ebrs
European Biological Rhythms Society

THESIS SUMMARY

Nemanja Milicevic
Department of Clinical Genetics, Amsterdam University Medical Center
1 April 2020

Circadian regulation of the retinal pigment epithelium - photoreceptor complex

Virtually all living organisms evolved internal-time keeping mechanisms in order to adapt to daily variations in the environment. In mammals, the master pacemaker responsible for harmonizing physiology and behavior to the rhythmic environment resides in the suprachiasmatic nucleus (SCN) in the brain. The SCN coordinates the circadian clocks in peripheral organs. The retina is considered as a peripheral oscillator that uniquely lies in direct contact with light. This enables the retina to adjust its molecular clockwork in response to input from the environment – i.e. "clock resetting / entrainment". On a molecular level, circadian clocks are composed of interlocking transcriptional and translational feedback loops. The "positive" arm of the loop, comprised of clock genes *Bmal1* and *Clock* and the "negative" arm comprised of *Per1-3*, *Cry1-2*, *Rev-Erb-β orchestrate,* via complex mechanisms of activation and repression, a 24h expression profile.

In the retina, a plethora of physiological, biochemical and cellular processes are under the control of the circadian clock. These clocks are present in virtually all retinal cell layers. The existence of an SCN independent, self-sustained circadian clock in the retinal pigment epithelium (RPE) is a relatively recent discovery. However, little is known about the functions and processes that are regulated by the RPE clock. The goal of this thesis is to increase our understanding of the regulation of the RPE clock and processes that are regulated by the RPE clock.

The retinal pigment epithelium (RPE) is a monolayer of cells that lies adjacent to the retinal photoreceptors. The RPE performs a variety of functions critical for retinal survival, such as removal of photo-oxidized photoreceptor outer segments (POS), transport of nutrients, removal of metabolic waste by-products, ion transport, secretion of essential factors, absorption of dispersed light, scavenging of reactive oxidative species and conversion of retinal in the visual cycle.

In **Chapter 1**, we reviewed the literature on the regulation of the circadian clock in the RPE and its (potential) role in RPE physiology and functions. First, we briefly summarized the literature on molecular mechanisms of the circadian clock and the regulation of the retinal clock. Next, we reviewed recent work on entrainment mechanisms of the RPE clock. We briefly summarized the current literature on the regulation of phagocytosis of photoreceptor outer segments, a process known to be regulated by the RPE clock. Emerging evidence suggests that the RPE clock is involved in regulating epithelial transport. Therefore, we provided a short summary of the epithelial transport mechanism of the RPE with a focus on ion, glucose and lactate transport. Results that support this hypothesis are provided in **Chapters 3** and **4**.

ebrs
European Biological Rhythms Society

THESIS SUMMARY

The phagocytosis of POS is critical function of the RPE which occurs in a daily peak. Despite decades of research, the cellular identity(s) and localization of the clock(s) driving this process remain unknown. In **Chapter 2**, we used a human RPE cell line – ARPE-19 as a model to study the regulation of the RPE circadian clock and phagocytosis machinery. In these (dispersed and monolayer) cells, we found that most known clock and phagocytosis genes were rhythmically expressed. In addition, cells cultured as monolayers tended to have shorter periods and showed rhythmic expression of the phagocytosis gene *PTK2*. Out of all clock genes, *REV-ERB* was among the most rhythmic in the RPE. We investigated the possibility that this gene is necessary for the rhythmicity of the RPE clock using *mPer2*^{Luc} knock-in mice. However, bioluminescence recordings of RPE-choroid explant preparations showed that *Rev-Erbα* knock-outs had relatively mild alterations of PER2 bioluminescence oscillations. Finally, results from our *in vitro* experiment using bovine POS and synchronized ARPE-19 monolayers suggested that POS might entrain the RPE clock and clock-controlled gene expression.

In **Chapter 3**, we investigated the possibility that the circadian clock regulates RPE-mediated ion transport. We used ARPE-19 cells cultured as monolayers as our RPE model. Using RT-PCR we showed that the mRNA expression of the Na^+ - K^+ - Cl^- cotransporter, *SLC12A2* is rhythmic in synchronized ARPE-19 monolayers. Immunohistochemistry and confocal microscopy revealed that the corresponding protein product, NKCC1 localized on the apical membrane of ARPE-19, similar to the *in vivo* condition. Next we found that the concentrations of Na^+ - K^+ - Cl^- ions oscillated in apical supernatants. We then used correlation analyses to determine the relationship between *SLC12A2* mRNA expression and ion concentration gradients. Our results suggested that the circadian clock might mediate in the transport of Na^+ - K^+ - Cl^- ions by the RPE.

Daily variations in glucose metabolism were described in the retina. The RPE supplies glucose to the retina and removes the glycolytic by-product, lactate, from the subretinal space to the bloodstream. Therefore, in **Chapter 4**, we proposed that the RPE-mediated transportation of glucose and lactate are under circadian control. To test this hypothesis we used our synchronized ARPE-19 monolayer cell culture model. We first characterized the glucose and lactate transporter mRNA expression profile in ARPE-19 monolayers and dispersed cell cultures. In both models, the highest expressed glucose transporter was *SLC2A1* (encoding GLUT1) and *SLC16A1* (encoding MCT1). We found that monolayers showed rhythmic expression of both *SLC2A1* and *SLC16A1*, in contrast to dispersed cells. The expression of these transporters responded to a POS challenge in a time-dependent manner suggesting that POS might serve as an entrainment-like stimulus for the rhythmic expression of *SLC2A1* and *SLC16A1*. Immunohistochemistry and confocal microscopy revealed that GLUT1 and MCT1 localized on the apical membrane of ARPE-19 monolayers. We then sampled the apical and basolateral supernatants from synchronized ARPE-19 monolayers. We found that lactate concentrations oscillated in apical supernatants, in contrast to glucose concentrations. We found that the mRNA of *SLC2A1* and *SLC16A1* correlated with the concentration gradients of glucose and lactate, respectively. These results suggest that the RPE-mediated transport of lactate is under the regulation of the circadian clock.

Finally, in **Chapter 5**, we investigated the possibility that the shedding (and phagocytosis) of POS might be driven by the circadian clock in the photoreceptors. For this study, we used *Per1 Per2* double mutant mice (*Per1*$^{-/-}$*Per2*^{Brdm1}) which are behaviorally arrhythmic in constant darkness (DD). Results of qPCR analyses revealed that *Per3* was rhythmic in *Per1*$^{-/-}$*Per2*^{Brdm1} retinas, in contrast to WT retinas, suggesting that *Per3* might compensate the deficiency of *Per1* and *Per2*. However, immunohistochemistry and microscopy revealed that, in DD conditions, *Per1*$^{-/-}$*Per2*^{Brdm1} mice did not show a peak in POS phagocytosis. We performed an RNA sequencing analysis of microdissected photoreceptors to reveal the transcriptional link

between the photoreceptor clock and POS phagocytosis. Analysis of the photoreceptor transcriptome obtained at time points before and during the peak in POS did not reveal any obvious transcriptional pathway. Results from this chapter contribute to the view that the clock-work controlling POS phagocytosis likely resides in the RPE.

In summary, this thesis sheds some light on the regulation of the RPE clock, POS phagocytosis and provides evidence that the RPE clock is involved in trans-epithelial transport. The phagocytosis machinery is rhythmic in the RPE, in contrast to the photoreceptors, suggesting that the RPE clock likely drives the daily peak in POS phagocytosis. Furthermore, results from our in vitro studies suggest that the RPE clock is involved in regulating the RPE-mediated trans-epithelial transport of ions, nutrients and waste by-products to and from the retina/bloodstream.