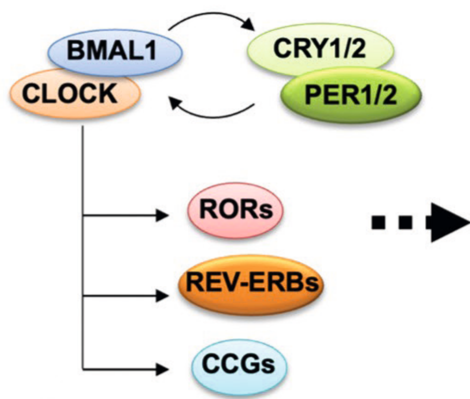
BIOLOGICAL RHYTHMS IN RETINAL DEVELOPMENT

Demystifying the eye

Udita Bagchi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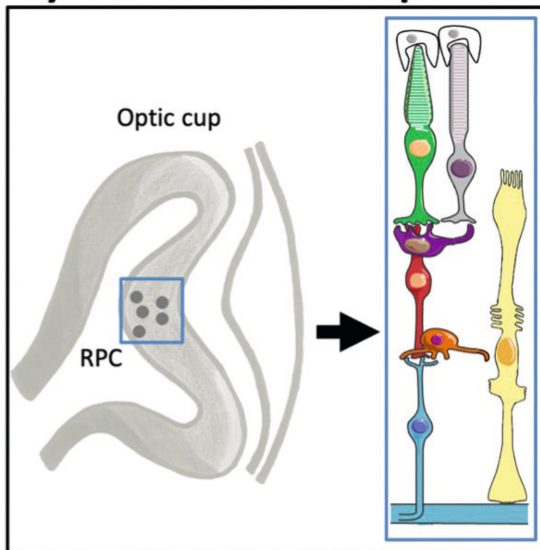
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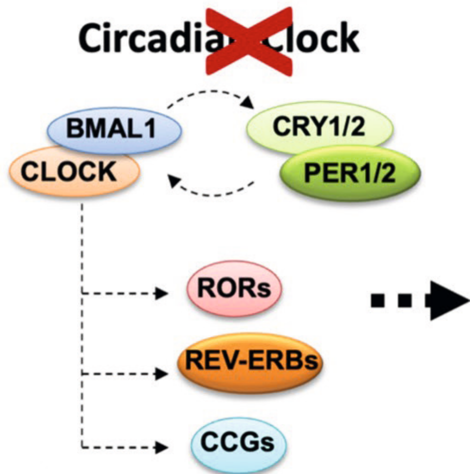
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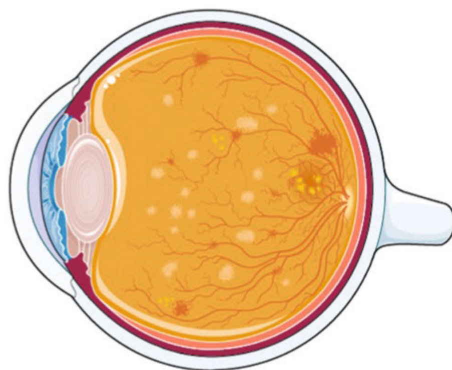
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Demystifying the eye

Udita Bagchi

We shall not cease from exploration, and the end of all of our exploring will be to arrive where we started and know the place for the first time.

- T.S Eliot

Biological Rhythms in Retinal Development.
Academic Thesis, Institute of Cellular and Integrative Neurosciences (INCI), Universitè de Strasbourg, France and Department of Clinical Genetics, University of Amsterdam, The Netherlands.

ISBN: 978-94-6458-704-3
Author: Udita Bagchi
Provided by thesis specialist Ridderprint, ridderprint.nl
Layout and design: Hans Schaapherder, persoonlijkproefschrift.nl

This work was financially supported by Neurotime Erasmus+, an Erasmus Mundus Program funded by the European Commission. Project number: 520124-1-2011-1-FR-ERA Mundus-EPJD. FPA: 2012-0026.

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UNIVERSITÉ DE STRASBOURG
France

UNIVERSITÉ D'AMSTERDAM
Pays-Bas

ÉCOLE DOCTORALE Sciences de la vie et de la santé de Strasbourg (ED414)

Institut des Neurosciences Cellulaires et Intégratives CNRS UPR3212

THÈSE présentée par :

Udita BAGCHI

Soutenue le : **19 décembre 2022**

pour obtenir le grade de : **Docteur de l'université de Strasbourg**
&
Docteur de l'université d'Amsterdam

Discipline/ Spécialité : Biologie/Neurosciences

Rôle de l'horloge circadienne dans le développement de la rétine

THÈSE dirigée par:

Dr. FELDER-SCHMITTBUHL Marie-Paule	Docteur, Université de Strasbourg
Prof. Dr. BERGEN Arthur A.	Professeur, Université d'Amsterdam

RAPPORTEURS:

Prof. Dr. KALSBEEK Andries	Professeur, Université d'Amsterdam
Dr. DKHISSI-BENYAHIA Ouria	Docteur, Université Claude Bernard Lyon 1

AUTRES MEMBRES DU JURY:

Dr. CHALLET Etienne	Docteur, Université de Strasbourg
Dr. ROUX Michel J.	Docteur, Université de Strasbourg
Prof. Dr. LA FLEUR Suzanne E.	Professeur, Université d'Amsterdam

Biological Rhythms in Retinal Development

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam

op gezag van de Rector Magnificus
prof. dr. ir. P.P.C.C. Verbeek

ten overstaan van een door het College voor Promoties ingestelde commissie,
in het openbaar te verdedigen in Institut des Neurosciences Cellulaires et Intégratives
op **maandag 19 december 2022**, te **14:00 uur**

door Udita Bagchi

geboren te Varanasi

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Promotores:	Prof. Dr. A.A.B. Bergen	AMC-UvA
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	Dr. M. J. Roux	IGBMC Strasbourg

Faculteit der Geneeskunde

Dit proefschrift is geschreven in het kader van het NeuroTime Erasmus Mundus Joint Doctorate Neuroscience PhD-programma, met als doel het behalen van een gezamenlijk doctoraat. Het proefschrift werd voorbereid in de Faculteit der Geneeskunde, van de Universiteit van Amsterdam; en in het Institut des Neurosciences Cellulaires et Intégratives, Université de Strasbourg.

This thesis has been written within the framework of the NeuroTime Erasmus Mundus Joint Doctorate Neuroscience PhD program, with the purpose of obtaining a joint doctorate degree. The thesis was prepared in the Faculty of Medicine at the University of Amsterdam and in the Institut des Neurosciences Cellulaires et Intégratives, Université de Strasbourg.

To suppose that ***the eye with all its inimitable contrivances*** *for* ***adjusting the focus to different distances****, for* ***admitting different amounts of light****, and for the* ***correction of spherical and chromatic aberration****, could have been formed by natural selection, seems, I freely confess, absurd in the highest degree.*

When it was first said that the sun stood still and the world turned round, the common sense of mankind declared the doctrine false; but the old saying of Vox populi, vox Dei, as every philosopher knows, cannot be trusted in science. ***Reason tells me, that if numerous gradations from a simple and imperfect eye to one complex and perfect can be shown to exist, each grade being useful to its possessor, as is certainly the case****; if further,* ***the eye ever varies and the variations be inherited****, as is likewise certainly the case; and if such variations should be useful to any animal under changing conditions of life, then the difficulty of believing that* ***a perfect and complex eye could be formed by natural selection****, though insuperable by our imagination, should not be considered as subversive of the theory.*

How a nerve comes to be sensitive to light*, hardly concerns us more than how life itself originated; but I may remark that, as some of the lowest organisms, in which nerves cannot be detected, are* ***capable of perceiving light****, it does not seem impossible that certain sensitive elements in their sarcode should become aggregated and developed into nerves,* ***endowed with this special sensibility****.*

-Charles Darwin (The Origin of Species)

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PART

Introduction and Outline

CHAPTER

General Introduction and Thesis Scope

CIRCADIAN RHYTHMS – THE BIOLOGICAL TIME-KEEPING SYSTEM

Circadian timing systems (CTS) are the oldest continuous endogenous timer, fundamental to all living organisms. These systems have developed throughout evolution from bacteria/unicellular algae to vertebrates, to respond and adapt optimally to the daily changes in the environmental cycles caused by the rotation of the earth around its axis (Ballesta et al. 2017; Takahashi 2017). A conserved model of the autoregulatory transcription/translation-based feedback loops is found across divergent phyla (Hardin et al. 1990; Loros and Dunlap 1991; Takahashi 2017). In the 1990s, discovery of clock genes (Takahashi 2004) confirmed the existence of the biological clock in mammals, organized in a hierarchical cluster of multiple clocks spread across the body. The capacity to generate circadian oscillations and clock gene expression in virtually all cells was subsequently reported (Lowrey et al. 2004; Takahashi et al. 2008).

At the apex of this hierarchical organization is the master clock located in the hypothalamus inside the suprachiasmatic nucleus (SCN), which acts as the central pacemaker to synchronize and entrain all peripheral clocks distributed throughout the body (Buhr and Takahashi 2013). The master clock oscillates with a period of approximately 24 hours, which coined the term ‘circadian’ (circa = about and dian = a day).

Societal clock alterations began over the last century with rapid industrialization and urbanization, leading to round the clock activities such as shift-work, jetlag, indoor working with little exposure to environmental light, over-exposure to digital screens and other light-emitting electronic devices late at night. These are common reasons for perturbed sleeping rhythms, daytime activity and eating disorders. Potential circadian disorders in humans mainly occur when this misalignment between the environmental rhythm (e.g., light–night cycle) and the endogenous circadian oscillators occur. For instance, severe imbalances in glucose homeostasis, insulin action, and appetite control have been reported in human subjects maintained in controlled circadian misalignment conditions (Buxton et al. 2012; McHill et al. 2014). The high redundancy in clock genes (often studied in animal models) might have a link in developing diseases due to clock gene mutations in humans, though such occurrences are few in humans so far. Examples of these mutations in clock genes include familial advanced sleep phase syndrome, which is induced by a missense mutation (S662G) of the core clock gene *PER2* (Jones et al. 1999; Xu et al. 2007). In addition, genome-wide association studies have identified several single nucleotide polymorphisms in clock genes associated with metabolic syndromes, hypertension, and diabetes mellitus (Saxena et al. 2007; Woon et al. 2007; Zeggini et al. 2007; Scott et al. 2008).

Mammalian circadian clock gene network

The molecular clockwork generating the circadian oscillations has been based on transcription/translation-based feedback loops involving "clock" gene encoded transcription factors (PER1-2, CRY1-2, BMAL1, and CLOCK). Over the 24h cycle, these feedback loops can transcriptionally regulate most cycling genes and gene expression programs (as shown in Figure 1).

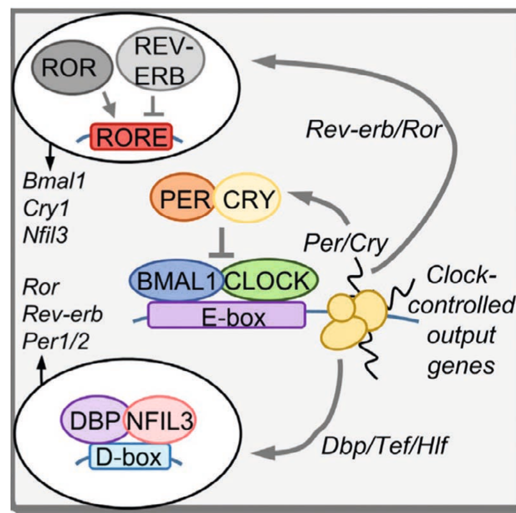


Figure 1. Simplified schematic representation of the mammalian molecular clock. The CLOCK:BMAL1 heterodimer binds to the E-boxes and initiates the transcription of clock target genes (primary loop). PER and CRY proteins form a heterodimer in the cytoplasm and translocate back to the nucleus when sufficiently high concentrations are reached. In the nucleus, the PER/CRY heterodimers inhibit CLOCK:BMAL1 from further activation of the transcription processes, which together constitute the primary negative feedback loop of the molecular clock. In particular, phosphorylation of PER and CRY proteins regulates nuclear entry of the PER/CRY heterodimer and its stability allowing the cycle to restart (hence also controlling the clock period). The *Bmal1* transcriptional activation by ROR, which is inhibited by REV, constitutes a secondary feedback loop. Also, NFIL3 and DBP or related factors (TEF/HLF) regulate the expression of *Ror*, *Rev-erb* and *Per1/2*, constituting another feedback to the clock. *Ror*, RAR-related orphan receptor; *Rev-erb*, the antisense *Rev-Erbα* in relation to the c-*ErbA-α/Thra* gene; RORE, ROR element; *Dbp*, albumin D-box binding protein; *Tef*, thyrotroph embryonic factor; *Hlf*, hepatic leukemia factor; *Nfil3*, nuclear factor, interleukin 3 regulated. Adapted from Koronowski and Sassone-Corsi, Science 2021.

At the core of the clockwork, the basic helix–loop–helix (bHLH)–PER-ARNT-SIM (PAS) transcription factors CLOCK and BMAL1 heterodimers bind and activate the E-box recognition sites of *Per1*, *Per2*, *Cry1*, and *Cry2* genes (Buhr and Takahashi 2013; Takahashi 2017). The protein products of these clock genes interact with each other: post-translational phosphorylation of the protein products regulates the entry and stability of PER/

CRY heterodimers into the nucleus, inhibiting the activity of the BMAL1/CLOCK complex and thereby repressing their own transcription. This allows the primary positive loop to restart gradually.

As part of the secondary loop, CLOCK and BMAL1 regulate the nuclear hormone receptors REV-ERBα and REV-ERBβ (encoded by *Nr1d1* and *Nr1d2*, respectively), which rhythmically represses the transcription of *Bmal1* and *Nfil3* (which encodes nuclear factor, interleukin-3 regulated). The activators driving this process are the retinoic acid-related orphan receptor-α (RORα), RORβ and RORγ (encoded by *Rora*, *Rorb* and *Rorc*, respectively) (Preitner et al. 2002; Sato et al. 2004; Guillaumond et al. 2005; reviewed in Takahashi 2017; and presented in Figure 1).

Besides the primary and the secondary loop, there exists another pathway that feeds back onto the other loops via D-boxes and involves the PAR-bZip factors DBP (D-box binding protein), TLF (thyrotroph embryonic factor) and HLF (hepatic leukemia factor), thus interacting with the NFIL3 repressor. These three feedback loops are known to transcriptionally regulate the clock output genes CCGs (clock-controlled genes) by acting on E-boxes, ROR-binding elements (ROREs), and D-boxes in the regulatory regions of target genes, thus regulating the activity and function of the clock itself.

The Eye: a multi-oscillatory circadian organization

The interplay between the brain and the light sensitive tissue lining the back of the eye – the retina transforms photons from the environment into electrical and neurochemical signals.

It is now known that besides retina, RPE (*rod and cone outer segment shedding and vulnerability to phototoxicity*), the iris/ciliary body (*aqueous humour production contributing to intraocular pressure-IOP*) and the cornea (*daily variation of thickness or mitotic rate*) also harbor autonomous clocks, that receive no or only restricted influence from the master circadian clock (Ikegami et al. 2020; Bobu et al. 2009; LaVail et al. 1980; Organisciak et al. 2000, Kikkawa et al. 1973; Xue et al. 2017; Lozano et al. 2015; Tsuchiya et al. 2017).

The entrainment of the RPE circadian clock must depend on signals coming from the retinal photoreceptors, it has been reported that dopamine—acting via D_2R—can entrain the RPE circadian clock (Baba et al. 2017, Goyal et al. 2020). Also, the activation of muscarinic receptors can induce a phase-shift in the human RPE circadian clock (Ikarashi et al. 2017). Interestingly—it has been reported that the peak of phagocytic activity by the RPE is advanced (about 3 hours) in mice lacking MT1 or MT2 receptors with respect to

control mice, although melatonin does not entrain the RPE circadian clock (Laurent et al. 2017). These data have indicated that the result of interactions between the retinal and the RPE circadian clocks suggests the timing of the daily burst in phagocytic activity (Felder-Schmittbuhl et al. 2018) but the rhythm is essentially determined by the RPE clock (De Verra et al. 2022). It has been reported that clock genes and proteins are expressed in the iris-ciliary body complex of mice (Dalvin et al. 2015) and cultured iris/ciliary body obtained from *mPer2*^{Luc} mice demonstrated a circadian rhythm in bioluminescence (Tsuchiya et al. 2017). Also, the circadian rhythm could not be entrained by light in the PER2::LUC bioluminescence from cultures of iris/ciliary body, thus highlighting that the entrainment of the circadian rhythm in these structures—and possibly the IOP—is mediated via neuronal or hormonal signaling outside the ciliary body and even from outside the eye (Felder-Schmittbuhl et al. 2018). Finally, it is worthwhile mentioning that the renewal of the corneal epithelium also showed a daily rhythm (Doughty et al. 1990) and the rate of mitosis is time of day dependent (highest during the night and lowest during the day) (Sandvig et al. 1994). A robust circadian rhythm in bioluminescence was obtained from the cultured corneas of *mPer2*^{Luc} mice (Yoo et al. 2004; Baba et al. 2015; Evans et al. 2015) that can be entrained by light via neuropsin or OPN5 (Buhr et al. 2015; Calligaro et al. 2019) or by melatonin via activation of MT2 receptors (Baba et al. 2015). It has been shown in the mice that circadian pattern of the corneal epithelial mitosis and clock gene expression can also be altered under constant light, constant dark, or jetlag conditions (Xue et al. 2017).

It is evident from the aforementioned evidence that many ocular structures contain circadian clocks; however, the cues used by these structures to entrain their circadian rhythms may vary (Felder-Schmittbuhl et al. 2018) and the interaction of these clocks also remains to be investigated.

The retina is a fascinating heterogeneous tissue, composed of an extraordinary cell circuitry of five neuronal cell types - photoreceptors, bipolar cells, retinal ganglion cells, horizontal cells and amacrine cells wired together into one of nature's most complex circuit boards. This circuitry is kept healthy by the retinal pigment epithelium (RPE) which is a honeycomb like structure sandwiched tightly between the photoreceptors and the Bruch's membrane. The RPE layer transports nutrients from the blood to the retina and hoovers up the toxic byproducts created by the photoreceptors as they react to light – a process more commonly known by phagocytosis. The retina is organized into discreet retinal cellular layers that include the RPE, outer nuclear layer (ONL), inner nuclear layer (INL) and the ganglion cell layer (GCL) separated by synaptic (plexiform) layers (Masland 2012 - presented in Figure 2A).

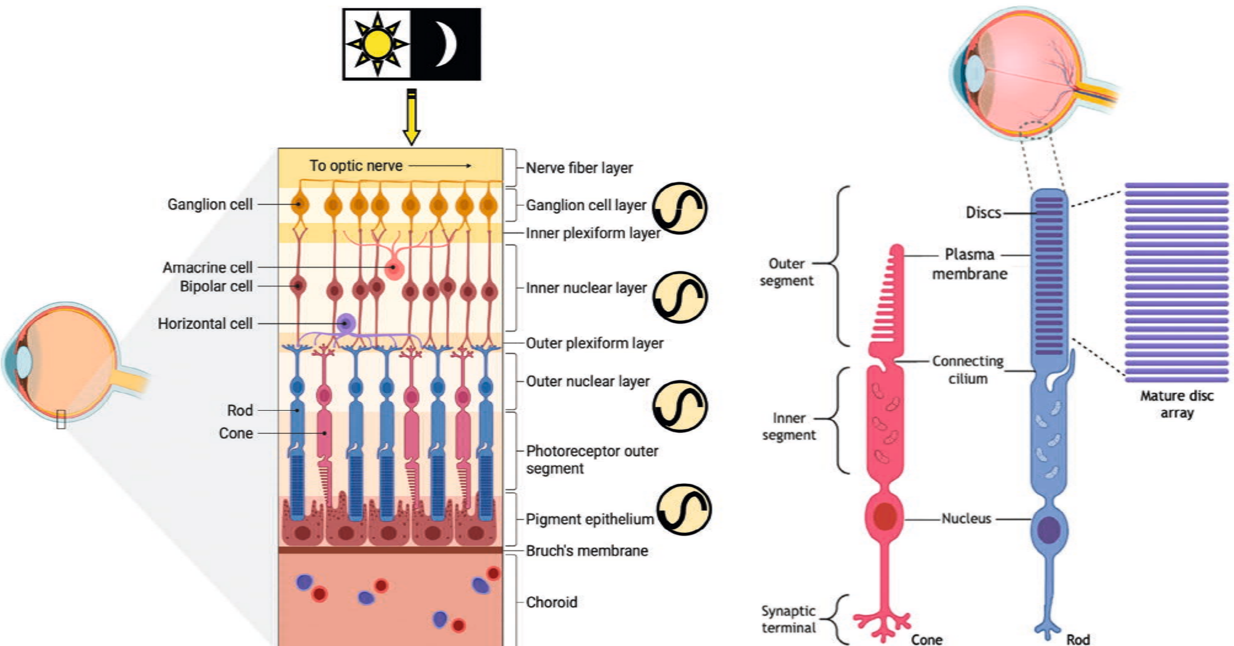


Figure 2. (A) Anatomical representation of the rodent retina and the localization of retinal clocks [adapted from Felder-Schmittbuhl et al. 2018]. The mammalian retina lies on the posterior part of the eye. The light-sensitive photoreceptors (rods and cones) are near the adjacent retinal pigment epithelium (RPE). Numerous retinal cell layers show rhythmic clock gene expression. These layers include the RPE, outer nuclear layer (ONL), inner nuclear layer (INL) and the ganglion cell layer (GCL). Axon-to-dendrite neural connections make up the plexiform layers. The inner and outer plexiform layers are known by abbreviations IPL and the OPL. The photoreceptors are segmented structures, having an inner segment (IS) connected through cilium to the outer segment (OS) that contains organized disc arrays in rods and membrane folds in cones [*presently OS is considered a specialized cilium (that exists in many cell types), even if the connecting cilium exists as well*] and ribbon synapses at their end-feet. Photo-transduction takes place in the outer segment in the presence of various photopigments like rhodopsin etc. **(B) An enlarged illustration of the segmented rod and cone photoreceptors.** Adapted from O'Hara-Wright and Cordero 2020. Created on biorender.com.

The Outer Nuclear Layer (ONL) consists of the cell bodies and nuclei of the photoreceptors (specialized light-sensitive segmented cells - Figure 2B); the Inner Nuclear Layer (INL) contains bipolar, horizontal, amacrine, nuclei of Müller glial cells and (displaced) ganglion cells; the Ganglion Cell Layer (GCL) contains ganglion and (displaced) amacrine cells. A detailed anatomical representation of the rodent retina is displayed in Figure 2A.

The retinal architecture and organization implicate that it can prepare itself to actively anticipate changes in the environmental light intensity, hence, most retinal cell types exhibit circadian clock properties outside of the SCN. The discovery of the independent retinal clock (Tosini & Menaker 1996, Besharse & McMahon 2016) in the mammalian eye accomplished the retina as a unique multi-oscillatory light-entraining circadian model. There is a complex grid of circadian oscillators distinctly powered by different retinal layers including the GCL and the RPE. Several biological functions in the eye have also been linked to the clock [reviewed in (McMahon et al. 2014; Felder-Schmittbuhl et al. 2017)]. These extend from the expression of photopigments (Bobu et al. 2013; von Schantz et al. 1999), visual sensitivity (Barnard et al. 2006; Cameron et al. 2008; Storch et al. 2007), photoreceptor outer segment phagocytosis by the RPE (the shedding is not regulated clearly by the clock based on present knowledge), susceptibility to phototoxicity (Bobu et al. 2009; LaVail 1980; Organisciak et al. 2000), daily variation of corneal thickness or mitotic rate (Kikkawa et al. 1973; Xue et al. 2017) and rhythmic processes in the ciliary body (Lozano et al. 2015; Tsuchiya et al. 2017). These data provide potential insight into the rhythmic physiology of the eye.

Organization of retinal rhythmicity at the level of the photoreceptor network

Temporal signal processing in the retina over the 24-hour period is influenced by light and modulated by circadian clock activity (McMahon et al. 2014; Barlow et al. 2001; Cahill et al. 1995; Green et al. 2004; Iuvone et al. 2005). Although clock gene expression is widespread in the retinal tissue and cells, we know little about individual contributions of each retinal cell type's clock to the overall daily changes in retinal function. It can be hypothesized that the functional organization in the retina at all levels is influenced by clocks, either through clock pathways in the direct vicinity or remotely via diffusible signals such as dopamine and melatonin (Felder-Schmittbuhl et al. 2018).

Accumulating evidence suggests that gap junctions play a prominent role in how illumination and circadian clock activity changes in the retinal function over 24h (Bloomfield et al. 2009; Ribelayga et al. 2017). The involvement of circadian clocks in the plasticity of gap junctions in retinal circuits remains unknown, but evidence has indicated that electrical coupling between photoreceptors is modulated by ambient light and circadian clocks. There is weak junctional conductance during the subjective day, much stronger in subjec-

tive night, and nearly abolished under bright light illumination, irrespective of the time in the daily cycle. These electrical coupling characteristics may contribute to the role of the melatonin/dopamine pathway, daily plasticity, and profound changes in the downstream circuits in the process of rod and cone light response properties (Felder-Schmittbuhl et al. 2018; Ribelayga et al. 2008; Jin et al. 2015 and 2016).

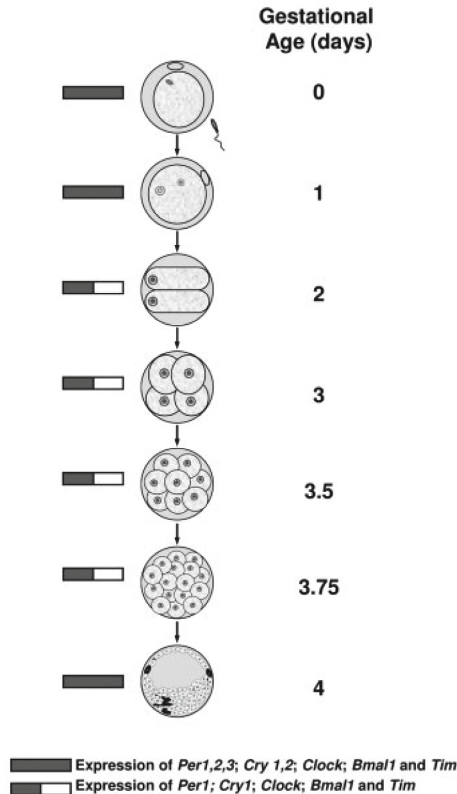


Figure 3. Core clock gene expression at different early embryonic stages in the quakenbush strain mice**.** The mRNA expression of the six canonical clock genes (*Per 1,2,3*; *Cry1,2*; *Clock*, *Bmal1*) were found in the unfertilized mouse oocyte at gestational days 0 and 1 (as shown by the black rectangles). Post fertilization, expression of these mRNAs decreases between the two-cell and 16-cell stages at gestational days 2, 3, 3.5 and 3.75 (as shown by the black-white rectangles) followed by a possible reinitiation at the blastocyst stage (gestational day 4). Adapted from Seron-Ferre et al. 2007 and Johnson et al. 2002.

Link between clock genes and retinal physiology

Over the years, it has been shown repeatedly that clock genes influence retinal physiology. For example, combined deletion of *Per1* and *Per2* has been correlated with a

reduced number of blue cone opsin (*Opn1sw*) expressing cells and decreased steady-state levels of blue cone opsin gene expression up to 1 year of age (Aït-Hmyed et al. 2013). Loss of *Rev-Erbα* (*Nr1d1*) expression results in retinal visual processing defects and disturbances in the retinal sensitivity to ambient light (Aït-Hmyed et al. 2016; Mollema et al. 2011). Spatial patterning of cone opsins and photoreceptor viability during aging in the retina is under *Bmal1* control (Sawant et al. 2017; Ash et al. (eds.) 2018). Finally, *Cry1/Cry2* knockout mice have compromised cone function (Wong et al. 2018).

Circadian clocks and development

During vertebrate development, some data suggest that the coordinated timing of cellular division and differentiation are under circadian control (Vallone et al. 2007). These timing cues are fed directly into the developmental program right from the embryonic stage progressively maturing during development, with attenuation of the clock function in the adult (Seron-Ferre et al. 2007 - presented in Figure 3). We have explored among vertebrates how the evolutionarily conserved gene regulatory networks controlling proliferation, specification, and differentiation of retinal precursors are interconnected with circadian mechanisms in – **Chapter 2**.

The circadian coordination of transcriptomic programs is also necessary during keratinocyte stem cell development (Janich et al. 2013). Also, in the multipotent neural precursor cells (NPCs), robust circadian oscillations of gene expression have been demonstrated (Dierickx et al. 2017; Weger et al. 2017; Draijer et al. 2018), though pluripotent embryonic stem (ES) cells show no visible rhythmicity (Kowalska et al. 2010; Yagita et al. 2010). Moreover, upon de-differentiation of NPCs back to ES cells, rhythms are lost but regained upon re-differentiation to NPCs (Yagita et al. 2010). We may probably suggest that the cell-intrinsic rhythms of circadian gene expression emerge during development. One of the very first study in mammals showed that soon after birth, overt rhythms of behaviour and physiology emerged (Dolatshad et al. 2010). The circadian control via the light dependent expression of the *BMAL1* and *PER1* clock genes is known to regulate the expression of melanogenic enzymes (TYR, TYRP1, DCT) and production of melanin in hair follicle melanocytes (Hardman et al. 2015). Possibly the circadian clock in the RPE could regulate L-DOPA and melanin production (Bakker et al. 2022). Although, it is currently unclear whether the aforementioned clock regulated processes act in sync with the retinal pigmentation pathway, nonetheless circadian timing should be considered during the development of pigmentation as well. From as early as embryonic day 13 (E13), there are visible signs of clock gene expression in rodent eyes – **Chapter 3**.

Impact of clock malfunction on development

Many physiological functions in mammals such as cell cycle progression, wakefulness and sleep, fluctuations in body temperature, alertness and motor ability, hormone secretion, immune regulation and cytokine release are under circadian clock regulation (Panda et al. 2002; Bass and Takahashi 2010). Circadian misalignment due to environmental (jetlag, shiftwork, or artificial light at night) changes or genetic defects is a common reason for dysfunction of various physiological processes during development till adulthood (Hastings et al. 2003). Circadian clock is essential for postnatal brain development (Kobayashi et al. 2015). During pubescence, sleep and circadian disruptions impacts brain development and causes susceptibility to mood disorders and inability to control the usage of legal or illegal drugs, alcohol, or medications leading to substance use disorders (Logan and McClung 2019).

Studies have shown that the clock component REV-ERBα directly inhibits the promoter of the *Fabp7* gene, a marker for neuronal progenitor cells, resulting in alterations in neuronal differentiation (Schnell et al. 2014), thus confirming the participation of circadian clocks in the regulation of neurogenesis. Furthermore, it was shown that in the absence of REV-ERBα, increased hippocampal neurogenesis and FABP7 expression were found, which was in connection to mood-related behavioral changes (Schnell et al. 2014). Persistent illumination during pregnancy resulted in impaired spatial memory in the hippocampus of rat adult progeny, due to the simultaneous inhibition of the genes encoding NMDA receptor subunits and clock genes (Vilches et al. 2014). Clock gene deletion has been linked with disturbed cell fate determination causing perturbed precursor proliferation in the early developing murine retina (Sawant et al. 2019; Bagchi et al. 2020). The absence of *Bmal1* alters rod bipolar cell development, as suggested by the abnormal presence of stunted dendritic processes of rod BCs already at 1 month (Baba et al. 2018a). To conclude, based on the subtle link between circadian clock and developmental physiology, this thesis has attempted to explore the role of the circadian clock in the retina as outlined below.

Thesis scope

The aim of the thesis is to understand how biological clock shapes eye development and retinal pathology. We have published our findings about the clock gene modulation effects in various signaling pathways during murine retinal development – **Chapter 3**. Also, we have focussed briefly about the clock links with ocular pathologies – **Chapter 2**. This thesis is composed of a series of experiments aimed to identify clock induced signals in rodents during retinal development.

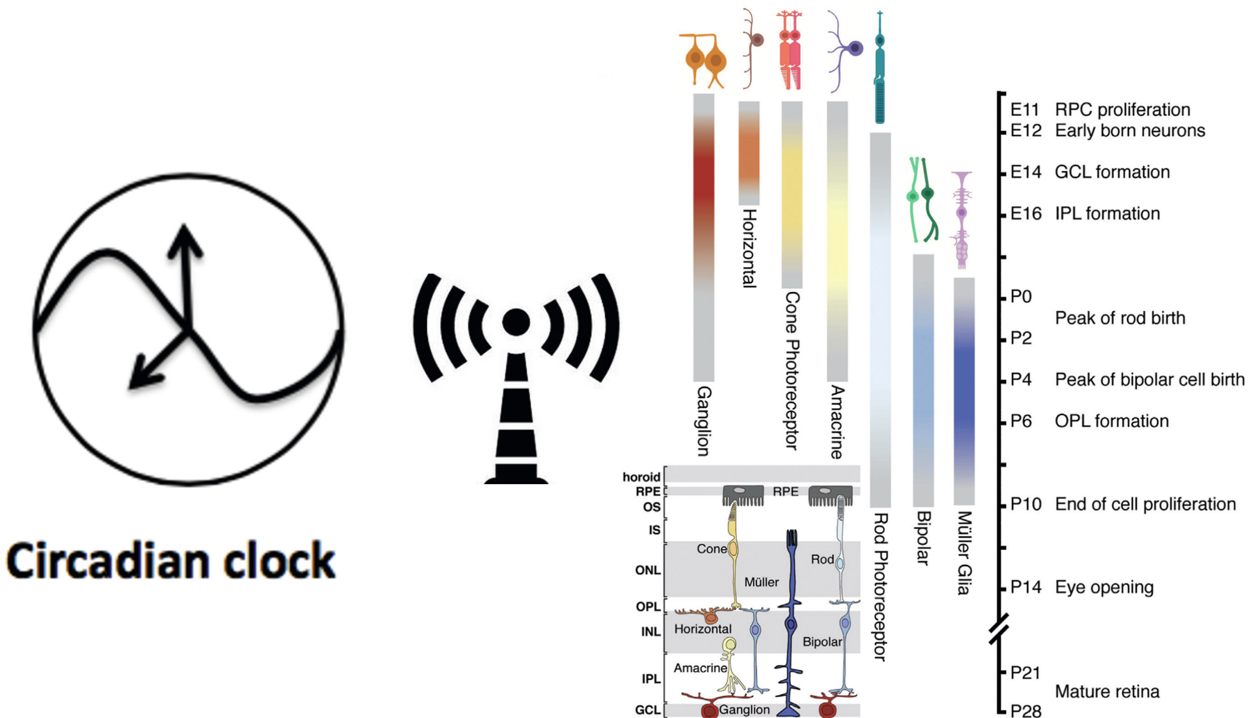


Figure 4. Hypothesis layout. Understanding the contribution of circadian clock mediated signals during retinal/photoreceptor development. Retinal neurogenesis occurs in a fixed histogenetic order as shown in the right image. Differentiation of the seven main retinal cell types from retinal progenitor cells (RPCs) proceeds sequentially in waves, with retinal ganglion cells, horizontal cells, cone photoreceptor cells and amacrine cells formed in an early retinogenesis wave, followed by the overlapping late-phase generation of rod photoreceptor, bipolar and Müller glia cells. The timing of RPE development is still unclear, it is a derivative of the optic neuroepithelium, which is initially specified as a patch of cells, the “eye field”, in the anterior neuroectoderm. The bars reflect the relative proportion of cells produced for each cell type. Adapted from O’Hara-Wright and Cordero 2020 and Brooks et al. 2019.

The main hypothesis of the thesis stems from the fact that there exists contribution of circadian clock mediated signals during retinal/photoreceptor development (presented in Figure 4) was generated from previous experiments when clock malfunction in the *Per1/Per2* double mutant mice showed a delay in general photoreceptor differentiation, along with reductions in cone opsin mRNA and protein levels (Aït-Hmyed et al. 2013). *Per1$^{-/-}$Per2^{Brdm1}* mutants carried the loss-of-function mutation of both genes (Zheng et al. 1999, 2001).

Part I, Chapter 1 is comprised of this general introduction to the field of chronobiology and summarizes previous research done in the field which paved the way to generate our main research question: Role of the biological clock in the development of the retina. **Chapter 2**, we briefly summarized the necessary physiological knowledge to comprehend and highlight how the circadian clock regulates vertebrate retinal development. Our review report is the first to investigate the importance of clock genes and circadian clocks in retinal development. We have also provided a detailed insight on ocular diseases that are associated with clock malfunction.

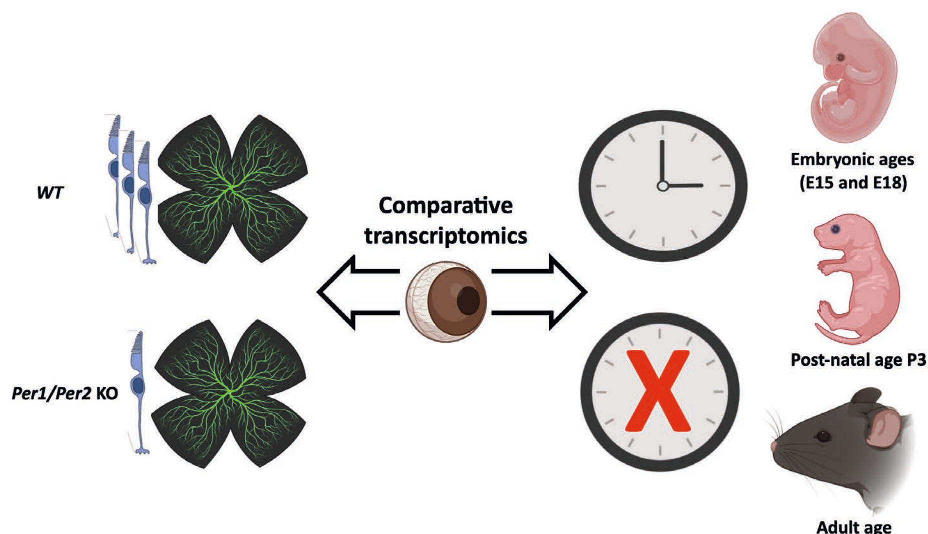


Figure 5. Research schematic: The retinas from Period 1 (*Per1*) and Period 2 (*Per2*) double-mutant *mice (Per1$^{-/-}$Per2^{Brdm1}) displaying retinal abnormalities (as shown by the dark whole retinal mount) versus the wild-type retina* were used to elucidate the downstream events that link circadian clock with retina/photoreceptor development. Comparative transcriptomics was employed to better understand the retinal physiology under both wild-type state and in clock dysfunction at embryonic ages (E15 and E18), post-natal age P3 and in the adult**.** Adapted from Aït-Hmyed et al. 2013.

The overall research schematic is illustrated in Figure 5. In **Part II**, we have explored the relevance of core-clock genes Period 1 and 2 on the developing and adult eye physiology using mouse models. In **Chapter 3**, 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 $Per1^{-/-}Per2^{Brdm1}$ mutants versus wild-type at E15, E18 and P3. In **Chapter 4**, we showed that under constant darkness, mice deficient for core circadian clock genes (*Per1* and *Per2*), lack a daily peak in POS phagocytosis. Compared to WT, $Per1^{-/-}Per2^{Brdm1}$ mutants were found to have a number of differentially expressed genes at the peak phagocytic timepoint. A potential group of interacting genes such as *Pacsin1*, *Syp*, *Camk2b* and *Camk2d* that might drive POS phagocytosis in the RPE were identified. In **Chapter 5**, impairment of *Per1* and *Per2* genes caused loss of 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.

In **Part III**, we have focussed on the clinical perspectives of the circadian clock system and clock genes in humans. In **Chapter 6**, we examined *BMAL1* as an active candidate gene of interest in the probands of consanguineous congenital cataract families by traditional Sanger sequencing. Over the past decade, numerous applications of chronobiology related health outcomes have been extensively explored by health care professionals (HCPs) in human clinical trials. In **Chapter 7**, we initiated a systematic analysis of all the current Patient-reported Outcome Measures (PROMs) associated with the circadian clock. PROMs or more commonly known by questionnaires are used in a variety of diseases in routine clinical practice. Our analysis was done to prepare an exhaustive list of questionnaires that address clock-related attributes like sleep, chronotype, circadian rhythms and shiftwork.

Lastly, **Chapter 8** takes a retrospective view of all the previous experimental chapters, integrating the acquired knowledge (**Chapter 3-7**) with the latest understanding in the field to better understand the vast implications of the circadian biology on eye physiology. Also, recommendations for future follow-up experiments and other possible research questions have been discussed in this section, including the limitations and strengths of this thesis.

REFERENCES

Ait-Hmyed Hakkari, O., Acar, N., Savier, E., Spinnhirny, P., Bennis, M., Felder- Schmittbuhl, M.P., Mendoza, J., Hicks, D., 2016. Rev-Erbalpha modulates retinal visual processing and behavioral responses to light. Faseb. J. 30, 3690–3701.

Ait-Hmyed O, Felder-Schmittbuhl MP, Garcia-Garrido M, Beck S, Seide C, Sothilingam V, Tanimoto N, Seeliger M, Bennis M, Hicks D. Mice lacking Period 1 and Period 2 circadian clock genes exhibit blue cone photoreceptor defects. Eur J Neurosci. 2013 Apr;37(7):1048-60.

B. Zheng, D.W. Larkin, U. Albrecht, Z.S. Sun, M. Sage, G. Eichele, C.C. Lee, A. Bradley, The mPer2 gene encodes a functional component of the mammalian circadian clock. Nature 400 (1999) 169–173.

B. Zheng, U. Albrecht, K. Kaasik, M. Sage, W. Lu, S. Vaishnav, Q. Li, Z.S. Sun, G. Eichele, A. Bradley, C.C. Lee, Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock. Cell 105 (2001) 683–694.

Baba K, Davidson AJ, Tosini G. Melatonin entrains PER2::LUC bioluminescence circadian rhythm in the mouse cornea. Invest Ophthalmol Vis Sci. 2015;56:4753–4758.

Baba K, DeBruyne JP, Tosini G. Dopamine 2 receptor activation entrains circadian clocks in mouse retinal pigment epithelium. Sci Rep. 2017;7:5103.

Baba, K., Piano, I., Lyuboslavsky, P., Chrenek, M.A., Sellers, J.T., Zhang, S., Gargini, C., He, L., Tosini, G., Iuvone, P.M., 2018a. Removal of clock gene Bmal1 from the retina affects retinal development and accelerates cone photoreceptor degeneration during aging. Proc. Natl. Acad. Sci. U. S. A. 115, 13099–13104.

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 Oct;1863(10):194623.

Bakker R, Wagstaff PE, Kruijt CC, Emri E, van Karnebeek CDM, Hoffmann MB, Brooks BP, Boon CJF, Montoliu L, van Genderen MM, Bergen AA. The retinal pigmentation pathway in human albinism: Not so black and white. Prog Retin Eye Res. 2022 Jun 18:101091.

Ballesta A, Innominato PF, Dallmann R, Rand DA, Lévi FA. Systems Chronotherapeutics. Pharmacol Rev. 2017 Apr;69(2):161-199.

Barlow R. Circadian and efferent modulation of visual sensitivity. Prog Brain Res. 2001;131:487–503.

Bass J, Takahashi JS. Circadian integration of metabolism and energetics. Science. 2010 Dec 3;330(6009):1349-54.

Besharse, J.C., Iuvone, P.M., 1983. Circadian clock in Xenopus eye controlling retinal serotonin N-acetyltransferase. Nature 305, 133–135.

Bloomfield SA, Volgyi B. The diverse functional roles and regulation of neuronal gap junctions in the retina. Nat Rev Neurosci. 2009;10:495–506.

Brooks MJ, Chen HY, Kelley RA, Mondal AK, Nagashima K, De Val N, Li T, Chaitankar V, Swaroop A. Improved Retinal Organoid Differentiation by Modulating Signaling Pathways Revealed by Comparative Transcriptome Analyses with Development In Vivo. Stem Cell Reports. 2019 Nov 12;13(5):891-905.

Buhr ED, Takahashi JS. Molecular components of the Mammalian circadian clock. Handb Exp Pharmacol. 2013;(217):3-27.

Buhr ED, Yue WW, Ren X, et al. Neuropsin (OPN5)-mediated photoentrainment of local circadian oscillators in mammalian retina and cornea. Proc Natl Acad Sci U S A. 2015;112: 13093–13098.

Buxton OM, Cain SW, O'Connor SP, Porter JH, Duffy JF, Wang W, Czeisler CA, Shea SA. Adverse metabolic consequences in humans of prolonged sleep restriction combined with circadian disruption. Sci Transl Med. 2012 Apr 11;4(129):129ra43.

C. Bobu, C. Sandu, V. Laurent, M.P. Felder-Schmittbuhl, D. Hicks, Prolonged light exposure induces widespread phase shifting in the circadian clock and visual pigment gene expression of the Arvicanthis ansorgei retina. Mol. Vis. 19 (2013) 1060–1073.

C. Bobu, D. Hicks, Regulation of retinal photoreceptor phagocytosis in a diurnal mammal by circadian clocks and ambient lighting. Invest. Ophthalmol. Vis. Sci. 50 (2009) 3495–3502.

Cahill GM, Besharse JC. Circadian rhythmicity in vertebrate retinas - regulation by a photoreceptor oscillator. Prog Retin Eye Res. 1995;14:267–291.

Calligaro H, Coutanson C, Najjar RP, Mazzaro N, Cooper HM, Haddjeri N, et al. (2019) Rods contribute to the light-induced phase shift of the retinal clock in mammals. PLoS Biol 17(3): e2006211.

D.G. McMahon, P.M. Iuvone, G. Tosini, Circadian organization of the mammalian retina: from gene regulation to physiology and diseases. Prog. Retin. Eye Res. 39 (2014) 58–76.

D.T. Organisciak, R.M. Darrow, L. Barsalou, R.K. Kutty, B. Wiggert, Circadian dependent retinal light damage in rats, Invest. Ophthalmol. Vis. Sci. 41 (2000) 3694–3701.

Dalvin LA, Fautsch MP. Analysis of circadian rhythm gene expression with reference to diurnal pattern of intraocular pressure in mice. Invest Ophthalmol Vis Sci. 2015;56:2657– 2663.

DeVera C, Dixon J, Chrenek MA, Baba K, Le YZ, Iuvone PM, Tosini G. The Circadian Clock in the Retinal Pigment Epithelium Controls the Diurnal Rhythm of Phagocytic Activity. Int J Mol Sci. 2022 May 10;23(10):5302.

Dierickx P, Vermunt MW, Muraro MJ, Creyghton MP, Doevendans PA, van Oudenaarden A, Geijsen N, Van Laake LW. Circadian networks in human embryonic stem cell-derived cardiomyocytes. EMBO Rep. 2017 Jul;18(7):1199-1212.

Dolatshad H, Cary AJ, Davis FC. Differential expression of the circadian clock in maternal and embryonic tissues of mice. PLoS One. 2010 Mar 24;5(3):e9855.

Doughty MJ. Morphometric analysis of the surface cells of rabbit corneal epithelium by scanning electron microscopy. Am J Anat. 1990;189:316–328.

Draijer S, Chaves I, Hoekman MFM. The circadian clock in adult neural stem cell maintenance. Prog Neurobiol. 2019 Feb;173:41-53.

Evans JA, Suen TC, Callif BL, et al. Shell neurons of the master circadian clock coordinate the phase of tissue clocks throughout the brain and body. BMC Biol. 2015;13:43.

Felder-Schmittbuhl, M.P., Buhr, E.D., Dkhissi-Benyahya, O., Hicks, D., Peirson, S.N., Ribelayga, C.P., Sandu, C., Spessert, R., Tosini, G., 2018. Ocular clocks: adapting mechanisms for eye functions and health. Invest. Ophthalmol. Vis. Sci. 59, 4856–4870.

Goyal V, DeVera C, Laurent V, Sellers J, Chrenek MA, Hicks D, Baba K, Iuvone PM, Tosini G. Dopamine 2 Receptor Signaling Controls the Daily Burst in Phagocytic Activity in the Mouse Retinal Pigment Epithelium. Invest Ophthalmol Vis Sci. 2020 May 11;61(5):10.

Green CB, Besharse JC. Retinal circadian clocks and control of retinal physiology. J Biol Rhythms. 2004;19:91–102.

Guillaumond F, Dardente H, Giguère V, Cermakian N. Differential control of Bmal1 circadian transcription by REV-ERB and ROR nuclear receptors. J Biol Rhythms. 2005 Oct;20(5):391-403.

H.C. Marie-Paule Felder-Schmittbuhl, Ouria Dkhissi-Benyahya, The retinal clock in mammals: role in health and disease. ChronoPhysiol. Ther. 7 (2017) 33–45.

Hardin, P., Hall, J. & Rosbash, M. Feedback of the Drosophila period gene product on circadian cycling of its messenger RNA levels. Nature 343, 536–540 (1990).

Hardman, J.A., Tobin, D.J., Haslam, I.S., Farjo, N., Farjo, B., Al-Nuaimi, Y., Grimaldi, B., Paus, R., 2015. The peripheral clock regulates human pigmentation. J. Invest. Dermatol. 135, 1053–1064.

Hastings MH, Reddy AB, Maywood ES. A clockwork web: circadian timing in brain and periphery, in health and disease. Nat Rev Neurosci. 2003 Aug;4(8):649-61.

Ikarashi R, Akechi H, Kanda Y, et al. Regulation of molecular clock oscillations and phagocytic activity via muscarinic Ca(2þ) signaling in human retinal pigment epithelial cells. Sci Rep. 2017;7:44175.

Iuvone PM, Tosini G, Pozdeyev N, Haque R, Klein DC, Chaurasia SS. Circadian clocks, clock networks, arylalkyl- amine N-acetyltransferase, and melatonin in the retina. Prog Retin Eye Res. 2005;24:433–456.

Janich P, Toufighi K, Solanas G, Luis NM, Minkwitz S, Serrano L, Lehner B, Benitah SA. Human epidermal stem cell function is regulated by circadian oscillations. Cell Stem Cell. 2013 Dec 5;13(6):745-53.

Jin NG, Chuang AZ, Masson PJ, Ribelayga CP. Rod electrical coupling is controlled by a circadian clock and dopamine in mouse retina. J Physiol. 2015;593:1597–1631.

Jin NG, Ribelayga CP. Direct evidence for daily plasticity of electrical coupling between rod photoreceptors in the mammalian retina. J Neurosci. 2016;36:178–184.

Johnson, M.H., Lim, A., Fernando, D., Day, M.L., 2002. Circadian clockwork genes are expressed in the reproductive tract and conceptus of the early pregnant mouse. Reprod. Biomed. Online 4, 140–145.

Jones CR, Campbell SS, Zone SE, Cooper F, DeSano A, Murphy PJ, Jones B, Czajkowski L, Ptácek LJ. Familial advanced sleep-phase syndrome: A short-period circadian rhythm variant in humans. Nat Med. 1999 Sep;5(9):1062-5.

K.F.Storch,C.Paz,J.Signorovitch,E.Raviola,B.Pawlyk,T.Li,C.J.Weitz,Intrinsic circadian clock of the mammalian retina: importance for retinal processing of visual information. Cell 130 (2007) 730–741.

Keisuke Ikegami, Yasufumi Shigeyoshi, Satoru Masubuchi; Circadian Regulation of IOP Rhythm by Dual Pathways of Glucocorticoids and the Sympathetic Nervous System. Invest. Ophthalmol. Vis. Sci. 2020;61(3):26.

Kikkawa Y. Diurnal variation in corneal thickness. Exp Eye Res. 1973;15:1–9.

Kobayashi Y, Ye Z, Hensch TK. Clock genes control cortical critical period timing. Neuron. 2015 Apr 8;86(1):264-75.

Koronowski KB, Sassone-Corsi P. Communicating clocks shape circadian homeostasis. Science. 2021 Feb 12;371(6530):eabd0951.

Kowalska E, Moriggi E, Bauer C, Dibner C, Brown SA. The circadian clock starts ticking at a developmentally early stage. J Biol Rhythms. 2010 Dec;25(6):442-9.

Laurent V, Sengupta A, Sanchez-Bretano A, Hicks D, Tosini G. Melatonin signaling affects the timing in the daily rhythm of phagocytic activity by the retinal pigment epithelium. Exp Eye Res. 2017;165:90–95.

Logan RW, McClung CA. Rhythms of life: circadian disruption and brain disorders across the lifespan. Nat Rev Neurosci. 2019 Jan;20(1):49-65.

Loros JJ, Dunlap JC (1991). Neurospora crassa clock-controlled genes are regulated at the level of transcription. Mol Cell Biol 11:558–563.

Lowrey PL, Takahashi JS. Mammalian circadian biology: elucidating genome-wide levels of temporal organization. Annu Rev Genomics Hum Genet. 2004;5:407-41.

Lozano, D.C., Hartwick, A.T., Twa, M.D., 2015. Circadian rhythm of intraocular pressure in the adult rat. Chronobiol. Int. 32, 513–523.

M. von Schantz, R.J. Lucas, R.G. Foster, Circadian oscillation of photopigment transcript levels in the mouse retina. Brain Res. Mol. Brain Res. 72 (1999) 108–114.

M.A. Cameron, A.R. Barnard, R.J. Lucas, The electroretinogram as a method for studying circadian rhythms in the mammalian retina. J. Genet. 87 (2008) 459–466.

M.M.LaVail, Circadian nature of rod outer segment disc shedding in the rat. Invest.Ophthalmol. Vis. Sci. 19 (1980) 407–411.

Masland RH. The neuronal organization of the retina. Neuron. 2012 Oct 18;76(2):266-80.

McHill AW, Melanson EL, Higgins J, Connick E, Moehlman TM, Stothard ER, Wright KP Jr. Impact of circadian misalignment on energy metabolism during simulated nightshift work. Proc Natl Acad Sci U S A. 2014 Dec 2;111(48):17302-7.

Mollema, N.J., Yuan, Y., Jelcick, A.S., Sachs, A.J., von Alpen, D., Schorderet, D., Escher, P., Haider, N.B., 2011. Nuclear receptor Rev-erb alpha (Nr1d1) functions in concert with Nr2e3 to regulate transcriptional networks in the retina. PLoS One 6, e17494.

O'Hara-Wright M, Gonzalez-Cordero A. Retinal organoids: a window into human retinal development. Development. 2020 Dec 24;147(24):dev189746.

Panda S, Antoch MP, Miller BH, et al. Coordinated transcription of key pathways in the mouse by the circadian clock. Cell. 2002;109:307–320.

Preitner, N. et al. The orphan nuclear receptor REV- ERBα controls circadian transcription within the positive limb of the mammalian circadian oscillator. Cell 110, 251–260 (2002).

R. Barnard, S. Hattar, M.W. Hankins, R.J. Lucas, Melanopsin regulates visual processing in the mouse retina. Curr. Biol. 16 (2006) 389–395.

Ribelayga C, Cao Y, Mangel SC. The circadian clock in the retina controls rod-cone coupling. Neuron. 2008;59:790– 801.

Ribelayga CP, O'Brien J. Circadian and light-adaptive control of electrical synaptic plasticity in the vertebrate retina. In: Jing J, ed. Network Functions and Plasticity: Perspectives from Studying Electrical Coupling in Microcircuits. Cam- bridge: Elsevier; 2017:209–241.

Sandvig KU, Haaskjold E, Refsum SB. Time dependency in the regenerative response to injury of the rat corneal epithelium. Chronobiol Int. 1994;11:173–179.

Sato, T. et al. A functional genomics strategy reveals Rora as a component of the mammalian circadian clock. Neuron 43, 527–537 (2004).

Sawant, O.B., Horton, A.M., Zucaro, O.F., Chan, R., Bonilha, V.L., Samuels, I.S., Rao, S., 2017. The circadian clock gene Bmal1 controls thyroid hormone-mediated spectral identity and cone photoreceptor function. Cell Rep. 21, 692–706.

Sawant, O.B., Jidigam, V.K., Fuller, R.D., Zucaro, O.F., Kpegba, C., Yu, M., Peachey, N.S., Rao, S., 2019. The circadian clock gene Bmal1 is required to control the timing of retinal neurogenesis and lamination of Muller glia in the mouse retina. Faseb. J. 33, 8745–8758.

Saxena MT, Aton SJ, Hildebolt C, et al. 2007. Bioluminescence imaging of period1 gene expression in utero. Mol Imaging 6:68–72.

Schnell A, Chappuis S, Schmutz I, Brai E, Ripperger JA, Schaad O, Welzl H, Descombes P, Alberi L, Albrecht U. The nuclear receptor REV-ERBα regulates Fabp7 and modulates adult hippocampal neurogenesis. PLoS One. 2014 Jun 16;9(6):e99883.

Scott, E. M., Carter, A. M., and Grant, P. J. (2008). Association between polymorphisms in the Clock gene, obesity and the metabolic syndrome in man. Int. J. Obes. 2005, 658–662.

Seron-Ferre M, Valenzuela GJ, Torres-Farfan C. Circadian clocks during embryonic and fetal development. Birth Defects Res C Embryo Today. 2007 Sep;81(3):204-14.

Takahashi JS. Finding new clock components: past and future. J Biol Rhythms. 2004 Oct;19(5):339-47.

Takahashi, J.S., 2017. Transcriptional architecture of the mammalian circadian clock. Nat. Rev. Genet. 18, 164–179.

Takahashi, J.S., Hong, H.K., Ko, C.H., McDearmon, E.L., 2008. The genetics of mammalian circadian order and disorder: implications for physiology and disease. Nat. Rev. Genet. 9, 764–775.

Tosini, G., Menaker, M., 1996. Circadian rhythms in cultured mammalian retina. Science 272, 419–421.

Tsuchiya S, Buhr ED, Higashide T, Sugiyama K, Van Gelder RN. Light entrainment of the murine intraocular pressure circadian rhythm utilizes non-local mechanisms. PLoS One. 2017;12:e0184790.

Vallone, D., Lahiri, K., Dickmeis, T., Foulkes, N.S., 2007. Start the clock! Circadian rhythms and development. Dev. Dynam. 236, 142–155.

Vilches N, Spichiger C, Mendez N, Abarzua-Catalan L, Galdames HA, Hazlerigg DG, Richter HG, Torres-Farfan C. Gestational chronodisruption impairs hippocampal expression of NMDA receptor subunits Grin1b/Grin3a and spatial memory in the adult offspring. PLoS One. 2014 Mar 24;9(3):e91313.

Weger M, Diotel N, Dorsemans AC, Dickmeis T, Weger BD. Stem cells and the circadian clock. Dev Biol. 2017 Nov 15;431(2):111-123.

Wong JCY, Smyllie NJ, Banks GT, Pothecary CA, Barnard AR, Maywood ES, Jagannath A, Hughes S, van der Horst GTJ, MacLaren RE, Hankins MW, Hastings MH, Nolan PM, Foster RG, Peirson SN. Differential roles for cryptochromes in the mammalian retinal clock. FASEB J. 2018 Aug;32(8):4302-4314.

Woon PY, Kaisaki PJ, Bragança J, Bihoreau MT, Levy JC, Farrall M, Gauguier D. Aryl hydrocarbon receptor nuclear translocator-like (BMAL1) is associated with susceptibility to hypertension and type 2 diabetes. Proc Natl Acad Sci U S A. 2007 Sep 4;104(36):14412-7.

Xu Y, Toh KL, Jones CR, Shin JY, Fu YH, Ptácek LJ. Modeling of a human circadian mutation yields insights into clock regulation by PER2. Cell. 2007 Jan 12;128(1):59-70.

Xue Y, Liu P, Wang H, et al. Modulation of circadian rhythms affects corneal epithelium renewal and repair in mice. Invest Ophthalmol Vis Sci. 2017;58:1865–1874.

Yagita K, Horie K, Koinuma S, Nakamura W, Yamanaka I, Urasaki A, Shigeyoshi Y, Kawakami K, Shimada S, Takeda J, Uchiyama Y. Development of the circadian oscillator during differentiation of mouse embryonic stem cells in vitro. Proc Natl Acad Sci U S A. 2010 Feb 23;107(8):3846-51.

Yoo SH, Yamazaki S, Lowrey PL, et al. PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. Proc Natl Acad Sci U S A. 2004;101:5339–5346.

Zeggini E, Weedon MN, Lindgren CM, Frayling TM, Elliott KS, Lango H, Timpson NJ, Perry JR, Rayner NW, Freathy RM, Barrett JC, Shields B, Morris AP, Ellard S, Groves CJ, Harries LW, Marchini JL, Owen KR, Knight B, Cardon LR, Walker M, Hitman GA, Morris AD, Doney AS; Wellcome Trust Case Control Consortium (WTCCC), McCarthy MI, Hattersley AT. Replication of genome-wide association signals in UK samples reveals risk loci for type 2 diabetes. Science. 2007 Jun 1;316(5829):1336-41.

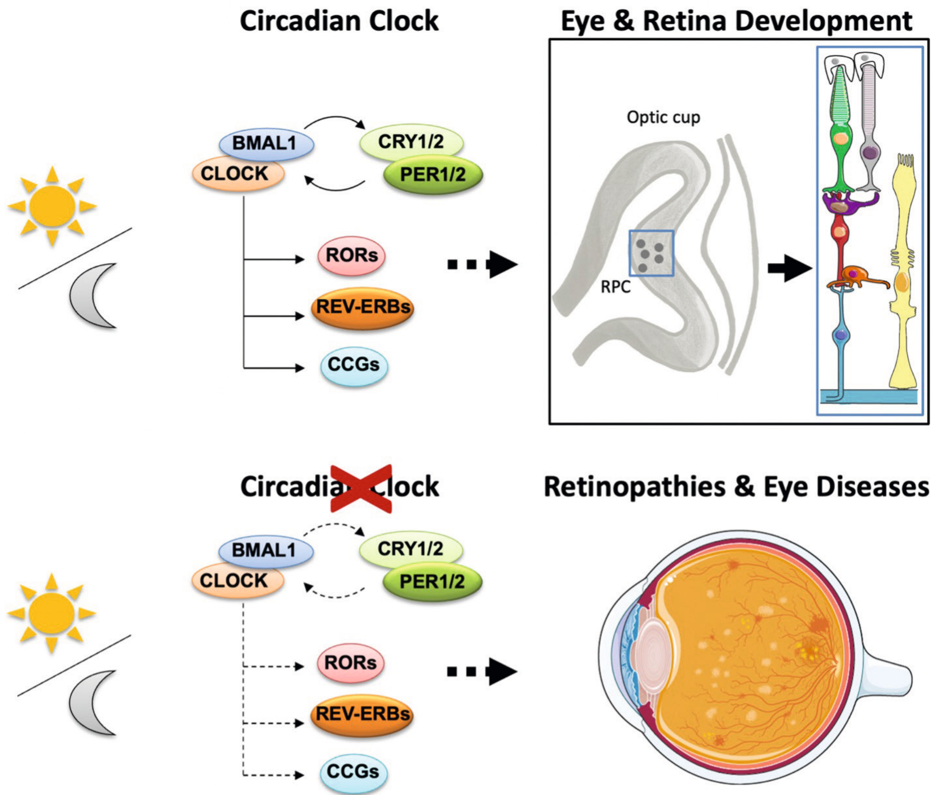
CHAPTER

*Amandine Bery**
*Udita Bagchi**
Arthur A. Bergen
Marie-Paule Felder-Schmittbuhl

*These authors contributed equally to this review

Developmental Biology 484 (2022) 40–56

Circadian clocks, retinogenesis and ocular health in vertebrates: new molecular insights



ABSTRACT

Circadian clocks are cell-autonomous, molecular pacemakers regulating a wide variety of behavioural and physiological processes in accordance with the 24 h light/dark cycle. The retina contains a complex network of cell-specific clocks orchestrating many biochemical and cellular parameters to adapt retinal biology and visual function to daily changes in light intensity. The gene regulatory networks controlling proliferation, specification, and differentiation of retinal precursors into the diverse retinal cell types are evolutionary conserved among vertebrates. However, how these mechanisms are interconnected with circadian clocks is not well-characterized. Here we explore the existing evidence for the regulation of retinal development by circadian clock-related pathways, throughout vertebrates. We provide evidence for the influence of clock genes, from early to final differentiation steps. In addition, we report that the clock, integrating environmental cues, modulates a number of pathological processes. We highlight its potential role in retinal diseases and its instructive function on eye growth and related disorders.

1. INTRODUCTION

The evolutionary force exerted by the Earth's 24 h rotation is responsible for the development of circadian clocks in living organisms. It drives rhythms in physiology and behaviour with an approximate period (circa) of 24 h (Pittendrigh, 1993). These rhythms are endogenously generated by conserved cell-autonomous mechanisms occurring in virtually every life-form from algae to mammals. Their chronic disruption predisposes to the failure of optimum physiological functions across a wide range of organs (Takahashi et al., 2008).

In mammals, the anticipation and response to light/dark (LD) and temperature cycles are regulated by daily rhythms in a variety of physiological functions including hormone levels and sleep (Roenneberg and Merrow, 2016). The 24 h rhythms are controlled by the hypothalamic structure named suprachiasmatic nuclei (SCN), acting as the central pacemaker for the whole body. These rhythms are retained in constant environmental conditions such as constant darkness (Hastings et al., 2018). The LD cycle is the most potent circadian synchroniser and acts via a unique light-sensing system located in the retina: the intrinsically photosensitive (melanopsin-expressing) retinal ganglion cells (ipRGC) transmit light-evoked neurochemical signals to the SCN and direct synchronization for peripheral (secondary) clocks. Indeed, cells of many different tissues and organs (skeletal muscle, heart, liver, kidney, bone/cartilage, among others) have such intrinsic secondary circadian clock (Albrecht, 2012). These drive many physiological functions, such as xenobiotic and endobiotic detoxification, carbohydrate and lipid metabolism, renal plasma flow and urine production, cardiovascular parameters, among others. Next to light, both food and body temperature are known to also synchronize circadian rhythms in peripheral tissues (Buhr et al., 2010; Damiola et al., 2000).

1.1 The clock machinery

Circadian clock organization at the cellular level is conserved across vertebrates (Bell-Pedersen et al., 2005; Dunlap, 1999). The functioning of circadian clocks is directed by clock gene-encoded transcription factors, mainly CLOCK, BMAL1, PER1, PER2, CRY1, CRY2. They interact closely in interlocked transcription/translation feedback loops. The CLOCK and BMAL1 proteins bind the E-box enhancer element of clock genes Period (*Per*) 1–2 (Shearman et al., 1997) and Cryptochrome 1–2 (*Cry 1–2*) (Kume et al., 1999), as well as that of Clock-Controlled Genes (CCG) and stimulate their expression (Koike et al., 2012) (Figure 1). The translocation of PER1-2 and CRY1-2 heterodimers back into the nucleus forms the primary core negative feedback loop, thus inhibiting CLOCK:BMAL1 transcriptional activity and hence the expression of *Per* and *Cry* genes [reviewed in (Takahashi, 2017)]. A second feedback loop involves the action of retinoic acid-related orphan nuclear receptor (ROR) and REV-ERB proteins, whose expression is also activated by CLOCK:BMAL1.

ROR and REV-ERB factors in turn regulate transcription of Bmal1 and distribute rhythms to CCG via ROR binding elements (RORE) (Preitner et al., 2002; Sato et al., 2004). In summary, the generation of tissue-specific rhythmic gene expression programs involves a two-branched process: (1) direct transcriptional activation of CCG due to the cumulative response of clock factors and tissue-specific regulators, and (2) indirect effect originating from the transcription factors (TF) that are clock targets (Miller et al., 2007). In this complex clockwork, Bmal1 is the only canonical clock gene whose deletion or mutation causes a complete loss of circadian rhythmicity (Bunger et al., 2000). The circadian core clock machinery is very similar between SCN and secondary oscillators, with slight differences. Besides, it profoundly influences tissue-specific gene expression programs throughout the body. For instance, the diurnal gene expression profiles isolated from 64 different central and peripheral tissues (including the eye) in the young male baboon, established that up to 81.7% protein-encoding genes display daily rhythmic expression in at least one organ (Mure et al., 2018).

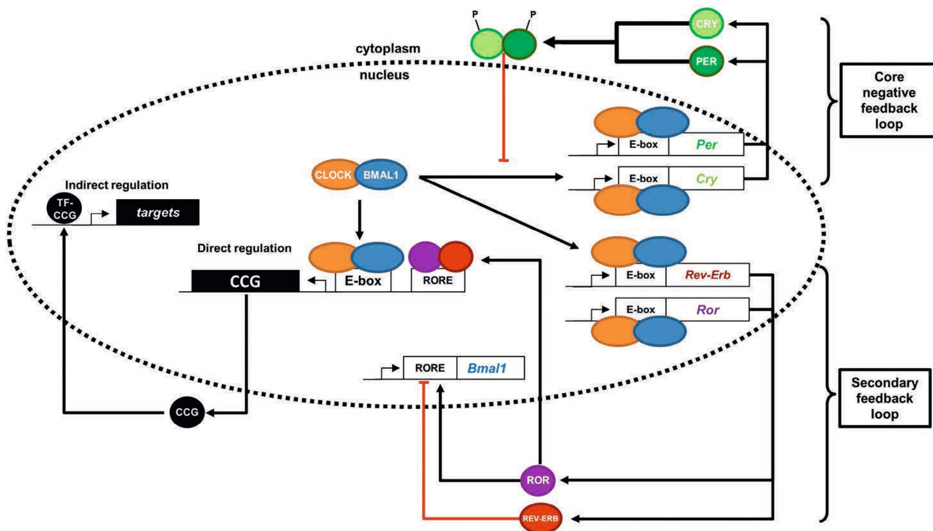


Figure 1. The circadian clock machinery. The endogenous molecular mechanism generating circadian rhythms is based on two main transcriptional–translational feedback loops involving clock genes. The CLOCK/BMAL1 heterodimer binds to an enhancer box (E-box) and drives the transcription of the clock genes Period (*Per1-2*), Cryptochrome (*Cry1-2*), *Rora-c*, and *Rev- Erbα-β*. PER and CRY (phosphorylated) proteins in turn inhibit CLOCK:BMAL1 transcriptional activity. By competitive binding to retinoid-related orphan receptors response elements (RORE), the REVERB and ROR proteins repress and activate Bmal1 transcription, respectively. The clock machinery modulates cell-specific gene expression via two transcriptional processes (direct and indirect), thus leading to 24 h rhythms in tissues. CCG, Clock Controlled Gene; TF, Transcription Factor [adapted from (Felder-Schmittbuhl et al., 2017)].

Among targets of the molecular clock, genes related to cell cycle regulation have been particularly well documented (Hunt and Sassone-Corsi, 2007). An intimate connection between the circadian clock, the cell cycle, and developmental processes has been reported but this phenomenon remains only partially characterized [reviewed in (Brown, 2014)]. This question has been partly addressed in the vertebrate retina, a tissue at the interface with the environment, in which the interaction between lighting conditions, clock function and development are particularly interesting to investigate.

1.2 The circadian clock in the retina

The neural retina is a fascinating heterogeneous tissue composed of a multitude of cell types, including glial cells and very specialized neurons such as the photoreceptors (PR). It is organized into three discreet cellular layers separated by synaptic (plexiform) layers and distinguished by the presence of specific cell types: the Outer Nuclear Layer (ONL) harbours the (cell bodies and nuclei of the) rod and cone PR; the Inner Nuclear Layer (INL) contains bipolar (BC), horizontal (HC), amacrine (AC), and (displaced) ganglion cells as well as nuclei of Müller glial cells (MGC) that stretch over several layers; and the Ganglion Cell Layer (GCL) contains large ganglion and (displaced) AC (Rodieck, 1973). At the back surface of the retina lies the Retinal Pigmented Epithelium (RPE), a non-neural layer of pigmented epithelial cells. All investigated retinal cells were shown to integrate and orchestrate circadian oscillations (Figure 2). Such organization implicates the retina in anticipating the environmental dawn/dusk cycles. During the early 80s, the circadian clock was found in both frog and avian eye (Besharse and Iuvone, 1983; Underwood et al., 1990). This clock was later shown to be located into light responsive cells of the retina, namely PR, and to drive rhythms of melatonin production, the core hormone of circadian rhythms (Cahill and Besharse, 1993; Thomas et al., 1993). Later, an autonomous clock was also evidenced in chick early embryonic, post-mitotic RGC, based on their capacity to generate self-sustained rhythms in arylalkylamine N-acetyltransferase (AA-NAT: the enzyme catalyzing the penultimate step in melatonin biosynthesis) expression (Garbarino-Pico et al., 2004b), metabolic labeling (Garbarino-Pico et al., 2004a) and melatonin (Contin et al., 2006).

In mammals, the existence of an independent circadian clock in the retina was suggested by experiments in animals with SCN lesions or severed optic nerves (Reme et al., 1991; Terman et al., 1993). The rhythm of melatonin release in cultured neural retinas of the golden hamster, and the functionalities of this rhythm finally established the mammalian retina as a bonafide circadian pacemaker: free running in constant darkness, entrainment by light, and temperature compensation (Tosini and Menaker, 1996, 1998).

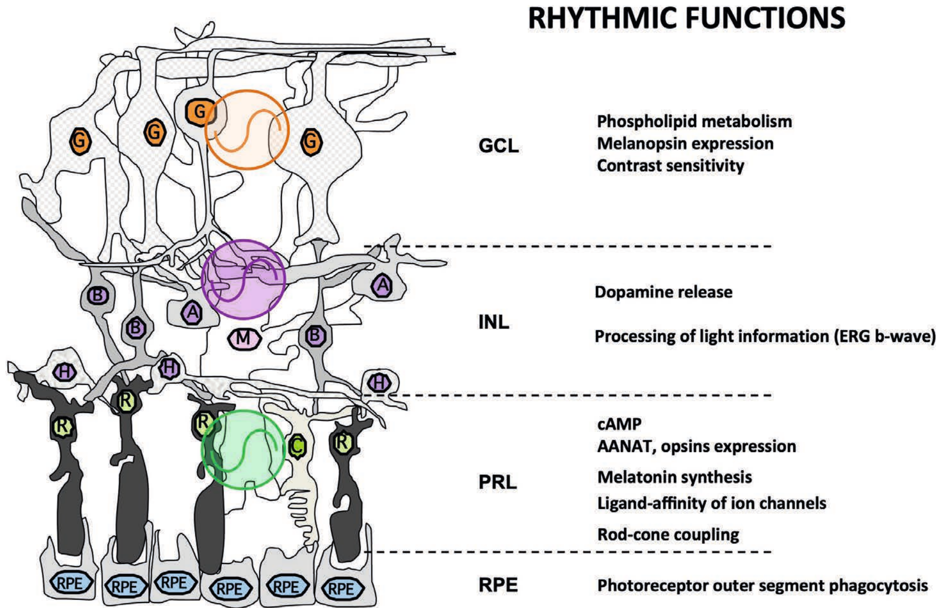


Figure 2. Rhythmic functions in the distinct retinal layers. A schematic transversal section through retinal layers and cell types is shown with the three main oscillators previously identified (Jaeger et al., 2015). Examples of mostly documented rhythmic, clock-controlled processes are listed on the right in RGC, INL, ONL and RPE [references in (Besharse and McMahon, 2016; Felder-Schmittbuhl et al., 2017, 2018)]. G: RGC; A: AC; B: BC; H: HC; M: MGC, R: Rods; C: Cones; RPE: Retinal Pigmented Epithelium.

Data from the literature suggest that the retinal clock controls the timing of a broad range of essential physiological and metabolic functions in the mature retina, allowing adaptation of vision to the daily changes in light intensity [for review, see (Besharse and McMahon, 2016; Felder-Schmittbuhl et al., 2018)]. More precisely, 90% of the (2670) genes that are rhythmically expressed in mouse eyes under LD condition undergo an alteration of their rhythmic mRNA levels in the *Bmal1* knock out (KO). These results indicate that even in the directly light-sensitive retinal tissue, the circadian clock mediates synchronization of gene expression programs by the LD cycle (Storch et al., 2007). Thus, the retina contains a unique combination of a circadian clock, light-entrainment capacity, and multiple clock target outputs. The cellular diversity of the mammalian retina, with significant dissimilarities in the clock gene expression patterns, phasing, and period between cells/layers, renders it challenging to assess the functioning of this network of circadian clocks at the whole tissue level. Kinetics of clock gene expression, at least in mice, showed that circadian rhythms are also generated in the retinal layers distinct from PR, including the RPE (Baba et al., 2010; Dkhissi-Benyahya et al., 2013; Gekakis et al., 1998; Hwang et al., 2013; Jaeger et al., 2015; Liu et al., 2012; Milicevic et al., 2021; Miyamoto and Sancar, 1998; Ruan et al., 2006, 2008; Witkovsky et al., 2003; Xu

et al., 2016). However, if rhythms of clock gene expression in whole retinas were mainly retained in constant dark conditions, this was less systematic when pure PR preparations were studied (Hiragaki et al., 2014; Sandu et al., 2011; Schneider et al., 2010; Tosini et al., 2007b). Nevertheless, rhythms in clock protein expression levels were found sustained in cones, in LD and constant darkness, contrasting with all other retinal cell types investigated in that study (Liu et al., 2012). Finally, it should be kept in mind that when comparing the distinct retinal layers, the more sustained clock gene oscillations appear to be generated in the inner retina (comprising the GCL and INL) (Jaeger et al., 2015; Ruan et al., 2008). Another challenge in the field of retinal clocks has been the characterization of their light entrainment pathway. Initial studies by Ruan and coworkers established the importance of dopamine in the phase shifting effect of light (Ruan et al., 2008). Although the signalling pathways have not been completely clarified, it appears that entrainment of the mouse retinal clock by light primarily involves rods (visible range) but also neuropsin-expressing ganglion cells (UV light) (Buhr et al., 2015; Calligaro et al., 2019).

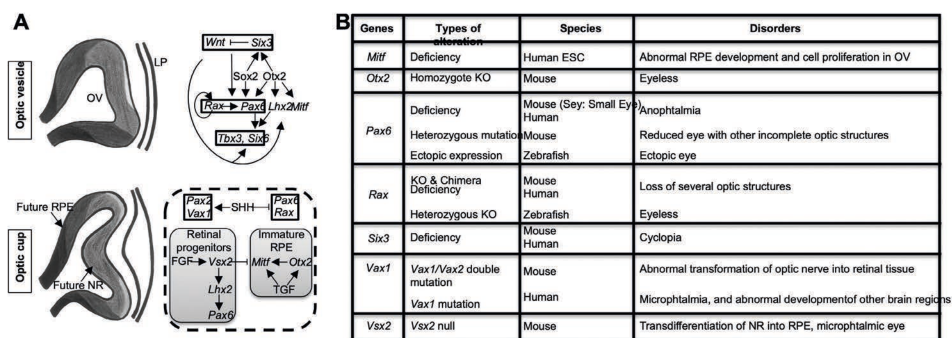
Hence, the retinal clock is a complex grid of circadian oscillators distinctly located in different cell layers and actively powered by synaptic connections and gap junctions (Jaeger et al., 2015). However, it is not yet known how such an oscillator network arises during retinal development. Conversely, the importance of clock genes and circadian clocks in retinal development has been rarely investigated.

Here we provide first a summarized description of molecular mechanisms regulating retinal development, second an outline of relevant studies that have investigated how circadian clocks intermingle with gene expression and morphogenesis in the developing retina and eye, and finally experimental evidence for the association between clock dysfunction and eye pathology.

2. RETINOGENESIS - AN EVOLUTIONARY PERSPECTIVE

Eye development and retinogenesis involve complex gene regulatory networks (GRN) that were characterized thanks to the identification of distinct mutants (Figure 3). Multiple well-conserved TF tightly regulate expansion and differentiation of neural progenitors during retinal neurogenesis until post-natal development (Heavner and Pevny, 2012; Vopalensky and Kozmik, 2009). They cluster into two major classes providing combinatorial influence on cell-fate specification during retinogenesis: the homeobox-containing class (ex.: *Crx*, *Otx2*, *Pax6*, *Rax*, *Six3/6*, *Vsx2*) and the basic helix-loop-helix (bHLH) TF (ex.: *Atoh7*, *Hes1*, *Hes5*, *Mitf*, *NeuroD*). (i) TF from the homeobox-containing class, whose mutation leads to absence of eyes (anophthalmia) or reduced eye size (microphthalmia) in human (Harding and Moosajee, 2019), control the specification of the optic primor-

dium and regulation of cell proliferation and differentiation (Dyer, 2003; Zagozewski et al., 2014b). For instance, pioneering studies from WJ Gehring and coworkers identified the master control gene *Pax6*, which when mutated leads to human Aniridia, mouse small eye and Drosophila eyeless phenotypes (Quiring et al., 1994). (ii) The bHLH TF ensure, in association with other TF, the competence states of progenitor cells [reviewed in (Hatakeyama and Kageyama, 2004)]. Additional control by *Shh* (Sonic Hedgehog) and Notch signalling specifies distinct cellular subtypes, through the symmetric vs. asymmetric division during the cell cycle [reviewed in (Agathocleous and Harris, 2009)].



Genes	Types of alteration	Species	Disorders
Mitf	Deficiency	Human ESC	Abnormal RPE development and cell proliferation in OV
Otx2	Homozygote KO	Mouse	Eyeless
Pax6	Deficiency	Mouse (Sey: Small Eye) Human	Anophtalmia
	Heterozygous mutation	Mouse	Reduced eye with other incomplete optic structures
	Ectopic expression	Zebrafish	Ectopic eye
Rax	KO & Chimera Deficiency	Mouse Human	Loss of several optic structures
	Heterozygous KO	Zebrafish	Eyeless
Six3	Deficiency	Mouse Human	Cyclopia
Vax1	*Vax1/Vax2* double mutation	Mouse	Abnormal transformation of optic nerve into retinal tissue
	Vax1 mutation	Human	Microphtalmia, and abnormal developmentof other brain regions
Vsx2	*Vsx2* null	Mouse	Transdifferentiation of NR into RPE, microphtalmic eye

Figure 3. The genetic signalling in the optic vesicle and optic cup. A) Diagram depicting the optic vesicle (OV) and the optic cup (left) with their corresponding Gene Regulatory Networks. B) Genes whose alteration has dramatic consequences in early development of the vertebrate eye [adapted from (Garita-Hernandez et al., 2016; Harding and Moosajee, 2019)]. KO: Knock Out; LP: Lens Placode; NR: Neural retina; OV: Optic Vesicle; RPE: Retinal Pigmented Epithelium.

Although retinogenesis is highly similar during eye development in many organisms, including chick, mouse and human, the percentage of each cell type in the fully differentiated retina varies from species to species, suggesting that retinal organization enables species-dependent visual adaptations [reviewed in (Lamb, 2009; Lamb et al., 2009)]. The present chapter describes principal steps leading to the determination of each cell type, based mainly on mouse model [reviewed in (Zagozewski et al., 2014a; Zhang et al., 2011)], with the mention of alternative processes in distinct vertebrates, when applicable. The intermingled GRN of rods and cones will be discussed in a subsequent section. Figure 4 summarizes the information contained in chapter 2.

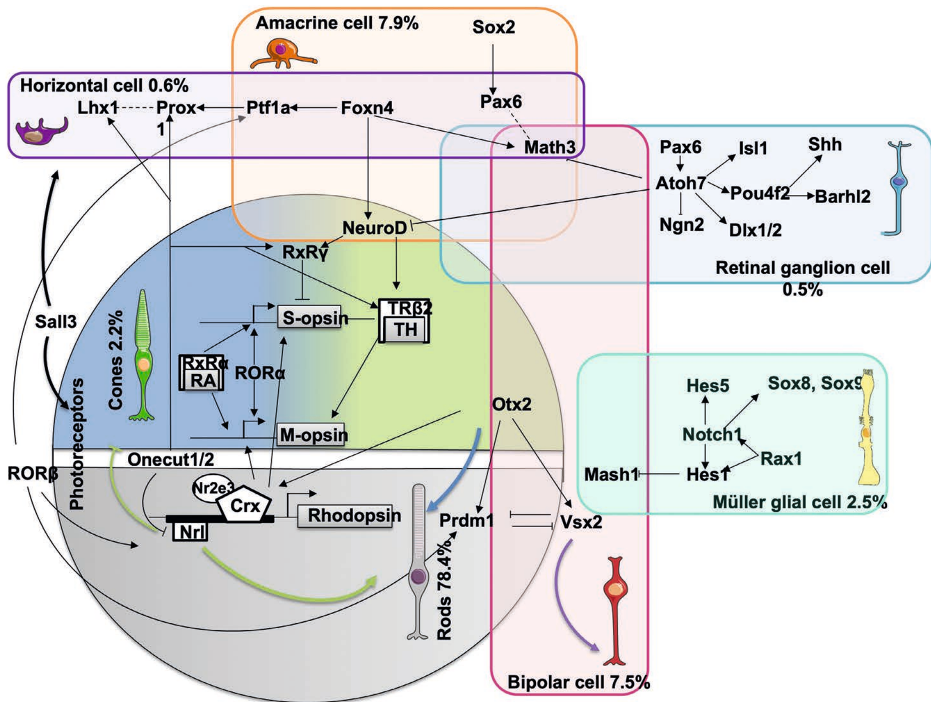


Figure 4. A network of genes essential for the development of retinal cell types in the mouse retina. Among photoreceptors, cones, and rods GRN are described in separate-colored hemispheres: grey for rods and multicolored (blue, green) for cones. Proportion of the seven retinal cell types are indicated [adapted from (Harada et al., 2007; Hughes et al., 2017)]. Dotted lines indicate indirect or poorly characterized gene relationships. Large colored arrows indicate that the TF directly activates generation of the cell type. [adapted from (Boije et al., 2015; Brzezinski and Reh, 2015; Ivanov, 2019; Zagozewski et al., 2014a; Zhang et al., 2011)]. Prepared with elements from Servier Medical Art https://smart.servier.com/.

2.1. Towards mature retina: retinal cell fate determination

The seven major retinal cell types are generated from common progenitors in an extensively conserved order, from ganglion cells (first) to MGC (last). The underlying genetic program is also well conserved among vertebrates and proceeds through a series of steps that increasingly restrict lineage choices and commit cells to a particular fate. One key event is the transition from the proliferating progenitors to cell fate specification. Especially, dynamics of variable *Pax6* expression during the cell cycle appear to play an instructive role in these processes, thus suggesting the cellular diversity in the retina essentially results from the modulation of key TF by extrinsic signalling cues.

The specification of retinal ganglion cells (RGC) requires *Pax6*, *Atoh7/Math5* signalling, *Pou4f2/Brn3b* and *Isl1* activity (Marquardt et al., 2001; Pan et al., 2008; Riesenberg et

al., 2009). *Atoh7* turns on ganglion cell development, while repressing the other retinal cell fates through repression of other proneural genes (*Math3*, *NeuroD*, *Ngn2*) (Le et al., 2006; Mu et al., 2005; Stenkamp, 2007; Yang et al., 2003). *Pou4f2* and *Isl1* together drive the ganglion cell differentiation program and upregulate *Shh*, which maintains retinal progenitor cells (RPC) proliferation in mice, in contrast with its pro-RGC differentiation effect in zebrafish (Wang et al., 2005). RGC development also involves the *Dlx1* and *Dlx2* homeobox genes, since their absence leads to increased apoptosis of late born RGC (de Melo et al., 2005). Finally, *Pou4f2* promotes RGC development via an Atoh7-independent pathway and induces RGC maturation and survival in the mouse via *Barhl2* activation (Ding et al., 2009).

Horizontal cell (HC) specification depends on *Foxn4* (Li et al., 2004; Marquardt and Gruss, 2002) which provides RPC competence to become HC or AC. Downstream of *Foxn4*, *Ptf1a* and *Hnf6/Oc1* (Onecut1) act together to specify the HC fate (Fujitani et al., 2006; Li et al., 2004; Wu et al., 2013). HC specification also requires *Prox1*, a gene essential for cell cycle exit of early RPC (Dyer et al., 2003). This followed from depletion of HC in the Prox1-null mouse, and enhanced HC production when mis-expressing *Prox1* (Dyer et al., 2003). Additional regulation by *Lim1/Lhx1* appears to control HC positioning (Fujitani et al., 2006; Li et al., 2004; Poche et al., 2007). A similar cascade was reported in zebrafish (Jusuf et al., 2011), frog (Dullin et al., 2007) and chicken retina (Boije et al., 2013; Suga et al., 2009). In particular, the appropriate positioning of HC in the INL involves the Spalt family TF *Sall3* (de Melo et al., 2011).

The specification of amacrine cells (AC) depends on *Foxn4* and its downstream targets *Math3* that works along with *NeuroD* (Li et al., 2004). *Math3* and *NeuroD* are essential, yet not sufficient for AC induction (Inoue et al., 2002). Downstream of *Foxn4* AC specification also involves *Ptf1a* (Fujitani et al., 2006; Nakhai et al., 2007). AC differentiation is also regulated by *Pax6* and its activator *Sox2*, which sustain early progenitor populations (Lin et al., 2009). Finally, differentiation of AC sub-populations involves notably *Ptf1a* and *Barhl2* for GABA-ergic cells and glycinergic cells (Ding et al., 2009; Nakhai et al., 2007) and *Isl1* for cholinergic cells (Elshatory et al., 2007).

Specification of the late-born Bipolar Cells (BC) requires both *Mash1* and *Math3* (Tomita et al., 2000). For the sake of completeness, it should be noted here that, before BC development, *Mash1* interacts with *Math3* in RPC to control the neurogenic-gliogenic balance. In retinal explants, deletion of *Mash1* delays the production of BC, rods and MGC, and reduces the number of BC at the expanse of MGCs (Tomita et al., 1996).

Another factor playing a crucial role in BC differentiation is a target of *Otx2* (Kim et al., 2008), *Vsx2/Chx10*, as shown by studies based on its mutation in mice (Burmeister et al., 1996; Koike et al., 2007) and its downregulation in zebrafish [reviewed in (Boije et al., 2015; Vitorino et al., 2009)].

The most numerous glial cells in the retina, Müller glial cells (MGC), arise from progenitor cells that followed the gliogenesis, not the neurogenesis pathway. A central cue in this process is Notch signalling, where *Hes5* promotes glial cell production, whereas *Hes1* maintains progenitor cell fate, notably by inhibiting the proneural *Mash1* gene (Hojo et al., 2000; Takatsuka et al., 2004; Tomita et al., 1996). In addition, *Notch* and *Hes1* are induced early in development by *Rax*, which controls early precursor proliferation and enables differentiation of late born cells (MGC, rods, BC) (Furukawa et al., 2000; Rodgers et al., 2018). The hierarchy of events required for MGC production further involves *Sox8* and *Sox9*, being induced downstream of Notch signalling (Muto et al., 2009). Interestingly, MGC have the capacity to regenerate retinal neurons in amphibians and zebrafish [reviewed in (Hamon et al., 2016)].

2

2.2. Photoreceptor differentiation

Mature PR handle phototransduction, converting photons to electrical and subsequent neurochemical signals. The general morphology of PR includes the outer segment (OS), the inner segment (IS), the cell body, and the synaptic terminal. Rods and cones are named according to their OS morphology and differ in electrophysiological properties. Both express visual protein pigments, the opsins. Vertebrates have five classes of opsins, a single rod class (RH1) and four cone opsin classes (SWS1, SWS2, RHB/RH2, LWS) that are all expressed in fish and exist in all lineages [reviewed in (Davies et al., 2012)]. Rods are responsible for vision at low light levels/dim light (scotopic vision) and display low spatial acuity. On the contrary, cones are active at higher light intensities (photopic vision) and provide high spatial acuity and color vision. Devoid of red or L-cones, the mouse retina has 5% of "pure" S-cones distributed across the whole retina whereas 95% cones express both M- and S-opsin in a dorso-ventral gradient (primarily S-opsin in the ventral part and M-opsin in the dorsal one) (S, M and L account for Short, Middle, and Long-wavelength-sensitive, respectively) (Applebury et al., 2000; Haverkamp et al., 2005).

Evolution has led to strong adaptations of vision. Especially, number, shape and size of PR sub-types, and molecular evolution of opsins differ among vertebrates, in line with the distinct demands of their operating environments. Hence, the timing of specification and differentiation, from PR precursors to distinct mature PR types, may slightly differ from species to species. In mice, cones are generated prenatally, peaking around E15. Rod generation takes place around birth (Brzezinski and Reh, 2015), their differentiation starting

from postnatal stage 5 (P5), and rhodopsin expression increasing rapidly. At around P10 OS form and eyes open by P13, after which the retinal visual activity matures (Findlater et al., 1993; Shen and Colonnese, 2016). Despite the functional differences between rods and cones, they share TF and cofactors that mutually regulate each other during specification and differentiation (ex: *NeuroD*, *Otx2*, *Crx*, *Nrl*, *Nr2e3*, *Nrl*, retinoic acid and thyroid hormones). For that reason, this chapter is organized according to the different TF and co- factors involved in the specification of both PR subtypes (reported in Figure 4).

2.2.1. TF1: early bHLH gene *NeuroD* is required for both rods and cones development

First involved in the neurogenic-gliogenic balance (Morrow et al., 1999), *NeuroD* is essential for rod development in the mouse, as shown by reduction of rods in *NeuroD* deficient mice and enhanced rod differentiation at the expense of BC after misexpressing *NeuroD* (Morrow et al., 1999). *NeuroD* also regulates cone differentiation, especially by modulating expression of the *TRβ2* (beta-2 subunit of thyroid receptor) encoding gene (see below) (Liu et al., 2008).

2.2.2. TF2: downstream of *Otx2*, the cone-rod homeobox gene, *Crx*, is central to PR differentiation

Otx2 triggers PR specification and activates *Crx* that will drive the formation of OS and expression of both cone- and rod-specific genes (Furukawa et al., 1999; Livesey et al., 2000; Nishida et al., 2003). In particular, *Crx* activation triggers a transcriptional cascade with consecutive activation of *Rorb* (encoding RORβ), *Nrl*, and *Nr2e3*, which leads to rod production (Corbo and Cepko, 2005; Haider et al., 2001; Jia et al., 2009; Kautzmann et al., 2011; Oh et al., 2007). Interestingly the development of the pineal gland, whose principal melatonin-synthetizing cells are very similar to retinal PR, also involves *Otx2* and *Crx*, thus suggesting a common ancestral origin (Furukawa et al., 1999; Nishida et al., 2003). Finally, the stabilization of the (late several phenotypes involves *Prdm1*, another downstream target of *Otx2* (Brzezinski et al., 2010; Mills et al., 2017; Wang et al., 2014).

2.2.3. TF3: *Nrl*, a rod specific gene with an evolutionary divergent role

Nrl (neural retina leucine zipper) is a *Maf* family member that is fully rod specific. In Nrl-deficient retinas prospective rods switch cell-fate to become S-cones (Mears et al., 2001). Further studies have emphasized that *Nrl* is responsible for rhodopsin expression (Kumar et al., 1996; Rehemtulla et al., 1996) and activates *Nr2e3* to suppress the expression of cone-specific genes, promoting rod development (Mears et al., 2001; Oh et al., 2008). Unlike human *NRL*, Xenopus *Nrl,* and likely zebrafish *Nrl*, are responsible for lens development (Coolen et al., 2005; McIlvain and Knox, 2007). In the avian lineage *Nrl* has been lost, hence PR differentiation (at least in chicken) involves other members of the *Maf* family: *MafA* for the regulation of the opsin in rods and *MafB* for one of the avian

green opsins (Enright et al., 2015). Finally, characterization of the *Nrl* KO mice showed a strong relationship between blue cones and rods specifically in mammals and suggested that *Nrl* evolution was instrumental in the advent of (nocturnal and rod-rich) mammals (Kim et al., 2016).

2.2.4. TF4: function of the nuclear receptor *Nr2e3* in PR differentiation

Rod differentiation relies on *Nrl* and its downstream target *Nr2e3*. Indeed, human *Nr2e3* or *Nrl* induce the differentiation of PR into rods after in vivo transfection in Xenopus, maximal effect being reached upon co-expression of both factors (McIlvain and Knox, 2007). Some studies in mice reported also that the NR2E3-CRX complex activates rhodopsin, whereas *NR2E3* alone repressed cone-specific genes (for instance S-opsin) in rods (Chen et al., 2005; Peng et al., 2005). Accordingly, human *NR2E3* mutation leads to enhanced S-cone syndrome (ESCS) associated with blue light hypersensitivity, early onset night blindness, varying degrees of L and M cone vision-loss and retinal degeneration (Haider et al., 2000). A similar phenotype was described in the *rd7* mouse model with *Nr2e3* deficiency: extra S-cones followed by degeneration of both cones and rods, plus other retinal abnormalities (Corbo and Cepko, 2005; Haider et al., 2001).

2.2.5. TF5-6: regulation of PR development by retinoic acid and thyroid hormones

Precise timing of PR production and maturation is regulated by members of the nuclear receptor TF family, especially receptors for retinoids and thyroid hormones.

Binding of retinoic acid (RA) to the RA nuclear receptor (RAR) triggers rod specification (Kelley et al., 1994). Closely related to RAR, the orphan nuclear receptors of the ROR family also control PR development: *RORα* (encoded by *Rora*), synergistically with *CRX*, activates transcription of S-opsin gene (Fujieda et al., 2009); *Rorb* gene is required for rod differentiation and function since its absence led to complete rod depletion and over-production of S-cones (Jia et al., 2009). *RORb* induces expression of *Nrl*, which in turn feeds back positively on *Rorb* (Fu et al., 2014), and expression of *Prdm1* (Wang et al., 2014). *Rorc* gene, as a target of *Rev-Erbα/Nr1d1*, is potentially involved in retinal differentiation at E18.5 in the mouse (Haider et al., 2009; Mollema et al., 2011).

The thyroid hormone (TH) plays a crucial role during cone development, since the lack of *TRβ2* in the mouse leads to depletion of M-opsin expression and concomitant widespread expression of S-opsin in cones (Ng et al., 2001). Hence, TH appears to activate the M-opsin gene and repress the S-opsin gene [as also does *RXRγ* (Roberts et al., 2005)]. In addition, the TH gradient occurring at the latest stages of cone development corresponds to the dorso-ventral gradient of M/S-opsins expression in cones (Roberts et al., 2006). Finally, M-cone development is also regulated by earlier activators of *Thrb*, such

as *NeuroD* (Liu et al., 2008) or *Otx2*, which acts in combination with *Hnf6/Oc1* (Emerson et al., 2013). However, the factor inducing cone fate, if any, remains to be identified.

To conclude, retinogenesis is regulated through a finely tuned GRN that is based on an ON/OFF program of several growth factors, TF and hormone signalling pathways. The general mechanism is well conserved among vertebrates, but some features differ between species and reflect species-specific development strategy, adaptations to environment and lifestyle, especially diurnal versus nocturnal animals. For example, AC subtypes are determined by *BhlhB5*, *Isl1* and *Barhl2* in Xenopus and mouse (Feng et al., 2006), whereas in human *BHLHB5/BHLHE22* expression is associated with emergence of human BC, thus suggesting divergence of evolutionary pathways between tetrapods and primates (Mellough et al., 2019). Furthermore, *Nrl* function is required for the development of mammalian rods, whereas it has been lost in the avian lineage and inversely *Nrl* is an important driver of ocular features in both Xenopus and zebrafish. Consequently, evolutionary divergence of molecular mechanisms exists during retinal cell differentiation and might be the result of circadian clock-related mechanisms, which are known to be involved in adaptation to environmental lighting conditions.

3. CLOCK GENES AND CLOCK-CONTROLLED GENES (CCG) DURING RETINAL DEVELOPMENT

Much is known presently about the functionality of circadian clocks during adult stem cell renewal, including neurogenesis (Terzibasi-Tozzini et al., 2017). However, when the circadian clock arises during development and how it acts have been long-standing questions (Agrawal et al., 2017; Landgraf et al., 2014; Seron-Ferre et al., 2012; Umemura et al., 2017; Yagita et al., 2010). Clock gene transcripts were repeatedly shown to be maternally inherited but their levels were not rhythmic (Amano et al., 2009; Curran et al., 2008; Dekens and Whitmore, 2008; Hamatani et al., 2004; Johnson et al., 2002).

In zebrafish, asynchronous, endogenously driven cellular oscillations of the zygotic *Per1* transcript were shown to start by 1-day post-fertilization (dpf). However, global rhythms of *Per1* expression in embryos were only observed when they were exposed to (synchronizing) LD cycles (Dekens and Whitmore, 2008). At these developmental stages, the induction of *Per1* rhythmicity required Clock gene, though Clock expression levels were constant (Dekens and Whitmore, 2008).

During early stages of development in mice, clock gene expression data indicate that their expression products are not fully matured yet to form a functional circadian circuit (Amano et al., 2009; Dolatshad et al., 2010). Interestingly, in mouse embryonic stem cells

(ESC), the all-trans retinoic acid induction towards a differentiated neural fate generates circadian oscillations. Conversely, loss of circadian oscillation is seen during the dedifferentiation of neural stem cells by the four major reprogramming factors (*Oct3/4*, *Sox2*, *Klf4*, and *c-Myc*) (Yagita et al., 2010). Thus, the current idea is that the functional clockwork machinery begins to operate as soon as embryonic differentiation starts (Umemura et al., 2017).

Nevertheless, a bidirectional co-regulation of the cell cycle and the circadian clock has also been proposed (Feillet et al., 2015). Furthermore, several cell cycle regulators are well-known to be clock-controlled: tumor suppressor p53, cyclins (such as *Ccnd1* and *Ccnd1b*), oncogenes like *c-Myc* and *Mdm2*, and *Sox9*, *Itga6*, *Wnt3*, *Smad7*, *Cdk4*, *Lhx2*, *Tcf4*, β-catenin (Fu and Kettner, 2013). It was proposed in zebrafish that the early cycling of the circadian clock, synchronized by the LD cycle, controls the timing of cell division (for instance in the gut and skin (Dekens et al., 2003)). This is also likely mediated by the circadian clock control of cell cycle genes (such as p20 and p21 during the G1 phase and genes expressed at G2/M checkpoint) [reviewed in (Brown, 2014; Laranjeiro and Whitmore, 2014)]. Perhaps surprisingly, the link between cell proliferation and the clock during organ development has been rarely reported in mammals (Bagchi et al., 2020; Li et al., 2007). Hence, the exact mechanisms for clocks to start ticking in early life are yet to be unravelled and the question remains whether circadian clocks are simply an evolutionary or also a developmental process, or both (Brown, 2014; Vallone et al., 2007).

Several cellular processes within the wild-type retina are regulated by clock genes (Figure 2) but the relevance of circadian rhythms during retinogenesis has only been rarely reported. Below we review the studies that have investigated the role of clock genes in the successive stages of retinal development in diverse vertebrates and the potential contribution of the circadian clock in generating the eye and retina.

3.1. Clock and clock-controlled genes in retinal progenitors and during cell fate determination

3.1.1. Clock in early eye development in xenopus and zebrafish

Insight into very early expression of clock factors has mainly been obtained in vertebrates with embryo transparency and external development such as amphibians and fish, and more recently in humans thanks to the development of organoids (Sridhar et al., 2020; Wagstaff et al., 2021).

In Xenopus, expression of the *XClock* gene was detected early during gastrulation in the anterior neural plate. It was later found expressed in the neural tube, with highest

expression in its anterior part, including developing eyes (Green et al., 2001). Expression of a dominant-negative mutant form of *XClock* during early development in Xenopus induced abnormal eye and head development, together with the downregulation of *Pax6* and *Otx2*, two (in-)direct transcriptional targets [(Morgan, 2002, 2004) also reviewed in (Vallone et al., 2007)]. Conversely, the over-expression of *XClock* gene in this dominant negative *XClock* mutant rescued *Otx2* levels and normal development (Morgan, 2002). Surprisingly, *XClock* expression is also directly upregulated by *Otx2* in Xenopus embryos (Green et al., 2001), suggesting a positive feedback loop between the two (Green et al., 2001; Morgan, 2002). Besides *Clock*, expression of *Per* genes was detected in the eye at the late Xenopus tailbud stage (stage 31, production of AC and BC), and rhythmic expression of *XBmal1* was determined at stage 41 (when the retina is differentiated and strong expression of rhodopsin is detected in PR layer) (Curran et al., 2008). Whether the early expression of *XClock* in the anterior embryonic region of Xenopus helps to coordinate local ontogenesis of the circadian clock with neural patterning remains to be established.

Intriguingly, (rhythmic) expression of clock genes *Per3* and *Rev-Erbα* was also described in the anterior neural region of the zebrafish embryo including the retina, already during 2-5dpf (when pigments are produced in the RPE) (Delaunay et al., 2000). Similarly, highest expression of *Clock1* and *Per1* transcripts occurred in the anterior region of embryos on second dpf (Dekens and Whitmore, 2008).

3.1.2. The clock during cell cycle and cell fate determination in the developing mouse retina

In mouse eyes, expression of most clock genes has been detected as early as E13 (embryonic day 13) and shows gene-specific and temporal regulated expression throughout development, until adulthood (Bagchi et al., 2020). Interestingly, in line with developmental events in zebrafish (described above), alteration of clock gene expression by knocking out *Bmal1* and *Per1/Per2* in genetically modified mice leads to cell cycle defects in the eye/retina (Bagchi et al., 2020; Sawant et al., 2019). Indeed, double mutation of *Per1* and *Per2* genes decreases expression of cell cycle genes whereas it increases that of phototransduction genes at P3 (Bagchi et al., 2020). In contrast, retina-specific (driven by a Chx10-Cre) loss of *Bmal1* during retinogenesis increased the number of cells entering the S-phase at E15 and reduced the number of cells exiting the cell-cycle (Sawant et al., 2019). This, most likely, underlies the observed reduction in early retinal (ganglion and amacrine) cell types observed in the same study (see below). This aspect was not specifically investigated in the *Per1*$^{-/-}$*Per2*^{Brdm1} mutant mice, but it is interesting to speculate that the observed increase of markers of late-born (rod) cells may be associated with a potential reduction in proliferative capacity (Bagchi et al., 2020). Taken together, the aforementioned studies suggest that the clock is important for the timing of cell division

during mouse retinal development and, when this is not optimal, cell fate specification is disturbed. It is also noteworthy from these studies that impairment of *Per1/Per2* vs *Bmal1* genes in mouse retina likely exerts opposite effects on precursor proliferation (respectively, decrease vs increase) (Bagchi et al., 2020; Sawant et al., 2019). In the molecular clockwork, *Per1/Per2* and *Bmal1* are involved in opposite regulatory arms and act, respectively, as repressors and activators of E box-containing promoters (see Figure 1). Thus, it is possible that the effect on cell division is a result of opposite regulatory effects. Importantly, these data also suggest that a molecular clockwork involving negative feedback between PER1/PER2 and BMAL1:CLOCK (or BMAL1:NPAS2) is already at play early in retinal development (Bagchi et al., 2020; Sawant et al., 2019) (model proposed in Figure 5). We previously hypothesized that extinction of cell cycle genes in the *Per1/Per2* mutant might be due to dysregulation of the Wnt and Hippo pathways (Bagchi et al., 2020). Although this link remains to be demonstrated in the retina, a strong interaction between Wnt signalling and the clock has been repeatedly documented, for instance in adipogenesis or myogenesis (Chatterjee et al., 2013, 2019; Guo et al., 2012).

As mentioned above, clock gene deletion affects cell fate determination in the early developing retina, an effect linked to disturbed precursor proliferation (Sawant et al., 2019). Indeed, Sawant and coworkers found reduced numbers of early-born cells in the *Bmal1* KO, between the mouse E15 and P9 stages, namely Brn3b-expressing RGC and a subset of AC (Calretinin+ and Choline acetyltransferase-ChAT+). Conversely, they observed increased numbers of late-born cells, such as (recoverin+) type II cone BC and (Sox9+) MGC. In addition, MGC were partially mislocalized into the ONL, leading to lamination defects in the retina (Sawant et al., 2019). This lack of *Bmal1* also leads to the abnormal presence of stunted dendritic processes of rod BC already at 1 month, thus suggesting that clock dysfunction also alters rod BCs development (Baba et al., 2018a).

3.2. Clock and clock-controlled genes during photoreceptor differentiation

Lighting conditions are suggested to regulate the dynamics of rod differentiation. For instance, mice pups (P5, P10, P17, P24) raised in constant darkness show alteration of rod development, visual function, and TH-induced-rhodopsin expression. Thus, light exposure and, most likely, circadian regulation, are both important during PR development, as early as P5 (Sawant et al., 2015). Through several well-documented examples described below, we provide an overview of the accumulating evidence that both the central and the secondary regulatory loops of the molecular clock play a role in PR differentiation.

3.2.1. The circadian clock regulates cone spectral identity in rodents

In mice, several studies have shown that the circadian clock is involved in cone differentiation. For instance, in a PR-specific deletion of *Bmal1* (Crx-Cre driven), S-opsin expression

was abnormally distributed over the whole retina and the number of M-opsin expressing cones was significantly reduced (Sawant et al., 2017). Approximately the same patterning was seen in *Clock* KO mice (Sawant et al., 2017). Similarly, *Per1*$^{-/-}$*Per2*^{Brdm1} mutant mice, that have a global clock defect with arrhythmicity in constant dark conditions (Zheng et al., 2001), are characterized by a number of developmental anatomical/histological eye defects. They display a reduced dorsal territory of blue cones, classically abundant in the ventral part of the retina. This is associated with reduced global mRNA expression of S- and M-opsin measured in whole retinas (Ait-Hmyed et al., 2013). Taken together, these data strongly suggest that the dorso-ventral distribution of cones is indeed regulated by the circadian clock.

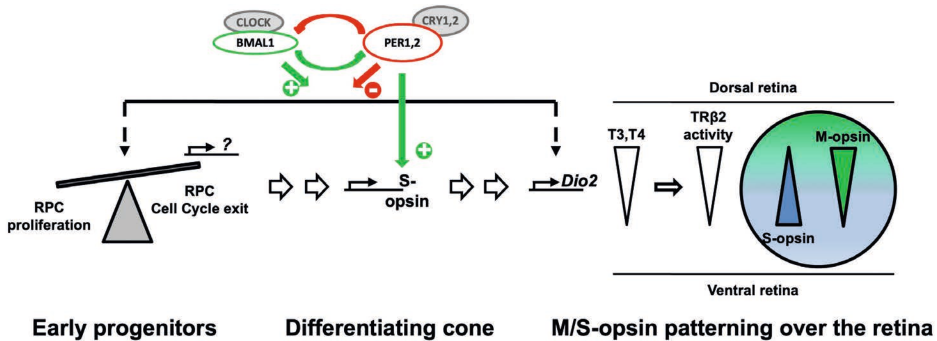


Figure 5. Schematic of the retinal developmental processes regulated by circadian clock (genes). The model proposes that the circadian clock, or at least the action of BMAL1 (transcriptional activator) or PER1, 2 (transcriptional inhibitors), regulates the balance between proliferation and cell cycle exit in early retinal progenitor cells (around E15), the expression of S-opsin (effect specifically observed in the absence of *Per2* or of both *Per1* and *Per2*) and the dorso-ventral patterning of M/S-opsin expression, by enhancing conversion of T4 (inactive) to T3 (active) versions of TH. After the data from (Ait-Hmyed et al., 2013; Bagchi et al., 2020; Sawant et al., 2017, 2019). RPC, early Retinal Progenitor Cells.

Further experimentation, with KO mice and chromatin immunoprecipitation (ChIP), has suggested that *Dio2* (the gene encoding TH-activating enzyme, type 2 iodothyronine deiodinase, which converts T3 to T4 form of TH) is the transcriptional target of BMAL1 during cone differentiation. Partial reversion of the *Bmal1* KO phenotype by T3 supplementation further provided evidence that the clock might regulate the cone-opsin gradient through TH signalling (Sawant et al., 2017). In this hypothesis, the dorso-ventral gradient of T3, the active form of TH, would be further enhanced by the induction of *Dio2* expression. No effect on *Dio2* mRNA was observed in the transcriptome analysis of the *Per1*$^{-/-}$*Per2*^{Brdm1} mutants, potentially because the study was performed (E15 to P3) before the induction of M-opsin expression, by P5 (Bagchi et al., 2020). As the level of

S-opsin expression appears to also be regulated by the Per2 gene (Sawant et al., 2017), it confirms the results in the *Per1*$^{-/-}$*Per2*^{Brdm1} mutants where retinal S-opsin mRNA levels are decreased (Ait-Hmyed et al., 2013) (model proposed Figure 5).

3.2.2. *Rev-Erbs* and *Rors* are at the interface between circadian clock and PR differentiation

In mammals, the main TF regulating the expression of PR genes are CRX, NRL, and NR2E3. A complex between NRL, NR2E3, CRX and REV-ERBα/NR1D1 has been shown to activate, in vitro, the expression of rod phototransduction genes, namely those coding for Rhodopsin (*Opn2*) and the alpha subunit of rod transducin *Gnat 1* (Cheng et al., 2004). Another example based on ChIP analysis from P2 and P21 C57BL/6 J mouse eyes provided evidence that *Rev-Erbα/Nr1d1* is itself a direct target of NR2E3, suggesting that Rev-Erbα/Nr1d1 and *Nr2e3* function in the same transcriptional network during PR development (Haider et al., 2009). Interestingly, in the degenerating retina of the *Nr2e3*-deficient *rd7* mouse, in vivo rescue experiments pointed to *Rev-Erbα/Nr1d1* as a candidate modifier gene for *Nr2e3* (Cruz et al., 2014). Furthermore, during retinal development NR2E3 and REV-ERBα/NR1D1 co-target phototransduction genes, such as Recoverin (*Rcvn*), whose expression is rhythmic in LD and under constant dark conditions (Mollema et al., 2011; Storch et al., 2007). Knock down of *Rev-Erbα/Nr1d1* induces pan-retinal spotting of the fundus associated with reduced light response as measured by electroretinography, but no obvious alteration of PR (Mollema et al., 2011). Neither did *Rev-Erbα/Nr1d1* KO mice show morphological PR defects, whereas they displayed altered light responses (Ait-Hmyed Hakkari et al., 2016). Another standing question is whether the *Nr2e3/Rev-Erbα* common signalling pathway has a link with a circadian clock controlling PR development. It is worth noting in this respect, that in fruit flies the orthologs of *Nr2e3* and of *Rev-Erbα/Nr1d1* together contribute to circadian clock function in the neuronal pacemaker, as shown in relevant fly mutants (Jaumouille et al., 2015).

During retinal development, the ROR clock factors have also been described as early actors (described above). Loss of *Rora* and *Rorb* in mice leads to, respectively, defective cone differentiation and complete depletion of rods (Fujieda et al., 2009; Jia et al., 2009). Interestingly, *Rorc* gene is over-expressed in the developing eyes of *Per1*$^{-/-}$*Per2*^{Brdm1} mutants (Bagchi et al., 2020).

Although precise mechanisms still need to be identified, the data described above suggest that clock genes control PR differentiation in mice. The clock might be involved in modulation of the balance between rod and S-cone genesis and the ratio of M-opsin vs S-opsin expression in cones. It has not been established whether these effects correlate with rhythmic expression of involved clock factors.

3.3. Is transcriptional regulation of retinal development more generally under clock control?

It is possible that the circadian clock has a more widespread link with development of retinal cell types. This is suggested by a number of studies summarized below. Using *Nr1d1* KO mice, Chavan and coworkers (Chavan et al., 2017) found that REV-ERBα/NR1D1 (that is abundantly expressed in the inner retina in addition to PR) can regulate transcription through interaction with other binding partners such as *Hnf6/Oc1* that also controls HC fate (Wu et al., 2013). Other clock-related factors, such as the BHLHE40 and BHLHE41 TF, compete with BMAL1:CLOCK for binding to the E-box. Both *Bhlhe40* and *Bhlhe41* genes have been associated with the generation of RPE lineage cells derived from human iPSC and *Bhlhe40* is an early marker of the presumptive RPE in zebrafish (Cechmanek and McFarlane, 2017; Chuang et al., 2018). A microarray analysis of the daily and circadian transcriptome conducted in larval zebrafish at 5 dpf allowed the identification of GRN underlying developmental effects of the clock (Li et al., 2013). This study pointed to a series of phase-specific oscillating TF genes controlling retinal fate. A number of first examples are genes involved in the developing retina, such as *Mitfa* and its downstream targets *Vsx1* and *Six3* (Li et al., 2013). Regarding the regulation of PR development, TF genes with oscillating expression include *Crx*, *Nr1d1*, and *Rorab*, the orthologue of mammalian *Rorb*. Interestingly in the ONL of 3 dpf and more mature zebrafish larvae, several TF regulating retinal development (Neurod followed by *Nr2e3*, and then *Rx1* and *Crx*) are rhythmically expressed (Laranjeiro and Whitmore, 2014). This suggests that TF which regulate developmental PR differentiation are later re-activated by the circadian clock to control phototransduction over the 24 h cycle.

Taken together, these data suggest a link between regulators of retinal development and the circadian clock. Furthermore, the clock might control retinal rhythmic physiology through these factors in the mature tissue. However, the exact relationship between circadian clock mechanisms, the LD cycle and retinal differentiation remains to be determined. A few data about light perception during differentiation provide some clues.

3.4. Ontogeny of circadian rhythms in the retina

3.4.1. Light perception and ontogeny of rhythms

In rats, light responses in the retina are observed immediately after birth (Mateju et al., 2010) while only at P4 in mice (Munoz Llamosas et al., 2000). In parallel, it was observed that melanopsin is already light-sensitive during fetal development in mice and has significant impact on the development of eye vasculature (Rao et al., 2013). It is also worth noting that in chicken, mRNAs for different circadian markers (*Bmal1*, *Clock*, *Per2*, *Cry1*), for the AANAT enzyme involved in melatonin synthesis, as well as for melanopsin (*Opn4*),

were found expressed in inner retinal cells as early as E8, when the distinct retinal layers are forming (Diaz et al., 2014). Furthermore, chick retinal cell cultures from E8 embryos are able to express 24 h rhythms in clock gene mRNAs, provided they have been exposed to daily LD cycle (Chaurasia et al., 2006; Lima et al., 2011). Taken together, these data suggest that cells are likely to acquire rather early the capacity to sense ambient lighting conditions and measure time. How this genetic effect gets harmonized with the synchronizing effect of the LD cycle is presently unknown. However, data obtained in chicken with photoreceptor-enriched embryonic retina cultures or with cultures of embryonic RGC suggest that light sensitive oscillations of cAMP, interconnected with light-affected Ca^{2+} levels might be the initial circadian regulators in the retina, that might prime the retina for clock-controlled gene regulations. Such model has been especially proposed for the regulation of rhythmic melatonin synthesis, that has been used since decades as a marker of rhythms in the circadian field.

3.4.2. Development of the rhythm in melatonin synthesis

Rhythmic synthesis of melatonin is one of the major functional outputs of the circadian clock in the vertebrate retina, especially in PR. In the PR, melatonin is primarily synthetized during darkness in most species (Cahill and Besharse, 1992; Tosini et al., 2007a; Zawilska and Iuvone, 1992). In addition to its role in controlling dark-adaptive retinal physiology, melatonin regulates cone viability (Gianesini et al., 2016). This rhythm mainly involves cyclic activity of AANAT. *Aanat* expression is regulated by the LD cycle but also by the circadian clock (Fukuhara et al., 2004; Tosini and Fukuhara, 2002). In Xenopus, rhythmic production of melatonin was greatly altered when a dominant-negative CLOCK protein was expressed specifically in PR (Hayasaka et al., 2002).

The onset of circadian rhythms in melatonin levels was first reported in chick retinas. Significant day/night differences in NAT activity emerged around E20, regulated by the LD cycle, to reach a maximum amplitude at post-hatch day 3 (3 dph). A circadian rhythm in NAT activity also appeared around hatching, indicating its regulation by the clock developed in ovo (Iuvone, 1990). In Xenopus embryos, a functional, photo-responsive circadian clock was shown to develop in the eye within the first days of life (Green et al., 1999). Optic vesicles of young Xenopus laevis embryos cultured in vitro slowly start releasing melatonin at developmental stage 26. The latter secretion becomes rhythmic in 2–3 days if optic vesicles are exposed to LD cycle but stays at constitutively high levels under constant darkness. In older Xenopus embryos (stage 47), melatonin is rhythmically released in vitro in the cultured mature eye/retina and shows similar amplitude when maintained in cyclic light or under continuous dark conditions (Green et al., 1999). In embryonic zebrafish, it is suggested that the first circadian melatonin rhythms are not of retinal but of pineal gland origin (Kazimi and Cahill, 1999). Indeed, a functional and

light-responsive circadian oscillator regulating melatonin synthesis in zebrafish becomes apparent just after 20–26 h post-fertilization, before retinal PR maturation (Kazimi and Cahill, 1999). More recently, the developmental rat retina expression pattern of *Aanat* mRNA was examined using Northern blot analysis (Sakamoto et al., 2002). The daily LD cycle-induced rhythmic changes in the *Aanat* mRNA levels began as early as P2. In contrast to LD, *Aanat* rhythms persisted for 1 day in constant darkness only after P14 (Sakamoto et al., 2002). Another study in rats, showed that maternal entrainment of circadian rhythms in pups persists during the first postnatal week (Duncan et al., 1986). However, the required signals from the mother were not sufficient to induce rhythmic expression of retinal AANAT during this period (Duncan et al., 1986). This indicates that the rhythm observed at P2 was essentially driven by the LD cycle and that the latter was required for later maturation of the circadian retinal system. Whether rhythmic melatonin release itself played a role in this maturation remains to be established.

In conclusion, circadian clock genes most likely fine tune retinal development at distinct steps, from very early progenitors to cell type-specific differentiation. These effects involve control of cell cycle, as well as direct control of target genes acting as regulators of patterning/differentiation, most of which have not yet been identified. It also remains to be determined when and how, within these processes, the retinal circadian clock starts ticking. Answering this long-standing question is hindered by the difficulty to track clock gene oscillations at the cellular level. Another question is how the clock in the developing retina gets synchronized by the LD cycle. In lower vertebrates such as zebrafish and Xenopus, these events are likely to occur rather early, facilitated by the embryo transparency. The data presently available indicate that they take place later in mammals. Moreover, the phenotypes of clock mutants in mice support the notion that the circadian clock only modestly contributes to retinal development. However, examination of the more general effects of clock disturbance on retinal and ocular health in adulthood provide a more comprehensive idea of the importance of clock regulation in the retina.

4. CIRCADIAN CLOCKS AND OCULAR HEALTH

Visual perception and light have a profound influence on human physiology, well-being and quality of life. According to the World Health Organization (WHO), an estimated 36 million people worldwide had blindness in 2015, a scale-up of 217 million individuals with moderate to severe visual impairments (Bourne et al., 2017). The role of the circadian clock in retinal development has been discussed in chapter 3 and a number of target developmental genes were listed. Interestingly, some of these genes are also involved in early-onset retinal diseases; however, a potential link between these diseases and

the circadian clock has not been documented yet. Conversely, due to their pervasive control of gene expression and to their role in homeostasis and long-term health, circadian clocks can possibly also modulate detrimental pathological effects of the aging retina. This can be illustrated by a number of examples from the literature: accelerated degeneration of (cone) PR has been reported upon aging in mice carrying *Bmal1* deletion (Baba et al., 2018a, 2018b). Also, a rhythmic component in retinal sensitivity to phototoxicity has been reported in rats and, of note, retinas were much more vulnerable at night (Organisciak et al., 2000). Thus, clock dysfunction might constitute a risk factor for blinding diseases. However, besides a few examples, the (long term) effects of circadian misalignment on human retinal disease remain essentially to be explored.

Below, we describe in more depth a few examples of a possible relationship between a disturbed circadian clock in the developing or aging eye and ocular defects, starting with the retina and extending to distinct eye compartments.

4.1. Clock dysfunction and retinal pathophysiology

4.1.1. Glaucoma

Glaucoma is a leading cause of irreversible blindness characterized by RGC degeneration resulting in visual field defects and optic nerve deterioration. Abnormal, high intraocular pressure (IOP) physically damaging the RGC is considered the main risk factor for glaucoma. The variations of IOP across the circadian cycle have been long recognized (Aihara et al., 2003; Lozano et al., 2015; Nickla et al., 1998; Read et al., 2008). IOP rhythm depends, at least in part, on the integrity of a circadian clock (Maeda et al., 2006). This rhythm derives notably from changes in the aqueous humor production by the ciliary body. Indeed, rhythmic expression of clock genes have been reported in the iris-ciliary body complex (Dalvin and Fautsch, 2015; Tsuchiya et al., 2017), and depends on signals from the SCN, likely through glucocorticoids and the sympathetic pathway activation (Ikegami et al., 2020). In humans, IOP is highest during the night (Liu et al., 1998).

It has been proposed that not the IOP alone, but the translaminar pressure over the optic nerve head determines RGC damage. This would explain the proportion of patients with a so-called normal-tension glaucoma. The translaminar pressure can then be defined as the difference of pressure between the IOP and the intracerebral pressure (ICP) (Janssen et al., 2013). Interestingly, not only IOP, but also ICP has been reported to be highest at night in rodents (Starcevic et al., 1988). Also, the choroid plexus, responsible for cerebrospinal fluid generation in the brain harbors a circadian clock (Myung et al., 2018). Finally, unlike the IOP and ICP, systemic blood pressure is minimal during the night [reviewed in (Bowe et al., 2015)]. This might increase the risk for reduced perfusion of eye or brain

tissues at night-time and potentially contribute to the progression of glaucoma [reviewed in (Ciulla et al., 2020)]. Clearly, the relationship between circadian controlled fluid pressure in the relevant body compartments in the context of glaucoma development is complex and warrants further investigation.

4.1.2. Diabetic retinopathy

Diabetic retinopathy (DR) is a complication of diabetes and considered a microvascular disease of the retina. Due to hyperglycemia, retinal vessels are damaged, leading to formation of ischemic zones and exudation from capillaries that contributes to macular edema. A strong link between DR and the circadian clock has been proposed in several studies. First, DR was associated with the reduction of clock gene expression in the rat retina (Busik et al., 2009). In 2013, Bhatwadekar and co-workers conversely showed in mice that circadian disruption by mutation of *Per2* induced DR, likely through a retinal microvasculature phenotype (Bhatwadekar et al., 2013; Jadhav et al., 2016). Later, the same authors corroborated these findings: conditional deletion of *Bmal1* in endothelial cells induces pathologic vascular and neuronal hallmarks of DR (Bhatwadekar et al., 2017). Finally, Vancura and coworkers found that, in mouse retina, disturbances in circadian and dopamine-directed regulation of fatty acid oxidation enzymes required for daily adaptation of energy metabolism, lead to DR pathology (Vancura et al., 2016). Unexpectedly, more recent data suggested that *Bmal1* KO does not pose a risk, but rather protects against DR (Vancura et al., 2021). Thus, the link between the clock and DR remains to be clarified.

4.1.3. Age-related macular degeneration (AMD)

AMD is a progressive chronic disease of the central retina. The disease affects 4% of the elderly, and no effective or patient-friendly cure exists. Subretinal drusen formation and neovascularization are clinical hallmarks of the disease (Bergen et al., 2019). AMD is caused by environmental and genetic factors. Mechanistically, the oxidative stress, the complement system, lipid metabolism and local extracellular matrix changes haven been implicated in the disease. So far, the potential role of the clock in AMD development has received little attention. However, because of the major importance of oxidative stress in AMD, and the fact that circadian clocks are major regulators of metabolism and cellular redox state, led to the hypothesis that clock dysfunction may contribute to AMD phenotypic variability (Fanjul-Moles and Lopez-Riquelme, 2016; Ruan et al., 2021). Also, biosynthesis and turnover of the oxidative stress-protective melanin pigment in the RPE (the primary retinal layer implicated in the pathology of AMD) is under circadian control (Bergen; personal communication). Genetic linkage, association and expression studies suggested the influence of genetic variation in the *Rora* clock gene on neovascular AMD (Jun et al., 2011; Silveira et al., 2010). Finally, it has been proposed that aberrant

upregulation of the Wnt/β-catenin signalling, controlled by clock genes and involved in exudative AMD, might be due to a disrupted circadian clock (Guo et al., 2012) [reviewed in (Vallee et al., 2020)].

4.2. Effects of clock dysfunction on the anterior eye segment: lens and cornea

Initial studies with *Bmal1* and *Clock* KO mice reported development of cataract, an abnormally cloudy lens. This phenotype has been linked to accelerated aging in the case of *Bmal1* KO (Dubrovsky et al., 2010; Kondratov et al., 2006). More recently, it was shown that *Bmal1* depletion induces a progressive decline of antioxidant-defence pathways in primary human lens epithelial cells and derived cell lines, making them vulnerable to reactive oxygen species (ROS) accumulation and apoptosis (Chhunchha et al., 2020). These processes might underlie the lens premature aging effect induced by *Bmal1* KO. Whether alteration of the circadian clock might also affect lens development was investigated in a genetic study of congenital cataract families. However, no genetic variation in BMAL1 could be linked to the disease (Bagchi et al., 2019).

The renewal and repair of the corneal epithelium are (also) influenced by the circadian clock (Lavker et al., 1991; Sandvig et al., 1994; Scheving and Pauly, 1967). Initial studies in mice showed that the ocular abnormalities induced by *Bmal1* or *Clock* deficiency also include corneal inflammation (Dubrovsky et al., 2010; Kondratov et al., 2006; Yang et al., 2016). Indeed, the whole cornea expresses clock genes that invoke circadian rhythms (Xue et al., 2017; Yoo et al., 2004). These rhythms, measured in whole corneas, are entrained by signals from the retina. For example, melatonin (most likely secreted by the retinal PR) is able to entrain the PER2-luciferase circadian rhythms in the mouse cornea (Baba et al., 2015). When normal environmental lighting cycles are replaced with altered light schedules (constant light, constant dark, reversed LD and jet-lag conditions), the homeostasis of the corneal epithelium, namely mitosis and wound healing, gets disturbed (Xue et al., 2017). Interestingly, analysis of the daily transcriptome in the mouse cornea showed significant enrichment in metabolic pathways and DNA replication during the day, consistent with the finding that corneal epithelial mitosis also peaks during the light phase (Jiao et al., 2019).

4.3. The retinal clock in refractive development and myopia

Myopia or near-sightedness is a prevalent vision disorder projected to affect 50% of the global population by 2050 (Holden et al., 2016). It is a complex disease caused by genetic and environmental factors. Myopia itself predisposes the eye to many other blinding conditions in adulthood. In the 19th century, inadequate lighting or insufficient exposure to the outdoors was suggested for the aetiology of myopia (Hobday, 2016). Indeed, outdoor, or natural light exposure partially protected children to develop myopia in the early

years of life (French et al., 2013). Today, it has become clear that light-induced retinal (dopamergic) signalling is an important driver for refractive error in myopia (Tedja et al., 2018; Wallman and Winawer, 2004; Zhou et al., 2017). A recent meta-GWAS analysis confirmed that refractive errors are associated with the two Gene Ontology Terms “circadian rhythms” and “circadian regulation of gene expression” (Hysi et al., 2020). Indeed, artificial lightning during dark hours, prolonged computer use (indoors) and irregular lifestyle contribute to the development and progression of the disease (Chakraborty et al., 2018).

Interestingly, eye dimensions undergo diurnal fluctuations both in humans (Burfield et al., 2018; Chakraborty et al., 2011; Stone et al., 2013) and in animal models [reviewed in (Nickla, 2013)]. The fluctuating ocular dimensions include axial length, vitreous chamber depth, and choroidal thickness (Chakraborty et al., 2018). Eye dimensions, and even their daily fluctuations, vary in response to imposed optical defocus (ie: artificially induced myopia or hyperopia) (Chakraborty et al., 2012; Nickla, 2013). At the same time, imposed optical defocus also disrupts rhythmic expression patterns of some clock genes in the retina/RPE and in the choroid (Stone et al., 2020). These data corroborate the potential link between refraction, circadian clock mechanisms, and the retina.

This link may also be important for eye development. In the retina-specific *Bmal1* KO mouse, the eyes developed longer axial length and an elongated vitreous chamber (Stone et al., 2019). Consequently, KO mice were significantly more myopic than control mice. Noteworthily, similar anatomical changes were reported in chicks when reared under constant light, suggesting the need of a proper day/night rhythm for optimal eye growth (Stone et al., 1995). Interestingly, it was also reported that brief bright light inhibits the growth of myopic eyes in the chick, when exposure takes place at a specific time of day in the evening (Sarfare et al., 2020). Taken together, these investigations support the idea that the effects of lighting on refractive development are linked to the circadian clock.

5. SUMMARY

The studies presented above suggest that vertebrate eye development is, in part, under circadian control. Indeed, the circadian clock (or at least clock genes) affects a number of steps of eye development, from the anterior neural tube to the optic cup and PR differentiation. Interestingly, in the same developmental timeframe, expression of cell cycle genes and S phase entry are also controlled by (mouse) clock genes. Moreover, in fish, where embryonic development occurs in transparent eggs (thus exposed to light), experimental results indicate that eye formation and circadian rhythms are settled synchronously and could influence each other. However, in higher vertebrates, besides proliferation of early precursors, solely PR differentiation appears to be regulated by

the circadian clock, especially in mammalian cones (summarized in Figure 5). We also report evidence that the clock, integrating environmental cues, modulates a number of pathological processes, not only in the retina, but also in other ocular compartments, suggesting that proper clock function may positively influence eye pathology.

6. CONCLUSIONS AND PERSPECTIVES

During retina embryonic and postnatal development, the circadian clock acts as a modulator of both precursor proliferation and differentiation, likely by defining the appropriate timing for these processes to occur. The results presented in this review suggest that the clock plays an important role in (24 h) eye physiology and homeostasis. Disruption of the clock most likely enhances eye pathology. These data also designate the eye as a perfect clock model to study these processes, both at the cellular, tissue and functional levels.

Further research in understanding the role of circadian clocks in retinal development, disease and therapeutics may include (1) a better understanding of the contribution of individual cell-specific clocks to retinal differentiation (2) a more thorough analysis of the clock in models for retinal disease, and (3) better understanding of a potential role of the clock in therapies for retinal disease. These therapeutic studies may include natural history studies in patients, time restricted application of drugs, and, more fundamentally, the role of the clock in stem cell development and cell replacement therapy.

Authors'contribution

AB and UB and MPFS wrote the manuscript. AAB and MPFS edited the manuscript.

Declaration of competing interest

The authors declare no conflicts of interest.

Acknowledgements

Supported by the Centre National de la Recherche Scientifique (UPR3212), Université de Strasbourg (UPR3212) and the NeuroTime Erasmus+ grant (European Commission).

REFERENCES

Agathocleous, M., Harris, W.A., 2009. From progenitors to differentiated cells in the vertebrate retina. Annu. Rev. Cell Dev. Biol. 25, 45–69. https://doi.org/10.1146/ annurev. cellbio.042308.113259.

Agrawal, P., Houl, J.H., Gunawardhana, K.L., Liu, T., Zhou, J., Zoran, M.J., Hardin, P.E., 2017. Drosophila CRY entrains clocks in body tissues to light and maintains passive membrane properties in a non-clock body tissue independent of light. Curr. Biol. 27, 2431–2441. https://doi.org/10.1016/j. cub.2017.06.064 e2433.

Aihara, M., Lindsey, J.D., Weinreb, R.N., 2003. Twenty-four-hour pattern of mouse intraocular pressure. Exp. Eye Res. 77, 681–686. https://doi.org/10.1016/ j.exer.2003.08.011.

Ait-Hmyed Hakkari, O., Acar, N., Savier, E., Spinnhirny, P., Bennis, M., Felder- Schmittbuhl, M.P., Mendoza, J., Hicks, D., 2016. Rev-Erbalpha modulates retinal visual processing and behavioral responses to light. Faseb. J. 30, 3690–3701. https:// doi.org/10.1096/fj.201600414R.

Ait-Hmyed, O., Felder-Schmittbuhl, M.P., Garcia-Garrido, M., Beck, S., Seide, C., Sothilingam, V., Tanimoto, N., Seeliger, M., Bennis, M., Hicks, D., 2013. Mice lacking Period 1 and Period 2 circadian clock genes exhibit blue cone photoreceptor defects. Eur. J. Neurosci. 37, 1048–1060. https://doi. org/10.1111/ejn.12103.

Albrecht, U., 2012. Timing to perfection: the biology of central and peripheral circadian clocks. Neuron 74, 246–260. https://doi.org/10.1016/j.neuron.2012.04.006.

Amano, T., Matsushita, A., Hatanaka, Y., Watanabe, T., Oishi, K., Ishida, N., Anzai, M., Mitani, T., Kato, H., Kishigami, S., Saeki, K., Hosoi, Y., Iritani, A., Matsumoto, K., 2009. Expression and functional analyses of circadian genes in mouse oocytes and preimplantation embryos: Cry1 is involved in the meiotic process independently of circadian clock regulation. Biol. Reprod. 80, 473–483. https://doi.org/10.1095/ biolreprod.108.069542.

Applebury, M.L., Antoch, M.P., Baxter, L.C., Chun, L.L., Falk, J.D., Farhangfar, F.,

Kage, K., Krzystolik, M.G., Lyass, L.A., Robbins, J.T., 2000. The murine cone photoreceptor: a single cone type expresses both S and M opsins with retinal spatial patterning. Neuron 27, 513–523. https://doi.org/10.1016/s0896-6273(00)00062-3.

Baba, K., Davidson, A.J., Tosini, G., 2015. Melatonin entrains PER2:LUC bioluminescence circadian rhythm in the mouse cornea. Invest. Ophthalmol. Vis. Sci. 56, 4753–4758. https://doi.org/10.1167/ iovs.15-17124.

Baba, K., Piano, I., Lyuboslavsky, P., Chrenek, M.A., Sellers, J.T., Zhang, S., Gargini, C., He, L., Tosini, G., Iuvone, P.M., 2018a. Removal of clock gene Bmal1 from the retina affects retinal development and accelerates cone photoreceptor degeneration during aging. Proc. Natl. Acad. Sci. U. S. A. 115, 13099–13104. https://doi.org/10.1073/ pnas.1808137115.

Baba, K., Ribelayga, C.P., Michael Iuvone, P., Tosini, G., 2018b. The retinal circadian clock and photoreceptor viability. Adv. Exp. Med. Biol. 1074, 345–350. https:// doi.org/10.1007/978-3-319-75402-4_42.

Baba, K., Sengupta, A., Tosini, M., Contreras-Alcantara, S., Tosini, G., 2010. Circadian regulation of the PERIOD 2:LUCIFERASE bioluminescence rhythm in the mouse retinal pigment epithelium-choroid. Mol. Vis. 16, 2605–2611.

Bagchi, U., Gegnaw, S.T., Milicevic, N., Sandu, C., Ten Brink, J.B., Jongejan, A., Hicks, D., Moerland, P.D., Felder-Schmittbuhl, M.P., Bergen, A.A., 2020. Core-clock genes Period 1 and 2 regulate visual cascade and cell cycle components during mouse eye development. Biochim Biophys Acta Gene Regul Mech 1863, 194623. https:// doi.org/10.1016/j.bbagrm.2020.194623.

Bagchi, U., M, S., S, Noorani S., Imran, K.M., Brink, T.B.J., Felder-Schmittbuhl, M.P., Bergen, A.A., 2019. Mutation analysis of circadian clock gene BMAL1 in 21 Pakistani congenital cataract families. Clin. Invest. 9, 1–3. https://doi.org/10.4172/Clinical- Investigation.1000144©2019.

Bell-Pedersen, D., Cassone, V.M., Earnest, D.J., Golden, S.S., Hardin, P.E., Thomas, T.L., Zoran, M.J., 2005. Circadian rhythms from multiple oscillators: lessons from diverse organisms. Nat. Rev. Genet. 6, 544–556. https://doi.org/10.1038/nrg1633.

Bergen, A.A., Arya, S., Koster, C., Pilgrim, M.G., Wiatrek-Moumoulidis, D., van der Spek, P.J., Hauck, S.M., Boon, C.J.F., Emri, E., Stewart, A.J., Lengyel, I., 2019. On the origin of proteins in human drusen: the meet, greet and stick hypothesis. Prog. Retin. Eye Res. 70, 55–84. https://doi.org/10.1016/j.preteyeres.2018.12.003.

Besharse, J.C., Iuvone, P.M., 1983. Circadian clock in Xenopus eye controlling retinal serotonin N-acetyltransferase. Nature 305, 133–135. https://doi.org/10.1038/ 305133a0.

Besharse, J.C., McMahon, D.G., 2016. The retina and other light-sensitive ocular clocks. J. Biol. Rhythm. 31, 223–243. https://doi.org/10.1177/0748730416642657.

Bhatwadekar, A.D., Beli, E., Diao, Y., Chen, J., Luo, Q., Alex, A., Caballero, S., Dominguez 2nd, J.M., Salazar, T.E., Busik, J.V., Segal, M.S., Grant, M.B., 2017. Conditional deletion of Bmal1 accentuates microvascular and macrovascular injury. Am. J. Pathol. 187, 1426–1435. https://doi.org/10.1016/j.ajpath.2017.02.014.

Bhatwadekar, A.D., Yan, Y., Qi, X., Thinschmidt, J.S., Neu, M.B., Li Calzi, S., Shaw, L.C., Dominiguez, J.M., Busik, J.V., Lee, C., Boulton, M.E., Grant, M.B., 2013. Per2 mutation recapitulates the vascular phenotype of diabetes in the retina and bone marrow. Diabetes 62, 273–282. https://doi.org/10.2337/db12-0172.

Boije, H., Rulands, S., Dudczig, S., Simons, B.D., Harris, W.A., 2015. The independent probabilistic firing of transcription factors: a paradigm for clonal variability in the zebrafish retina. Dev. Cell 34, 532–543. https://doi.org/10.1016/ j.devcel.2015.08.011.

Boije, H., Shirazi Fard, S., Ring, H., Hallbook, F., 2013. Forkheadbox N4 (FoxN4) triggers context-dependent differentiation in the developing chick retina and neural tube. Differentiation 85, 11–19. https://doi.org/10.1016/j.diff.2012.12.002.

Bourne, R.R.A., Flaxman, S.R., Braithwaite, T., Cicinelli, M.V., Das, A., Jonas, J.B., Keeffe, J., Kempen, J.H., Leasher, J., Limburg, H., Naidoo, K., Pesudovs, K., Resnikoff, S., Silvester, A., Stevens, G.A., Tahhan, N., Wong, T.Y., Taylor, H.R., Bourne, R., Ackland, P., Arditi, A., Barkana, Y., Bozkurt, B., Braithwaite, T., Bron, A., Budenz, D., Cai, F., Casson, R., Chakravarthy, U., Choi, J., Cicinelli, M.V., Congdon, N., Dana, R., Dandona, R., Dandona, L., Das, A., Dekaris, I., Del Monte, M., Deva, J., Dreer, L., Ellwein, L., Frazier, M., Frick, K., Friedman, D., Furtado, J., Gao, H., Gazzard, G., George, R., Gichuhi, S., Gonzalez, V., Hammond, B.,Hartnett, M.E., He, M., Hejtmancik, J., Hirai, F., Huang, J., Ingram, A., Javitt, J., Jonas, J., Joslin, C., Keeffe, J., Kempen, J., Khairallah, M., Khanna, R., Kim, J., Lambrou, G., Lansingh, V.C., Lanzetta, P., Leasher, J., Lim, J., Limburg, H., Mansouri, K., Mathew, A., Morse, A., Munoz, B., Musch, D., Naidoo, K., Nangia, V., Palaiou, M., Parodi, M.B., Pena, F.Y., Pesudovs, K., Peto, T., Quigley, H., Raju, M., Ramulu, P., Resnikoff, S., Robin, A., Rossetti, L., Saaddine, J., Sandar, M.Y.A., Serle, J., Shen, T., Shetty, R., Sieving, P., Silva, J.C., Silvester, A., Sitorus, R.S., Stambolian, D., Stevens, G., Taylor, H., Tejedor, J., Tielsch, J., Tsilimbaris, M., van Meurs, J., Varma, R., Virgili, G., Volmink, J., Wang, Y.X., Wang, N.-L., West, S., Wiedemann, P., Wong, T., Wormald, R., Zheng, Y., 2017. Magnitude, temporal trends, and projections of the global prevalence of blindness and distance and near vision impairment: a systematic review and meta-analysis. Lancet Global Health 5, e888–e897. https://doi.org/10.1016/s2214-109x(17)30293-0.

Bowe, A., Grunig, M., Schubert, J., Demir, M., Hoffmann, V., Kutting, F., Pelc, A., Steffen, H.M., 2015. Circadian variation in arterial blood pressure and glaucomatous optic neuropathy-A systematic review and meta-analysis. Am. J. Hypertens. 28, 1077–1082. https://doi.org/10.1093/ajh/hpv016.

Brown, S.A., 2014. Circadian clock-mediated control of stem cell division and differentiation: beyond night and day. Development 141, 3105–3111. https:// doi.org/10.1242/dev.104851.

Brzezinski, J.A., Reh, T.A., 2015. Photoreceptor cell fate specification in vertebrates. Development 142, 3263–3273. https://doi.org/10.1242/dev.127043.

Brzezinski, J.A.t., Lamba, D.A., Reh, T.A., 2010. Blimp1 controls photoreceptor versus bipolar cell fate choice during retinal development. Development 137, 619–629. https://doi.org/10.1242/dev.043968.

Buhr, E.D., Yoo, S.H., Takahashi, J.S., 2010. Temperature as a universal resetting cue for mammalian circadian oscillators. Science 330, 379–385. https://doi.org/10.1126/ science.1195262.

Buhr, E.D., Yue, W.W., Ren, X., Jiang, Z., Liao, H.W., Mei, X., Vemaraju, S., Nguyen, M.T., Reed, R.R., Lang, R.A., Yau, K.W., Van Gelder, R.N., 2015. Neuropsin (OPN5)- mediated photoentrainment of local circadian oscillators in mammalian retina and cornea. Proc. Natl. Acad. Sci. U. S. A. 112, 13093–13098. https://doi.org/10.1073/ pnas.1516259112.

Bunger, M.K., Wilsbacher, L.D., Moran, S.M., Clendenin, C., Radcliffe, L.A., Hogenesch, J.B., Simon, M.C., Takahashi, J.S., Bradfield, C.A., 2000. Mop 3 is an essential component of the master circadian pacemaker in mammals. Cell 103, 1009–1017. https://doi.org/10.1016/s0092-8674(00)00205-1.

Burfield, H.J., Patel, N.B., Ostrin, L.A., 2018. Ocular biometric diurnal rhythms in emmetropic and myopic adults. Invest. Ophthalmol. Vis. Sci. 59, 5176–5187. https:// doi.org/10.1167/iovs.18-25389.

Burmeister, M., Novak, J., Liang, M.Y., Basu, S., Ploder, L., Hawes, N.L., Vidgen, D., Hoover, F., Goldman, D., Kalnins, V.I., Roderick, T.H., Taylor, B.A., Hankin, M.H., McInnes, R.R., 1996. Ocular retardation mouse caused by Chx10 homeobox null allele: impaired retinal progenitor proliferation and bipolar cell differentiation. Nat. Genet. 12, 376–384. https://doi.org/10.1038/ng0496-376.

Busik, J.V., Tikhonenko, M., Bhatwadekar, A., Opreanu, M., Yakubova, N., Caballero, S., Player, D., Nakagawa, T., Afzal, A., Kielczewski, J., Sochacki, A., Hasty, S., Li Calzi, S., Kim, S., Duclas, S.K., Segal, M.S., Guberski, D.L., Esselman, 34. W.J., Boulton, M.E., Grant, M.B., 2009. Diabetic retinopathy is associated with bone marrow neuropathy and a depressed peripheral clock. J. Exp. Med. 206, 2897–2906. https://doi.org/ 10.1084/jem.20090889.

Cahill, G.M., Besharse, J.C., 1992. Light-sensitive melatonin synthesis by Xenopus photoreceptors after destruction of the inner retina. Vis. Neurosci. 8, 487–490. https://doi.org/10.1017/s0952523800005009.

Cahill, G.M., Besharse, J.C., 1993. Circadian clock functions localized in xenopus retinal photoreceptors. Neuron 10, 573–577. https://doi.org/10.1016/0896-6273(93) 90160-s.

Calligaro, H., Coutanson, C., Najjar, R.P., Mazzaro, N., Cooper, H.M., Haddjeri, N., Felder- Schmittbuhl, M.P., Dkhissi-Benyahya, O., 2019. Rods contribute to the light-induced phase shift of the retinal clock in mammals. PLoS Biol. 17, e2006211. https:// doi.org/10.1371/journal.pbio.2006211.

Cechmanek, P.B., McFarlane, S., 2017. Retinal pigment epithelium expansion around the neural retina occurs in two separate phases with distinct mechanisms. Dev. Dynam. 246, 598–609. https://doi.org/10.1002/dvdy.24525.

Chakraborty, R., Ostrin, L.A., Nickla, D.L., Iuvone, P.M., Pardue, M.T., Stone, R.A., 2018. Circadian rhythms, refractive development, and myopia. Ophthalmic Physiol. Opt. 38, 217–245. https://doi.org/10.1111/opo.12453.

Chakraborty, R., Read, S.A., Collins, M.J., 2011. Diurnal variations in axial length, choroidal thickness, intraocular pressure, and ocular biometrics. Invest. Ophthalmol. Vis. Sci. 52, 5121–5129. https://doi.org/10.1167/iovs.11-7364.

Chakraborty, R., Read, S.A., Collins, M.J., 2012. Monocular myopic defocus and daily changes in axial length and choroidal thickness of human eyes. Exp. Eye Res. 103, 47–54. https://doi.org/10.1016/j.exer.2012.08.002.

Chatterjee, S., Nam, D., Guo, B., Kim, J.M., Winnier, G.E., Lee, J., Berdeaux, R., Yechoor, V.K., Ma, K., 2013. Brain and muscle Arnt-like 1 is a key regulator of myogenesis. J. Cell Sci. 126, 2213–2224. https://doi.org/10.1242/jcs.120519.

Chatterjee, S., Yin, H., Li, W., Lee, J., Yechoor, V.K., Ma, K., 2019. The nuclear receptor and clock repressor rev-erbalpha suppresses myogenesis. Sci. Rep. 9, 4585. https:// doi.org/10.1038/s41598-019-41059-7.

Chaurasia, S.S., Pozdeyev, N., Haque, R., Visser, A., Ivanova, T.N., Iuvone, P.M., 2006. Circadian clockwork machinery in neural retina: evidence for the presence of functional clock components in photoreceptor-enriched chick retinal cell cultures. Mol. Vis. 12, 215–223.

Chavan, R., Preitner, N., Okabe, T., Strittmatter, L.M., Xu, C., Ripperger, J.A., Pitteloud, N., Albrecht, U., 2017. REV-ERBalpha regulates Fgf 21 expression in the liver via hepatic nuclear factor 6. Biol Open 6, 1–7. https://doi.org/10.1242/ bio.021519.

Chen, J., Rattner, A., Nathans, J., 2005. The rod photoreceptor-specific nuclear receptor Nr2e3 represses transcription of multiple cone-specific genes. J. Neurosci. 25, 118–129. https://doi.org/10.1523/JNEUROSCI.3571-04.2005.

Cheng, H., Khanna, H., Oh, E.C., Hicks, D., Mitton, K.P., Swaroop, A., 2004. Photoreceptor-specific nuclear receptor NR2E3 functions as a transcriptional activator in rod photoreceptors. Hum. Mol. Genet. 13, 1563–1575. https://doi.org/ 10.1093/hmg/ddh173.

Chhunchha, B., Kubo, E., Singh, D.P., 2020. Clock protein Bmal1 and Nrf 2 cooperatively control aging or oxidative response and redox homeostasis by regulating rhythmic expression of Prdx6. Cells 9. https://doi.org/10.3390/cells9081861.

Chuang, J.H., Yarmishyn, A.A., Hwang, D.K., Hsu, C.C., Wang, M.L., Yang, Y.P.,

Chien, K.H., Chiou, S.H., Peng, C.H., Chen, S.J., 2018. Expression profiling of cell- intrinsic regulators in the process of differentiation of human iPSCs into retinal lineages. Stem Cell Res. Ther. 9, 140. https://doi.org/10.1186/s13287-018-0848-7.

Ciulla, L., Moorthy, M., Mathew, S., Siesky, B., Verticchio Vercellin, A.C., Price, D., Januleviciene, I., Harris, A., 2020. Circadian rhythm and glaucoma: what do we know? J. Glaucoma 29, 127–132. https://doi.org/10.1097/IJG.0000000000001402.

Contin, M.A., Verra, D.M., Guido, M.E., 2006. An invertebrate-like phototransduction cascade mediates light detection in the chicken retinal ganglion cells. Faseb. J. 20, 2648–2650. https://doi.org/10.1096/fj.06-6133fje.

Coolen, M., Sii-Felice, K., Bronchain, O., Mazabraud, A., Bourrat, F., Retaux, S., Felder- Schmittbuhl, M.P., Mazan, S., Plouhinec, J.L., 2005. Phylogenomic analysis and expression patterns of large Maf genes in Xenopus tropicalis provide new insights into the functional evolution of the gene family in osteichthyans. Dev. Gene. Evol. 215, 327–339. https://doi.org/10.1007/s00427-005-0476-y.

Corbo, J.C., Cepko, C.L., 2005. A hybrid photoreceptor expressing both rod and cone genes in a mouse model of enhanced S-cone syndrome. PLoS Genet. 1, e11. https:// doi.org/10.1371/journal.pgen.0010011.

Cruz, N.M., Yuan, Y., Leehy, B.D., Baid, R., Kompella, U., DeAngelis, M.M., Escher, P., Haider, N.B., 2014. Modifier genes as therapeutics: the nuclear hormone receptor Rev Erb alpha (Nr1d1) rescues Nr2e3 associated retinal disease. PLoS One 9, e87942. https://doi.org/10.1371/journal.pone.0087942.

Curran, K.L., LaRue, S., Bronson, B., Solis, J., Trow, A., Sarver, N., Zhu, H., 2008. Circadian genes are expressed during early development in Xenopus laevis. PLoS One 3, e2749. https://doi.org/10.1371/journal.pone.0002749.

Dalvin, L.A., Fautsch, M.P., 2015. Analysis of circadian rhythm gene expression with reference to diurnal pattern of intraocular pressure in mice. Invest. Ophthalmol. Vis. Sci. 56, 2657–2663. https://doi.org/10.1167/iovs.15-16449.

Damiola, F., Le Minh, N., Preitner, N., Kornmann, B., Fleury-Olela, F., Schibler, U., 2000. Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. Genes Dev. 14, 2950–2961. https://doi.org/10.1101/gad.183500.

Davies, W.I., Collin, S.P., Hunt, D.M., 2012. Molecular ecology and adaptation of visual photopigments in craniates. Mol. Ecol. 21, 3121–3158. https://doi.org/10.1111/ j.1365-294X.2012.05617.x.

de Melo, J., Du, G., Fonseca, M., Gillespie, L.A., Turk, W.J., Rubenstein, J.L.,

Eisenstat, D.D., 2005. Dlx1 and Dlx2 function is necessary for terminal differentiation and survival of late-born retinal ganglion cells in the developing mouse retina. Development 132, 311–322. https://doi.org/10.1242/dev.01560.

de Melo, J., Peng, G.H., Chen, S., Blackshaw, S., 2011. The Spalt family transcription factor Sall3 regulates the development of cone photoreceptors and retinal horizontal interneurons. Development 138, 2325–2336. https://doi.org/10.1242/dev.061846.

Dekens, M.P., Santoriello, C., Vallone, D., Grassi, G., Whitmore, D., Foulkes, N.S., 2003. Light regulates the cell cycle in zebrafish. Curr. Biol. 13, 2051–2057. https://doi.org/ 10.1016/j.cub.2003.10.022.

Dekens, M.P., Whitmore, D., 2008. Autonomous onset of the circadian clock in the zebrafish embryo. EMBO J. 27, 2757–2765. https://doi.org/10.1038/ emboj.2008.183.

Delaunay, F., Thisse, C., Marchand, O., Laudet, V., Thisse, B., 2000. An inherited functional circadian clock in zebrafish embryos. Science 289, 297–300. https:// doi.org/10.1126/science.289.5477.297.

Diaz, N.M., Morera, L.P., Verra, D.M., Contin, M.A., Guido, M.E., 2014. Early appearance of nonvisual and circadian markers in the developing inner retinal cells of chicken. BioMed Res. Int. 646847. https://doi.org/10.1155/2014/646847, 2014.

Ding, Q., Chen, H., Xie, X., Libby, R.T., Tian, N., Gan, L., 2009. BARHL2 differentially regulates the development of retinal amacrine and ganglion neurons. J. Neurosci. 29, 3992–4003. https://doi.org/10.1523/JNEUROSCI.5237-08.2009.

Dkhissi-Benyahya, O., Coutanson, C., Knoblauch, K., Lahouaoui, H., Leviel, V., Rey, C., Bennis, M., Cooper, H.M., 2013. The absence of melanopsin alters retinal clock function and dopamine regulation by light. Cell. Mol. Life Sci. 70, 3435–3447. https://doi.org/10.1007/s00018-013-1338-9.

Dolatshad, H., Cary, A.J., Davis, F.C., 2010. Differential expression of the circadian clock in maternal and embryonic tissues of mice. PLoS One 5, e9855. https://doi.org/ 10.1371/journal.pone.0009855.

Dubrovsky, Y.V., Samsa, W.E., Kondratov, R.V., 2010. Deficiency of circadian protein CLOCK reduces lifespan and increases age-related cataract development in mice. Aging 2, 936–944. https://doi.org/10.18632/aging.100241.

Dullin, J.P., Locker, M., Robach, M., Henningfeld, K.A., Parain, K., Afelik, S., Pieler, T., Perron, M., 2007. Ptf1a triggers GABAergic neuronal cell fates in the retina. BMC Dev. Biol. 7, 110. https://doi.org/10.1186/1471-213X-7-110.

Duncan, M.J., Banister, M.J., Reppert, S.M., 1986. Developmental appearance of light- dark entrainment in the rat. Brain Res. 369, 326–330. https://doi.org/10.1016/ 0006-8993(86)90544-5.

Dunlap, J.C., 1999. Molecular bases for circadian clocks. Cell 96, 271–290. https:// doi.org/10.1016/s0092-8674(00)80566-8.

Dyer, M.A., 2003. Regulation of proliferation, cell fate specification and differentiation by the homeodomain proteins Prox1, Six3, and Chx10 in the developing retina. Cell Cycle 2, 350–357.

Dyer, M.A., Livesey, F.J., Cepko, C.L., Oliver, G., 2003. Prox1 function controls progenitor cell proliferation and horizontal cell genesis in the mammalian retina. Nat. Genet. 34, 53–58. https://doi.org/10.1038/ng1144.

Elshatory, Y., Everhart, D., Deng, M., Xie, X., Barlow, R.B., Gan, L., 2007. Islet-1 controls the differentiation of retinal bipolar and cholinergic amacrine cells. J. Neurosci. 27, 12707–12720. https://doi.org/10.1523/JNEUROSCI.3951-07.2007.

Emerson, M.M., Surzenko, N., Goetz, J.J., Trimarchi, J., Cepko, C.L., 2013. Otx2 and Onecut1 promote the fates of cone photoreceptors and horizontal cells and repress rod photoreceptors. Dev. Cell 26, 59–72. https://doi.org/10.1016/ j.devcel.2013.06.005.

Enright, J.M., Lawrence, K.A., Hadzic, T., Corbo, J.C., 2015. Transcriptome profiling of developing photoreceptor subtypes reveals candidate genes involved in avian photoreceptor diversification. J. Comp. Neurol. 523, 649–668. https://doi.org/ 10.1002/cne.23702.

Fanjul-Moles, M.L., Lopez-Riquelme, G.O., 2016. Relationship between oxidative stress, circadian rhythms, and AMD. Oxid. Med. Cell. Longev. 7420637. https://doi.org/ 10.1155/2016/7420637, 2016.

Feillet, C., van der Horst, G.T., Levi, F., Rand, D.A., Delaunay, F., 2015. Coupling between the circadian clock and cell cycle oscillators: implication for healthy cells and malignant growth. Front. Neurol. 6, 96. https://doi.org/10.3389/fneur.2015.00096.

Felder-Schmittbuhl, M.-P., Calligaro, H., Dkhissi-Benyahya, O., 2017. The retinal clock in mammals: role in health and disease. ChronoPhysiol. Ther. 7, 33–45. https://doi.org/ 10.2147/cpt.s115251.

Felder-Schmittbuhl, M.P., Buhr, E.D., Dkhissi-Benyahya, O., Hicks, D., Peirson, S.N., Ribelayga, C.P., Sandu, C., Spessert, R., Tosini, G., 2018. Ocular clocks: adapting mechanisms for eye functions and health. Invest. Ophthalmol. Vis. Sci. 59, 4856–4870. https://doi.org/10.1167/iovs.18-24957.

Feng, L., Xie, X., Joshi, P.S., Yang, Z., Shibasaki, K., Chow, R.L., Gan, L., 2006. Requirement for Bhlhb5 in the specification of amacrine and cone bipolar subtypes in mouse retina. Development 133, 4815–4825. https://doi.org/10.1242/dev.02664.

Findlater, G.S., McDougall, R.D., Kaufman, M.H., 1993. Eyelid development, fusion and subsequent reopening in the mouse. J. Anat. 183 (Pt 1), 121–129.

French, A.N., Ashby, R.S., Morgan, I.G., Rose, K.A., 2013. Time outdoors and the prevention of myopia. Exp. Eye Res. 114, 58–68. https://doi.org/10.1016/ j.exer.2013.04.018.

Fu, L., Kettner, N.M., 2013. The circadian clock in cancer development and therapy. Prog Mol Biol Transl Sci 119, 221–282. https://doi.org/10.1016/B978-0-12-396971- 2.00009-9.

Fu, Y., Liu, H., Ng, L., Kim, J.W., Hao, H., Swaroop, A., Forrest, D., 2014. Feedback induction of a photoreceptor-specific isoform of retinoid-related orphan nuclear receptor beta by the rod transcription factor NRL. J. Biol. Chem. 289, 32469–32480. https://doi.org/10.1074/jbc.M114.605774.

Fujieda, H., Bremner, R., Mears, A.J., Sasaki, H., 2009. Retinoic acid receptor-related orphan receptor alpha regulates a subset of cone genes during mouse retinal development. J. Neurochem. 108, 91–101. https://doi.org/10.1111/j.1471- 4159.2008.05739.x.

Fujitani, Y., Fujitani, S., Luo, H., Qiu, F., Burlison, J., Long, Q., Kawaguchi, Y., Edlund, H., MacDonald, R.J., Furukawa, T., Fujikado, T., Magnuson, M.A., Xiang, M.,Wright, C.V., 2006. Ptf1a determines horizontal and amacrine cell fates during mouse retinal development. Development 133, 4439–4450. https://doi.org/10.1242/ dev.02598.

Fukuhara, C., Liu, C., Ivanova, T.N., Chan, G.C., Storm, D.R., Iuvone, P.M., Tosini, G., 2004. Gating of the cAMP signaling cascade and melatonin synthesis by the circadian clock in mammalian retina. J. Neurosci. 24, 1803–1811. https://doi.org/10.1523/ JNEUROSCI.4988-03.2004.

Furukawa, T., Morrow, E.M., Li, T., Davis, F.C., Cepko, C.L., 1999. Retinopathy and attenuated circadian entrainment in Crx-deficient mice. Nat. Genet. 23, 466–470. https://doi.org/10.1038/70591.

Furukawa, T., Mukherjee, S., Bao, Z.Z., Morrow, E.M., Cepko, C.L., 2000. rax, Hes1, and notch 1 promote the formation of Muller glia by postnatal retinal progenitor cells. Neuron 26, 383–394. https://doi.org/10.1016/s0896-6273(00)81171-x.

Garbarino-Pico, E., Carpentieri, A.R., Castagnet, P.I., Pasquare, S.J., Giusto, N.M., Caputto, B.L., Guido, M.E., 2004a. Synthesis of retinal ganglion cell phospholipids is under control of an endogenous circadian clock: daily variations in phospholipid- synthesizing enzyme activities. J. Neurosci. Res. 76, 642–652. https://doi.org/ 10.1002/jnr.20126.

Garbarino-Pico, E., Carpentieri, A.R., Contin, M.A., Sarmiento, M.I., Brocco, M.A., Panzetta, P., Rosenstein, R.E., Caputto, B.L., Guido, M.E., 2004b. Retinal ganglion cells are autonomous circadian oscillators synthesizing N-acetylserotonin during the day. J. Biol. Chem. 279, 51172–51181. https://doi.org/10.1074/jbc.M309248200.

Garita-Hernandez, M., Goureau, O., Dalkara, D., 2016. Gene and Cell Therapy for Inherited Retinal Dystrophies, pp. 1–16. https://doi.org/10.1002/ 9780470015902.a0026565.

Gekakis, N., Staknis, D., Nguyen, H.B., Davis, F.C., Wilsbacher, L.D., King, D.P., Takahashi, J.S., Weitz, C.J., 1998. Role of the CLOCK protein in the mammalian circadian mechanism. Science 280, 1564–1569. https://doi.org/10.1126/ science.280.5369.1564.

Gianesini, C., Hiragaki, S., Laurent, V., Hicks, D., Tosini, G., 2016. Cone viability is affected by disruption of melatonin receptors signaling. Invest. Ophthalmol. Vis. Sci. 57, 94–104. https://doi.org/10.1167/iovs.15-18235.

Green, C.B., Durston, A.J., Morgan, R., 2001. The circadian gene Clock is restricted to the anterior neural plate early in development and is regulated by the neural inducer noggin and the transcription factor Otx2. Mech. Dev. 101, 105–110. https://doi.org/ 10.1016/s0925-4773(00)00559-1.

Green, C.B., Liang, M.Y., Steenhard, B.M., Besharse, J.C., 1999. Ontogeny of circadian and light regulation of melatonin release in Xenopus laevis embryos. Brain Res Dev Brain Res 117, 109–116. https://doi.org/10.1016/s0165-3806(99)00109-1.

Guo, B., Chatterjee, S., Li, L., Kim, J.M., Lee, J., Yechoor, V.K., Minze, L.J., Hsueh, W., Ma, K., 2012. The clock gene, brain and muscle Arnt-like 1, regulates adipogenesis via Wnt signaling pathway. Faseb. J. 26, 3453–3463. https://doi.org/10.1096/fj.12- 205781.

Haider, N.B., Jacobson, S.G., Cideciyan, A.V., Swiderski, R., Streb, L.M., Searby, C., Beck, G., Hockey, R., Hanna, D.B., Gorman, S., Duhl, D., Carmi, R., Bennett, J., Weleber, R.G., Fishman, G.A., Wright, A.F., Stone, E.M., Sheffield, V.C., 2000. Mutation of a nuclear receptor gene, NR2E3, causes enhanced S cone syndrome, a disorder of retinal cell fate. Nat. Genet. 24, 127–131. https://doi.org/10.1038/ 72777.

Haider, N.B., Mollema, N., Gaule, M., Yuan, Y., Sachs, A.J., Nystuen, A.M., Naggert, J.K., Nishina, P.M., 2009. Nr2e3-directed transcriptional regulation of genes involved in photoreceptor development and cell-type specific phototransduction. Exp. Eye Res. 89, 365–372. https://doi.org/10.1016/j.exer.2009.04.006.

Haider, N.B., Naggert, J.K., Nishina, P.M., 2001. Excess cone cell proliferation due to lack of a functional NR2E3 causes retinal dysplasia and degeneration in rd7/rd7 mice. Hum. Mol. Genet. 10, 1619–1626. https://doi.org/10.1093/hmg/10.16.1619.

Hamatani, T., Carter, M.G., Sharov, A.A., Ko, M.S., 2004. Dynamics of global gene expression changes during mouse preimplantation development. Dev. Cell 6, 117–131. https://doi.org/10.1016/s1534-5807(03)00373-3.

Hamon, A., Roger, J.E., Yang, X.J., Perron, M., 2016. Muller glial cell-dependent regeneration of the neural retina: an overview across vertebrate model systems. Dev. Dynam. 245, 727–738. https://doi.org/10.1002/dvdy.24375.

Harada, T., Harada, C., Parada, L.F., 2007. Molecular regulation of visual system development: more than meets the eye. Genes Dev. 21, 367–378. https://doi.org/ 10.1101/gad.1504307.

Harding, P., Moosajee, M., 2019. The molecular basis of human anophthalmia and microphthalmia. J. Dev. Biol. 7. https://doi.org/10.3390/jdb7030016.

Hastings, M.H., Maywood, E.S., Brancaccio, M., 2018. Generation of circadian rhythms in the suprachiasmatic nucleus. Nat. Rev. Neurosci. 19, 453–469. https://doi.org/ 10.1038/s41583-018-0026-z.

Hatakeyama, J., Kageyama, R., 2004. Retinal cell fate determination and bHLH factors. Semin. Cell Dev. Biol. 15, 83–89. https://doi.org/10.1016/j.semcdb.2003.09.005.

Haverkamp, S., Wassle, H., Duebel, J., Kuner, T., Augustine, G.J., Feng, G., Euler, T., 2005. The primordial, blue-cone color system of the mouse retina. J. Neurosci. 25, 5438–5445. https://doi.org/10.1523/JNEUROSCI.1117-05.2005.

Hayasaka, N., LaRue, S.I., Green, C.B., 2002. In vivo disruption of Xenopus CLOCK in the retinal photoreceptor cells abolishes circadian melatonin rhythmicity without affecting its production levels. J. Neurosci. 22, 1600–1607.

Heavner, W., Pevny, L., 2012. Eye development and retinogenesis. Cold Spring Harbor Perspect. Biol. 4. https://doi.org/10.1101/cshperspect.a008391.

Hiragaki, S., Baba, K., Coulson, E., Kunst, S., Spessert, R., Tosini, G., 2014. Melatonin signaling modulates clock genes expression in the mouse retina. PLoS One 9, e106819. https://doi.org/10.1371/journal.pone.0106819.

Hobday, R., 2016. Myopia and daylight in schools: a neglected aspect of public health? Perspect Public Health 136, 50–55. https://doi.org/10.1177/1757913915576679.

Hojo, M., Ohtsuka, T., Hashimoto, N., Gradwohl, G., Guillemot, F., Kageyama, R., 2000. Glial cell fate specification modulated by the bHLH gene Hes5 in mouse retina. Development 127, 2515–2522.

Holden, B.A., Fricke, T.R., Wilson, D.A., Jong, M., Naidoo, K.S., Sankaridurg, P., Wong, T.Y., Naduvilath, T.J., Resnikoff, S., 2016. Global prevalence of myopia and high myopia and temporal trends from 2000 through 2050. Ophthalmology 123, 1036–1042. https://doi.org/10.1016/j.ophtha.2016.01.006.

Hughes, A.E., Enright, J.M., Myers, C.A., Shen, S.Q., Corbo, J.C., 2017. Cell type-specific epigenomic analysis reveals a uniquely closed chromatin architecture in mouse rod photoreceptors. Sci. Rep. 7, 43184. https://doi.org/10.1038/srep43184.

Hunt, T., Sassone-Corsi, P., 2007. Riding tandem: circadian clocks and the cell cycle. Cell 129, 461–464. https://doi.org/10.1016/j.cell.2007.04.015.

Hwang, C.K., Chaurasia, S.S., Jackson, C.R., Chan, G.C., Storm, D.R., Iuvone, P.M., 2013. Circadian rhythm of contrast sensitivity is regulated by a dopamine-neuronal PAS- domain protein 2-adenylyl cyclase 1 signaling pathway in retinal ganglion cells. J. Neurosci. 33, 14989–14997. https://doi.org/10.1523/JNEUROSCI.2039-13.2013.

Hysi, PG, Choquet, H, Khawaja, AP, Wojciechowski, R, Tedja, MS, Yin, J, Simcoe, MJ, Patasova, K, Mahroo, OA, Thai, KK, Cumberland, PM, Melles, RB, Verhoeven, VJM, Vitart, V, Segre, A, Stone, RA, Wareham, N, Hewitt, AW, Mackey, DA, Klaver, CCW, MacGregor, S, Khaw, PT, Foster, PJ, Guggenheim, JA, Rahi, JS, Jorgenson, E, Hammond, CJ, 2020. Meta-analysis of 542,934 subjects of European ancestry identifies new genes and mechanisms predisposing to refractive error and myopia. Nat. Genet. 52, 401–407. https://doi.org/10.1038/s41588-020-0599-0.

Ikegami, K., Shigeyoshi, Y., Masubuchi, S., 2020. Circadian regulation of IOP rhythm by dual pathways of glucocorticoids and the sympathetic nervous system. Invest. Ophthalmol. Vis. Sci. 61, 26. https://doi.org/10.1167/iovs.61.3.26.

Inoue, T., Hojo, M., Bessho, Y., Tano, Y., Lee, J.E., Kageyama, R., 2002. Math3 and NeuroD regulate amacrine cell fate specification in the retina. Development 129, 831–842.

Iuvone, P.M., 1990. Development of melatonin synthesis in chicken retina: regulation of serotonin N-acetyltransferase activity by light, circadian oscillators, and cyclic AMP. J. Neurochem. 54, 1562–1568. https://doi.org/10.1111/j.1471- 4159.1990.tb01205.x.

Ivanov, D., 2019. Notch signaling-induced oscillatory gene expression may drive neurogenesis in the developing retina. Front. Mol. Neurosci. 12, 226. https://doi.org/ 10.3389/fnmol.2019.00226.

Jadhav, V., Luo, Q., Dominguez, J.M., Al-Sabah, J., Chaqour, B., Grant, M.B., Bhatwadekar, A.D., 2016. Per2-Mediated vascular dysfunction is caused by the upregulation of the connective tissue growth factor (CTGF). PLoS One 11, e0163367. https://doi.org/10.1371/journal.pone.0163367.

Jaeger, C., Sandu, C., Malan, A., Mellac, K., Hicks, D., Felder-Schmittbuhl, M.P., 2015. Circadian organization of the rodent retina involves strongly coupled, layer-specific oscillators. Faseb. J. 29, 1493–1504. https://doi.org/10.1096/fj.14-261214.

Janssen, S.F., Gorgels, T.G., Ramdas, W.D., Klaver, C.C., van Duijn, C.M., Jansonius, N.M., Bergen, A.A., 2013. The vast complexity of primary open angle glaucoma: disease genes, risks, molecular mechanisms and pathobiology. Prog. Retin. Eye Res. 37, 31–67. https://doi.org/10.1016/j.preteyeres.2013.09.001.

Jaumouille, E., Machado Almeida, P., Stahli, P., Koch, R., Nagoshi, E., 2015. Transcriptional regulation via nuclear receptor crosstalk required for the Drosophila circadian clock. Curr. Biol. 25, 1502–1508. https://doi.org/10.1016/ j.cub.2015.04.017.

Jia, L., Oh, E.C., Ng, L., Srinivas, M., Brooks, M., Swaroop, A., Forrest, D., 2009. Retinoid- related orphan nuclear receptor RORbeta is an early-acting factor in rod photoreceptor development. Proc. Natl. Acad. Sci. U. S. A. 106, 17534–17539. https://doi.org/10.1073/pnas.0902425106.

Jiao, X., Wu, M., Lu, D., Gu, J., Li, Z., 2019. Transcriptional profiling of daily patterns of mRNA expression in the C57BL/6J mouse cornea. Curr. Eye Res. 44, 1054–1066. https://doi.org/10.1080/02713683.2019.1625408.

Johnson, M.H., Lim, A., Fernando, D., Day, M.L., 2002. Circadian clockwork genes are expressed in the reproductive tract and conceptus of the early pregnant mouse. Reprod. Biomed. Online 4, 140–145. https://doi.org/10.1016/s1472-6483(10) 61931-1.

Jun, G., Nicolaou, M., Morrison, M.A., Buros, J., Morgan, D.J., Radeke, M.J., Yonekawa, Y., Tsironi, E.E., Kotoula, M.G., Zacharaki, F., Mollema, N., Yuan, Y., Miller, J.W., Haider, N.B., Hageman, G.S., Kim, I.K., Schaumberg, D.A., Farrer, L.A., DeAngelis, M.M., 2011. Influence of ROBO1 and RORA on risk of age-related macular degeneration reveals genetically distinct phenotypes in disease pathophysiology. PLoS One 6, e25775. https://doi.org/10.1371/journal.pone.0025775.

Jusuf, P.R., Almeida, A.D., Randlett, O., Joubin, K., Poggi, L., Harris, W.A., 2011. Origin and determination of inhibitory cell lineages in the vertebrate retina. J. Neurosci. 31, 2549–2562. https://doi.org/10.1523/JNEUROSCI.4713-10.2011.

Kautzmann, M.A., Kim, D.S., Felder-Schmittbuhl, M.P., Swaroop, A., 2011. Combinatorial regulation of photoreceptor differentiation factor, neural retina leucine zipper gene NRL, revealed by in vivo promoter analysis. J. Biol. Chem. 286, 28247–28255. https://doi.org/10.1074/jbc.M111.257246.

Kazimi, N., Cahill, G.M., 1999. Development of a circadian melatonin rhythm in embryonic zebrafish. Brain Res Dev Brain Res 117, 47–52. https://doi.org/10.1016/ s0165-3806(99)00096-6.

Kelley, M.W., Turner, J.K., Reh, T.A., 1994. Retinoic acid promotes differentiation of photoreceptors in vitro. Development 120, 2091–2102.

Kim, D.S., Matsuda, T., Cepko, C.L., 2008. A core paired-type and POU homeodomain- containing transcription factor program drives retinal bipolar cell gene expression. J. Neurosci. 28, 7748–7764. https://doi.org/10.1523/JNEUROSCI.0397-08.2008.

Kim, J.W., Yang, H.J., Oel, A.P., Brooks, M.J., Jia, L., Plachetzki, D.C., Li, W., Allison, W.T., Swaroop, A., 2016. Recruitment of rod photoreceptors from short- wavelength-sensitive cones during the evolution of nocturnal vision in mammals. Dev. Cell 37, 520–532. https://doi.org/10.1016/j.devcel.2016.05.023.

Koike, C., Nishida, A., Ueno, S., Saito, H., Sanuki, R., Sato, S., Furukawa, A., Aizawa, S., Matsuo, I., Suzuki, N., Kondo, M., Furukawa, T., 2007. Functional roles of Otx2 transcription factor in postnatal mouse retinal development. Mol. Cell Biol. 27, 8318–8329. https://doi.org/10.1128/MCB.01209-07.

Koike, N., Yoo, S.H., Huang, H.C., Kumar, V., Lee, C., Kim, T.K., Takahashi, J.S., 2012. Transcriptional architecture and chromatin landscape of the core circadian clock in mammals. Science 338, 349–354. https://doi.org/10.1126/science.1226339.

Kondratov, R.V., Kondratova, A.A., Gorbacheva, V.Y., Vykhovanets, O.V., Antoch, M.P., 2006. Early aging and age-related pathologies in mice deficient in BMAL1, the core componentof the circadian clock. Genes Dev. 20, 1868–1873. https://doi.org/ 10.1101/gad.1432206.

Kumar, R., Chen, S., Scheurer, D., Wang, Q.L., Duh, E., Sung, C.H., Rehemtulla, A., Swaroop, A., Adler, R., Zack, D.J., 1996. The bZIP transcription factor Nrl stimulates rhodopsin promoter activity in primary retinal cell cultures. J. Biol. Chem. 271, 29612–29618. https://doi.org/10.1074/jbc.271.47.29612.

Kume, K., Zylka, M.J., Sriram, S., Shearman, L.P., Weaver, D.R., Jin, X., Maywood, E.S., Hastings, M.H., Reppert, S.M., 1999. mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. Cell 98, 193–205. https:// doi.org/10.1016/s0092-8674(00)81014-4.

Lamb, T.D., 2009. Evolution of vertebrate retinal photoreception. Philos. Trans. R. Soc. Lond. B Biol. Sci. 364, 2911–2924. https://doi.org/10.1098/rstb.2009.0102.

Lamb, T.D., Arendt, D., Collin, S.P., 2009. The evolution of phototransduction and eyes. Philos. Trans. R. Soc. Lond. B Biol. Sci. 364, 2791–2793. https://doi.org/10.1098/ rstb.2009.0106.

Landgraf, D., Koch, C.E., Oster, H., 2014. Embryonic development of circadian clocks in the mammalian suprachiasmatic nuclei. Front. Neuroanat. 8, 143. https://doi.org/ 10.3389/fnana.2014.00143.

Laranjeiro, R., Whitmore, D., 2014. Transcription factors involved in retinogenesis are co- opted by the circadian clock following photoreceptor differentiation. Development 141, 2644–2656. https://doi.org/10.1242/dev.104380.

Lavker, R.M., Dong, G., Cheng, S.Z., Kudoh, K., Cotsarelis, G., Sun, T.T., 1991. Relative proliferative rates of limbal and corneal epithelia. Implications of corneal epithelial migration, circadian rhythm, and suprabasally located DNA-synthesizing keratinocytes. Invest. Ophthalmol. Vis. Sci. 32, 1864–1875.

Le, T.T., Wroblewski, E., Patel, S., Riesenberg, A.N., Brown, N.L., 2006. Math5 is required for both early retinal neuron differentiation and cell cycle progression. Dev. Biol. 295, 764–778. https://doi.org/10.1016/j.ydbio.2006.03.055.

Li, S., Mo, Z., Yang, X., Price, S.M., Shen, M.M., Xiang, M., 2004. Foxn4 controls the genesis of amacrine and horizontal cells by retinal progenitors. Neuron 43, 795–807. https://doi.org/10.1016/j.neuron.2004.08.041.

Li, Y., Li, G., Wang, H., Du, J., Yan, J., 2013. Analysis of a gene regulatory cascade mediating circadian rhythm in zebrafish. PLoS Comput. Biol. 9, e1002940. https:// doi.org/10.1371/journal.pcbi.1002940.

Li, Z., Ruan, L., Lin, S., Gittes, G.K., 2007. Clock controls timing of mouse pancreatic differentiation through regulation of Wnt- and Notch-based and cell division components. Biochem. Biophys. Res. Commun. 359, 491–496. https://doi.org/ 10.1016/j.bbrc.2007.05.156.

Lima, L.H., Santos, K.P., Lauro Castrucci, A.M., 2011. Clock genes, melanopsins, melatonin, and dopamine key enzymes and their modulation by light and glutamate in chicken embryonic retinal cells. Chronobiol. Int. 28, 89–100. https://doi.org/ 10.3109/07420528.2010.540685.

Lin, Y.P., Ouchi, Y., Satoh, S., Watanabe, S., 2009. Sox2 plays a role in the induction of amacrine and Muller glial cells in mouse retinal progenitor cells. Invest. Ophthalmol. Vis. Sci. 50, 68–74. https://doi.org/10.1167/iovs.07-1619.

Liu, H., Etter, P., Hayes, S., Jones, I., Nelson, B., Hartman, B., Forrest, D., Reh, T.A., 2008. NeuroD1 regulates expression of thyroid hormone receptor 2 and cone opsins in the developing mouse retina. J. Neurosci. 28, 749–756. https://doi.org/10.1523/ JNEUROSCI.4832-07.2008.

Liu, J.H., Kripke, D.F., Hoffman, R.E., Twa, M.D., Loving, R.T., Rex, K.M., Gupta, N., Weinreb, R.N., 1998. Nocturnal elevation of intraocular pressure in young adults. Invest. Ophthalmol. Vis. Sci. 39, 2707–2712.

Liu, X., Zhang, Z., Ribelayga, C.P., 2012. Heterogeneous expression of the core circadian clock proteins among neuronal cell types in mouse retina. PLoS One 7, e50602. https://doi.org/10.1371/journal.pone.0050602.

Livesey, F.J., Furukawa, T., Steffen, M.A., Church, G.M., Cepko, C.L., 2000. Microarray analysis of the transcriptional network controlled by the photoreceptor homeobox gene Crx. Curr. Biol. 10, 301–310. https://doi.org/10.1016/s0960-9822(00)00379- 1.

Lozano, D.C., Hartwick, A.T., Twa, M.D., 2015. Circadian rhythm of intraocular pressure in the adult rat. Chronobiol. Int. 32, 513–523. https://doi.org/10.3109/ 07420528.2015.1008135.

Maeda, A., Tsujiya, S., Higashide, T., Toida, K., Todo, T., Ueyama, T., Okamura, H., Sugiyama, K., 2006. Circadian intraocular pressure rhythm is generated by clock genes. Invest. Ophthalmol. Vis. Sci. 47, 4050–4052. https://doi.org/10.1167/ iovs.06-0183.

Marquardt, T., Ashery-Padan, R., Andrejewski, N., Scardigli, R., Guillemot, F., Gruss, P., 2001. Pax6 is required for the multipotent state of retinal progenitor cells. Cell 105, 43–55. https://doi.org/10.1016/s0092-8674(01)00295-1.

Marquardt, T., Gruss, P., 2002. Generating neuronal diversity in the retina: one for nearly all. Trends Neurosci. 25, 32–38. https://doi.org/10.1016/s0166-2236(00)02028-2.

Mateju, K., Sumova, A., Bendova, Z., 2010. Expression and light sensitivity of clock genes Per1 and Per2 and immediate-early gene c-fos within the retina of early postnatal Wistar rats. J. Comp. Neurol. 518, 3630–3644. https://doi.org/10.1002/cne.22421.

McIlvain, V.A., Knox, B.E., 2007. Nr2e3 and Nrl can reprogram retinal precursors to the rod fate in Xenopus retina. Dev. Dynam. 236, 1970–1979. https://doi.org/10.1002/ dvdy.21128.

Mears, A.J., Kondo, M., Swain, P.K., Takada, Y., Bush, R.A., Saunders, T.L., Sieving, P.A., Swaroop, A., 2001. Nrl is required for rod photoreceptor development. Nat. Genet. 29, 447–452. https://doi.org/10.1038/ng774.

Mellough, C.B., Bauer, R., Collin, J., Dorgau, B., Zerti, D., Dolan, D.W.P., Jones, C.M., Izuogu, O.G., Yu, M., Hallam, D., Steyn, J.S., White, K., Steel, D.H., Santibanez- Koref, M., Elliott, D.J., Jackson, M.S., Lindsay, S., Grellscheid, S., Lako, M., 2019. An integrated transcriptional analysis of the developing human retina. Development 146. https://doi.org/10.1242/dev.169474.

Milicevic, N., Ait-Hmyed Hakkari, O., Bagchi, U., Sandu, C., Jongejan, A., Moerland, P.D., Ten Brink, J.B., Hicks, D., Bergen, A.A., Felder-Schmittbuhl, M.P., 2021. Core circadian clock genes Per1 and Per2 regulate the rhythm in photoreceptor outer segment phagocytosis. Faseb. J. 35, e21722. https://doi.org/10.1096/ fj.202100293RR.

Miller, B.H., McDearmon, E.L., Panda, S., Hayes, K.R., Zhang, J., Andrews, J.L., Antoch, M.P., Walker, J.R., Esser, K.A., Hogenesch, J.B., Takahashi, J.S., 2007. Circadian and CLOCK-controlled regulation of the mouse transcriptome and cell proliferation. Proc. Natl. Acad. Sci. U. S. A. 104, 3342–3347. https://doi.org/ 10.1073/pnas.0611724104.

Mills, T.S., Eliseeva, T., Bersie, S.M., Randazzo, G., Nahreini, J., Park, K.U.,

Brzezinski, J.A.t., 2017. Combinatorial regulation of a Blimp1 (Prdm1) enhancer in the mouse retina. PLoS One 12, e0176905. https://doi.org/10.1371/ journal.pone.0176905.

Miyamoto, Y., Sancar, A., 1998. Vitamin B2-based blue-light photoreceptors in the retinohypothalamic tract as the photoactive pigments for setting the circadian clock in mammals. Proc. Natl. Acad. Sci. U. S. A. 95, 6097–6102. https://doi.org/10.1073/ pnas.95.11.6097.

Mollema, N.J., Yuan, Y., Jelcick, A.S., Sachs, A.J., von Alpen, D., Schorderet, D., Escher, P., Haider, N.B., 2011. Nuclear receptor Rev-erb alpha (Nr1d1) functions in concert with Nr2e3 to regulate transcriptional networks in the retina. PLoS One 6, e17494. https://doi.org/10.1371/journal.pone.0017494.

Morgan, R., 2002. The circadian gene Clock is required for the correct early expression of the head specific gene Otx2. Int. J. Dev. Biol. 46, 999–1004.

Morgan, R., 2004. Pax6 is a direct, positively regulated target of the circadian gene Clock. Dev. Dynam. 230, 643–650. https://doi.org/10.1002/dvdy.20097.

Morrow, E.M., Furukawa, T., Lee, J.E., Cepko, C.L., 1999. NeuroD regulates multiple functions in the developing neural retina in rodent. Development 126, 23–36.

Mu, X., Fu, X., Sun, H., Beremand, P.D., Thomas, T.L., Klein, W.H., 2005. A gene network downstream of transcription factor Math5 regulates retinal progenitor cell competence and ganglion cell fate. Dev. Biol. 280, 467–481. https://doi.org/ 10.1016/j.ydbio.2005.01.028.

Munoz Llamosas, M., Huerta, J.J., Cernuda-Cernuda, R., Garcia-Fernandez, J.M., 2000. Ontogeny of a photic response in the retina and suprachiasmatic nucleus in the mouse. Brain Res Dev Brain Res 120, 1–6. https://doi.org/10.1016/s0165-3806(99) 00175-3.

Mure, L.S., Le, H.D., Benegiamo, G., Chang, M.W., Rios, L., Jillani, N., Ngotho, M., Kariuki, T., Dkhissi-Benyahya, O., Cooper, H.M., Panda, S., 2018. Diurnal transcriptome atlas of a primate across major neural and peripheral tissues. Science 359. https://doi.org/10.1126/science.aao0318.

Muto, A., Iida, A., Satoh, S., Watanabe, S., 2009. The group E Sox genes Sox8 and Sox9 are regulated by Notch signaling and are required for Muller glial cell development in mouse retina. Exp. Eye Res. 89, 549–558. https://doi.org/10.1016/ j.exer.2009.05.006.

Myung, J., Schmal, C., Hong, S., Tsukizawa, Y., Rose, P., Zhang, Y., Holtzman, M.J., De Schutter, E., Herzel, H., Bordyugov, G., Takumi, T., 2018. The choroid plexus is an important circadian clock component. Nat. Commun. 9, 1062. https://doi.org/ 10.1038/s41467-018-03507-2.

Nakhai, H., Sel, S., Favor, J., Mendoza-Torres, L., Paulsen, F., Duncker, G.I., Schmid, R.M., 2007. Ptf1a is essential for the differentiation of GABAergic and glycinergic amacrine cells and horizontal cells in the mouse retina. Development 134, 1151–1160. https:// doi.org/10.1242/dev.02781.

Ng, L., Hurley, J.B., Dierks, B., Srinivas, M., Salto, C., Vennstrom, B., Reh, T.A., Forrest, D., 2001. A thyroid hormone receptor that is required for the development of green cone photoreceptors. Nat. Genet. 27, 94–98. https://doi.org/10.1038/83829.

Nickla, D.L., 2013. Ocular diurnal rhythms and eye growth regulation: where we are 50 years after Lauber. Exp. Eye Res. 114, 25–34. https://doi.org/10.1016/ j.exer.2012.12.013.

Nickla, D.L., Wildsoet, C., Wallman, J., 1998. The circadian rhythm in intraocular pressure and its relation to diurnal ocular growth changes in chicks. Exp. Eye Res. 66, 183–193. https://doi.org/10.1006/exer.1997.0425.

Nishida, A., Furukawa, A., Koike, C., Tano, Y., Aizawa, S., Matsuo, I., Furukawa, T., 2003. Otx2 homeobox gene controls retinal photoreceptor cell fate and pineal gland development. Nat. Neurosci. 6, 1255–1263. https://doi.org/10.1038/nn1155.

Oh, E.C., Cheng, H., Hao, H., Jia, L., Khan, N.W., Swaroop, A., 2008. Rod differentiation factor NRL activates the expression of nuclear receptor NR2E3 to suppress the development of cone photoreceptors. Brain Res. 1236, 16–29. https://doi.org/ 10.1016/j.brainres.2008.01.028.

Oh, E.C., Khan, N., Novelli, E., Khanna, H., Strettoi, E., Swaroop, A., 2007. Transformation of cone precursors to functional rod photoreceptors by bZIP transcription factor NRL. Proc. Natl. Acad. Sci. U. S. A. 104, 1679–1684. https:// doi.org/10.1073/pnas.0605934104.

Organisciak, D.T., Darrow, R.M., Barsalou, L., Kutty, R.K., Wiggert, B., 2000. Circadian- dependent retinal light damage in rats. Invest. Ophthalmol. Vis. Sci. 41, 3694–3701.

Pan, L., Deng, M., Xie, X., Gan, L., 2008. ISL1 and BRN3B co-regulate the differentiation of murine retinal ganglion cells. Development 135, 1981–1990. https://doi.org/10.1242/dev.010751.

Peng, G.H., Ahmad, O., Ahmad, F., Liu, J., Chen, S., 2005. The photoreceptor-specific nuclear receptor Nr2e3 interacts with Crx and exerts opposing effects on the transcription of rod versus cone genes. Hum. Mol. Genet. 14, 747–764. https:// doi.org/10.1093/hmg/ddi070.

Pittendrigh, C.S., 1993. Temporal organization: reflections of a Darwinian clock-watcher. Annu. Rev. Physiol. 55, 16–54. https://doi.org/10.1146/ annurev.ph.55.030193.000313.

Poche, R.A., Kwan, K.M., Raven, M.A., Furuta, Y., Reese, B.E., Behringer, R.R., 2007. Lim1 is essential for the correct laminar positioning of retinal horizontal cells. J. Neurosci. 27, 14099–14107. https://doi.org/10.1523/JNEUROSCI.4046-07.2007.

Preitner, N., Damiola, F., Lopez-Molina, L., Zakany, J., Duboule, D., Albrecht, U., Schibler, U., 2002. The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. Cell 110, 251–260. https://doi.org/10.1016/s0092-8674(02)00825-5.

Quiring, R., Walldorf, U., Kloter, U., Gehring, W.J., 1994. Homology of the eyeless gene of Drosophila to the Small eye gene in mice and Aniridia in humans. Science 265, 785–789. https://doi.org/10.1126/science.7914031.

Rao, S., Chun, C., Fan, J., Kofron, J.M., Yang, M.B., Hegde, R.S., Ferrara, N., Copenhagen, D.R., Lang, R.A., 2013. A direct and melanopsin-dependent fetal light response regulates mouse eye development. Nature 494, 243–246. https://doi.org/ 10.1038/nature11823.

Read, S.A., Collins, M.J., Iskander, D.R., 2008. Diurnal variation of axial length, intraocular pressure, and anterior eye biometrics. Invest. Ophthalmol. Vis. Sci. 49, 2911–2918. https://doi.org/10.1167/iovs.08-1833.

Rehemtulla, A., Warwar, R., Kumar, R., Ji, X., Zack, D.J., Swaroop, A., 1996. The basic motif-leucine zipper transcription factor Nrl can positively regulate rhodopsin gene expression. Proc. Natl. Acad. Sci. U. S. A. 93, 191–195. https://doi.org/10.1073/ pnas.93.1.191.

Reme, C.E., Wirz-Justice, A., Terman, M., 1991. The visual input stage of the mammalian circadian pacemaking system: I. Is there a clock in the mammalian eye? J. Biol. Rhythm. 6, 5–29. https://doi.org/10.1177/074873049100600104.

Riesenberg, A.N., Le, T.T., Willardsen, M.I., Blackburn, D.C., Vetter, M.L., Brown, N.L., 2009. Pax6 regulation of Math5 during mouse retinal neurogenesis. Genesis 47, 175–187. https://doi.org/10.1002/dvg.20479.

Roberts, M.R., Hendrickson, A., McGuire, C.R., Reh, T.A., 2005. Retinoid X receptor (gamma) is necessary to establish the S-opsin gradient in cone photoreceptors of the developing mouse retina. Invest. Ophthalmol. Vis. Sci. 46, 2897–2904. https:// doi.org/10.1167/iovs.05-0093.

Roberts, M.R., Srinivas, M., Forrest, D., Morreale de Escobar, G., Reh, T.A., 2006. Making the gradient: thyroid hormone regulates cone opsin expression in the developing mouse retina. Proc. Natl. Acad. Sci. U. S. A. 103, 6218–6223. https://doi.org/ 10.1073/pnas.0509981103.

Rodgers, H.M., Huffman, V.J., Voronina, V.A., Lewandoski, M., Mathers, P.H., 2018. The role of the Rx homeobox gene in retinal progenitor proliferation and cell fate specification. Mech. Dev. 151, 18–29. https://doi.org/10.1016/j.mod.2018.04.003.

Rodieck, R.W., 1973. The Vertebrate Retina; Principles of Structure and Function. Freeman, San Francisco.

Roenneberg, T., Merrow, M., 2016. The circadian clock and human health. Curr. Biol. 26, R432–R443. https://doi.org/10.1016/j.cub.2016.04.011.

Ruan, G.X., Allen, G.C., Yamazaki, S., McMahon, D.G., 2008. An autonomous circadian clock in the inner mouse retina regulated by dopamine and GABA. PLoS Biol. 6, e249. https://doi.org/10.1371/journal.pbio.0060249.

Ruan, G.X., Zhang, D.Q., Zhou, T., Yamazaki, S., McMahon, D.G., 2006. Circadian organization of the mammalian retina. Proc. Natl. Acad. Sci. U. S. A. 103, 9703–9708. https://doi.org/10.1073/pnas.0601940103.

Ruan, Y., Jiang, S., Gericke, A., 2021. Age-related macular degeneration: role of oxidative stress and blood vessels. Int. J. Mol. Sci. 22. https://doi.org/10.3390/ijms22031296. Sakamoto, K., Oishi, K., Ishida, N., 2002. Ontogeny of circadian expression of serotonin N- acetyltransferase mRNA in the rat retina. Neurosci. Lett. 317, 53–55. https://doi.org/10.1016/s0304-3940(01)02407-7.

Sandu, C., Hicks, D., Felder-Schmittbuhl, M.P., 2011. Rat photoreceptor circadian oscillator strongly relies on lighting conditions. Eur. J. Neurosci. 34, 507–516. https://doi.org/10.1111/j.1460-9568.2011.07772.x.

Sandvig, K.U., Haaskjold, E., Refsum, S.B., 1994. Time dependency in the regenerative response to injury of the rat corneal epithelium. Chronobiol. Int. 11, 173–179. https://doi.org/10.3109/07420529409057237.

Sarfare, S., Yang, J., Nickla, D.L., 2020. The effects of brief high intensity light on ocular growth in chicks developing myopia vary with time of day. Exp. Eye Res. 195, 108039. https://doi.org/10.1016/j.exer.2020.108039.

Sato, T.K., Panda, S., Miraglia, L.J., Reyes, T.M., Rudic, R.D., McNamara, P., Naik, K.A., FitzGerald, G.A., Kay, S.A., Hogenesch, J.B., 2004. A functional genomics strategy reveals Rora as a component of the mammalian circadian clock. Neuron 43, 527–537. https://doi.org/10.1016/j.neuron.2004.07.018.

Sawant, O., Horton, A.M., Shukla, M., Rayborn, M.E., Peachey, N.S., Hollyfield, J.G., Rao, S., 2015. Light-regulated thyroid hormone signaling is required for rod photoreceptor development in the mouse retina. Invest. Ophthalmol. Vis. Sci. 56, 8248–8257. https://doi.org/10.1167/iovs.15-17743.

Sawant, O.B., Horton, A.M., Zucaro, O.F., Chan, R., Bonilha, V.L., Samuels, I.S., Rao, S., 2017. The circadian clock gene Bmal1 controls thyroid hormone-mediated spectral identity and cone photoreceptor function. Cell Rep. 21, 692–706. https://doi.org/ 10.1016/j.celrep.2017.09.069.

Sawant, O.B., Jidigam, V.K., Fuller, R.D., Zucaro, O.F., Kpegba, C., Yu, M., Peachey, N.S., Rao, S., 2019. The circadian clock gene Bmal1 is required to control the timing of retinal neurogenesis and lamination of Muller glia in the mouse retina. Faseb. J. 33, 8745–8758. https://doi.org/10.1096/fj.201801832RR.

Scheving, L.E., Pauly, J.E., 1967. Circadian phase relationships of thymidine-3H uptake, labeled nuclei, grain counts, and cell division rate in rat corneal epithelium. J. Cell Biol. 32, 677–683. https://doi.org/10.1083/jcb.32.3.677.

Schneider, K., Tippmann, S., Spiwoks-Becker, I., Holthues, H., Wolloscheck, T., Spatkowski, G., Engel, L., Frederiksen, U., Spessert, R., 2010. Unique clockwork in photoreceptor of rat. J. Neurochem. 115, 585–594. https://doi.org/10.1111/j.1471-4159.2010.06953.x.

Seron-Ferre, M., Mendez, N., Abarzua-Catalan, L., Vilches, N., Valenzuela, F.J., Reynolds, H.E., Llanos, A.J., Rojas, A., Valenzuela, G.J., Torres-Farfan, C., 2012. Circadian rhythms in the fetus. Mol. Cell. Endocrinol. 349, 68–75. https://doi.org/ 10.1016/j.mce.2011.07.039.

Shearman, L.P., Zylka, M.J., Weaver, D.R., Kolakowski Jr., L.F., Reppert, S.M., 1997. Two period homologs: circadian expression and photic regulation in the suprachiasmatic nuclei. Neuron 19, 1261–1269. https://doi.org/10.1016/s0896-6273(00)80417-1.

Shen, J., Colonnese, M.T., 2016. Development of activity in the mouse visual cortex.

J. Neurosci. 36, 12259–12275. https://doi.org/10.1523/JNEUROSCI.1903-16.2016. Silveira, A.C., Morrison, M.A., Ji, F., Xu, H., Reinecke, J.B., Adams, S.M., Arneberg, T.M.,

Janssian, M., Lee, J.E., Yuan, Y., Schaumberg, D.A., Kotoula, M.G., Tsironi, E.E., Tsiloulis, A.N., Chatzoulis, D.Z., Miller, J.W., Kim, I.K., Hageman, G.S., Farrer, L.A., Haider, N.B., DeAngelis, M.M., 2010. Convergence of linkage, gene expression and association data demonstrates the influence of the RAR-related orphan receptor alpha (RORA) gene on neovascular AMD: a systems biology based approach. Vis. Res. 50, 698–715. https://doi.org/10.1016/j.visres.2009.09.016.

Sridhar, A., Hoshino, A., Finkbeiner, C.R., Chitsazan, A., Dai, L., Haugan, A.K., Eschenbacher, K.M., Jackson, D.L., Trapnell, C., Bermingham-McDonogh, O., Glass, I., Reh, T.A., 2020. Single-cell transcriptomic comparison of human fetal retina, hPSC-derived retinal organoids, and long-term retinal cultures. Cell Rep. 30, 1644–1659. https://doi.org/10.1016/j.celrep.2020.01.007 e1644.

Starcevic, V.P., Morrow, B.A., Farner, L.A., Keil, L.C., Severs, W.B., 1988. Long-term recording of cerebrospinal fluid pressure in freely behaving rats. Brain Res. 462, 112–117. https://doi.org/10.1016/0006-8993(88)90592-6.

Stenkamp, D.L., 2007. Neurogenesis in the Fish Retina, vol. 259, pp. 173–224. https:// doi.org/10.1016/s0074-7696(06)59005-9.

Stone, R.A., Lin, T., Desai, D., Capehart, C., 1995. Photoperiod, early post-natal eye growth, and visual deprivation. Vis. Res. 35, 1195–1202. https://doi.org/10.1016/ 0042-6989(94)00232-b.

Stone, R.A., McGlinn, A.M., Chakraborty, R., Lee, D.C., Yang, V., Elmasri, A., Landis, E., Shaffer, J., Iuvone, P.M., Zheng, X., Sehgal, A., Pardue, M.T., 2019. Altered ocular parameters from circadian clock gene disruptions. PLoS One 14, e0217111. https:// doi.org/10.1371/journal.pone.0217111.

Stone, R.A., Pardue, M.T., Iuvone, P.M., Khurana, T.S., 2013. Pharmacology of myopia and potential role for intrinsic retinal circadian rhythms. Exp. Eye Res. 114, 35–47. https://doi.org/10.1016/j.exer.2013.01.001.

Stone, R.A., Wei, W., Sarfare, S., McGeehan, B., Engelhart, K.C., Khurana, T.S., Maguire, M.G., Iuvone, P.M., Nickla, D.L., 2020. Visual image quality impacts circadian rhythm-related gene expression in retina and in choroid: a potential mechanism for ametropias. Invest. Ophthalmol. Vis. Sci. 61, 13. https://doi.org/ 10.1167/iovs.61.5.13.

Storch, K.F., Paz, C., Signorovitch, J., Raviola, E., Pawlyk, B., Li, T., Weitz, C.J., 2007. Intrinsic circadian clock of the mammalian retina: importance for retinal processing of visual information. Cell 130, 730–741. https://doi.org/10.1016/ j.cell.2007.06.045.

Suga, A., Taira, M., Nakagawa, S., 2009. LIM family transcription factors regulate the subtype-specific morphogenesis of retinal horizontal cells at post-migratory stages. Dev. Biol. 330, 318–328. https://doi.org/10.1016/j.ydbio.2009.04.002.

Takahashi, J.S., 2017. Transcriptional architecture of the mammalian circadian clock. Nat. Rev. Genet. 18, 164–179. https://doi.org/10.1038/nrg.2016.150.

Takahashi, J.S., Hong, H.K., Ko, C.H., McDearmon, E.L., 2008. The genetics of mammalian circadian order and disorder: implications for physiology and disease. Nat. Rev. Genet. 9, 764–775. https://doi.org/10.1038/nrg2430.

Takatsuka, K., Hatakeyama, J., Bessho, Y., Kageyama, R., 2004. Roles of the bHLH gene Hes1 in retinal morphogenesis. Brain Res. 1004, 148–155. https://doi.org/10.1016/ j.brainres.2004.01.045.

Tedja, M.S., Wojciechowski, R., Hysi, P.G., Eriksson, N., Furlotte, N.A.,Verhoeven, V.J.M., Iglesias, A.I., Meester-Smoor, M.A., Tompson, S.W., Fan, Q., Khawaja, A.P., Cheng, C.Y., Hohn, R., Yamashiro, K., Wenocur, A., Grazal, C., Haller, T., Metspalu, A., Wedenoja, J., Jonas, J.B., Wang, Y.X., Xie, J., Mitchell, P., Foster, P.J., Klein, B.E.K., Klein, R., Paterson, A.D., Hosseini, S.M., Shah, R.L., Williams, C., Teo, Y.Y., Tham, Y.C., Gupta, P., Zhao, W., Shi, Y., Saw, W.Y., Tai, E.S., Sim, X.L., Huffman, J.E., Polasek, O., Hayward, C., Bencic, G., Rudan, I., Wilson, J.F., Consortium, C., andMe Research, T., Eye, U.K.B., Vision, C., Joshi, P.K., Tsujikawa, A., Matsuda, F., Whisenhunt, K.N., Zeller, T., van der Spek, P.J., Haak, R., Meijers-Heijboer, H., van Leeuwen, E.M., Iyengar, S.K., Lass, J.H., Hofman, A., Rivadeneira, F., Uitterlinden, A.G., Vingerling, J.R., Lehtimaki, T., Raitakari, O.T., Biino, G., Concas, M.P., Schwantes-An, T.H., Igo Jr., R.P., Cuellar-Partida, G., Martin, N.G., Craig, J.E., Gharahkhani, P., Williams, K.M., Nag, A., Rahi, J.S., Cumberland, P.M., Delcourt, C., Bellenguez, C., Ried, J.S., Bergen, A.A., Meitinger, T., Gieger, C., Wong, T.Y., Hewitt, A.W., Mackey, D.A., Simpson, C.L., Pfeiffer, N., Parssinen, O., Baird, P.N., Vitart, V., Amin, N., van Duijn, C.M., Bailey-Wilson, J.E., Young, T.L., Saw, S.M., Stambolian, D., MacGregor, S., Guggenheim, J.A., Tung, J.Y., Hammond, C.J., Klaver, C.C.W., 2018. Genome-wide association meta-analysis highlights light-induced signaling as a driver for refractive error. Nat. Genet. 50, 834–848. https://doi.org/10.1038/s41588-018-0127-7.

Terman, J.S., Reme, C.E., Terman, M., 1993. Rod outer segment disk shedding in rats with lesions of the suprachiasmatic nucleus. Brain Res. 605, 256–264. https://doi.org/ 10.1016/0006-8993(93)91748-h.

Terzibasi-Tozzini, E., Martinez-Nicolas, A., Lucas-Sanchez, A., 2017. The clock is ticking. Ageing of the circadian system: from physiology to cell cycle. Semin. Cell Dev. Biol. 70, 164–176. https://doi.org/10.1016/j.semcdb.2017.06.011.

Thomas, K.B., Tigges, M., Iuvone, P.M., 1993. Melatonin synthesis and circadian tryptophan hydroxylase activity in chicken retina following destruction of serotonin immunoreactive amacrine and bipolar cells by kainic acid. Brain Res. 601, 303–307. https://doi.org/10.1016/0006-8993(93)91725-8.

Tomita, K., Moriyoshi, K., Nakanishi, S., Guillemot, F., Kageyama, R., 2000. Mammalian achaete-scute and atonal homologs regulate neuronal versus glial fate determination in the central nervous system. EMBO J. 19, 5460–5472. https://doi.org/10.1093/ emboj/19.20.5460.

Tomita, K., Nakanishi, S., Guillemot, F., Kageyama, R., 1996. Mash1 promotes neuronal differentiation in the retina. Gene Cell. 1, 765–774. https://doi.org/10.1111/j.1365- 2443.1996.tb00016.x.

Tosini, G., Davidson, A.J., Fukuhara, C., Kasamatsu, M., Castanon-Cervantes, O., 2007a. Localization of a circadian clock in mammalian photoreceptors. Faseb. J. 21, 3866–3871. https://doi.org/10.1096/fj.07-8371com.

Tosini, G., Fukuhara, C., 2002. The mammalian retina as a clock. Cell Tissue Res. 309, 119–126. https://doi.org/10.1007/s00441-002-0578-z.

Tosini, G., Kasamatsu, M., Sakamoto, K., 2007b. Clock gene expression in the rat retina: effects of lighting conditions and photoreceptor degeneration. Brain Res. 1159, 134–140. https://doi.org/10.1016/j.brainres.2007.05.023.

Tosini, G., Menaker, M., 1996. Circadian rhythms in cultured mammalian retina. Science 272, 419–421. https://doi.org/10.1126/science.272.5260.419.

Tosini, G., Menaker, M., 1998. The tau mutation affects temperature compensation of hamster retinal circadian oscillators. Neuroreport 9, 1001–1005. https://doi.org/ 10.1097/00001756-199804200-00009.

Tsuchiya, S., Buhr, E.D., Higashide, T., Sugiyama, K., Van Gelder, R.N., 2017. Light entrainment of the murine intraocular pressure circadian rhythm utilizes non-local mechanisms. PLoS One 12, e0184790. https://doi.org/10.1371/ journal.pone.0184790.

Umemura, Y., Koike, N., Ohashi, M., Tsuchiya, Y., Meng, Q.J., Minami, Y., Hara, M., Hisatomi, M., Yagita, K., 2017. Involvement of posttranscriptional regulation of Clock in the emergence of circadian clock oscillation during mouse development. Proc. Natl. Acad. Sci. U. S. A. 114, E7479–E7488. https://doi.org/10.1073/ pnas.1703170114.

Underwood, H., Barrett, R.K., Siopes, T., 1990. The quail's eye: a biological clock. J. Biol. Rhythm. 5, 257–265. https://doi.org/10.1177/074873049000500307.

Vallee, A., Lecarpentier, Y., Vallee, R., Guillevin, R., Vallee, J.N., 2020. Circadian rhythms in exudative age-related macular degeneration: the key role of the canonical WNT/ beta-Catenin pathway. Int. J. Mol. Sci. 21. https://doi.org/10.3390/ijms21030820.

Vallone, D., Lahiri, K., Dickmeis, T., Foulkes, N.S., 2007. Start the clock! Circadian rhythms and development. Dev. Dynam. 236, 142–155. https://doi.org/10.1002/ dvdy.20998.

Vancura, P., Oebel, L., Spohn, S., Frederiksen, U., Schafer, K., Sticht, C., Spessert, R., 2021. Evidence for a dysfunction and disease-promoting role of the circadian clock in the diabetic retina. Exp. Eye Res. 211, 108751. https://doi.org/10.1016/ j.exer.2021.108751.

Vancura, P., Wolloscheck, T., Baba, K., Tosini, G., Iuvone, P.M., Spessert, R., 2016. Circadian and dopaminergic regulation of fatty acid oxidation pathway genes in retina and photoreceptor cells. PLoS One 11, e0164665. https://doi.org/10.1371/ journal.pone.0164665.

Vitorino, M., Jusuf, P.R., Maurus, D., Kimura, Y., Higashijima, S., Harris, W.A., 2009. Vsx2 in the zebrafish retina: restricted lineages through derepression. Neural Dev. 4, 14. https://doi.org/10.1186/1749-8104-4-14.

Vopalensky, P., Kozmik, Z., 2009. Eye evolution: common use and independent recruitment of genetic components. Philos. Trans. R. Soc. Lond. B Biol. Sci. 364, 2819–2832. https://doi.org/10.1098/rstb.2009.0079.

Wagstaff, P.E., Heredero Berzal, A., Boon, C.J.F., Quinn, P.M.J., Ten Asbroek, A., Bergen, A.A., 2021. The role of Small molecules and their effect on the molecular mechanisms of early retinal organoid development. Int. J. Mol. Sci. 22. https:// doi.org/10.3390/ijms22137081.

Wallman, J., Winawer, J., 2004. Homeostasis of eye growth and the question of myopia. Neuron 43, 447–468. https://doi.org/10.1016/j.neuron.2004.08.008.

Sengel, C., Emerson, M.M., Cepko, C.L., 2014. A gene regulatory network controls the binary fate decision of rod and bipolar cells in the vertebrate retina. Dev. Cell 30, 513–527. https://doi.org/10.1016/j.devcel.2014.07.018.

Wang, Y., Dakubo, G.D., Thurig, S., Mazerolle, C.J., Wallace, V.A., 2005. Retinal ganglion cell-derived sonic hedgehog locally controls proliferation and the timing of RGC development in the embryonic mouse retina. Development 132, 5103–5113. https:// doi.org/10.1242/dev.02096.

Witkovsky, P., Veisenberger, E., LeSauter, J., Yan, L., Johnson, M., Zhang, D.Q., McMahon, D., Silver, R., 2003. Cellular location and circadian rhythm of expression of the biological clock gene Period 1 in the mouse retina. J. Neurosci. 23, 7670–7676.

Wu, F., Li, R., Umino, Y., Kaczynski, T.J., Sapkota, D., Li, S., Xiang, M., Fliesler, S.J., Sherry, D.M., Gannon, M., Solessio, E., Mu, X., 2013. Onecut1 is essential for horizontal cell genesis and retinal integrity. J. Neurosci. 33, 13053–13065. https:// doi.org/10.1523/JNEUROSCI.0116-13.2013, 13065a.

Xu, L., Ruan, G., Dai, H., Liu, A.C., Penn, J., McMahon, D.G., 2016. Mammalian retinal Muller cells have circadian clock function. Mol. Vis. 22, 275–283.

Xue, Y., Liu, P., Wang, H., Xiao, C., Lin, C., Liu, J., Dong, D., Fu, T., Yang, Y., Wang, Z., Pan, H., Chen, J., Li, Y., Cai, D., Li, Z., 2017. Modulation of circadian rhythms affects corneal epithelium renewal and repair in mice. Invest. Ophthalmol. Vis. Sci. 58, 1865–1874. https://doi.org/10.1167/iovs.16-21154.

Yagita, K., Horie, K., Koinuma, S., Nakamura, W., Yamanaka, I., Urasaki, A., Shigeyoshi, Y., Kawakami, K., Shimada, S., Takeda, J., Uchiyama, Y., 2010. Development of the circadian oscillator during differentiation of mouse embryonic stem cells in vitro. Proc. Natl. Acad. Sci. U. S. A. 107, 3846–3851. https://doi.org/ 10.1073/pnas.0913256107.

Yang, G., Chen, L., Grant, G.R., Paschos, G., Song, W.L., Musiek, E.S., Lee, V., McLoughlin, S.C., Grosser, T., Cotsarelis, G., FitzGerald, G.A., 2016. Timing of expression of the core clock gene Bmal1 influences its effects on aging and survival. Sci. Transl. Med. 8. https://doi.org/10.1126/scitranslmed.aad3305, 324ra316.

Yang, Z., Ding, K., Pan, L., Deng, M., Gan, L., 2003. Math5 determines the competence state of retinal ganglion cell progenitors. Dev. Biol. 264, 240–254. https://doi.org/10.1016/j.ydbio.2003.08.005.

Yoo, S.H., Yamazaki, S., Lowrey, P.L., Shimomura, K., Ko, C.H., Buhr, E.D., Siepka, S.M., Hong, H.K., Oh, W.J., Yoo, O.J., Menaker, M., Takahashi, J.S., 2004. PERIOD2: LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. Proc. Natl. Acad. Sci. U. S. A. 101, 5339–5346. https://doi.org/10.1073/pnas.0308709101.

Zagozewski, J.L., Zhang, Q., Eisenstat, D.D., 2014a. Genetic regulation of vertebrate eye development. Clin. Genet. 86, 453–460. https://doi.org/10.1111/cge.12493.

Zagozewski, J.L., Zhang, Q., Pinto, V.I., Wigle, J.T., Eisenstat, D.D., 2014b. The role of homeobox genes in retinal development and disease. Dev. Biol. 393, 195–208. https://doi.org/10.1016/j.ydbio.2014.07.004.

Zawilska, J.B., Iuvone, P.M., 1992. Melatonin synthesis in chicken retina: effect of kainic acid-induced lesions on the diurnal rhythm and D2-dopamine receptor-mediated regulation of serotonin N-acetyltransferase activity. Neurosci. Lett. 135, 71–74. https://doi.org/10.1016/0304-3940(92)90138-w.

Zhang, X., Serb, J.M., Greenlee, M.H., 2011. Mouse retinal development: a dark horse model for systems biology research. Bioinf. Biol. Insights 5, 99–113. https://doi.org/ 10.4137/BBI.S6930.

Zheng, B., Albrecht, U., Kaasik, K., Sage, M., Lu, W., Vaishnav, S., Li, Q., Sun, Z.S., Eichele, G., Bradley, A., Lee, C.C., 2001. Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock. Cell 105, 683–694. https://doi.org/ 10.1016/s0092-8674(01)00380-4.

Zhou, X., Pardue, M.T., Iuvone, P.M., Qu, J., 2017. Dopamine signaling and myopia development: what are the key challenges. Prog. Retin. Eye Res. 61, 60–71. https:// doi.org/10.1016/j.preteyeres.2017.06.003.

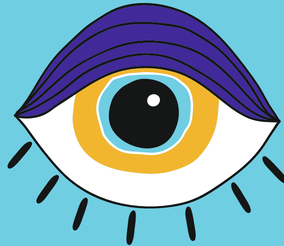
PART

The relevance of core-clock genes Period 1 and 2 on the developing and adult eye physiology

CHAPTER

Udita Bagchi
Shumet Gegnaw
Nemanja Milićević
Cristina Sandu
Jacoline B. ten Brink
Aldo Jongejan
David Hicks
Perry D. Moerland
Marie-Paule Felder-Schmittbuhl
Arthur A. Bergen

BBA - Gene Regulatory Mechanisms 1863 (2020) 194623



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

ABSTRACT

The circadian clock regulates mammalian retina development. We previously observed that retinas from Period1 (*Per1*) and Period2 (*Per2*) double mutant mice mutant (*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 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) and also determined expression profiles of clock genes during early development. All clock genes displayed changes in transcript levels along eye development. RNA-Seq data show major gene expression changes between WT and mutant eyes, with number of differentially expressed genes (DEG) increasing with developmental age. Functional annotation of the genes showing the most significant changes in expression levels in mutant mice concerned major molecular pathways relating to circadian rhythm signaling at E15 and E18 and to the visual cascade and the cell cycle at P3. Our data indicate that clock transcription factors display pleiotropic functions ensuring proper timing of cell-type generation during eye development. Overall, our study provides new insights into signaling 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 the nuclear receptor families of REV-ERBs (encoded by *Nr1d1*, *Nr1d2*) as well as the RORs (encoded by *Rora*, *Rorb*, *Rorc*). In addition, the core clock network has many other putative regulatory genes like *Dbp*, *Hlf*, *Tef*, *Ciart*, *Csnk1ε*, *Csnk1δ*, *Fbxl3*, *Fbxl21*, *Nfil3*, *Bhlhe40, Bhlhe41,* and *Arntl2* (Takahashi et al. 2017).

It is 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 et al. 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 (reviewed in [Reppert et al. 2002; Tosini et al. 1996; Felder-Schmittbuhl et al. 2018]). Not surprisingly, the only mammalian tissue which is necessary for the synchronization of circadian rhythms generated within the SCN with the light/dark cycle, also happens to be the eye (Lee et al. 2003; Yamazaki et al. 2002). Several functions in the mammalian eye are controlled by the circadian clock (reviewed in [McMahon et al. 2014; Felder-Schmittbuhl et al. 2017]) extending from the expression of photopigments (Bobu et al. 2013; von Schantz et al. 1999) to visual sensitivity (Barnard et al. 2006; Cameron et al. 2008; Storch et al. 2007). Notably, retina survival processes such as outer segment disk shedding by rods and cones, phagocytosis by RPE cells and the susceptibility to phototoxicity are linked with the clock (Bobu et al. 2009; LaVail et al. 1980; Organisciak et al. 2000). In addition, the rate of mitosis measured in the cornea (Kikkawa et al. 1973; Xue et al. 2017) along with the daily production of aqueous humor in the ciliary body contributing to intraocular pressure (Tsuchiya et al. 2017; Lozano et al. 2015), 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 et al. 2020). Protein products of clock genes regulate cellular processes within the eye all along development till adulthood (Storch et al. 2007; Liu et al. 2012; Sawant et al. 2019; Baba et al. 2018). Loss of *Rev-Erbα* (*Nr1d1*) expression has detailed several defects during retinal visual processing and in the adjustment of retinal sensitivity to ambient light (Aït-Hmyed Hakkari et al. 2016; Mollema et al. 2011). We previously reported developmental defects related to eye physiology up to 1 year of age in the *Per1*$^{-/-}$*Per2*^{Brdm1} mutant mice (Zheng et al. 2001; Zheng et al. 1999). 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 (Aït-Hmyed Hakkari et al. 2013). The circadian clock gene *Bmal1* was recently shown to affect spatial patterning of cone opsins in the retina, thus corroborating the idea that circadian clock genes are necessary for adequate cone development (Sawant et al. 2017). Furthermore, in *Cry1/Cry2* double mutant animals, cone function was also suggested to be compromised (Wong et al. 2018). However, the molecular mechanism linking clock genes and cone development remains unclear.

Understanding how the circadian clock controls cell biology is essential to apprehend how its disturbance could lead to pathogenicity (Chaix et al. 2016). So far, the emergence of genome-wide transcriptomic approaches has allowed systematic analysis of temporal gene expression in specific cell-types/organs (Panda et al. 2002; Trott and Menet 2018; Beytebiere et al. 2019; Zhang et al. 2014; Mure et al. 2018; Cepko et al. 1996). For example, transcriptomes of 12 mouse organs over time revealed 43% of protein-encoding genes possessing a rhythmic expression profile (Carter-Dawson and LaVail 1979). 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% (Sandu et al. 2011). 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; Sandu et al. 2011), yet the understanding of developmental signaling pathways potentially under the control of clock gene expression is 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.

Here, we present the developmental expression profiles of clock transcripts in mouse 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*$^{-/-}$*Per-*

2^{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 and 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. 2001; Zheng et al. 1999) 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 lux during the light phase, dim red light <5 lux 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.

3

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′-TTTGTTCTGTGAGCTC CTGAACGC-3′ and Per2 KO rev: 5′-ACTTCCATTTGTCACGTCCTGCAC-3′; (Zheng et al. 2001; Zheng et al. 1999); Per1 WT fwd: 5′-GTCTTGGTCTCATTCTAGGACACC-3′ and Per1 WT rev: 5′-AACATGAGAGCTTCCAGTCCTCTC-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.

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 developmental stages, embryonic (E) (E13, E15, E18) and postnatal (P) (P0, P3, P13, P24), were reached. All the timepoints were used for

qRT-PCR estimation (section 2.5) in the wild-type whereas RNA-seq was performed on both wild-type and mutant for E15, E18 and P3 (section 2.6). For very young mice, decapitation was used. Cervical dislocation was performed only for older animals. Eye sampling was performed at the Zeitgeber time (ZT4), 4 h 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 Figure 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, 2ml; QuantaBio, Beverly, MA, USA) at 12000 x 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-1000V 3.5 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA; A_{260}/A_{280} and A_{260}/A_{230} 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)

Total RNA (500 µg) was used to synthesize cDNA using the High-Capacity 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 (Hellemans et al. 2007). 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 sec and annealing–elongation at 60 °C for 1 min. The PCR conditions were 1 x TaqMan Universal PCR Master Mix, No AMPErase UNG (Thermofisher); 1 x 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; [Adachi et al. 2015]) was used for data analysis. Transcript levels were normalized to *Gapdh* and *Sdha* (Sayols et al. 2016). 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 Mg^{2+}. 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 (2X) and Lib. Amp. Primer Mix (10X) (Roche Sequencing Solutions, Pleasanton, CA USA) to produce strand-specific PCR products. The library amplification process was verified using flash gel visualization along with cleanup 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 [Bolger et al. 2014], Picard Tools), trimmed using Trimmomatic v0.32 (Kim et al. 2015) and aligned to the reference mm10 mouse genome (Ensembl GRCm38 v87) using HISAT2 (v2.0.4) (Anders et al. 2015). Counts were obtained using HTSeq (v0.6.1) (Robinson et al. 2010a) 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 (Ritchie et al. 2015) and limma (Robinson et al. 2010b) 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 (Law et al. 2014) and precision weighted using voom (S.M.-I. Consortium 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.

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, at each age (Ingenuity Systems, version 24718999, accessed in November 2018). The list of all identified transcripts in our RNA seq dataset was used as a reference background. 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 control mouse whole eyes during development

We performed a temporal analysis of clock gene expression in developing eyes under light/dark (LD) cycle conditions at Zeitgeber time 4 (ZT4: four hours after lights on) for seven different age time points (E13, E15, E18, P0, P3, P13, P24), mostly confined to the period of photoreceptor genesis and maturation. We quantified the gene expression of core clock genes (*Bmal1* or *Arntl*, *Clock*, *Per1*, *Per2*, *Per3*, *Cry1*, *Cry2*, *Nr1d1* and *Rorb*) by quantitative RT-PCR (qRT-PCR) on the RNA extracted from whole eyes. Distinct gene expression dynamics were revealed for core clock components at the defined ages during eye development (Figure 1 and Supplementary Table S2).

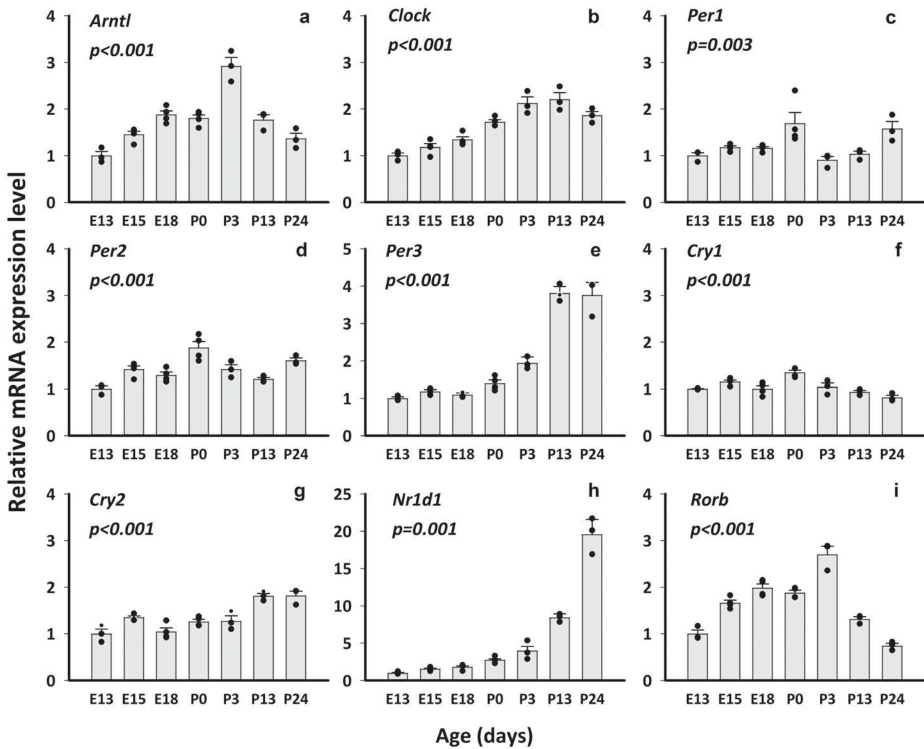


Figure 1. Relative mRNA expression profiles of circadian clock genes in the WT whole eyes across eye developmental time points (n=3-4/age). Bar graphs show mean ±SEM mRNA expression levels of (a) *Arntl* (*Bmal1*) (b) *Clock* (c) *Per1* (d) *Per2* (e) *Per3* (f) *Cry1* (g) *Cry2* (h) *Nr1d1* and (i) *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.

Transcripts of all clock genes examined are already detected at E13 and their abundance shows significant age effect using one-way ANOVA (Figure 1). The relative expression level of *Bmal1* (*Arntl*) mRNA reached a peak just after birth at P3 while maintaining moderate expression at other ages (Figure 1a). Similarly, *Rorb* mRNA had significantly highest expression around P3 (Figure 1i). A steady increase in gene expression was maintained by *Clock* gene from E13 until P3 (Figure 1b). Among *Period* genes, *Per1* and *Per2* expression peaked at birth (P0) whereas *Per3* expression culminated only at the postnatal ages: P13 and P24 (Figure 1c-e). *Cry2* also reached the highest expression levels close to eye opening at P13 (Figure 1f). *Cry1* mRNA had a peak in gene expression at birth (P0) (Figure 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 (Figure 1h).

3.2 RNA-Seq analysis of differentially expressed genes in control and *Per1*$^{-/-}$*Per2*^{Brdm1} mutants

3.2.1 Whole transcriptome changes

We performed RNA sequencing to compare the whole eye gene expression profiles between wild-type and *Per1*$^{-/-}$*Per2*^{Brdm1} mice, as a function of time during embryonic (E15, E18) and postnatal (P3) ages. Principal component analysis showed clear age and genotype effects in the whole eye transcriptome (Figure 2).

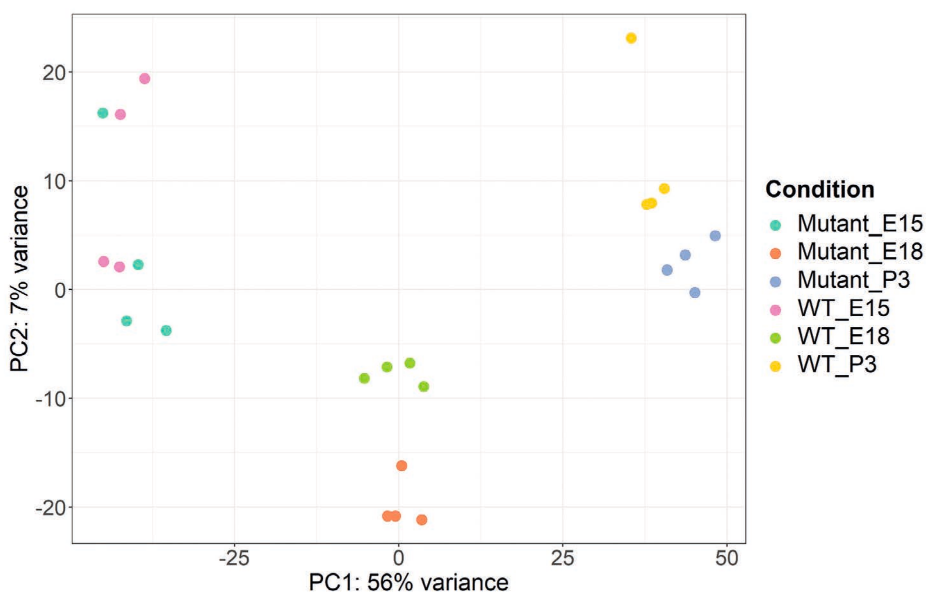


Figure 2. Principal component analysis of eye transcriptomes in WT and mutant eyes at E15, E18 and P3. The percentage with which a principal component (PC) accounts for the variability in the data is indicated on the corresponding axis. Each circle represents an individual RNA sample from WT or mutants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

Age-related effects were more pronounced and segregated on the first principal component (PC), which explained most of the variance in the data (PC1: 56%). Indeed, both in wild-type and mutant mice, many genes (respectively 14046 and 15445) were significantly differentially expressed (adjusted p-value <0.05) between different developmental stages, reflecting the extensive changes occurring in eye development between E15

and P3. Genotype-related effects segregated on the second principal component, which explained 7% of the variance in the data. Gene expression profiles cluster together for mutants and wild-type at E15, whereas clear differences can be observed between WT and mutant expression profiles at E18 and P3.

3.2.2 Specific transcriptome changes (in) between developmental stages

Analysis of differentially expressed genes (DEGs; adjusted p-value <0.05, and fold change ≤-1.5 or ≥1.5) of the *Per1*$^{-/-}$ *Per2*^{Brdm1} versus WT whole eyes show increasing number of DEGs during development with 36, 200 and 367 genes showing significantly increased expression and 18, 90 and 273 genes significantly decreased expression at E15, E18 and P3 respectively in the mutant mice (Figure 3a).

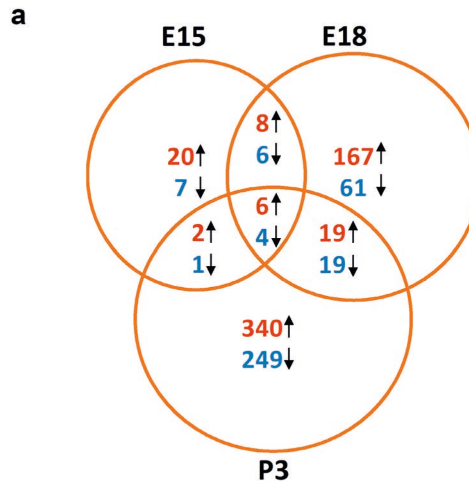


Figure 3. (a) Venn diagram of differentially expressed genes in mutant versus WT eyes. The total numbers of differentially expressed genes (increased or decreased) in each age group and the overlaps are indicated. Upregulated (higher in mutant) genes are marked in red and down-regulated (higher in WT) genes are marked in blue (adjusted p-value <0.05, and fold change ≤-1.5 or ≥1.5).

It is noteworthy that the overall number of upregulated genes was higher, potentially because *Per1* and *Per2* are transcriptional repressors. Only limited numbers of genes are differentially expressed at more than one developmental stage.

Of the 23 genes which were shown to be involved in the molecular clockwork (Takahashi et al. 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 (Figure 3b).

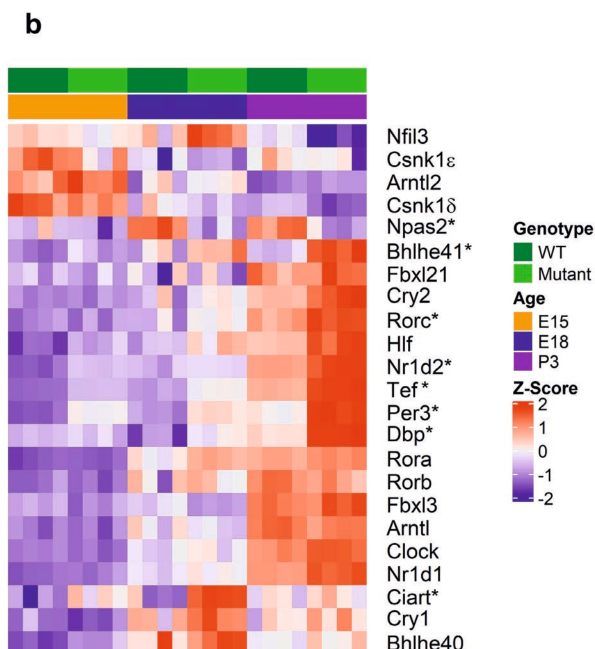


Figure 3. (b) Heatmap of clock and clock-related gene expression during embryonic (E15 and E18) and postnatal (P3) ages of *Per1$^{-/-}$Per2^{Brdm1}* mutants versus WT. Genes were clustered using Euclidean distance and complete linkage. Color-coding corresponds to z-score of logCPM values from blue (lowest 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, $\log_2$ counts per million.

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 consistently increased at P3. In the E18 and P3 mutants, *Npas2* expression was markedly reduced whereas *Dbp* was significantly upregulated. Thus, mutation of *Per1* and *Per2* genes profoundly affects expression levels of other clock genes part of regulatory feedback loops, in particular genes which are targets of the BMAL1/CLOCK dimer.

All the differentially expressed genes at E15 within the cut-off (FDR < 0.05, FC ≥ 1.5) are marked out (Figure 4a). Only selected DEGs are highlighted in the corresponding volcano plots for E18 and P3 age groups (Figure 4b and c), mostly related to the eye, many being photoreceptor-specific. Besides the eye, genes belonging to clock and neuronal development are also shown. 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.

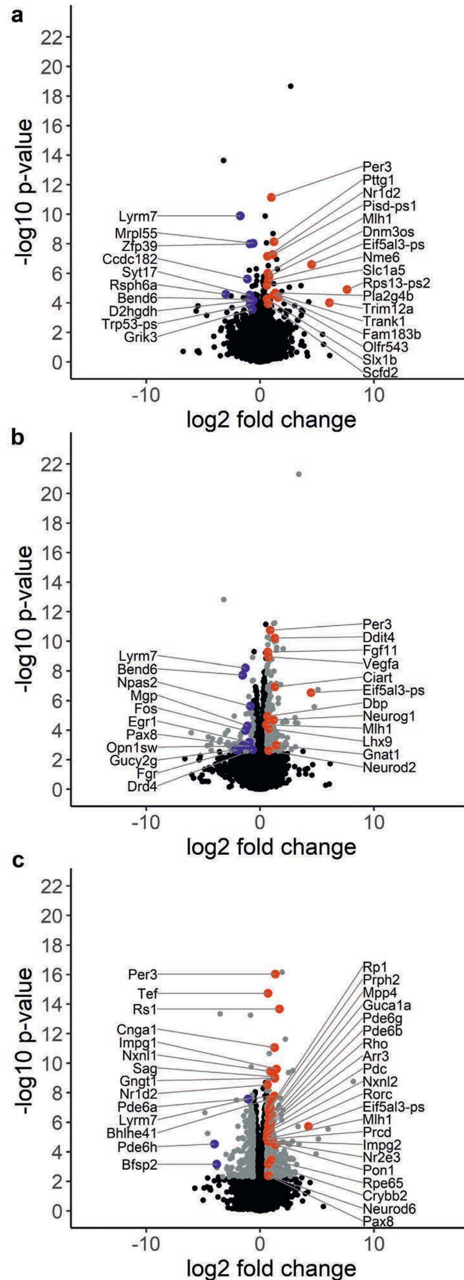
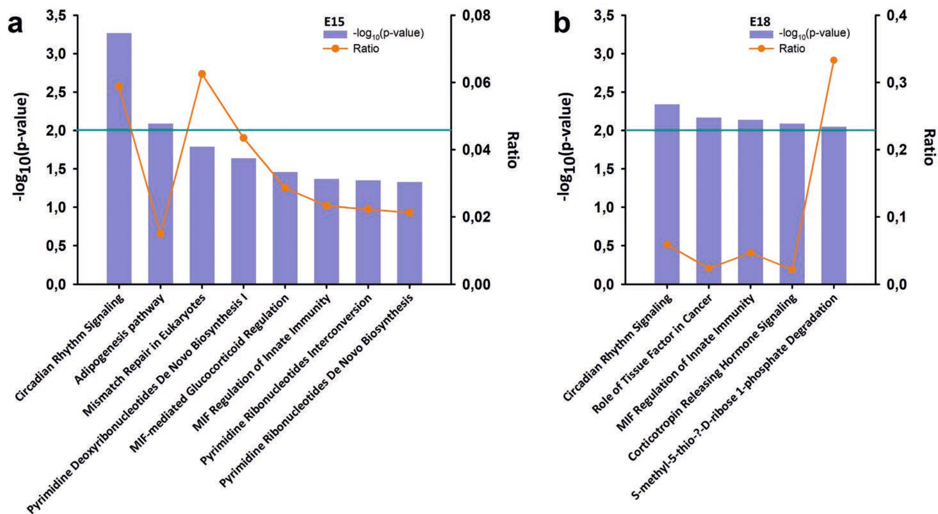


Figure 4. Volcano plots for the genes found enriched in *Per1$^{-/-}$Per2^{Brdm1}* versus WT whole eyes at E15, E18 and P3. The plot shows the enrichment (x axis, log2-transformed fold change) of the genes in mutant versus WT eyes against the significance (y axis, p-value, -log10 scale). Genes with adjusted 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. Top canonical pathways associated with genes differentially expressed at E15 are related to circadian rhythm signaling and adipogenesis pathway (Figure 5a). Interestingly, circadian rhythm signaling is also the top-ranked canonical pathway at E18 followed by four other pathways suggesting enrichment in signaling molecules (Figure 5b). Ten canonical pathways were significantly enriched with genes differentially expressed between *Per1*$^{-/-}$*Per2*^{Brdm1} mutants and wild-type at the postnatal age P3 (Figure 5c). The topmost canonical pathway was related to Phototransduction, which agrees with the phenotype (alteration of photoreceptor development at early postnatal age) previously found in the mutant (Aït-Hmyed Hakkari et al. 2013). The following pathways were mainly related to cell cycle.



In addition to the components of phototransduction cascade which were mainly upregulated in the mutant as highlighted in Figure 6a,b (see also Supplementary Figure S2 and S3a,c), there was a global increase in the 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 palmitoy-

lated protein 4) (details in Supplementary Table S5), indicating the differentiation of the photoreceptor complex.

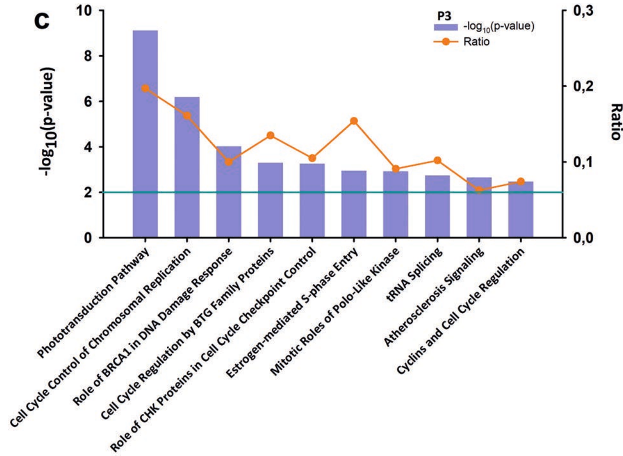
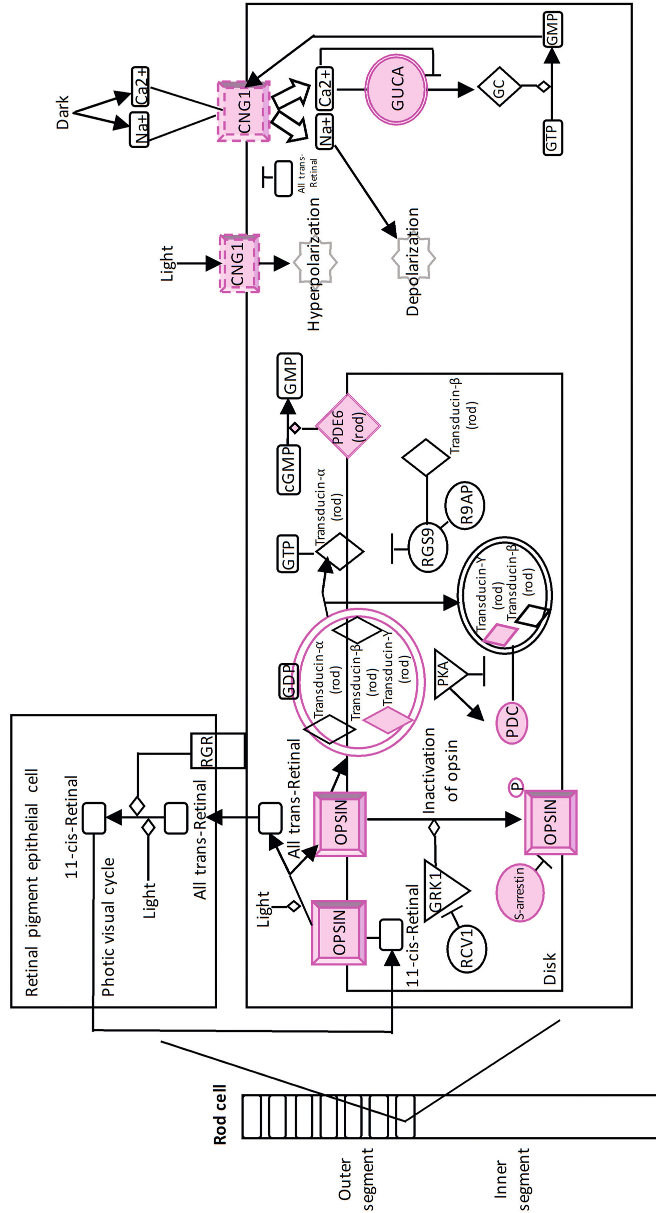


Figure 5. (a) (b) and (c) Top-ranked canonical pathways at E15, E18 and P3 for DEGs in *Per1*$^{-/-}$ *Per2*^{Brdm1} mutants versus WT. Bars represent the -$\log_{10}$ adjusted p-value (left y-axis). Orange line represents the ratio of the number of differentially expressed genes present in the canonical 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.

A substantial fraction of genes previously linked to ocular pathology were found in P3 mutants with increased expression levels such as *Rpe65* (retinal pigment epithelium 65), *Bbs7* (Bardet-Biedl syndrome 7), *Rp1* (retinitis pigmentosa 1), *Pon1* (serum paraoxonase/ arylesterase 1), *Nxnl1* and *Nxnl2* (Rod-derived cone viability factors), *Ptgds* (prostaglandin D2 synthase), *Rx1* (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 (Figure 5c and 7a, b, c). All genes which are part of the `Cell Cycle Control of Chromosomal Replication' pathway such as 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* showed a clear down-regulation of gene expression (Figure 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*, followed by the cyclins B1 (*Ccnb1*) and D1 (*Ccnd1*) (Figure 7b).



Retinal pigment epithelial cell
Photic visual cycle
11-cis-Retinal
Light
All trans-Retinal
RGR
Light
OPSIN
OPSIN
All trans-Retinal
11-cis-Retinal
GRK1
RCV1
Inactivation of opsin
P
OPSIN
S-arrestin
Disk
GDP
Transducin-α (rod)
Transducin-β (rod)
Transducin-γ (rod)
PKA
PDC
GTP
Transducin-α (rod)
cGMP
GMP
PDE6 (rod)
RGS9
R9AP
Transducin-β (rod)
Transducin-γ (rod)
Transducin-β (rod)
Light
CNG1
Hyperpolarization
Depolarization
All trans-Retinal
Dark
Na+
Ca2+
CNG1
Na+
Ca2+
GUCA
GC
GTP
GMP
Rod cell
Outer segment
Inner segment

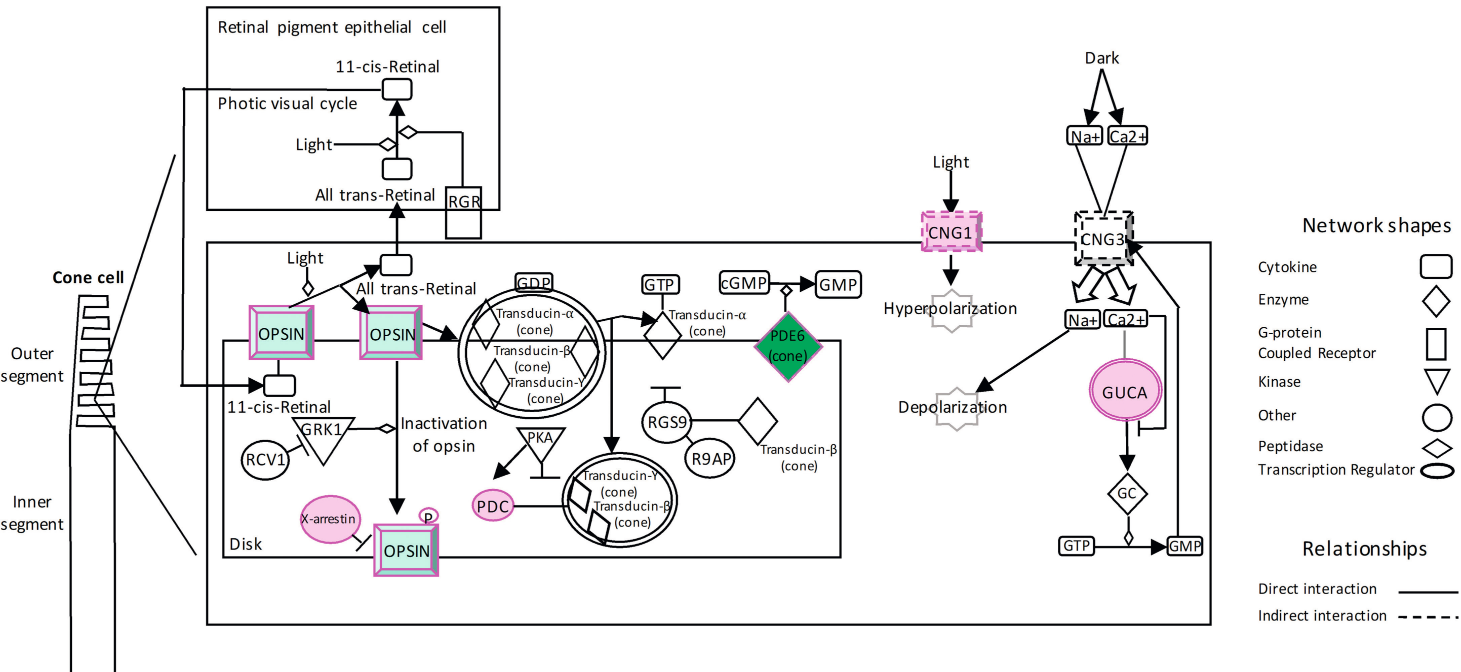


Figure 6. Ingenuity pathway analysis identified Phototransduction Pathway to be associated with differentially expressed genes at P3. The phototransduction pathways of rods and cones are shown respectively in a and b. Shapes of the network elements refer to the functional category of the gene product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

3

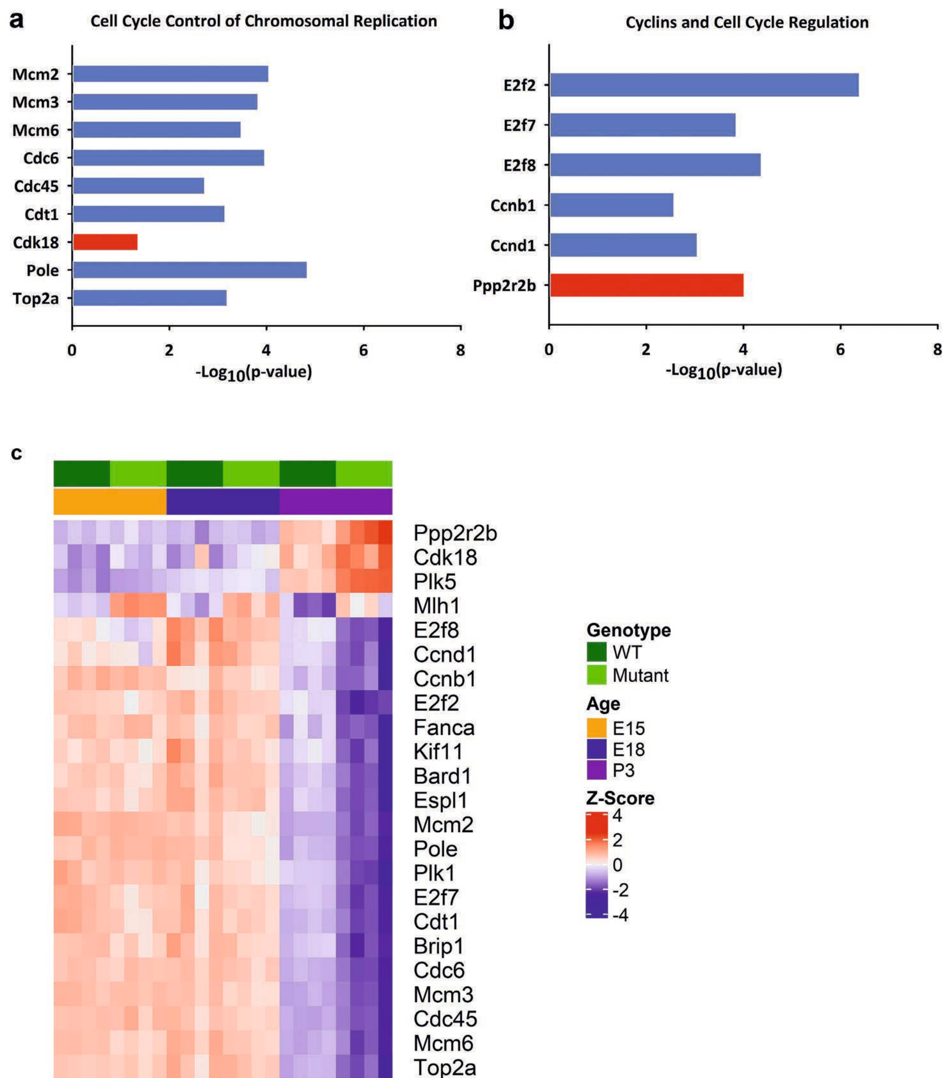


Figure 7. IPA analysis of DEG at P3 showed enrichment in the (a) Cell Cycle Control of Chromosomal Replication genes and (b) Cyclins and Cell Cycle Regulation genes pathways. Significance for the differential expression between mutant and WT eyes is shown as $-\text{Log}_{10}$ (Adj p-value) (x-axis). 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) Heatmap 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.

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. DNA replication and Cell cycle are the major GO terms among the downregulated genes in WT eyes between E18 and P3 (Supplementary Figure S3d) indicating repression of cell division at this period of development. However, downregulation of these genes appears even more prominent in the mutant eyes, based on the p values of these canonical pathways (Supplementary Figure S3d).

4. DISCUSSION

The circadian clock is a major regulator of physiology and behavior through the timed 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 (Bennis et al. 2017; Levine et al. 2004; Logan and McClung 2019; Zhao et al. 2018a; Zhao et al. 2018b; Jenson et al. 2012). Here we show that clock genes are expressed throughout eye development in mice from E13 onwards, with gene-specific, time-associated gene expression profiles. By comparing the eye transcriptome of WT and *Per1*$^{-/-}$ *Per2*^{Brdm1} mice at E15, E18 and P3, we show alteration in expression of clock factors from the regulatory feedback loops. At P3 specifically, mutant eyes display the most extensive alterations: major upregulation of phototransduction-related genes, together with reduction in cell cycle related transcripts. Our work provides 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.

We previously described that retina from *Per1*$^{-/-}$*Per2*^{Brdm1} mutant mice display altered photoreceptor differentiation with reduced blue cone numbers and opsin expression (Aït-Hmyed Hakkari et al. 2013). To reveal the underlying molecular mechanisms of this observation, here we used comparative transcriptome analysis of the developing whole eye, including (premature) retinal photoreceptors, in animals from the same mutant line. We found that visual and sensory perception of light stimulus are the major GO terms found enriched in the upregulated genes in WT eyes between E15 and P3 (Supplementary Figure S4, see also Figure S3a, c). This indicates that our analyses are relevant for identifying related changes in the mutants.

Several groups have reported association between clock gene mutation(s) and abnormal eye/retinal development (Baba et al. 2018; Aït-Hmyed Hakkari et al. 2013; Sawant et al. 2017; Aït-Hmyed Hakkari et al. 2016; Mollema et al. 2011; McQueen et al. 2018; Li et al. 2007) but it is not clear whether the observed defects are due to an abnormal clock

or to non-circadian function of the clock genes. Here we show robust changes in clock gene expression in the *Per1$^{-/-}$Per2^{Brdm1}* eyes, within the distinct arms of the regulatory loops, in all age groups. In addition, using RT-PCR and RNAseq, we provide evidence that the main clock genes are already expressed at E13, with extensive changes throughout development. Thus, our data suggests that some regulatory mechanisms driven by the clock are already at play at E13. Among these, the upregulation of *Per3* mRNA in mutant vs WT eyes is striking (FC > 1.9 at E15, > 1.8 at E18 and > 2.5 at P3): whether this results from relieving BMAL1/CLOCK repression by PER1/PER2 or from a compensatory developmental effect induced by *Per1/Per2* absence remains to be determined.

Adaptation of vision to the light/dark cycle has been proposed to involve the rhythmic expression of phototransduction components such as opsins (Stone et al. 2019; Bobu et al. 2013) and transducin (Stone et al. 2013), including their shuttling between the inner segment and the outer segment (Stone et al. 2013) and the post-translational modulation of ion channel sensitivity (Aldiri et al. 2017). Mechanisms underlying rhythmic transcription of genes from the phototransduction cascade are not yet understood. In zebrafish, key developmental, retina-specific transcription factors driving photoreceptor differentiation, such as NR2E3, are co-regulated by the circadian clock in the adult for the control of daily photoreceptor physiology (Pearring et al. 2013). In our study, the upregulation of phototransduction related genes (19.7% of the genes associated with this pathway) in *Per1$^{-/-}$Per2^{Brdm1}* mice supports the idea that an important part of the phototransduction component might also be the target of the clock in mammals.

It is presently unknown how the clock might control phototransduction genes in the mammalian retina. Ingenuity pathway analysis indicates NRL and CRX are the major regulators of the differentially expressed genes at P3. *Nr2e3*, which acts as a co-activator of rod-genes with NRL, CRX and REV-ERBα (NR1D1) (Ko 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 (Laranjeiro et al. 2014). *Nr2e3* mutations cause enhanced S-cone syndrome among humans (Corbo et al. 2010). 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 (Aït-Hmyed Hakkari et al. 2013). We believe that NR2E3-mediated regulation might be one of the causes of the changes in the phototransduction machinery observed in the mutant at P3. However, further studies are needed to determine the precise mechanisms involved. Another transcription factor gene displaying upregulation (FC>1.5) in the mutant eye at P3 is *Rorc*, which codes for the ROR gamma clock factor and is itself a target of *Nr2e3* during retinal development (Hao et al. 2012; Mollema et al. 2011). However, its function

in the retina is not known. It was previously shown 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 (Cheng et al. 2004). In the absence of *Rorb*, complete loss of rods and over-production of blue cones occurs during retinal development (Peng et al. 2005). It will be interesting to understand if the retinal architecture is disrupted in the absence/ mutation of *Rorc* as well.

Effects of clock alteration on blue cones has been previously described (Sawant et al. 2017). Namely, photoreceptor-specific loss of *Bmal1* induces extended distribution of the s-opsin expressing cones along the dorso-ventral axis and this was shown to involve alteration of 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, like that of m-opsin (Sawant et al. 2017). By contrast, we found a decrease in the expression levels of s-opsin at E18 and P3, which is retained in the adult (Aït-Hmyed Hakkari et al. 2013). This is like the phenotype induced by a photoreceptor-specific knock-out 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.

3

The link between circadian clock and cell cycle regulation has long been established (Cheng et al. 2006). However, there are only few studies of clock regulation, cell division and cellular differentiation together. The loss of *Bmal1* causes cell cycle arrest with an upregulation of *p21* and a block in the G1 phase in the hair follicles (Haider et al. 2000). The gene expression levels of *Wee1* (cell cycle checkpoint kinase) and *p21* (cyclin-dependent kinase inhibitor) are also regulated by *Bmal1* in the liver (Cheng et al. 2006; Haider et al. 2001). *Cry2* promotes the circadian regulation of myogenic differentiation and regeneration in the mouse (Haider et al. 2009) whereas *Rev-Erbα* (*Nr1d1*) acts as an inhibitor of the same processes (Fujieda et al. 2009). Also, *Clock* gene silencing induced spontaneous differentiation of mouse embryonic stem cells indicating an earlier exit from their pluripotent state (Jia et al. 2009). In our study, the cell cycle appears perturbed in the eyes from *Per1*$^{-/-}$*Per2*^{Brdm1} animals at P3 (Figure 7 and Supplementary Figure S3d), an age at which a substantial part of precursors for late-born retinal cell types are still dividing (reviewed in [Masri et al. 2013]). In general, cell-cycle specific markers have been strongly connected with retina development (Lin et al. 2009). E2f family transcription factors (Matsuo et al. 2003; Lowe et al. 2018) and cyclin D1 (Chatterjee et al. 2019; Lu et al. 2016; Dyer et al. 2001; Dagnino et al. 1997) are well known to regulate embryonic

aspects of retinal development (Vuong et al. 2012). 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. 2009a; Das et al. 2012b; Fantl et al. 1995; Ma et al. 1998): 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 (Baba et al. 2018). 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. We do not know how the absence of *Per1* and *Per2* shuts down the expression of cell cycle-related genes. Pathway analysis of the DEG in WT and mutant eyes indicates that Wnt and Hippo pathways are downregulated between E15 and E18 in WT but these pathways are absent or underrepresented in the mutant DEG at the same age (Supplementary Figure S3b). Moreover, The PI3K-AKT signaling pathway, which includes many growth factors and associated receptors, is significant among downregulated DEG in the *Per1*$^{-/-}$*Per2*^{Brdm1} eyes but absent in the WT (Supplementary Figure S3b). It might be that the combination of these enriched and underrepresented pathways between E15 and E18 in the mutant eyes drives 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 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
Conceptualization: Udita Bagchi, Marie-Paule Felder-Schmittbuhl, Arthur A. Bergen
Software: Aldo Jongejan, Perry D. Moerland
Validation: Udita Bagchi, Cristina Sandu, Marie-Paule Felder-Schmittbuhl
Formal analysis: Udita Bagchi, Aldo Jongejan, Perry D. Moerland
Investigation: Udita Bagchi, Shumet Gegnaw
Resources: Udita Bagchi, Cristina Sandu, Nemanja Milićević, Jacoline B. ten Brink

Data curation: Udita Bagchi, Shumet Gegnaw, Cristina Sandu, Aldo Jongejan
Writing – original draft: Udita Bagchi
Writing – review & editing: Udita Bagchi, Nemanja Milicevic, Perry D. Moerland, David Hicks, Marie-Paule Felder-Schmittbuhl, Arthur A. Bergen
Visualization: Udita Bagchi, Cristina Sandu, Aldo Jongejan
Supervision: Marie-Paule Felder-Schmittbuhl, Perry D. Moerland, Arthur A. Bergen
Project administration: Udita Bagchi, Cristina Sandu, Marie-Paule Felder-Schmittbuhl
Funding acquisition: Marie-Paule Felder-Schmittbuhl, Arthur A. Bergen

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.

Acknowledgements

The authors would like to thank Dr U. Albrecht (University of Fribourg, Switzerland) for providing the *Per1*$^{-/-}$*Per2*^{Brdm1} mice. This work was carried out in Academic Medical Center (AMC) Amsterdam, The Netherlands, in close cooperation with CNRS/University of Strasbourg, France. The authors confirm no conflict of interests in the execution of this work.

Funding

This study was part of Neurotime Erasmus Mundus Project number: 520124-1-2011-1-FR-ERA Mundus-EPJD, FPA: 2012-0026, supported by a generous grant from the European Union. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at –
https://doi.org/10.1016/j.bbagrm.2020.194623

REFERENCES

A. Bennis, J.B. Ten Brink, P.D. Moerland, V.M. Heine, A.A. Bergen, Comparative gene expression study and pathway analysis of the human iris- and the retinal pigment epithelium, PLoS One 12 (2017) e0182983.

A. Chaix, A. Zarrinpar, S. Panda, The circadian coordination of cell biology, J. Cell Biol. 215 (2016) 15–25.

A. Sumova, M. Sladek, L. Polidarova, M. Novakova, P. Houdek, Circadian system from conception till adulthood, Prog. Brain Res. 199 (2012) 83–103.

A.J. Trott, J.S. Menet, Regulation of circadian clock transcriptional output by CLOCK:BMAL1, PLoS Genet. 14 (2018) e1007156.

A.R. Barnard, S. Hattar, M.W. Hankins, R.J. Lucas, Melanopsin regulates visual processing in the mouse retina, Curr. Biol. 16 (2006) 389–395.

B. Zheng, D.W. Larkin, U. Albrecht, Z.S. Sun, M. Sage, G. Eichele, C.C. Lee, A. Bradley, The mPer2 gene encodes a functional component of the mammalian circadian clock, Nature 400 (1999) 169–173.

C. Bobu, C. Sandu, V. Laurent, M.P. Felder-Schmittbuhl, D. Hicks, Prolonged light exposure induces widespread phase shifting in the circadian clock and visual pigment gene expression of the Arvicanthis ansorgei retina, Mol. Vis. 19 (2013) 1060–1073.

C. Bobu, D. Hicks, Regulation of retinal photoreceptor phagocytosis in a diurnal mammal by circadian clocks and ambient lighting, Invest. Ophthalmol. Vis. Sci. 50 (2009) 3495–3502.

C. Lu, Y. Yang, R. Zhao, B. Hua, C. Xu, Z. Yan, N. Sun, R. Qian, Role of circadian gene clock during differentiation of mouse pluripotent stem cells, Prot. Cell 7 (2016) 820–832.

C. Ma, D. Papermaster, C.L. Cepko, A unique pattern of photoreceptor degeneration in cyclin D1 mutant mice, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 9938–9943.

C.L. Cepko, C.P. Austin, X. Yang, M. Alexiades, D. Ezzeddine, Cell fate determination in the vertebrate retina, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 589–595. L.D. Carter-Dawson, M.M. LaVail, Rods and cones in the mouse retina. II. Autoradiographic analysis of cell generation using tritiated thymidine, J. Comp. Neurol. 188 (1979) 263–272.

C.M. McQueen, E.E. Schmitt, T.R. Sarkar, J. Elswood, R.P. Metz, D. Earnest, M. Rijnkels, W.W. Porter, PER2 regulation of mammary gland development, Development 145 (2018) dev157966.

C.Sandu, D.Hicks, M.P.Felder-Schmittbuhl, Rat photoreceptor circadian oscillator strongly relies on lighting conditions, Eur. J. Neurosci. 34 (2011) 507–516.

C.W. Law, Y. Chen, W. Shi, G.K. Smyth, voom: precision weights unlock linear model analysis tools for RNA-seq read counts, Genome Biol. 15 (2014) R29. S.M.-I. Consortium, A comprehensive assessment of RNA-seq accuracy, reproducibility and information content by the Sequencing Quality Control Consortium, Nat. Biotechnol. 32 (2014) 903–914.

D. Kim, B. Langmead, S.L. Salzberg, HISAT: a fast spliced aligner with low memory requirements, Nat. Methods 12 (2015) 357–360.

D.C. Lozano, A.T. Hartwick, M.D. Twa, Circadian rhythm of intraocular pressure in the adult rat, Chronobiol. Int. 32 (2015) 513–523.

D.G. McMahon, P.M. Iuvone, G. Tosini, Circadian organization of the mammalian retina: from gene regulation to physiology and diseases, Prog. Retin. Eye Res. 39 (2014) 58–76.

D.T. Organisciak, R.M. Darrow, L. Barsalou, R.K. Kutty, B. Wiggert, Circadian-dependent retinal light damage in rats, Invest. Ophthalmol. Vis. Sci. 41 (2000) 3694–3701.

E.M. Levine, E.S. Green, Cell-intrinsic regulators of proliferation in vertebrate retinal progenitors, Semin. Cell Dev. Biol. 15 (2004) 63–74.

G. Das, A.M. Clark, E.M. Levine, Cyclin D1 inactivation extends proliferation and alters histogenesis in the postnatal mouse retina, Dev. Dyn. 241 (2012) 941–952.

G. Das, Y. Choi, P. Sicinski, E.M. Levine, Cyclin D1 fine-tunes the neurogenic output of embryonic retinal progenitor cells, Neural Dev. 4 (2009) 15.

G. Tosini, M. Menaker, Circadian rhythms in cultured mammalian retina, Science 272 (1996) 419–421.

G.H. Peng, O. Ahmad, F. Ahmad, J. Liu, S. Chen, The photoreceptor-specific nuclear receptor Nr2e3 interacts with Crx and exerts opposing effects on the transcription of rod versus cone genes, Hum. Mol. Genet. 14 (2005) 747–764.

G.Y. Ko, M.L. Ko, S.E. Dryer, Circadian regulation of cGMP-gated channels of vertebrate cone photoreceptors: role of cAMP and Ras, J. Neurosci. 24 (2004) 1296–1304.

H. Adachi, H. Tominaga, Y. Maruyama, K. Yoneda, K. Maruyama, K. Yoshii,

S. Kinoshita, M. Nakano, K. Tashiro, Stage-specific reference genes significant for quantitative PCR during mouse retinal development, Genes Cells 20 (2015) 625–635.

H. Cheng, H. Khanna, E.C. Oh, D. Hicks, K.P. Mitton, A. Swaroop, Photoreceptor-specific nuclear receptor NR2E3 functions as a transcriptional activator in rod photoreceptors, Hum. Mol. Genet. 13 (2004) 1563–1575.

H. Cheng, T.S. Aleman, A.V. Cideciyan, R. Khanna, S.G. Jacobson, A. Swaroop, In vivo function of the orphan nuclear receptor NR2E3 in establishing photoreceptor identity during mammalian retinal development, Hum. Mol. Genet. 15 (2006) 2588–2602.

H. Fujieda, R. Bremner, A.J. Mears, H. Sasaki, Retinoic acid receptor-related orphan receptor alpha regulates a subset of cone genes during mouse retinal development, J. Neurochem. 108 (2009) 91–101.

H. Hao, D.S. Kim, B. Klocke, K.R. Johnson, K. Cui, N. Gotoh, C. Zang, J. Gregorski, L. Gieser, W. Peng, Y. Fann, M. Seifert, K. Zhao, A. Swaroop, Transcriptional regulation of rod photoreceptor homeostasis revealed by in vivo NRL targetome analysis, PLoS Genet. 8 (2012) e1002649.

H.C. Marie-Paule Felder-Schmittbuhl, Ouria Dkhissi-Benyahya, The retinal clock in mammals: role in health and disease, ChronoPhysiol. Ther. 7 (2017) 33–45.

H.S.Lee, J.L.Nelms, M.Nguyen, R.Silver, M.N.Lehman, The eye is necessary for a circadian rhythm in the suprachiasmatic nucleus, Nat. Neurosci. 6 (2003) 111–112.

I. Aldiri, B. Xu, L. Wang, X. Chen, D. Hiler, L. Griffiths, M. Valentine, A. Shirinifard, S. Thiagarajan, A. Sablauer, M.E. Barabas, J. Zhang, D. Johnson, S. Frase, X. Zhou, J. Easton, J. Zhang, E.R. Mardis, R.K. Wilson, J.R. Downing, M.A. Dyer,P. St. Jude Children's Research Hospital-Washington University Pediatric Cancer Genome, The dynamic epigenetic landscape of the retina during development, reprogramming, and tumorigenesis, Neuron 94 (2017) 550–568 (e510).

J. Hellemans, G. Mortier, A. De Paepe, F. Speleman, J. Vandesompele, qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data, Genome Biol. 8 (2007) R19.

J. Zhao, X. Zhou, Q. Tang, R. Yu, S. Yu, Y. Long, C. Cao, J. Han, A. Shi, J.J. Mao, X. Chen, L. Chen, BMAL1 deficiency contributes to mandibular dysplasia by upregulating MMP3, Stem Cell Rep. 10 (2018) 180–195.

J.A.Mohawk, C.B.Green, J.S.Takahashi, Central and peripheral circadian clocks in mammals, Annu. Rev. Neurosci. 35 (2012) 445–462.

J.C. Corbo, K.A. Lawrence, M. Karlstetter, C.A. Myers, M. Abdelaziz, W. Dirkes, K. Weigelt, M. Seifert, V. Benes, L.G. Fritsche, B.H. Weber, T. Langmann, CRX ChIP-seq reveals the cis-regulatory architecture of mouse photoreceptors, Genome Res. 20 (2010) 1512–1525.

J.C.Y.Wong,N.J.Smyllie,G.T.Banks,C.A.Pothecary,A.R.Barnard,E.S.Maywood, A. Jagannath, S. Hughes, G.T.J. van der Horst, R.E. MacLaren, M.W. Hankins, M.H. Hastings, P.M. Nolan, R.G. Foster, S.N. Peirson, Differential roles for cryptochromes in the mammalian retinal clock, FASEB J. 32 (2018) 4302–4314.

J.N. Pearring, R.Y. Salinas, S.A. Baker, V.Y. Arshavsky, Protein sorting, targeting and trafficking in photoreceptor cells, Prog. Retin. Eye Res. 36 (2013) 24–51.

J.R. Beytebiere, B.J. Greenwell, A. Sahasrabudhe, J.S. Menet, Clock-controlled rhythmic transcription: is the clock enough and how does it work? Transcription 10 (2019) 212–221.

J.S.Takahashi, Transcriptional architecture of the mammalian circadian clock, Nat. Rev. Genet. 18 (2017) 164–179.

K. Baba, I. Piano, P. Lyuboslavsky, M.A. Chrenek, J.T. Sellers, S. Zhang, C. Gargini, L. He, G. Tosini, P.M. Iuvone, Removal of clock gene Bmal1 from the retina affects retinal development and accelerates cone photoreceptor degeneration during aging, Proc. Natl. Acad. Sci. U. S. A. 115 (2018) 13099–13104.

K.F.Storch,C.Paz,J.Signorovitch,E.Raviola,B.Pawlyk,T.Li,C.J.Weitz,Intrinsic circadian clock of the mammalian retina: importance for retinal processing of visual information, Cell 130 (2007) 730–741.

K.K. Lin, V. Kumar, M. Geyfman, D. Chudova, A.T. Ihler, P. Smyth, R. Paus,

J.S. Takahashi, B. Andersen, Circadian clock genes contribute to the regulation of hair follicle cycling, PLoS Genet. 5 (2009) e1000573.

L. Dagnino, C.J. Fry, S.M. Bartley, P. Farnham, B.L. Gallie, R.A. Phillips, Expression patterns of the E2F family of transcription factors during mouse nervous system development, Mech. Dev. 66 (1997) 13–25.

L. Jia, E.C. Oh, L. Ng, M. Srinivas, M. Brooks, A. Swaroop, D. Forrest, Retinoid-related orphan nuclear receptor ROR beta is an early-acting factor in rod photoreceptor development, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 17534–17539.

L. Vuong, D.E. Brobst, A. Saadi, I. Ivanovic, M.R. Al-Ubaidi, Pattern of expression of p53, its family members, and regulators during early ocular development and in the post-mitotic retina, Invest. Ophthalmol. Vis. Sci. 53 (2012) 4821–4831.

L.D. Jensen, Z. Cao, M. Nakamura, Y. Yang, L. Brautigam, P. Andersson, Y. Zhang, E. Wahlberg, T. Lanne, K. Hosaka, Y. Cao, Opposing effects of circadian clock genes bmal1 and period2 in regulation of VEGF-dependent angiogenesis in developing zebrafish, Cell Rep. 2 (2012) 231–241.

L.S. Mure, H.D. Le, G. Benegiamo, M.W. Chang, L. Rios, N. Jillani, M. Ngotho,

T. Kariuki, O. Dkhissi-Benyahya, H.M. Cooper, S. Panda, Diurnal transcriptome atlas of a primate across major neural and peripheral tissues, Science 359 (2018) eaao0318.

M. Lowe, J. Lage, E. Paatela, D. Munson, R. Hostager, C. Yuan, N. Katoku-Kikyo, M. Ruiz-Estevez, Y. Asakura, J. Staats, M. Qahar, M. Lohman, A. Asakura, N. Kikyo, Cry2 is critical for circadian regulation of myogenic differentiation by Bclaf1- mediated mRNA stabilization of cyclin D1 and Tmem176b, Cell Rep. 22 (2018) 2118–2132.

M. Mirmiran, L. Bernardo, S.L. Jenkins, X.H. Ma, J.T. Brenna, P.W. Nathanielsz, Growth, neurobehavioral and circadian rhythm development in newborn baboons, Pediatr. Res. 49 (2001) 673–677.

M. von Schantz, R.J. Lucas, R.G. Foster, Circadian oscillation of photopigment transcript levels in the mouse retina, Brain Res. Mol. Brain Res. 72 (1999) 108–114.

M.A. Cameron, A.R. Barnard, R.J. Lucas, The electroretinogram as a method for studying circadian rhythms in the mammalian retina, J. Genet. 87 (2008) 459–466.

M.A. Dyer, C.L. Cepko, Regulating proliferation during retinal development, Nat. Rev. Neurosci. 2 (2001) 333–342.

M.D. Robinson, A. Oshlack, A scaling normalization method for differential expression analysis of RNA-seq data, Genome Biol. 11 (2010) R25.

M.D. Robinson, D.J. McCarthy, G.K. Smyth, edgeR: a bioconductor package for differential expression analysis of digital gene expression data, Bioinformatics 26 (2010) 139–140.

M.E. Ritchie, B. Phipson, D. Wu, Y. Hu, C.W. Law, W. Shi, G.K. Smyth, limma powers differential expression analyses for RNA-sequencing and microarray studies, Nucleic Acids Res. 43 (2015) e47.

M.M.LaVail, Circadian nature of rod outer segment disc shedding in the rat, Invest.Ophthalmol. Vis. Sci. 19 (1980) 407–411.

M.P. Felder-Schmittbuhl, E.D. Buhr, O. Dkhissi-Benyahya, D. Hicks, S.N. Peirson, C.P. Ribelayga, C. Sandu, R. Spessert, G. Tosini, Ocular clocks: adapting mechanisms for eye functions and health, Invest. Ophthalmol. Vis. Sci. 59 (2018) 4856–4870.

N.B. Haider, J.K. Naggert, P.M. Nishina, Excess cone cell proliferation due to lack of a functional NR2E3 causes retinal dysplasia and degeneration in rd7/rd7 mice, Hum. Mol. Genet. 10 (2001) 1619–1626.

N.B. Haider, N. Mollema, M. Gaule, Y. Yuan, A.J. Sachs, A.M. Nystuen, J.K. Naggert, P.M. Nishina, Nr2e3-directed transcriptional regulation of genes involved in photoreceptor development and cell-type specific phototransduction, Exp. Eye Res. 89 (2009) 365–372.

N.B. Haider, S.G. Jacobson, A.V. Cideciyan, R. Swiderski, L.M. Streb, C. Searby, G. Beck, R. Hockey, D.B. Hanna, S. Gorman, D. Duhl, R. Carmi, J. Bennett,

R.G. Weleber, G.A. Fishman, A.F. Wright, E.M. Stone, V.C. Sheffield, Mutation of a nuclear receptor gene, NR2E3, causes enhanced S cone syndrome, a disorder of retinal cell fate, Nat. Genet. 24 (2000) 127–131.

N.J. Mollema, Y. Yuan, A.S. Jelcick, A.J. Sachs, D. von Alpen, D. Schorderet,

P. Escher, N.B. Haider, Nuclear receptor Rev-erb alpha (Nr1d1) functions in concert with Nr2e3 to regulate transcriptional networks in the retina, PLoS One 6 (2011) e17494.

O. Ait-Hmyed Hakkari, N. Acar, E. Savier, P. Spinnhirny, M. Bennis, M.P. Felder- Schmittbuhl, J. Mendoza, D. Hicks, Rev-Erbalpha modulates retinal visual processing and behavioral responses to light, FASEB J. 30 (2016) 3690–3701.

O. Ait-Hmyed, M.P. Felder-Schmittbuhl, M. Garcia-Garrido, S. Beck, C. Seide, V. Sothilingam, N. Tanimoto, M. Seeliger, M. Bennis, D. Hicks, Mice lacking Period 1 and Period 2 circadian clock genes exhibit blue cone photoreceptor defects, Eur. J. Neurosci. 37 (2013) 1048–1060.

O.B. Sawant, V.K. Jidigam, R.D. Fuller, O.F. Zucaro, C. Kpegba, M. Yu,

N.S. Peachey, S. Rao, The circadian clock gene Bmal1 is required to control the timing of retinal neurogenesis and lamination of Muller glia in the mouse retina, FASEB J 33 (2019) 8745–8758.

O.B.Sawant, A.M.Horton, O.F.Zucaro, R.Chan, V.L.Bonilha, I.S.Samuels, S.Rao, The circadian clock gene Bmal1 controls thyroid hormone-mediated spectral identity and cone photoreceptor function, Cell Rep. 21 (2017) 692–706.

R. Laranjeiro, D. Whitmore, Transcription factors involved in retinogenesis are co-opted by the circadian clock following photoreceptor differentiation, Development 141 (2014) 2644–2656.

R.A. Stone, A.M. McGlinn, R. Chakraborty, D.C. Lee, V. Yang, A. Elmasri, E. Landis, J. Shaffer, P.M. Iuvone, X. Zheng, A. Sehgal, M.T. Pardue, Altered ocular parameters from circadian clock gene disruptions, PLoS One 14 (2019) e0217111.

R.A. Stone, M.T. Pardue, P.M. Iuvone, T.S. Khurana, Pharmacology of myopia and potential role for intrinsic retinal circadian rhythms, Exp. Eye Res. 114 (2013) 35–47.

R.W. Logan, C.A. McClung, Rhythms of life: circadian disruption and brain disorders across the lifespan, Nat. Rev. Neurosci. 20 (2019) 49–65.

R.Zhang, N.F.Lahens, H.I.Ballance, M.E.Hughes, J.B.Hogenesch, A circadian gene expression atlas in mammals: implications for biology and medicine, Proc. Natl. Acad. Sci. U. S. A. 111 (2014) 16219–16224.

S. Anders, P.T. Pyl, W. Huber, HTSeq—a Python framework to work with high-throughput sequencing data, Bioinformatics 31 (2015) 166–169.

S. Chatterjee, H. Yin, W. Li, J. Lee, V.K. Yechoor, K. Ma, The nuclear receptor and clock repressor rev-erb alpha suppresses myogenesis, Sci. Rep. 9 (2019) 4585.

S. Honma, Development of the mammalian circadian clock, Eur. J. Neurosci. 51 (2020) 182–193.

S. Masri, M. Cervantes, P. Sassone-Corsi, The circadian clock and cell cycle: inter-connected biological circuits, Curr. Opin. Cell Biol. 25 (2013) 730–734.

S. Panda, M.P. Antoch, B.H. Miller, A.I. Su, A.B. Schook, M. Straume, P.G. Schultz, S.A. Kay, J.S. Takahashi, J.B. Hogenesch, Coordinated transcription of key path- ways in the mouse by the circadian clock, Cell 109 (2002) 307–320.

S. Sayols, D. Scherzinger, H. Klein, dupRadar: a Bioconductor package for the assessment of PCR artifacts in RNA-Seq data, BMC Bioinformatics 17 (2016) 428. A.M. Bolger, M. Lohse, B. Usadel, Trimmomatic: a flexible trimmer for Illumina sequence data, Bioinformatics 30 (2014) 2114–2120.

S. Tsuchiya, E.D. Buhr, T. Higashide, K. Sugiyama, R.N. Van Gelder, Light entrainment of the murine intraocular pressure circadian rhythm utilizes non-local mechanisms, PLoS One 12 (2017) e0184790.

S.H.Yoo, S.Yamazaki, P.L.Lowrey, K.Shimomura, C.H.Ko, E.D.Buhr, S.M.Siepka, H.K. Hong, W.J. Oh, O.J. Yoo, M. Menaker, J.S. Takahashi, PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 5339–5346.

S.M. Reppert, D.R. Weaver, Coordination of circadian timing in mammals, Nature 418 (2002) 935–941.

S.Yamazaki, V.Alones, M.Menaker, Interaction of the retina with suprachiasmatic pacemakers in the control of circadian behavior, J. Biol. Rhythm. 17 (2002) 315–329.

T. Matsuo, S. Yamaguchi, S. Mitsui, A. Emi, F. Shimoda, H. Okamura, Control mechanism of the circadian clock for timing of cell division in vivo, Science 302 (2003) 255–259.

V. Fantl, G. Stamp, A. Andrews, I. Rosewell, C. Dickson, Mice lacking cyclin D1 are small and show defects in eye and mammary gland development, Genes Dev. 9 (1995) 2364–2372.

X. Liu, Z. Zhang, C.P. Ribelayga, Heterogeneous expression of the core circadian clock proteins among neuronal cell types in mouse retina, PLoS One 7 (2012) e50602.

X. Zhou, R. Yu, Y. Long, J. Zhao, S. Yu, Q. Tang, L. Chen, BMAL1 deficiency promotes skeletal mandibular hypoplasia via OPG downregulation, Cell Prolif. 51 (2018) e12470.

Y. Kikkawa, Diurnal variation in corneal thickness, Exp. Eye Res. 15 (1973) 1–9.

Y. Xue, P. Liu, H. Wang, C. Xiao, C. Lin, J. Liu, D. Dong, T. Fu, Y. Yang, Z. Wang, H. Pan, J. Chen, Y. Li, D. Cai, Z. Li, Modulation of circadian rhythms affects corneal epithelium renewal and repair in mice, Invest. Ophthalmol. Vis. Sci. 58 (2017) 1865–1874.

Y.Kobayashi, Z.Ye, T.K.Hensch, Clock genes control cortical critical period timing, Neuron 86 (2015) 264–275.

Z. Li, L. Ruan, S. Lin, G.K. Gittes, Clock controls timing of mouse pancreatic differentiation through regulation of Wnt- and Notch-based and cell division components, Biochem. Biophys. Res. Commun. 359 (2007) 491–496.

Zheng, U. Albrecht, K. Kaasik, M. Sage, W. Lu, S. Vaishnav, Q. Li, Z.S. Sun, G. Eichele, A. Bradley, C.C. Lee, Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock, Cell 105 (2001) 683–694.

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

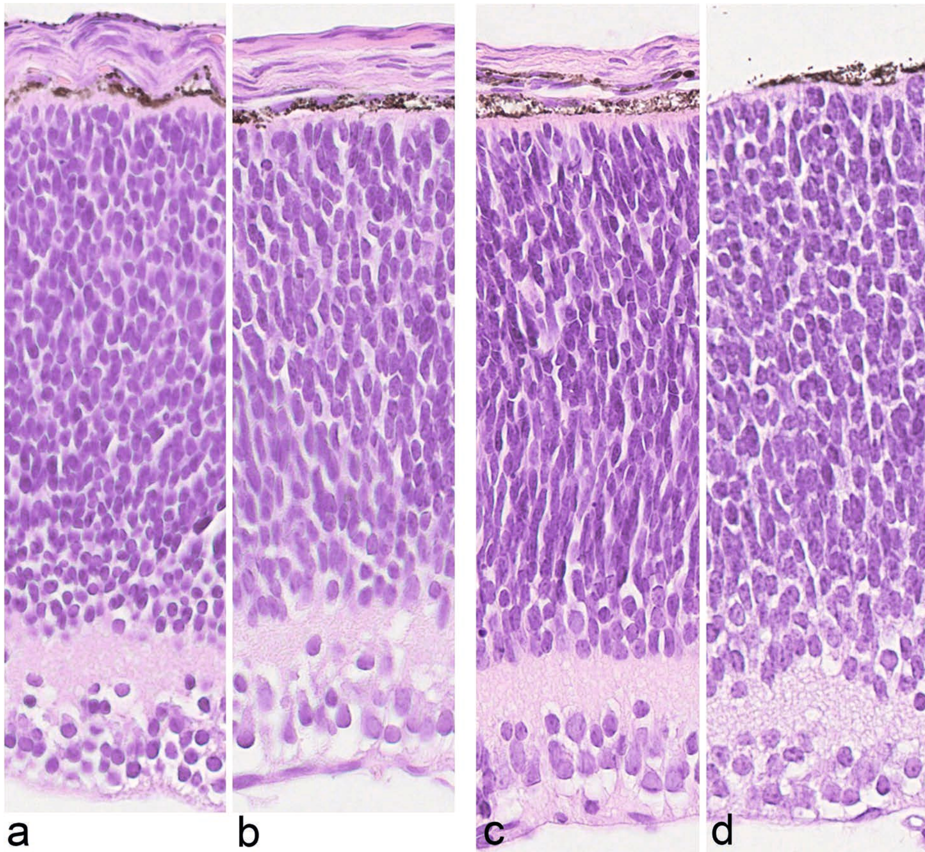


Figure S1-Photomicrographs of retinal sections from WT (a, c) and mutant (b, d) eyes at P0 (a, b) and P3 (c, d) showing no major structural differences between genotypes at these developmental ages.

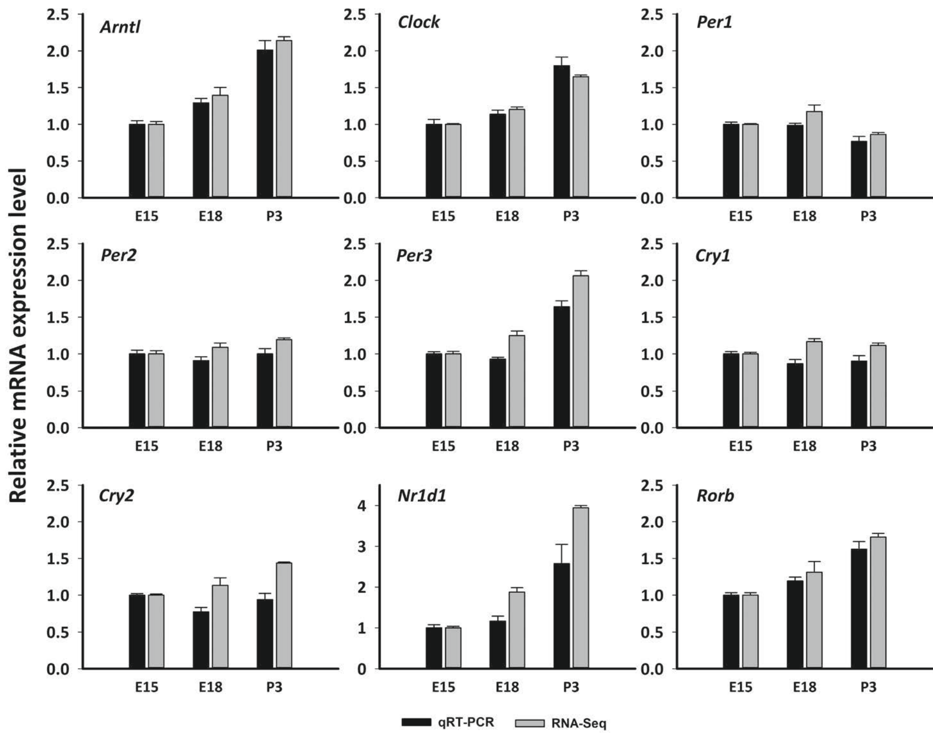
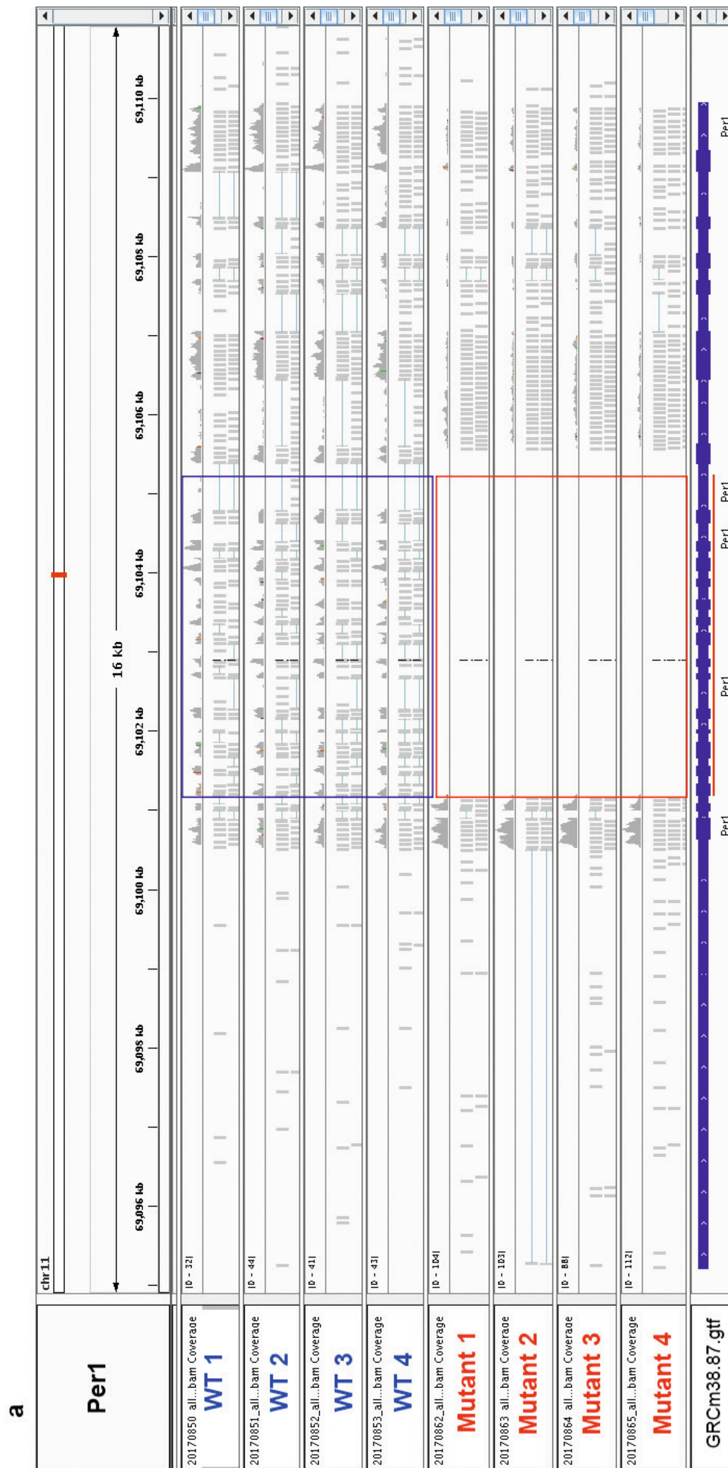


Figure S2-Comparative mRNA expression profiles of circadian clock genes in the WT whole eyes across eye developmental time points (n=3-4/age) of qRT-PCR versus RNA-seq experiments. Bar graphs show mean ±SEM mRNA expression levels of *Arntl* (*Bmal1*), *Clock*, *Per1*, *Per2*, *Per3*, *Cry1*, *Cry2*, *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
WT 1
WT 2
WT 3
WT 4
Mutant 1
Mutant 2
Mutant 3
Mutant 4
GRCm38.87.gtf
Per1

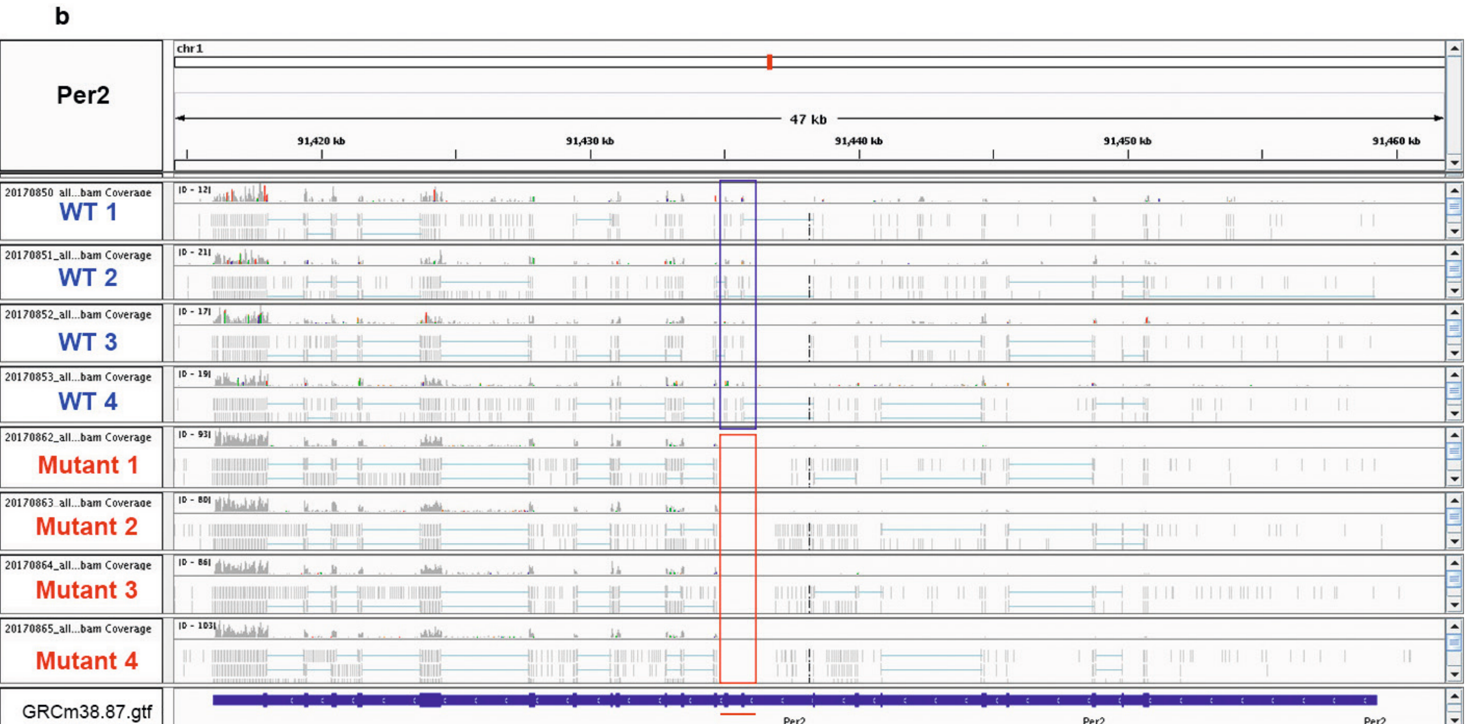


Figure S3-IGV images depicting RNA sequencing reads as obtained for wild type (WT) and mutant mice for both the (a) *Per1* and (b) *Per2* gene at developmental timepoint E15. Per sample tracks with the read coverage (top) and the first few RNA Seq reads are shown (bottom). Each rectangle (grey by default) 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.

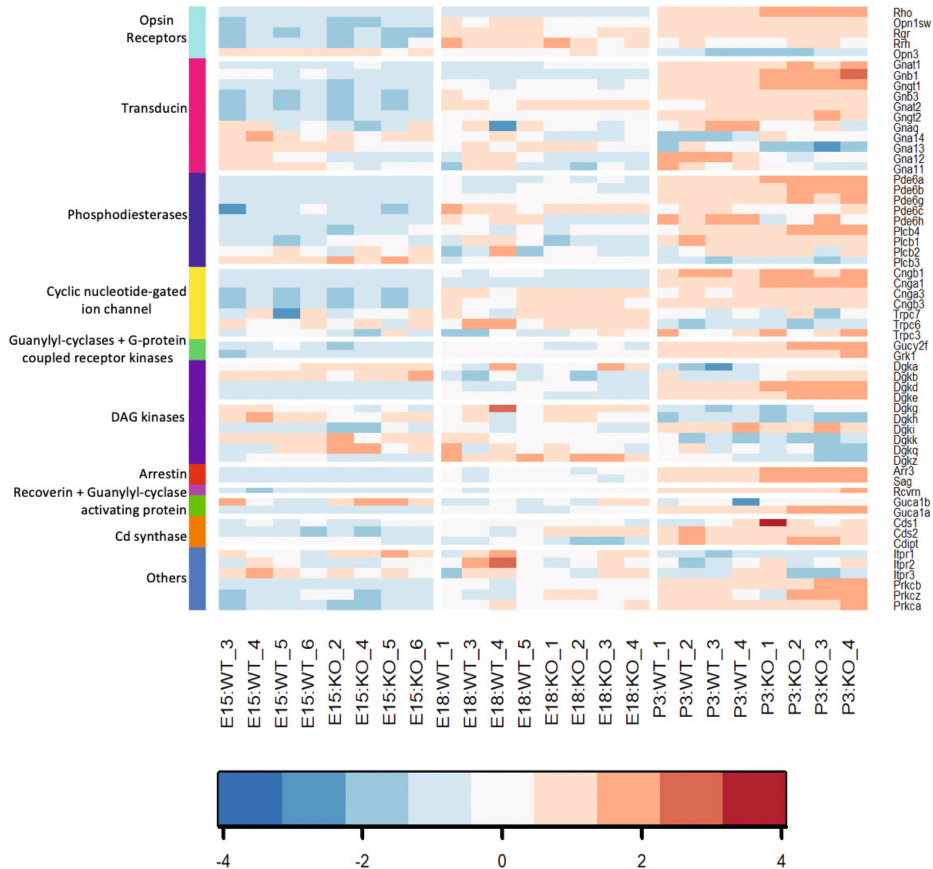


Figure S4-Heatmap of phototransduction genes during embryonic (E15 and E18) and postnatal (P3) ages of *Per1*$^{-/-}$*Per2*^{Brdm1} mutants versus WT. Genes were clustered using Euclidean distance and complete linkage. Color-coding corresponds to z- score of logCPM values from blue (lowest expression) 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.

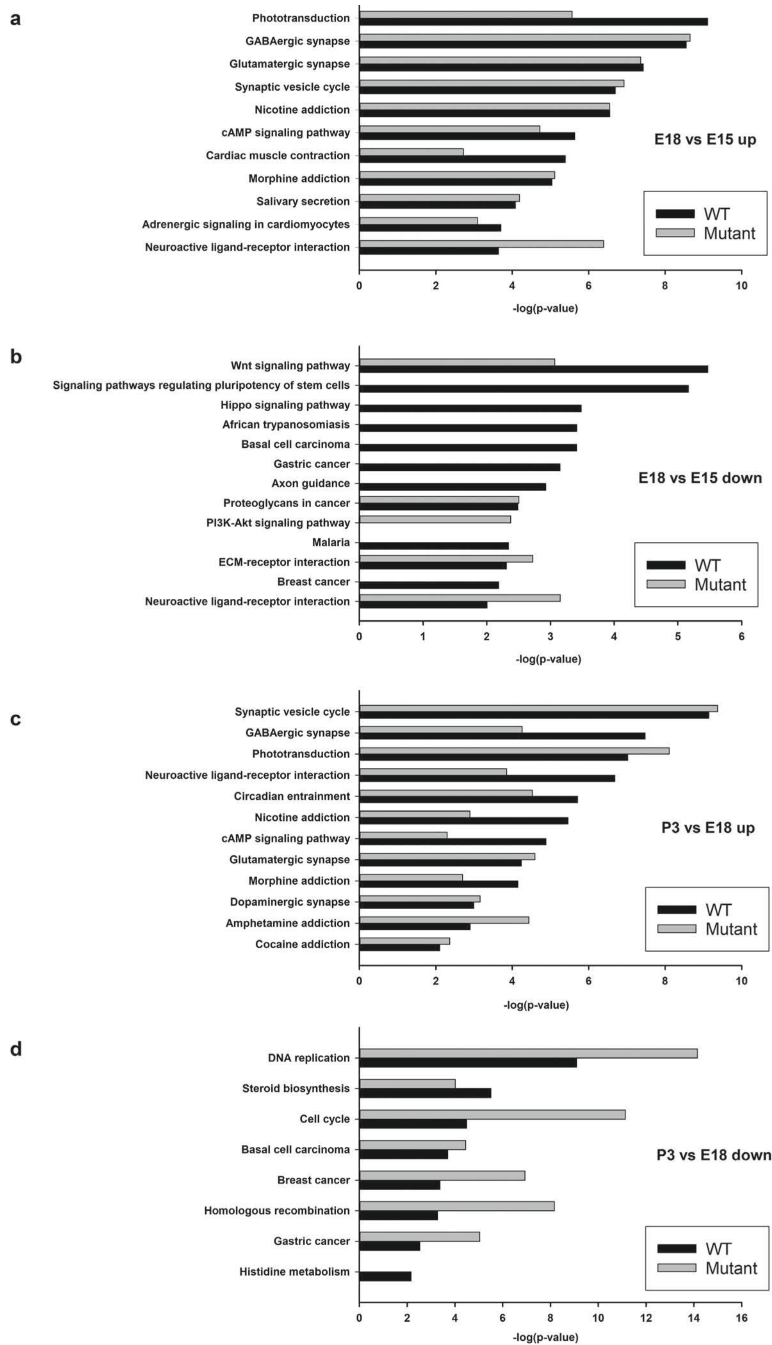


Figure S5-Canonical pathway analysis of DEG in WT and *Per1$^{-/-}$Per2^{Brdm1}* eyes between E15 and E18 (a and b for DEG which are respectively up or down regulated) and between E18 and P3 (c and d for DEG which are respectively up or down regulated). Bars (black for WT,grey for mutant) represent the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

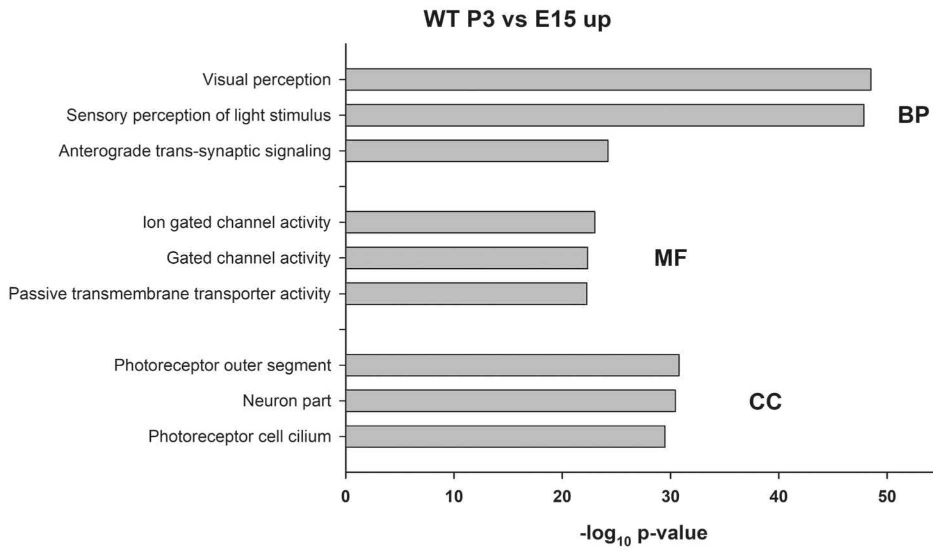


Figure S6-Canonical pathway analysis of upregulated DEGs between E15 and P3 in WT. Bars represent the -log10 adjusted p-value (x-axis). BP stands for Biological Process, MF for Molecular Function and CC for Cellular Component.

CHAPTER

Nemanja Milićević[*]
Ouafa Ait-Hmyed Hakkari[*]
Udita Bagchi
Cristina Sandu
Aldo Jongejan
Perry D. Moerland
Jacoline B. ten Brink
David Hicks
Arthur A. Bergen
Marie-Paule Felder-Schmittbuhl

[*]These authors contributed equally to this review

The FASEB Journal. 2021;35:e21722

Core Circadian Clock Genes *Per1* and *Per2* regulate the Rhythm in Photoreceptor Outer Segment Phagocytosis

ABSTRACT

Retinal photoreceptors undergo daily renewal of their distal outer segments, a process indispensable for maintaining retinal health. Photoreceptor Outer Segment (POS) phagocytosis occurs as a daily peak, roughly about one hour after light onset. However, the underlying cellular and molecular mechanisms which initiate this process are still unknown. Here we show that, under constant darkness, mice deficient for core circadian clock genes (*Per1* and *Per2*), lack a daily peak in POS phagocytosis. By qPCR analysis we found that core clock genes were rhythmic over 24h in both WT and *Per1, Per2* double mutant whole retinas. More precise transcriptomics analysis of laser capture microdissected WT photoreceptors revealed no differentially expressed genes between time-points preceding and during the peak of POS phagocytosis. By contrast, we found that microdissected WT retinal pigment epithelium (RPE) had a number of genes that were differentially expressed at the peak phagocytic time-point compared to adjacent ones. We also found a number of differentially expressed genes in *Per1*, *Per2* double mutant RPE compared to WT ones at the peak phagocytic time-point. Finally, based on STRING analysis we found a group of interacting genes which potentially drive POS phagocytosis in the RPE. This potential pathway consists of genes such as: *Pacsin1*, *Syp*, *Camk2b* and *Camk2d* among others. Our findings indicate that *Per1* and *Per2* are necessary clock components for driving POS phagocytosis and suggest that this process is transcriptionally driven by the RPE.

Keywords: photoreceptor, retinal pigment epithelium, circadian rhythm, phagocytosis, clock gene, photoreceptor outer segment

Abbreviations

Bmal1	Brain and Muscle ARNT-Like 1
BP	biological process
CPM	counts per million
Cry	Cryptochrome
DD	constant darkness
DEGs	differentially expressed genes
FDR	false discovery rate
Hprt	Hypoxanthine Phosphoribosyltransferase
KEGG	Kyoto encyclopedia of genes and genomes
LCM	laser capture microdissection
LD	light-dark cycle
MF	molecular function
Per	Period
POS	photoreceptor outer segment
ROS	rod outer segment
Ror	RAR-related orphan receptor
RPE	retinal pigment epithelium
SCN	suprachiasmatic nucleus
Tbp	TATA-Box binding Protein
WP	WikiPathways
WT	wild type
ZT	Zeitgeber time

INTRODUCTION

Light/dark transitions are one of the hallmarks of life on Earth. Living organisms adapt their behavior and physiology according to cyclic changes in environmental conditions. In mammals, these rhythmic adjustments in molecular and cellular physiology are enabled through a hierarchical network of oscillators, encompassing a “central clock” located in the suprachiasmatic nucleus (SCN) in the brain and peripheral oscillators (Stratmann & Schibler, 2006). The core molecular components generating these oscillations are comprised of interlocking transcriptional-translational feedback loops involving “clock” transcription factors such as PER1-2, CLOCK, BMAL1, CRY1-2, REV-ERBs and RORs (Ko & Takahashi, 2006). These factors drive rhythmic expression of “clock-controlled genes” thereby enabling rhythmic adaptations in physiology.

The retina stands out as a peripheral oscillator as it lies in direct contact with the main environmental synchronizing stimulus – light (Terman, Reme et al., 1993). This light-sensitive organ is composed of multiple layers of cells, all of which were shown to oscillate in a layer-specific manner and are strongly coupled (Jaeger, Sandu et al., 2015). Numerous aspects of retinal physiology and functions were shown to be rhythmic (McMahon, Iuvone et al., 2014) such as melatonin release (Besharse & Iuvone, 1983, Tosini & Menaker, 1996), rod-cone coupling (Ribelayga, Cao et al., 2008, Ribelayga & Mangel, 2010), visual sensitivity (Barnard, Hattar et al., 2006, Bassi & Powers, 1986) and photoreceptor disc shedding (LaVail, 1976). Of all retinal cells, circadian oscillations in photoreceptors have been most extensively studied (reviewed by (Ko, 2018)).

Retinal photoreceptors are specialized, light-sensitive neuronal cells. They are metabolically highly active cells in which homeostasis is tightly controlled. They consist of a cell body, a specialized synapse, inner and outer segments. Together with the adjacent retinal pigment epithelium (RPE), the POS contain the molecular machinery that sustains phototransduction. Excessive light exposure can damage these cells. A mechanism that prevents the accumulation of photo-oxidative compounds is rapid POS renewal (Kevany & Palczewski, 2010). This turnover involves several critical steps. At the proximal POS end, these steps include synthesis and intracellular transport of structural and functional proteins. At the distal end, POS fragments are shed and subsequently phagocytosed by the RPE. Impairment of phagocytosis was previously implicated in photoreceptor degeneration in both animal models (D’Cruz, Yasumura et al., 2000) and humans (Gal, Li et al., 2000). Despite many studies devoted to the subject, the molecular mechanisms that control POS phagocytosis remain elusive (Mazzoni, Safa et al., 2014, McMahon et al., 2014, Müller & Finnemann, 2020). Phagocytosis of POS was shown to be highly cyclic, taking place in rods as a daily peak occurring about one hour after light is turned on in

both nocturnal and diurnal mammals (Bobu, Craft et al., 2006, Krigel, Felder-Schmittbuhl et al., 2010, LaVail, 1976). This peak is maintained under constant darkness, implicating circadian control. However, little is known about the transcriptional events that occur prior and during the peak of POS phagocytosis.

In the present study, we tested the hypothesis that *Per1* and *Per2* are necessary clock components for initiating the phagocytosis of rod outer segment in mice. We investigated the transcriptional changes that occur in the RPE and photoreceptors prior and during the peak in POS phagocytosis. Finally, we proposed a potential pathway for initiating POS phagocytosis based on our transcriptomics data obtained from multiple time-points, purest possible microdissected sample material and phagocytically arrhythmic *Per1, Per2* mouse double knockout model.

METHODS

Animals

Experiments were conducted using homozygote double mutant mice carrying the loss-of-function mutation of *mPer1* gene ($Per1^{-/-}$; (Zheng, Albrecht et al., 2001)) and mutation of the *mPer2* gene ($Per2^{Brdm1}$, (Zheng, Larkin et al., 1999); hereafter defined as $Per1^{-/-}Per2^{Brdm1}$ or KO). Intercrosses between heterozygous (C57BL/6/J x 129 SvEvBrd) F1 offspring gave rise to F2 homozygous mutants. Mutant and wild-type (WT) animals on this mixed background were used in this study, maintained as described in (Albrecht, Zheng et al., 2001). Mice were maintained in our animal facilities (Chronobiotron, UMS3415, Strasbourg, France) on a 12h light/12h dark (LD) cycle (300 lux during the light phase), with an ambient temperature of 22 ± 1 °C. The animals were given free access to food and water. In all experiments, control and mutant mice were age-matched. Only male mice were used for the RNAseq study, but both males and females were used for qPCR experiments and phagocytosis analysis. All experimental procedures were performed in accordance with the Association for Research in Vision and Ophthalmology Statement on Use of Animals in Ophthalmic and Vision Research, as well as with the European Union Directive (2010/63/EU). Age-matched WT and $Per1^{-/-}Per2^{Brdm1}$ mice (6 weeks old) were sacrificed in constant darkness (dark/dark, DD) at time-points (expressed in circadian time (CT); CT0 – time when lights were on during LD conditions, CT12 – lights off in LD conditions) specific to each experiment. Sacrifice was performed under complete darkness by using night-vision goggles ATN NVG-7 (American Technologies Network Corp., San Francisco, CA, USA) and eye sampling was done under dim red light (< 5 lux). Animals were anesthetized by CO_2 inhalation and subsequently killed by cervical dislocation.

Genotyping

Mice were genotyped by PCR amplification of tail DNA with 4 sets of primers specific either for the genomic regions that were deleted in mutants but present in WT (5′-GTCTTGGTCTCATTCTAGGACACC and 5′-AACATGAGAGCTTCCAGTCCTCTC for *Per1* gene; 5′-AGTAGGTCGTCTTCTTTATGCCCC and 5′-CTCTGCTTTCAACTCCTGTGTCTG for *Per2* gene), or for the recombinant alleles present in mutants only (5′-ACAAACTCACAGAGCCCATCC and 5′-ACTTCCATTTGTCACGTCCTGCAC for $Per1^{-/-}$, 5′-TTTGTTCTGTGAGCTCCTGAACGC and 5′-ACTTCCATTTGTCACGTCCTGCAC for $Per2^{Brdm1}$).

Immunohistochemistry

Eye globes were immersion-fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C. Eyeballs were rinsed in PBS, cut into two hemispheres and cryoprotected upon transfer to an ascending series of sucrose solutions (10%, 20% and 30% each for 1h) and then embedded (Tissue-Tek OCT compound; Thermo-Shandon, Pittsburg, PA, USA). Cryostat sections (10 µm thick) were permeabilized for 5 min with 0.1% Triton X-100 and saturated with PBS containing 0.1% bovine serum albumin, 0.1% Tween-20 and 0.1% sodium azide for 30 min. Sections were incubated overnight at 4°C with monoclonal anti-rhodopsin antibody Rho-4D2 (Hicks & Molday, 1985). Secondary antibody incubation was performed at room temperature for 2h with Alexa 488 anti-mouse IgG-conjugated antibodies (Molecular Probes Inc., Eugene, OR, USA). Cell nuclei were stained with DAPI (Molecular Probes). Slides were washed thoroughly, mounted in PBS/glycerol (1:1), and observed by an epifluorescence microscope (Nikon Optiphot 2). The number of phagosomes was quantified, as described previously by us (Bobu et al., 2006). Transverse sections (n=4/animal) were obtained from the central retina, covering the whole width of the retina from one periphery to the other. Taking the POS/RPE interface as a baseline, any immunopositive inclusion exceeding 1 µm lying within the RPE subcellular space was scored as a phagosome. Phagosomes were counted by aligning a 150 x 150 μm^2 grid parallel with the RPE layer and displacing it dorsally and ventrally with respect to the optic nerve, along the POS/RPE interface, from the posterior to the superior margin. The phagosome counts are expressed as the sum of all 4 sections/eye.

RT-qPCR gene expression analysis

Retinas were sampled immediately after sacrifice. A small incision was performed on the cornea with a sterile blade, lens and vitreous were discarded, and the retina was directly collected with sterile forceps and immediately frozen in liquid nitrogen and stored at -80 °C.

Retinas were homogenized in the RNable (Eurobio Scientific, France) solution by using a 23-gauge sterile needle and 1 ml syringe and mRNA extracted according to the manu-

facturer's recommendations. Resuspended RNA was treated with DNAse I (0.1 U/µl, 30 min, 37°C - Fermentas) followed by phenol/chloroform/isoamylalcohol extraction and sodium acetate/isopropanol precipitation. RNA concentration and purity were measured using NanoDrop ND-1000V 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 (Agilent Technologies; RNA integrity numbers were between 7.8 and 9).

500 ng of total RNA were reverse transcribed by using random primers and the "High-Capacity RNA-to-cDNA" kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. qPCR was performed using the 7300 Real-Time PCR System (Applied Biosystems) and the hydrolyzed probe-based TaqMan chemistry, with optimized Gene Expression Assays designed for specific mRNA amplification (Table S1). We used the TaqMan Universal PCR Master Mix with No AMPErase UNG (Applied Biosystems) and 1µl of cDNA in a total volume of 20 µl. The PCR program was; 10 min at 95°C and then 40 cycles of 15 s at 95°C and 1 min at 60°C. The fluorescence acquisition was performed at the end of the elongation step (7300 System Sequence Detection Software V 1.3.1 - Applied Biosystems). Each PCR reaction was done in duplicate. A dilution curve of the pool of all cDNA samples from one series was used to determine working dilution and to calculate the amplification efficiency for each assay (values were between 1.8 and 2 for all assays). No-template control reactions were performed as negative controls for each assay. One 96-well plate corresponded to the analysis of one gene. Data analysis was performed with qBase software (free v1.3.5) (Hellemans, Mortier et al., 2007) and transcript levels were normalized using *Hprt* and *Tbp* that showed constant expression in their mRNA during the 24-h cycle (data not shown). Average gene expression levels within one experiment (one genotype) were set to 1, so that amplitudes (representing the maximal deviation from this 100% mean) could be compared between groups as was previously performed by Hiragaki and colleagues (Hiragaki, Baba et al., 2014).

Laser Capture Microdissection

Eye globes were enucleated under dim red light (<5 lux), embedded in OCT, snap-frozen and stored at -80°C until use. Eyes were cryosectioned at 10 µm thickness. Each eye provided 116 – 258 sections. All sections were dehydrated with ethanol and stained with Cresyl Violet staining (LCM Staining Kit, Ambion) and air-dried before microdissection with a Laser Microdissection System (LCM; PALM, Bernried, Germany). The RPE and photoreceptors were isolated with LCM (Figure S1). The number of eye sections used for LCM RPE and photoreceptor isolation between genotypes was similar with 183 ± 10.18 (mean ± SD) slices used from WT eyes, whereas 201.3 ± 8.91 slices were used from double mutants ($P = 0.19$, Student's *t*-test).

RNA isolation for RNA sequencing

Total RNA was isolated using a RNeasy Micro kit (Qiagen Benelux, Venlo, The Netherlands), quantified with a Nanodrop (Isogen Life Science B.V., The Netherlands) and the quality was checked on a Bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands). Sample RNA integrity (RIN) values for photoreceptors ranged from 7 to 9.8, except for 3 samples (RIN = 3.2, 4.1, 4.1). For RPE samples, RIN values ranged from 5 – 9.5.

Library preparation and RNA sequencing

We used the KAPA mRNA HyperPrep kit (Illumina Platforms). For generating libraries, we used one batch of 20 ng of total photoreceptor (n = 8) RNA and 30 ng for the other three batches (n = 24) according to the manufacturer's protocol (Illumina Platforms). For generating libraries from RPE samples we used 20 ng of RNA. RPE samples with low RNA yield were pooled. RPE libraries were generated in three batches. The presence of cDNA was confirmed using flash gels (cat No. 57032, Lonza, Rockland, ME, USA). Libraries were 50 bp single-end sequenced using the Illumina HiSeq 4000 platform.

Bioinformatics

The photoreceptor and RPE RNA-seq data were analyzed separately, but with the same software versions and parameter settings unless indicated otherwise. Raw sequencing data were subjected to quality control using FastQC (v.0.11.15), Picard Tools, and dupRadar (Sayols, Scherzinger et al., 2016). All samples were of sufficient quality. Reads were trimmed for adapter sequences using Trimmomatic (v0.32) (Bolger, Lohse et al., 2014). Trimmed reads were aligned to the mouse genome (Ensembl GRCm38.p6) using HISAT2 (v2.1.0) (Kim, Langmead et al., 2015). Gene level counts were obtained using HTSeq (v0.11) (Anders, Pyl et al., 2015) with default parameters except --stranded=reverse and the mouse GTF from Ensembl (release 93). Statistical analyses were performed using the edgeR (Robinson, McCarthy et al., 2010) and limma R (v3.5.0)/Bioconductor (v3.7) packages (Ritchie, Phipson et al., 2015). Genes with more than 2 counts in 4 or more samples (photoreceptors) or in 3 or more samples (RPE) were retained. Count data were transformed to log2-counts per million (logCPM), normalized by applying the trimmed mean of M-values method and precision weighted using voom (Law, Chen et al., 2014). Pairwise differential expression between the conditions of interest was assessed using an empirical Bayes moderated t-test within limma's linear model framework, including the precision weights estimated by voom. Both for WT and *Per1*$^{-/-}$*Per2*^{Brdm1} a moderated F-test was used to determine which genes are differentially expressed between timepoints. Resulting p-values were corrected for multiple testing using the Benjamini-Hochberg false discovery rate (FDR). An adjusted p-value < 0.05 was considered significant for photoreceptors. For the RPE an adjusted p-value of < 0.1 was considered significant. Additional gene annotation was retrieved from Ensembl (photoreceptors: release 94,

RPE: release 98) using the biomaRt R/Bioconductor package. Gene ontology and pathway enrichment analysis was performed using g:Profiler (Reimand, Kull et al., 2007). We set all identified transcripts in our RNA-seq dataset as a reference background. We set an adjusted $P < 0.05$ as a threshold for significantly enriched pathways using the g:SCS method to correct for multiple testing (Reimand et al., 2007). We investigated interactions between protein products of the list of potential POS phagocytosis candidate genes by STRING analysis (Szklarczyk, Franceschini et al., 2015). The 57 candidate genes encode for 49 proteins represented as nodes in the STRING network analysis. By setting the threshold to 0.25, we found 32 edges in the STRING network. Non-interacting nodes were not shown.

Statistics

Data are represented as means ± SEM. Plots were generated using GraphPad Prism (La Jolla, CA, USA), SigmaPlot (Systat Software, San Jose, CA, USA) or R (Bell Labs, Murray Hill, NJ, USA). Normality of distribution was tested using the Shapiro-Wilk test. In case of non-normal distribution, the analysis was performed using ANOVA on ranks. Circadian expression profiles were determined using non-linear regression fitting to the equation $y = y0 + c \cdot \cos[2\pi (t-\varphi)/24]$, where y0 represents mesor, c amplitude and φ acrophase (Nelson, Tong et al., 1979, Sandu, Hicks et al., 2011). The function featured the following constraints: $\varphi < 24$, $\varphi > 0$ and $c > 0$. Gene expression profiles were considered to be rhythmic when significant fitting ($P < 0.05$) was observed to the equation $y = y0 + c \cdot \cos[2\pi (t-\varphi)/24]$. Further analyses, where indicated, were performed using 1-way or 2-way ANOVA analysis followed by Holm-Sidak's post hoc tests.

RESULTS

Peak of rod outer segment phagocytosis is blunted in the retinas of *Per1$^{-/-}$ Per2^{Brdm1}* mice

The phagocytosis of photoreceptor outer segments is a highly rhythmic process occurring in a daily peak. This process persists in constant darkness, suggesting that it is driven by the circadian clock (Bobu & Hicks, 2009, LaVail, 1976). We tested the hypothesis that intact clockwork is required to sustain a rhythm of POS phagocytosis in constant darkness (DD). To that end, we used the *Per1$^{-/-}$Per2^{Brdm1}* clock mutant mice which are behaviorally arrhythmic in DD (Zheng et al., 2001).

We used age-matched (2 months old) wild-type and *Per1$^{-/-}$Per2^{Brdm1}* mice, harvested eye globes at 8 time points over 24 h and analyzed anti-rhodopsin-stained phagosomes in the RPE (Figure 1a). We quantified POS phagosomes at various time-points under DD conditions (n= 3 animals per genotype and per time point). A 2-way ANOVA analysis

showed that the number of POS phagosomes was affected by genotype (WT versus *Per1*$^{-/-}$*Per2*^{Brdm1}, $P < 0.001$), time ($P < 0.001$) and an interaction between genotype and time ($P < 0.001$). Post-hoc analysis showed that phagocytic activity was rhythmic in wild type mice only, with 3-4 times more phagosomes at time-point CT1 compared with baseline ($P < 0.001$ for all time point comparisons) (Figure 1b, c; also confirmed by 1-way ANOVA, $F_{7,16} = 34.49$; $P < 0.001$). In contrast, in *Per1*$^{-/-}$*Per2*^{Brdm1} mice, there was no obvious peak (1-way ANOVA, $F_{7,16} = 2.35$; $P = 0.075$). These results suggest that *Per1* and/or *Per2* is required for rhythmic POS phagocytosis.

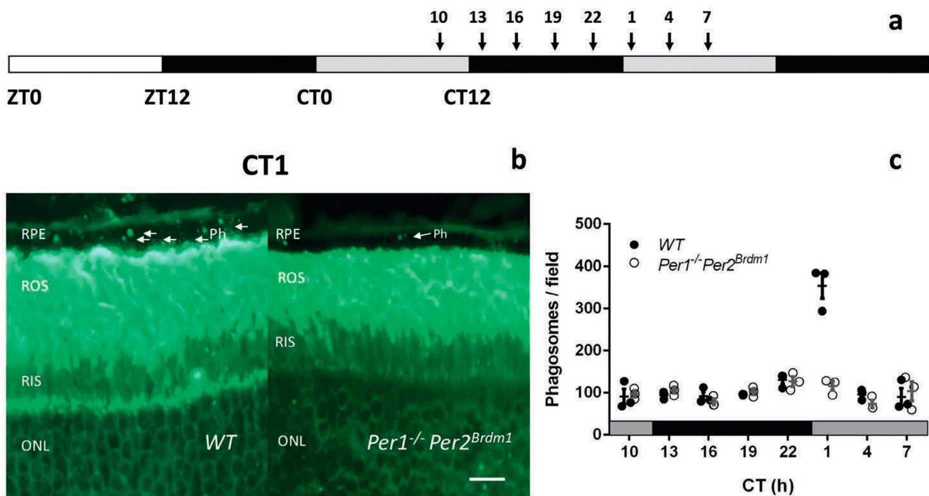


Figure 1. Mice lacking *Per1* and *Per2* show an impaired peak in POS phagocytosis. (a) WT and *Per1*$^{-/-}$*Per2*^{Brdm1} mice maintained under 12h light (white bar) - dark (black bar) conditions were placed under constant darkness (DD, grey - black bars) and sacrificed at time-points indicated by arrows. (b) Representative image of Rho-4D2 stained phagosomes of WT and *Per1*$^{-/-}$*Per2*^{Brdm1} retinas obtained at CT1 during the peak in phagocytosis in DD conditions. RPE – retinal pigment epithelium, ROS – rod outer segments, RIS – rod inner segments, ONL – outer nuclear layer, Ph – phagosomes. The scale bar is 10 µm. (c) Quantification of phagosomes in WT and *Per1*$^{-/-}$*Per2*^{Brdm1} retinas under DD showed that *Per1*$^{-/-}$*Per2*^{Brdm1} mice had no detectable peak in ROS phagocytosis. N = 3 / genotype / time-point. Graphs show mean ± SEM and values from individual samples are shown as dots.

Molecular makeup of the retinal clock in absence of *Per1* and *Per2*

Since the peak of phagocytosis is attenuated in the mutant mice in DD, we hypothesized that the molecular clockwork is impaired in *Per1*$^{-/-}$*Per2*^{Brdm1} retinas. To test this hypothesis, we sampled retinas from WT and *Per1*$^{-/-}$*Per2*^{Brdm1} mice every 4h over 24h under DD, and quantified relative mRNA levels of clock genes by qPCR (Figure 2a). Rhythmicity in expression profiles was assessed by cosinor analysis. These changes over the 24h cycle

was mainly confirmed by 1-way ANOVA analysis (Table S2). Under DD conditions we found rhythmic clock gene expression for *Bmal1*, *Per1*, *Per2*, *Rev-Erbα* and *Rorb* in WT whole retinas (Figure 2b, Table S2). Unexpectedly, in DD conditions, we found that in *Per1*$^{-/-}$*Per2*^{Brdm1} mouse retinas also five clock genes were rhythmic: *Bmal1*, *Per3*, *Cry1*, *Cry2* and *Rev-Erbα.* Therefore, in contrast to our hypothesis, these results suggest *Per1* and *Per2* mutations do not significantly impair the rhythmicity of whole retinas in mice.

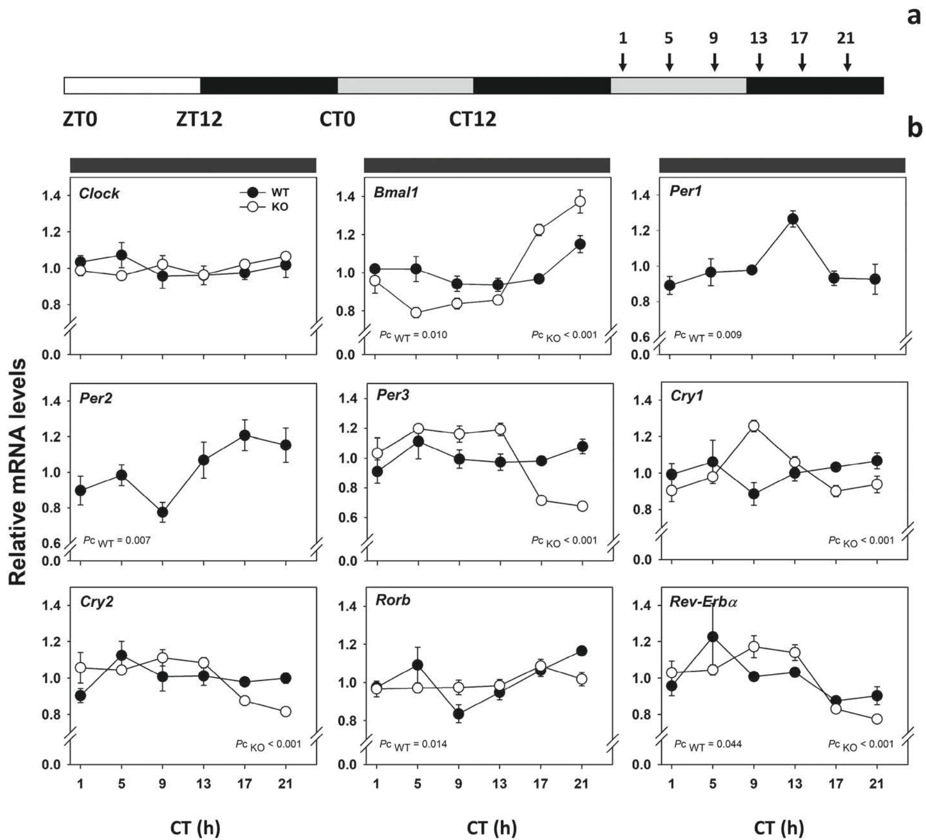


Figure 2. Clock gene expression profiles in WT (black dots) and *Per1*$^{-/-}$*Per2*^{Brdm1} (white dots; KO) whole retinas under DD conditions. (a) Mice were placed under DD conditions, sacrificed at time-points indicated by arrows and their whole retinas were harvested. (b) QPCR analysis revealed that rhythmic gene expression was observed for *Bmal1*, *Per1*, *Per2*, *Rev-Erbα* and *Rorb* in WT retinas. Rhythmic expression was found for *Bmal1*, *Per3, Cry1, Cry2* and *Rev-Erbα* in *Per1*$^{-/-}$*Per2*^{Brdm1} retinas. Values represent mean ± SEM. Significant temporal variations are indicated ($P < 0.05$). *Pc* – *P*-value of cosinor non-linear regression fitting to the equation $y = y0 + c \cdot \cos [2\pi (t-\Phi)/24]$, with y0 – mesor, c – amplitude and Φ – acrophase. N = 3-4 for WT and 4-5 for double mutants / time-point.

Transcriptomics analysis of WT mouse RPE and photoreceptors

To characterize the potential link between the circadian clock and the peak in POS phagocytosis, we first sought to characterize the time-affected transcriptomes of the RPE and photoreceptors. We harvested WT and $Per1^{/}Per2^{Brdm1}$ mouse eyes kept in DD at 4 time-points (CT19, 22, 1 and 10) (Figure 3a). We laser-capture-microdissected the RPE and photoreceptors from each mouse eye (n = 4 / genotype / time-point), extracted RNA and performed RNA sequencing. In the RPE and photoreceptors, respectively, a total of 24 382 and 22 694 genes had sufficiently large counts to be retained in the statistical analysis. Next, we performed a pair-wise comparison of WT RPE and photoreceptor transcriptomes between consecutive time-points (Figure 3b and c). In WT RPE, we found a large number of differentially expressed genes in comparisons between the expected peak in phagocytosis time-point CT1 and adjacent time-points (CT22 and CT10, respectively, Figure 3b). In WT photoreceptors, most genes are differentially expressed in comparisons between CT10 and adjacent time points (CT1 and CT19, respectively, Figure 3c). By contrast, in all pair-wise comparisons we found that only 3 genes differed significantly between time-points (i.e. were up-regulated at CT10 vs 19) in $Per1^{-/-}Per2^{Brdm1}$ RPE. We found that 1 gene was down-regulated at CT19 vs CT10 in $Per1^{-/-}Per2^{Brdm1}$ photoreceptors. Thus, these results suggest that the transcriptional program for initiating POS phagocytosis is likely in the RPE and not photoreceptors.

Our differential expression analysis showed that 594 genes in WT RPE (=2.44% of all genes retained in the analysis) and 2 372 genes in WT photoreceptors (=10.45% of retained genes) varied over time-points (Figure 3d, Table S3, S4). Among them are components of the circadian clock network (Table S3, S4). Pathway analysis of time-affected genes in WT mice RPE revealed that, in addition to circadian pathways, phototransduction and metabolic-related pathways were functionally enriched (Table S5). Time-affected WT photoreceptor genes were enriched in circadian, metabolic, neurotransmission and DNA repair-related pathways (Table S6). Interestingly, 119 time-affected genes overlap in RPE and photoreceptors (Table S7) and are functionally enriched in glucose metabolism and neurotransmitter release-related pathways (Figure 3e, Table S8). We also found that, respectively, 32 and 48 time-affected genes in the RPE and photoreceptors overlap with the RetNet list of eye disease-related genes (SP Daiger, 1998) (Figure 3d, Table S9). Thus, our results show that in the RPE and photoreceptors, a large number of genes and pathways vary in a time-dependent manner, a number of which are implicated in eye diseases.

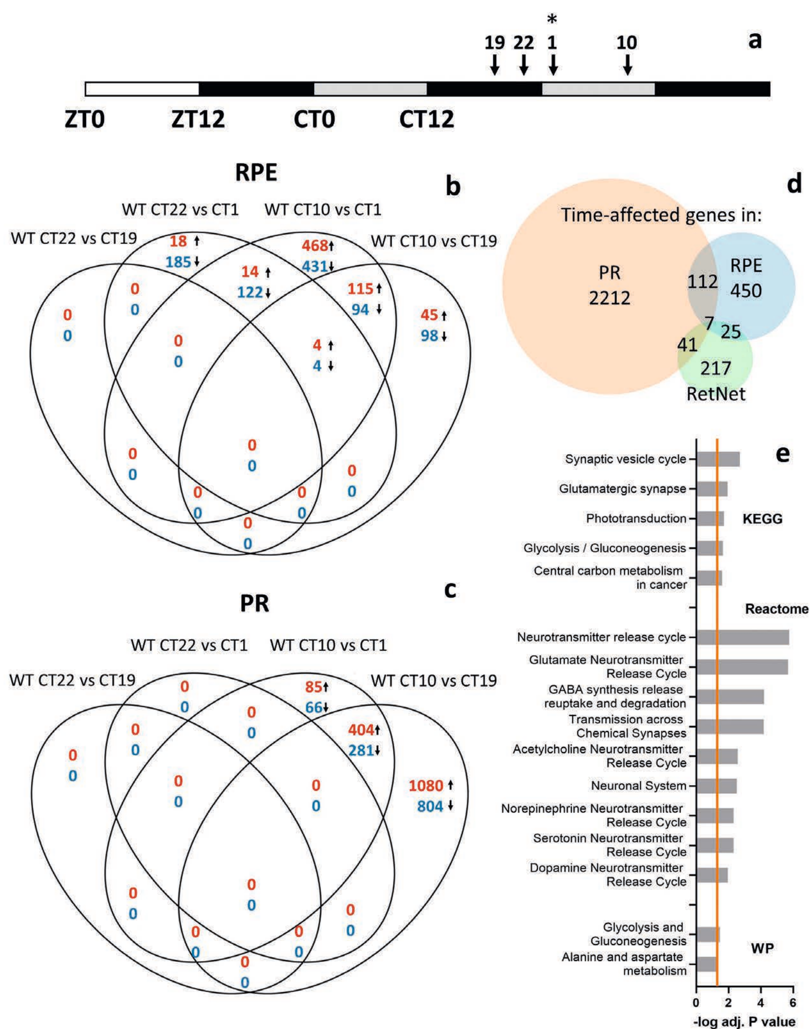


Figure 3. Transcriptional profiling of WT mouse RPE and photoreceptors. (a) Eyes were obtained under DD conditions from 4 successive time-points: before (CT19, CT22), during (CT1) and after (CT10) the expected peak in POS phagocytosis (n = 4 / time-point). RPE and photoreceptors were meticulously laser-capture-microdissected from each mouse eye, RNA was extracted and the transcriptomes were determined using RNA-sequencing. (b) In the RPE, a substantial number of genes were differentially expressed at time-points adjacent to the peak in POS phagocytosis – CT1. (c) By contrast, in photoreceptors (PR) most differential gene expression occurs around CT10. Red numbers represent the number of up-regulated differentially expressed genes, whereas blue ones are down-regulated. (d) A substantial number of identified transcripts showed a time effect in WT PR and RPE. There is considerable overlap (n=119) between time affected genes in these two tissues, a number of which overlap with the RetNet list of eye disease-related genes (SP Daiger, 1998). (e) Functional annotation (performed using g:Profiler) revealed that overlapping time-affected genes in RPE and PR are enriched in glucose metabolism and neurotransmission-related pathways. The orange line represents the significance level cut-off (adjusted $P < 0.05$). WikiPathways, Reactome and KEGG are databases of biological pathways.

Potential molecular pathway that initiates POS phagocytosis

Our results suggested that the transcriptional events in the RPE might initiate POS phagocytosis (Figure 3b). Our results also suggested that *Per1* and/or *Per2* are necessary for driving the peak in POS phagocytosis under DD (Figure 1), but the molecular link is unclear. To characterize this link, we performed pair-wise comparisons between WT and $Per1^{-/-}Per2^{Brdm1}$ RPE transcriptomes. We found a substantial number of genes that were differentially expressed in $Per1^{-/-}Per2^{Brdm1}$ RPE compared to WT ones at the peak POS phagocytosis time-point CT1 (Figure 4a). Next, we defined selection criteria for genes that potentially initiate POS phagocytosis (Figure 4b). Considering that the peak in POS phagocytosis is lacking in $Per1^{-/-}Per2^{Brdm1}$ mice, we assumed that the genes that initiate POS phagocytosis are down-regulated in double mutant RPE compared to WT ones at CT1. POS phagocytosis occurs as a peak in WT mice on a molecular and functional level (Law, Parinot et al., 2015, Nandrot, Kim et al., 2004). Thus, we selected genes that are both up-regulated at CT1 vs CT22 and down-regulated at CT10 vs CT1 in WT RPE. We removed possible photoreceptor “contaminants” from this list by using mouse signature cone and rod genes (Macosko, Basu et al., 2015) and the Gene Ontology database (POS cellular component, GO:0001750). Using this strategy, we obtained a list of 57 candidate genes (Figure 4b, Table S10). These genes are functionally enriched in neurotransmission related pathways (Figure 4c, Table S11). To reveal the interactions that protein products of these genes are involved in, we constructed a protein-protein interaction network using STRING (Szklarczyk et al., 2015) (Figure 4d). Our list revealed a number of functional associations in which the protein products of candidate genes are involved in, most of which are associated with the term cell junction (highlighted in red in Figure 4d). This cluster involves the interactions of *Syp*, *Gnaz*, *Pacsin1*, *Snap91*, *Camk2d* and *Camk2b* as identified in our STRING analysis. Thus, it is possible that POS phagocytosis might be initiated by the largest cluster identified in this analysis.

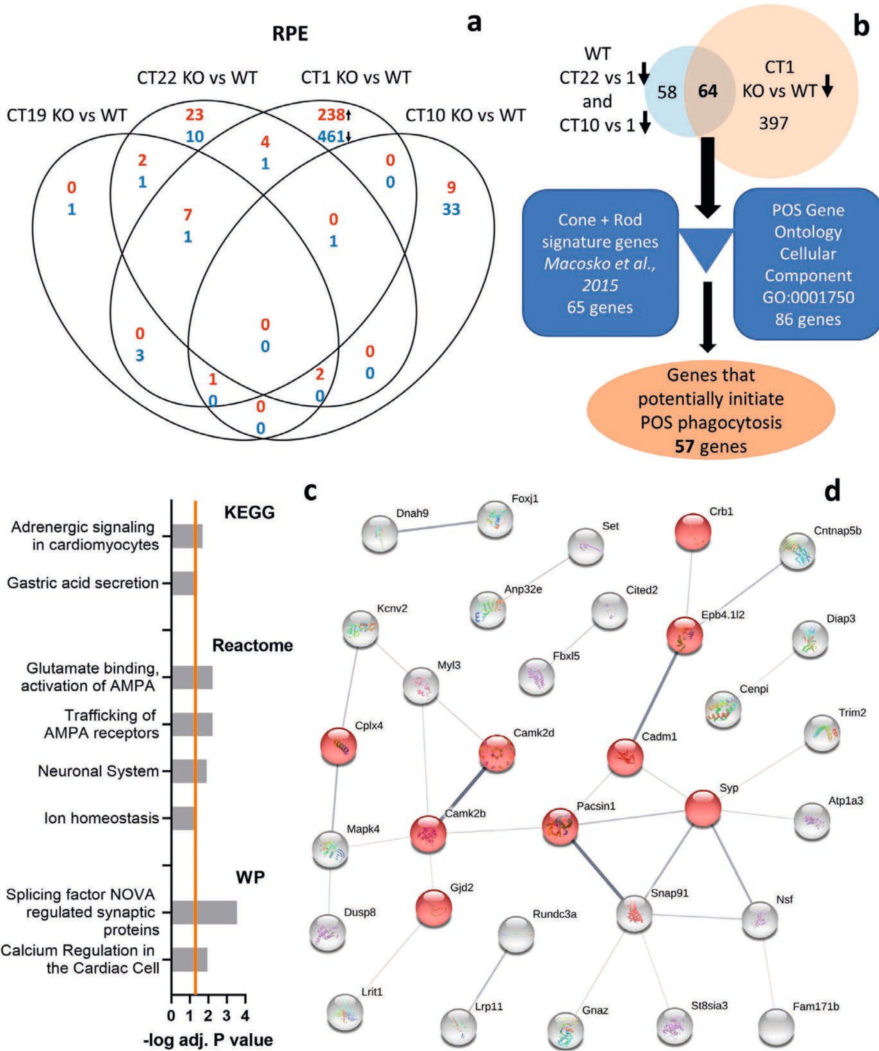


Figure 4. Identification of potential phagocytic pathways in RPE. (a) A comparison of WT and *Per1*$^{-/-}$*Per2*^{Brdm1} (KO) RPE transcriptomes within each time-point revealed that most genes were differentially expressed during the peak phagocytosis time point - CT1. Red numbers represent the number of up-regulated differentially expressed genes, whereas blue ones are down-regulated. (b) Selection strategy for compiling the list of genes in the RPE possibly implicated in regulating POS phagocytosis. Signature rod and cone genes (Macosko et al., 2015) and the Gene Ontology term "Photoreceptor Outer Segment" were used to remove photoreceptor genes from the list of genes that potentially regulate POS phagocytosis. (c) Functional enrichment analysis using g:Profiler showed that these genes are enriched in neurotransmission related pathways from the WikiPathways (WP), Reactome, KEGG databases. The orange line represents the significance cut-off (adjusted $P < 0.05$). (d) STRING network analysis of protein functional associations of products of RPE genes implicated in initiating phagocytosis. Nodes represent protein products (n=57). Disconnected nodes are not shown. Edges represent protein functional associations. Interaction confidence scores range 0.25 - 0.99.

DISCUSSION

In the present study, we found no peak in POS phagocytosis in retinas of mice carrying a combined *Per1* and *Per2* mutation under constant darkness. Unexpectedly, gene expression analysis revealed that mutant retinas remained rhythmic under constant darkness, in contrast to mutant RPE and photoreceptors which showed no temporal variation. Using the purest possible RPE and photoreceptor sample material obtained by microdissection, we found significant differential gene expression in WT RPE at the peak phagocytosis time-point, but not in photoreceptors. Our results suggest a network of genes that potentially initiates POS phagocytosis in the RPE. These data challenge the view that molecular events in photoreceptors drive POS phagocytosis (via expression of phosphatidylserine “eat-me” signals) (Müller & Finnemann, 2020).

Retinal clocks are present in virtually all retinal layers (Jaeger et al., 2015, Liu, Zhang et al., 2012, Ruan, Allen et al., 2008, Ruan, Zhang et al., 2006) and are tightly coupled (Jaeger et al., 2015). Coupling between retinal clocks contributes towards the precise timing of physiology within the retina (Felder-Schmittbuhl, Buhr et al., 2018) . In our study in $Per1^{/}Per2^{Brdm1}$ mice, constant darkness prevented any increased phagocytosis following subjective onset of day, a process known to be clock-regulated (Fisher, Pfeffer et al., 1983, LaVail, 1976, LaVail, 1980, Young, 1971, Young, 1978). Thus, we speculated that constant darkness might impair the clockwork in $Per1^{/}Per2^{Brdm1}$ whole retinas. The literature is not consistent regarding the effects of lighting conditions on clock gene expression in the whole retina. Studies either report no effects of DD on global retinal oscillations (Liu et al., 2012, Zhang, Zhou et al., 2005) or suggest that DD conditions dampen retinal rhythmicity (Bobu, Sandu et al., 2013, Sandu et al., 2011, Storch, Paz et al., 2007). Unexpectedly, our qPCR study revealed that clock gene expression remained rhythmic in both WT and $Per1^{/}Per2^{Brdm1}$ whole retinas. The origin of rhythmicity in mutant whole retinas is not known. It is most likely not due to input from the central clock, because retinal clocks are known to be independent from the SCN (Terman et al., 1993) and the SCN is considered arrhythmic based on locomotor activity of $Per1^{/}Per2^{Brdm1}$ mice in DD (Zheng et al., 2001). The source is most likely not in photoreceptors because in this study transcriptomics analysis of LCM-isolated $Per1^{-/-}Per2^{Brdm1}$ photoreceptors showed no temporal variations. Therefore, it is likely that rhythms in mutant whole retinas originate from retinal layers which display the most robust rhythms: e.g. the inner retina (Jaeger et al., 2015, Ruan et al., 2008, Ruan et al., 2006, Sandu et al., 2011, Zhang et al., 2005). Considering that the number of oscillating genes differs considerably across mouse organs/tissues (Zhang, Lahens et al., 2014), it is possible that *Per1* and/or *Per2* mutations impact the RPE and photoreceptor clocks disproportionally more than the clockwork of other retinal cells.

Regardless of the reasons, these results suggest that (global) retinal rhythmicity is not sufficient for driving the peak of POS phagocytosis.

The phagocytosis of POS is a rhythmic process occurring roughly one-hour after light onset (Fisher et al., 1983, LaVail, 1976, LaVail, 1980, Young, 1971, Young, 1978). This process is critical for retinal health as demonstrated by retinal degeneration displayed in both human patients (Gal et al., 2000) and animal models (D'Cruz et al., 2000, Laurent, Sengupta et al., 2017, Muller, Mas Gomez et al., 2014). Some literature stresses the importance of precise timing of POS phagocytosis in maintaining retinal health (Laurent et al., 2017, Muller et al., 2014). This view is corroborated by our finding that a number of eye disease-related genes vary across time-points in the RPE and photoreceptors. However, it was recently reported that dopamine D2 receptor knockout mice had no peak in POS phagocytosis and displayed no apparent retinal pathologies (Goyal, DeVera et al., 2020). Regardless, the molecular pathways responsible for driving this peak are not known (Mazzoni et al., 2014, McMahon et al., 2014, Müller & Finnemann, 2020). By using immunohistochemistry and quantifying ingested POS in clock mutant mouse retinas we showed that *Per1* and/or *Per2* are necessary (molecular clock) components for the transient surge in POS phagocytosis.

The prevailing view is that POS phagocytosis is initiated by the externalization of phosphatidylserine "eat-me" signals on the POS membrane (Mazzoni et al., 2014, Müller & Finnemann, 2020). However, we found that microdissected WT photoreceptors did not differ in gene expression 3h or 6h before the peak in POS phagocytosis. By contrast, in WT RPE, we found that a number of genes were differentially expressed at the phagocytic peak time-point compared to the 3h earlier one. In addition, at the peak phagocytosis time-point, we found a vast number of differentially expressed genes in $Per1^{/}Per2^{Brdm1}$ RPE compared to WT ones. These results suggest that POS phagocytosis is initiated by the RPE. This possibility is indeed plausible because the RPE was shown to display sustained rhythms in various models: in vivo (DeVera & Tosini, 2020, Goyal et al., 2020, Louer, Gunzel et al., 2020, Louer, Yi et al., 2020, Mure, Le et al., 2018); ex vivo (Baba, DeBruyne et al., 2017, Baba, Sengupta et al., 2010, Milicevic, Mazzaro et al., 2019b) and in cell culture models (Ikarashi, Akechi et al., 2017, Milicevic, Duursma et al., 2019a, Milicevic et al., 2019b, Milicevic, Ten Brink et al., 2020, Morioka, Kanda et al., 2018). Importantly, the phagocytic machinery is rhythmic in these cells (Goyal et al., 2020, Ikarashi et al., 2017, Milicevic et al., 2019b, Nandrot et al., 2004). Furthermore, in an arrhythmic BMAL1 knockout cell culture model there was no rhythm of phagocytic activity (Ikarashi et al., 2017).

Finally, we proposed a network of genes for regulating ROS phagocytosis in the RPE. The candidate genes in this list are enriched in the ion homeostasis pathway. This is expected as previous studies implicated ion channels in POS phagocytosis such as voltage gated sodium channels (Johansson, Karema-Jokinen et al., 2019) and the L-type calcium channel $Ca_v1.3$ (Muller et al., 2014). The list also contains known genes implicated in POS phagocytosis such as *Mfge8* (Nandrot, Anand et al., 2007) and *Myl3* (Tang, Lu et al., 2015). Cell junctions were also enriched in the candidate gene list, among which *Gjd2* encodes for a gap junction protein. It is possible that increased gap junction expression enhances the connectivity of the RPE at the peak phagocytic time-point. That might, in turn, lead to a synchronized and sharp phagocytic peak across the whole RPE. However, it should also be noted that a number of genes in the list have not been sufficiently characterized e.g. *Gm13112*, *Gm13735*, *Gm16701*, etc. Therefore, our list of candidate genes provides ample opportunities for investigation for the research community.

The strength of this approach is the use of the purest possible sample material obtained from LCM. In addition, we considered the rhythmic nature of POS phagocytosis by using samples from multiple time points. We also compared our results with an arrhythmic mouse model that lacked this peak phagocytic activity. There are some limitations in our approach. For example, the genes implicated in initiating phagocytosis might not be down-regulated after the peak phagocytic time-point. It might be that at the peak phagocytic time-points, the down-regulated genes repress RPE phagocytic activity. It is also possible that genes in the list might be "contaminants" originating from POS fragments that are ingested by the RPE. Despite the imperfections, this list will be a valuable tool for studying the POS phagocytosis pathway.

In conclusion, our study reveals that *Per1*/*Per2* are necessary circadian clock components for driving the rhythm of POS phagocytosis. Our results show that *Per1* and *Per2* mutation does not impair the rhythmicity of the whole retina. Our data suggests that the molecular pathways that initiate POS phagocytosis are most likely initiated by the RPE by genes functionally enriched in neurotransmission related pathways.

Declarations

Funding: This project has been funded with support from the NeuroTime Erasmus+ grant (European Union), Rotterdamse Stichting Blindenbelangen (Netherlands), Nelly Reef fund (Netherlands), Stichting voor Ooglijders (Netherlands), Stichting tot Verbetering van het Lot der Blinden (Netherlands) and Retina France (France).

Conflicts of interest/Competing interests: The authors declare no competing interests.

Availability of data and material: Data supporting the conclusions of this article are included within the article and are available from the corresponding authors on reasonable request.

Code availability: The R code for analysis is available from the corresponding authors on reasonable request.

Ethics approval: All experimental procedures were performed in accordance with the Association for Research in Vision and Ophthalmology Statement on Use of Animals in Ophthalmic and Vision Research, as well as with the European Union Directive (2010/63/EU).

Consent to participate: Not applicable

Consent for publication: All authors read and approve of the contents of this manuscript.

Author contributions: N.M. performed experiments, analysis, prepared figures, wrote the manuscript and obtained funding. O.A.H.H. performed experiments, data analysis, prepared figures and obtained funding. P.D.M. and A.J. performed bioinformatics analysis and edited the manuscript. U.B., J.B.T.B. and C.S. provided technical assistance, performed experiments, prepared figures and edited the manuscript. D.H., A.A.B. and M.P.F.S. conceptualized and directed the project, obtained funding, provided resources, performed analysis and edited the manuscript.

ACKNOWLEDGEMENTS

We thank Anneloor L.M.A. ten Asbroek and Nguyen-Vy Vo for technical assistance. We thank Dr. D. Sage, Dr. S. Reibel and N. Lethenet from the Chronobiotron (UMS 3415) for animal care and Dr. U. Albrecht (University of Freiburg) for the *Per1*$^{-/-}$*Per2*^{Brdm1} mice. This project has been funded with support from the NeuroTime Erasmus+ grant (European Union), Rotterdamse Stichting Blindenbelangen (Netherlands), Nelly Reef fund (Netherlands), Stichting voor Ooglijders (Netherlands), Stichting tot Verbetering van het Lot der Blinden (Netherlands) and Retina France (France).

SUPPORTING INFORMATION

Additional supporting Information may be found online in the Supporting Information section.

REFERENCES

Albrecht U, Zheng B, Larkin D, Sun ZS, Lee CC (2001) MPer1 and mper2 are essential for normal resetting of the circadian clock. Journal of biological rhythms 16: 100-4.

Anders S, Pyl PT, Huber W (2015) HTSeq--a Python framework to work with high-throughput sequencing data. Bioinformatics (Oxford, England) 31: 166-9.

Baba K, DeBruyne JP, Tosini G (2017) Dopamine 2 Receptor Activation Entrains Circadian Clocks in Mouse Retinal Pigment Epithelium. Scientific reports 7: 5103.

Baba K, Sengupta A, Tosini M, Contreras-Alcantara S, Tosini G (2010) Circadian regulation of the PERIOD 2::LUCIFERASE bioluminescence rhythm in the mouse retinal pigment epithelium-choroid. Molecular vision 16: 2605-11.

Barnard AR, Hattar S, Hankins MW, Lucas RJ (2006) Melanopsin regulates visual processing in the mouse retina. Current biology : CB 16: 389-95.

Bassi CJ, Powers MK (1986) Daily fluctuations in the detectability of dim lights by humans. Physiology & behavior 38: 871-7.

Besharse JC, Iuvone PM (1983) Circadian clock in Xenopus eye controlling retinal serotonin N-acetyltransferase. Nature 305: 133-5.

Bobu C, Craft CM, Masson-Pevet M, Hicks D (2006) Photoreceptor organization and rhythmic phagocytosis in the nile rat Arvicanthis ansorgei: a novel diurnal rodent model for the study of cone pathophysiology. Investigative ophthalmology & visual science 47: 3109-18.

Bobu C, Hicks D (2009) Regulation of retinal photoreceptor phagocytosis in a diurnal mammal by circadian clocks and ambient lighting. Investigative ophthalmology & visual science 50: 3495-502.

Bobu C, Sandu C, Laurent V, Felder-Schmittbuhl MP, Hicks D (2013) Prolonged light exposure induces widespread phase shifting in the circadian clock and visual pigment gene expression of the Arvicanthis ansorgei retina. Molecular vision 19: 1060-73.

Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics (Oxford, England) 30: 2114-20.

D'Cruz PM, Yasumura D, Weir J, Matthes MT, Abderrahim H, LaVail MM, Vollrath D (2000) Mutation of the receptor tyrosine kinase gene Mertk in the retinal dystrophic RCS rat. Human molecular genetics 9: 645-51.

DeVera C, Tosini G (2020) Circadian analysis of the mouse retinal pigment epithelium transcriptome. Experimental eye research 193: 107988.

Felder-Schmittbuhl MP, Buhr ED, Dkhissi-Benyahya O, Hicks D, Peirson SN, Ribelayga CP, Sandu C, Spessert R, Tosini G (2018) Ocular Clocks: Adapting Mechanisms for Eye Functions and Health. Investigative ophthalmology & visual science 59: 4856-4870.

Fisher SK, Pfeffer BA, Anderson DH (1983) Both rod and cone disc shedding are related to light onset in the cat. Investigative ophthalmology & visual science 24: 844-56.

Gal A, Li Y, Thompson DA, Weir J, Orth U, Jacobson SG, Apfelstedt-Sylla E, Vollrath D (2000) Mutations in MERTK, the human orthologue of the RCS rat retinal dystrophy gene, cause retinitis pigmentosa. Nature genetics 26: 270-1.

Goyal V, DeVera C, Laurent V, Sellers J, Chrenek MA, Hicks D, Baba K, Iuvone PM, Tosini G (2020) Dopamine 2 Receptor Signaling Controls the Daily Burst in Phagocytic Activity in the Mouse Retinal Pigment Epithelium. Investigative ophthalmology & visual science 61: 10.

Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J (2007) qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome biology 8: R19.

Hicks D, Molday RS (1985) Localization of lectin receptors on bovine photoreceptor cells using dextran-gold markers. Investigative ophthalmology & visual science 26: 1002-13.

Hiragaki S, Baba K, Coulson E, Kunst S, Spessert R, Tosini G (2014) Melatonin signaling modulates clock genes expression in the mouse retina. PloS one 9: e106819.

Ikarashi R, Akechi H, Kanda Y, Ahmad A, Takeuchi K, Morioka E, Sugiyama T, Ebisawa T, Ikeda M, Ikeda M (2017) Regulation of molecular clock oscillations and phagocytic activity via muscarinic Ca (2+) signaling in human retinal pigment epithelial cells. Scientific reports 7: 44175.

Jaeger C, Sandu C, Malan A, Mellac K, Hicks D, Felder-Schmittbuhl MP (2015) Circadian organization of the rodent retina involves strongly coupled, layer-specific oscillators. FASEB journal: official publication of the Federation of American Societies for Experimental Biology 29: 1493-504.

Johansson JK, Karema-Jokinen VI, Hakanen S, Jylhä A, Uusitalo H, Vihinen-Ranta M, Skottman H, Ihalainen TO, Nymark S (2019) Sodium channels enable fast electrical signaling and regulate phagocytosis in the retinal pigment epithelium. BMC biology 17: 63.

Kevany BM, Palczewski K (2010) Phagocytosis of retinal rod and cone photoreceptors. Physiology (Bethesda, Md) 25: 8-15.

Kim D, Langmead B, Salzberg SL (2015) HISAT: a fast-spliced aligner with low memory requirements. Nature methods 12: 357-60.

Ko CH, Takahashi JS (2006) Molecular components of the mammalian circadian clock. Human molecular genetics 15 Spec No 2: R271-7.

Ko GY (2018) Circadian regulation in the retina: From molecules to network. The European journal of neuroscience.

Krigel A, Felder-Schmittbuhl MP, Hicks D (2010) Circadian-clock driven cone-like photoreceptor phagocytosis in the neural retina leucine zipper gene knockout mouse. Molecular vision 16: 2873-81.

Laurent V, Sengupta A, Sanchez-Bretano A, Hicks D, Tosini G (2017) Melatonin signaling affects the timing in the daily rhythm of phagocytic activity by the retinal pigment epithelium. Experimental eye research 165: 90-95.

LaVail MM (1976) Rod outer segment disk shedding in rat retina: relationship to cyclic lighting. Science (New York, NY) 194: 1071-4.

LaVail MM (1980) Circadian nature of rod outer segment disc shedding in the rat. Investigative ophthalmology & visual science 19: 407-11.

Law AL, Parinot C, Chatagnon J, Gravez B, Sahel JA, Bhattacharya SS, Nandrot EF (2015) Cleavage of Mer tyrosine kinase (MerTK) from the cell surface contributes to the regulation of retinal phagocytosis. The Journal of biological chemistry 290: 4941-52.

Law CW, Chen Y, Shi W, Smyth GK (2014) voom: Precision weights unlock linear model analysis tools for RNA-seq read counts. Genome biology 15: R29.

Liu X, Zhang Z, Ribelayga CP (2012) Heterogeneous expression of the core circadian clock proteins among neuronal cell types in mouse retina. PloS one 7: e50602.

Louer EMM, Gunzel D, Rosenthal R, Carmone C, Yi G, Stunnenberg HG, den Hollander AI, Deen PMT (2020) Differential day-night expression of tight junction components in murine retinal pigment epithelium. Experimental eye research 193: 107985.

Louer EMM, Yi G, Carmone C, Robben J, Stunnenberg HG, den Hollander AI, Deen PMT (2020) Genes Involved in Energy Metabolism Are Differentially Expressed During the Day-Night Cycle in Murine Retinal Pigment Epithelium. Investigative ophthalmology & visual science 61: 49.

Macosko EZ, Basu A, Satija R, Nemesh J, Shekhar K, Goldman M, Tirosh I, Bialas AR, Kamitaki N, Martersteck EM, Trombetta JJ, Weitz DA, Sanes JR, Shalek AK, Regev A, McCarroll SA (2015) Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets. Cell 161: 1202-1214.

Mazzoni F, Safa H, Finnemann SC (2014) Understanding photoreceptor outer segment phagocytosis: use and utility of RPE cells in culture. Experimental eye research 126: 51-60.

McMahon DG, Iuvone PM, Tosini G (2014) Circadian organization of the mammalian retina: from gene regulation to physiology and diseases. Progress in retinal and eye research 39: 58-76.

Milicevic N, Duursma A, Ten Asbroek A, Felder-Schmittbuhl MP, Bergen AA (2019a) Does the circadian clock make RPE-mediated ion transport “tick” via SLC12A2 (NKCC1)? Chronobiology international 36: 1592-1598.

Milicevic N, Mazzaro N, de Bruin I, Wils E, Ten Brink J, Asbroek AT, Mendoza J, Bergen A, Felder-Schmittbuhl MP (2019b) Rev-Erbalpha and Photoreceptor Outer Segments modulate the Circadian Clock in Retinal Pigment Epithelial Cells. Scientific reports 9: 11790.

Milicevic N, Ten Brink JB, Ten Asbroek A, Bergen AA, Felder-Schmittbuhl MP (2020) The circadian clock regulates RPE-mediated lactate transport via SLC16A1 (MCT1). Experimental eye research 190: 107861.

Morioka E, Kanda Y, Koizumi H, Miyamoto T, Ikeda M (2018) Histamine Regulates Molecular Clock Oscillations in Human Retinal Pigment Epithelial Cells via H1 Receptors. Frontiers in endocrinology 9: 108.

Müller C, Finnemann SC (2020) RPE Phagocytosis. In Retinal Pigment Epithelium in Health and Disease, Klettner AK, Dithmar S (eds) pp 47-63. Cham: Springer International Publishing.

Muller C, Mas Gomez N, Ruth P, Strauss O (2014) CaV1.3 L-type channels, maxiK Ca(2+)-dependent K(+) channels and bestrophin-1 regulate rhythmic photoreceptor outer segment phagocytosis by retinal pigment epithelial cells. Cellular signalling 26: 968-78.

Mure LS, Le HD, Benegiamo G, Chang MW, Rios L, Jillani N, Ngotho M, Kariuki T, Dkhissi-Benyahya O, Cooper HM, Panda S (2018) Diurnal transcriptome atlas of a primate across major neural and peripheral tissues. Science (New York, NY) 359.

Nandrot EF, Anand M, Almeida D, Atabai K, Sheppard D, Finnemann SC (2007) Essential role for MFG-E8 as ligand for alphavbeta5 integrin in diurnal retinal phagocytosis. Proceedings of the National Academy of Sciences of the United States of America 104: 12005-10.

Nandrot EF, Kim Y, Brodie SE, Huang X, Sheppard D, Finnemann SC (2004) Loss of synchronized retinal phagocytosis and age-related blindness in mice lacking alphavbeta5 integrin. The Journal of experimental medicine 200: 1539-45.

Nelson W, Tong YL, Lee JK, Halberg F (1979) Methods for cosinor-rhythmometry. Chronobiologia 6: 305-23.

Reimand J, Kull M, Peterson H, Hansen J, Vilo J (2007) g:Profiler--a web-based toolset for functional profiling of gene lists from large-scale experiments. Nucleic acids research 35: W193-200.

Ribelayga C, Cao Y, Mangel SC (2008) The circadian clock in the retina controls rod-cone coupling. Neuron 59: 790-801.

Ribelayga C, Mangel SC (2010) Identification of a circadian clock-controlled neural pathway in the rabbit retina. PloS one 5: e11020.

Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK (2015) limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic acids research 43: e47.

Robinson MD, McCarthy DJ, Smyth GK (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics (Oxford, England) 26: 139-40.

Ruan GX, Allen GC, Yamazaki S, McMahon DG (2008) An autonomous circadian clock in the inner mouse retina regulated by dopamine and GABA. PLoS biology 6: e249.

Ruan GX, Zhang DQ, Zhou T, Yamazaki S, McMahon DG (2006) Circadian organization of the mammalian retina. Proceedings of the National Academy of Sciences of the United States of America 103: 9703-8.

Sandu C, Hicks D, Felder-Schmittbuhl MP (2011) Rat photoreceptor circadian oscillator strongly relies on lighting conditions. The European journal of neuroscience 34: 507-16.

Sayols S, Scherzinger D, Klein H (2016) dupRadar: a Bioconductor package for the assessment of PCR artifacts in RNA-Seq data. BMC bioinformatics 17: 428.

SP Daiger BR, J Greenberg, A Christoffels, W Hide (1998) RetNet, the Retinal Information Network. In University of Texas-Houston Health Science Center.

Storch KF, Paz C, Signorovitch J, Raviola E, Pawlyk B, Li T, Weitz CJ (2007) Intrinsic circadian clock of the mammalian retina: importance for retinal processing of visual information. Cell 130: 730-741.

Stratmann M, Schibler U (2006) Properties, entrainment, and physiological functions of mammalian peripheral oscillators. Journal of biological rhythms 21: 494-506.

Szklarczyk D, Franceschini A, Wyder S, Forslund K, Heller D, Huerta-Cepas J, Simonovic M, Roth A, Santos A, Tsafou KP, Kuhn M, Bork P, Jensen LJ, von Mering C (2015) STRING v10: protein-protein interaction networks, integrated over the tree of life. Nucleic acids research 43: D447-52.

Tang Y, Lu Q, Wei Y, Han L, Ji R, Li Q, Lu Q (2015) Mertk deficiency alters expression of micro-RNAs in the retinal pigment epithelium cells. Metabolic Brain Disease 30: 943-950.

Terman JS, Reme CE, Terman M (1993) Rod outer segment disk shedding in rats with lesions of the suprachiasmatic nucleus. Brain research 605: 256-64.

Tosini G, Menaker M (1996) Circadian rhythms in cultured mammalian retina. Science (New York, NY) 272: 419-21.

Young RW (1971) The renewal of rod and cone outer segments in the rhesus monkey. The Journal of cell biology 49: 303-18.

Young RW (1978) The daily rhythm of shedding and degradation of rod and cone outer segment membranes in the chick retina. Investigative ophthalmology & visual science 17: 105-16.

Zhang DQ, Zhou T, Ruan GX, McMahon DG (2005) Circadian rhythm of Period1 clock gene expression in NOS amacrine cells of the mouse retina. Brain research 1050: 101-9.

Zhang R, Lahens NF, Ballance HI, Hughes ME, Hogenesch JB (2014) A circadian gene expression atlas in mammals: implications for biology and medicine. Proceedings of the National Academy of Sciences of the United States of America 111: 16219-24.

Zheng B, Albrecht U, Kaasik K, Sage M, Lu W, Vaishnav S, Li Q, Sun ZS, Eichele G, Bradley A, Lee CC (2001) Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock. Cell 105: 683-94.

Zheng B, Larkin DW, Albrecht U, Sun ZS, Sage M, Eichele G, Lee CC, Bradley A (1999) The mPer2 gene encodes a functional component of the mammalian circadian clock. Nature 400: 169-73.

SUPPLEMENTARY RESULT

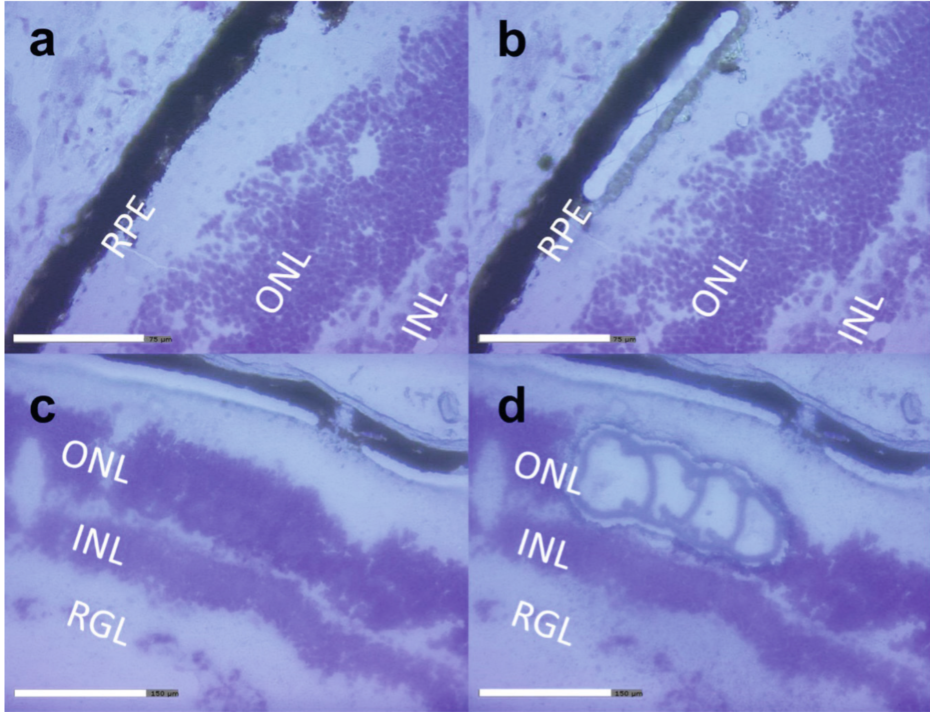


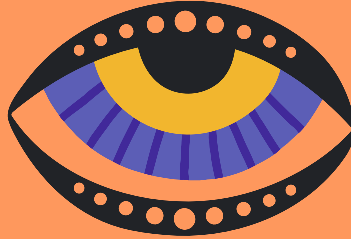
Figure S1. Laser capture microdissection of photoreceptors from WT and *Per1$^{-/-}$Per2^{Brdm1}* mouse eyes. Eyes were cryosectioned at 10 µm thickness. Nuclei were stained using cresyl violet. (a) Micrographs of an eye section before and (b) after laser capture microdissection of the RPE are shown. The scale bar is 75 µm. (c) Micrographs are shown of slice before and (d) after microdissection of photoreceptors. The scale bar is 150 µm. ONL – outer nuclear layer; INL – inner nuclear layer and RGL – retinal ganglion layer.

CHAPTER

Udita Bagchi*
Shumet Gegnaw*
Cristina Sandu
Jacoline B. ten Brink
David Hicks
Marie-Paule Felder-Schmittbuhl
Arthur A. Bergen

**These authors contributed equally to this article*

To be submitted



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

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 24 h. Surprisingly upon aging, we detected no marked decrease in visual sensitivity in the *Per1/Per2* mutants, and no histological changes. Taken together, these data suggest that *Per1/Per2* are indispensable for the rhythm of light processing in photoreceptors but are not essential for retinal health during aging.

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; Sakamoto et al. 2006; 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 (Ruan et al. 2008; Dkhissi-Benyahya et al. 2013; Jaeger et al. 2015; Tosini et al. 2007). 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 (Aït-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) (Bagchi et al. 2020; Sawant et al. 2019); 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 (Aït-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 (*Per2*$^{m/m}$) 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 clock autonomy and the circadian visual processing in mammals.

MATERIALS AND METHODS

All animal experiments were authorized by the French Ministry for Higher Education, Research, and Innovation (APAFIS#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.

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. 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 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.

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 re-exposed or not to light and tested the following day according to protocol 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}, $3{\times}10^{-2}$, 10^{-1}, $3{\times}10^{-1}$, 1, 3, and 10 cd s/m^2.

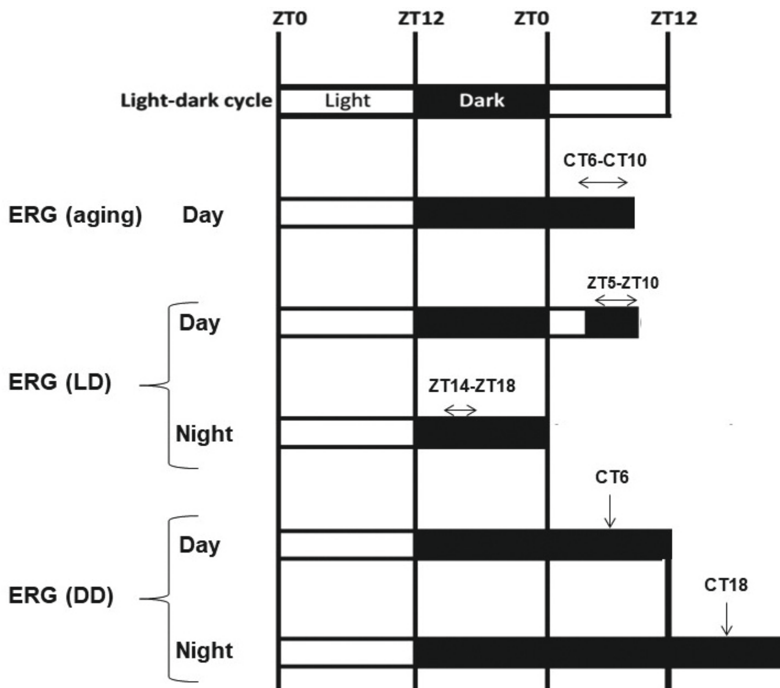


Figure 1. Protocol of light exposure during animal housing (upper line) and the distinct ERG protocols. Effect of aging (8-9, 13-14 and 18 months) was evaluated in the afternoon following overnight dark-adaptation (n = 3 to 6 per genotype). Effect of time of day in LD was analyzed during the night (ZT 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).

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 (Aït-Hmyed 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).

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) (Figure 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) (Figure 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).

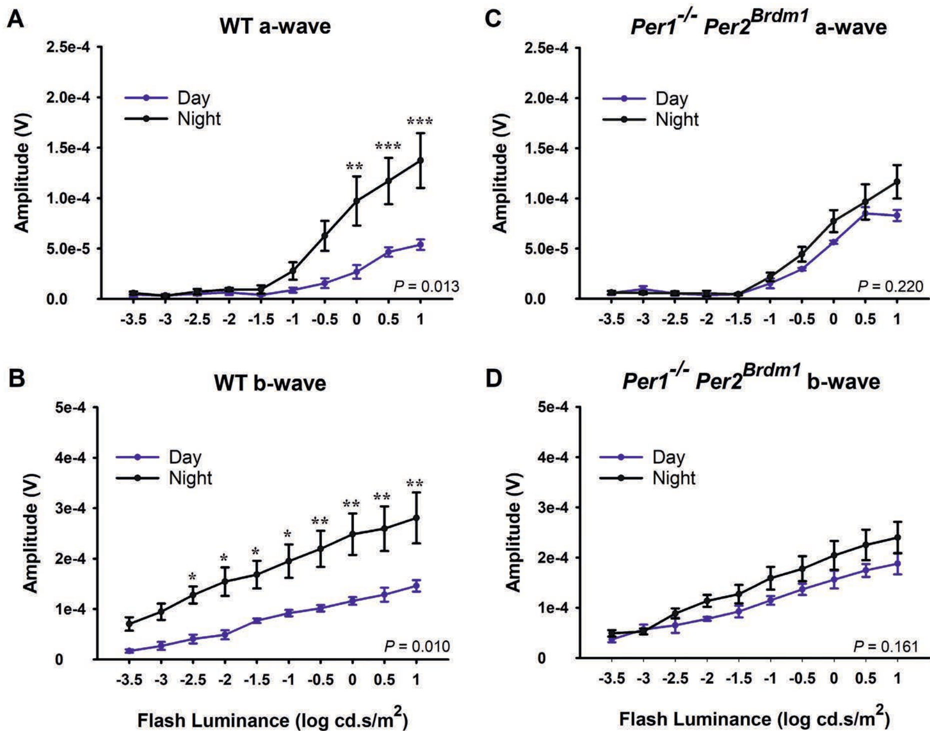


Figure 2. Scotopic ERGs in WT and *Per1*$^{-/-}$*Per2*^{Brdm1} dark-adapted mice in day and night under LD condition. In control mice, the scotopic a- and b-wave amplitudes significantly differed over the LD cycle, with values larger during the night than at day (Figure 2A, B). These day/night differences were reduced and not significant in *Per1*$^{-/-}$*Per2*^{Brdm1} mutant mice (Figure 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) (Figure 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) (Figure 3C and 3D).

These results in WT animals in DD conditions are like what we previously 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).

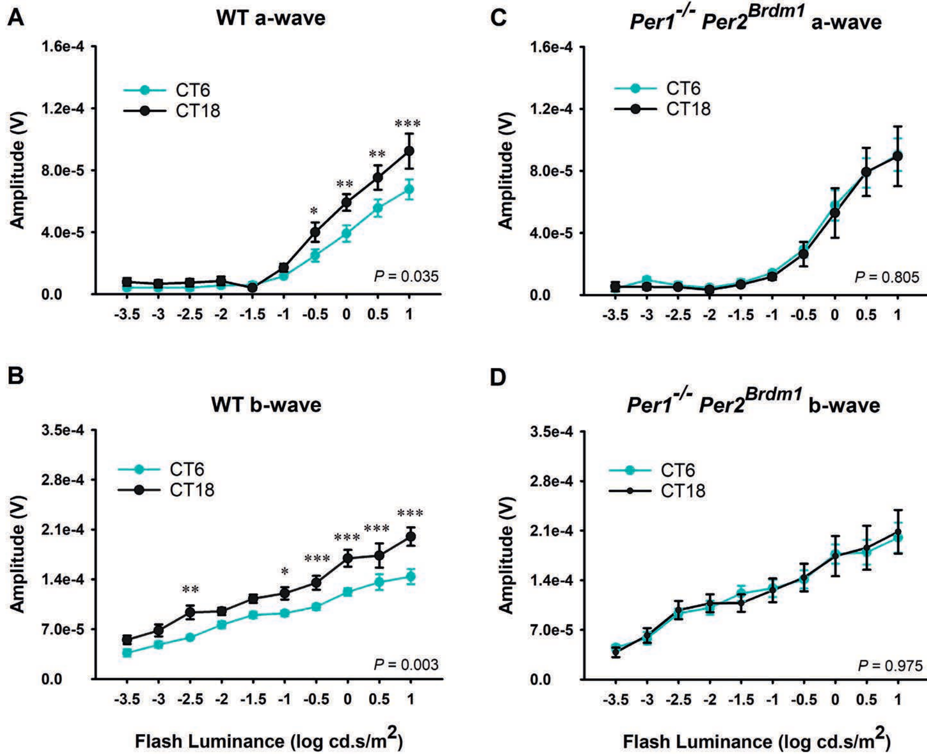


Figure 3. Scotopic ERG a- and b-wave amplitudes are controlled by the circadian clock. The WT mice exhibit significantly larger scotopic a- and b-wave amplitudes at CT18 (black) compared to CT6 (blue). The curves for both a- and b-wave amplitudes showed time of day dependence (specifically at higher 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).

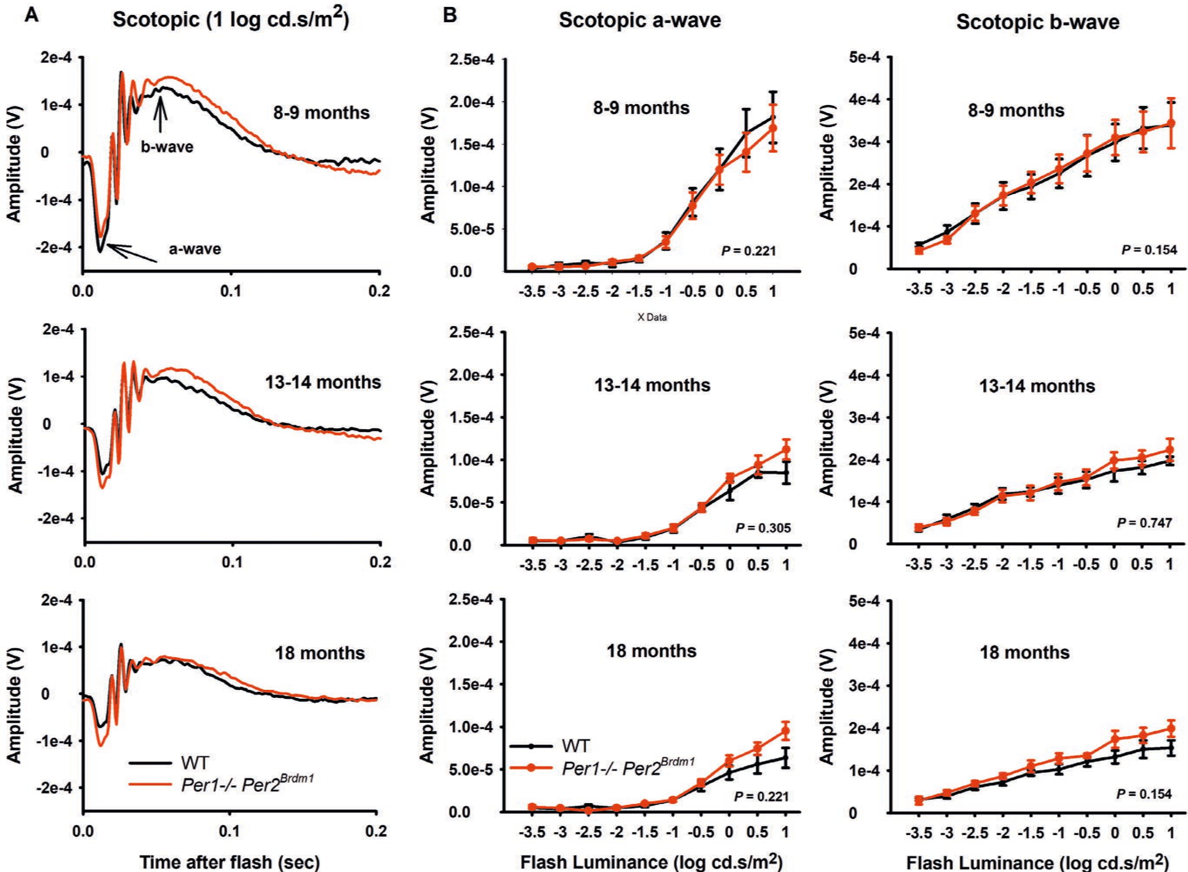
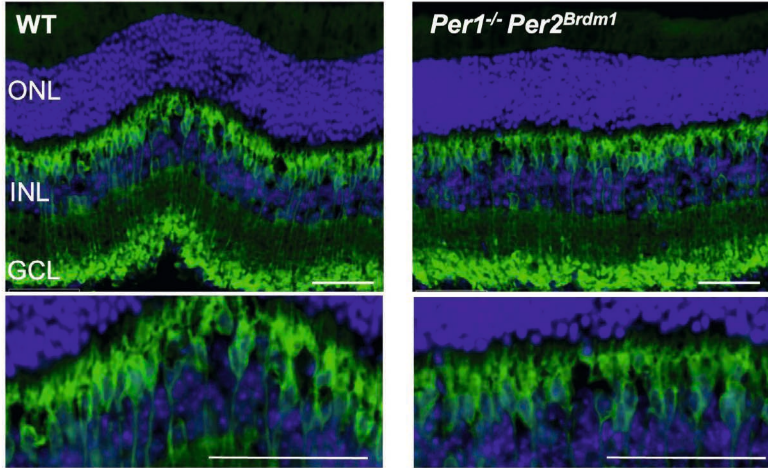
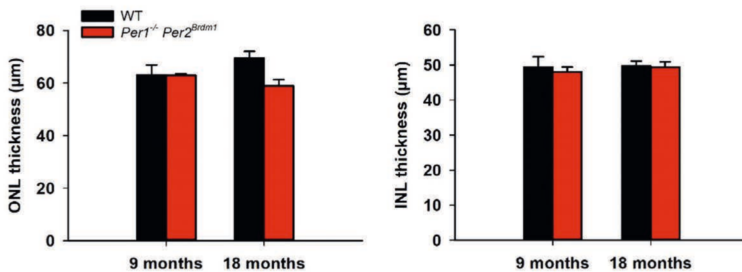


Figure 4. No major effect of aging in *Per1*$^{-/-}$*Per2*^{Brdm1} mutants in scotopic ERG responses. (A) Scotopic ERG average waveforms at 1 log cd·s/m^2 **flash.** These average waveforms were obtained from *Per1*$^{-/-}$*Per2*^{Brdm1} mutant mice (red lines, n= 3-6 per age) and WT (black lines, n= 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).





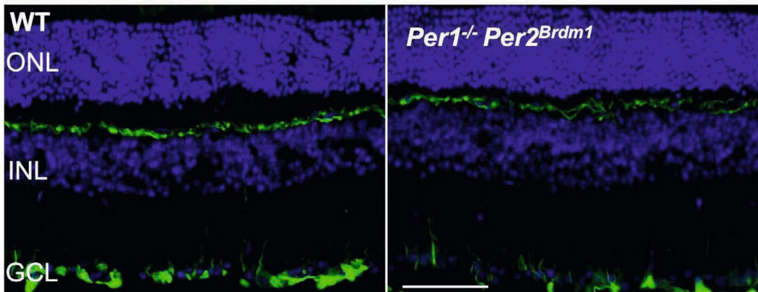


Figure 5. Immunostaining and morphological analysis of WT and mutant retinal sections. (A) Representative micrographs of retinal sections stained with anti-PKCα antibody and higher magnifications of the INL region showing the detail of dendritic processes from rod-bipolar cells in the outer plexiform 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.

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 (Figure 4A, B).

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 (Aït-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).

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 (Rosenwasser et al. 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 whose expression is decreased at CT10 vs CT19 in the WT and that are increased 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 at daytime.

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 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 (de Vera 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 (Milicevic 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 during the night, 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.

Acknowledgements

We thank Dr. Dominique Sage, Dr. Sophie Reibel, and Nicolas Lethenet for animal care. This project has been funded with support from the NeuroTime Erasmus+ of the European Commission and the Centre National pour la Recherche Scientifique, France.

REFERENCES

Ait-Hmyed Hakkari, O., Acar, N., Savier, E., Spinnhirny, P., Bennis, M., Felder- Schmittbuhl, M.P., Mendoza, J., Hicks, D., 2016. Rev-Erbalpha modulates retinal visual processing and behavioral responses to light. Faseb. J. 30, 3690–3701.

Ait-Hmyed O, Felder-Schmittbuhl MP, Garcia-Garrido M, et al. Mice lacking period 1 and period 2 circadian clock genes exhibit blue cone photoreceptor defects. Eur J Neurosci. 2013;37:1048–1060.

Albrecht, U., Zheng, B., Larkin, D., Sun, Z.S. & Lee, C.C. (2001) MPer1 and mPer2 are essential for normal resetting of the circadian clock. J. Biol. Rhythms, 16, 100–104.

B. Zheng, D.W. Larkin, U. Albrecht, Z.S. Sun, M. Sage, G. Eichele, C.C. Lee,A. Bradley, The mPer2 gene encodes a functional component of the mammalian circadian clock, Nature 400 (1999) 169–173.

B. Zheng, U. Albrecht, K. Kaasik, M. Sage, W. Lu, S. Vaishnav, Q. Li, Z.S. Sun,G. Eichele, A. Bradley, C.C. Lee, Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock, Cell 105 (2001) 683–694.

Baba, K., Ribelayga, C.P., Michael Iuvone, P., Tosini, G., 2018a. The retinal circadian clock and photoreceptor viability. Adv. Exp. Med. Biol. 1074, 345–350.

Bagchi, U., Gegnaw, S.T., Milicevic, N., Sandu, C., Ten Brink, J.B., Jongejan, A.,

Hicks, D., Moerland, P.D., Felder-Schmittbuhl, M.P., Bergen, A.A., 2020. Core-clock genes Period 1 and 2 regulate visual cascade and cell cycle components during mouse eye development. Biochim Biophys Acta Gene Regul Mech 1863, 194623.

Barnard, A.R., Hattar, S., Hankins, M.W., Lucas, R.J., 2006. Melanopsin regulates visual processing in the mouse retina. Curr. Biol. 16, 389–395.

Bassi, C.J., Powers, M.K., 1986. Daily fluctuations in the detectability of dim lights by humans. Physiol. Behav. 38, 871–877.

Bobu, C., Sandu, C., Laurent, V., Felder-Schmittbuhl, M.P., Hicks, D., 2013. Prolonged light exposure induces widespread phase shifting in the circadian clock and visual pigment gene expression of the Arvicanthis ansorgei retina. Mol. Vis. 19, 1060–1073.

Brann, M.R., Cohen, L.V., 1987. Diurnal expression of transducin mRNA and translocation of transducin in rods of rat retina. Science 235, 585–587.

Cameron, M.A., Barnard, A.R., Hut, R.A., Bonnefont, X., van der Horst, G.T., Hankins, M. W., Lucas, R.J., 2008. Electroretinography of wild-type and Cry mutant mice reveals circadian tuning of photopic and mesopic retinal responses. J. Biol. Rhythm. 23, 489–501.

DeVera C, Dixon J, Chrenek MA, Baba K, Le YZ, Iuvone PM, Tosini G. The Circadian Clock in the Retinal Pigment Epithelium Controls the Diurnal Rhythm of Phagocytic Activity. Int J Mol Sci. 2022 May 10;23(10):5302.

Di, R., Luo, Q., Mathew, D., Bhatwadekar, A.D., 2019. Diabetes alters diurnal rhythm of electroretinogram in db/db mice. Yale J. Biol. Med. 92, 155–167.

Dkhissi-Benyahya, O., Coutanson, C., Knoblauch, K., Lahouaoui, H., Leviel, V., Rey, C., Bennis, M., Cooper, H.M., 2013. The absence of melanopsin alters retinal clock function and dopamine regulation by light. Cell. Mol. Life Sci. 70, 3435–3447.

Felder-Schmittbuhl, M.P., Buhr, E.D., Dkhissi-Benyahya, O., Hicks, D., Peirson, S.N., Ribelayga, C.P., Sandu, C., Spessert, R., Tosini, G., 2018. Ocular clocks: adapting mechanisms for eye functions and health. Invest. Ophthalmol. Vis. Sci. 59, 4856–4870.

Gegnaw, S.T., Sandu, Cristina, Mendoza, Jorge, Bergen A.A., Felder-Schmittbuhl, M.P., 2021. Dark-adapted light response in mice is regulated by a circadian clock located in rod photoreceptors. Exp. Eye Res., Vol. 213, 108807, ISSN 0014-4835.

Gustincich, S., Contini, M., Gariboldi, M., Puopolo, M., Kadota, K., Bono, H., LeMieux, J., Walsh, P., Carninci, P., Hayashizaki, Y., Okazaki, Y., Raviola, E., 2004. Gene discovery in genetically labeled single dopaminergic neurons of the retina. Proc. Natl. Acad. Sci. U. S. A. 101, 5069–5074.

Jadhav V, Luo Q, M. Dominguez J 2nd, Al-Sabah J, Chaqour B, et al. (2016) Per2-Mediated Vascular Dysfunction Is Caused by the Upregulation of the Connective Tissue Growth Factor (CTGF). PLOS ONE 11(9): e0163367.

Jaeger, C., Sandu, C., Malan, A., Mellac, K., Hicks, D., Felder-Schmittbuhl, M.P., 2015. Circadian organization of the rodent retina involves strongly coupled, layer-specific oscillators. Faseb. J. 29, 1493–1504.

Kunst, S., Wolloscheck, T., Hölter, P., Wengert, A., Grether, M., Sticht, C., Weyer, V., Wolfrum, U., Spessert, R., 2013. Transcriptional analysis of rat photoreceptor cells reveals daily regulation of genes important for visual signaling and light damage susceptibility. J. Neurochem. 124, 757–769.

Liu, X., Zhang, Z., Ribelayga, C.P., 2012. Heterogeneous expression of the core circadian clock proteins among neuronal cell types in mouse retina. PLoS One 7, e50602.

McMahon, D.G., Iuvone, P.M., Tosini, G., 2014. Circadian organization of the mammalian retina: from gene regulation to physiology and diseases. Prog. Retin. Eye Res. 39, 58–76.

Milicevic, N., Ait-Hmyed Hakkari, O., Bagchi, U., Sandu, C., Jongejan, A., Moerland, P. D., Ten Brink, J.B., Hicks, D., Bergen, A.A., Felder-Schmittbuhl, M.P., 2021. Core circadian clock genes Per1 and Per2 regulate the rhythm in photoreceptor outer segment phagocytosis. Faseb. J. 35, e21722.

Rosenwasser, A.M., Raibert, M., Terman, J.S., Terman, M., 1979. Circadian rhythm of luminance detectability in the rat. Physiol. Behav. 23, 17–21.

Ruan, G.X., Allen, G.C., Yamazaki, S., McMahon, D.G., 2008. An autonomous circadian clock in the inner mouse retina regulated by dopamine and GABA. PLoS Biol. 6, e249.

Ruan, G.X., Zhang, D.Q., Zhou, T., Yamazaki, S., McMahon, D.G., 2006. Circadian organization of the mammalian retina. Proc. Natl. Acad. Sci. U. S. A. 103, 9703–9708.

Sakamoto, K., Liu, C., Kasamatsu, M., Iuvone, P.M., Tosini, G., 2006. Intraocular injection of kainic acid does not abolish the circadian rhythm of arylalkylamine N- acetyltransferase mRNA in rat photoreceptors. Mol. Vis. 12, 117–124.

Sandberg, M.A., Pawlyk, B.S., Berson, E.L., 1986. Electroretinogram (ERG) sensitivity and phagosome frequency in the normal pigmented rat. Exp. Eye Res. 43, 781–789.

Sawant, O.B., Jidigam, V.K., Fuller, R.D., Zucaro, O.F., Kpegba, C., Yu, M., Peachey, N.S., Rao, S., 2019. The circadian clock gene Bmal1 is required to control the timing of retinal neurogenesis and lamination of Müller glia in the mouse retina. Faseb. J. 33, 8745–8758.

Sengupta, A., Baba, K., Mazzoni, F., Pozdeyev, N.V., Strettoi, E., Iuvone, P.M., Tosini, G., 2011. Localization of melatonin receptor 1 in mouse retina and its role in the circadian regulation of the electroretinogram and dopamine levels. PLoS One 6, e24483.

Storch, K.F., Paz, C., Signorovitch, J., Raviola, E., Pawlyk, B., Li, T., Weitz, C.J., 2007. Intrinsic circadian clock of the mammalian retina: importance for retinal processing of visual information. Cell 130, 730–741.

Tanimoto, N., Muehlfriedel, R.L., Fischer, M.D., Fahl, E., Humphries, P., Biel, M., Seeliger, M.W., 2009. Vision tests in the mouse: functional phenotyping with electroretinography. Front. Biosci. 14, 2730–2737.

Terman, J.S., Reme, C.E., Terman, M., 1993. Rod outer segment disk shedding in rats with lesions of the suprachiasmatic nucleus. Brain Res. 605, 256–264.

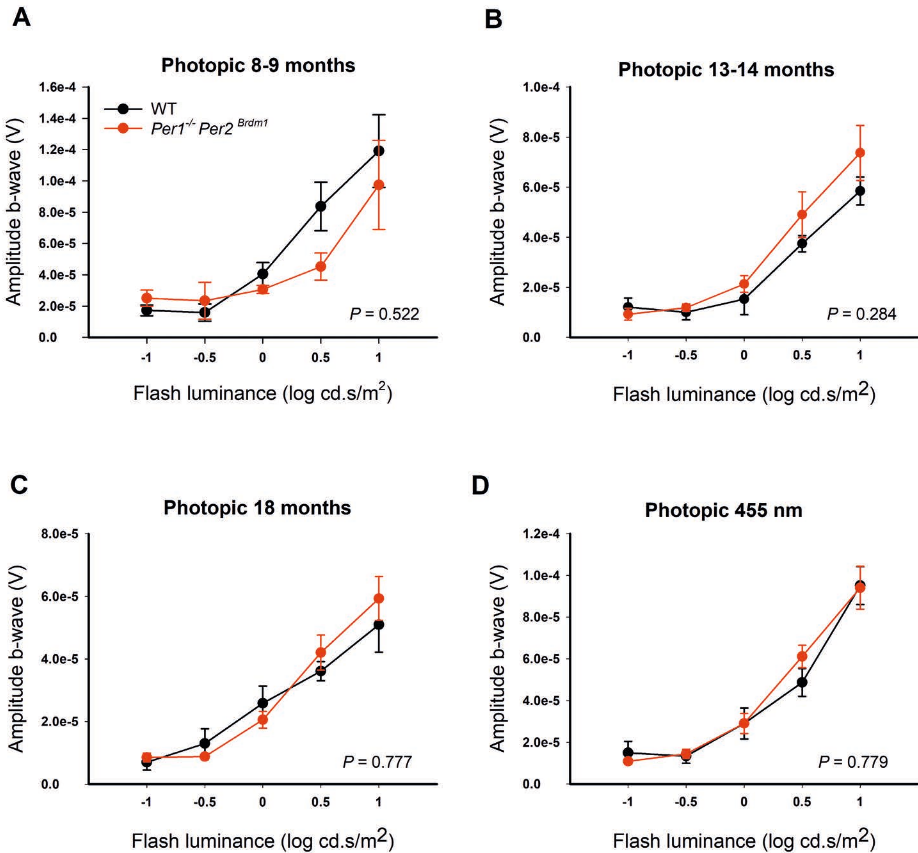
Tosini, G., Davidson, A.J., Fukuhara, C., Kasamatsu, M., Castanon-Cervantes, O., 2007. Localization of a circadian clock in mammalian photoreceptors. Faseb. J. 21, 3866–3871.

Tosini, G., Menaker, M., 1996. Circadian rhythms in cultured mammalian retina. Science 272, 419–421.

von Schantz, M., Lucas, R.J., Foster, R.G., 1999. Circadian oscillation of photopigment transcript levels in the mouse retina. Brain Res Mol Brain Res 72, 108–114.

Wong, J.C.Y., Smyllie, N.J., Banks, G.T., Pothecary, C.A., Barnard, A.R., Maywood, E.S.,Jagannath, A., Hughes, S., van der Horst, G.T.J., MacLaren, R.E., Hankins, M.W., Hastings, M.H., Nolan, P.M., Foster, R.G., Peirson, S.N., 2018. Differential roles for cryptochromes in the mammalian retinal clock. Faseb. J. 32, 4302–4314.

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.

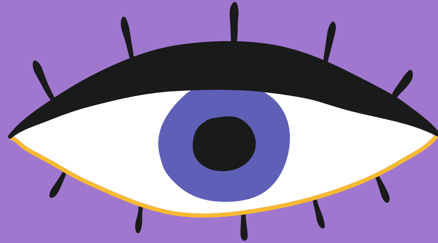
PART

The clinical implications of circadian clock system in humans

CHAPTER

Udita Bagchi
Shazia Micheal
Sorath Noorani Siddiqui
Muhammad Imran Khan
Jacoline B. ten Brink
Marie-Paule Felder-Schmittbuhl
Arthur A. Bergen

Clin. Invest. (Lond.) (2019) 9(1), 1-3
ISSN 2041-6792



Mutation analysis of circadian clock gene *BMAL1* in 21 Pakistani congenital cataract families

ABSTRACT

Background

Given the cataract phenotype in *Bmal1*$^{-/-}$ mice, we aimed to identify potential disease-causing variants in the human circadian clock candidate gene *BMAL1* in 21 probands of consanguineous Pakistani congenital cataract families.

Methods

Ophthalmic examinations were performed for the probands and available family members. Genomic DNA was isolated from a volume of 5 ml of peripheral blood. The entire coding region of the candidate gene *BMAL1* was analyzed in the probands of 21 families with targeted Sanger sequencing.

Results

A heterozygous missense variant c.41A>T; p.(Asp14Val) was detected in 1 of the 21 patients, which has a rare allele frequency of 0.000065 (2/30576 individuals) exclusively in the South-Asian population. The variant did not co-segregate with the disease phenotype in the family. A non-synonymous variant (rs2290037) in the heterozygous state was also identified in 5 out of 21 probands with a higher allele frequency of 0.1190 as compared to the global population (0.06626; 15570/234984 individuals).

Conclusion

Our study is the first to investigate the core circadian clock gene *BMAL1* in humans for their association with congenital cataract. Unfortunately, no clear association between human genetic *BMAL1* variants and cataract was found. Compared with targeted NGS technologies, traditional Sanger sequencing remains an indispensable cost-effective tool specially to report mutation profiles in small study cohorts. Our study may act to guide further studies in the molecular clockwork pathway from other (disease) populations.

Keywords: congenital cataract, consanguineous, circadian, clockwork, infantile, mutation

BACKGROUND

Congenital or infantile cataract is defined by the opacity of the crystalline lens resulting in the (predisposition for) partial to complete pediatric visual disability. The World Health Organization (WHO) describes cataract as the primary cause of blindness throughout the world affecting 16 million people worldwide (Thylefors et al. 1995). The incidence of congenital cataract (CC) is estimated to be 1–6 cases per 10,000 live births in developed countries, and 5–15 cases per 10,000 in the underdeveloped countries (Apple et al. 2000). Approximately 200,000 children every year are affected by lifelong vision impairment due to congenital cataract (Foster et al. 1997). Inherited cataracts represent a significant contribution to CC (Stoll et al. 1992; Gregg 1991; Blohme and Tornqvist 1997). Currently, over 48 genes have been delineated in the Cat-Map database (**https://cat-map.wustl.edu/**) underlying the pathogenesis of congenital cataract. Nearly 50 % of the disease is accounted for by mutations in the crystalline genes (Sun et al. 2017; Bateman et al. 2007). Connexin genes comprise approximately a share of 25% (AlFadhli et al. 2012; Shen et al. 2017; Mohebi et al. 2017) along with the causative gene mutations described in other structural proteins, namely beaded filament structural protein 2 (*BFSP2*) (Berthoud and Ngezahayo 2017), lens intrinsic membrane protein (*LIM2*) (Jakobs et al. 2000; Irum et al. 2016), aquaporin0 (*MIP*) (Pras et al. 2004; Berry et al. 2000), enzymes like glucosaminyl (N-acetyl) transferase 2 (*GCNT2*) (Irum et al. 2016) and in transcription factors such as paired-like homeodomain 3 (*PITX3*) (Qin et al. 2016), avian musculoaponeurotic fibrosarcoma (*MAF*) (Semina et al. 1998), heat shock transcription factor 4 gene (*HSF4*) (Vanita et al. 2006).

Identification of new genetic mutations in cataract patients will improve our understanding of cataractogenesis during childhood and could provide further insights into lens biology. There is mounting experimental evidence that suggests a direct or indirect involvement of the circadian clock in cataract (Behnam et al. 2015; Khorsand et al. 2016; Yan and Wang 2016; Nishi et al. 2015; Saeki et al. 2014; Bai et al. 2013). Circadian rhythms are 24-hour temporal programmes, widely distributed in mammalian tissues and synchronized by a master-hypothalamic clock (Ostrin 2018). In mammals, the core clock genes, including *Bmal1*, *Clock*, *Cry*, and *Per*, are rhythmically expressed in the suprachiasmatic nucleus (SCN) - the master clock in the hypothalamus and in almost all peripheral cells/tissues, including the lens and retina of the eyes (Ostrin 2018). The clock transcription factors control the expression of numerous target genes in a circadian manner, influencing many physiological and biochemical processes (Ko and Takahashi 2006), including those in the eye (Dibner et al. 2010). Clock factors act upstream of or in cooperation with tissue-specific transcription factors to temporally modulate RNA polymerase II loading, histone modification or three-dimensional conformation of the

chromatin (Felder-Schmittbuhl et al. 2018). Thus, cycling transcriptomes are rather tissue-specific (Vakili et al. 2016). In addition, the clock orchestrates the temporal expression of genes during the development of the eye *per se* (Lech et al. 2016; Curran et al. 2008; Kobayashi et al. 2015; Sawant et al. 2017). Notably, circadian clock genes *Bmal1* and *Clock* have been observed to be involved in the pathophysiology of cataract in mice (Vallone et al. 2007; Kondratov et al. 2006; Kondratov et al. 2009). It has been reported that genes implicated in cataract development in humans, may also be the key players in animal models like rodents and vice versa (Dubrovsky et al. 2010; Graw 2009). Daily variations in levels of crystalline mRNAs and proteins in the retinal photoreceptor cells of rats highlight the role of circadian processes in retinal crystalline synthesis (Churchill and Graw 2011). In humans, decreased potential for circadian photo-entrainment is known to be associated with cataract development (Organisciak et al. 2011). Moreover, visual acuity and circadian photoreception via the photosensitive retinal ganglion cells is profoundly impaired in cataract patients (Kessel et al. 2011; Brondsted et al. 2015; Gimenez et al. 2016). Finally, progressive loss of vision leading to blindness fails to render the required input signals to the biological clock (Turner et al. 2008; Skene et al. 1999a). These data prompted us to investigate the possible association between cataract development and the clock.

The primary transcriptional regulator of the circadian clock, the brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein BMAL1 is implicated in the regulation of early to premature ocular aging (Vallone et al. 2007; Kondratov et al. 2006; Skene et al. 1999b). More than 50 % of *Bmal1* deficient mice developed cataract before the 40th week of life (Vallone et al. 2007). Also, deletion of BMAL1 disrupts clock-dependent oscillatory gene expression and behavioral rhythmicity coincident with eye pathologies, reduced body weight, impaired hair growth, abnormal bone calcification, neurodegeneration, and a shortened lifespan (Skene et al. 1999b; Yang et al. 2016; Geyfman and Andersen 2010; McDearmon et al. 2006; Samsa et al. 2016). Recently, the conditional deletion of BMAL1 in endothelial and hematopoietic cells of the murine retina (Bhatwadekar et al. 2017) demonstrated pathologic hallmarks of diabetic retinopathy, thereby expanding on the ocular pathology because of molecular BMAL1 defects. It has been identified that BMAL1-mediated activation of the DNA repair system can render remedial mechanisms for treating photodamage, including photoaging (Joo et al. 2018).

Due to the heterogeneous nature of congenital cataracts, the involvement of additional genetic and environmental factors cannot be dismissed. In this study, we aimed to identify the disease-causing variants in the *BMAL1* gene associated with the CC phenotype in the consanguineous Pakistani families to explore any existing links between the circadian clock and ocular abnormalities.

MATERIALS AND METHODS

Subjects

The patients were recruited at the pediatric ophthalmology department of Al-Shifa Eye Trust Hospital, Rawalpindi, Pakistan. The study was approved by the Institutional Review Board of the Al-Shifa Eye Trust Hospital (Rawalpindi, Pakistan), and adhered to the tenets of the Declaration of Helsinki with the approval code PK2014:102. Written informed consent was obtained for study participation from the participants and/or their parents, as appropriate. Comprehensive, ocular, medical, and family histories were obtained from the parents/available family member. A detailed ophthalmic examination was performed for both affected and unaffected individuals of families. Blood samples were collected from affected and unaffected siblings, and from the parents. Genomic DNA was extracted using QIAGEN DNA Blood Midi Kit (QIAGEN, Germantown, Maryland, USA).

PCR and Sanger Sequencing

PCR amplification of the 16 coding exons of the *BMAL1* gene was performed in the (n=21) probands of the consanguineous cataract families using a PE 9700 thermocycler (Applied Biosystems, Foster City, CA). Primers for the *BMAL1* gene (NM_001351814.1) were designed using Primer 3 (Yu and Weaver 2011) (**http://bioinfo.ut.ee/primer3-0.4.0/**) to cover exon/intron boundaries up to 100 base pairs into introns and are presented in Table 1. All amplicons were subjected to the following cycling conditions: initial denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 64 °C for 30s, and 72 °C for 30 s. PCR products were analyzed on 2% agarose gels followed by Sanger sequencing using ABI BigDye chemistry (Applied Biosystems Inc., Foster City, CA, USA), and were processed through an automated ABI 3730 Sequencer (Applied Biosystems, Inc.) using standard protocols.

Table 1: Primer sequences of *BMAL1 (ARNTL)*.

Primer Name	Sequence 5′-3′	Product size
ARNTL_E5_Forward	GCTCTTTCCATTCTATCACATGC	476
ARNTL_E5_Reverse	TGTCGCCACCTAGAGTTGG	
ARNTL_E6-7_Forward	TGGCTGTTCGAACTTTATGA	567
ARNTL_E6-7_Reverse	ACAGGACCAAACATGCAGAG	
ARNTL_E8_Forward	TGCCTTGTCAGATGAACATTGA	568
ARNTL_E8_Reverse	GGCATACTACTGAAGGCTACAT	
ARNTL_E9_Forward	AGAGACTAGGCCACTTACAGA	345
ARNTL_E9_Reverse	AGAAATGTGAAGCCTGTCCA	
ARNTL_E10_Forward	TCCTGTGCTTTGGATGCTT	442
ARNTL_E10_Reverse	TGCAGCAATAGAAGAAAGCCA	
ARNTL_E11_Forward	AACCTCCAGATGCCTCCTTC	456
ARNTL_E11_Reverse	GCCAAAGATAGCTCTGGTGC	
ARNTL_E12_Forward	AGTGAGGCAGGCAAGAAAAG	390
ARNTL_E12_Reverse	AGCCAGAAACCATGGAACC	
ARNTL_E13_Forward	TCCCTACCTACATCCCATCC	487
ARNTL_E13_Reverse	TTCTTAGAAAAGCCAGCTGATG	
ARNTL_E14_Forward	GCAGCTTTGACCTTGCTCTC	347
ARNTL_E14_Reverse	GGCTGGCTGACTCTACATCC	
ARNTL_E15_Forward	CTAAAGAGCGATGTCGTTGG	415
ARNTL_E15_Reverse	AGCTTCTGCCAGTCCTGAG	
ARNTL_E16_Forward	ACCTCTGCTGAACTGTGTCC	470
ARNTL_E16_Reverse	GAAATCCGCACATCATCC	
ARNTL_E17_Forward	ACTGCAAATGGATCATGGGA	383
ARNTL_E17_Reverse	TGTTTAACAAGCAGCATCCCT	
ARNTL_E18_Forward	GCTTGCCAAACCCTAATCTAGAT	349
ARNTL_E18_Reverse	CCTCACACAGATGCATTTACTTC	
ARNTL_E19_Forward	AGAAAACTGAAGCCATTTGAAGC	399
ARNTL_E19_Reverse	CTCCACCAAAACTCAAATACTGG	
ARNTL_E20_Forward	AAGCAGCATCTCACCCTACC	423
ARNTL_E20_Reverse	TCAATGGCTCTGAGATGGCT	

Data processing

The obtained sequences were aligned with the reference sequence (NM_001351814.1) using CodonCode Aligner (version 6.1) (CodonCode Co., Centerville, MA, USA). Intra-familial segregation analysis was also performed upon the identification of variant in the exon 5 of the *BMAL1* gene in the respective family.

Potential pathogenicity of the identified DNA variants was evaluated by publicly available tools including PhyloP, Grantham and polymorphism phenotyping v-2 (PolyPhen-2) (version 2.1.0 r367) (**http://genetics.bwh.harvard.edu/pph2/**), MutationTaster (**http://www.mutationtaster.org/**), and sorting intolerant from tolerant (SIFT, **http://sift.bii.a-star.edu.sg/**) to predict the functional impact of the sequence variants on the encoded protein. To determine the amino acid conservation among different species, protein sequences were obtained from UniProt (**https://www.uniprot.org/uniprot/O00327**) database. Kalign (2.0) was used for the multiple nucleotide and amino acid sequence alignment.

RESULTS

Mutation detection

All coding sequences of *BMAL1* of 21 cataract probands were screened. In one proband, we identified a c.41A>T; p.(Asp14Val) variant; in five probands, we detected a non-synonymous variant (rs2290037). The remainder of the sequences were wild-type.

In one proband, a *BMAL1* c.41A>T; p.(Asp14Val) a missense variant in exon 5 was present in a heterozygous form (Figure 1B). This particular variant c.41A>T; p.(Asp14Val) was bioinformatically predicted to be deleterious by SIFT, damaging by PolyPhen-2 and disease-causing by Mutation Taster. The wildtype nucleotide and amino acid residues were highly conserved with a PhyloP score of 4.73, and a grantham score 152 respectively. The nucleotide and amino acid residues were found to be highly conserved among different orthologous species (Figure 1C and 1D). The p.Asp14Val variant was present with a rare allele frequency of 0.000065 (2/30576 individuals) exclusively in the South-Asian population (**https://www.uniprot.org/uniprot/O00327**). Segregation analysis of the variant was performed, but the c.41A>T; p.(Asp14Val) variant did not co-segregate with the disease phenotype in the family (Figure 1A).

In addition, we detected a non-synonymous variant (rs2290037) in *BMAL1* intron 7 in 5 out of 21 probands. In our patient set, the variant occurred with a much higher allele frequency of 0.1190 than the general population.

Figure 1

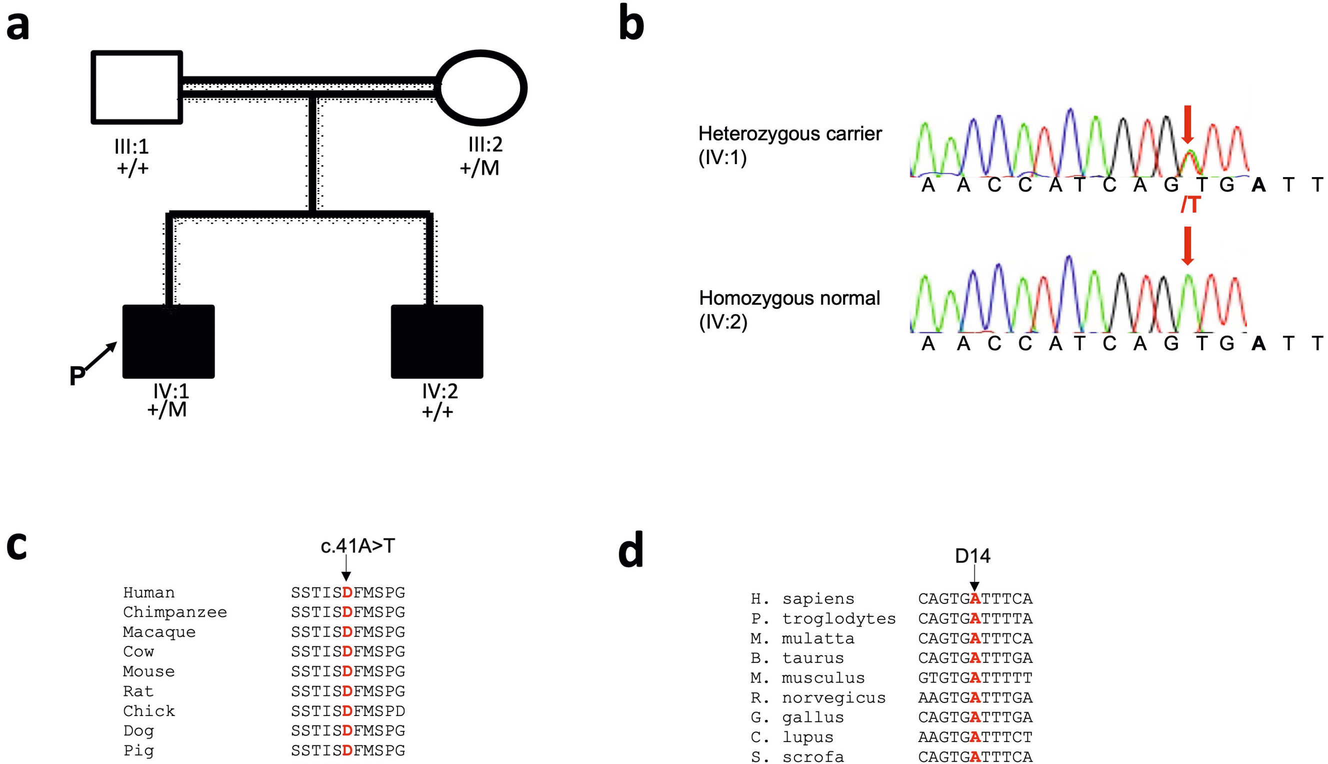


Figure 1: (A) Pedigree and non-segregation of a missense mutation c.41A>T; p. (Asp14Val) in the BMAL1 gene in a recessive congenital cataract family; **(B) DNA chromatogram of the BMAL1 fragment** for the affected individual (IV:1) carrying heterozygous genetic mutation; **(C,D) Multiple sequence alignment** showing the nucleotide and amino acid conservation in different species for the c.41A>T; p. (Asp14Val). The wild-type nucleotide (D) and amino acid (A) are indicated with an arrow and in red color.

DISCUSSION AND CONCLUSION

Circadian clock genes influence disease susceptibility due to their pleiotropic activities on gene expression by involvement in multiple pathways or via direct involvement with circadian clock function (http://gnomad.broadinstitute.org). Earlier studies have reported *BMAL1* gene variants in humans contributing to fertility and seasonality (Kovanen et al. 2010) as well as in hypertension and type-2 diabetes (Woon et al. 2007). Given the occurrence of cataract in *Bmal1*-/- and *Clock*-/- mice, we tested the hypothesis that human *BMAL1* mutations could be involved in human cataract.

In this study, we showed that genetic variations in *BMAL1* in 21 patients with CC disease did not account for the disease phenotype. Although the identified missense variant c.41A>T; p.(Asp14Val) altered the wild-type amino acid sequence, occurred in a highly evolutionary conserved residue, and was also determined to be “probably pathogenic” by bioinformatics, it happened in the heterozygous state in a single patient and did not segregate in the family. Thus, we could not correlate the Asp14Val variant with the occurrence of congenital cataract. We did not exclude potential additional pathogenic mutations in our CC probands occurring outside of the coding exons and the flanking intron splice sites. A non-synonymous heterozygous variant (rs2290037) with a higher allele frequency was detected in the intronic region. It has been previously estimated that 5% of the rare non-synonymous heterozygous variants carry at least ~22 pathogenic derived alleles, which if turns out to be homozygous due to consanguineous marriages, can lead to recessive diseases (Li et al. 2013).

To the best of our knowledge, our study is the first attempt to evaluate the presence of genetic variants in the *BMAL1* gene for congenital cataract. The samples were identified from a well-characterized epidemiological cohort, which has a high degree of genetic heterogeneity (Chen et al. 2017). Based on results in the mice, it will be apt to state that the human circadian clock genes may not be such an attractive target for mutation analysis in cataract families. Yet, we cannot entirely exclude the involvement of *BMAL1* in human cataract. *BMAL1* gene is an intricate member of the clockwork web, and since *BMAL1* is not a sole member, *BMAL1-CLOCK* complex drives the clockwork machinery. Therefore, some more specific, and additional screening of congenital cataract patients with (other) circadian clock genes (sequences) may be justified in subsequent studies.

Conflict of Interest
The authors declare no competing conflict of interests.

Additional Funding

The study was supported by the Algemene Nederlandse Vereniging ter Voorkoming van Blindheid.

Availability of Data and Materials

All relevant datasets are used in the manuscript. The analyzed data is available from the corresponding author upon request.

Authors' Contributions

U.B and A.A.B.B. conceived and designed the experiments; U.B. performed the experiments; S.M, M.I and S.N.S. recruited patients and collected samples; J.B, M.P.F.S., and A.A.B.B. contributed reagents/materials/analysis tools; and U.B. wrote the manuscript. All authors have read and approved the final manuscript.

Ethics Approval and Consent to Participate

The study was approved by the Institutional Review Board of the Al-Shifa Eye Trust Hospital (Rawalpindi, Pakistan), adhering to the tenets of the Declaration of Helsinki with the approval code PK2014:102.

Consent for Publication

The family members of the probands signed a written informed consent form for publication of all data.

Acknowledgments

The authors thank the family members for their cooperation during the study. The entire study is funded and supported by the European Research Council (European Commission) under Erasmus Mundus Project number: 520124-1-2011-1-FR-ERA Mundus-EPJD, FPA: 2012-0026.

REFERENCES

AlFadhli S, Abdelmoaty S, Al-Hajeri A, Behbehani A, Alkuraya F. Novel crystallin gamma B mutations in a Kuwaiti family with autosomal dominant congenital cataracts reveal genetic and clinical heterogeneity. *Mol Vis* 8: 2931-2936 (2012).

Apple DJ, Ram J, Foster A, Peng Q. Elimination of cataract blindness: A global perspective entering the new millenium. *Surv Ophthalmol* 45: 196 (2000).

Bai J, Dong L, Song Z, et al. The role of melatonin as an antioxidant in human lens epithelial cells. *Free Radic Res* 47: 635-642 (2013).

Bateman JB, von-Bischhoffshaunsen FR, Richter L, Flodman P, Burch D, Spence MA. Gene conversion mutation in crystallin, beta-B2 (CRYBB2) in a Chilean family with autosomal dominant cataract. *Ophthalmol* 114: 425-432 (2007).

Behnam M, Imagawa E, Chaleshtori ARS, et al. A novel homozygous mutation in HSF4 causing autosomal recessive congenital cataract. *J Hum Genet* 61: 177 (2015).

Berry V, Francis P, Kaushal S, Moore A, Bhattacharya S. Missense mutations in MIP underlie autosomal dominant 'polymorphic' and lamellar cataracts linked to 12q. *Nat Genet* 25: 15-17 (2000).

Berthoud VM, Ngezahayo A. Focus on lens connexins. *BMC Cell Biol* 18: 6 (2017).

Bhatwadekar AD, Beli E, Diao Y, et al. Conditional deletion of Bmal1 accentuates microvascular and macrovascular injury. *Am J Pathol* 187: 1426-1435 (2017).

Blohme J, Tornqvist K. Visual impairment in Swedish children: III. Diagnoses. *Acta Ophthalmol Scand* 75: 681-687 (1997). https://cat-map.wustl.edu/.

Brondsted AE, Sander B, Haargaard B, et al. The effect of cataract surgery on circadian photoentrainment: a randomized trial of blue-blocking versus neutral intraocular lenses. *Ophthalmol* 122: 2115-2124 (2015).

Chen J, Wang Q, Cabrera PE, et al. Molecular genetic analysis of Pakistani families with autosomal recessive congenital cataracts by homozygosity screening. *Invest Ophthalmol Vis Sci* 58: 2207-2217 (2017).

Churchill A, Graw J. Clinical and experimental advances in congenital and paediatric cataracts. *Philos Trans R Soc Lond B Biol Sci* 366: 1234-1249 (2011).

Curran KL, LaRue S, Bronson B, et al. Circadian genes are expressed during early development in Xenopus laevis. *PLoS One* 3: e2749 (2008).

Dibner C, Schibler U, Albrecht U. The mammalian circadian timing system: Organization and coordination of central and peripheral clocks. *Annu Rev Physiol* 72: 517-549 (2010).

Dubrovsky YV, Samsa WE, Kondratov RV. Deficiency of circadian protein CLOCK reduces lifespan and increases age- related cataract development in mice. *Aging (Albany NY)* 2: 936- 944 (2010).

Felder-Schmittbuhl MP, Buhr ED, Dkhissi-Benyahya O, et al. Ocular clocks: Adapting mechanisms for eye functions and health. *Invest Ophthalmol Vis Sci* 59: 4856-4870 (2018).

Foster A, Gilbert C, Rahi J. Epidemiology of cataract in childhood: A global perspective. *J Cataract Refract Surg* 23: 601- 604 (1997).

Geyfman M, Andersen B. Clock genes, hair growth and aging. *Aging (Albany NY)* 2: 122-128 (2010).

Gimenez M, Beersma D, Daan S, et al. Melatonin and sleep- wake rhythms before and after ocular lens replacement in elderly humans. *Biology (Basel)* 5 (2016).

Graw J. Mouse models of cataract. *J Genet* 88: 469-486 (2009).

Gregg NM. Congenital cataract following German measles in the mother 1941. *Epidemiol Infect* 170: 3-14 (1991).

http://bioinfo.ut.ee/primer3-0.4.0/

http://genetics.bwh.harvard.edu/pph2/

http://gnomad.broadinstitute.org

http://sift.bii.a-star.edu.sg/

http://www.mutationtaster.org/

https://www.uniprot.org/uniprot/O00327

Irum B, Khan SY, Ali M, et al. Mutation in LIM2 is responsible for autosomal recessive congenital cataracts. *PloS One* 11: e0162620 (2016).

Jakobs PM, Hess JF, FitzGerald PG, Kramer P, Weleber RG, Litt M. Autosomal-dominant congenital cataract associated with a deletion mutation in the human beaded filament protein gene BFSP2. *Am J Hum Genet* 66: 1432-1436 (2000).

Joo JH, Hong IK, Kim NK, Choi E. Trichosanthes kirilowii extract enhances repair of UVB radiation-induced DNA damage by regulating BMAL1 and miR1423p in human keratinocytes. *Mol Med Rep* 17: 877-883 (2018).

Kessel L, Siganos G, Jorgensen T, Larsen M. Sleep disturbances are related to decreased transmission of blue light to the retina caused by lens yellowing. *Sleep* 34: 1215-1219 (2011).

Khorsand M, Akmali M, Sharzad S, Beheshtitabar M. Melatonin reduces cataract formation and aldose reductase activity in lenses of streptozotocin-induced diabetic rat. *Iran J Med Sci* 41: 305-313 (2016).

Ko CH, Takahashi JS. Molecular components of the mammalian circadian clock. *Hum Mol Genet* 15: R271-R277 (2006).

Kobayashi Y, Ye Z, Hensch TK. Clock genes control cortical critical period timing. *Neuron* 86: 264-275 (2015).

Kondratov RV, Kondratova AA, Gorbacheva VY, Vykhovanets OV, Antoch MP. Early aging and age-related pathologies in mice deficient in BMAL1, the core componentof the circadian clock. *Genes Dev* 20: 1868-1873 (2006).

Kondratov RV, Vykhovanets O, Kondratova AA, Antoch MP. Antioxidant N-acetyl-L-cysteine ameliorates symptoms of premature aging associated with the deficiency of the circadian protein BMAL1. *Aging (Albany NY)* 1: 979-987 (2009).

Kovanen L, Saarikoski ST, Aromaa A, Lonnqvist J, Partonen T. ARNTL (BMAL1) and NPAS2 gene variants contribute to fertility and seasonality. *PLoS One* 5: e10007 (2010).

Lech K, Ackermann K, Revell VL, Lao O, Skene DJ, Kayser M. Dissecting daily and circadian expression rhythms of clock- controlled genes in human blood. *J Biol Rhythms* 31: 68-81 (2016).

Li MX, Kwan JS, Bao SY, et al. Predicting mendelian disease- causing non-synonymous single nucleotide variants in exome sequencing studies. *PLoS Genet* 9: e1003143 (2013).

McDearmon EL, Patel KN, Ko CH, et al. Dissecting the functions of the mammalian clock protein BMAL1 by tissue- specific rescue in mice. *Science* 314: 1304-1308 (2006).

Mohebi M, Chenari S, Akbari A, et al. Mutation analysis of connexin 50 gene among Iranian families with autosomal dominant cataracts. *Iran J Basic Med Sci* 20: 288-93 (2017).

Nishi T, Saeki K, Obayashi K, et al. The effect of blue-blocking intraocular lenses on circadian biological rhythm: protocol for a randomised controlled trial (CLOCK-IOL colour study). *BMJ Open* 5: e007930 (2015).

Organisciak D, Darrow R, Barsalou L, Rapp C, McDonald B, Wong P. Light induced and circadian effects on retinal photoreceptor cell crystallins. *Photochem Photobiol* 87: 151-159 (2011).

Ostrin LA. Ocular and systemic melatonin and the influence of light exposure. *Clin Exp Optom* (2018).

Pras E, Raz J, Yahalom V, et al. A nonsense mutation in the glucosaminyl (N-acetyl) transferase 2 gene (GCNT2): Association with autosomal recessive congenital cataracts. *Invest Ophthalmol Vis Sci* 45: 1940-1945 (2004).

Qin L, Guo L, Wang H, et al. A novel MIP mutation in familial congenital nuclear cataracts. *Eur J Med Genet* 59: 488-491 (2016).

Saeki K, Obayashi K, Nishi T, et al. Short-term influence of cataract surgery on circadian biological rhythm and related health outcomes (CLOCK-IOL trial): study protocol for a randomized controlled trial. *Trials* 15: 514 (2014).

Samsa WE, Vasanji A, Midura RJ, Kondratov RV. Deficiency of circadian clock protein BMAL1 in mice results in a low bone mass phenotype. *Bone* 84: 194-203 (2016).

Sawant OB, Horton AM, Zucaro OF, et al. the circadian clock gene bmal1 controls thyroid hormone-mediated spectral identity and cone photoreceptor function. *Cell Rep* 21: 692-706 (2017).

Semina EV, Ferrell RE, Mintz-Hittner HA, et al. A novel homeobox gene PITX3 is mutated in families with autosomal- dominant cataracts and ASMD. *Nat Genet* 19: 167 (1998).

Shen C, Wang J, Wu X, et al. Next-generation sequencing for D47N mutation in Cx50 analysis associated with autosomal dominant congenital cataract in a six-generation Chinese family. *BMC Ophthalmol* 17: 73 (2017).

Skene DJ, Lockley SW, Arendt J. Melatonin in circadian sleep disorders in the blind. *Biol Signals Recept* 8: 90-95 (1999).

Skene DJ, Lockley SW, Thapan K, Arendt J. Effects of light on human circadian rhythms. *Reprod Nutr Dev* 39: 295-304 (1999).

Stoll C, Alembik Y, Dott B, Roth MP. Epidemiology of congenital eye malformations in 131,760 consecutive births. *Ophthalmic Paediatr Genet* 13:179-186 (1992).

Sun Z, Zhou Q, Li H, Yang L, Wu S, Sui R. Mutations in crystallin genes result in congenital cataract associated with other ocular abnormalities. *Mol Vis* 23: 977-986 (2017).

Thylefors B, AD Negrel, Ramachandra Pararajasegaram, KY Dadzie. Global data on blindness. *Bull World Health Organ* 73: 115-121 (1995).

Turner PL, Mainster MA. Circadian photoreception: ageing and the eye's important role in systemic health. *Br J Ophthalmol* 92: 1439-1444 (2008).

Vakili H, Jin Y, Cattini PA. Evidence for a circadian effect on the reduction of human growth hormone gene expression in response to excess caloric intake. *J Biol Chem* 291: 13823-13833 (2016).

Vallone D, Lahiri K, Dickmeis T, Foulkes NS. Start the clock! Circadian rhythms and development. *Dev Dyn* 236: 142-155 (2007).

Vanita V, Singh D, Robinson PN, Sperling K, Singh JR. A novel mutation in the DNA-binding domain of MAF at 16q23. 1 associated with autosomal dominant "cerulean cataract" in an Indian family. *Am J Med Genet A* 140: 558-566 (2006).

Woon PY, Kaisaki PJ, Braganca J, et al. Aryl hydrocarbon receptor nuclear translocator-like (BMAL1) is associated with susceptibility to hypertension and type 2 diabetes. *Proc Natl Acad Sci* 104: 14412-14417 (2007).

Yan SS, Wang W. The effect of lens aging and cataract surgery on circadian rhythm. *Int J Ophthalmol* 9: 1066-1074 (2016).

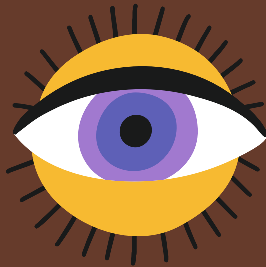
Yang G, Chen L, Grant GR, et al. Timing of expression of the core clock gene Bmal1 influences its effects on aging and survival. *Sci Transl Med* 8: 324ra16 (2016).

Yu EA, Weaver DR. Disrupting the circadian clock: gene-specific effects on aging, cancer, and other phenotypes. *Aging (Albany NY)* 3: 479-493 (2011).

CHAPTER

Udita Bagchi
Arthur A. Bergen
Marie-Paule Felder-Schmittbuhl

Under preparation



Patient-Reported Outcome Measures (PROMs) associated with the human circadian clock: a first clinical inventory exploring clock-related parameters

ABSTRACT

We made a first clinical inventory aimed at summarizing existing evidence of patient-reported outcomes focused on the circadian clock during clinical drug trials. We performed an extensive literature review based on Medline, Embase, Cochrane and PsycINFO, using the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) methodology to identify all studies that used circadian clock associated PROMs to assess the quality of life (QoL) in healthy individuals to diseased patients. Original articles reporting data from non-invasive interventions such as patient-reported outcome measures on circadian clock related parameters were included. We excluded review articles, articles on cost-effectiveness/utility without patient-reported outcome measures (PROM) data and articles not in the English language. Initially, 376 articles were identified; 197 of which were included in the final review. We found a robust evidence of PROMs referring to the evaluation of circadian disturbances in the literature review. A total of 85 PROMs were used to assess QoL in the dataset, out of which 62 PROMs were frequently administered to healthy individuals. This search identified PROMs that were specifically designed to assess sleep and chronotype. Most studies were found to have respectively employed the Pittsburgh Sleep Quality Index (PSQI) and Horne-Östberg Morningness-Eveningness Questionnaire (HO-MEQ). The Circadian Type Inventory (CTI), Bergen Shift Work Sleep Questionnaire (BSWSQ), Standard Shiftwork Index (SSI), Sleep, Circadian Rhythms and Mood (SCRAM) and Sleep-Wake Activity Inventory (SWAI) were also found. As compared to other PROMs, the latter ones were specifically developed for shiftwork and sleep-wake activities. The QoL assessment made using these PROMs must be largely considered qualitative, not quantitative. Nonetheless, our hypothesis is that circadian clock associated PROMs will be useful in clinical settings. They can be useful to assess and compare QoL outcomes from the patient perspective given societal clock alterations, medication effectiveness and surgical outcomes based on the time of day.

KeyWords: circadian rhythms, patient-reported outcomes, quality of life, clinical inventory

INTRODUCTION

The circadian system relates to the activity of an internal time-keeping system that persists even in the absence of external stimuli as an automated replay of biological functions to the light/dark cycle. It acts via the master clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Pittendrigh 1993; Reppert and Weaver 2002). The zeitgebers (German word for “time-givers”) tend to synchronize the neuronal signals in the SCN with the environment, predominantly by daylight (Arendt and Middleton 2017; Kyriacou and Hastings 2010). Molecular mechanisms generating the internal day is based upon a transcriptional-translational regulatory feedback loop of canonical clock genes and their products (Young et al. 2001). From cyanobacteria to vertebrates, circadian rhythms have been demonstrated in all phyla (Roenneberg et al. 2005). Over the last three decades, genetic studies have also identified unique species-specific clock genes (Vitaterna et al. 1994; Crosthwaite et al. 1995; Hunter-Ensor et al. 1996; Bunger et al. 2000; Bae et al. 2001; Dudley et al. 2003; Siepka et al. 2007).

The assumptions regarding the mechanisms of the molecular clock remain hypothetical in humans, as human clock genes and their function have been predominantly inferred through DNA sequence similarities with animal models. These clock gene homologs have been found with rhythmic expression patterns over 24h in blood cells, oral mucosa and hair follicles (Teboul et al. 2005; Bjarnason et al. 2001; Akashi et al. 2010). Also, cultured primary human fibroblasts tranduced with a *Bmal1*-luciferase vector express sustained rhythms of bioluminescence (Brown 2005). Studies of diseases implicating genetic variants in clock genes in humans are still rare (Xie et al. 2019): For instance, previous genome-wide association studies (GWAS) identified several clock gene (*CLOCK*, *PER2*, *RGS16*, *FBXL13*, *CRY1*, *CRY2* and *BMAL1*) single nucleotide polymorphisms associated with sleep, obesity, psychiatric disorders, and metabolic syndrome (Riestra et al. 2017; Schuch et al. 2018; Lane et al. 2016; Salazar et al. 2021). In addition, a missense mutation (S662G) of the core clock gene PER2 has been documented to induce familial advanced sleep phase syndrome among humans (Jones et al. 1999). Taken together, these results indicate that mechanisms generating rhythms in human are similar to other mammalian species.

Association studies of circadian phenotypes with naturally occurring genetic variants have characterized the relevance of the inferred mammalian clock genes in humans (Allebrandt and Roenneberg 2008). These efforts were primarily based on a phenotyping instrument focussed on time-of-day preferences, and resulted in a score of the patient-reported outcome measure (PROM), rather than the actual timing (like phase of entrainment and chronotype). One of the earliest and most prominent circadian phenotyping instrument being used to date is the Horne-Østberg Morningness-Eveningness

Questionnaire - MEQ (Horne and Østberg 1976). These phenotyping instruments are essential clinical outcome measures used to evaluate disease burden or quality of clinical care from a patient's perspective. PROMs, as per definition, provide a meaningful measure of patient experience by evaluating the patient's QoL. Therefore, PROMs are required by regulatory authorities during clinical trials, ethics committees and audits in guiding service provision.

The aim of the present inventory is to provide an informative summation of the existing literature regarding different circadian rhythm related patient-reported outcome measures (CR-PROMs). More specifically, the goal was to (a) focus on some of the pivotal clock-related parameters such as chronotype, sleep, sleep and insomnia-related cognition, sleep quality, sleep disturbance, sleep reactivity, sleep habits, sleep quality's impact on daily functions, circadian rhythms, diagnosis of sleep disorders, insomnia, night-time sleep and daytime sleepiness, shiftwork adaptation, shiftwork screening, shiftwork impact on psychological and physiological well-being and sleep-wake activities, (b) in addition, reporting all study populations including healthy individuals used for administering CR-PROMs and (c) identifying the most common questionnaires associated with circadian rhythms (based on frequency of occurrence score).

Measures that describe or reflect how a patient feels or functions as a result of circadian rhythm perturbation during clinical trials has not yet been documented. To the best of our knowledge, this is the first inventory of circadian biology-associated PROMs that suggest a potential role in supporting clinical trial endpoints.

METHODS

The present inventory has been performed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (Moher et al. 2009) and will, in its future final form, be registered with the International prospective register of systematic reviews (https://prisma-statement.org).

Literature search

Quantitative inventory literature searches were conducted in relevant databases: Medline, PsychINFO, Pubmed, Embase, Clinical Trials.gov and Cochrane libraries. The search was conducted in May 2021 covering MeSH terms synonymous with "circadian clocks", "chronobiology disorders", "jetlag syndrome", "sleep disorders" and "smith-magenis syndrome" including free text phrases such as "morningness eveningness questionnaire", "chronotype", "basic language morningness scale" and "circadian rhythm". No limit was set on the language and the date of publication.

Study selection

We defined several different circadian parameters including mood, behaviour, sleep, chronotype, and/or circadian rhythmicity appropriate for the assessment of circadian clock attributes in clinical populations.

Only quantitative studies where the usage of standardized questionnaires according to the Food and Drug Administration (FDA) December 2009 guidance (https://www.fda.gov/regulatory-information/search-fda-guidance-documents/patient-reported-outcome-measures-use-medical-product-development-support-labeling-claims) was specified, were included. Literature presenting non-clinical reviews, case studies, letters to the editor, guidelines, protocols, chronicles, legal documents, molecular and genetic studies in animal models as well as non-English publications were excluded. Based on these criteria in a double-blind format, the content from potential manuscripts at the abstract level were extracted and subsequently the inclusion and exclusion criteria were applied (U.B., see Figure 1).

Study scope

Literature identifying patient-reported outcome measures (PROMs) only in neurological disabilities were examined in order to maintain a pragmatic and lean approach for the generation of the present clinical inventory. Other COAs such as Clinician-reported Outcomes (ClinRO), Performance-reported Outcomes (PerfO) and Observer-reported Outcomes (ObsRO) were excluded. The distribution of COAs in the inventory review has been illustrated in Figure 2.

Data extraction

To address the objectives of this inventory review, the following data was extracted: full reference, name of clinical outcome assessments (questionnaires), questionnaire characteristics, place of clinical study, study participants, therapeutic indication and control condition and measures of circadian parameters.

RESULTS

Qualitative inventory search results

A total of 2402 records were identified from the initial search, out of which 1857 records were removed given our exclusion criteria. This yielded 545 records. After reading the titles, abstracts and full-texts, a further 179 records were excluded as it included articles identifying COAs other than PROMs. The remaining 366 records were listed for further evaluation and a further 179 articles were excluded as they did not meet the selection criteria. The final inventory included 187 records (see flowchart in Figure 1).

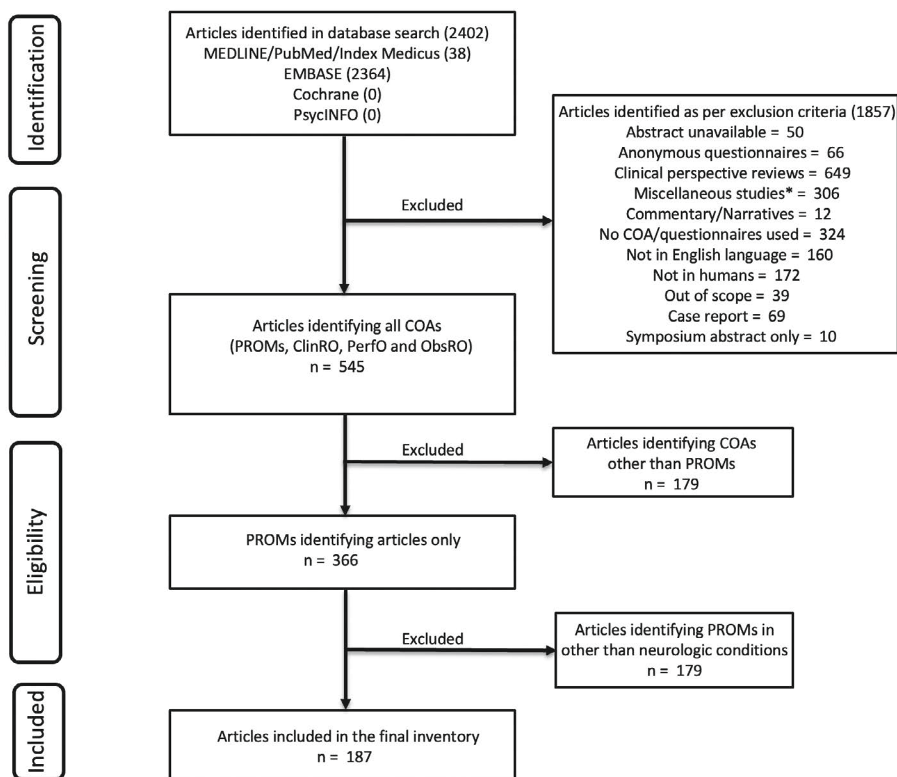


Figure 1. Flow diagram of steps involved in the study inclusion process. A total of 2402 records were identified, out of which 1857 records were excluded. This yielded 545 records. After reading the titles and abstracts, a further 179 records were removed. The remaining 366 records were listed for further evaluation. A further 179 articles were excluded as they did not match the selection criteria. The final inventory included 187 records.

Content results

A summary of all the clinical outcome assessments (COA) associated with circadian clock resulting from our first inventory based on final review of 187 articles is presented in **Tables 1 – 4**. The emphasis is on patient-reported outcome measures (Figure 2) and a categorization of specific outcomes to different clock parameters and population cohorts.

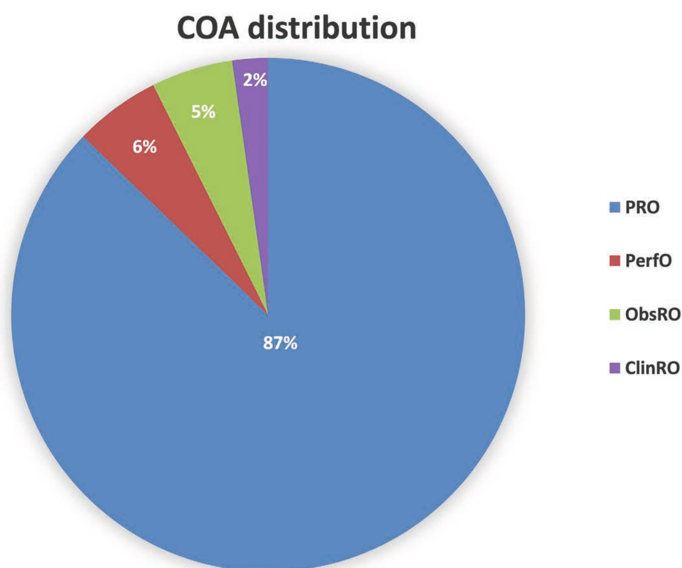


Figure 2: Distribution of COAs in the inventory review. PRO: Patient-reported outcomes, PerfO: Performance-reported outcomes, ObsRO: Observer-reported outcomes and ClinRO: Clinician-reported outcomes

Table 1: Exhaustive list of all Clinical Outcome Assessments (COAs) associated with the circadian clock found in the inventory review. Pittsburgh Sleep Quality Index (PSQI) was the most frequently used questionnaire as investigators examined sleep quality via PSQI in different clinical trials. Frequency of occurrence was empirically calculated through the Pivot table function of MS Excel on the basis of the number of times the "clock-associated domain of interest" was repeated in the 187 articles. The top five identified COAs are annotated in *italics*.

Clinical Outcome Assessment (COA) associated with circadian clock	Frequency of Occurrence
Pittsburgh Sleep Quality Index (PSQI)	160
Epworth Sleepiness Scale (ESS)	124
Horne-Östberg Morningness-Eveningness Questionnaire (HO-MEQ)	123
Munich Chrono-Type Questionnaire (MCTQ)	45
Multiple Sleep Latency Test (MSLT)	40
Insomnia Severity Index (ISI)	35
Karolinska Sleepiness Scale (KSS)	33
Children's Sleep Habits Questionnaire (CSHQ)	15
SLEEP-50	14
Children's ChronoType Questionnaire (CCTQ)	11
Composite Scale of Morningness (CSM)	10
Reduced Horne and Ostberg Morningness and Eveningness Questionnaire (rMEQ)	8

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Table 1: *[continued]*

Clinical Outcome Assessment (COA) associated with circadian clock	Frequency of Occurrence
Sleep Disturbance Scale for Children (SDSC)	8
Biological Rhythms Interview of Assessment in Neuropsychiatry (BRIAN)	7
Berlin questionnaire (BQ)	7
Visual Analog Scale (VAS) assessing subjective sleepiness	7
Athens Insomnia Scale (AIS)	7
Stanford Sleepiness Scale (SSS)	6
Morningness-Eveningness Scale for Children (MESC)	6
Consensus Sleep Diary (CSD)	5
Patient-Reported Outcomes Information System Short Form v1.0 Sleep Disturbance (PROMIS Short Form v1.0 Sleep Disturbance)	5
Pediatric Sleep Questionnaire (PSQ)	4
Pediatric Daytime Sleepiness Scale (PDSS)	4
Circadian Type Inventory (CTI)	3
Women’s Health Initiative Insomnia Rating Scale (WHIIRS)	3
Parkinson’s Disease Sleep Scale (PDSS)	3
Children’s Sleep Habits Questionnaire (CSHQ)	3
Standard Shiftwork Index (SSI)	3
Patient-Reported Outcomes Information System Short Form v1.0 Sleep-Related Impairment (PROMIS Short Form v1.0 Sleep-Related Impairment)	3
Sleep Hygiene Index (SHI)	3
Shift Work Disorder Questionnaire (SWDQ)	3
Munich ChronoType Questionnaire for Shift-Workers (MCTQShift)	3
Verran and Snyder-Halpern Sleep Scale (VSH)	2
Johns Drowsiness Scale (JDSTM)	2
Sleep Regularity Index (SRI)	2
Innsbruck REM Sleep Behavior Disorder Inventory (Innsbruck RBD-I)	2
Sleep Disorders Inventory (SDI)	2
Global Sleep Assessment Questionnaire (GSAQ)	2
Brief Infant Sleep Questionnaire (BISQ)	2
Dysfunctional Beliefs and Attitudes about Sleep (DBAS)	2
Bergen Insomnia Scale (BIS)	2
Munich Parasomnia Screening (MUPS)	2
Auckland Sleep Questionnaire (ASQ)	2
5-point Likert scale assessing misconceptions about sleep	2
The Great British Sleep Survey	1
Circadian Type Questionnaire (CTQ)	1
Duke Structured Interview for Sleep Disorders (DSISD)	1
Sleep Disorders Questionnaire (SDQ)	1
Visual Analog Scale (VAS) assessing change in sleep quality	1
Sleep Hygiene Self-Test	1

[continued on next page]

Table 1: *[continued]*

Clinical Outcome Assessment (COA) associated with circadian clock	Frequency of Occurrence
Sleep-Wake Problems Behaviour Scale (SWPBS)	1
Sleep Condition Indicator (SCI)	1
Consensus Sleep Diary for Morning (CSD-M)	1
Sleep Apnea Clinical Score (SACS)	1
Chronic Sleep Restriction Questionnaire (CSRQ)	1
Owl and Lark Questionnaire	1
Questionnaire de sommeil de Spiegel	1
Nightmare Experience Scale (NExS)	1
Athlete Morningness Eveningness Scale (AMES)	1
Shift Work Disorder Screening Questionnaire (SWDSQ)	1
Richards-Campbell Sleep Questionnaire (RCSQ)	1
Sleep Habit Questionnaire (SHQ)	1
Delayed Sleep Phase Disorder (DSPD) symptom screening survey	1
Munich ChronoType Questionnaire for Shift-Workers (MCTQShift)	1
Sleep-Wake Activity Inventory (SWAI)	1
St. Mary's Hospital Sleep Questionnaire (SMH)	1
Circadian Rhythm Assessment Questionnaire	1
Snoring, Tiredness, Observed apnea, and high blood Pressure - Body mass index, Age, Neck circumference, and Gender (STOP-BANG)	1
Children's Morningness-Eveningness Preferences Scale (CMEP)	1
Morningness-Eveningness Stability Scale improved (MESSi)	1
Child and Adolescent Sleep Checklist (CASC)	1
School Sleep Habits Survey (SSHS)	1
Sleep Problems Index II (SPI II)	1
Morningness-Eveningness Questionnaire for Self-Assessment (MEQ-SA)	1
Basic Language Morningness (BALM) scale	1
Medical Outcomes Study Sleep scale-Revised (MOS Sleep Scale-R)	1
Albany Sleep Problems Scale (ASPS)	1
Medical Outcomes Study Sleep scale (MOS Sleep)	1
Echols Sleep Behavior Observation Tool (EBOT)	1
Leeds Sleep Evaluation Questionnaire (LSEQ)	1
Duke Structured Interview Schedule for Sleep Disorders (DSISD)	1
Sleepiness Scale (SLS)	1
Diagnostic Interview for Sleep Patterns and Disorders (DISP)	1
School Sleep Habits Survey	1
Daily Cognitive Communicative and Sleep Profile (DCCASP)	1
Johns Drowsiness Scale (JDS)	1
Richards Campbell Sleep Questionnaire (RCSQ)	1
Insomnia Symptom Composite Score (ICS)	1
Cleveland Adolescent Sleepiness Questionnaire (CASQ)	1

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Table 1: *[continued]*

Clinical Outcome Assessment (COA) associated with circadian clock	Frequency of Occurrence
7-point Likert scale assessing sleep disturbances	1
REM Sleep Behavior Disorder Screening Questionnaire (RBDSQ)	1
Sleep in the Intensive Care Unit Questionnaire	1
Chronotype Questionnaire (ChQ)	1
Insomnia Interview Schedule (IIS)	1
Children’s Sleep Hygiene Scale (CSHS)	1
Scales for Outcomes in Parkinson’s Disease - Sleep Disturbances (SCOPA-SLEEP)	1
Reduced Horne and Ostberg Morningness and Eveningness Questionnaire (rMEQ)	1
Hyperarousal Scale (HAS)	1
Sleep Self Report (SSR)	1
Sleep, Circadian Rhythms and Mood (SCRAM)	1
Caen Chronotype Questionnaire (CCQ)	1
Holland Sleep Disorders Questionnaire (HSDQ)	1
Pre-Sleep Arousal Scale (PSAS)	1
Groningen Sleep Quality Scale (GSQS)	1
Bergen Shift Work Seep Questionnaire (BSWSQ)	1
Scale of Older Adults Routine (SOAR)	1
Basic Nordic Sleep Questionnaire (BNSQ)	1
General Sleep Disturbance Scale (GSDS)	1
Sleep Disorders Symptom Checklist-25 (SDS-CL-25)	1
General Behavior Inventory Sleep Subscale (GBISS)	1
Sleep Inertia Questionnaire (SIQ)	1
Functional Outcomes of Sleep Questionnaire (FOSQ)	1
Pediatric Sleep Medication Survey	1
Ford Insomnia Response to Stress Test (FIRST)	1
Insomnia Sleep Questionnaire Packet	1
Sleep/Wake-Behaviours-App (SWAPP)	1
Grand Total	**807**

Table 2: Exhaustive list of only Circadian Clock associated Patient-Reported Outcome Measures (CR-PROMs) found in the inventory review. Pittsburgh Sleep Quality Index (PSQI) was the most frequently used questionnaire as investigators examined sleep quality via PSQI in different clinical trials. Frequency of occurrence was empirically calculated through the Pivot table function of MS Excel on the basis of the number of times the “clock-associated domain of interest” was repeated in the 187 articles. The top five identified CR-PROMs are annotated in *italics*.

Circadian Clock associated Patient-Reported Outcome Measures (CR-PROMs)	Frequency of Occurrence
Pittsburgh Sleep Quality Index (PSQI)	160
Epworth Sleepiness Scale (ESS)	124
Horne-Östberg Morningness-Eveningness Questionnaire (HO-MEQ)	123

[continued on next page]

Table 2: *[continued]*

Circadian Clock associated Patient-Reported Outcome Measures (CR-PROMs)	Frequency of Occurrence
Munich Chrono-Type Questionnaire (MCTQ)	45
Karolinska Sleepiness Scale (KSS)	33
SLEEP-50	14
Composite Scale of Morningness (CSM)	10
Reduced Horne and Ostberg Morningness and Eveningness Questionnaire (rMEQ)	8
Biological Rhythms Interview of Assessment in Neuropsychiatry (BRIAN)	7
Visual Analog Scale (VAS) assessing subjective sleepiness	7
Berlin questionnaire (BQ)	7
Athens Insomnia Scale (AIS)	7
Stanford Sleepiness Scale (SSS)	6
Morningness-Eveningness Scale for Children (MESC)	6
Consensus Sleep Diary (CSD)	5
Patient-Reported Outcomes Information System Short Form v1.0 Sleep Disturbance (PROMIS Short Form v1.0 Sleep Disturbance)	5
Pediatric Daytime Sleepiness Scale (PDSS)	4
Circadian Type Inventory (CTI)	3
Women's Health Initiative Insomnia Rating Scale (WHIIRS)	3
Standard Shiftwork Index (SSI)	3
Sleep Hygiene Index (SHI)	3
Patient-Reported Outcomes Information System Short Form v1.0 Sleep-Related Impairment (PROMIS Short Form v1.0 Sleep-Related Impairment)	3
Parkinson's Disease Sleep Scale (PDSS)	3
Munich ChronoType Questionnaire for Shift-Workers (MCTQShift)	3
Auckland Sleep Questionnaire (ASQ)	2
Dysfunctional Beliefs and Attitudes about Sleep (DBAS)	2
Global Sleep Assessment Questionnaire (GSAQ)	2
Verran and Snyder-Halpern Sleep Scale (VSH)	2
Bergen Insomnia Scale (BIS)	2
Munich Parasomnia Screening (MUPS)	2
Johns Drowsiness Scale (JDSTM)	2
5-point Likert scale assessing misconceptions about sleep	2
Delayed Sleep Phase Disorder (DSPD) symptom screening survey	1
Basic Language Morningness (BALM) scale	1
Chronotype Questionnaire (ChQ)	1
St. Mary's Hospital Sleep Questionnaire (SMH)	1
Functional Outcomes of Sleep Questionnaire (FOSQ)	1
Pediatric Sleep Medication Survey	1
Visual Analog Scale (VAS) assessing change in sleep quality	1
Snoring, Tiredness, Observed apnea, and high blood Pressure - Body mass index, Age, Neck circumference, and Gender (STOP-BANG)	1

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Table 2: *[continued]*

Circadian Clock associated Patient-Reported Outcome Measures (CR-PROMs)	Frequency of Occurrence
Sleep Condition Indicator (SCI)	1
Sleepiness Scale (SLS)	1
Pre-Sleep Arousal Scale (PSAS)	1
Sleep, Circadian Rhythms and Mood (SCRAM)	1
Sleep in the Intensive Care Unit Questionnaire	1
Sleep-Wake Problems Behaviour Scale (SWPBS)	1
Consensus Sleep Diary for Morning (CSD-M)	1
Owl and Lark Questionnaire	1
Circadian Type Questionnaire (CTQ)	1
Nightmare Experience Scale (NExS)	1
Caen Chronotype Questionnaire (CCQ)	1
Richards-Campbell Sleep Questionnaire (RCSQ)	1
Scales for Outcomes in Parkinson’s Disease - Sleep Disturbances (SCOPA-SLEEP)	1
Sleep-Wake Activity Inventory (SWAI)	1
Athlete Morningness Eveningness Scale (AMES)	1
Munich ChronoType Questionnaire for Shift-Workers (MCTQShift)	1
General Sleep Disturbance Scale (GSDS)	1
The Great British Sleep Survey	1
Ford Insomnia Response to Stress Test (FIRST)	1
Morningness-Eveningness Stability Scale improved (MESSi)	1
Reduced Horne and Ostberg Morningness and Eveningness Questionnaire (rMEQ)	1
Sleep Self Report (SSR)	1
Daily Cognitive Communicative and Sleep Profile (DCCASP)	1
Morningness-Eveningness Questionnaire for Self-Assessment (MEQ-SA)	1
Sleep Hygiene Self-Test	1
Medical Outcomes Study Sleep scale-Revised (MOS Sleep Scale-R)	1
Cleveland Adolescent Sleepiness Questionnaire (CASQ)	1
Medical Outcomes Study Sleep scale (MOS Sleep)	1
Sleep Habit Questionnaire (SHQ)	1
Leeds Sleep Evaluation Questionnaire (LSEQ)	1
Chronic Sleep Restriction Questionnaire (CSRQ)	1
Sleep Problems Index II (SPI II)	1
Sleep Disorders Symptom Checklist-25 (SDS-CL-25)	1
Richards Campbell Sleep Questionnaire (RCSQ)	1
Bergen Shift Work Seep Questionnaire (BSWSQ)	1
7-point Likert scale assessing sleep disturbances	1
Basic Nordic Sleep Questionnaire (BNSQ)	1
School Sleep Habits Survey	1
Questionnaire de sommeil de Spiegel	1
Sleep Inertia Questionnaire (SIQ)	1

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Table 2: *[continued]*

Circadian Clock associated Patient-Reported Outcome Measures (CR-PROMs)	Frequency of Occurrence
School Sleep Habits Survey (SSHS)	1
Groningen Sleep Quality Scale (GSQS)	1
REM Sleep Behavior Disorder Screening Questionnaire (RBDSQ)	1
Insomnia Sleep Questionnaire Packet	1
Hyperarousal Scale (HAS)	1
Grand Total	**661**

Table 3: Categorization of Patient-Reported Outcome Measures (PROMs) on the basis of biological rhythm characteristics. Pittsburgh Sleep Quality Index (PSQI) was the most frequently used questionnaire as investigators examined sleep quality via PSQI in different clinical trials. Frequency of occurrence was empirically calculated through the Pivot table function of MS Excel on the basis of the number of times the "clock-associated domain of interest" was repeated in the 187 articles. The top five identified PROMs are annotated in *italics*.

Patient-reported Outcome Measures (PROMs) separated as per circadian characteristics	Frequency of Occurrence
Sleep	**400**
Pittsburgh Sleep Quality Index (PSQI)	160
Epworth Sleepiness Scale (ESS)	124
Karolinska Sleepiness Scale (KSS)	33
SLEEP-50	14
Berlin questionnaire (BQ)	7
Athens Insomnia Scale (AIS)	7
Visual Analog Scale (VAS) assessing subjective sleepiness	7
Stanford Sleepiness Scale (SSS)	6
Consensus Sleep Diary (CSD)	5
Pediatric Daytime Sleepiness Scale (PDSS)	4
Sleep Hygiene Index (SHI)	3
Parkinson's Disease Sleep Scale (PDSS)	3
5-point Likert scale assessing misconceptions about sleep	2
Auckland Sleep Questionnaire (ASQ)	2
Verran and Snyder-Halpern Sleep Scale (VSH)	2
Johns Drowsiness Scale (JDSTM)	2
Richards-Campbell Sleep Questionnaire (RCSQ)	1
Sleep Hygiene Self-Test	1
Questionnaire de sommeil de Spiegel	1
Visual Analog Scale (VAS) assessing change in sleep quality	1
St. Mary's Hospital Sleep Questionnaire (SMH)	1
Pediatric Sleep Medication Survey	1
7-point Likert scale assessing sleep disturbances	1
Sleep Inertia Questionnaire (SIQ)	1

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Table 3: *[continued]*

Patient-reported Outcome Measures (PROMs) separated as per circadian characteristics	Frequency of Occurrence
Consensus Sleep Diary for Morning (CSD-M)	1
Sleep Habit Questionnaire (SHQ)	1
Cleveland Adolescent Sleepiness Questionnaire (CASQ)	1
Nightmare Experience Scale (NExS)	1
Basic Nordic Sleep Questionnaire (BNSQ)	1
The Great British Sleep Survey	1
Sleepiness Scale (SLS)	1
Sleep Self Report (SSR)	1
Richards Campbell Sleep Questionnaire (RCSQ)	1
Global Sleep Assessment Questionnaire (GSAQ)	1
Sleep in the Intensive Care Unit Questionnaire	1
Chronotype	**212**
Horne-Östberg Morningness-Eveningness Questionnaire (HO-MEQ)	123
Munich Chrono-Type Questionnaire (MCTQ)	45
Composite Scale of Morningness (CSM)	10
Reduced Horne and Ostberg Morningness and Eveningness Questionnaire (rMEQ)	8
Biological Rhythms Interview of Assessment in Neuropsychiatry (BRIAN)	7
Morningness-Eveningness Scale for Children (MESC)	6
Munich ChronoType Questionnaire for Shift-Workers (MCTQShift)	3
Athlete Morningness Eveningness Scale (AMES)	1
Chronotype Questionnaire (ChQ)	1
Circadian Type Questionnaire (CTQ)	1
Reduced Horne and Ostberg Morningness and Eveningness Questionnaire (rMEQ)	1
Caen Chronotype Questionnaire (CCQ)	1
Owl and Lark Questionnaire	1
Basic Language Morningness (BALM) scale	1
Morningness-Eveningness Stability Scale improved (MESSi)	1
Munich ChronoType Questionnaire for Shift-Workers (MCTQShift)	1
Morningness-Eveningness Questionnaire for Self-Assessment (MEQ-SA)	1
Insomnia	**20**
Patient-Reported Outcomes Information System Short Form v1.0 Sleep Disturbance (PROMIS Short Form v1.0 Sleep Disturbance)	5
Women's Health Initiative Insomnia Rating Scale (WHIIRS)	3
Patient-Reported Outcomes Information System Short Form v1.0 Sleep-Related Impairment (PROMIS Short Form v1.0 Sleep-Related Impairment)	3
Bergen Insomnia Scale (BIS)	2
Munich Parasomnia Screening (MUPS)	2
Chronic Sleep Restriction Questionnaire (CSRQ)	1
Leeds Sleep Evaluation Questionnaire (LSEQ)	1
Sleep Condition Indicator (SCI)	1

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Table 3: *[continued]*

Patient-reported Outcome Measures (PROMs) separated as per circadian characteristics	Frequency of Occurrence
Insomnia Sleep Questionnaire Packet	1
Functional Outcomes of Sleep Questionnaire (FOSQ)	1
Diagnosis of Sleep Disorders	**5**
Hyperarousal Scale (HAS)	1
Sleep Disorders Symptom Checklist-25 (SDS-CL-25)	1
Global Sleep Assessment Questionnaire (GSAQ)	1
REM Sleep Behavior Disorder Screening Questionnaire (RBDSQ)	1
Pre-Sleep Arousal Scale (PSAS)	1
Sleep Habits	**5**
School Sleep Habits Survey	1
Snoring, Tiredness, Observed apnea, and high blood Pressure - Body mass index, Age, Neck circumference, and Gender (STOP-BANG)	1
Delayed Sleep Phase Disorder (DSPD) symptom screening survey	1
Sleep-Wake Problems Behaviour Scale (SWPBS)	1
School Sleep Habits Survey (SSHS)	1
Shift Work Adaptation	**4**
Circadian Type Inventory (CTI)	3
Bergen Shift Work Seep Questionnaire (BSWSQ)	1
Shiftwork impact on psychological and physiological well-being	**3**
Standard Shiftwork Index (SSI)	3
Sleep Quality	**3**
Sleep Problems Index II (SPI II)	1
Groningen Sleep Quality Scale (GSQS)	1
Medical Outcomes Study Sleep scale (MOS Sleep)	1
Sleep and Insomnia-related Cognition	**2**
Dysfunctional Beliefs and Attitudes about Sleep (DBAS)	2
Sleep Disturbance	**2**
General Sleep Disturbance Scale (GSDS)	1
Medical Outcomes Study Sleep Scale-Revised (MOS Sleep Scale-R)	1
Sleep Quality & Daily Functions	**1**
Daily Cognitive Communicative and Sleep Profile (DCCASP)	1
Circadian Rhythms	**2**
Sleep, Circadian Rhythms and Mood (SCRAM)	1
Sleep-Wake Activity Inventory (SWAI)	1
Night-time Sleep and Daytime Sleepiness	**1**
Scales for Outcomes in Parkinson's Disease - Sleep Disturbances (SCOPA-SLEEP)	1
Sleep Reactivity	**1**
Ford Insomnia Response to Stress Test (FIRST)	1
Grand Total	**661**

Table 4: Segregation of Circadian Clock associated Patient-Reported Outcome Measures (CR-PROMs) on the basis of population cohort - healthy participants and patients with neurological conditions. In the healthy individuals, Horne-Östberg Morningness-Eveningness Questionnaire (HO-MEQ) was the most frequently used questionnaire followed by Pittsburgh Sleep Quality Index (PSQI) as investigators examined chronotype versus sleep quality in different clinical trials. In patients with neurological conditions, variety of different questionnaires were used such as Karolinska Sleepiness Scale (KSS), Horne-Östberg Morningness-Eveningness Questionnaire (HO-MEQ), Pittsburgh Sleep Quality Index (PSQI), Epworth Sleepiness Scale (ESS), Visual Analog Scale (VAS) assessing subjective sleepiness, Munich Chrono-Type Questionnaire (MCTQ) and Biological Rhythms Interview of Assessment in Neuropsychiatry (BRIAN). Frequency of occurrence was empirically calculated through the Pivot table function of MS Excel on the basis of the number of times the "clock-associated domain of interest" was repeated in the 187 articles. The top five identified PROMs are annotated in *italics*.

Circadian Clock associated Patient-Reported Outcome Measures (CR-PROMs) in healthy participants and in patients with neurological conditions	Frequency of Occurrence
Healthy Participants	**134**
Horne-Östberg Morningness-Eveningness Questionnaire (HO-MEQ)	40
Pittsburgh Sleep Quality Index (PSQI)	13
Epworth Sleepiness Scale (ESS)	11
SLEEP-50	11
Munich Chrono-Type Questionnaire (MCTQ)	11
Karolinska Sleepiness Scale (KSS)	10
Composite Scale of Morningness (CSM)	4
Reduced Horne and Ostberg Morningness and Eveningness Questionnaire (rMEQ)	4
Auckland Sleep Questionnaire (ASQ)	2
Athens Insomnia Scale (AIS)	2
5-point Likert scale assessing misconceptions about sleep	2
Patient-Reported Outcomes Information System Short Form v1.0 Sleep Disturbance (PROMIS Short Form v1.0 Sleep Disturbance)	2
Consensus Sleep Diary (CSD)	2
Delayed Sleep Phase Disorder (DSPD) symptom screening survey	1
Stanford Sleepiness Scale (SSS)	1
Pittsburgh Sleep Quality Index (PSQI)	1
Sleep-Wake Problems Behaviour Scale (SWPBS)	1
School Sleep Habits Survey	1
Caen Chronotype Questionnaire (CCQ)	1
Consensus Sleep Diary for Morning (CSD-M)	1
Pediatric Sleep Medication Survey	1
Morningness-Eveningness Stability Scale improved (MESSi)	1
Cleveland Adolescent Sleepiness Questionnaire (CASQ)	1
Morningness-Eveningness Scale for Children (MESC)	1
Berlin questionnaire (BQ)	1
Leeds Sleep Evaluation Questionnaire (LSEQ)	1
Athlete Morningness Eveningness Scale (AMES)	1
Visual Analog Scale (VAS) assessing subjective sleepiness	1
7-point Likert scale assessing sleep disturbances	1

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Table 4: *[continued]*

Circadian Clock associated Patient-Reported Outcome Measures (CR-PROMs) in healthy participants and in patients with neurological conditions	Frequency of Occurrence
Johns Drowsiness Scale (JDSTM)	1
Chronotype Questionnaire (ChQ)	1
Sleep Habit Questionnaire (SHQ)	1
School Sleep Habits Survey (SSHS)	1
Shift Workers	**40**
Karolinska Sleepiness Scale (KSS)	12
Pittsburgh Sleep Quality Index (PSQI)	9
Epworth Sleepiness Scale (ESS)	5
Munich Chrono-Type Questionnaire (MCTQ)	4
Horne-Östberg Morningness-Eveningness Questionnaire (HO-MEQ)	3
Munich ChronoType Questionnaire for Shift-Workers (MCTQShift)	2
Stanford Sleepiness Scale (SSS)	2
Bergen Shift Work Seep Questionnaire (BSWSQ)	1
Standard Shiftwork Index (SSI)	1
Munich ChronoType Questionnaire for Shift-Workers (MCTQShift)	1
Bipolar Disorder	**16**
Pittsburgh Sleep Quality Index (PSQI)	8
Munich Chrono-Type Questionnaire (MCTQ)	2
Horne-Östberg Morningness-Eveningness Questionnaire (HO-MEQ)	2
Athens Insomnia Scale (AIS)	1
Basic Language Morningness (BALM) scale	1
Visual Analog Scale (VAS) assessing change in sleep quality	1
Epworth Sleepiness Scale (ESS)	1
Circadian Rhythm Sleep-Wake Disorders (CRSWDs)	**14**
Horne-Östberg Morningness-Eveningness Questionnaire (HO-MEQ)	7
Epworth Sleepiness Scale (ESS)	3
Karolinska Sleepiness Scale (KSS)	1
Pittsburgh Sleep Quality Index (PSQI)	1
Sleep Disorders Symptom Checklist-25 (SDS-CL-25)	1
Munich Chrono-Type Questionnaire (MCTQ)	1
Sleep Disorders	**13**
Epworth Sleepiness Scale (ESS)	6
Horne-Östberg Morningness-Eveningness Questionnaire (HO-MEQ)	2
Pittsburgh Sleep Quality Index (PSQI)	2
Consensus Sleep Diary (CSD)	1
Richards Campbell Sleep Questionnaire (RCSQ)	1
Hyperarousal Scale (HAS)	1
Depressive Disorders	**11**
Horne-Östberg Morningness-Eveningness Questionnaire (HO-MEQ)	3
Biological Rhythms Interview of Assessment in Neuropsychiatry (BRIAN)	2
Munich Chrono-Type Questionnaire (MCTQ)	2

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Table 4: *[continued]*

Circadian Clock associated Patient-Reported Outcome Measures (CR-PROMs) in healthy participants and in patients with neurological conditions	**Frequency of Occurrence**
Pittsburgh Sleep Quality Index (PSQI)	2
Sleep Hygiene Index (SHI)	1
Sleep, Circadian Rhythms and Mood (SCRAM)	1
Epilepsy	**7**
Pittsburgh Sleep Quality Index (PSQI)	4
Epworth Sleepiness Scale (ESS)	2
Horne-Östberg Morningness-Eveningness Questionnaire (HO-MEQ)	1
Parkinson's Disease	**7**
Pittsburgh Sleep Quality Index (PSQI)	2
Horne-Östberg Morningness-Eveningness Questionnaire (HO-MEQ)	2
Parkinson's Disease Sleep Scale (PDSS)	2
Epworth Sleepiness Scale (ESS)	1
Traumatic Brain Injuries	**8**
Horne-Östberg Morningness-Eveningness Questionnaire (HO-MEQ)	2
Daily Cognitive Communicative and Sleep Profile (DCCASP)	1
Pittsburgh Sleep Quality Index (PSQI)	2
Athens Insomnia Scale (AIS)	1
Sleep Self Report (SSR)	1
Epworth Sleepiness Scale (ESS)	1
Huntington's Disease	**5**
Pittsburgh Sleep Quality Index (PSQI)	3
Consensus Sleep Diary (CSD)	1
Epworth Sleepiness Scale (ESS)	1
Alzheimer's Disease	**4**
Pittsburgh Sleep Quality Index (PSQI)	2
Functional Outcomes of Sleep Questionnaire (FOSQ)	1
Horne-Östberg Morningness-Eveningness Questionnaire (HO-MEQ)	1
Seasonal Affective Disorder	3
Visual Analog Scale (VAS) assessing subjective sleepiness	1
Karolinska Sleepiness Scale (KSS)	1
Pittsburgh Sleep Quality Index (PSQI)	1
Schizophrenia	**3**
Pittsburgh Sleep Quality Index (PSQI)	3
Spinal Cord Injury	**2**
Epworth Sleepiness Scale (ESS)	1
Pittsburgh Sleep Quality Index (PSQI)	1
Eye Lens Opacity	**2**
Pittsburgh Sleep Quality Index (PSQI)	2
Optic Nerve Disorders	**1**
Pittsburgh Sleep Quality Index (PSQI)	1
Retinitis Pigmentosa	**1**

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Table 4: *[continued]*

Circadian Clock associated Patient-Reported Outcome Measures (CR-PROMs) in healthy participants and in patients with neurological conditions	Frequency of Occurrence
Pittsburgh Sleep Quality Index (PSQI)	1
Mood Disorders	**1**
Horne-Östberg Morningness-Eveningness Questionnaire (HO-MEQ)	1
Migraine	**1**
Epworth Sleepiness Scale (ESS)	1
Primary Glaucoma	**1**
Pittsburgh Sleep Quality Index (PSQI)	1
Obstructive Sleep Apnea	**1**
Epworth Sleepiness Scale (ESS)	1
Multiple Sclerosis	**1**
Epworth Sleepiness Scale (ESS)	1
Narcolepsy	**1**
Epworth Sleepiness Scale (ESS)	1
Obstructive Sleep Apnea and Parkinson's Disease	**1**
Epworth Sleepiness Scale (ESS)	1
Depressive Disorder with Sensorineural Hearing Loss	**1**
Pittsburgh Sleep Quality Index (PSQI)	1
Depressive Disorder with Sleep Onset Insomnia	**1**
Visual Analog Scale (VAS) assessing subjective sleepiness	1
Memory Decline	**1**
Epworth Sleepiness Scale (ESS)	1
Depressive and Anxiety Disorders	**1**
Munich Chrono-Type Questionnaire (MCTQ)	1
Idiopathic REM Sleep Behavior Disorder and Neurodegenerative Synucleinopathy	**1**
Epworth Sleepiness Scale (ESS)	1
Blindness	**1**
Pittsburgh Sleep Quality Index (PSQI)	1
Idiopathic Hypersomnia	**1**
Epworth Sleepiness Scale (ESS)	1
Autism Spectrum Disorder	**1**
Horne-Östberg Morningness-Eveningness Questionnaire (HO-MEQ)	1
Sleep and Psychiatric Disorders	**1**
Epworth Sleepiness Scale (ESS)	1
Healthy Participants, Depressive Disorders + Bipolar Disease	**1**
Biological Rhythms Interview of Assessment in Neuropsychiatry (BRIAN)	1
Meningococcal Meningitis	**1**
Epworth Sleepiness Scale (ESS)	1
Grand Total	**289**

RESULTS

Synthesis of Outcomes

The included studies used to generate **Tables 1-4** had considerable methodological heterogeneity, varying effect sizes and are reported per intervention or control group. Therefore, the results obtained should be mostly viewed as indicative.

Patient-Reported Outcome Measures

We have enlisted from our inventory review a total of 116 clinical outcome assessments (COAs) or questionnaires, out of which 85 are patient-reported outcome measures in **Table 1**. The exhaustive distribution of all the four different types of COAs found in our inventory review of 187 articles is illustrated as a pie diagram in Figure 2.

The top five patient-reported outcome measures found in our inventory analysis corresponded to the circadian parameters of sleep and chronotype as depicted in **Table 2** (generated after the exclusion of other COAs such as ClinRO, ObsRO and PerfO): Pittsburgh Sleep Quality Index (PSQI) - gathers consistent information over a 1-month time interval about the subjective nature of sleep habits, Epworth Sleepiness Scale (ESS) - very short questionnaire measuring daytime sleepiness, Horne-Östberg Morningness-Eveningness Questionnaire (HO-MEQ) - measures peak alertness of a person in the morning, in the evening, or in between, Munich Chrono-Type Questionnaire (MCTQ) - estimates chronotype in individuals on the basis of the midpoint between sleep onset and offset on work-free days based on the accumulated sleep debt over the workweek and Karolinska Sleepiness Scale (KSS) – measures situational sleepiness or the subjective nature of sleep at a particular time during the day. The heterogeneous occurrence of the questionnaires does not allow for a direct comparison, instead the intent of the inventory analysis was to reiterate the frequency of usage of CR-PROMs in the last 2 decades in routine clinical practice.

In **Table 3**, we have categorized 85 Patient-Reported Outcome Measures (PROMs) on the basis of health-related quality of life domains portraying circadian characteristics such as: sleep, chronotype, insomnia, diagnosis of sleep disorders, sleep quality & habits, sleep reactivity, shiftwork adaptation, shiftwork impact on psychological and physiological well-being, sleep and insomnia-related cognition, sleep disturbance, sleep quality & daily functions, circadian rhythms, night-time sleep and daytime sleepiness. In our dataset, the most frequently occurring domain of interest was sleep followed by chronotype and insomnia.

Reference (Country)	Total Sample Size (Numbers/in Ratio)/Mean Age in years	Diagnosis/indication/Population cohort	PSQI results in the control/intervention group
Dumitraș et al. 2021 (Moldova)	118 High school students (grades 10-12), from 7 lyceums of Chisinau and Balti, 17 +/- 1.1 years, male to female ratio 1:2.6	Healthy participants were registered with higher normal daytime sleepiness (6-10 points on ESS).	Mean PSQI score was 7.2 +/- 0.5, and the prevalence of 'poor' sleepers (PSQI score > 5) was 69.5% (82/118)
Toscano-Hermoso et al. 2020 (Spain)	855 university students (476 women, 55.7% and 378 men, 44.3%) with an average age of 22.55 years	Healthy participants were registered using the PSQI, Nightmare Frequency Scale, Nightmare Proneness Scale, and the Composite Morningness Scale	Women show a higher risk [OR = 2.61] of presenting poor sleep quality (> 5 points PSQI) compared with men (p < 0.001)
Sun et al. 2019 (China)	5497 medical students	Healthy participants were registered using the PSQI, MEQ and Health-promoting Lifestyle Profile	Sleep quality total score in evening-types = 5.43 +/- 2.66 was higher than morning-types = 3.88 +/- 2.20, P < .001. Morning-type students (OR = 0.40, 95%CI = 0.29-0.55) and intermediate-type students (OR = 0.53, 95%CI = 0.41-0.69) had a lower risk of poor sleep quality compared to evening-types
Lim et al. 2020 (Korea)	951 pre-employed firefighters, 602 were included in the study	Healthy participants were registered for the cross-sectional survey using Insomnia Severity Index (ISI)	Good sleep quality = 347 (57.6%) and Poor sleep quality = 255 (42.4%). PSQI score was 3.29 ± 1.41) and 7.87 ± 2.20)
Tsanas et al. 2020 (United Kingdom)	Non-traumatized controls = 30, Traumatized without PTSD = 43 and 42 with PTSD. 115 participants aged between 18 - 65 years	Non-traumatized controls, PTSD patients and traumatized without PTSD completed daily sleep diary over 7 days and PSQI	PTSD group = 91% (38/42) had clinically marked sleep problems at baseline (total PSQI>5). PSQI was statistically significantly different between PTSD participants and nontraumatized controls (*P*<.001)
Ikeda et al. 2012 (Japan)	100 caregivers of disabled children, including 96 mothers, 2 fathers and 2 grandmothers. 66 boys and 34 girls = 88 children (1-17 years)	Healthy participants including children and caregivers were registered for PSQI and the Japanese version of Zarit Burden Interview (ZBI)	PSQI scores were significantly higher in caregivers of children with "Problems with sleep-related breathing" and "Problems with circadian rhythm". A significant correlation was identified between perceived ZBI and caregiver PSQI.

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Table 5: *[continued]*

Reference (Country)	Total Sample Size (Numbers/in Ratio)/Mean Age in years	Diagnosis/indication/Population cohort	PSQI results in the control/intervention group
Mishra et al. 2020 (India)	120 schizophrenia patients on Ramelteon therapy	Schizophrenia patients categorized on Positive and Negative Syndrome Scale (PANSS) scoring and randomized into control (haloperidol/risperidone) or test (add-on ramelteon) groups. PSQI is evaluated besides baseline serum melatonin, serum AANAT and urinary melatonin	Decrease in PSQI scores (Positive group: -1·57; 95%CI: -2·59 to -0·55; p = 0·003; Negative group: -2·49; 95%CI: -4·59 to -0·39; p = 0·021
Hajaghazadeh et al. 2019 (Iran)	120 nurses, 23 males and 97 females	120 nurses completed the cross-sectional survey using PSQI, ESS and MEQ	Mean (±SD) of PSQI 6.88(2.18), about 74% nurses reported poor sleep quality. The mean PSQI was significantly different between shift work and non-shift work nurses. The mean PSQI of three main chronotypes was statistically different.
Cruz et al. 2019 (Spain)	99 people older than 50 years (69 women and 30 men; mean age, 68.74±7.18 years) with no associated diseases	Elderly participants were registered with the digital versions of the Word Learning and Visual Paired Associates tests and PSQI	PSQI was negatively correlated with Visual Paired Associates and Word Learning test performance. Performance decreased in line with sleep quality. Participants sex showed a weak effect on sleep latency.
Vijaykumar et al. 2018 (India)	Group I = Nurses doing night shift from past 1 week (n = 50) of age group (25–50). Group II = Nurses doing day shift from past 1 week (n = 50) of the age group (25–50). Group III (controls) = Females who are not exposed to shiftwork (n = 50) of the age group (25–50).	150 hospital shift working nurses registered with a cross-sectional study to assess sleep quality with cognition	Global score of PSQI, subjective sleep quality, sleep duration, and sleep medication was statistically high among night shift nurses suggesting poor sleep quality compared to day shift and controls (P = 0.021, P = 0.021, P = 0.00, P = 0.00). Positive correlation was found between global PSQI score and reaction time (r = 0.096).

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Table 5: *[continued]*

Reference (Country)	Total Sample Size (Numbers/in Ratio)/Mean Age in years	Diagnosis/indication/Population cohort	PSQI results in the control/intervention group
Zare et al. 2017 (Iran)	400 randomly selected employees of an Iranian gas company (179 office workers and 221 operational workers) with at least 1 year of job tenure	Healthy participants registered with a cross sectional study to assess the relationship between circadian rhythm and contributing factors of sickness absence	There was a significant relationship between short-term absenteeism and amplitude of circadian rhythm [odds ratio (OR) = 6.13], sleep quality (OR = 14.46) and sleepiness (OR = 2.08). Long-term absenteeism was also significantly associated with amplitude of circadian rhythm (OR = 2.42), sleep quality (OR = 21.56) and sleepiness (OR = 6.44).
Bukowska-Damska et al. 2017 (Poland)	710 nurses and midwives (347 working on rotating nights and 363 working only during the day) aged 40-60 years.	Heathy participants were registered in a cross-sectional study to assess the potential association between poor sleep quality or sleep duration with DNA methylation profiles of clock genes considering rotating night work and chronotype as modifiers using PSQI and MEQ	A positive association was found between average sleep duration of less than 6 hours and the methylation level of PER2 morning chronotype subjects, and an inverse association for CRY2 intermediate chronotype subjects only among day workers. Both the system of work and the chronotype turned out to be important modifiers.
Choi et al. 2016 (South Korea)	160 consecutive patients with epilepsy (aged 20-49 years, focal epilepsy, FE: generalized epilepsy, GE=127:33) and 130 age-gender matched healthy controls (HC).	Patients with epilepsy (PWE) completed a sleep diary for more than 2 weeks, PSQI, ESS, and the MEQ to understand the association of sleep-wake pattern, sleep quality and chronotype with seizures.	Sleep-wake patterns on workdays were different between patients with epilepsy and HC ($p<0.001$), although PSQI scores did not differ. Social jetlag (difference of mid-sleep time between workdays and free days) was more evident in PWE (1.4h) than HC (0.7h, $p<0.001$). Higher seizure frequency was positively correlated with higher PSQI after adjusting for age, gender, and number of antiepileptic drugs ($p<0.05$).

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Table 5: *[continued]*

Reference (Country)	Total Sample Size (Numbers/in Ratio)/Mean Age in years	Diagnosis/indication/Population cohort	PSQI results in the control/intervention group
Unterberger et al. 2015 (Austria)	100 consecutive patients with idiopathic, generalized epilepsy, and 100 consecutive patients with partial epilepsy were compared with 100 gender- and age matched (+/− 2.5 years) healthy controls.	Patients with epilepsy assessed for association between sleep disorders and circadian rhythm	There was a negative correlation between the duration of epilepsy and sleep quality, as assessed with PSQI (Spearman rho = −0.256, p < 0.001).
Wang et al. 2013 (China)	92 primary open angle glaucoma (POAG) patients, 48 primary angle-closure glaucoma (PACG) patients and 199 controls	Glaucoma patients were evaluated with PSQI to determine if glaucoma affects the sleep quality or not	Prevalence of sleep disturbances was higher (PSQI score >7) in patients with PACG compared to POAG patients in the age interval of 61-80.
Schneider et al. 2011 (Brazil)	372 students (66.7% females on average 21.6 years old). 92.2% non-smokers, 58.9% engaged in physical activities and 19.7% were night-shift workers	Sample of undergraduate students used to assess the association of the morningness-eveningness dimension with behavioral and health aspects by employing PSQI and MEQ questionnaires	Poor sleep quality (OR = 1.89), minor psychiatric disorders (OR = 1.92), and tobacco consumption (OR = 3.65) predominated among evening types.
Giglio et al. 2010 (Brazil)	81 outpatients with bipolar disease in remission and 79 control subjects	Bipolar patients and control subjects evaluated for circadian preference and sleep pattern by using the PSQI questionnaire	Circadian preference was associated only with sleep latency (p=0.01) in bipolar patients. No associations with any PSQI subscales (sleep quality, duration, efficiency, disturbances, use of medication or daytime disturbances) or the whole PSQI (p>0.05 for all) were found. Chronotype was not associated with PSQI subscales in control subjects (p>0.05 for all).

The type of study participants who were administered Circadian Clock associated Patient-Reported Outcome Measures (CR-PROMs) were reported in **Table 4**. Healthy individuals constituted the most frequently occurring population followed by shift workers. Our inventory review identified other notable illnesses as well, where CR-PROMs are actively used to evaluate patient's quality of life such as (according to decreasing occurrence) bipolar disease, circadian rhythm sleep-wake disorders (CRSWDs), sleep and depressive disorders, epilepsy, Parkinson's disease, traumatic brain and spinal cord injuries, Huntington's disease, Alzheimer's disease, schizophrenia, eye lens opacity, optic nerve disorders, retinitis pigmentosa, mood disorders, migraine, primary glaucoma, obstructive sleep apnea, multiple sclerosis, narcolepsy, depressive disorder with sensorineural hearing loss, depressive disorder with sleep onset insomnia, anxiety and psychiatric disorders, idiopathic REM sleep behavior disorder and neurodegenerative synucleinopathy, blindness, idiopathic hypersomnia, autism spectrum disorder and meningococcal meningitis.

Finally, we have listed detailed characteristics of included studies (sample sizes of ≥ 100) for Pittsburgh Sleep Quality Index (PSQI) – being the questionnaire with the highest frequency of occurrence in our dataset, in **Table 5**.

DISCUSSION

To our knowledge, this is the first inventory on the circadian clock associated patient-reported outcomes. The results of our literature review might serve as a valuable indicator when more systematic (prospective) studies are done using similar parameters, along with clinical outcomes, for assessing health care quality during clinical trials.

The strength of the literature review is that only well-designed (validated) questionnaires have been included. The objective criteria of the Patient-Reported Outcomes Measurement Information System (PROMIS) have been defined by the National Institute of Health's Roadmap Initiative employing techniques of psychometrics such as item response theory (IRT) and computerized adaptive testing (CAT). Besides NIH, US Food and Drug Administration (FDA), European Medicines Agency (EMA), and scientific consortia from professional societies have also provided consistent recommendations for the selection, validation and quality assessment of PROMs. Recently, it has been concluded that there is increasing consistency in recommendations to guide PROM selection for clinical trials (Crossnohere et al. 2021).

In the present inventory review, 85 PROMs were identified, from which 16 PROMs (frequency of occurrence ≥ 5) were found specifically designed to assess sleep and

chronotype. Those were: Pittsburgh Sleep Quality Index (PSQI), Horne-Östberg Morningness-Eveningness Questionnaire (HO-MEQ), Munich Chrono-Type Questionnaire (MCTQ), Karolinska Sleepiness Scale (KSS), SLEEP-50, Composite Scale of Morningness (CSM), Reduced Horne and Ostberg Morningness and Eveningness Questionnaire (rMEQ), Biological Rhythms Interview of Assessment in Neuropsychiatry (BRIAN), Visual Analog Scale (VAS) assessing subjective sleepiness, Berlin questionnaire (BQ), Athens Insomnia Scale (AIS), Stanford Sleepiness Scale (SSS), Morningness-Eveningness Scale for Children (MESC), Consensus Sleep Diary (CSD) and Patient-Reported Outcomes Information System Short Form v1.0 Sleep Disturbance (PROMIS Short Form v1.0 Sleep Disturbance). Moreover, 5 PROMs (frequency of occurrence ≤ 3) were also found in our dataset specifically developed for shiftwork and sleep-wake activities, for instance Circadian Type Inventory (CTI), Bergen Shift Work Sleep Questionnaire (BSWSQ), Standard Shiftwork Index (SSI), Sleep, Circadian Rhythms and Mood (SCRAM) and Sleep-Wake Activity Inventory (SWAI).

Sleep quality is a common clinical construct and has been associated as an important symptom in psychiatry and other medical conditions. In our inventory, PSQI was frequently administered to measure the sleep quality in patients of Bipolar disorder, Epilepsy, Parkinson's disease, Huntington's disease, Alzheimer's disease, Schizophrenia and in eye lens opacity. PSQI was originally used to assess sleep quality in psychiatric patients and consisted of 19 self-rated questions grouped into 7 components, each weighing equally on a 0-3 scale (Buysse et al. 1989). The seven component scores are summed to yield a global PSQI score, which has a range of 0-21. Higher score indicates worse quality of sleep. In **Table 5**, we have extracted PSQI scores as per different population cohorts. Fundamentally, PSQI scores have been used in the included studies as a simple screening measure to identify "poor" and "good" sleepers. Also, the PSQI could be a great tool in identifying patients with sleep disturbance concomitant with other symptoms. As a longitudinal application, PSQI has been used to examine the course and natural history of sleep/wake disorders.

Limitations of the current inventory include methodological drawbacks such as dropouts, variable study length, small sample size, selection bias, inconsistencies in QoL due to the use of non-disease-specific and generic PROMs, heterogeneity of identified questionnaires and lack of blinding procedures regarding participants. When taking the global impact of circadian rhythms into account, it can be concluded that research regarding the measurement of clock effects on patient-reported outcomes is far from achieving its full potential. Currently, PROMs are complemented with wearable technology like wrist actigraphy (Innominato et al. 2018; Komarzynski et al. 2019) so that continuous assessment of circadian biomarkers such as those provided by the rest/activity, body position,

distal skin temperature and light exposure considering the status and phase of individual circadian systems can be done in clinical trials. Also, implementation of CR-PROMs in healthy and diseased subjects have not been able to highlight the gaps regarding the circadian clock influence (cause versus consequence relationship).

This inventory implies that a robust systematic review of randomized controlled trials (RCT) integrating CR-PROMs could gain a better understanding of the connection between the patient's psychosocial/emotional domains with the chronotype and sleep-wake aspects. Future studies could focus on not only health-related quality of life but also in mental health and vision-related quality of life. Overall, the main conclusion of this inventory review is a first attempt of conducting a narrative synthesis of the presence of circadian clock associated outcomes in the clinical scenario.

Author contributions

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

Acknowledgments

The authors are grateful for all the help with the literature search from the University of Amsterdam UMC and Strasbourg libraries in the Netherlands and in France. The European Research Council (European Commission) under NeuroTime Erasmus+ Mundus Project number: 520124-1-2011-1-FR-ERA Mundus-EPJD, FPA: 2012-0026 has funded and supported the study.

REFERENCES

Akashi M, Soma H, Yamamoto T, Tsugitomi A, Yamashita S, Yamamoto T, Nishida E, Yasuda A, Liao JK, Node K. Noninvasive method for assessing the human circadian clock using hair follicle cells. Proc Natl Acad Sci U S A. 2010 Aug 31;107(35):15643-8.

Allebrandt KV, Roenneberg T. The search for circadian clock components in humans: new perspectives for association studies. Braz J Med Biol Res. 2008 Aug;41(8):716-21.

Arendt J, Middleton B. Human seasonal and circadian studies in Antarctica (Halley, 75°S). Gen Comp Endocrinol. 2018 Mar 1;258:250-258.

Bae K, Jin X, Maywood ES, Hastings MH, Reppert SM, Weaver DR. Differential functions of mPer1, mPer2, and mPer3 in the SCN circadian clock. Neuron 2001; 30: 525- 536.

Bjarnason GA, Jordan RC, Wood PA, Li Q, Lincoln DW, Sothern RB, Hrushesky WJ, Ben-David Y. Circadian expression of clock genes in human oral mucosa and skin: association with specific cell-cycle phases. Am J Pathol. 2001 May;158(5):1793-801.

Bukowska-Damska A, Reszka E, Kaluzny P, Wieczorek E, Przybek M, Zienolddiny S, Peplonska B. Sleep quality and methylation status of core circadian rhythm genes among nurses and midwives. Chronobiol Int. 2017;34(9):1211-1223.

Bunger MK, Wilsbacher LD, Moran SM, Clendenin C, Radcliffe LA, Hogenesch JB, et al. Mop3 is an essential component of the master circadian pacemaker in mammals. Cell 2000; 103: 1009-1017.

Buysse,D.J., Reynolds,C.F., Monk,T.H., Berman,S.R., & Kupfer,D.J. (1989). The Pittsburgh Sleep Quality Index (PSQI): A new instrument for psychiatric research and practice. Psychiatry Research, 28(2), 193-213.

Choi SJ, Joo EY, Hong SB. Sleep-wake pattern, chronotype and seizures in patients with epilepsy. Epilepsy Res. 2016 Feb;120:19-24.

Crossnohere NL, Brundage M, Calvert MJ, King M, Reeve BB, Thorner E, Wu AW, Snyder C. International guidance on the selection of patient-reported outcome measures in clinical trials: a review. Qual Life Res. 2021 Jan;30(1):21-40.

Crosthwaite SK, Loros JJ, Dunlap JC. Light-induced resetting of a circadian clock is mediated by a rapid increase in frequency transcript. Cell 1995; 81: 1003-1012.

Cruz T, García L, Álvarez MA, Manzanero AL. Sleep quality and memory function in healthy ageing. Neurologia (Engl Ed). 2019 Apr 11:S0213-4853(19)30003-9. English, Spanish.

Dudley CA, Erbel-Sieler C, Estill SJ, Reick M, Franken P, Pitts S, et al. Altered patterns of sleep and behavioral adaptability in NPAS2-deficient mice. Science 2003; 301: 379- 383.

Dumitras M; Lupusor A, Vovc V. The quality of sleep-in high school urban students. Journal of Sleep Research. 2021, nr. S1(29), p. 281. ISSN 1365-2869.

Giglio LM, Magalhães PV, Andersen ML, Walz JC, Jakobson L, Kapczinski F. Circadian preference in bipolar disorder. Sleep Breath. 2010 Jun;14(2):153-5.

Hajaghazadeh M, Zamanzadeh V, Ghofrani M, Orujlu S (2019). Morningness-Eveningness Chronotypes, Sleep Quality, and Sleepiness Among Nurses. The Open Public Health Journal. 12. 414-419.

Horne JA, Ostberg O. A self-assessment questionnaire to determine morningness-eveningness in human circadian rhythms. Int J Chronobiol. 1976;4(2):97-110.

Hunter-Ensor M, Ousley A, Sehgal A. Regulation of the Drosophila protein timeless suggests a mechanism for resetting the circadian clock by light. Cell 1996; 84: 677-685.

Ikeda T, Nagai T, Kato-Nishimura K, Mohri I, Taniike M. Sleep problems in physically disabled children and burden on caregivers. Brain Dev. 2012 Mar;34(3):223-9.

Innominato PF, Komarzynski S, Palesh OG, Dallmann R, Bjarnason GA, Giacchetti S, Ulusakarya A, Bouchahda M, Haydar M, Ballesta A, Karaboué A, Wreglesworth NI, Spiegel D, Lévi FA. Circadian rest-activity rhythm as an objective biomarker of patient-reported outcomes in patients with advanced cancer. Cancer Med. 2018 Sep;7(9):4396-4405.

Jones CR, Campbell SS, Zone SE, Cooper F, DeSano A, Murphy PJ, Jones B, Czajkowski L, Ptácek LJ. Familial advanced sleep-phase syndrome: A short-period circadian rhythm variant in humans. Nat Med. 1999 Sep;5(9):1062-5.

Komarzynski S, Huang Q, Lévi FA, Palesh OG, Ulusakarya A, Bouchahda M, Haydar M, Wreglesworth NI, Morère JF, Adam R, Innominato PF. The day after: correlates of patient-reported outcomes with actigraphy-assessed sleep in cancer patients at home (inCASA project). Sleep. 2019 Oct 9;42(10):zsz146.

Kyriacou CP, Hastings MH. Circadian clocks: genes, sleep, and cognition. Trends Cogn Sci. 2010 Jun;14(6):259-67.

Lane, J., Vlasac, I., Anderson, S. et al. Genome-wide association analysis identifies novel loci for chronotype in 100,420 individuals from the UK Biobank. Nat Commun 7, 10889 (2016).

Lim M, Lee S, Seo K, Oh HJ, Shin JS, Kim SK, Kang HT, Jeong KS, Oh SS, Koh SB, Ahn YS. Psychosocial factors affecting sleep quality of pre-employed firefighters: a cross-sectional study. Ann Occup Environ Med. 2020 May 8;32:e12.

Mishra A, Maiti R, Mishra BR, Jena M, Nath S, Sahu P. Effect of add-on ramelteon therapy on sleep and circadian rhythm disruption in patients with schizophrenia: A randomized controlled trial. Eur Neuropsychopharmacol. 2020 Feb;31:109-118.

Moher D, Liberati A, Tetzlaff J, Altman DG; PRISMA Group. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. PLoS Med. 2009 Jul 21;6(7):e1000097.

Pittendrigh CS. Temporal organization: reflections of a Darwinian clock-watcher. Annu Rev Physiol. 1993;55:16-54.

Reppert SM, Weaver DR. Coordination of circadian timing in mammals. Nature. 2002 Aug 29;418(6901):935-41.

Riestra P, Gebreab SY, Xu R, Khan RJ, Gaye A, Correa A, Min N, Sims M, Davis SK. Circadian CLOCK gene polymorphisms in relation to sleep patterns and obesity in African Americans: findings from the Jackson heart study. BMC Genet. 2017 Jun 23;18(1):58.

Roenneberg T, Merrow M. Circadian clocks - the fall and rise of physiology. Nat Rev Mol Cell Biol 2005; 6: 965-971.

Salazar P, Konda S, Sridhar A, Arbieva Z, Daviglus M, Darbar D, Rehman J. Common genetic variation in circadian clock genes are associated with cardiovascular risk factors in an African American and Hispanic/Latino cohort. Int J Cardiol Heart Vasc. 2021 Jun 3;34:100808.

Schneider ML, Vasconcellos DC, Dantas G, Levandovski R, Caumo W, Allebrandt KV, Doring M, Hidalgo MP. Morningness-eveningness, use of stimulants, and minor psychiatric disorders among undergraduate students. Int J Psychol. 2011 Feb 1;46(1):18-23.

Schuch JB, Genro JP, Bastos CR, Ghisleni G, Tovo-Rodrigues L. The role of CLOCK gene in psychiatric disorders: Evidence from human and animal research. Am J Med Genet B Neuropsychiatr Genet. 2018 Mar;177(2):181-198.

Siepka SM, Yoo SH, Park J, Song W, Kumar V, Hu Y, et al. Circadian mutant Overtime reveals F-box protein FBXL3 regulation of cryptochrome and period gene expression. Cell 2007; 129: 1011-1023.

Sun J, Chen M, Cai W, Wang Z, Wu S, Sun X, Liu H. Chronotype: implications for sleep quality in medical students. Chronobiol Int. 2019 Aug;36(8):1115-1123.

Teboul M, Barrat-Petit MA, Li XM, Claustrat B, Formento JL, Delaunay F, Lévi F, Milano G. Atypical patterns of circadian clock gene expression in human peripheral blood mononuclear cells. J Mol Med (Berl). 2005 Sep;83(9):693-9.

Toscano-Hermoso MD, Arbinaga F, Fernández-Ozcorta EJ, Gómez-Salgado J, Ruiz-Frutos C. Influence of Sleeping Patterns in Health and Academic Performance Among University Students. Int J Environ Res Public Health. 2020 Apr 16;17(8):2760.

Tsanas A, Woodward E, Ehlers A. Objective Characterization of Activity, Sleep, and Circadian Rhythm Patterns Using a Wrist-Worn Actigraphy Sensor: Insights Into Posttraumatic Stress Disorder. JMIR Mhealth Uhealth. 2020 Apr 20;8(4):e14306.

Unterberger I, Gabelia D, Prieschl M, Chea K, Hofer M, Högl B, Luef G, Frauscher B. Sleep disorders and circadian rhythm in epilepsy revisited: a prospective controlled study. Sleep Med. 2015 Feb;16(2):237-42.

Vijaykumar N, Kiran S, Karne SL. Influence of altered circadian rhythm on quality of sleep and its association with cognition in shift nurses. Natl J Physiol Pharm Pharmacol 2018;8(5):643-649.

Vitaterna MH, King DP, Chang AM, Kornhauser JM, Lowrey PL, McDonald JD, et al. Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior. Science 1994; 264: 719-725.

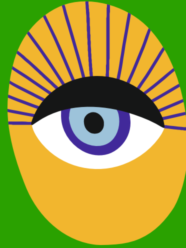
Wang H, Zhang Y, Ding J, Wang N. Changes in the circadian rhythm in patients with primary glaucoma. PLoS One. 2013 Apr 29;8(4):e62841.

Xie Y, Tang Q, Chen G, Xie M, Yu S, Zhao J, Chen L. New Insights Into the Circadian Rhythm and Its Related Diseases. Front Physiol. 2019 Jun 25;10:682.

Young MW, Kay SA. Time zones: a comparative genetics of circadian clocks. Nat Rev Genet 2001; 2: 702-715.

Zare R, Choobineh A, Keshavarzi S. Association of Amplitude and Stability of Circadian Rhythm, Sleep Quality, and Occupational Stress with Sickness Absence among a Gas Company Employees-A Cross Sectional Study from Iran. Saf Health Work. 2017 Sep;8(3):276-281.

CHAPTER



General Discussion, Limitations & Strengths, and Future Perspectives

GENERAL DISCUSSION

Within the retina, the photoreceptors are directly sensitive to light and enable vision. The mammalian retina exerts a number of mechanisms and functions, such as expression of photopigments, processing of visual information, synthesis and release of melatonin, photoreceptor outer segment disk shedding, RPE mediated phagocytosis and resistance against phototoxicity (reviewed in [McMahon et al. 2014; Felder-Schmittbuhl et al. 2017]). All these are modulated by circadian clocks. Emerging experimental evidence indicates circadian dysfunction due to genetic mutations or environmental factors (i.e., jet lag or shiftwork) may influence the expression of retinal diseases (Jauregui-Lozano et al. 2022; Hodge et al. 2022; Jidigam et al. 2022).

So far, circadian clock involvement in eye development has not been studied extensively. In this thesis, we provide data that suggest a potential correlation between circadian clock malfunction and changes in function/turnover of photoreceptors. We approached the objective by investigating the detailed molecular mechanisms linking clock genes and retinal development as well as rhythmic functions, in *Per1*$^{-/-}$*Per2*^{Brdm1} mice.

Summary of our findings about role of the clock in retinal physiology

We recently reviewed the evidence that clock genes influence development, maturation, and pathology of the retina, most likely via complex genetically driven regulatory networks (GRN) (Bery, Bagchi et al. 2022). Our updated schematic model of retinal development highlights the clock regulation of the delicate balance between proliferation and cell cycle exit in early retinal progenitor cells. This regulation by circadian clock genes also includes S-opsin expression and dorso-ventral patterning of M/S-opsin expression. Interestingly, literature studies showed that the clock modulates several pathological processes in the eye including those associated with glaucoma, diabetic retinopathy, age-related macular degeneration, cataract, and myopia. These data suggests that the circadian clock plays a fundamental role in eye physiology and homeostasis.

The primary focus of this thesis is to investigate the potential relevance of *Per1* and *Per2* genes on the developing and adult eye physiology. Clock perturbations in the relevant mouse eyes were studied through comparative temporal transcriptomics during development and adulthood. Firstly, transcriptional changes across ocular developmental stages (E15, E18 and P3) between wild-type (WT) and *Per1*$^{-/-}$*Per2*^{Brdm1} mice were investigated by means of RNA sequencing. Major gene expression changes were observed between the phenotypes, with the number of differentially expressed genes (DEG) increasing with developmental age. After functional gene annotation, mutant mice at E15 and E18 showed the most significant changes in expression levels relating to circadian rhythm sig-

naling pathways. At P3, the gene expression levels of the visual cascade and the cell cycle related molecular pathways were respectively higher and lower compared to WT eyes. In mouse WT eyes between E13 and P24, the developmental expression profiles of clock transcripts displayed changes in transcript levels along with normal eye development.

Secondly, a similar experimental set-up was used to study the transcriptional changes that occur under constant dark conditions in retinal photoreceptors together with the adjacent RPE prior and during the peak of photoreceptor outer segment (POS) phagocytosis in 6 weeks old age-matched WT and *Per1*$^{-/-}$*Per2*^{Brdm1} mice. Various potential pathways associated with phototransduction, DNA synthesis, neurotransmission and POS phagocytosis orchestrating interactive crosstalk with clockwork genes were identified. Our comprehensive list of identified candidate genes could provide ample opportunities for the research community to sufficiently characterize precise mechanisms linking all retinal clocks in rodents [virtually present in all retinal layers] (Jaeger et al. 2015; Ruan et al. 2006, 2008; Liu et al. 2012) to cell-cycle and neurotransmission-related pathways.

Finally, we partly addressed functional biological readouts of *Per1* and *Per2* inactivation for visual processing. The day/night rhythms in dark-adapted light response were lost, but no major detectable effects on aging in the retina was observed.

The potential clinical implications of the circadian clock system in early-onset retinal diseases have been recently documented (Bakker et al. 2022). Bery, Bagchi et al. 2022 reviewed retinal pathophysiology caused due to clock dysfunction, starting with the retina, and extending it to distinct eye compartments. We investigated the potential involvement of the clock gene *BMAL1* in congenital cataract, Given the occurrence of cataract in *Bmal1*$^{-/-}$ and *Clock*$^{-/-}$ mice, we aimed to test the hypothesis that *BMAL1* mutations could also play a role in human cataract. As such, we sequenced the human *BMAL1* gene in the DNA of our (relatively small) study cohort of 21 patients with congenital cataract. We found that genetic variation in *BMAL1* did not have an impact on the disease phenotype. However, we did identify a *BMAL1* missense variant that alters the WT amino acid sequence in a highly evolutionary conserved residue. This variant was annotated as "probably pathogenic" in bioinformatic analysis. We reported our result as a rare variant as it only happened in the heterozygous state in a single patient, and did not segregate in the family. Evidence obtained from rodent models complemented with the screening of (other) circadian clock genes (sequences) might be justified to understand whether the clockwork machinery is involved in childhood cataract as *BMAL1* is not a sole member of the clock network.

We have also shortly explored clinical trial end results [only questionnaires/Patient-Reported Outcome Measures (PROMs)] pertaining to the measurement of clock attributes such as sleep, chronotype, circadian rhythms, shiftwork, and sleep disorders across variety of disease populations. We found Pittsburgh Sleep Quality Index (PSQI) and Horne-Östberg Morningness-Eveningness Questionnaire (HO-MEQ) as the frequently used questionnaires assessing sleep and chronotype among healthy individuals. Other questionnaires pertaining to shiftwork and sleep-wake activities were also identified in our literature review such as Circadian Type Inventory (CTI), Bergen Shift Work Sleep Questionnaire (BSWSQ), Standard Shiftwork Index (SSI), Sleep, Circadian Rhythms and Mood (SCRAM) and Sleep-Wake Activity Inventory (SWAI).

Strengths and limitations

In this section, we describe number of strengths and limitations of our studies in this thesis.

One of the strengths in our studies as discussed in **Chapters 3-5** was the use of very well characterized *Per1*$^{-/-}$*Per2*^{Brdm1} double mutant rodent model for phenotypical (RNA expression, immunohistochemistry), structural (histology, in vivo imaging), and functional attributes by Aït-Hmyed et al. 2013 prior to the experiments conducted in this thesis. Also, in **Chapters 3 and 4**, the usage of high-quality RNA in the experiments from mouse whole eyes and meticulously microdissected photoreceptors and RPE and enabled minimal disruption of the cellular transcriptional machinery. Our work in **Chapter 3** provided evidence that clock genes take part in eye development, likely via signaling pathways between differentiation programs and regulation of cell division. We observed that gene expression differences between WT and mutant eyes are much more pronounced at P3 than at embryonic stages. Moreover, there was an upregulation of *Per3* mRNA in mutant vs WT eyes (FC > 2.5 at P3 but also FC > 1.9 at E15, and > 1.8 at E18). The developmental expression kinetics of *Per3* indicates a role in adult eyes rather than during development therefore it is not unlikely that *Per3* could act as a potential compensatory clock gene modulator at this stage. Also, the fact that the maximum expression of *Per1* and *Per2* occurs around P0 - the peak of their biological effect. Centering further studies around this time point might provide additional answers about the pathways regulated by *Per1/Per2* in eye development.

Another strength of the thesis was the choice of congenital cataract for our study in **Chapter 6** due to the developmental implications of the disease that *Bmal1* and *Clock* knockout mice induces premature aging in the eye. In our study, we could not see a clear association between human genetic *BMAL1* variants and cataract. Nevertheless, as described in **Chapter 2** (Bery, Bagchi et al. 2022) there are several examples of clock

malfunction possibly modulating detrimental pathological effects of aging in the retina and early-onset retinal diseases: this is maybe the entry point of clock defects in the pathogenesis of cataract.

We used the *Per1*$^{-/-}$*Per2*^{Brdm1} double mutant (considered as a double knockout Zheng et al. 2001, 1999) rodent instead of a rod-specific model in **Chapters 3-5** to study a range of detectable retinal phenotypes and its transcriptional signatures in the retinal physiology. Global knockout mice, as compared to conditional knockouts, are obtained through the global elimination of a gene's function, and are not limited to a certain tissue within the body. A rod-specific model could have provided a more localized response of how the retinal tissue behaves in the absence of certain clock genes. The expression patterns of *Per1* and *Per2* within the retina remains controversial, as there is not much consistent expression observed within rod photoreceptors versus inner retinal neurons including retinal ganglion cells (RGCs) (Ruan et al. 2006, 2008). Only *Per1* gene expression is reported but not *Per2* in rods by Tosini et al. 2007; on the other hand, in isolated rat rod photoreceptor layers, definite but low *Per2* expression was shown (Sandu et al. 2011). Yet, the data of **Chapters 3-5** demonstrate that use of this global mutant was efficient in highlighting a number of clock-controlled processes in the eye/retina, even if a cell autonomous effect could not be demonstrated.

As with limitations of the thesis, given the redundancy of the 3 Period genes in the molecular clockwork, **Chapter 3** indicates that part of the biological properties of *Per* genes could not be determined in our setup and future studies should investigate the effects on the eye of triple knockouts. Another potential limitation of this study is that we did not observe an a-priori expected enrichment for BMAL1/CLOCK targets in our DEG set (one-sided Fisher's exact test, $p = 0.74$). This most likely indicates that differentially expressed genes between WT and mutant eyes were both direct (for instance CLOCK/BMAL1 but also DBP, NR1D1/2) and (essentially) indirect clock targets. Even if this screen does not reveal the direct clock-controlled genes, the extent of transcriptional changes in mutant eyes, in particular at P3, demonstrate the far-reaching effects of invalidating *Per1/2*.

Chapter 4 provided strong evidence that the rhythm in phagocytosis is not controlled by the photoreceptor clock, as confirmed by the recent publication (De Vera et al. 2022) but did not precisely reveal the clock targets that might regulate the phagocytosis peak within the RPE. Functional studies with the list of potential genes of interest upregulated at the peak and downregulated in the mutant, as well as in silico analyses of their regulatory sequences should be used in the future to further identify the node regulators of phagocytosis downstream of the clock. By contrast, our data of **Chapter 5** indicate the circadian rhythm of rod response to light in scotopic condition is regulated by the

clock and that no premature aging of the retina was detected [whereas it does occur when *Bmal1* or *Clock* are deleted (whole knockout or retina-specific KO for *Bmal1*)]. This contrasts with data regarding other organs/physiological functions that were markedly disturbed, in the same *Per1*$^{-/-}$*Per2*^{Brdm1} mutant mouse line (for instance, Ma et al. 2009; Zheng et al. 2019; Adebiyi et al. 2019).

Future perspectives

The contribution of rods, cones, and intrinsically photosensitive melanopsin-containing ganglion cells (ipRGCs) in shaping the visual response has been studied in the retinal clock field. Moreover, recent studies have proposed entrainment of the mouse retinal clock by a UV-sensitive photopigment called neuropsin or OPN5 (Buhr et al. 2015; Calligaro et al. 2019; Barnard et al. 2006). Recent data also indicate that the effects of violet light on retinal OPN5, specifically in the evening protect eyes from lens-induced myopia in 3-week-old mice (Jiang et al. 2021). OPN5 is also involved in the regulation of light-dependent vascular development in the eye (Nguyen et al. 2019). It will be interesting to note if there are any clock genes who cross-talk between the violet light–OPN5–retinal circadian clock and developmental pathways in the mammalian retina.

In Chapter 3, we speculated one of the causes of the morphological/functional changes in the phototransduction machinery observed in the mutant at P3 might be due to NR2E3-mediated regulation. Our hypothesis was that clock might control photo-transduction gene expression in the mammalian retina (Corbo et al. 2010; Hao et al. 2012) at a node between the clock and retinal development because *Nr2e3* (FC > 1.6 at P3 in the *Per1*$^{-/-}$*Per2*^{Brdm1} mouse eyes) co-activates rod-genes with NRL, CRX and REV-ERBα (NR1D1) (Cheng et al. 2004). Recently, the efficacy of *Nr2e3* delivery to the sub-retinal space of murine models of retinitis pigmentosa demonstrated overall rescue of retinal degeneration and restoration of retinal homeostasis (Li et al. 2021). Combining our results with those of the literature, it will be interesting to understand the role of *Nr2e3* by means of immunohistochemical staining precisely at the regulatory node during retinal development of *Per1*$^{-/-}$*Per2*^{Brdm1}, *Bmal1* and *Clock* deficient mice.

Yet another aspect worth considering is the effect of absence/mutation of *Rorc* (a target of *Nr2e3*) in the retinal architecture. It can be studied by examining the tissue expression of *Rorc* in the post-natal retina as *Rorc* displayed upregulation in our data (FC > 1.5 at P3 in the mutant eyes). Recent findings targeting the *Rorc* and its downstream effector, interleukin (IL)-17A axis in primary cultures of rat retinal microglia influenced VEGF production and suppressed neovascular retinopathy (Talia et al. 2016). Moreover, loss of other gene paralogs like *Rora* leads to defective cone differentiation (Fujieda et al. 2009) and *Rorb* causes complete loss of rods and over-production of blue cones during retinal

development (Jia et al. 2009), confirming the idea that ROR factors might be at the note between clock and retinal development.

In our study in the *Per1$^{-/-}$Per2^{Brdm1}* double mutants, the cell cycle appeared reduced at P3 - an age at which a substantial part of precursors for late-born retinal cell types are still dividing. Labelling of WT and *Per1/Per2* mutant eye sections with markers for proliferating cells (KI67 for instance) at the P3 stage but also before, would be of particular interest in this mapping.

Investigation of conditional retina-specific knockout model of *Per1* and *Per2* could check if the retinal circuitry is hampered and if the clock cell-autonomously controls photoreceptor generation during development. The scotopic and photopic ERG light-responses could be recorded in the young and adult mice, this would allow precise evaluation of the effects on PR viability during aging.

The rise of increased light pollution and humans being overexposed to light at night opens an interesting paradigm to study further – understanding the additional effect of light input during retinal development/maturation (likely mediated through the clock). It had been shown earlier that circadian light cycles during postnatal period in the mice had an enduring influence on retinal physiology and a marked impact on vision in adulthood (Jackson et al. 2014). In rats, constant light exposure was shown to terminate pregnancy at embryo stage, associated with low melatonin levels in the mothers (Berbets et al. 2019). Implications of evening light exposure on sleep characteristics have been explored in human pregnancy too (Liset et al. 2021). Modern lifestyles often lack regular rhythms of sleeping, eating, and working. Given the links between circadian dysregulation and increased susceptibility to cancer, cardio-metabolic, digestive, immune as well as neuropsychiatric disorders, the potential impact of chronomedicine in improving health and fitness is a therapeutic approach worth exploring.

It is discussed in the clock field that successful targeting of the circadian output pathways might be better with drug targets instead of the core clock components, for example via melatonin cycling, modulating casein kinases, REV-ERBs, RORs and CRYs (Lee et al. 2021). Kramer et al. 2022 states individual physiological rhythms could be tailored to time-of-day adapted treatment regimens (exploiting the clock); by identifying disrupted rhythms through improvement/resynchronization interventions in the clock (targeting the clock); and by personalized interventions according to the individual's chronotype (detecting the clock). Chrono-Exercise is one of the few emerging non-invasive circadian interventions shown to improve retinal degeneration and promotes retinal astrocyte plasticity in adult male BALB/c mice (Bales et al. 2022).

Extensive research in circadian biology in the past 25 years has expanded our understanding of the implications of circadian clockwork in human health and disease. The application of circadian medicine in the treatment/prevention of human diseases remains open and is worth exploring as a reasonable therapeutic recommendation in clinical practice.

REFERENCES

Adebiyi MG, Zhao Z, Ye Y, Manalo J, Hong Y, Lee CC, Xian W, McKeon F, Culp-Hill R, D' Alessandro A, Kellems RE, Yoo SH, Han L, Xia Y. Circadian period 2: a missing beneficial factor in sickle cell disease by lowering pulmonary inflammation, iron overload, and mortality. FASEB J. 2019 Sep;33(9):10528-10537.

Ait-Hmyed, O., Felder-Schmittbuhl, M.P., Garcia-Garrido, M., Beck, S., Seide, C., Sothilingam, V., Tanimoto, N., Seeliger, M., Bennis, M., Hicks, D., 2013. Mice lacking Period 1 and Period 2 circadian clock genes exhibit blue cone photoreceptor defects. Eur. J. Neurosci. 37, 1048–1060.

B. Zheng, D.W. Larkin, U. Albrecht, Z.S. Sun, M. Sage, G. Eichele, C.C. Lee, A. Bradley, The mPer2 gene encodes a functional component of the mammalian circadian clock, Nature 400 (1999) 169–173.

B. Zheng, U. Albrecht, K. Kaasik, M. Sage, W. Lu, S. Vaishnav, Q. Li, Z.S. Sun, G. Eichele, A. Bradley, C.C. Lee, Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock, Cell 105 (2001) 683–694.

Bakker R, Wagstaff PE, Kruijt CC, Emri E, van Karnebeek CDM, Hoffmann MB, Brooks BP, Boon CJF, Montoliu L, van Genderen MM, Bergen AA. The retinal pigmentation pathway in human albinism: Not so black and white. Prog Retin Eye Res. 2022 Jun 18:101091.

Bales KL, Chacko AS, Nickerson JM, Boatright JH, Pardue MT. Treadmill exercise promotes retinal astrocyte plasticity and protects against retinal degeneration in a mouse model of light-induced retinal degeneration. J Neurosci Res. 2022 Sep;100(9):1695-1706.

Barnard AR, Hattar S, Hankins MW, Lucas RJ. Melanopsin regulates visual processing in the mouse retina. Curr Biol. 2006 Feb 21;16(4):389-95.

Berbets AM, Barbe AM, Yuzko OM. Constant light exposure terminates pregnancy in rats with pineal gland dysfunction, low melatonin level and pro-inflammatory response. Melatonin Research. 2019; 2 (4):9–24.

Bery A, Bagchi U, Bergen AA, Felder-Schmittbuhl MP. Circadian clocks, retinogenesis and ocular health in vertebrates: new molecular insights. Dev Biol. 2022 Apr;484:40-56.

Buhr ED, Yue WW, Ren X, et al. Neuropsin (OPN5)-mediated photoentrainment of local circadian oscillators in mammalian retina and cornea. Proc Natl Acad Sci U S A. 2015;112: 13093–13098.

Calligaro H, Coutanson C, Najjar RP, Mazzaro N, Cooper HM, Haddjeri N, et al. (2019) Rods contribute to the light-induced phase shift of the retinal clock in mammals. PLoS Biol 17(3): e2006211.

DeVera C, Dixon J, Chrenek MA, Baba K, Le YZ, Iuvone PM, Tosini G. The Circadian Clock in the Retinal Pigment Epithelium Controls the Diurnal Rhythm of Phagocytic Activity. Int J Mol Sci. 2022 May 10;23(10):5302.

Felder-Schmittbuhl, M.P., Calligaro, H., Dkhissi-Benyahya, O., 2017. The retinal clock in mammals: role in health and disease. Chronophysiology Ther 7, 33–45.

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 Dec;213:108807.

H. Cheng, H. Khanna, E.C. Oh, D. Hicks, K.P. Mitton, A. Swaroop, Photoreceptor- specific nuclear receptor NR2E3 functions as a transcriptional activator in rod photoreceptors, Hum. Mol. Genet. 13 (2004) 1563–1575.

H. Fujieda, R. Bremner, A.J. Mears, H. Sasaki, Retinoic acid receptor-related orphan receptor alpha regulates a subset of cone genes during mouse retinal development, J. Neurochem. 108 (2009) 91–101.

H. Hao, D.S. Kim, B. Klocke, K.R. Johnson, K. Cui, N. Gotoh, C. Zang, J. Gregorski, L. Gieser, W. Peng, Y. Fann, M. Seifert, K. Zhao, A. Swaroop, Transcriptional regulation of rod photoreceptor homeostasis revealed by in vivo NRL targetome ana- lysis, PLoS Genet. 8 (2012) e1002649.

Hodge BA, Meyerhof GT, Katewa SD, Lian T, Lau C, Bar S, Leung NY, Li M, Li-Kroeger D, Melov S, Schilling B, Montell C, Kapahi P. Dietary restriction and the transcription factor clock delay eye aging to extend lifespan in Drosophila Melanogaster. Nat Commun. 2022 Jun 7;13(1):3156.

J.C. Corbo, K.A. Lawrence, M. Karlstetter, C.A. Myers, M. Abdelaziz, W. Dirkes, K. Weigelt, M. Seifert, V. Benes, L.G. Fritsche, B.H. Weber, T. Langmann, CRX ChIP- seq reveals the cis-regulatory architecture of mouse photoreceptors, Genome Res. 20 (2010) 1512–1525.

Jackson CR, Capozzi M, Dai H, McMahon DG. Circadian perinatal photoperiod has enduring effects on retinal dopamine and visual function. J Neurosci. 2014 Mar 26;34(13):4627-33.

Jaeger, C., Sandu, C., Malan, A., Mellac, K., Hicks, D., Felder-Schmittbuhl, M.P., 2015. Circadian organization of the rodent retina involves strongly coupled, layer-specific oscillators. Faseb. J. 29, 1493–1504.

Jauregui-Lozano J, Hall H, Stanhope SC, Bakhle K, Marlin MM, Weake VM. The Clock:Cycle complex is a major transcriptional regulator of Drosophila photoreceptors that protects the eye from retinal degeneration and oxidative stress. PLoS Genet. 2022 Jan 31;18(1):e1010021.

Jiang X, Pardue MT, Mori K, Ikeda SI, Torii H, D'Souza S, Lang RA, Kurihara T, Tsubota K. Violet light suppresses lens-induced myopia via neuropsin (OPN5) in mice. Proc Natl Acad Sci U S A. 2021 Jun 1;118(22):e2018840118.

Jidigam VK, Sawant OB, Fuller RD, Wilcots K, Singh R, Lang RA, Rao S. Neuronal Bmal1 regulates retinal angiogenesis and neovascularization in mice. Commun Biol. 2022 Aug 6;5(1):792.

Kramer A, Lange T, Spies C, Finger A-M, Berg D, Oster H (2022) Foundations of circadian medicine. PLoS Biol 20(3): e3001567.

L. Jia, E.C. Oh, L. Ng, M. Srinivas, M. Brooks, A. Swaroop, D. Forrest, Retinoid- related orphan nuclear receptor RORbeta is an early-acting factor in rod photoreceptor development, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 17534–17539.

Lee Y, Field JM, Sehgal A. Circadian Rhythms, Disease and Chronotherapy. J Biol Rhythms. 2021 Dec;36(6):503-531.

Li, S., Datta, S., Brabbit, E. et al. Nr2e3 is a genetic modifier that rescues retinal degeneration and promotes homeostasis in multiple models of retinitis pigmentosa. Gene Ther 28, 223–241 (2021).

Liset R, Grønli J, Henriksen RE, Henriksen TEG, Nilsen RM, Pallesen S. Sleep, evening light exposure and perceived stress in healthy nulliparous women in the third trimester of pregnancy. PLoS One. 2021 Jun 3;16(6):e0252285.

Liu, X., Zhang, Z., Ribelayga, C.P., 2012. Heterogeneous expression of the core circadian clock proteins among neuronal cell types in mouse retina. PLoS One 7, e50602.

Ma K, Xiao R, Tseng H-T, Shan L, Fu L, Moore DD (2009) Circadian Dysregulation Disrupts Bile Acid Homeostasis. PLoS ONE 4(8): e6843.

McMahon, D.G., Iuvone, P.M., Tosini, G., 2014. Circadian organization of the mammalian retina: from gene regulation to physiology and diseases. Prog. Retin. Eye Res. 39, 58–76.

Nguyen MT, Vemaraju S, Nayak G, Odaka Y, Buhr ED, Alonzo N, Tran U, Batie M, Upton BA, Darvas M, Kozmik Z, Rao S, Hegde RS, Iuvone PM, Van Gelder RN, Lang RA. An opsin 5-dopamine pathway mediates light-dependent vascular development in the eye. Nat Cell Biol. 2019 Apr;21(4):420-429.

Ruan, G.X., Allen, G.C., Yamazaki, S., McMahon, D.G., 2008. An autonomous circadian clock in the inner mouse retina regulated by dopamine and GABA. PLoS Biol. 6, e249.

Ruan, G.X., Zhang, D.Q., Zhou, T., Yamazaki, S., McMahon, D.G., 2006. Circadian organization of the mammalian retina. Proc. Natl. Acad. Sci. U. S. A. 103, 9703–9708.

Sandu, C., Hicks, D., Felder-Schmittbuhl, M.P., 2011. Rat photoreceptor circadian oscillator strongly relies on lighting conditions. Eur. J. Neurosci. 34, 507–516.

Talia DM, Deliyanti D, Agrotis A, Wilkinson-Berka JL. Inhibition of the Nuclear Receptor RORγ and Interleukin-17A Suppresses Neovascular Retinopathy: Involvement of Immunocompetent Microglia. Arterioscler Thromb Vasc Biol. 2016 Jun;36(6):1186-96.

Tosini, G., Davidson, A.J., Fukuhara, C., Kasamatsu, M., Castanon-Cervantes, O., 2007. Localization of a circadian clock in mammalian photoreceptors. Faseb. J. 21, 3866–3871.

Zheng Y, Liu C, Li Y, Jiang H, Yang P, Tang J, Xu Y, Wang H, He Y. Loss-of-function mutations with circadian rhythm regulator Per1/Per2 lead to premature ovarian insufficiency†. Biol Reprod. 2019 Apr 1;100(4):1066-1072.

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SUMMARY

The 24-hour rotation of the Earth around its axis evolved life and established a perpetual cycle of light-dark. As a result, intrinsic ≈ 24h rhythms became a fundamental hallmark of every cellular and physiological process throughout the body. These biological mechanisms are conserved across all photosensitive life-forms thereby harmoniously monitoring our geophysical time. The first emergence of a timed molecular oscillator was seen ~2.1 billion years ago in cyanobacteria. In mammalian tissues, rhythmicity is programmed by widely distributed molecular clocks and synchronized by a master, hypothalamic clock residing in the suprachiasmatic nucleus (SCN) of the brain. The master clock oscillates with a period of approximately 24 hours, which introduced the term ‘circadian’ (circa = about and dian = a day).

Circadian clocks keep time by using complex transcriptional/post-translational feedback loops involving transcription factors encoded by clock genes (mainly *Bmal1*, *Clock*, *Per1-3*, *Cry1-2*) which transmit their cycling patterns to their target genes, hence to gene expression programs. A secondary feedback loop involves the retinoic acid-related orphan receptor (ROR) and REV-ERB, a nuclear hormone receptor–encoding gene, which are both are CLOCK/BMAL1 targets distributes rhythms to CCGs via ROR binding elements (RORE). Genome-wide transcriptome profiling studies 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 different temporally controlled tasks.

The retina contains an endogenous circadian clock which regulates many physiological processes in a recurrent 24-hour rhythm allowing adaptation of the visual function to daily changes in light intensity. It also surprisingly harbors a widely distributed network of circadian clocks within distinct retinal cellular layers. Thus, the retinal clock is involved in diverse cyclic functional processes, such as melatonin synthesis, dopamine synthesis, the sensitivity of ion channels, and photoreceptor renewal. Although progress has been made in characterizing the molecular makeup and cellular localization of the retinal clock, little is known about the downstream events that link the clock to retinal physiology. Evidence for a causative effect of circadian clock disruption has been provided by diverse studies in the eye on aging, visual processing, sensitivity to ambient light including developmental defects relating to opsins. Likewise, several data have linked the clock to tissue differentiation, such as in the muscle.

The objective of this PhD thesis was to identify the molecular machinery underlying the influence of the circadian clock during eye development. This thesis has been divided into

different sections. In **Part I**, **Chapter 1** includes a brief introduction to circadian rhythms and eye development, illustrating the necessary principles for understanding the basis of our research. At the end of this chapter, the scope of the thesis is described.

In **Chapter 2**, we have included an exhaustive review of animal and human studies describing the circadian control in eye development. To better analyze the reviewed studies, we first reported about the intimate relationship shared between the circadian clock, the cell cycle, and developmental processes. A separate section describing the historical timeline of the retinal clock in different species has also been included. Furthermore, we have also thrown some light on when the clock starts ticking in vertebrates. We have segregated review into different sub-sections pertaining to retinogenesis, clock genes and clock-controlled genes (CCG) during retinal development and circadian implications of ocular health. Our overview from the reviewed studies showed a dynamic regulatory landscape of retina development across different retinal cell types employing several bHLH, homeobox and clock genes. Assimilating the existing literature on the circadian control of gene expression in the developing zebrafish, chick, and xenopus retina; we have proposed a gene regulatory network (GRN) for the mouse retinal clock. A recurrent question in the field regarding the transcriptional regulation of retinal development under clock control has also been answered. In addition, we have indicated that the effect of clock dysfunction on retinal development and its links with chronic eye diseases provides a strong association of the clock machinery in maintaining ocular health. Our large-scale targeted literature review highlights further research in the field, especially to determine other intrinsic/extrinsic factors and the upstream role of the circadian clock in the embryonic eye by utilizing *in vitro* 3-D environments of stem cell population.

In **Part II**, we have explored the relevance of core-clock genes Period 1 and 2 on the developing and adult eye physiology. 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. In accordance with this phenotype, in **Chapter 3**, 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 $Per1^{-/-}Per2^{Brdm1}$ mutants versus wild-type at E15, E18 and P3. RNA-Seq data showed major gene expression changes between WT and mutant eyes, with the number of differentially expressed genes (DEG) increasing with developmental age. We have characterized the developing whole eye transcriptomes that are differentially expressed in $Per1^{-/-}Per2^{Brdm1}$ mutant versus wild-type and have assessed thousands of transcripts into functional categories by integrative dataset analysis. Functional annotation of the genes showed that the most significant changes in expression levels in mutant mice involve molecular pathways relating to circadian

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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. We have also carried out the developmental expression profiles of clock transcripts between embryonic day 13 (E13) and postnatal day 24 (P24) in mouse WT eyes. All clock genes displayed changes in transcript levels along with normal eye development. Based on the genomic differences in the timing of developmental processes along with the functional implications that arise in various biological pathways due to clock perturbations, we have concluded 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.

Daily renewal of the distal outer segments of the retinal photoreceptors is indispensable for maintaining retinal health. The underlying cellular and molecular mechanisms which initiate the photoreceptor outer segment (POS) phagocytosis are not known. Currently, it is evidenced that a daily peak of POS phagocytosis roughly occurs about one hour after light onset. In **Chapter 4**, we have shown that under constant darkness, mice deficient for core circadian clock genes (*Per1* and *Per2*), lack a daily peak in POS phagocytosis. By qPCR analysis we found that core clock genes were rhythmic over 24h in both WT and $Per1^{-/-}Per2^{Brdm1}$ mutant whole retinas. Precision transcriptomics analysis of laser capture microdissected WT photoreceptors revealed no differentially expressed genes between time-points preceding and during the peak of POS phagocytosis. By contrast, we found that microdissected WT retinal pigment epithelium (RPE) had several genes that were differentially expressed at the peak phagocytic time-point compared to adjacent ones. We also found several differentially expressed genes in $Per1^{-/-}Per2^{Brdm1}$ mutant RPE compared to WT ones at the peak phagocytic time-point. Finally, based on STRING analysis we found a group of interacting genes which potentially drive POS phagocytosis in the RPE. This potential pathway consists of genes such as: *Pacsin1*, *Syp*, *Camk2b* and *Camk2d* among others. Our findings indicated that *Per1* and *Per2* are necessary clock components for driving POS phagocytosis including that, it is transcriptionally driven by the RPE. Adding to the assertion, In **Chapter 5**, mice deficient for core circadian clock genes (*Per1* and *Per2*), showed enhanced nocturnal light sensitivity and a modest yet significant reduction in the outer nuclear layer (ONL) thickness as compared to the wild-type mice.

In **Part III**, we have focussed on the clinical perspective or the disease accountability of core-clock gene BMAL1 in human cataract. The primary transcriptional regulator of the circadian clock, the brain, and muscle aryl hydrocarbon receptor nuclear translocator-like protein BMAL1 is implicated in the regulation of early to premature ocular aging. More than 50% of *Bmal1* deficient mice are known to develop cataract before the 40th week of life. Recently, the conditional deletion of *Bmal1* in endothelial and hematopoietic cells

of the murine retina demonstrated pathologic hallmarks of diabetic retinopathy, thereby expanding on the ocular pathology because of molecular *Bmal1* defects. In agreement with these observations, in **Chapter 6** we examined *BMAL1* as an active candidate gene of interest in congenital cataract. Our study was the first to investigate the core circadian clock gene *BMAL1* in humans for their association with congenital cataract. We aimed to identify the disease-causing variants in the *BMAL1* gene associated with the CC phenotype in the consanguineous Pakistani families to explore any existing links between the circadian clock and ocular abnormalities. In the era of exome sequencing, we have used traditional Sanger sequencing as an indispensable cost-effective tool to report mutation profiles in our small clinical study cohort. We identified missense variant c.41A>T; p.(Asp14Val) altered the wild-type amino acid sequence, occurred in a highly evolutionary conserved residue, and was also determined to be "probably pathogenic" by bioinformatics, it happened in the heterozygous state in a single patient and did not segregate in the family. Thus, we could not correlate the Asp14Val variant with the occurrence of congenital cataract. A non-synonymous heterozygous variant (rs2290037) with a higher allele frequency was detected in the intronic region. It has been previously estimated that 5% of the rare non-synonymous heterozygous variants carry at least ~22 pathogenic derived alleles, which if turns out to be homozygous due to consanguineous marriages, can lead to recessive diseases. Although, no clear association between human genetic *BMAL1* variants and cataract was found, yet our study was informative and acts as a prelude to considering further studies in the clockwork pathway from other populations (**Chapter 6**). *BMAL1* gene is an intricate member of the clockwork web, and since *BMAL1* is not a sole member, BMAL1-CLOCK complex drives the clockwork machinery. Therefore, additional screening of congenital cataract patients with (other) circadian clock genes (sequences) may be justified in subsequent studies. Over the past decade, numerous applications of chronobiology have surfaced in disease treatment. In **Chapter 7**, we have highlighted how circadian biology related Patient-reported Outcome Measures (PROMs) play a potential role in supporting human clinical trial endpoints. We performed a systematic analysis and disease mapping of PROMs associated with the circadian clock in the last 20 years.

As a conclusion, this PhD thesis reveals various facets of the ubiquitous relevance of circadian clock in eye physiology. Although, more studies will be needed to determine the connection between the circadian phenotype and retinal abnormalities, still our findings reveal potential biological mechanisms specifically signaling pathways potentially linking the clock, photoreceptor generation and control of the cell cycle in the developing retina. Future studies are needed to make sure that the downregulation of cell-cycle-related pathways occurs in the retina exclusively and not elsewhere in the eye. Furthermore, because of clock malfunction the phagocytosis machinery is compromised in adult mice, yet another result confirming clock components as integral players in maintaining the daily turnover of outer segments.

RÉSUMÉ DE LA THÈSE

La rotation de 24 heures de la Terre autour de son axe a fait évoluer la vie et a établi un cycle perpétuel de lumière-obscurité. Par conséquent, les rythmes intrinsèques ≈ 24h sont devenus une caractéristique fondamentale de chaque processus cellulaire et physiologique dans tout le corps. Ces mécanismes biologiques sont conservés à travers toutes les formes de vie photosensibles surveillant ainsi harmonieusement notre temps géophysique. La première émergence d'un oscillateur moléculaire rythmé a été observée il y a ~2,1 milliards d'années chez les cyanobactéries. Dans les tissus des mammifères, la rythmicité est programmée par des horloges moléculaires largement distribuées et synchronisées par une horloge maîtresse, hypothalamique, résidant dans le noyau supra-chiasmatique (SCN) du cerveau. L'horloge maîtresse oscille avec une période d'environ 24 heures, ce qui a introduit le terme «circadien» (circa = environ et dian = un jour).

Les horloges circadiennes gardent le temps en utilisant des boucles de rétroaction transcriptionnelles/post-traductionnelles complexes impliquant des facteurs de transcription codés par des gènes d'horloge (principalement *Bmal1*, *Clock*, *Per1-3*, *Cry1-2*) qui transmettent leurs schémas de cycle à leurs gènes cibles, donc aux programmes d'expression génétique. Une boucle de rétroaction secondaire implique le récepteur orphelin lié à l'acide rétinoïque (ROR) et REV-ERB, un gène codant pour un récepteur d'hormone nucléaire, qui sont tous deux des cibles de CLOCK/BMAL1 distribue les rythmes aux CCGs via des éléments de liaison ROR (RORE). Les études de profilage du transcriptome à l'échelle du génome ont mis en évidence un large éventail (environ 43% de tous les gènes codant pour des protéines) de gènes ubiquitaires et spécifiques aux tissus sous contrôle circadien, en accord avec la nécessité, pour différents organes, de remplir différentes tâches contrôlées dans le temps.

La rétine contient une horloge circadienne endogène qui régule de nombreux processus physiologiques selon un rythme récurrent de 24 heures permettant l'adaptation de la fonction visuelle aux changements quotidiens d'intensité lumineuse. De manière surprenante, elle abrite également un réseau largement distribué d'horloges circadiennes au sein de couches cellulaires rétiniennes distinctes. Ainsi, l'horloge rétinienne est impliquée dans divers processus fonctionnels cycliques, tels que la synthèse de mélatonine, la synthèse de dopamine, la sensibilité des canaux ioniques et le renouvellement des photorécepteurs. Bien que des progrès aient été réalisés dans la caractérisation de la composition moléculaire et de la localisation cellulaire de l'horloge rétinienne, on sait peu de choses sur les événements en aval qui relient l'horloge à la physiologie rétinienne. Des preuves d'un effet causal de la perturbation de l'horloge circadienne ont été fournies par diverses études dans l'œil sur le vieillissement, le traitement visuel, la sensibilité à

la lumière ambiante, y compris les défauts de développement relatifs aux opsines. De même, plusieurs données ont lié l'horloge à la différenciation des tissus, comme dans le muscle.

L'objectif de cette thèse de doctorat était d'identifier la machinerie moléculaire qui sous-tend l'influence de l'horloge circadienne au cours du développement de l'œil. Cette thèse a été divisée en différentes parties. Dans la première partie, le **chapitre 1** comprend une brève introduction aux rythmes circadiens et au développement de l'œil, illustrant les principes nécessaires à la compréhension de la base de notre recherche. À la fin de ce chapitre, la portée de la thèse est décrite.

Dans le **chapitre 2**, nous avons inclus une revue exhaustive des études animales et humaines décrivant le contrôle circadien dans le développement des yeux. Pour mieux analyser les études examinées, nous avons d'abord fait état de la relation intime partagée entre l'horloge circadienne, le cycle cellulaire et les processus de développement. Une section distincte décrivant la chronologie historique de l'horloge rétinienne chez différentes espèces a également été incluse. En outre, nous avons également jeté un peu de lumière sur le moment où l'horloge commence à faire tic-tac chez les vertébrés. Nous avons séparé l'examen en différentes sous-sections relatives à la rétinogenèse, aux gènes de l'horloge et aux gènes contrôlés par l'horloge (CCG) pendant le développement rétinien et aux implications circadiennes de la santé oculaire. Notre aperçu des études examinées a montré un paysage réglementaire dynamique du développement de la rétine à travers différents types de cellules rétiniennes employant plusieurs gènes bHLH, homeobox et d'horloge. Assimilant la littérature existante sur le contrôle circadien de l'expression des gènes dans le développement de la rétine du poisson zèbre, du poussin et du xénope ; nous avons proposé un réseau de régulation des gènes (GRN) pour l'horloge rétinienne de la souris. Nous avons également répondu à une question récurrente dans le domaine concernant la régulation transcriptionnelle du développement de la rétine sous le contrôle de l'horloge. En outre, nous avons indiqué que l'effet du dysfonctionnement de l'horloge sur le développement rétinien et ses liens avec les maladies oculaires chroniques fournit une forte association de la machinerie de l'horloge dans le maintien de la santé oculaire. Notre revue de littérature ciblée à grande échelle met en évidence les recherches à venir dans le domaine, notamment pour déterminer d'autres facteurs intrinsèques/extrinsèques et le rôle en amont de l'horloge circadienne dans l'œil embryonnaire en utilisant des environnements 3D in vitro de la population de cellules souches.

Dans la deuxième partie, nous avons exploré la pertinence des gènes de l'horloge centrale Période 1 et 2 sur la physiologie de l'œil en développement et adulte. Les rétines

de souris doublement mutantes Période 1 (*Per1*) et Période 2 (*Per2*) (*Per1*$^{-/-}$*Per2*^{Brdm1}) présentent une distribution anormale des cônes bleus associée à une réduction des niveaux d'ARNm et de protéines de l'opsine conique, jusqu'à l'âge de 1 an. Conformément à ce phénotype, dans le **chapitre 3**, au moyen d'une approche transcriptomique fonctionnelle utilisant l'abondance de l'ARNm comme lecture primaire, nous avons comparé l'expression génique différentielle à l'échelle du génome dans l'œil entier des mutants *Per1*$^{-/-}$*Per2*^{Brdm1} par rapport au type sauvage à E15, E18 et P3. Les données RNA-Seq ont montré des changements majeurs dans l'expression des gènes entre les yeux WT et mutants, le nombre de gènes différentiellement exprimés (DEG) augmentant avec l'âge de développement. Nous avons caractérisé les transcriptomes de l'œil entier en développement qui sont exprimés de manière différentielle chez le mutant *Per1*$^{-/-}$*Per2*^{Brdm1} par rapport au type sauvage, et nous avons évalué des milliers de transcrits en catégories fonctionnelles par une analyse intégrative des ensembles de données. L'annotation fonctionnelle des gènes a montré que les changements les plus significatifs des niveaux d'expression chez les souris mutantes impliquent des voies moléculaires liées à la signalisation du rythme circadien à E15 et E18. À P3, la cascade visuelle et le cycle cellulaire étaient respectivement plus et moins exprimés par rapport aux yeux WT. Nous avons également réalisé les profils d'expression développementale des transcriptions d'horloge entre le jour embryonnaire 13 (E13) et le jour postnatal 24 (P24) dans les yeux de souris WT. Tous les gènes d'horloge ont montré des changements dans les niveaux de transcription au cours du développement normal de l'œil. Sur la base des différences génomiques dans la synchronisation des processus de développement ainsi que des implications fonctionnelles qui surviennent dans diverses voies biologiques en raison des perturbations de l'horloge, nous avons conclu que l'ablation de *Per1/Per2* affecte de manière significative l'expression génétique des membres de la boucle de régulation secondaire de l'horloge circadienne, de la voie de phototransduction ainsi que des composants du cycle cellulaire pendant le développement.

Le renouvellement quotidien des segments externes distaux des photorécepteurs de la rétine est indispensable au maintien de la santé de la rétine. Les mécanismes cellulaires et moléculaires sous-jacents qui initient la phagocytose du segment externe des photorécepteurs (POS) ne sont pas connus. Actuellement, il est prouvé qu'un pic quotidien de phagocytose du POS se produit approximativement une heure après le début de la lumière. Dans le **chapitre 4**, nous avons montré que dans une obscurité constante, les souris déficientes pour les gènes centraux de l'horloge circadienne (*Per1* et *Per2*), n'ont pas de pic quotidien de phagocytose POS. Par analyse qPCR, nous avons constaté que les gènes de l'horloge centrale étaient rythmés sur 24h dans les rétines entières WT et des mutants *Per1*$^{-/-}$*Per2*^{Brdm1}. L'analyse transcriptomique de précision des photorécepteurs WT microdisséqués par capture laser n'a révélé aucun gène exprimé de manière

différentielle entre les points de temps précédant et pendant le pic de phagocytose POS. En revanche, nous avons constaté que l'épithélium pigmentaire rétinien (RPE) WT microdisséqué présentait un certain nombre de gènes différentiellement exprimés au moment du pic de phagocytose par rapport aux points de temps adjacents. Nous avons également trouvé un certain nombre de gènes exprimés de manière différentielle dans l'RPE des mutants *Per1*$^{-/-}$*Per2*^{Brdm1} par rapport à celui des WT au moment du pic phagocytaire. Enfin, sur la base de l'analyse STRING, nous avons trouvé un groupe de gènes en interaction qui conduisent potentiellement la phagocytose POS dans l'RPE. Cette voie potentielle se compose de gènes tels que : *Pacsin1*, *Syp*, *Camk2b* et *Camk2d* entre autres. Nos résultats indiquent que *Per1* et *Per2* sont des composants d'horloge nécessaires pour piloter la phagocytose des POS, y compris qu'elle est pilotée transcriptionnellement par l'RPE. En plus de l'affirmation, dans le **chapitre 5**, les souris déficientes pour les principaux gènes de l'horloge circadienne (*Per1* et *Per2*), ont montré une sensibilité accrue à la lumière nocturne et une réduction modeste mais significative de l'épaisseur de la couche nucléaire externe (ONL) par rapport aux souris de type sauvage.

Dans la troisième partie, nous nous sommes concentrés sur la perspective clinique ou l'imputabilité à la maladie du gène d'horloge central BMAL1 dans la cataracte humaine. Le principal régulateur transcriptionnel de l'horloge circadienne, du cerveau et de la protéine de type translocateur nucléaire du récepteur des hydrocarbures aryliques des muscles, BMAL1, est impliqué dans la régulation du vieillissement oculaire précoce à prématuré. On sait que plus de 50 % des souris déficientes en Bmal1 développent une cataracte avant la 40e semaine de vie. Récemment, la délétion conditionnelle de Bmal1 dans les cellules endothéliales et hématopoïétiques de la rétine murine a démontré les caractéristiques pathologiques de la rétinopathie diabétique, élargissant ainsi la pathologie oculaire comme conséquence des défauts moléculaires de Bmal1. En accord avec ces observations, dans le **chapitre 6**, nous avons examiné BMAL1 comme un gène candidat actif d'intérêt dans la cataracte congénitale. Notre étude a été la première à examiner le gène central de l'horloge circadienne BMAL1 chez l'homme pour son association avec la cataracte congénitale. Nous avons cherché à identifier les variantes pathogènes du gène BMAL1 associées au phénotype CC dans les familles pakistanaises consanguines afin d'explorer tout lien existant entre l'horloge circadienne et les anomalies oculaires. À l'ère du séquençage de l'exome, nous avons utilisé le séquençage Sanger traditionnel comme outil indispensable et rentable pour rapporter les profils de mutation dans notre petite cohorte d'étude clinique. Nous avons identifié la variante faux-sens c.41A>T ; p.(Asp14Val) qui modifie la séquence d'acides aminés de type sauvage, se produit dans un résidu hautement conservé au cours de l'évolution, et a également été déterminée comme étant «probablement pathogène» par la bioinformatique, elle s'est produite à l'état hétérozygote chez un seul patient et ne s'est pas ségréguée dans la famille. Nous

n'avons donc pas pu établir de corrélation entre le variant Asp14Val et l'apparition de la cataracte congénitale. Une variante hétérozygote non synonyme (rs2290037) avec une fréquence allélique plus élevée a été détectée dans la région intronique. Il a été estimé précédemment que 5 % des variantes hétérozygotes non synonymes rares portent au moins ~22 allèles dérivés pathogènes, qui, s'ils s'avèrent homozygotes en raison de mariages consanguins, peuvent entraîner des maladies récessives. Bien qu'aucune association claire entre les variants génétiques humains de BMAL1 et la cataracte n'ait été trouvée, notre étude est néanmoins instructive et sert de prélude à l'examen d'autres études sur la voie de l'horloge dans d'autres populations (**chapitre 6**). Le gène BMAL1 est un membre complexe de la toile d'araignée de l'horloge, et puisque BMAL1 n'est pas un membre unique, le complexe BMAL1-CLOCK dirige la machinerie de l'horloge. Par conséquent, un dépistage supplémentaire de (d'autres) gènes (séquences) de l'horloge circadienne chez les patients atteints de cataracte congénitale pourrait être justifié dans des études ultérieures. Au cours de la dernière décennie, de nombreuses applications de la chronobiologie ont fait surface dans le traitement des maladies. Dans le **chapitre 7**, nous avons mis en évidence la manière dont les mesures des résultats rapportés par les patients (PROM), liées à la biologie circadienne, jouent un rôle potentiel dans le soutien des critères d'évaluation des essais cliniques chez l'homme. Nous avons effectué une analyse systématique et une cartographie des maladies des PROMs associés à l'horloge circadienne au cours des 20 dernières années.

En conclusion, cette thèse de doctorat révèle diverses facettes de la pertinence omniprésente de l'horloge circadienne dans la physiologie oculaire. Bien que d'autres études seront nécessaires pour déterminer le lien entre le phénotype circadien et les anomalies rétiniennes, nos résultats révèlent néanmoins des mécanismes biologiques potentiels, notamment des voies de signalisation reliant potentiellement l'horloge, la génération de photorécepteurs et le contrôle du cycle cellulaire dans la rétine en développement. De futures études sont nécessaires pour s'assurer que la régulation négative des voies liées au cycle cellulaire se produit exclusivement dans la rétine et non ailleurs dans l'œil. En outre, en raison du dysfonctionnement de l'horloge, le mécanisme de phagocytose est compromis chez les souris adultes, un autre résultat confirmant que les composants de l'horloge sont des acteurs intégraux dans le maintien du renouvellement quotidien des segments externes.

SAMENVATTING

De 24-uurs omwenteling van de aarde om haar as heeft het leven ontwikkeld en een eeuwigdurende cyclus van licht-donker ingesteld. Als gevolg daarvan werden intrinsieke ≈ 24-uurs ritmen een fundamenteel kenmerk van elk cellulair en fysiologisch proces in het hele lichaam. Deze biologische mechanismen worden in alle lichtgevoelige levensvormen in stand gehouden en bewaken zo op harmonische wijze onze geofysische tijd. Het eerste ontstaan van een getimede moleculaire oscillator werd ~2,1 miljard jaar geleden gezien in cyanobacteriën. In de weefsels van zoogdieren wordt de ritmiciteit geprogrammeerd door wijd verspreide moleculaire klokken en gesynchroniseerd door een hypothalamische hoofdklok in de suprachiasmatische kern (SCN) van de hersenen. De hoofdklok oscilleert met een periode van ongeveer 24 uur, waarmee de term "circadiaans" (circa = ongeveer en dian = een dag) is geïntroduceerd.

Circadiane klokken houden de tijd bij door gebruik te maken van complexe transcriptionele/post-translationele terugkoppelingslussen waarbij transcriptiefactoren betrokken zijn die gecodeerd worden door klokgenen (hoofdzakelijk *Bmal1*, *Clock*, *Per1-3*, *Cry1-2*), die hun cyclische patronen doorgeven aan hun doelgenen, vandaar aan genexpressieprogramma's. Een secundaire terugkoppelingslus betreft de retinoïnezuur-gerelateerde weesreceptor (ROR) en REV-ERB, een nucleair hormoonreceptor-coderend gen, die beide CLOCK/BMAL1-doelwitten zijn, verdeelt ritmen naar CCG's via ROR-bindingselementen (RORE). Genoombrede transcriptoom profileringsstudies hebben een breed scala (ongeveer 43% van alle eiwit-coderende genen) van ubiquitaire en weefselspecifieke genen onder circadiane controle aan het licht gebracht, in overeenstemming met de noodzaak, voor verschillende organen, om verschillende temporeel gecontroleerde taken te vervullen.

Het netvlies bevat een endogene circadiane klok die vele fysiologische processen regelt in een terugkerend 24-uurs ritme dat aanpassing van de visuele functie aan dagelijkse veranderingen in lichtintensiteit mogelijk maakt. Verrassend genoeg herbergt het ook een wijd verspreid netwerk van circadiane klokken binnen verschillende netvliescellagen. De netvliesklok is dus betrokken bij diverse cyclische functionele processen, zoals de melatoninesynthese, de dopaminesynthese, de gevoeligheid van ionenkanalen, en de vernieuwing van de fotoreceptoren. Hoewel er vooruitgang is geboekt bij de karakterisering van de moleculaire opbouw en de cellulaire lokalisatie van de netvliesklok, is er weinig bekend over de downstream gebeurtenissen die de klok met de netvliesfysiologie verbinden. Bewijzen voor een oorzakelijk effect van verstoring van de circadiane klok zijn geleverd door diverse studies in het oog over veroudering, visuele verwerking, gevoeligheid voor omgevingslicht, waaronder ontwikkelingsdefecten met betrekking tot opsines.

A

Evenzo hebben verschillende gegevens de klok in verband gebracht met de differentiatie van weefsels, zoals in de spier.

Het doel van deze doctoraalscriptie was de moleculaire machinerie te identificeren die ten grondslag ligt aan de invloed van de circadiane klok tijdens de ontwikkeling van het oog. Deze dissertatie is in verschillende delen verdeeld. In deel I bevat **hoofdstuk 1** een korte inleiding over circadiane ritmen en de ontwikkeling van het oog, die de noodzakelijke beginselen illustreert om de basis van ons onderzoek te begrijpen. Aan het eind van dit hoofdstuk wordt de reikwijdte van het proefschrift beschreven.

In **hoofdstuk 2** hebben wij een uitputtend overzicht opgenomen van dierlijke en menselijke studies die de circadiane controle in de oogontwikkeling beschrijven. Om de bestudeerde studies beter te kunnen analyseren, hebben wij eerst gerapporteerd over de intieme relatie die gedeeld wordt tussen de circadiane klok, de celcyclus, en ontwikkelingsprocessen. Er is ook een apart gedeelte opgenomen waarin de historische tijdlijn van de netvliesklok in verschillende soorten wordt beschreven. Verder hebben wij ook enig licht geworpen op het tijdstip waarop de klok begint te tikken bij gewervelde dieren. Wij hebben het overzicht onderverdeeld in verschillende subsecties die betrekking hebben op retinogenese, klokgenen en klokgestuurde genen (CCG) tijdens de retinale ontwikkeling en circadiane implicaties van de gezondheid van de ogen. Ons overzicht van de besproken studies toonde een dynamisch regulerend landschap van de netvliesontwikkeling over verschillende netvliesceltypes heen, waarbij verschillende bHLH-, homeobox- en klokgenen gebruikt worden. Door de bestaande literatuur over de circadiane controle van genexpressie in het zich ontwikkelende netvlies van de zebravis, de kuiken en de xenopus te assimileren, hebben wij een genregulerend netwerk (GRN) voorgesteld voor de netvliesklok van de muis. Een steeds terugkerende vraag in het veld betreffende de transcriptionele regulatie van de netvliesontwikkeling onder klokcontrole is ook beantwoord. Bovendien hebben wij aangegeven dat het effect van klokdisfunctie op de ontwikkeling van het netvlies en de verbanden met chronische oogziekten een sterke associatie van de klokmachinerie bij het behoud van de gezondheid van de ogen oplevert. Ons grootschalig gericht literatuuroverzicht wijst op verder onderzoek op dit gebied, vooral om andere intrinsieke/extrinsieke factoren en de stroomopwaartse rol van de circadiane klok in het embryonale oog te bepalen door gebruik te maken van in vitro 3-D omgevingen van stamcelpopulaties.

In deel II hebben wij de relevantie van de kern-klokgenen Periode 1 en 2 op de zich ontwikkelende en volwassen oogfysiologie onderzocht. De retina's van Periode 1 (*Per1*) en Periode 2 (*Per2*) dubbel-mutante muizen (*Per1*$^{-/-}$*Per2*^{Brdm1}) vertonen een abnormale verdeling van de blauwe kegel, geassocieerd met een vermindering van het kegel opsine

mRNA en eiwit niveau, tot op de leeftijd van 1 jaar. In overeenstemming met dit fenotype vergeleken wij in **hoofdstuk 3**, door middel van een functionele transcriptomics-benadering met mRNA-abundantie als primaire uitlezing, genoombreed differentiële genexpressie in het hele oog van *Per1*$^{-/-}$*Per2*^{Brdm1} mutanten versus wild-type op E15, E18 en P3. RNA-Seq gegevens toonden grote genexpressie veranderingen tussen WT en mutant ogen, waarbij het aantal differentieel tot expressie komende genen (DEG) toenam met de ontwikkelingsleeftijd. Wij hebben de transcriptomen van de zich ontwikkelende hele ogen gekarakteriseerd die differentieel tot expressie komen in *Per1*$^{-/-}$*Per2*^{Brdm1} mutant versus wild-type, en hebben duizenden transcripten in functionele categorieën ingedeeld door middel van integratieve dataset-analyse. Functionele annotatie van de genen toonde aan dat de meest significante veranderingen in expressieniveaus bij de mutantmuizen betrekking hebben op moleculaire pathways die te maken hebben met circadiane ritmesignalering op E15 en E18. Op P3 waren de visuele cascade en de celcyclus respectievelijk hoger en lager uitgedrukt in vergelijking met WT-ogen. Wij hebben ook de ontwikkelingsexpressieprofielen van kloktranscripten tussen embryonale dag 13 (E13) en postnatale dag 24 (P24) in de WT-ogen van de muis uitgevoerd. Alle klokgenen vertoonden veranderingen in transcriptniveaus samen met de normale ontwikkeling van de ogen. Gebaseerd op de genomische verschillen in de timing van ontwikkelingsprocessen, samen met de functionele implicaties die ontstaan in verschillende biologische routes als gevolg van klokverstoringen, hebben wij geconcludeerd dat de *Per1/Per2* ablatie de genexpressie van de secundaire regelkringleden van het circadiane uurwerk, van de fototransductieroute en van celcycluscomponenten tijdens de ontwikkeling aanzienlijk beïnvloedt.

Dagelijkse vernieuwing van de distale buitenste segmenten van de retinale fotoreceptoren is onontbeerlijk voor de gezondheid van het netvlies. De onderliggende cellulaire en moleculaire mechanismen die de fagocytose van het fotoreceptor buitensegment (POS) in gang zetten, zijn niet bekend. Momenteel is aangetoond dat een dagelijkse piek van POS fagocytose ruwweg ongeveer één uur na het begin van het licht optreedt. In **hoofdstuk 4** hebben wij aangetoond dat onder constante duisternis, muizen deficiënt voor de kern circadiane klokgenen (*Per1* en *Per2*), een dagelijkse piek in POS fagocytose missen. Door qPCR analyse vonden wij dat de kernklokgenen over 24 uur ritmisch waren in zowel WT als *Per1*$^{-/-}$*Per2*^{Brdm1} mutant hele netvliezen. Precisie transcriptomics analyse van laser capture microdissected WT fotoreceptoren toonde geen differentieel tot expressie komende genen tussen tijdstippen voorafgaand aan en tijdens de piek van POS fagocytose. Daarentegen vonden wij dat gemicrodissecteerd WT retinaal pigment epitheel (RPE) een aantal genen had die differentieel tot expressie kwamen op het piek fagocytose tijdstip, vergeleken met de aangrenzende. Wij vonden ook een aantal differentieel tot expressie komende genen in *Per1*$^{-/-}$*Per2*^{Brdm1} gemuteerde RPE vergeleken

met die van WT op het piek-tijdstip van de fagocytose. Tenslotte vonden wij op basis van STRING analyse een groep van op elkaar inwerkende genen die mogelijk de POS fagocytose in de RPE aansturen. Deze potentiële pathway bestaat uit genen zoals: *Pacsin1*, *Syp*, *Camk2b* en *Camk2d*, onder andere. Onze bevindingen wijzen erop dat *Per1* en *Per2* noodzakelijke klokcomponenten zijn voor het aansturen van POS fagocytose, ook dat, het transcriptioneel door de RPE aangestuurd wordt. Toevoegen aan de bewering, in **hoofdstuk 5**, muizen met een tekort aan core circadiane klokgenen (*Per1* en *Per2*), vertoonden een verbeterde nachtelijke lichtgevoeligheid en een bescheiden maar significante vermindering van de dikte van de buitenste nucleaire laag (ONL) in vergelijking met de wild-type muizen.

In deel III hebben wij ons geconcentreerd op het klinische perspectief of de ziektetoerekeningsvatbaarheid van het kern-klokgen BMAL1 in menselijke cataract. De primaire transcriptionele regulator van de circadiane klok, de hersenen, en de spieren arylkoolwaterstofreceptor nucleair translocator-achtig eiwit BMAL1 is betrokken bij de regulatie van vroege tot vroegtijdige veroudering van de ogen. Van meer dan 50% van de Bmal1 deficiënte muizen is bekend dat zij cataract ontwikkelen vóór de 40e levensweek. Onlangs heeft de voorwaardelijke deletie van Bmal1 in endotheel- en hematopoietische cellen van het muizennetvlies pathologische kenmerken van diabetische retinopathie aangetoond, waarmee de oculaire pathologie als gevolg van moleculaire Bmal1 defecten uitgebreid wordt. In overeenstemming met deze waarnemingen hebben wij in **Hoofdstuk 6** BMAL1 onderzocht als een actief kandidaat-gen dat van belang is bij congenitale cataract. Onze studie was de eerste die het kern-circadiane klokgen BMAL1 bij mensen onderzocht op hun associatie met congenitale cataract. Wij trachtten de ziekte-veroorzakende varianten in het BMAL1 gen te identificeren die geassocieerd zijn met het CC fenotype in de consanguine Pakistaanse families om eventuele bestaande verbanden tussen de circadiane klok en oogafwijkingen te onderzoeken. In het tijdperk van exoom sequencing hebben wij traditionele Sanger sequencing gebruikt als een onmisbaar kosteneffectief hulpmiddel om mutatieprofielen in ons kleine klinische studiecohort te rapporteren. Wij identificeerden een missense variant c.41A>T; p.(Asp14Val) die de wild-type aminozuursequentie veranderde, voorkwam in een zeer evolutionair geconserveerd residu, en ook door bio-informatica als “waarschijnlijk pathogeen” werd vastgesteld, het gebeurde in de heterozygote toestand bij één enkele patiënt en segregeerde niet in de familie. Wij konden de Asp14Val variant dus niet in verband brengen met het optreden van congenitale cataract. Een niet-synonieme heterozygote variant (rs2290037) met een hogere allelfrequentie werd in de intronic regio ontdekt. Men heeft eerder geschat dat 5% van de zeldzame niet-synonieme heterozygote varianten minstens ~22 pathogene afgeleide allelen dragen, die, als ze homozygoot blijken te zijn als gevolg van consanguine huwelijken, tot recessieve ziekten kunnen leiden. Hoewel er geen duidelijke associatie tussen

menselijke genetische BMAL1-varianten en cataract gevonden werd, was onze studie toch informatief en vormt zij een voorbode om verdere studies in het klokwerkpad van andere bevolkingsgroepen te overwegen (**Hoofdstuk 6**). Het BMAL1 gen is een ingewikkeld lid van het uurwerkweb, en aangezien BMAL1 niet het enige lid is, drijft het BMAL1-CLOCK complex de uurwerkmachine aan. Daarom kan extra screening van congenitale cataractpatiënten met (andere) circadiane klokgenen (sequenties) in volgende studies gerechtvaardigd zijn. In het afgelopen decennium zijn er talloze toepassingen van chronobiologie opgedoken in de behandeling van ziekten. In **hoofdstuk 7** hebben wij belicht hoe circadiane biologie gerelateerde Patient-reported Outcome Measures (PROMs) een potentiële rol spelen bij de ondersteuning van menselijke klinische proefeindpunten. Wij hebben een systematische analyse en het in kaart brengen van ziekten uitgevoerd van PROMs die verband houden met de circadiane klok in de afgelopen 20 jaar.

Als conclusie kan worden gesteld dat dit proefschrift verschillende facetten onthult van de alomtegenwoordige relevantie van de circadiane klok in de oogfysiologie. Hoewel er meer studies nodig zullen zijn om het verband tussen het circadiane fenotype en netvliesafwijkingen vast te stellen, onthullen onze bevindingen toch potentiële biologische mechanismen, met name signaalwegen die mogelijk een verband leggen tussen de klok, de fotoreceptorgeneratie en de controle van de celcyclus in het zich ontwikkelende netvlies. Toekomstige studies zijn nodig om er zeker van te zijn dat de downregulatie van celcyclus-gerelateerde pathways uitsluitend in het netvlies optreedt en niet elders in het oog. Bovendien wordt als gevolg van de klokstoring de fagocytose-machinerie bij volwassen muizen gecompromitteerd, nog een resultaat dat bevestigt dat klokcomponenten een integrale rol spelen bij het in stand houden van de dagelijkse omzet van buitensegmenten.

Udita BAGCHI

Rôle de l'horloge circadienne dans le développement de la rétine

Résumé

Cette thèse a étudié le rôle de l'horloge circadienne dans la rétine de souris, du développement jusqu'à l'âge adulte. Les gènes Period1 (*Per1*) et Period2 (*Per2*) qui sous-tendent l'horloge circadienne sont impliqués dans la division cellulaire ainsi que dans l'ontogenèse de la rétine. Les souris doublement mutantes *Per1/Per2* présentent un retard dans la différenciation générale des photorécepteurs, ainsi que des réductions des niveaux d'ARNm et de protéines des opsines de cônes. À la lumière de ces observations, nous avons montré que, pendant le développement, l'ablation de *Per1/Per2* affecte de manière significative l'expression génique de composants clés de la cascade de phototransduction et du cycle cellulaire, ainsi que les membres de la boucle de régulation secondaire de l'horloge circadienne. Nous avons également montré que les souris déficientes en *Per1* et *Per2,* si soumises à l'obscurité constante, ne présentent pas de pic quotidien de phagocytose du segment externe des photorécepteurs. Nous avons également trouvé que la modulation quotidienne de la sensibilité visuelle est altérée par rapport aux souris de type sauvage, lorsque les gènes *Per1* et *Per2* sont invalidés. Finalement, nous avons également exploré les perspectives cliniques en examinant les mutations du gène *BMAL1* chez les probands de familles porteuses de cataracte congénitale consanguine et avons réalisé une analyse systématique des mesures de résultats rapportés par les patients (PROM) en lien avec le système circadien et actuellement utilisées dans les essais cliniques chez l'homme.

Mots-clés: *Per1*, *Per2*, circadien, photorécepteur, développement, différenciation, essais cliniques

Résumé en anglais

This thesis investigated the role of the circadian clock during the development of the mouse retina until adulthood. The *Period1* (*Per1*) and *Period2* (*Per2*) genes underlying the retinal clock have been involved in cell division as well as in the retinal ontogeny modus operandi. The *Per1/Per2* double mutant mice show a delay in general photoreceptor differentiation, along with reductions in cone opsin mRNA and protein levels. In light of these observations, we further provided the evidence that during development *Per1/Per2* ablation significantly affects gene expression of the phototransduction pathway and cell cycle components, as well as the secondary regulatory loop members of the circadian clockwork. We showed that mice deficient for core circadian clock genes (*Per1* and *Per2*) lack a daily peak in photoreceptor outer segment (POS) phagocytosis under constant darkness. We have also found an impaired daily modulation of visual sensitivity during the inactivation of *Per1* and *Per2* genes as compared to the wild-type mice. Besides, we have also explored clinical perspectives by examining *Bmal1* gene mutations in probands of consanguineous congenital cataract families and generated a systematic analysis of the current patient-reported outcome measures (PROM) used in human clinical trials.

Keywords: *Per1*, *Per2*, circadian, photoreceptor, development, differentiation, clinical trials

A

PHD PORTFOLIO

Name PhD student: ***Udita Bagchi***
PhD period: ***October 2015 – June 2019***
Name of PhD supervisor: ***Marie-Paule Felder-Schmittbuhl***
Name of co-supervisor: ***Arthur A. Bergen***

1. PhD training	**Year**	**ECTS***
General courses		
- The Amsterdam UMC World of Science	2016	0.7
- Practical Biostatistics	2016	1.3
- Scientific Writing in English for Publication	2016	1.4
- Bioinformatics Sequence Analysis	2017	1.0
- Project Management	2017	0.5
- Effective Writing course	2017	1.4
Specific courses		
- Intellectual Property Rights	2015	0.1
- Measuring the impact of mechanical forces in morphogenetic processes	2015	0.1
- Advanced qPCR	2016	0.7
- Artificial Intelligence Foundations: Neural Networks	2020	0.1
- Leveraging Neuroscience in the Workplace	2020	0.1
Seminars, workshops, and master classes		
- Winter school “The attentive brain, the deluded brain” what is reality?	2015	1.1
- MAPS 2015	2015	1.1
- A peek into the Council of Europe	2016	0.1
- Summer School course for PhD students- LabEx Net RNA	2017	0.7
Presentations		
- Neurotime Annual Meeting	2016	0.1
- Ecole Doctoral (ED days)	2016	0.4
- Neurotime Annual Meeting	2017	0.1
- XV European Biological Rhythms Society Congress	2017	0.6
(Inter)national conferences		
- Young Researcher Vision Camp, Germany	2016	0.9
- The Sleep and Circadian Neuroscience Summer School, Oxford, UK	2016	1.3
- Summer school in “*Light, Time, Frontiers, Innovation*” at Nagoya, Japan	2017	1.8
- Novartis Regional Biotechnology Leadership Camp at Ljubljana, Slovenia	2019	0.9
- Catania International Summer School of Neuroscience at Catania, Sicily	2019	1.3

Other	Year	ECTS*
- French Language for Foreigners (Beginners Group-A1/A2 level)	2016	6.7
- Roche Continents Workshop in Salzburg, Austria	2016	1.3
- Council of Europe – PhD Student Delegation Visit	2016	0.1
- European Parliament – PhD Student Delegation Visit	2016	0.1
- French Language for Foreigners (Beginners Group-A1/A2 level)	2018	2.4
- MOOC “Research Integrity in Scientific Professions” e-learning course (provided by the University of Bordeaux)	2019	0.3
- IMD MBA ‘Create the Future’ Asia Assessment Challenge	2020	0.2
- Sojourn French course A2	2020	6.7
- MOOC “Research Integrity in Scientific Professions” e-learning course (provided by the University of Bordeaux)	2020	0.3
- Fundamental Training Course in International Conference on Harmonisation- Good Clinical Practice (ICH-GCP) 4.0	2020	0.1
- Goethe Institute German course B2	2021	0.6

2. Parameters of Esteem	Year
Grants	
- NeuroTime Erasmus+ Mundus Joint Doctoral Fellowship	2015 - 2019
Awards and Prizes	
- BioCamp Winner (1st prize) at Novartis Regional Biotechnology Leadership Camp	2019
- 500€ travel grant recipient for the Catania International Summer School of Neuroscience	2019

3. Industry experience	Year
Clinical Research Organization (CRO)	
- Research Associate II (ICON plc.)	2019 – 2020
- Clinical Trial Site Support Lead (IQVIA Holdings Inc.)	2020 – 2021
Pharmaceutical	
- Global Scientific Project Manager TA Oncology (Boehringer Ingelheim)	2021 – Present

4. Publications	Year
Peer reviewed	
- Publications in Thesis	2019 - 2022
Other	
- Publications in English literature books (voluntary work), online newspapers, etc.	2015, 2019

ACKNOWLEDGEMENTS

Life can only be understood backwards; but it must be lived forwards - Soren Kierkegaard

I am greatly pleased to have met such incredible supervisors in the very early phase of my scientific research career. I owe my eternal recognition and thankfulness to my thesis advisors: **Dr. Marie-Paule Felder-Schmittbuhl** and **Dr. Arthur A. Bergen**.

My sincere appreciation and gratitude for **Dr. Marie-Paule Felder-Schmittbuhl** for guiding me right from the first day of my arrival in her laboratory at Institut des Neurosciences Cellulaires et Intégratives (INCI), Strasbourg, France on 10 September 2015. Your insightful discussions and suggestions throughout the course of the PhD thesis have been instrumental in helping me solve complex issues during experiments and during the tedious long writing phase of all the research papers. These 7 years of my PhD thesis would not have been possible without your consistent support and collaboration through skype, SMS, phone calls, emails etc. I was fascinated and humbled to be your mentee. Your leadership style, your stern and stoic demeanor, your perfectionism, and empathy will forever make me think very fondly of you. Transitioning from a different scientific background into the world of chronobiology, was a smooth sail only due to your extraordinary, brilliant teaching tactics. Your assistance aided me to prepare excellent PowerPoint presentations and motivated me for better public speaking.

I deeply acknowledge **Dr. Arthur A. Bergen** for giving me an outstanding platform at Academic Medical Center (AMC) to perform a great PhD research project. It was under your tutelage that I developed my first research proposal for undertaking a small patient cohort study. Also, I am very grateful for the constant source of unconditional support and guidance I received from your current and former team members such as **Nemanja, Anneloor, Jacoline and Shazia**. Your positive outlook and agreeing to attend countless meetings for the finalization of the RNAseq experimental design with the Bioinformatics team members – **Aldo and Perry**, has been a real cardinal point during the PhD. It was a privilege to work with **Aldo and Perry**, no matter whatever the request was, the results were always delivered promptly. An excellent liaison! I also appreciate that you approved the skilled handling of my RNAseq samples by the sequencing facility team members at AMC: **Linda, Marja and Patrick**, finally this made it possible to obtain excellent results with delicate samples.

As a new PhD student in the laboratory, **Cristina Sandu**'s research expertise, understanding of lab materials and methods added considerably to my PhD graduate experience. A good support system is always the most important part amidst the chaotic experiments, poor results, delays, and uncertainties. Your guidance voluminously contributed and encouraged

me at every step of the PhD writing process. I will never forget those long conversations in the evening in your office and at the lab. Your manual finesse in multiplex assays (qPCR, ELISA etc.) are something to yearn for. Your incredible intelligence in making perfectly aligned scientific figures are exemplary. I could not have done without your ideas for all the figures in my paper (Bagchi et al. 2020). You became my primary resource for getting my science questions answered. Your willingness and love for teaching was contagious.

I am totally indebted to **Dr. Paul Pevet** and **Dr. Domitille Boudard**, who kindly selected me to participate in the NEUROTIME PhD Programme. I am also obliged to thank **Dr. Andries Kalsbeek (Dries)**, an impeccable personality whose instant replies to almost any email at any time of the day, generosity and wisdom totally makes me bow with gratitude each time. Hats off to your hard work and constant support, makes me feel truly beholden.

I would also like to thank my mid-thesis jury committee members: **Dr. Etienne Challet** and **Dr. Michel Roux**. Your encouragement and open-ness were very helpful.

A big shout out (THANKS!!!) also goes to **Shumet Tenaw** who made sure that we had a great experimental result, despite multiple repetitions and sample scarcity. It is because of you, that we could provide a perfect qPCR analysis diagram to the journal reviewers on time for the paper Bagchi et al. 2020.

I extend my special mention also to **Lamis Saad, Himanshu William**, **Anamitra Bagchi, Neha Sharma** and **my parents** who cheered and celebrated each accomplishment of mine, like their own. You all made me feel so special, you always went through the hard times together with me. Your unfailing emotional support and outstanding companionship reminds me of how a journey is easier when we travel together. No matter how far we are! You all taught me that *distance always endears and sweetens relationships*.

An honorable mention to the former Alma mater who paved the route to the attainment of a doctoral degree – **Banasthali University, India**; **Kantonsschule Solothurn, Switzerland**; **Albert-Ludwigs-Universität (ALU) Freiburg, Germany** and **Universidad de Buenos Aires (UBA), Argentina**.

Charles Darwin rightly said – "*The love for all living creatures is the most noble attribute of man*", yet we do require animals frequently for scientific research. Therefore, last, and not the least, my heartfelt gratitude goes to all the laboratory animals who were sacrificed for this study including pregnant mice and embryos, this research would not have been possible without them. My profound thanks go to **Dr. Dominique Sage**, **Dr. Sophie Reibel** and **Nicolas Lethenet** for animal care.

ABOUT THE AUTHOR

Udita Bagchi is born on December 12, 1989, in a small town of Northern India called Varanasi. From a marvelously nomadic existence in her childhood, having changed eight schools in twelve years in various cities across India, she ended up with an academic roller-coaster ride spanning a diversity of perspectives. Her first exposure to the vast field of medicine, biology, and chemistry happened at Banasthali University, India during the four years (2008 to 2012) of undergraduation in pharmaceutical sciences. Being an enthusiast of learning foreign languages, she immersed herself in learning German language at Banasthali University, India. Following this, unique life-changing experiences occurred when she was selected to represent her country twice in “International Student Cultural Exchange Programmes” at Kantonsschule Solothurn, Switzerland, and University of Weingarten, Germany in the year 2009 and 2011 respectively. The international exchange programs were facilitated through DAAD (Deutscher Akademischer Austauschdienst) – A New Passage to India and Banasthali university merit scholarships. After finishing her Bachelor of Pharmacy (B. Pharm) with a thesis specializing in medicinal chemistry entitled on the ‘*Synthesis of a pyrazole derivative by the principles of green chemistry through microwave heating*’, she decided to embark on the road less traveled by. Ever since she has been a globetrotter and embraced a world citizen lifestyle. From July to November 2013, she was a recipient of another DAAD scholarship to pursue a Diploma of Advanced Studies (DAS) in Biomedical Sciences from Albert-Ludwigs-Universität (ALU) Freiburg, Germany. The Diploma project in Atherosclerosis entitled – *The functional role of Tumor necrosis factor receptor-associated factor 1 (TRAF-1) in platelets* was undertaken in the laboratory of Dr. Andreas Zirlik at Universitäts-Herzzentrum Freiburg for 3 months. As a result of bilateral partnership between Albert-Ludwigs-Universität (ALU) Freiburg, Germany, and Universidad de Buenos Aires (UBA), Argentina, she got a unique opportunity to participate in a Master of Science (MSc.) program in Biomedical Sciences at the Facultad de Farmacia y Bioquímica - Universidad de Buenos Aires (UBA), Argentina from December 2013 to June 2014. The MSc. thesis was performed in the laboratory of Dr. Andreas Herrlich at Harvard Institute of Medicine (HIM), Boston USA from July 2014 – June 2015 in Triple-Negative Breast Cancer. The MSc. thesis identified *cleavage regulatory mechanisms and phenotypic consequences to the targeting of the ADAM17/EGFR axis in Triple-negative breast cancer (TNBC)*. From October 2015 onwards, she was awarded the Erasmus Mundus Joint Doctorate Fellowship in Neurosciences (NeuroTime Program - Neural Processing of Time) with Université de Strasbourg, France, and Universiteit van Amsterdam (UvA), The Netherlands. The joint Ph.D. project entitled – *Role of the biological clock in retinal development* was closely undertaken between two prestigious research institutes - Institut des Neurosciences Cellulaires et Intégratives (INCI) Strasbourg and Academic Medical Center (Amsterdam UMC) in the

laboratories of Dr. Marie-Paule Felder-Schmittbuhl and Dr. Arthur A. Bergen respectively. The first few months of doctoral training from October 2015 – April 2016 was spent in INCI, Strasbourg followed by a two-year tenure in Amsterdam UMC from May 2016 – May 2018. During this period, she presented scientific posters and delivered talks on the Ph.D. thesis results at the Sleep and Circadian Neuroscience Institute (SCNI), Oxford UK; Young Researcher Vision Camp, Germany; Neurotime Annual Meetings, Ecole Doctoral (ED) days' workshop, XV European Biological Rhythms Society Congress, Summer school in "Light, Time, Frontiers, Innovation" at Nagoya, Japan and at the Catania International Summer School of Neuroscience at Catania, Sicily. She was also selected from a large pool of top European doctoral students in various leadership discourses like in the 10th anniversary of Roche Continents–Youth! Arts! Science! at Salzburg, Austria, and in Novartis Regional BioCamp at Ljubljana, Slovenia. While actively engaged in writing the Ph.D. thesis and in the peer-reviewing of different scientific articles on a day-to-day basis, she worked as a corporate employee from July 2019 – November 2020 in a Clinical Research Organization (CRO) called ICON plc. in the Department of Health Economics and Outcomes Research (HEOR) at Lyon, France. She also led the clinical trials site optimization and patient recruitment at IQVIA Holdings Inc. in the Department of Global Patient and Site Solutions (GPSS) from November 2020 – September 2021 at Lyon, France.

Since October 2021, she is contributing to the field of Oncology as a Global Scientific Project Manager in the Department of Medical Affairs in Boehringer Ingelheim, Germany.

The 2017 Nobel Prize in physiology or medicine awarded to
Jeffrey Hall, Michael Rosbash and Michael Young
"for their discoveries of molecular mechanisms controlling the circadian rhythm"
provided widespread acknowledgement of the
fundamental importance of circadian clocks.

It's only after accepting body clocks as a profoundly biological function
that one can fully appreciate how big a role they play in our health and well-being.

Many thanks in advance.

Udita

BIOLOGICAL RHYTHMS IN RETINAL DEVELOPMENT

Demystifying the eye

Udita Bagchi

A neuroscientist by training, a poet/writer at heart, an avid reader, and a nature lover. Literary creations by the subtle interplay of words and emotions are her secret haven. As a staunch believer in the power of dreams, twenty years down the line, she envisions living each moment just like the way she lives it today. Eager to learn, study the world and engage with it. She aspires on - *gravitate to people and what is best for you will gravitate to you.*