorylation, and NADH regeneration, indicating these processes might be particularly dysfunctional in the DM. In addition, these genes are enriched in binding sites for the GABPA metabolism regulator. Key metabolism regulators such as *Sirt1* and *Pgc-1α* display daily mRNA expression rhythms in the retina (Ban et al., 2013; Kunst et al., 2013). Also, circadian time-affected genes in mouse photoreceptors are enriched in biological pathways related to metabolism (Milićević et al., 2021). However, the exact BMAL1 metabolic targets involved in the retinal phenotype of the DM remain presently to be identified.

The strength of our study is the use of a conditional, highly cell-specific knock out of *Bmal1*, the central actor of the molecular clock, combined with both functional and molecular approaches to understand interaction between clock and rod health. Unlike what has been proposed in the (Liu et al., 2012; Baba et al., 2018a), our data argue in favour of the existence of a circadian clock in rods, similar to cones, that not only controls their daily light sensitivity but also confers a neuroprotective role. Interestingly, our results are comparable to similar data obtained in drosophila. Indeed, Jauregui-Lozano showed the critical role of CLOCK:CYCLE complex in conteracting oxidative stress and ensuring photoreceptor homeostasis upon aging (Jauregui-Lozano et al., 2022). There are, however, also some limitations in our approach. Indeed, performing RNA-seq in retinas from 4 month animals likely precluded the identification early degenerative events induced by absence of BMAL1. In spite of that, our data establish the DM as a valuable, innovative experimental model to understand the importance of the circadian clock in photoreceptors.

5. Conclusion

Our results offer new molecular mechanisms about the role of the core circadian clock in retinal degeneration. It more specifically points to molecular mechanisms potentially linking clock, phototransduction cascade and/or metabolism in healthy and diseased retina. The uncovered molecular mechanisms linking circadian clock and retinal degeneration could inspire life style advice or may open new future treatment approaches for blinding disease.

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Supplementary methods

Locomotor activity recording

For behavioural recordings, animals were housed in individual standard cages equipped with infrared detectors placed above the cage and linked to an automated recording system (CAMS, Circadian Activity Monitoring System, Lyon, France) as previously described (Salaberry et al., 2019). Data were collected in 5 min bins and analysed with the ClockLab Software (Actimetrics, Wilmette, IL, USA). Locomotor activity data were represented as double-plotted in actograms.

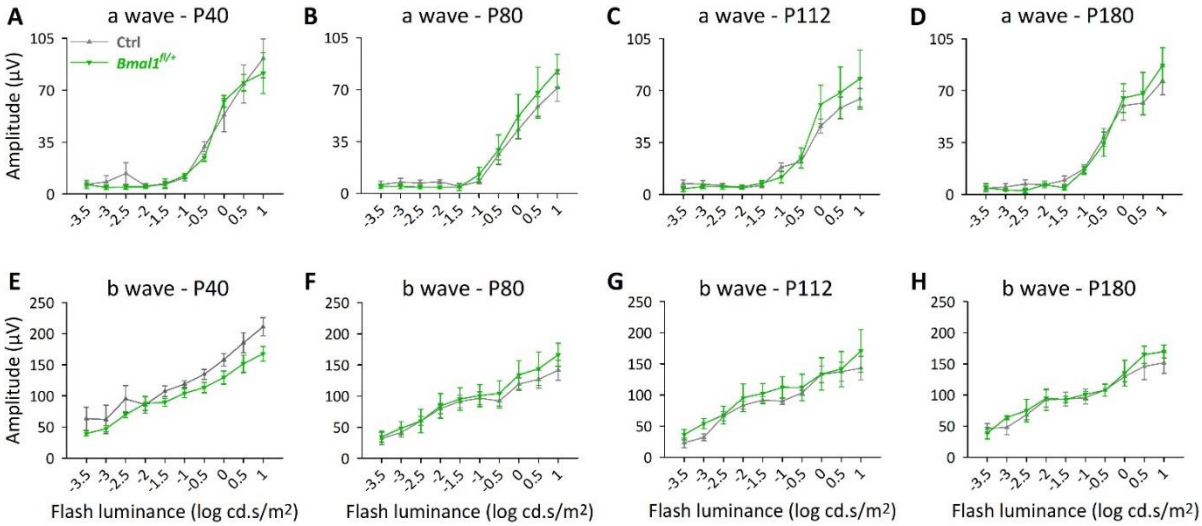
Bioluminescence recording

PER2::luciferase bioluminescence recordings were carried out on whole retina explants prepared from 1.5-3 month-old mice from the four genotype groups (*Per2*$^{Luc/Luc}$ background) as previously described (Jaeger et al., 2015). Eyeballs were dissected at room temperature in 1 x HBSS containing antibiotics (100 U/mL penicillin and 100 mg/ml streptomycin), 100 mM HEPES and 4.2 mM sodium bicarbonate. Retinas were carefully detached from the retinal pigment epithelium, flattened with small radial incisions and placed, photoreceptors down, onto a semipermeable membrane (Millipore, Billerica, MA, USA) in a 35 mm culture dish containing pre-incubation medium [1 ml neurobasal A medium (Gibco, Invitrogen, Life Technologies, Carlsbad, CA, USA) supplemented with antibiotics (25 U/ml penicillin and 25 mg/mL streptomycin, Sigma-Aldrich), 2% B27 (Invitrogen, Life Technologies, Grand Island, NY, USA), and 2 mM L-glutamine (Gibco, Life Technologies, Carlsbad, CA, USA). Samples were kept 24 h at 37°C in a humidified 5% CO2 incubator then the medium was changed with pre-warmed (37°C) 199 recording medium [1 mL medium 199 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with antibiotics (25 U/mL penicillin and 25 mg/mL streptomycin, Sigma-Aldrich), 4 mM sodium bicarbonate, 20 mM D(+)-glucose (Sigma-Aldrich), 2% B27 (Invitrogen), 0.7 mM L-glutamine (Gibco), and 100 mM beetle luciferin (Promega, Fitchburg, WI, USA)]. The medium change was performed under dim red light. Dishes were sealed with high-vacuum grease (Dow Corning; Midland, MI, USA) and placed into the LumiCycle (Actimetrix, Wilmette, IL, USA) heated at 36°C. Samples were recorded during 6-8 days and the photons were integrated for 112 s every 15 min.

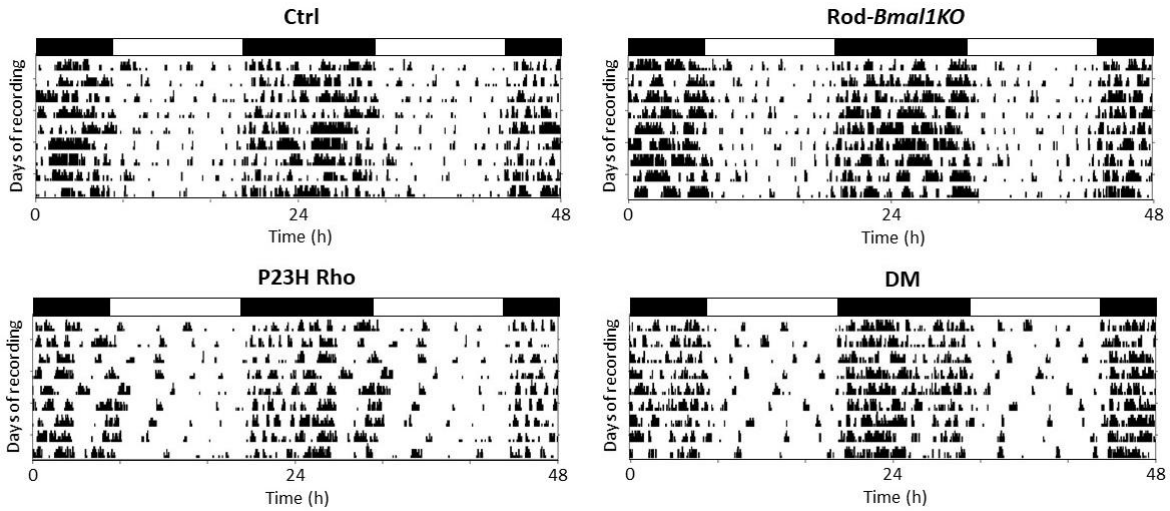
RT-qPCR analysis

150 ng total RNA from whole retinas (see RNAsequencing) 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). Statistical analysis of relative expression levels was performed by One-way ANOVA and Holm-Sidak's post hoc testing (Sigmaplot V12, Systat Software Inc.)

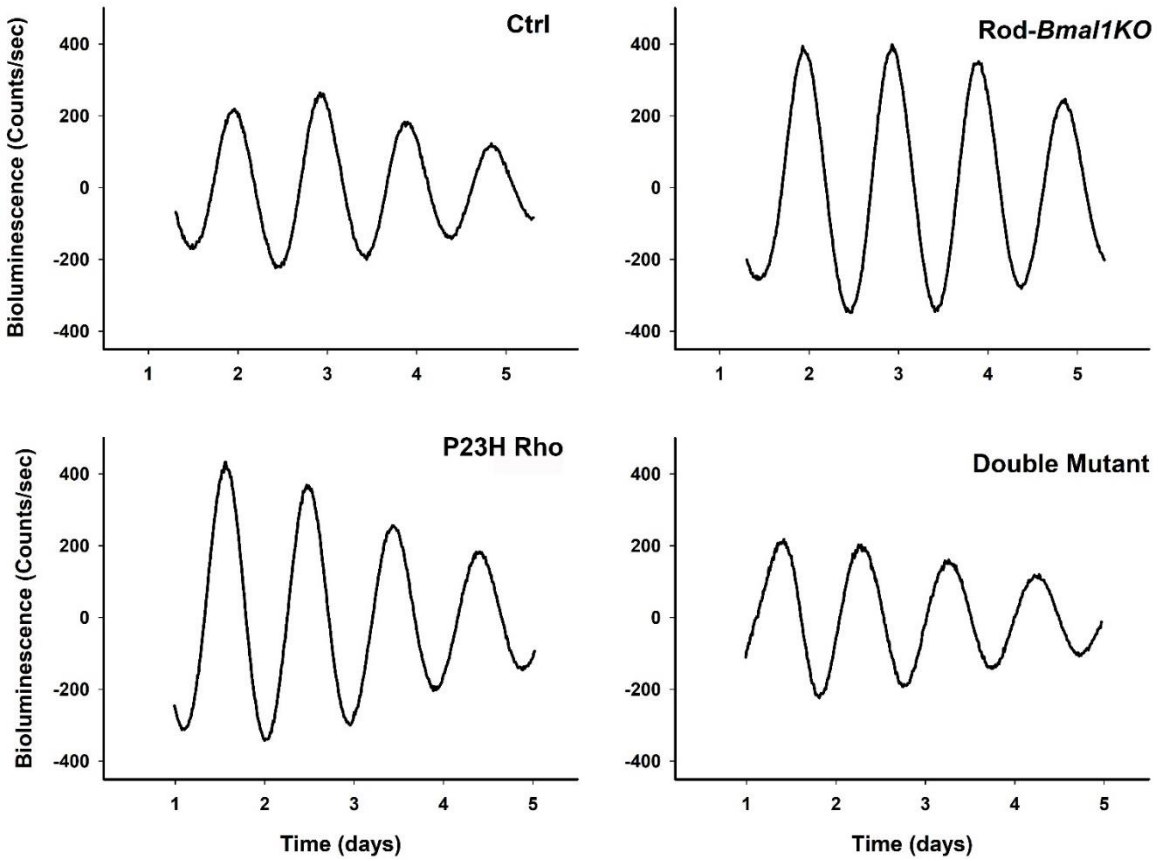
Supplementary material



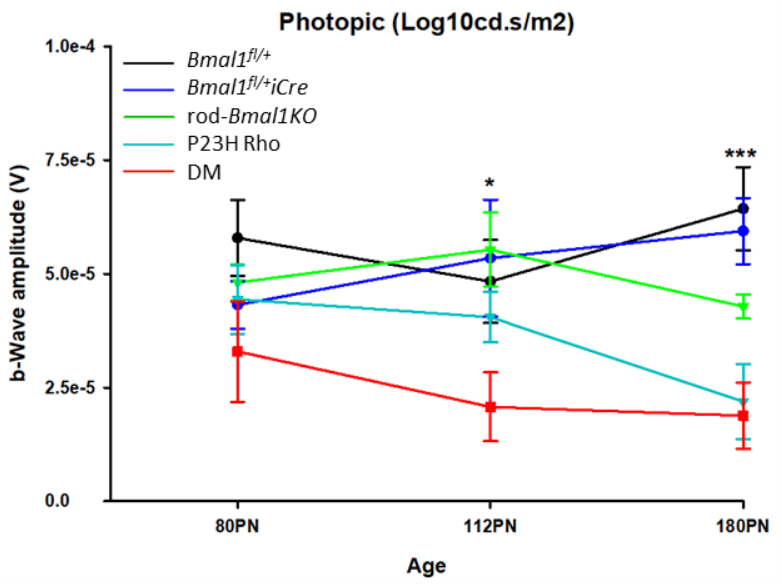
Supplementary Figure 1. Comparison of scotopic ERG amplitudes in Ctrl and *Bmal1*$^{fl/+}$ mice shows that the loss of one *Bmal1* allele does not impact light response between P40 and P180. Amplitudes of a- (A-D) and b-(E-H) waves are presented according to intensity of the light stimulus in Ctrl (*Bmal1*$^{fl/+}$*; Rho-iCre/+*, gray) and *Bmal1*$^{fl/+}$ (green) mice. Comparison between genotypes was performed by 2-way repeated measures ANOVA (n = 6-7, n = 6-8, n = 6-8, and n = 5/genotype at P40, 80, 112, and 180, respectively).



Supplementary Figure 2. Representative actograms of mice from the different genotype groups, exposed to a 12h/12h LD cycle, showing their capacity to entrain to this environmental synchronizer. White and black bars indicate day and night periods respectively.

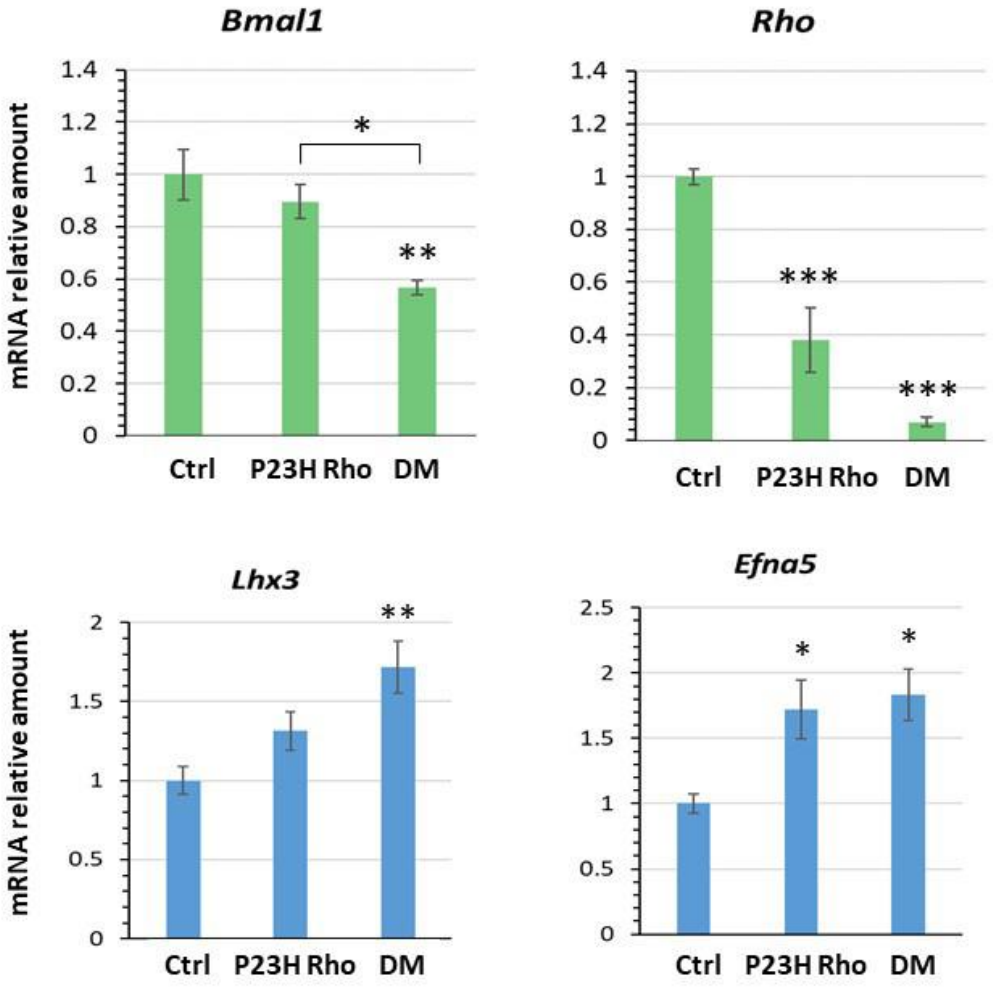


Supplementary Figure 3. Whole retina explants from the distinct genotype groups show intact PER2::Luciferase rhythmicity. PER2::Luciferase activity was recorded in real-time from retinal explants sampled from mice on the *Per2*$^{Luc\ KIKI}$ background, aged 1.5 to 3 months. Representative bioluminescence traces from Ctrl, rod-*Bmal1KO*, P23H Rho, and Double mutant retinas are shown.



*P<0.05 (DM Vs *Bmal1*$^{fl/+}$; *Bmal1*$^{fl/+}$*iCre*)
***P<0.001 (P23H Rho Vs *Bmal1*$^{fl/+}$; *Bmal1*$^{fl/+}$*iCre*; rod-*Bmal1KO*)
(DM Vs *Bmal1*$^{fl/+}$; *Bmal1*$^{fl/+}$*iCre*; rod-*Bmal1KO*)

Supplementary Figure 4. Light adapted (Photopic) ERG was performed on all genotype groups at P80, P112 and P180. Amplitudes of the b-wave at 10 cd.s/m^2 are shown. Significant differences between genotypes are indicated (n=5-8 per genotype). *Bmal1*$^{fl/+}$*iCre/+* = Ctrl.



Supplementary Figure 5. RT-qPCR analyses of a panel of genes to validate the results from RNA-seq analyses (same mRNAs) for the P23H Rho and DM retinas. mRNA levels were compared to retinas from Ctrl mice. Expression patterns all showed significant genotype effect by One way ANOVA analysis ($p < 0.05$). Significant differences between genotypes, based on Holm-Sidak post hoc analysis, are indicated: * $p<0.05$, * $p<0.01$, * $p<0.001$ (n=4 per genotype).

Part III

Roles of rod photoreceptors to the retina clock network

Chapter 6

Enhanced robustness of the mouse retinal circadian clock upon inherited retina degeneration

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Enhanced robustness of the mouse retinal circadian clock upon inherited retina degeneration

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Abstract

Daily biological rhythms are fundamental to retinal physiology and visual function. They are generated by a local circadian clock composed of a network of cell type/layer-specific, coupled oscillators. Animal models of retinal degeneration have been instrumental in characterizing the anatomical organization of the retinal clock. However, it is still unclear, among the multiple cell-types composing the retina, which ones are essential for proper circadian function. In the present study, we used a previously well-characterized mouse model for autosomal dominant retinitis pigmentosa to examine the relationship between rod degeneration and the retinal circadian clock. This model carries the P23H mutation in rhodopsin, which induces mild rod degeneration in heterozygous and rapid loss of photoreceptors in homozygous genotypes. By measuring PER2::LUC bioluminescence rhythms, we show that the retinal clock in *P23H/+* heterozygous mice displays circadian rhythms with significantly increased robustness and amplitude. By treating retinal explants with L-α aminoadipic acid, we further provide evidence that this enhanced rhythmicity might involve activation of Müller glial cells.

Key words: retinitis pigmentosa, circadian clock, retina, photoreceptor, P23H, PER2::LUC bioluminescence

Introduction

Twenty-four hour biological rhythms are a fundamental hallmark of every cellular and physiological process, running in synchrony with the light/dark (LD) cycle (Albrecht, 2012). These rhythms are generated by molecular clocks widely distributed in tissues and orchestrated by the principal pacemaker in the suprachiasmatic nuclei (SCN). The main synchronizing agent to the SCN is environmental light, perceived by classical retinal photoreceptors (PRs: rods, cones) and melanopsin-containing photosensitive retinal ganglion cells. Intracellular circadian clocks generate timing through transcriptional/translational feedback loops involving transcription factors encoded by clock genes (e.g., *Bmal1*, *Clock*, *Per1-3*, *Cry1-2*). This leads to rhythmic expression of clock factors with a ~24h period, driving gene expression programs and cellular functions along with the LD cycle. In the vertebrate retina, many rhythmic processes have been documented. Examples that optimize vision and retinal survival include electroretinogram (ERG) amplitudes, melatonin and dopamine release, shedding and phagocytosis of PR outer segments (McMahon et al., 2014). Daily retinal rhythms are controlled by a self-sustained circadian clock system distributed across the distinct cellular layers, including PR and pigmented epithelium, and that is entrained by light (Tosini and Menaker, 1996; Jaeger et al., 2015; Milicevic et al., 2021). We recently reported that a circadian clock located in rods regulates visual light processing in a cell autonomous manner (Gegnaw et al., 2021). However, how individual cell types are connected within the retinal clock network and contribute to the overall rhythmic retinal function is not yet understood.

Animal models of retinal degeneration (rd) have been widely used to understand light entrainment mechanisms in SCN and retina (Calligaro et al., 2019; Foster et al., 2020). Indeed, retinal degenerations are genetically and clinically heterogeneous, affecting various cell types. This provides the opportunity to investigate distinct aspects of rod/cone function in the circadian system. Photoreceptor degeneration also triggers substantial changes in the inner retina, in particular in Müller glial cells (MGC) that modify their gene expression programs as a cellular attempt to slow down photoreceptor death and to promote tissue repair. This activation of MGC, or gliotic response, includes metabolic alterations such as

increased glial fibrillary acidic protein (GFAP) expression and altered glutamate uptake, and release of neuroprotective factors and antioxydants, among others (Fletcher, 2000; Nagar et al., 2009; Bringmann and Wiedemann, 2012; Roesch et al., 2012; Jones et al., 2016; Tomita et al., 2021). In this study we questioned the role of rods in the retinal circadian network by using a mouse knock-in model of the human P23H rhodopsin mutation, associated with autosomal dominant retinitis pigmentosa (Sakami et al., 2011). We show that mildly degenerating retinal explants carrying the heterozygous P23H mutation display enhanced circadian rhythmicity of PER2 protein expression that might involve glial cell activation.

Materials and Methods

All experimental protocols were carried out according to the European Parliament and The Council of the European Union Directive (2010/63/EU) and institutional ethical guidelines (APAFIS#10213-2017060920001367-v3). Animals were maintained in the Chronobiotron animal facility (UMS 3415, Strasbourg) under 12h/12h LD cycle (300 lx and < 5 lx dim red light during light and dark, respectively) at a 22 ± 1°C ambient temperature, with free access to food and water. Mice carrying the P23H mutation knock-in in the rhodopsin gene (Sakami et al., 2011) and the PER2::Luciferase reporter gene (Yoo et al., 2004) (Jackson Laboratories, USA) were bred together to generate following genotypes: *P23H/P23H* homozygotes ($Rho^{P23H/P23H}$), *P23H/+* heterozygotes ($Rho^{P23H/+}$) and their wild type (WT) littermates, all on the C57BL/6J and $Per2^{Luc/+}$ backgrounds. Animals were genotyped by PCR on tail genomic DNA (Yoo et al., 2004); primers for P23H knock-in detection: 5'-GCCTGTTTAGCTGAGAAAAC-3' and 5'-GACCACGTAACAAACTTCTG-3'. The retinal status in the distinct genotype/age groups was assessed by morphological analysis using hematoxylin/eosin staining (Supplementary Figure S1). The results are in agreement with what was previously described for mice carrying heterozygous and homozygous versions of the P23H mutation at various ages (Sakami et al., 2011).

Whole retina circadian rhythms were assessed by PER2::Luciferase bioluminescence recordings carried out at postanatal days 36 (P36) and 70 (P70) as previously described (Jaeger et al., 2015). Oscillations were analysed using the Lumicycle Analysis software (Actimetrics, USA). Raw data (counts per second, cps) were baseline-subtracted (24h running average). Periods and amplitudes (height of the 3 first peaks) were determined by using LMFit (damped sine). The relative rhythmic power was determined by using Periodogram function

of Lumicycle Analysis to evaluate robustness of rhythms (Ruan et al., 2012; Buonfiglio et al., 2014; Herzog et al., 2015). Analyses were performed on 3 complete cycles after exclusion of the first oscillation. Glial cell involvement was assessed using the metabolic inhibitor of glial cells, L-α aminoadipic acid (L-AAA), on retinal explants from 3-month-old mice (Jablonski and Iannaccone, 2000; Thangaraj et al., 2015). After 3 complete cycles of control recording (baseline), medium was replaced with a fresh medium containing L-AAA [0.4mM; (Brown and Kretzschmar, 1998)] or vehicle (treatment; 3 complete cycles) and finally the medium was refreshed (washout). L-AAA effects were evaluated by calculating the ratio of first peak amplitudes for treatment or washout vs baseline. The lack of effect of the gliotoxin on cell survival was assessed by TUNEL assay on WT retina explants (Supplementary Figure S4).

To assess central clock rhythms, 1 mm2 SCN-containing explants were dissected from fresh brain coronal sections and cultured as described (Salaberry et al., 2017). Period, amplitude, phase were determined with the Sin Fit (damped) function (Lumicycle Analysis).

General locomotor activity of 8-month-old mice was recorded using infrared motion captors placed above cages, connected to a Circadian Activity Monitoring System (CAMS, INSERM, France), 12 days in LD, then 12 days under constant darkness conditions (DD). Data were analysed using ClockLab (Actimetrics).

Results are presented as means ± SEM. Statistical analysis was performed as described in figure legends (Sigma Plot 13, Systat Software, USA). When required, ANOVAs were followed by post hoc Holm-Sidak multiple comparisons tests. Significance level was set at $P < 0.05$.

Results

We characterized the effects of distinct degrees of rod degeneration on the retinal clock activity in WT, *P23H/+* and *P23H/P23H* mice sampled at two ages. First, we measured at P36, the time point at which P23H/+ mice display minor visual impairment (supplementary Figure S1) and retain ≈50% of their photoreceptors (Sakami et al., 2011). Interestingly, retinal explants from all genotypes displayed sustained oscillations in PER2::Luciferase activity (Figure 1a-c), with similar periods close to the one previously reported in *mPer2*^{Luc} mice (Jaeger et al., 2015) (WT 22.79 ± 0.07 h, *P23H/+* 22.76 ± 0.15 h, *P23H/P23H* 23.08 ± 0.26 h) (Figure 1d). Unexpectedly, retinas from *P23H/+* heterozygous mutants showed the most robust oscillations, as exemplified by their rhythmic power (1.96 ± 0.02) that was significantly

higher than in WT (1.75 ± 0.05) (Figure 1e). This result was corroborated by the amplitude values, significantly higher in *P23H/+* retinas than in WT (Figure 1f). The *P23H/P23H* mutants, which lose 90% photoreceptors by P36 (Sakami et al., 2011), showed a relative rhythmic power comparable to WT retinas (1.79 ± 0.05) (Figure 1e).

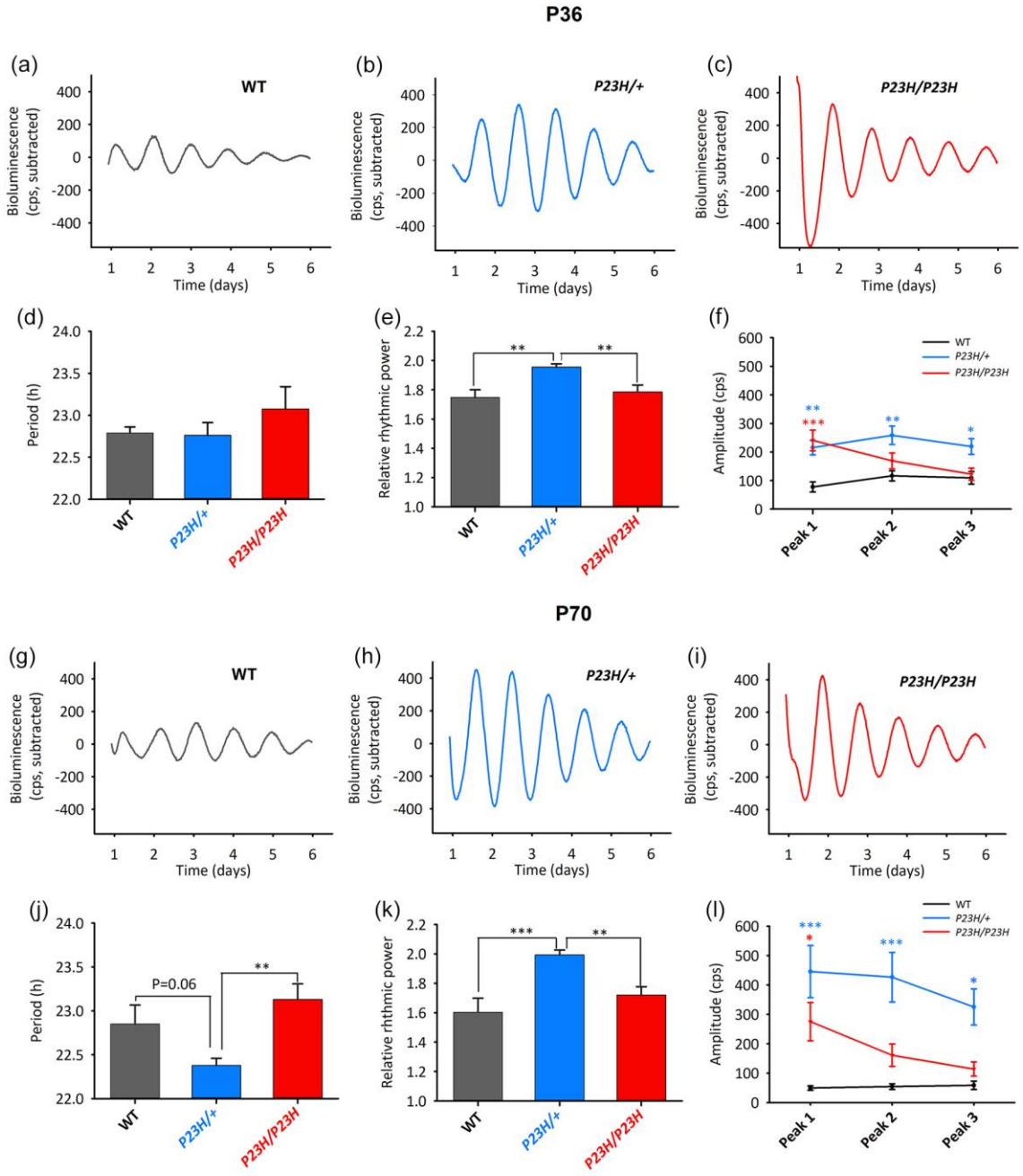

Figure 1. Rod degeneration affects robustness of the retinal circadian clock. PER2::Luciferase activity was analyzed in retinal explants sampled at P36 (a-f) and P70 (g-l). Representative bioluminescence traces from WT (a, g), *P23H/+* (b, h), and *P23H/P23H* (c, i) retinas. At P36 (N=11-16 per genotype) explants from all groups exhibited similar periods (p=0.406, one-way ANOVA) (d) but *P23H/+* samples showed highest relative rhythmic power (e) (genotype effect, p<0.001). (f) Genotype effects were also observed for amplitudes of the three first peaks (genotype, p= 0.003; Genotype × Peak, p<0.001; two-way repeated measures ANOVA). At P70 (N=13-17 per genotype), there was a genotype effect on the period (p=0.002,

one-way ANOVA) (j), the relative rhythmic power ($p<0.001$) (k), and the amplitudes at the distinct peaks (genotype, $p=0.001$; Genotype × Peak, $p<0.001$) (l). WT=wild-type. ***$p<0.001$; **$p<0.01$; *$p<0.05$.

We next analyzed bioluminescence activity in P70 retinas (Figure 1g-i). Compared to P36, visual ERG response at P70 is further impaired in *P23H/+* mutants (supplementary Figure S2). Nonetheless, at this age the *P23H/+* retinas displayed circadian oscillations similar to those at P36, yielding the largest robustness. This was shown by relative rhythmic power values (1.99 ± 0.03) which were significantly higher than in WT (1.71 ± 0.06) and homozygotes (1.79 ± 0.03) (Figure 1k) and by amplitudes being significantly larger in heterozygotes than in WT retinas (Figure 1l).

At both P36 and P70, amplitudes of the *P23H/P23H* samples were decreasing from high at peak 1 (similar to *P23H/+*) to low (similar to WT) at peak 2 and 3 (Figure 1f,l). Uniquely at P70, the heterozygous mutant retinas also displayed a trend for shortened period (22.38 ± 0.08 h) with respect to WT (22.65 ± 0.19 h) and were significantly shorter with respect to homozygous retinas (23.10 ± 0.15 h) (Figure 1j). Interestingly, retinas carrying another type of severe rod degeneration induced by the *rd10* recessive mutation of the rod phosphodiesterase beta subunit (Chang et al., 2002; Gargini et al., 2007), display similar circadian activity as the *P23H/P23H* retinas; e.g. increased amplitudes but similar periods and rhythmic power with respect to their controls (supplementary Figure S3). Taken together, the above results suggest that the retinal circadian clock becomes more robust upon ongoing rod degeneration.

We hypothesized that activation of Müller glial cells (MGC) might be involved in the enhanced oscillating capacity of the *P23H/+* mutant. We treated retinal explants after 3 days of baseline oscillations with the L-AAA (0.4 mM) gliotoxin. During the 3 days of drug-exposure oscillations of WT and *P23H/P23H* explants displayed dramatic amplitude reduction and, accordingly, period could not be determined (Figure 2a, c, d, f). In contrast, rhythms of *P23H/+* retinas were barely affected by the drug, suggesting that activation of MGC in the degenerating retina, might compensate for the inhibitory effect of L-AAA (Figure 2b, d, f). Likewise, *P23H/+* retinas mostly behaved like vehicle controls upon drug washout, whereas WT and *P23H/P23H* samples retained reduced amplitudes and showed altered periods (Figure 2e,g).

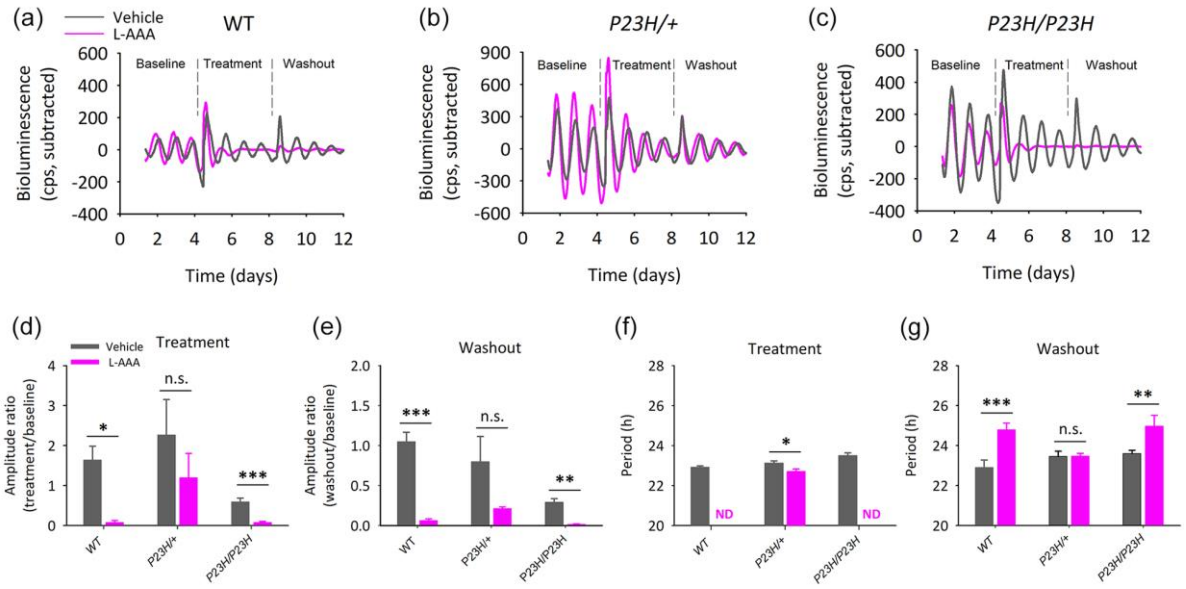

Figure 2. PER2::Luciferase oscillations are not affected by L-AAA treatment in *P23H/+* retinas. (a-c) Representative bioluminescence profiles in retinal explants from 3-month-old WT (a), P23H/+ (b) and *P23H/P23H* (c) mice upon baseline, treatment with 0.4 mM L-AAA and washout (N=5-6 per genotype and treatment group). (d) L-AAA strongly affected oscillation amplitudes (effect of treatment, p=0.019; genotype, p=0.026, two-way ANOVA). It induced marked amplitude reduction in WT and *P23H/P23H* retinas but had no significant effect in *P23H/+* samples (p= 0.362) (Student t test). (e) Differential effects of L-AAA on the three genotypes persisted upon washout (effect of treatment, p<0.001; genotype, p=0.015, two-way ANOVA and Student t tests). (f) Oscillations were abolished in WT and *P23H/P23H* samples during L-AAA treatment, but were retained in *P23H/+* retinas with minor period alteration (p=0.041, Student t test). (g) upon washout PER2 oscillations were measurable in retinas from all genotypes but were differentially affected by previous L-AAA treatment (treatment effect, p<0.001; Treatment × Genotype, p=0.029, two-way ANOVA). n.s.=not significant; ND=not determined; WT=wild-type; ANOVA= Analysis of variance; L-AAA =L-α aminoadipic acid. ***p<0.001; **p<0.01; *p<0.05.

We finally evaluated whether a retina with increased clock robustness would affect the SCN clock functionality. We first analyzed locomotor activity rhythms in WT and *P23H/+* mice (Figure 3a-b). Animals from both genotypes were synchronized to the LD cycle, and showed similar periods of circadian locomotor activity under DD conditions (WT 23.92 ± 0.00 h; *P23H/+* 23.95 ± 0.02 h). Likewise, SCN explants did not show any significant difference in period, amplitude or phase of PER2::Luciferase oscillations (Figure 3c-f). Thus, increased robustness of the retinal clock does not influence the central clock and the downstream rest/activity cycle.

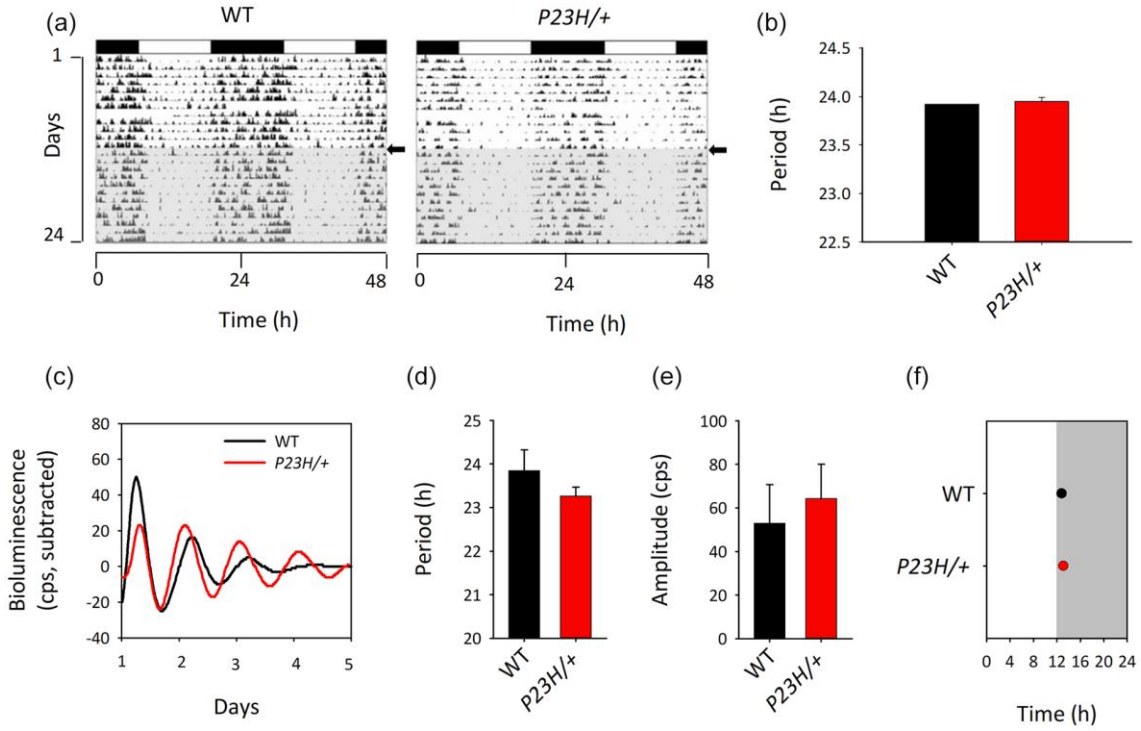

Figure 3: The P23H/+ mutation does not induce alterations of the central clock. (a) Representative actograms of *P23H/+* and WT littermates in LD (white/black bars: light/dark cycle) and DD (gray background) showing similar light-entrained behavior and endogenous rhythmic activity, as confirmed by periods in DD (p=0.730, N=4-5) (b). (c-f) SCN sampled at the end of the behavioral experiment showed no significant difference in bioluminescence profile (c), period (d), amplitude (e) and phase (f) (N=3-6). WT=wild-type; DD= constant darkness; SCN=suprachiasmatic nuclei; LD= light/dark.

Discussion

We recently reported evidence indicating that rods harbor a circadian clockwork that regulates daily visual light processing in a cell autonomous manner, since rod-specific Bmal1 disruption abolishes the circadian day–night differences in dark-adapted ERG a- and b-wave amplitudes (Gegnaw et al., 2021). By contrast, absence of clock in rods did not exert major effect on the global retinal clock, as assessed by PER2::Luciferase bioluminescence in explants. To get further insight into the contribution of rods to the clock network, we here addressed the potential relationship between degeneration of these cells and retinal circadian clock activity. We provide evidence that mice with a heterozygous P23H rhodopsin mutation, that induces slow rod degeneration, display increased robustness and amplitude of the retinal clock at both P36 and P70. Previous histological analyses in this model showed that degeneration at these ages is progressing but not complete (Sakami et al., 2011), as currently confirmed by our histological analysis (supplementary Figure S1) and ERG recordings (supplementary Figure S2). It was reported that PR affect retinal clock activity but the

underlying mechanisms remain unknown. In another animal model, the RCS/N-rdy rats displaying extensive PR degeneration, clock gene expression measured in whole retinas was perturbed but no major change was observed for *Per2* mRNA rhythms (Tosini et al., 2007b). In yet another study, retinal explants from rd1 homozygous mice displayed sustained rhythms in PER2::Luciferase activity, whereas their heterozygous littermates (with normal retinas) were reported not to show retinal rhythms (Ruan et al., 2006). Our results provide a more complex picture of PR contribution to the retinal clock network, pointing at a large, likely transient, enhancing effect when their degeneration is ongoing. The enhanced robustness of the *P23H/+* retinal clock (higher rhythmic power than in the other genotypes) might be due to a stimulatory effect induced by dying rods on gene expression in inner retinal cells, at this early stage of retinal remodeling (likely phase 1 of retinal degeneration) (Pfeiffer et al., 2020). The moderate phenotype of the homozygous mutant retinas indicates that this enhancing effect might fade once most PR are gone (likely phase 2).

It is indeed well-known that remodeling of the retinal (clock) network occurs upon PR death (Marc et al., 2003), which might also affect rhythms in the remaining cells. MGC are first line reactive cells following PR degeneration, with notably gliosis and early phagocytosis of dying cells (Bringmann and Wiedemann, 2012; Sakami et al., 2019). Acute PR degeneration in zebrafish triggers MGC to regenerate neurons (Hamon et al., 2016). This has been associated with induction of a specific gene expression program supporting the regenerative capacity, that includes activation of clock genes (Sifuentes et al., 2016). Basing on these results, we here questioned whether the enhancement of retinal PER2 oscillations in the *P23H/+* mutant, at least in part, might be due to activation of MGC and of their clock. We reasoned that MGC stimulation might counterbalance the deleterious effects of the L-AAA gliotoxin. Indeed, L-AAA treatment drastically reduced oscillating capacity in WT retinas, but did not affect rhythms in *P23H/+* retinas. The effect of L-AAA on rhythms in WT is in agreement with the fact that MGC contain a circadian clock and supports the idea that this clock contributes to the overall oscillating capacity of the retina (Xu et al., 2016). By contrast, retinas from *P23H/+* mice appear resilient to 0.4mM L-AAA treatment. This corroborates our hypothesis that early rod degeneration induces early stage retinal remodeling with glial cell activation that can counterbalance the deleterious effects of the drug. Strikingly, retinas from homozygous *P23H/P23H* mice were affected by L-AAA treatment similar to WT. One explanation for this

finding is that in *P23H/P23H* retinas, that have lost almost all photoreceptors at 3 months, the early glial cell activation is over and the drug can further exert its deleterious effect on glial cells and the global retinal clock. A limitation of our study is that, besides MGC stimulation, rod degeneration also activates more global phenotypic changes in the retina, in particular inflammatory/immune response and activation of microglial cells (Olivares-Gonzalez et al., 2021) and rewiring inside the retinal network (Marc et al., 2003). Thus, we cannot exclude that enhanced rhythmicity in *P23H/+* retinas also involves activation of other clock-carrying retinal cell type(s).

We also assessed whether this enhanced rhythmicity of *P23H/+* retinas affects the circadian system more generally but we did not find any difference with controls, neither in locomotor activity rhythms nor in PER2 oscillations in the SCN. Previously, Storch and coworkers reported that behavioral rhythms are normal in mice with retina-specific *Bmal1* deletion (Storch et al., 2007). Thus, even if cellular content of the retina can profoundly impact the SCN (Lupi et al., 1999; Tosini et al., 2007a), its circadian clock itself does not appear to play an overt role. In agreement with this result, our preliminary data indicate minor effect of the *P23H/+* mutation on melanopsin expression (data not shown), which confirms previous conclusion from investigation of rodless/coneless mice (Semo et al., 2003).

In conclusion, retinal clock rhythmicity is enhanced when rods are degenerating, which likely involves activation of glial cells and suggests that rods contribute minorily to the robustness of the retinal clock network. These results provide new insights into the cellular/molecular events that are turned on in mice, when PR degenerate.

Acknowledgements

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Conflicting interest statement

The authors declare that there is no conflict of interest.

NOTE: Supplementary material is available for this article online.

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Supplementary material

Supplementary methods

Histological examination of retinas by light microscopy was performed as previously described (Ait-Hmyed et al., 2013). Briefly, eyes previously fixed in 4% paraformaldehyde, were dehydrated in an ascending ethanol series, transferred to toluene and embedded in paraffin wax by conventional techniques. Sections (5 µm) were cut on a microtome, collected on microscope slides and subjected to staining with Mayer's Hemalum and counterstaining with Eosin Y. Images were captured from the posterior eyecup at 1 mm from the optic nerve head by using a NanoZoomer S60 Digital slide scanner C13210 (Hamamatsu) at 40x, in bright field.

The rd10/rd10 (Chang et al., 2002) mouse line (Pde6brd10, The Jackson Laboratory, Bar Harbor, ME, USA, kindly provided by Dr. Jérôme Roger, Centre d'Etude et de Recherche Thérapeutique en Ophtalmologie, Retina France, Orsay, France) was maintained on the C57BL/6J and heterozygous mPer2Luc background and raised in the Chronobiotron animal facility (CNRS UMS 3415, Strasbourg) under 12h/12h LD, ambient temperature of 22 ± 1°C, and with free access to food and water.

Dark-adapted (scotopic) ERG recordings were performed according to previously described procedures (Tanimoto et al., 2009; Ait-Hmyed Hakkari et al., 2016), on three groups of mice (n = 4-5 per group): one month-old (1m) and 2month-old (2m) RhoP23H/+ and 2.5 month-old wild type mice. Mice were dark-adapted overnight and anesthetized with a combination of xylazine and ketamine in Ringer solution at 30 µl/g [Rompun 2% = 2.0g/100ml (Bayer, Puteau, France); Imalgene 1000 = 100mg/ml (Merial, Lyon, France)]. Scotopic ERG was recorded from both eyes using corneal/active electrodes (thin gold-wire with a 2-mm ring end). Ocrygel (Virbac, Carros, France) eye drop was applied to ensure good electrical contact and to keep the cornea hydrated during the entire procedure. Both eyes were exposed to white flashes of ascending intensities.

Apoptosis was assessed using TUNEL (Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labelling) assay in whole retina explants cultured for 4 days in standard bioluminescence recording conditions followed by 24h in the presence of 0.4 mM L-AAA. Retinas were fixed on membrane for 1h in 4% PFA, gently washed with PBS,

embedded in OCT and frozen for cryostat sectioning (-22°C). 10 µm transversal sections were pretreated 30 min at room temperature with Proteinase K (10 µg/ml in PBS, Roche) and permeabilized with Triton X-100 0.1%/ sodium citrate 0.1%/PBS (8 min, room temperature). Sections were incubated with solution mixture (In situ Cell Death Detection Kit-TMR red, Roche Diagnostics, Basel, Switzerland) in a dark humid chamber at 37 °C for 1 h according to the manufacturer's instructions; positive and negative controls were included. Cell nuclei were also stained with DAPI. Sections were mounted with PBS/glycerol 1:1 and then observed under a fluorescence microscope (Nikon). The total number of apoptotic cells in the three different nuclear layers was manually counted.

Supplementary data

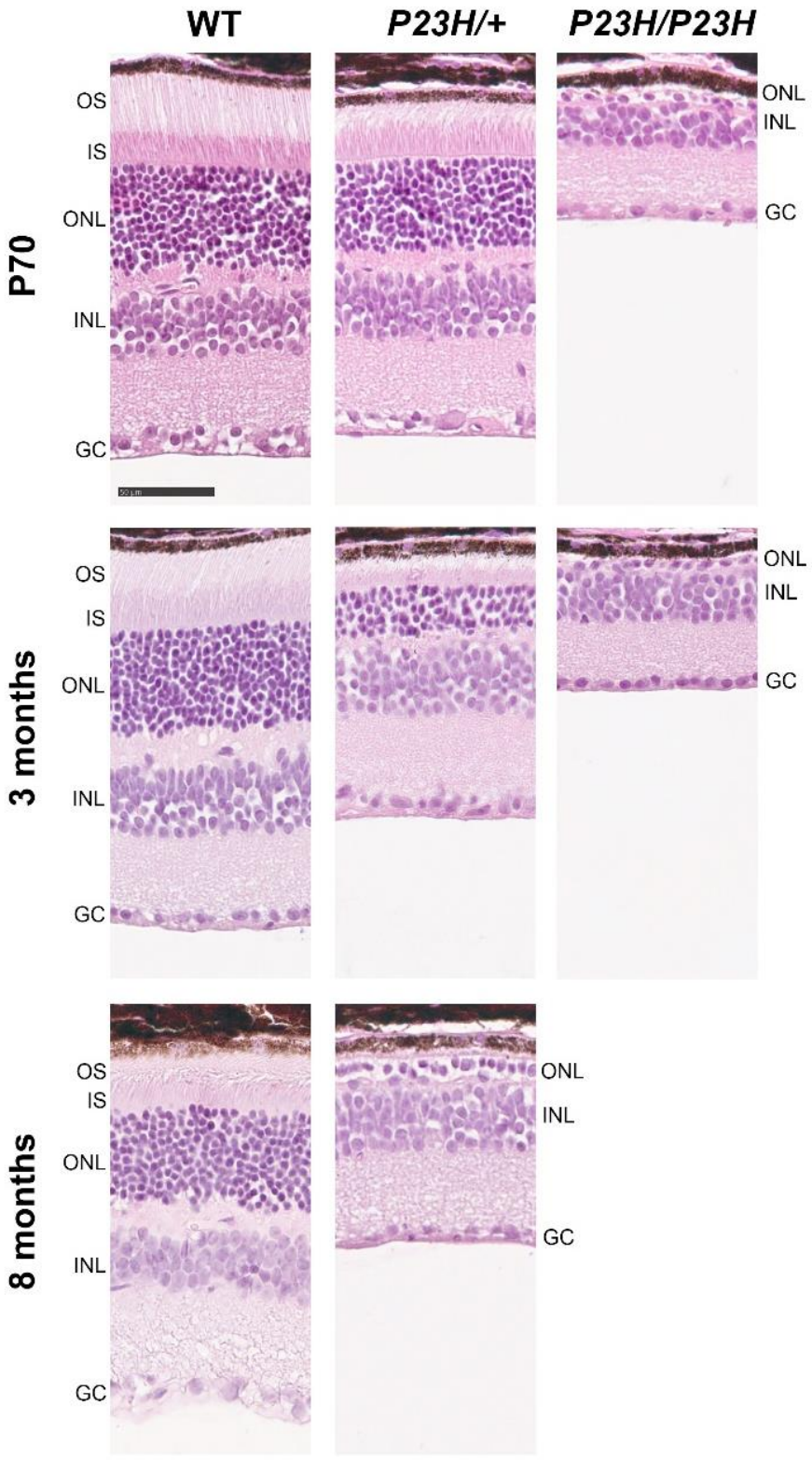

Figure S1. Retinal morphology in WT, heterozygous and homozygous P23H mutant mice aged P70, 3 months and 8 months. Sections were collected from the posterior eyecup at 1 mm from the optic nerve head and subjected to hematoxylin/eosin staining. A reduction in the outer segment (OS) length and outer

nuclear layer (ONL) thickness is already visible in *P23H/+* mice at P70 and gets more pronounced at 3 months, resulting in an almost complete absence of the ONL at 8 months. In homozygous *P23H/P23H* mutant retinas the ONL is already mostly lost at P70. Retinal sections from 8 month-old mice were collected at the end of locomotor activity recordings (Figure 3A). IS, inner segment; INL, inner nuclear layer; GC, ganglion cells. Scale bar: 50 µm.

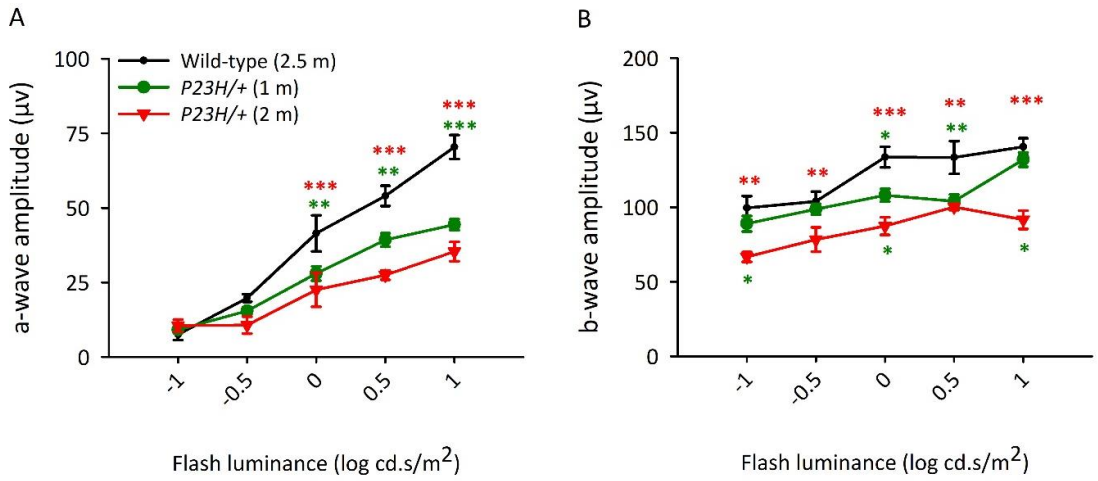

Figure S2. Dark-adapted ERG analysis. (A) and (B) show the mean a-wave and mean b-wave ERG amplitudes (respectively) measured by dark-adapted flash intensity series on 1 month old (1m, green lines) and 2 month-old (2m, red lines) *P23H/+* mice and 2.5 month-old (2.5m, black lines) wild-type mice (N = 4-5 per group). There was as significant effect of group and group x intensity interaction for amplitudes of both a-wave ($P < 0.001$ and $P < 0.001$, respectively) and b-wave ($P < 0.001$ and $P = 0.018$ respectively). 1m and 2m *P23H/+* mice displayed a significant decrease in amplitudes of both a-wave and b-wave, with respect to wild type mice (a wave: $P = 0.004$, $P < 0.001$ at 1m and 2m, respectively; b-wave: $P = 0.026$, $P < 0.001$, at 1m and 2m, respectively). 2m *P23H/+* mice also displayed a significant decrease in b-wave amplitude ($P = 0.014$) compared to 1m P23H/+ mice, but the difference in a-wave amplitude did not reach statistical significance ($P = 0.074$). ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. Data are presented as means ± SEM. 1m, 1-month-old; 2m, 2-month-old; 2.5m, 2.5-month-old.

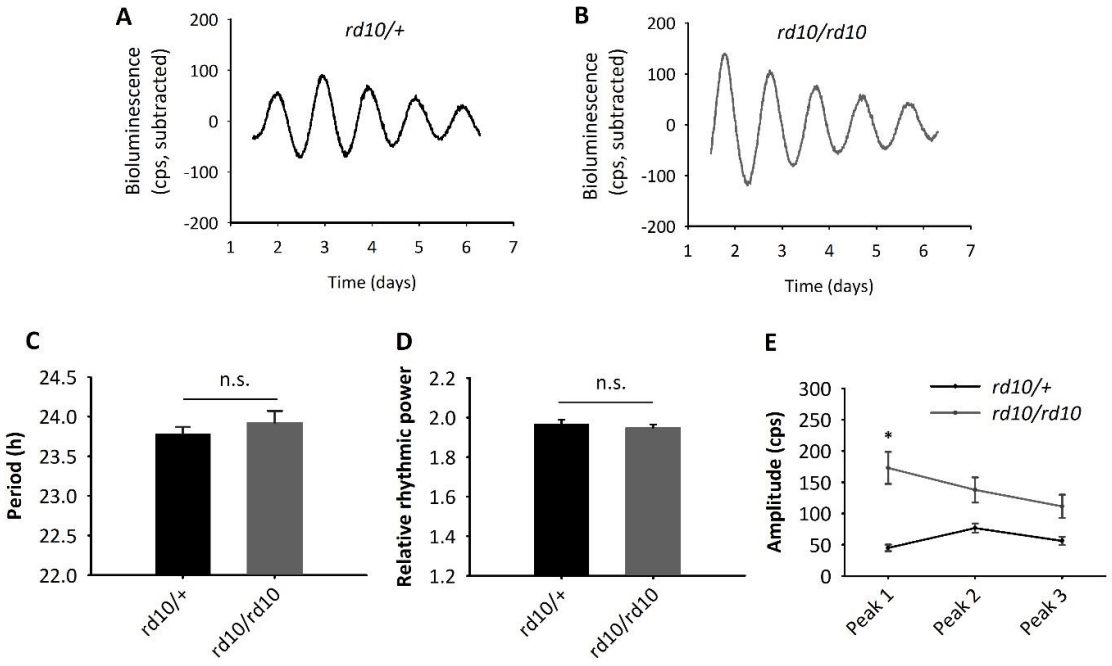

Figure S3. PER2::Luciferase activity was measured in retinal explants from 2 month-old *rd10/rd10* and *rd10/+* littermate control mice. Representative bioluminescence rhythms from rd10/+ (A) and *rd10/rd10* (B) retinas are shown. The retinas from *rd10/+* and *rd10/rd10* mice exhibited the same periods (C) and relative rhythmic power (D). (E) The amplitude of bioluminescence rhythms recorded from homozygous *rd10/rd10* mice was significantly higher than observed in control *rd10/+* mice (genotype P = 0.026, genotype x peak P < 0.001, *rd10/+* N = 4, rd10/rd10 N = 8, two-way RM ANOVA). *P < 0.05; n.s. not significant. cps, counts per second. Data are presented as means ± SEM.

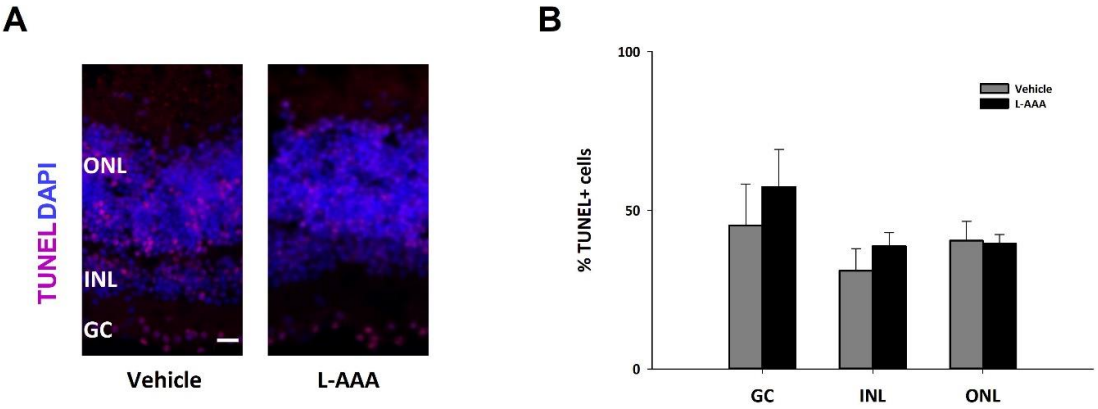

Figure S4. L-AAA treatment does not induce apoptosis in whole retina explants. (A) Representative sections showing the results of terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate nick end labeling (TUNEL, red) staining in WT retina explants treated with 0.4 mM L-AAA, as in Figure 2. Nuclei were also stained with DAPI (blue). Scale bar: 20 µm (B) TUNEL staining reveals no difference in percentage of apoptotic cells between vehicle and L-AAA treated retinas in the three different retina layers (N=5 per group). GC, ganglion cells; INL, inner nuclear layer; ONL, outer nuclear layer. Data are presented as means ± SEM.

Chapter 7

General discussion and future perspectives

General discussion

In the retina, photoreceptor cells are highly specialized sensory cells that sense light and initiate vision. These cells include rods and cones and which possess a cell-intrinsic molecular circadian clock mechanism. This clock temporally regulates several physiological processes that include expression of photopigments and phototransduction-related genes, (nocturnal) synthesis and release of melatonin, metabolism, and visual sensitivity and processing of light information. Photoreceptor cells clock also regulates processes that are directly linked to retinal survival such as vulnerability of photoreceptors to degeneration following light damage (Felder-Schmittbuhl et al., 2018; Bhoi et al., 2023).

Data from a number of studies support the hypothesis that deficits in retinal circadian clocks modulate various retinal pathologies such as glaucoma, diabetic retinopathy, and age related macular degeneration (Felder-Schmittbuhl et al., 2017; Bery et al., 2022). Thus, the retinal circadian misregulation may constitute a risk factor for aforementioned retinal diseases. Nevertheless, the underlying mechanisms remain to be examined. In this thesis, we provide data that support the view that the misregulation of circadian clock increases the susceptibility of photoreceptor to degeneration in retinitis pigmentosa, one of the most genetically heterogeneous inherited diseases.

The data of this thesis provide more extensive links between circadian clocks and photoreceptor biology that were hardly investigated before: examples are photoreceptor differentiation and the daily variations in rod light response. The potential mechanisms involved in these distinct processes are discussed below, see below the sections “The role of the circadian clock in the developing eye” and “Circadian regulation of visual light processing”. In addition, the strengths and limitations of our studies will also be addressed, see below the section “Strengths and limitations of our studies in this thesis”. Finally, potential future research directions will be presented, see below the section “Future directions”.

The role of the circadian clock in the developing eye

Data from a number of literatures suggest that the clock plays an important role in eye physiology and homeostasis (reviewed in Felder-Schmittbuhl et al., 2017; Bery et al., 2022). So far, transcriptomic studies in ocular tissues provided an extensive list of cyclic processes in various compartments of the eye (Storch et al., 2007; Mure et al., 2018). However, the

mechanism how the clock genes regulate developmental molecular pathways is still not well understood. In chapter 2, we investigated how *Per1* and *Per2* clock genes, previously shown to regulate the ratio of s- versus m-cones in the retina (Ait-Hmyed et al., 2013), are involved in signalling and transcriptional regulation during eye development (Bagchi et al., 2020). By comparing the whole-eye transcriptome of wild type (WT) and *Per1*$^{-/-}$*Per2*^{Brdm1} mutant mice, at E15, E18 and at P3, we show major gene expression changes between genotypes, with the number of differentially expressed genes (DEGs) increasing with developmental age. At P3 specifically, mutant eyes show major upregulation of phototransduction related genes, together with reduction in cell cycle related genes.

We detected expression of clock genes during early eye development (E13) with later variation in expression levels along with age (chapter 2). Recent studies confirmed this results and showed a rhythm in both *Bmal1* and *Cry1* expression in retinas from foetuses whose mothers were maintained in LD. Thus a clock-related rhythmic process might be involved in the transcriptional changes we found in our study. The fact that *Bmal1* ko in photoreceptor has a phenotypic effect opposite (an expansion of s-cone territory) to the one of the *Per1-Per2* mutant (or of *Per2* ko alone) further confirmed this idea (Sawant et al., 2017).

It is not clear whether the clock acts mostly on gene expression programs related to mitosis or to differentiation or both. The link with cell cycle has been largely described, with effects of the clock at distinct checkpoints (review by Bedont et al., 2020). It might be that the circadian clock regulates such steps of retinal development, notably at the level of cyclins or Wnt signalling, as reported in our study.

The clock might also control the differentiation rate of photoreceptor during development. Rhythmic expression of several phototransduction genes has been reported, but the mechanisms underlying this phenomenon are not yet understood. In zebrafish, key retina-specific transcription factors, such as NR2E3, drive photoreceptor differentiation and are co-regulated by the circadian clock to regulate daily photoreceptor physiology (Laranjeiro and Whitmore, 2014). *Nr2e3* expression in the mouse retina is also rhythmic and clock regulated (Kunst et al., 2015). In our transcriptomics study of *Per1*$^{-/-}$*Per2*^{Brdm1} mutant mice, we observed an alteration of phototransduction related transcripts. All data together supports the idea that an important part of the phototransduction machinery might also be the target of the clock in mammals.

Our ingenuity pathway analysis of differentially expressed genes of the *Per1*$^{-/-}$*Per2*^{Brdm1} versus WT whole eyes indicated NRL and CRX are the top regulators of the differentially expressed genes at P3. NR2E3, which co-activates rod photoreceptor genes with NRL, CRX and REV-ERBα (NR1D1) (Cheng et al., 2004), has a FC > 1.6 in *Per1*$^{-/-}$*Per2*^{Brdm1} eyes at P3. NR2E3 is a photoreceptor-specific nuclear receptor that activates several rod photoreceptor-specific genes during retinal development, at the expense of the s-cone cell fate (Cheng et al., 2011). This is in agreement with the phenotype of the *Per1*$^{-/-}$*Per2*^{Brdm1} mutant eyes (Ait-Hmyed et al., 2013) and with our molecular data showing an increase in expression of rhodopsin at P3 (FC>1.6) and decrease (2 and 2.4 fold at E18 and P3 respectively) for s-opsin. We speculate that NR2E3-mediated regulation might be one of the causes of the changes in the phototransduction machinery observed in the mutant at P3. Further studies are necessary to determine the precise mechanisms involved.

Circadian regulation of visual light processing

A plethora of rhythmic processes has been described in the mammalian eye. Most of them are controlled by the retinal circadian clock (Felder-Schmittbuhl et al., 2018). However, the links between clock genes, retinal cell-specific clocks and visual light processing is limited. Circadian rhythms in retinal (bright light) photopic responses have been demonstrated in several studies (Barnard et al., 2006; Storch et al., 2007; Cameron et al., 2008; Wong et al., 2018), but have been barely evaluated for (low light intensity) scotopic responses.

In chapter 3, we investigated the possibility that retinal responses at scotopic light intensities are under control of circadian clock. We observed a significant increase of responses to scotopic (and mesopic) light stimulations at subjective night as compared to subjective day in WT mice, based on the amplitude of the (measurable) b-wave. This indicated that the rod cell response to scotopic stimulation is clock regulated. This pattern in light sensitivity was abolished in *Per1*$^{-/-}$*Per2*^{Brdm1} mutants on the same mixed C57BL/6J x 129 SvEvBrd background; thus, our electroretinogram (ERG) studies provide further evidence for the functional biological readouts of *Per1* and *Per2* inactivation in visual processing. The ERG is a commonly used method to assess retinal functioning. In the scotopic ERG, the b-wave is mainly attributed to rod bipolar cells and the a-wave represents the response of rods to a flash of light, and thus a- and b-wave amplitudes can be used to examine the effect of time of day on specific retinal cell-type light response (Cameron et al., 2006; Robson and Frishman, 2014).

However the day/night difference observed in response to mesopic light could be attributed to rods or cones.

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). More recent investigations in mice suggested that the sensitivity to scotopic light stimulation displays a circadian pattern (Di et al., 2019). Thus, based on literature and our work, we conclude that the retinal scotopic light response is clock-controlled.

In chapter 4, we investigated the possibility that the day-night differences in scotopic ERG light response might be generated by the clock in rods. For this study, we used the rod-specific knockout (KO) of Bmal1 that carries an inactivated clock in rods and has a C57BL/6J background, using similar experimental set-up as used in the studies described in chapter 3. Again, we observed rhythmic scotopic ERG a- and b-waves in control mice, with higher amplitudes during the subjective night than subjective day (Gegnaw et al., 2021). These data corroborate our findings from chapter 3, that there is an increase in ERG scotopic (and mesopic) light-response during the subjective night with WT mice on two distinct mouse backgrounds, together providing convincing evidence that this property is clock regulated. We observed dys-rhythmic a- and b-wave amplitudes in rod-specific *Bmal1* knockout mice. Based on these data, we conclude that rods do contain a circadian clockwork that regulates their sensitivity to scotopic and mesopic light. Interestingly, recent data exploring specifically the mesopic range of light stimulation partially confirmed our results by showing a marked rhythm in rod-related b-wave, with highest response at subjective night (Allen, 2022).

The mechanism underlying the increase of rod light response at night is presently unknown. The mRNA expression of several genes of the phototransduction components (*Pde6b, Sag, Cnga1, Rcvrn* encoding subunits of the phosphodiesterase and of the cGMP-gated cation channel, and rod-arrestin) and of a number of ion channels change over the 24 h cycle in mouse photoreceptors (Milićević et al., 2021). Based on the rhythmic expression of these genes in photoreceptors, we hypothesize that at least some of them might be directly controlled by the molecular clock. For instance, BMAL1, as a transcriptional activator, trans-activates clock genes and clock-controlled genes by binding to E-box sequences. Therefore, the observed increase in rod light response during the subjective night might be a direct clock/E-box effect involving some of the aforementioned genes. Importantly, retinal

photoreceptor sensitivity to light has also been described to be modulated daily by post-translational processes, such as changes in the affinity of cGMP-gated channels in chicken retinas (Ko et al., 2001). The affinity of cone cGMP-gated channels was found to be increased during the subjective night. This effect was modulated at least in part by circadian rhythms in the activation of the ERK mitogen-activated protein kinase and of the Ca2+/calmodulin-dependent protein kinase II (CaMKII).

We speculate that the observed increase in light response triggered by rods during the subjective night could also be due to functional and electrical coupling between rods and cones mediated by rod/cone gap junctions through which secondary messengers such as Ca2+ and cGMP diffuse. These gap junctions are likely functionally open at night in the dark, as the functional and electrical coupling between rods and cones is shown to be increased during the subjective night (Ribelayga et al., 2008). It might be that this increased rod-cone coupling promotes the second-order rod pathway in nocturnal condition (Tsukamoto et al., 2001; Völgyi et al., 2004). Interestingly, it was shown that the rhythm in rod mesopic response might be due to increased gap junctions between rods and cones during the night, because the night increase in b-wave is blunted under pharmacological treatment (Meclofenamic acid) blocking gap junctions (Allen, 2022). Accordingly, gap junction (connexion 36 (*Cx36*)) levels and phosphorylation (controlling connectivity) are clock regulated (Katti et al., 2013; Li et al., 2013; Zhang et al., 2015). Based on these reports and our results, we propose a potential molecular mechanism for the circadian regulation of retinal sensitivity to scotopic light (Figure 1).

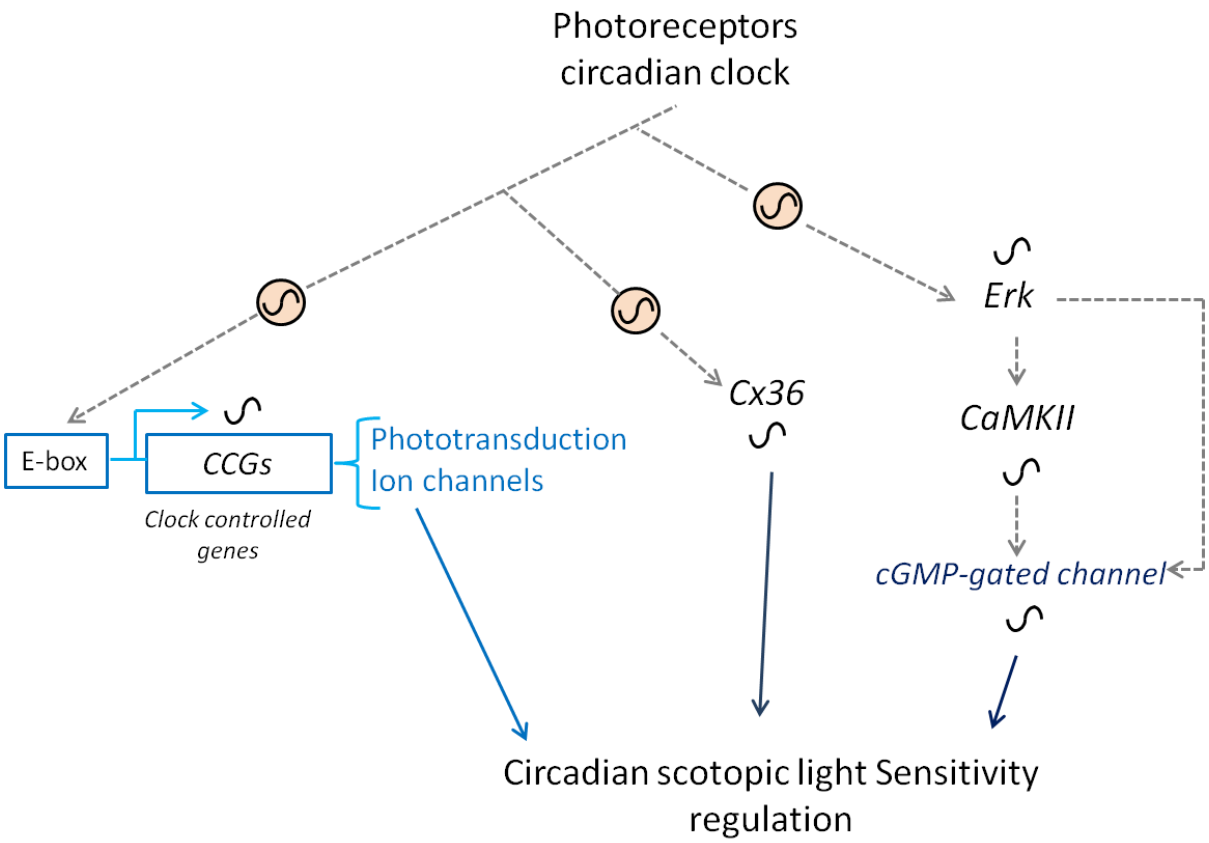

Figure 1. A model for retinal scotopic light sensitivity regulation. A circadian oscillator in photoreceptors regulates the rhythmic expression of phototransduction cascade and ion channel genes in photoreceptors, which ultimately might directly modulate light sensitivity to scotopic light. It also regulates the transcript levels of Cx36 and thus contributes to retinal photoreceptors light response. It also regulates the rhythmic activation of ERK MAP kinase, and the ERK rhythm drives an antiphase rhythm in CaMKII activation. Both kinases were depicted as regulating the cGMP-gated channels, which might also (in part) regulate the photoreceptors sensitivity to light.

Does the disturbed photoreceptor clock modulate retinal disease?

As reviewed by Bery and co-workers, experimental evidence suggests that deficits in retinal clocks modulate retinal pathology in glaucoma, diabetic retinopathy, and age-related macular degeneration (Bery et al., 2022). The expression of circadian clock proteins has been described in most retinal neurons of the mouse (Liu et al., 2012) but evidence from the literature suggests that only cone type photoreceptor contain a functional circadian clock. In chapter 3 of this thesis, using ERG, we provided the first functional evidence that rod photoreceptors contain a functional clock (Gegnaw et al., 2021). Considering that this clock is a very recent discovery, the potential link between the regulation and function of this clock and with retinal disease has not been investigated yet.

It is possible that based on the work of Baba and Tosini that during aging the circadian clock cycles dampen in the retina (Baba and Tosini, 2018). This might lead to electrical decoupling between rod and cone photoreceptors, ultimately leading to age-related retinal decline (Baba et al., 2018b; Baba and Tosini, 2018). Interestingly, many retinal diseases, including RP, arise later in life, when circadian function might be compromised (Welz and Benitah, 2020). However, the question does the disease comprise the clock or vice versa; or does this process enhances/influences the pathology remains open. Individuals with the similar RP disease gene mutations can have different retinal phenotypes that affect the timing of disease onset and rate of progression. This variability may be induced by environmental factors, epigenetic factors, genetic modifier loci or light damage (Ferrari et al., 2011; Contín et al., 2016) but also a role of temporally or chronically disturbed circadian rhythms has not been excluded.

Thus, in chapter 5, we investigated this hypothesis by combining rod specific-*Bmal1* clock gene ko with the P23H Rho mutant mouse, whose retinas were studied through comparative temporal transcriptomics in 4 months old mice. Our whole retina RNA seq analysis identified 889 genes differentially expressed in retinas from rod-specific *Bmal1KO*/P23H Rho (DM) vs P23H Rho mice that are potentially responsible for the exacerbation of photoreceptor degeneration in P23H Rho retinas. It is likely that these genes are involved in maintenance of photoreceptor cells and retinal homeostasis. Therefore, the genes in retinal cells controlled by the clock machinery should be considered.

Loss of function of multiple visual transduction cascade genes has been associated with loss of phototransduction function which in turn causes degeneration of photoreceptors (van Soest et al., 1999 for review). Indeed, we found a significant decrease in transcript levels of several phototransduction genes in the DM with respect to control or P23H retinas. However, the opposite hypothesis (phototransduction gene expression is reduced because of photoreceptor death) is likely true as well. Inability to modulate the daily adjustment of visual processing may underlie the accelerated photoreceptor degeneration in the DM. Pittendrigh's "escape the light" hypothesis speculates that circadian systems evolved as a means for cells/organisms to anticipate and manage the effects of light damage (Pittendrigh, 1993; Baik et al., 2018). Our results revealed a circadian pattern of rod light response, that peaks at night and is blunted in circadian clock mutants, as also seen in drosophila (Nippe et al., 2017; Gegnaw et al., 2021). *Grk1* is among the down-regulated genes in DM retinas; it

encodes a kinase that phosphorylates rhodopsin and contributes to the termination of phototransduction (reviewed in Arshavsky, 2002) and maintenance of rod sensitivity (Cideciyan et al., 1998; Sakurai et al., 2011). Deficiency of *Grk1* causes light-dependent retinal degeneration (Chen et al., 1999; Yetemian et al., 2010) and is responsible for the Oguchi form of congenital stationary night blindness (Hayashi et al., 2007; Oishi et al., 2007; Azam et al., 2009). Concurrent circadian oscillations of *Grk1* transcripts (specifically in PR: (Kunst et al., 2013), protein phosphorylation (Horner et al., 2005; Osawa et al., 2011) and abundance of the *Grk1* inhibitor recoverin (Zernii et al., 2011; Grigoriev et al., 2012) are consistent with a key role of *Grk1* in regulating the daily adjustment of light processing and sensitivity (reviewed in Barlow, 2001) and whose loss might contribute to PR loss in the DM. Interestingly, a circadian component in retinal sensitivity to light-mediated damage has been reported in rats: rat retinas were much more vulnerable to the damaging effects of a 1200-1400 lux light pulse during the dark phase, thus highlighting the physiologic role of the circadian clocks in suppressing sensitivity to phototoxicity during the day (Organisciak et al., 2000).

The circadian clock plays a role in healthy aging because mice deficient in *Bmal1* display reduced lifespan and symptoms of premature aging including cataracts and neurodegeneration (Kondratov et al., 2006; Kunst et al., 2013). Similarly, circadian disruption induced by exposure to chronic jet lag reduces lifespan and alters brain activity in animal models (Davidson et al., 2006; Gao et al., 2020). In human, circadian misalignment or clock (BMAL1) gene mutation has been associated with occurrence of neurodegenerative diseases such as Alzheimer's and Parkinson's disease (Chen et al., 2015; Gu et al., 2015; Bokenberger et al., 2018). We identified genes associated with metabolic pathways to be down regulated in DM vs P23H retinas, suggesting that *Bmal1* ko also influences homeostatic mechanisms such as regulation of metabolism and of oxidative stress, that play crucial roles in rod health and disease (Newton and Megaw, 2020).

The link between BMAL1 mutation and neuronal pathology in mice involves impairment of redox homeostasis (Kunst et al., 2013). Oxidative and reductive cycles synchronize to 24h rhythms through tight regulation of metabolism by circadian clocks, at least in the periphery, but this relationship has not been clearly demonstrated in the central nervous system (reviewed by Smith and Musiek, 2020).

In peripheral cells, a strong reciprocal link exists between clock factors and metabolic processes, at several levels. Among these, NAD(P)(H) is at the interphase between metabolism, circadian rhythms and aging (Levine et al., 2022). Also, mitochondria metabolic activity and nutrient use are under circadian control (Jacobi et al., 2015; Neufeld-Cohen et al., 2016). Taken together, literature data suggest that the metabolic processes in the peripheral cells are most likely controlled by the circadian clock.

In our study, when analysing biological pathways that are specifically downregulated in DM retinas with respect to P23H Rho mutants, we found highly significant enrichment in metabolic pathways such as glycolysis, oxidative phosphorylation, and NADH regeneration, indicating these processes might be particularly dysfunctional in the DM. Key metabolism regulators such as *Sirt1* and *Pgc-1α* display daily mRNA expression rhythms in the retina (Ban et al., 2013; Kunst et al., 2013). Also, circadian time-affected genes in mouse photoreceptors are enriched in biological pathways related to metabolism (Milićević et al., 2021). Thus, our data corroborates evidence in the literature that disruption of normal circadian function in the context of retinal disease might make the retina more susceptible to metabolic deficit and thereby enhance photoreceptor degeneration. Nonetheless, the exact BMAL1 metabolic targets involved in the retinal phenotype of the DM remain presently to be identified.

The role of photoreceptor cells in retinal clock activity

As argued in chapter 4, rods contain a circadian clock that regulates daily visual light processing (Gegnaw et al., 2021). By contrast, absence of clock in these cells did not exert any major effect on the global retinal clock, as assessed by PER2::Luciferase bioluminescence in explants. Nonetheless, we did find altered retinal levels of mRNAs for several clock genes (*Cry1*, *Nr1d2*) (Gegnaw et al., 2021). To get more insights into the contribution of rods to the retinal clock network we studied the (potential) relationship between degeneration of these cells and the retinal circadian clock activity in Chapter 6.

In chapter 6, we investigated the impact of rod degeneration on the activity of the retinal clock. The retinal clock in *Rho*$^{P23H/+}$ heterozygous mice (but not homozygotes) displayed more robust PER2::Luciferase rhythms than in wild-type controls (Gegnaw et al., 2022). It was reported that photoreceptor affect the activity of the retinal clock, but the responsible mechanisms were yet to be elucidated. Indeed, in the RCS/N-rdy rats displaying extensive

photoreceptor loss, clock gene expression analysed in whole retinas was affected but no major change was observed for *Per2* mRNA rhythms (Tosini et al., 2007). In another study, however, retinal explants from homozygous *rd1* mutant mice displayed sustained rhythms in PER2::Luciferase activity, whereas their heterozygous littermates (with normal retinas) were reported not to show retinal rhythms (Ruan et al., 2006). Also, in retinas devoid of rods that are totally converted into cones, no major effect on the retinal clock was observed (Sandu et al., 2021). Thus, our results combined with those of the literature suggest a complex picture of the photoreceptor role in the retinal clock network that might not easily be defined by whole retina bioluminescence studies.

Strengths and limitations of our studies in this thesis

One of the strengths in this thesis was the use of the well characterized *Per1*$^{-/-}$*Per2*^{Brdm1} mutant mouse model for molecular characterization, in combination with found structural and functional readouts previously described by Ait-Hmyed and coworkers (Ait-Hmyed et al., 2013). Also, we generated a rod-specific *Bmal1* knockout model by combining the well-characterized *Rho-iCre* mouse (Li et al., 2005) and the widely used floxed *Bmal1* allele (Storch et al., 2007) to study the role of the rod molecular clockwork in the day/night differences of retinal function, and in retinal disease and pathology. This model, together with the cone-specific knockout of *Bmal1* (Bhoi et al., 2021) was the first to assess functional clock properties in a specific retinal cell type. It also answered the controversial question of clock existence in rods albeit their low level of clock gene expression (Liu et al., 2012; Baba et al., 2018a; Bhoi et al., 2021) by providing molecular (BMAL1 expression detected by IHC) and functional evidence (see above the section ''Circadian regulation of visual light processing'').

There are also a number of limitations or our studies in this thesis: in chapters 2 and 3, we used *Per1*$^{-/-}$*Per2*^{Brdm1} double mutant model in our experiments. Given the redundancy of the three Period (*Per*) genes in the molecular clockwork, part of the physiological properties of *Per* genes could not be determined in our experimental setup. For example, we observed an upregulation of *Per3* expression in eyes from *Per1-Per2* mutant mice (relative to WT mice) (FC > 2.5 at P3; but also FC > 1.9 and 1.8 at E15 and E18 respectively). The developmental *Per3* gene expression kinetics point to a role in adult eyes rather than at embryonic stages. Thus it is likely that *Per3* displays compensatory effect for *Per1* and *Per2* absence. Future studies

should preferably investigate how inactivation of *Per1*, *Per2*, and *Per3* genes (triple mutants) impact eye development.

Our data of chapter 3 reveal a circadian rhythm of rod light response, abolished in *Per1* and *Per2* double mutant mice. Surprisingly, upon aging we detected no decrease in visual sensitivity/retinal integrity in the mutants. In contrast, it does occur with other circadian clock mutants, such as *Clock* or *Bmal1* mutants (whole knockout or retina-specific knockout for *Bmal1*) (Baba et al., 2018a; Baba et al., 2018b). Therefore, the lack of effect on aging retina observed in *Per1* and *Per2* double mutants might also be due to the upregulation of *Per3*.

In chapter 5, our ability to analyse the P23H vs Ctrl or DM vs Ctrl transcriptomes in the whole retina allowed uncovering the most altered pathways and (new) clock influenced disease genes in the whole retina. A limitation of our analysis is that they are likely under-powered to identify the full spectrum of (rod) photoreceptor specific transcriptomes, because our mRNA samples were isolated from whole-retina lysates. In addition retinas were collected for only one age time point (4 months) whereas our histological staining results (data not shown) provide evidence for aggravated photoreceptor loss already at 1 month. Analysis of PR specific transcriptomics, performed from mRNA collected from micro dissected PRs at multiple time points, indicated that neurotransmission components were among the most time affected pathways, thus highlighting the importance of circadian regulation within these cells (Milićević et al., 2021). Similar study could be performed in these retinas. Alternatively, single cell RNAseq could provide a powerful alternative for identifying the early events of *Bmal1* knockout and P23H synergy.

Future directions

In chapter 2, we speculated that the functional changes in photoreceptors phototransduction machinery observed in $Per1^{-/-}Per2^{Brdm1}$ mice at P3 might mediated by NR2E3. We hypothesised that clock could regulate the expression of rod phototransduction genes in mammalian retina (Hao et al., 2012) at a node between clock and retinal development because Nr2e3 (FC > 1.6 at P3 in eyes from $Per1^{-/-}Per2^{Brdm1}$ mice relative to WT mice) activates rod phototransduction genes synergistically with REV-ERBα, NRL, and CRX (Cheng et al., 2004). Recently, the efficacy of AAV mediated Nr2e3 administration to the sub-retinal space of mouse models of retinitis pigmentosa demonstrated overall rescue of retinal degeneration

as observed by increase in photoreceptor cells (Li et al., 2021). Thus, based on our results and literature, it will be interesting to understand the potential role of *Nr2e3* at the regulatory node during photoreceptor differentiation of *Per1*$^{-/-}$*Per2*^{Brdm1}, *Bmal1* and *Rev-Erb-α* knockout mice.

Our findings in chapter 5 establish the intrinsic circadian clock in rods as a regulator of susceptibility of photoreceptor degeneration in retinitis pigmentosa, potentially through the regulation of phototransduction and metabolism related genes by BMAL1. Given that BMAL1 transcriptionally controls circadian and non-circadian transcripts, future studies at multiple time points may determine whether the time-of-day regulation of these genes by BMAL1 is important to promoting the survival of photoreceptors in retinal disease.

Humans in the modern lifestyle, being overexposed to light at night, opens an interesting paradigm to study for further-understanding the effect of this abnormal light exposure during healthy retinal aging and disease. Such detrimental effects might modulated by retinal clock dysfunction itself. However, considering the strong detrimental impact that circadian dysregulation exerts on all physiological functions, it is not unlikely that chronic desynchronization of circadian rhythms negatively modulates our retinal health. The aforementioned hypothesis is corrorborated by the observed links between circadian dysregulation and several disease pathologies that include retinopathies, neurodegenerative diseases, cancer, and retinitis pigmentosa (Musiek et al., 2013; Sulli et al., 2019; Bhatwadekar and Rameswara, 2020; Nassan and Videnovic, 2022; chapter 5 of this thesis). We conclude that the potential impact of improving lifestyle behaviour by a healthy sleep pattern in improving human health is a therapeutic approach worth investigating.

Finally, it is also important to note that (while helpful) current transgenic rodent models of retinal diseases, including models of retinitis pigmentosa, may not fully capture the mechanistic complexity involved; further translational methods such as in vitro on biopsy-derived (human) cell lines and clinical investigations on health control and retinal disorder subjects are much needed.

Conclusion

In summary, the data obtained in this thesis provide evidence that light processing by rods, under scotopic-mesopic condition, is cell-autonomously regulated by a circadian clock with

an increase in visual sensitivity, specifically during the night. The latter also seems essential to rod homeostasis, since its dysregulation synergizes with photoreceptor degeneration of genetic origin and enhances photoreceptor degeneration. The underlying mechanisms, suggested by our results, remain to be functionally demonstrated. The relationship between circadian clocks and retinal disease seems complex since, conversely, the rhythmic properties of the retina are also transiently activated during degeneration.

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Chapter 8

Thesis summary

Nederlandse Samenvatting

Résumé de thèse

Thesis Summary

Daily rhythmicity is a central hallmark of vision encompassing the adaptation of retinal physiology and light information processing to the day/night cycle. These cyclic processes are regulated within the retina by an internal circadian clock whose cellular localization remains to be determined. The retinal clock controls, for example, the mRNA expression of photopigments, visual sensitivity, phagocytosis of photoreceptor outer segments by the underlying pigment epithelium and sensitivity to phototoxicity. Different studies have shown that disruption of the retinal circadian clock affects sensitivity to light, processing of visual information and photoreceptor cell viability during aging. Recently, hundreds of genetic retinal disease genes have been identified (www.retnet.org). These diseases primarily affect the photoreceptor and/or the retinal pigmented epithelium cells, typically involving multiple pathogenic molecular alterations and subsequent cell death and vision loss. Although many of genes associated with retinal degeneration have been identified, no link has been established between the circadian clock and genetic retinal diseases.

Thus, the objective of this thesis was to explore the functional and molecular link between the circadian clock and retinal genetic diseases.

The description of the results obtained during this thesis is divided into three parts in the manuscript. In **part I**, the molecular mechanisms of the circadian clock and the regulation of the retinal clock are briefly introduced, as well as the regulation of gene expression by the circadian clock. Genetic retinal diseases, in particular retinitis pigmentosa (RP) and their impact on circadian clock function are described. Finally, the end of this chapter presents the scope of the thesis (**Chapter 1**).

In **part II**, we explored the effects of circadian clock dysfunction on retinal physiology and disease. First, we investigated the effects of Period1 (*Per1*) and Period2 (*Per2*) clock genes inactivation on the retina. *Per1* and *Per2* are key components of the mammalian circadian clock. Both genes are expressed in the retina and exhibit cyclic expression.

In **chapter 2**, we explored the role of *Per 1* and *Per 2* clock genes for eye development. Indeed, we had observed that the retinas of adult $Per1^{-/-}Per2^{Brdm1}$ mutant mice show an abnormal distribution of the blue cones, suggesting an alteration in the development of the retina (Aït-

Hmyed et al., 2013). We have characterized the expression profiles of clock genes between E13 (13th day of embryonic development) and P24 (24th postnatal day) in the eyes of wild-type mice. All clock genes (including *Per1* and *Per2*) are expressed and show significant changes in their level of expression during normal eye development. We next compared whole-eye transcriptomes between *Per1*$^{-/-}$*Per2*^{Brdm1} mutants and wild-type (WT) mice, at E15, E18 and at P3. RNA-Sequencing data showed major differences between genotypes, with the number of differentially expressed genes (DEGs) increasing with developmental age. Functional analysis of DEGs shows that the most significant changes concern clock genes and circadian rhythms at E15 and E18. At P3, there is a strong increase, in the mutant eyes, in expression phototransduction cascade genes, while genes related to the cell cycle are strongly repressed in the mutants. Our results suggest a role of the clock during retinal development, in the transition between proliferation and differentiation, likely at a critical moment for the genesis of photoreceptors (Bagchi et al., 2020).

Although previous results from our laboratory with the same line of *Per1*$^{-/-}$*Per2*^{Brdm1} mutant mice did not reveal any significant impairment of visual function (electroretinography (ERG) under scotopic and photopic conditions; Aït-Hmyed et al., 2013) the properties of the response to light over 24 hours had not been studied in this mutant, which no longer exhibits a sleep/activity rhythm under constant conditions. In **Chapter 3**, we explored the effects of these mutations on retinal rhythmicity. We performed functional and phenotypical analysis by ERG and immunohistochemistry respectively. As a first result in this study, we showed that light response in scotopic and mesopic ERG recordings shows a day/night difference, with increase during the night that is retained in constant dark conditions. By contrast, inactivation of *Per1* and *Per2* genes totally impairs this daily modulation of visual response as exemplified by the effects on the amplitudes of a- and b-waves in scotopic ERG condition. In the mutant, the light sensitivity appears constitutively enhanced, like in the wild type night-time state. Surprisingly, upon aging, there is no marked decrease in visual sensitivity/retinal integrity in the *Per1*$^{-/-}$*Per2*^{Brdm1} mutant, indicating that the circadian clock is dispensable for general retinal health, or that the presence of *Per3* compensates for *Per1* and *Per2* absence. The conclusion of this chapter is that light processing at the level of photoreceptors under scotopic and mesopic conditions in mice is regulated by a circadian clock. It supports the view that rods (photoreceptors active at low light intensity) display increased sensitivity during the

night, likely providing optimal performance in nocturnal species (Gegnaw *et al*., in preparation). In **Chapter 4**, to further decipher how/where the day-night differences in ERG light response are generated, we used the rod-specific knockout (KO) of *Bmal1*, the main gene of the molecular clock, that carry an inactivated clock in rod photoreceptor cells. We conducted analyses of the light response over the circadian cycle similar to those of chapter 3. Again, we observed circadian day-night differences in ERG a- and b-waves in control mice, with higher amplitudes during the subjective night. These differences were however abolished in rod-specific *Bmal1* KO mice, whose ERG light-responses remained constitutively low (day-like). We generated mice carrying the together with the Per2-Luciferase reporter knock in to evaluate how *Bmal1* deletion in rods could affect the global rhythmic capacity of the retina. Analysis of whole retina bioluminescence recordings showed that PER2::Luciferase rhythmicity was not defective in rod-specific *Bmal1* KO mice but was characterized by longer period and increased rhythmic power compared to control retinas. These results confirm those from the preceding chapter and point to the essential role played by the clock located in rods in adjusting visual response around the 24h cycle. They also prompted us to further investigate the regulation and function of rod-specific clock genes in the context of retinal health and disease (Gegnaw et al., 2021 Experimental Eye Research).

Next, in **Chapter 5**, we explored the potential links between dysfunction of the rod circadian clock and retinal disease. We performed this study in mice, combining rod-specific *Bmal1* KO (rod-*Bmal1KO*) with the P23H mutation of rhodopsin (rod visual pigment), one of the major causes of autosomal dominant retinitis pigmentosa (RP) in human. We used mice of 4 genotypes: 1, carrying the (heterozygous) P23H rhodopsin mutation; 2, rod-*Bmal1KO*; 3, double mutants of these strains and control (Ctrl) mice. We show by structural (histology, immunohistochemistry) and functional (electroretinography) analyses that combining the rod-*Bmal1KO* with the P23H rhodopsin line, dramatically exacerbates the RP phenotype induced by the P23H mutation. Indeed, we observed accelerated reduction of ERG amplitude, loss of photoreceptor cells and gliosis in the double mutants. These observations were corroborated by RNA-Sequencing analysis, where we found major gene expression changes between the double mutant and P23H retinas confirming the more advanced state of degeneration in the double mutant. The functional analysis of differentially expressed genes obtained in this data set (double mutant vs P23H retinal transcriptomes) reveal

transcriptional pathways related with development and metabolic events. These results indicate that the circadian clock in rods regulates homeostatic mechanisms (related in particular to metabolism but which remain to be precisely identified) whose alteration induces a synergistic effect with retinal degeneration (Gegnaw et al., in preparation).

In **part III**, we analysed the effects of photoreceptor degeneration on the retinal clock (**Chapter 6**). We used mice carrying the P23H mutation of rhodopsin; either in the heterozygous state, which induces mild degeneration of the rods, or in the homozygous state, responsible for a rapid loss of all the photoreceptors. As in Chapter 3, we also introduced the PER2::Luciferase reporter into these mice to examine how rod degeneration impacts the overall rhythmic capacity of the retina. Bioluminescence was recorded on explants of whole retinas (WT, $Rho^{P23H/+}$, and $Rho^{P23H/P23H}$) taken at P36 or P70, 2 key stages of degeneration in mutants. Surprisingly, our data show that the retinal clock in $Rho^{P23H/+}$ heterozygous mice (but not homozygotes) displays more robust circadian rhythms than in wild-type controls. By contrast, increased robustness of retinal rhythms does not affect the central clock in the brain, as assessed by bioluminescence of explanted suprachiasmatic nuclei and locomotor activity recording. By treating retinal explants with L-α aminoadipic acid, we further provide evidence that this enhanced rhythmicity might involve activation of Müller glial cells downstream of the degenerating photoreceptors (Gegnaw et al., 2022 Journal of Biological Rhythms).

In conclusion, the data obtained in this thesis provide evidence that light processing, under scotopic and mesopic conditions, is regulated by a circadian clock which induces an increase in visual sensitivity specifically during the night. This regulation takes place in the rods themselves, by the clock they contain. The latter also seems essential to rod homeostasis, since its invalidation produces a synergistic effect with degeneration of genetic origin. The underlying mechanisms, suggested by our results, remain to be functionally demonstrated. The relationship between circadian clocks and retinal disease seems complex since, conversely, the rhythmic properties of the retina are transiently activated during degeneration. Finally, the role of the circadian clock in regard to photoreceptors is already expressed during embryonic development, by regulating a key step in their ontogenesis; the transition between division and differentiation of precursors.

Nederlandse Samenvatting

Dagelijkse ritmiek is een centraal kenmerk van het gezichtsvermogen en omvat zowel de aanpassing van de fysiologie van het netvlies en de verwerking van lichtinformatie aan de dag/nacht-cyclus. Deze cyclische processen worden binnen het netvlies gereguleerd door een interne circadiane klok waarvan de cellulaire lokalisatie nog moet worden vastgesteld. De netvlies klok regelt bijvoorbeeld: de mRNA-expressie van fotopigmenten, visuele gevoeligheid, fagocytose van de buitenste segmenten van de fotoreceptor door het onderliggende pigmentepitheel en gevoeligheid voor fototoxiciteit. Verschillende onderzoeken hebben aangetoond dat verstoring van de circadiane klok van het netvlies de gevoeligheid voor licht, de verwerking van visuele informatie en de levensvatbaarheid van fotoreceptorcellen tijdens het ouder worden beïnvloedt.

De afgelopen decennia zijn honderden genen betrokken bij netvliesziekten geïdentificeerd (www.retnet.org). Deze ziekten tasten voornamelijk de fotoreceptor en/of de retinale gepigmenteerde epitheelcellen aan, meestal door meerdere pathogene moleculaire veranderingen en daaropvolgende celdood en dus verlies van gezichtsvermogen. Hoewel veel van de genen die verband houden met netvliesdegeneratie zijn geïdentificeerd, is er nog geen verband gelegd tussen de circadiane klok en genetische aandoeningen van het netvlies.Het belangrijkste doel van dit proefschrift was dan ook om de functionele en moleculaire link tussen de klok en netvliesziekte te onderzoeken.

De beschrijving van de tijdens dit proefschrift verkregen resultaten is in het manuscript in drie delen verdeeld. In **deel I** worden de moleculaire mechanismen van de circadiane klok en de regulatie van de netvlies klok kort geïntroduceerd, evenals de regulatie van genexpressie door de circadiane klok. Genetische netvliesziekten, in het bijzonder retinitis pigmentosa (RP), en hun impact op de circadiane klokfunctie worden beschreven. Ten slotte presenteert het einde van dit hoofdstuk de reikwijdte van het proefschrift (**hoofdstuk 1**).

In **deel II** onderzochten we de effecten van circadiane klokdisfunctie op de fysiologie en ziekte van het netvlies. Eerst onderzochten we de effecten van de inactivatie van Period1 (*Per1*) en Period2 (Per2) klokgenen op het netvlies. *Per1* en *Per2* zijn sleutelcomponenten van de circadiane klok van zoogdieren. Beide genen komen tot expressie in het netvlies en vertonen cyclische expressie.

In **hoofdstuk 2** hebben we de rol van *Per 1* en *Per 2* klokgenen voor de oogontwikkeling onderzocht. We hadden inderdaad waargenomen dat de netvliezen van volwassen *Per1*$^{-/-}$ *Per2*^{Brdm1}-mutante muizen een abnormale verdeling van de blauwe kegeltjes vertonen, wat duidt op een verandering in de ontwikkeling van het netvlies (Aït-Hmyed et al., 2013). We hebben de expressieprofielen van klokgenen tussen E13 (13e dag van embryonale ontwikkeling) en P24 (24e postnatale dag) gekarakteriseerd in de ogen van wildtype muizen. Alle klokgenen (inclusief *Per1* en *Per2*) komen tot expressie en vertonen significante veranderingen in hun expressieniveau tijdens normale oogontwikkeling. Vervolgens vergeleken we transcriptomen van het hele oog tussen *Per1*$^{-/-}$*Per2*^{Brdm1}-mutanten en wildtype (WT) muizen, op E15, E18 en op P3. RNA-sequencing-gegevens lieten grote verschillen tussen genotypen zien, waarbij het aantal differentieel tot expressie gebrachte genen (DEG's) toenam met de ontwikkelingsleeftijd. Functionele analyse van DEG's laat zien dat de belangrijkste veranderingen betrekking hebben op klokgenen en circadiane ritmes op E15 en E18. Op P3 is er in de mutante ogen een sterke toename van de cascadegenen voor expressie-fototransductie, terwijl genen die verband houden met de celcyclus sterk worden onderdrukt in de mutanten. Onze resultaten suggereren een rol van de klok tijdens de ontwikkeling van het netvlies, in de overgang tussen proliferatie en differentiatie, mogenlijk op een cruciaal moment voor het ontstaan van fotoreceptoren (Bagchi et al., 2020).

Hoewel eerdere resultaten van ons laboratorium met dezelfde lijn *Per1*$^{-/-}$*Per2*^{Brdm1}-mutante muizen geen significante verslechtering van de visuele functie aan het licht brachten (elektroretinografie (ERG) onder scotopische en fotopische omstandigheden; Aït-Hmyed et al., 2013) zijn de eigenschappen van de reactie op licht gedurende 24 uur was niet onderzocht bij deze mutant, die onder constante omstandigheden geen slaap-activiteitsritme meer vertoont. In **Hoofdstuk 3** hebben we de effecten van deze mutaties op de ritmiek van het netvlies onderzocht. We voerden functionele en fenotypische analyses uit met respectievelijk ERG en immunohistochemie. Als eerste resultaat van deze studie hebben we aangetoond dat de lichtrespons in scotopische en mesopische ERG-opnamen een dag/nacht-verschil laat zien, met een toename gedurende de nacht die behouden blijft onder constante donkere omstandigheden. Daarentegen schaadt inactivatie van *Per1*- en *Per2*-genen deze dagelijkse modulatie van de visuele respons volledig, zoals geïllustreerd door de effecten op de amplitudes van a- en b-golven in scotopische ERG-toestand. Bij de mutant lijkt de

lichtgevoeligheid constitutief verbeterd, net als in de wildtype nachtelijke toestand. Verrassend genoeg is er bij veroudering geen duidelijke afname in visuele gevoeligheid/ netvlies integriteit in de *Per1*$^{-/-}$*Per2*^{Brdm1}-mutant, wat aangeeft dat de circadiane klok overbodig is voor de algemene gezondheid van het netvlies, of dat de aanwezigheid van *Per3* de afwezigheid van *Per1* en *Per2* compenseert. De conclusie van dit hoofdstuk is dat de lichtverwerking op het niveau van fotoreceptoren onder scotopische en mesopische omstandigheden bij muizen wordt gereguleerd door een circadiane klok. Het ondersteunt de opvatting dat staafjes (fotoreceptoren die actief zijn bij lage lichtintensiteit) 's nachts een verhoogde gevoeligheid vertonen, waardoor ze waarschijnlijk optimale prestaties leveren bij nachtelijke soorten (Gegnaw et al., in voorbereiding).

Om verder te ontcijferen hoe/waar de dag-nachtverschillen in de ERG-lichtrespons worden gegenereerd, hebben we in **hoofdstuk 4** de staafspecifieke knock-out (KO) muis van *Bmal1* gebruikt, het belangrijkste gen van de moleculaire klok, dat een geïnactiveerde klok in de staaf draagt. fotoreceptorcellen. We voerden analyses van de licht respons over de circadiane cyclus uit, vergelijkbaar met die uit hoofdstuk 3. Opnieuw observeerden we circadiane dag-nachtverschillen in ERG a- en b-golven bij controlemuizen, met hogere amplitudes tijdens de subjectieve nacht. Deze verschillen werden echter opgeheven bij staafspecifieke Bmal1 KO-muizen, waarvan de ERG-lichtreacties constitutief laag bleven (dagachtig). We hebben muizen gegenereerd die samen met de Per2-Luciferase-reporter de knock-in muizen om te evalueren hoe *Bmal1*-deletie in staafjes de globale ritmische capaciteit van het netvlies zou kunnen beïnvloeden. Analyse van bioluminescentieopnamen van het hele netvlies toonde aan dat de PER2::Luciferase-ritmiek niet defect was in staafspecifieke Bmal1 KO-muizen, maar werd gekenmerkt door een langere periode en een verhoogd ritmisch vermogen vergeleken met controle-netvliezen. Deze resultaten bevestigen die uit het voorgaande hoofdstuk en wijzen op de essentiële rol die de klok in staafjes speelt bij het aanpassen van de visuele respons rond de 24-uurscyclus. Ze hebben ons er ook toe aangezet om de structuur en functie van staafspecifieke klokgenen verder te onderzoeken in de context van de gezondheid en ziekte van het netvlies (Gegnaw et al., 2021 Experimental Eye Research).

Vervolgens onderzochten we in **Hoofdstuk 5** de mogelijke verbanden tussen disfunctie van de circadiane klok en retinale ziekten. We hebben deze studie uitgevoerd bij muizen, waarbij staafspecifieke *Bmal1* KO (staaf-*Bmal1KO*) werd gecombineerd met de P23H-mutatie van

rodopsine (staafjesvisueel pigment), een van de belangrijkste oorzaken van autosomaal dominante retinitis pigmentosa (RP) bij de mens. We gebruikten muizen van 4 genotypen: 1) die de (heterozygote) P23H-rodopsine mutatie droegen; 2) staaf-Bmal1KO; 3) dubbele mutanten van deze stammen en controle muizen (Ctrl). We laten door structurele (histologie, immunohistochemie) en functionele (elektroretinografie) analyses zien dat het combineren van de staaf-*Bmal1KO* met de P23H-rodopsine lijn het RP-fenotype geïnduceerd door de P23H-mutatie dramatisch verergert. We observeren een versnelde vermindering van de ERG-amplitude, verlies van fotoreceptorcellen en het oprtreden van gliosis in de dubbele mutanten. Deze waarnemingen werden bevestigd door RNA-sequencing-analyse, waarbij we grote veranderingen in de genexpressie tussen de dubbele mutant en de single mutant P23H-netvliezen vonden, wat de meer gevorderde staat van degeneratie in de dubbele mutant bevestigde. De bioinformatische analyse van differentieel tot expressie gebrachte genen verkregen in deze dataset (dubbele mutant versus P23H-retinale transcriptomen) identificeert transcriptionele routes die verband houden met ontwikkeling en metabolische gebeurtenissen. Deze resultaten geven aan dat de circadiane klok in staafjes homeostatische mechanismen reguleert (in het bijzonder gerelateerd aan het metabolisme maar die nog precies moeten worden geïdentificeerd) waarvan de verandering een synergetisch effect induceert met retinale degeneratie (Gegnaw et al., in voorbereiding).

In **deel III** analyseerden we de effecten van fotoreceptor degeneratie op de retinale klok (**Hoofdstuk 6**). We gebruikten muizen die de P23H-mutatie van rodopsine droegen; hetzij in de heterozygote toestand, die milde degeneratie van de staafjes veroorzaakt, hetzij in de homozygote toestand, verantwoordelijk voor een snel verlies van alle fotoreceptoren. Net als in Hoofdstuk 3 hebben we ook de PER2::Luciferase-reporter in deze muizen geïntroduceerd om te onderzoeken hoe staafdegeneratie de algehele ritmische capaciteit van het netvlies beïnvloedt. Bioluminescentie werd geregistreerd op explantaten van hele netvliezen (WT, RhoP23H/+ en RhoP23H/P23H) genomen op P36 of P70, 2 sleutelstadia van degeneratie bij mutanten. Verrassend genoeg laten onze gegevens zien dat de retinale klok bij RhoP23H/+ heterozygote muizen (maar niet homozygoten) robuustere circadiane ritmes vertoont dan bij wildtype controles. Daarentegen heeft de verhoogde robuustheid van de retinale ritmes in heterozygoten geen invloed op de centrale klok in de hersenen, zoals beoordeeld door bioluminescentie van geëxplanteerde suprachiasmatische kernen en registratie van

bewegingsactiviteit. Door retinale explantaten vervolgens te behandelen met L-α-aminoadipinezuur leveren we verder bewijs dat deze verbeterde ritmiek de activering van Müller-gliale cellen stroomafwaarts van de degenererende fotoreceptoren zou kunnen inhouden (Gegnaw et al., 2022 Journal of Biological Rhythms).

Conclusie

Concluderend leveren de gegevens verkregen in dit proefschrift bewijs dat, lichtverwerking, onder scotopische en mesopische omstandigheden, wordt gereguleerd door een circadiane klok die specifiek tijdens de nacht een toename van de visuele gevoeligheid induceert. Deze regeling vindt plaats in de staven zelf, door de klok die ze bevatten. Dit laatste lijkt ook essentieel voor de homeostase, omdat de invalidatie ervan een synergetisch effect oplevert met degeneratie van genetische oorsprong. De onderliggende mechanismen, gesuggereerd door onze resultaten, moeten nog functioneel worden aangetoond. De relatie tussen circadiane klokken en netvliesaandoeningen lijkt complex, omdat omgekeerd de ritmische eigenschappen van het netvlies tijdelijk worden geactiveerd tijdens degeneratie. Ten slotte komt de rol van de circadiane klok met betrekking tot fotoreceptoren al tot uiting tijdens de embryonale ontwikkeling, door een sleutelstap in hun ontogenese te reguleren; de overgang tussen verdeling en differentiatie van voorlopers.

Résumé de thèse

La rythmicité journalière est une caractéristique fondamentale de la vision qui englobe l'adaptation au cycle jour/nuit de la physiologie rétinienne et du traitement de l'information lumineuse. Ces processus cycliques sont régulés au sein de la rétine par une horloge circadienne dont la localisation cellulaire reste à déterminer. L'horloge rétinienne contrôle, par exemple, l'expression de l'ARNm des photopigments, la sensibilité visuelle et la phagocytose des segments externes des photorécepteurs par l'épithélium pigmentaire sous-jacent et la sensibilité à la phototoxicité. Différentes études ont montré que la perturbation de l'horloge circadienne rétinienne affecte la sensibilité à la lumière, le traitement de l'information visuelle et la viabilité des cellules photoréceptrices au cours du vieillissement. Des centaines de gènes impliqués dans des maladies génétiques de la rétine ont été identifiés (www.retnet.org). Ces maladies affectent principalement les photorécepteurs et/ou les cellules de l'épithélium pigmenté rétinien, et induisent de profonds changements d'expression génique puis de multiples événements moléculaires potentiellement néfastes, jusqu'à l'apoptose et la mort cellulaire des photorécepteurs et la perte de vision. Bien que des centaines de gènes associés à la dégénérescence rétinienne aient été identifiés, aucun lien n'a été établi entre l'horloge circadienne et les maladies génétiques de la rétine.

Ainsi, l'objectif de cette thèse était d'explorer le lien fonctionnel et moléculaire entre l'horloge et les maladies génétiques rétiniennes.

La description des résultats obtenus est divisée en trois parties dans le manuscrit. Dans la **partie I**, sont brièvement introduits les mécanismes moléculaires de l'horloge circadienne et de la régulation de l'horloge rétinienne, ainsi que la régulation de l'expression des gènes par l'horloge circadienne. Les maladies génétiques rétiniennes, en particulier la rétinite pigmentaire (RP) et leur impact sur la fonction de l'horloge circadienne sont décrits par la suite. Enfin, la fin de ce chapitre présente les objectifs généraux de la thèse (**Chapitre 1**).

Dans la **partie II**, nous avons exploré les effets du dysfonctionnement de l'horloge circadienne sur la physiologie et la physiopathologie rétiniennes. Tout d'abord, nous avons étudié les effets sur la rétine de la double inactivation des gènes d'horloge Period1 (*Per1*) et Period2 (*Per2*). *Per1* et *Per2* sont des composants clés de l'horloge circadienne des mammifères. Les deux gènes sont exprimés dans la rétine et présentent une expression rythmique.

Dans le **chapitre 2**, nous avons exploré le rôle des gènes d'horloge *Per1* et *Per2* dans le développement de l'œil. En effet, nous avions observé que les rétines de souris adultes doubles mutantes pour les gènes *Per1* et *Per2* présentent une distribution anormale des cônes bleus, suggérant une altération du développement de la rétine (Aït-Hmyed et al., 2013). Nous avons caractérisé les profils d'expression des gènes horloge entre E13 (13e jour de développement embryonnaire) et P24 (24e jour postnatal) dans les yeux de souris sauvages. Tous ces gènes (y compris *Per1* et *Per2*) sont exprimés et présentent des changements significatifs dans leur niveau d'expression au cours du développement normal de l'œil. Nous avons ensuite comparé les transcriptomes de l'œil entier entre les mutants *Per1*$^{-/-}$*Per2*^{Brdm1} et les souris sauvages à E15, E18 et P3. Les données de RNA-Seq ont montré des différences majeures entre les génotypes, qui s'accentuent entre E15 et P3. A E15 et E18 les différences les plus significatives concernent les gènes horloge, en lien avec la mutation de *Per1* et *Per2*. A P3, il y a une augmentation majeure de l'expression des gènes de la cascade de phototransduction chez les mutants, alors que les gènes liés au cycle cellulaire sont fortement réprimés. Nos résultats suggèrent qu'au cours du développement de la rétine, l'horloge circadienne joue un rôle dans la régulation de la transition entre sortie du cycle cellulaire et différenciation, à un moment critique pour la genèse des photorécepteurs (Bagchi et al., 2020).

Bien que des résultats antérieurs de notre laboratoire avec la même lignée de souris *Per1*$^{-/-}$ *Per2*^{Brdm1} n'aient révélé aucune altération significative de la fonction visuelle (électrorétinographie en conditions scotopiques et photopiques ; Aït-Hmyed et al., 2013) les propriétés de la réponse à la lumière sur 24h n'avaient pas été étudiées chez ce mutant, qui ne présente plus de rythme sommeil/activité dans des conditions constantes. Dans le **chapitre 3**, nous avons continué à explorer les effets de l'altération de l'horloge circadienne (lignée *Per1*$^{-/-}$*Per2*^{Brdm1}) sur la rythmicité rétinienne. Nous avons effectué l'analyse fonctionnelle et phénotypique en utilisant respectivement l'électrorétinographie (ERG) et l'immunohistochimie. Les résultats de cette étude montrent que l'inactivation des gènes *Per1* et *Per2* abolit la modulation journalière de la réponse visuelle, comme en témoignent les effets sur les amplitudes des ondes a et b dans l'ERG scotopique. Chez le mutant, la sensibilité à la lumière apparaît constitutivement augmentée, comme dans l'état nocturne des souris de type sauvage. Étonnamment, lors du vieillissement, il n'y a pas de diminution marquée de la

sensibilité visuelle ou de l'intégrité du tissu rétinien chez les mutants *Per1*$^{-/-}$*Per2*^{Brdm1}, indiquant que l'horloge circadienne n'est pas indispensable pour la santé rétinienne générale, ou que la présence du gène Per3 compense les absences de *Per1* et *Per2*. La conclusion de ce chapitre est que le traitement de la lumière par les photorécepteurs, dans des conditions scotopiques et mésopiques, chez la souris est contrôlé par une horloge circadienne. L'augmentation de la sensibilité des bâtonnets durant la nuit est en accord avec les contraintes de la vision nocturne, du moins chez la souris, espèce nocturne (Gegnaw et al., en préparation). Dans le **chapitre 4**, pour comprendre où sont générées les différences jour-nuit dans la réponse ERG, nous avons utilisé des souris présentant une inactivation (rod-*Bmal1KO*) du gène horloge Bmal1 (un gène clé de l'horloge circadienne) spécifiquement dans les bâtonnets pour faire le même type d'analyse ERG. Nous avons observé des différences (circadiennes) jour-nuit dans les ondes a et b de la réponse ERG chez des souris témoins (exprimant un allèle de Bmal1 dans des bâtonnets), avec des amplitudes plus élevées pendant la nuit subjective. Ces différences ont été abolies chez les souris rod-*Bmal1KO*, dont les réponses à la lumière ERG sont restées constitutivement réduites (comme pendant le jour). Nous avons introduit dans les souris rod-*Bmal1KO* le gène rapporteur Per2-Luciférase afin d'évaluer comment la perte de *Bmal1* dans les bâtonnets pourrait affecter la capacité rythmique globale de la rétine. Les enregistrement de bioluminescence générée par des explants de rétine entière de souris rod-*Bmal1KO* révèlent une période plus longue et une puissance rythmique plus élevée par rapport aux rétines dont le gène *Bmal1* est intact. La conclusion de ce chapitre est que le traitement de la lumière en conditions scotopiques et mésopiques chez la souris est régulé par l'horloge circadienne. Ils soutiennent l'idée que les bâtonnets contiennent une horloge circadienne qui permet une optimisation de la réponse à la lumière au cours du cycle jour/nuit (Gegnaw et al., 2021 Experimental Eye Research).

Ensuite, dans le **chapitre 5**, nous avons exploré les liens potentiels entre dysfonctionnement de l'horloge circadienne des bâtonnets et pathologie rétinienne. Nous avons réalisé cette étude chez la souris, en combinant le KO de *Bmal1* spécifique aux bâtonnets (rod-*Bmal1KO*) avec la mutation P23H de la rhodopsine (pigment visuel des bâtonnets), l'une des principales causes de rétinite pigmentaire (RP) autosomique dominante chez l'humain. Nous avons utilisé des souris de 4 génotypes : 1, portant la mutation (hétérozygote) P23H de la rhodopsine ; 2, rod-Bmal1KO; 3, des doubles mutants de ces souches et des souris témoins Ctrl. Nous

montrons par des analyses structurales (histologie, immunohistochimie) et fonctionnelles (électrorétinographie) que la délétion de Bmal1 dans les bâtonnets affectés par la mutation P23H exacerbe fortement le phénotype RP rétinien. En effet, nous avons observé une réduction de l'amplitude de l'ERG, une perte de cellules photoréceptrices et une gliose plus précoces chez les doubles mutants. Ces observations ont été corroborées par l'analyse RNA-Seq, qui révèle des changements majeurs dans l'expression génique entre les rétines double mutantes et simple mutantes P23H confirmant l'état de dégénérescence plus avancé dans le double mutant. Ces résultats indiquent que l'horloge circadienne des bâtonnets régule des mécanismes homéostasiques (liés notamment au métabolisme mais qui restent à identifier précisément) dont l'altération induit un effet synergique avec la dégénérescence rétinienne (Gegnaw et al., 2021 en préparation).

Dans la **partie III**, j'ai analysé les effets de la dégénérescence des photorécepteurs sur l'horloge rétinienne (**Chapitre 6**). J'ai utilisé les souris porteuses de la mutation P23H de la rhodopsine; soit à l'état hétérozygote, qui induit une dégénérescence modérée des bâtonnets, soit à l'état homozygote, responsable d'une perte rapide de l'ensemble des photorécepteurs. Comme dans le chapitre 3, nous avons aussi introduit dans ces souris le rapporteur Per2-luciférase pour examiner comment la dégénérescence des bâtonnets impacte la capacité rythmique globale de la rétine. La bioluminescence a été enregistrée sur des explants de rétines entières prélevées à P36 ou P70, 2 étapes clés de la dégénérescence chez les mutants. De manière surprenante, nos données montrent que l'horloge rétinienne chez les souris hétérozygotes P23H/+ (mais pas les homozygotes) présentent des rythmes circadiens plus robustes que chez les contrôles sauvages. Les résultats du traitement de ces explants rétiniens avec de l'acide L-α-aminoadipique, gliotoxine inhibant l'activité des cellules gliales, suggèrent que la rythmicité accrue observée chez les hétérozygotes P23H pourrait impliquer l'activation des cellules de Müller, principal type de cellule gliale dans la rétine (Gegnaw et al., 2022 Journal of Biological Rhythms).

En conclusion, les données obtenues dans cette thèse montrent que le traitement de la lumière par la rétine de souris, dans des conditions scotopiques et mésopiques, est régulé par une horloge circadienne qui induit une augmentation de la sensibilité visuelle spécifiquement pendant la nuit. Cette régulation a lieu dans les bâtonnets proprement dits, par l'horloge qu'ils contiennent. Cette dernière semble de plus indispensable à l'homéostasie des

bâtonnets, puisque son invalidation produit un effet synergique avec une dégénérescence d'origine génétique. Les mécanismes sous-jacents, suggérés par nos résultats, restent à démontrer de manière fonctionnelle. La relation entre horloges et pathologie rétinienne semble complexe puisque à l'inverse, les propriétés rythmiques de la rétine sont transitoirement activées pendant la dégénérescence. Enfin, l'importance de l'horloge circadienne vis-à-vis des photorécepteurs s'exprime déjà au cours du développement embryonnaire, en régulant une étape clé de leur ontogenèse ; la transition entre division et différenciation des précurseurs.

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The connection between circadian clock (impairment) and retinal disease

Résumé

Cette thèse a étudié comment une dérégulation de l'horloge circadienne, qui n'avait pas été clairement associée à une maladie rétinienne jusqu'à présent, pourrait contribuer à la dégénérescence et influencer le développement et la fonction de la rétine. L'inactivation spécifique du gène horloge *Bmal1* (rod-*Bmal1KO*) dans la lignée de souris portant la mutation P23H de la rhodopsine aggrave les symptômes de dégénérescence rétinienne, tels que la réduction de la réponse ERG et la perte de bâtonnets, induits par la seule mutation P23H. Ces observations ont été corroborées par l'analyse RNA-Seq qui a révélé des changements majeurs dans l'expression des gènes, liés à la phototransduction et aux processus métaboliques, entre le double mutant (rod-*Bmal1KO*/P23H) et les rétines P23H. Nous avons montré qu'au cours du développement, l'invalidation des gènes horloge *Per1* et *Per2* chez la souris affecte de manière significative l'expression des gènes de la phototransduction et du cycle cellulaire. Nous avons observé que les souris adultes déficientes en *Per1* et *Per2* ne modulent pas quotidiennement leur sensibilité à la lumière, dans des conditions scotopiques et mésopiques. Nous avons également constaté une altération de la régulation journalière de la sensibilité à la lumière chez les souris déficientes en gène d'horloge *Bmal1* dans les bâtonnets. De plus, nous avons investigué comment la dégénérescence des bâtonnets pourrait influencer la capacité rythmique globale de la rétine en mesurant les rythmes de bioluminescence PER2::LUC chez des souris P23H. Nos résultats montrent que l'horloge rétinienne chez les souris hétérozygotes *P23H/+* présente des rythmes circadiens avec une robustesse et une amplitude significativement accrues. Ces effets impliquent probablement l'activation des cellules gliales.

Mots clés: Rétine, Circadien, maladie rétinienne, développement, Scotopique

Résumé en anglais

This thesis investigated how circadian clock misregulation, which has not been clearly associated with retinal genetic disease so far, could contribute to degeneration and influence development and function in the retina. The rod-specific knockout of *Bmal1* clock gene (rod-*Bmal1KO*) from the mouse line carrying the P23H mutation of rhodopsin exacerbated the retinal degeneration phenotypes, such as reduction in ERG response and rods loss, induced by the P23H mutation alone. These observations were corroborated by RNA-Seq analysis, where we found major changes in expression of genes related to phototransduction and metabolic processes, between the (rod-*Bmal1KO*/P23H) double mutant and P23H retinas. We showed that during development, *Per1* and *Per2* clock genes deficiency in mice significantly affects gene expression of phototransduction and cell cycle components. We found that adult mice deficient for *Per1* and *Per2* genes lack a daily modulation of light sensitivity, under scotopic and mesopic conditions. We also found an impaired daily modulation of light sensitivity in mice deficient for *Bmal1* clock gene in rods. Additionally, we investigated how rod degeneration could impact on the global rhythmic capacity of the retina by measuring PER2::LUC bioluminescence rhythms in P23H mice. We showed that the retinal clock in *P23H/+* heterozygous mice displays circadian rhythms with significantly increased robustness and amplitude. These effects likely involve activation of glial cells.

Key words: Retina, Circadian, retinal disease, development, Scotopic

Appendix

PhD portfolio

PhD portfolio

Name PhD candidate: Shumet Tenaw Gegnaw

PhD period: October 2016 – December 2023

Name PhD supervisors: Marie-Paule Felder-Schmittbuhl, Arthur A. Bergen

1. PhD Training

Training name	Year	Workload (hours)
Scientific		
- Electrophysiology School of Strasbourg	2017	35
- Basic and Clinical aspects of neurobiology of rhythms Summer School	2017	14
- Animal experimentation	2018	30
- European Biological Rhythms Society Congress -Lyon	2019	35
Socio professional		
- Réunion de rentrée pour les doctorants de 1ère année	2016	2
- Electrophysiology School of Strasbourg	2017	3
- MOOC Intégrité scientifique dans les métiers de la recherche (Bordeaux)	2019	10
- Virtual visit of the Council of Europe and the European Parliament	2021	1:30
- Hack your Numistral	2021	3
- Integrity charter in Scientific profession	2021	3
- Identifying postdoctoral opportunities at international level and writing a proposal	2021	6
	Total	**141 hours**

2. Conferences

Poster presentations XVI European Biological Rhythms Society Congress: Circadian Clock Disruption Negatively Modulates Retinal Photoreceptor Degeneration.	2019	Lyon, France
NeuroFrance 2021: Circadian Clock Disruption Negatively Modulates Retinal Photoreceptor Degeneration.	2021	Strasbourg, France
Oral presentation Annual Neurotime meeting: Does a poorly functioning circadian clock constitutes a risk factor for (genetically determined) retinal disorders?	2017	Amsterdam, the Netherlands

3. Grant

Travel Grant from the Société Francophone de Chronobiologie to attend the European Biological Rhythms Society congress, 2019, Lyon, France.

4. Publications

Peer reviewed

Gegnaw ST, Sandu C, Mendoza J, Bergen AA, Felder-Schmittbuhl MP. Dark-adapted light response in mice is regulated by a circadian clock located in rod photoreceptors. ***Exp Eye Res*** (2021) 213:108807. doi:10.1016/j.exer.2021.108807.

Gegnaw ST, Sandu C, Mazzaro N, Mendoza J, Bergen AA, Felder-Schmittbuhl MP. Enhanced Robustness of the Mouse Retinal Circadian Clock Upon Inherited Retina Degeneration. ***J Biol Rhythms*** (2022) 37(5):567-574. doi: 10.1177/07487304221112845. Epub.2022.35912966.

Bagchi U, Gegnaw ST, Milićević N, Sandu C, Ten Brink JB, Jongejan A, Hicks D, Moerland PD, Felder-Schmittbuhl MP, Bergen AA. Core-clock genes Period 1 and 2 regulate visual cascade and cell cycle components during mouse eye development. ***Biochim Biophys Acta Gene Regul Mech*** (2020) 1863:194623. doi: 10.1016/j.bbagrm.2020.194623.

4. Manuscripts in preparation

Shumet T. Gegnaw, Cristina Sandu, Milicevic N, Jacoline B. ten Brink, Aldo Jongejan, Perry D. Moerland, Marie Paule Felder-Schmittbuhl, Arthur A. Bergen. Circadian Clock Disruption Negatively Modulates Retinal Photoreceptor Degeneration.

Shumet T. Gegnaw, Udita Bagchi, Nemanja Milićević, Arthur A. Bergen, Marie-Paule Felder-Schmittbuhl. Loss of daily rhythm in treatment of light information in the *Per1/Per2* mutant mouse.

Appendix

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Above all, don't fear difficult moments. The best comes from them. – Rita Levi-Montalcini

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