Circadian rhythms in diabetic retinopathy

Abstract

Mounting evidence suggests that the circadian system is integral to the development and management of diabetic complications. Circadian disruption increases the risk of diabetes, which, in turn, causes desynchrony in the circadian system and the molecular clock that exists in the cell. Despite several of the underlying pathologies being either controlled by or linked to the circadian clock, little is known about the impact of a dysfunctional circadian system in the most common complication of diabetes, diabetic retinopathy (DR). For example, hypoxia, a major therapeutic target for DR, alters the expression of the core genes in the molecular clock in endothelial cells. This presents a novel mechanistic hypothesis for the role of a disrupted circadian clock in DR.

Introduction

The rotation of the Earth imposes daily environmental cycles on the organisms on its surface. The circadian system has evolved to anticipate these patterns to help organisms survive and thrive. This system integrates the rhythms of the environment, especially light, to modulate our physiology between day and night. Unfortunately, in modern society, we often inadvertently confuse the circadian system. Longer time spent indoors, artificially extended days, and more extreme changes, such as shift work and jet lag, all challenge the synchrony of the circadian system. The immediate symptom of this disruption is poor sleep and groggy waking hours, but the actual impact on the body is much more widespread. Circadian rhythms exist in diverse processes across all organs in the body. Their centrality to human health was highlighted with the 2017 Nobel Prize in Physiology or Medicine awarded to Dr. Jeffrey C. Hall, Dr. Michael Rosbash, and Dr. Michael W. Young for their discoveries of molecular mechanisms controlling circadian rhythms. It makes sense then that circadian disruption has been implicated in a multitude of diseases, with diabetes as a prominent example. Circadian disruption increases the risk of developing diabetes; diabetes, in turn, diminishes the robustness of circadian rhythms. Many crucial functions of the retina are regulated by the circadian system,[1] so understanding the roles of circadian rhythms in diabetic retinopathy (DR) is greatly important.

The circadian system

The retina is the sole source of light to the circadian master clock, the suprachiasmatic nucleus in the hypothalamus. Light is the most important timing cue for this master clock. As such, the retina is uniquely important, and blinding diseases like DR might have implications far beyond the eye. The master clock uses light information from the retina to synchronize every other clock in the body except the retina's own circadian clock since it receives its own light information (**Figure 1**). This is one reason why the retina clock is less studied than others, with current research mostly exploring the master clock or larger tissue clocks. The retina clock is also very complex, with distinct clocks on each retinal layer including the inner nuclear and vascular layers,[2] further complicating the elucidation of its clock system.

The smallest level of the circadian system is a molecular clock, found in almost all cells and acting in concert to drive daily rhythmic processes. The molecular clock is a set of interlocked loops of genes that interact to control their own expression. The main loop in this molecular clock has two pairs of genes. The first pair form the positive arm, so called because together they create a positive transcription factor that initiates the expression of other genes in the nucleus. The second set of genes is initiated by this positive arm,[4] and once they are expressed, they inhibit further action of the positive arm. Therefore, they are called the negative arm. In this way the molecular clock regulates its own expression in an ongoing cycle, where one turn takes around 24 hours, depending on genetics, age, and environmental cues (**Figure 2**). This clock is important because the positive arm does not just target other clock genes, it also triggers transcription

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Key points:

- General clock gene expression is altered in induced, pluripotent stem cell-derived endothelial cells from diabetes patients.
- Hypoxia, but not hyperglycemia, acutely changes the amplitude and patterns of expression of core circadian clock genes in retinal endothelial cells.
- A core circadian transcription factor is upregulated and peaks earlier in hypoxia.
- Conversely, an important negative regulator in the molecular clock is downregulated in hypoxia.

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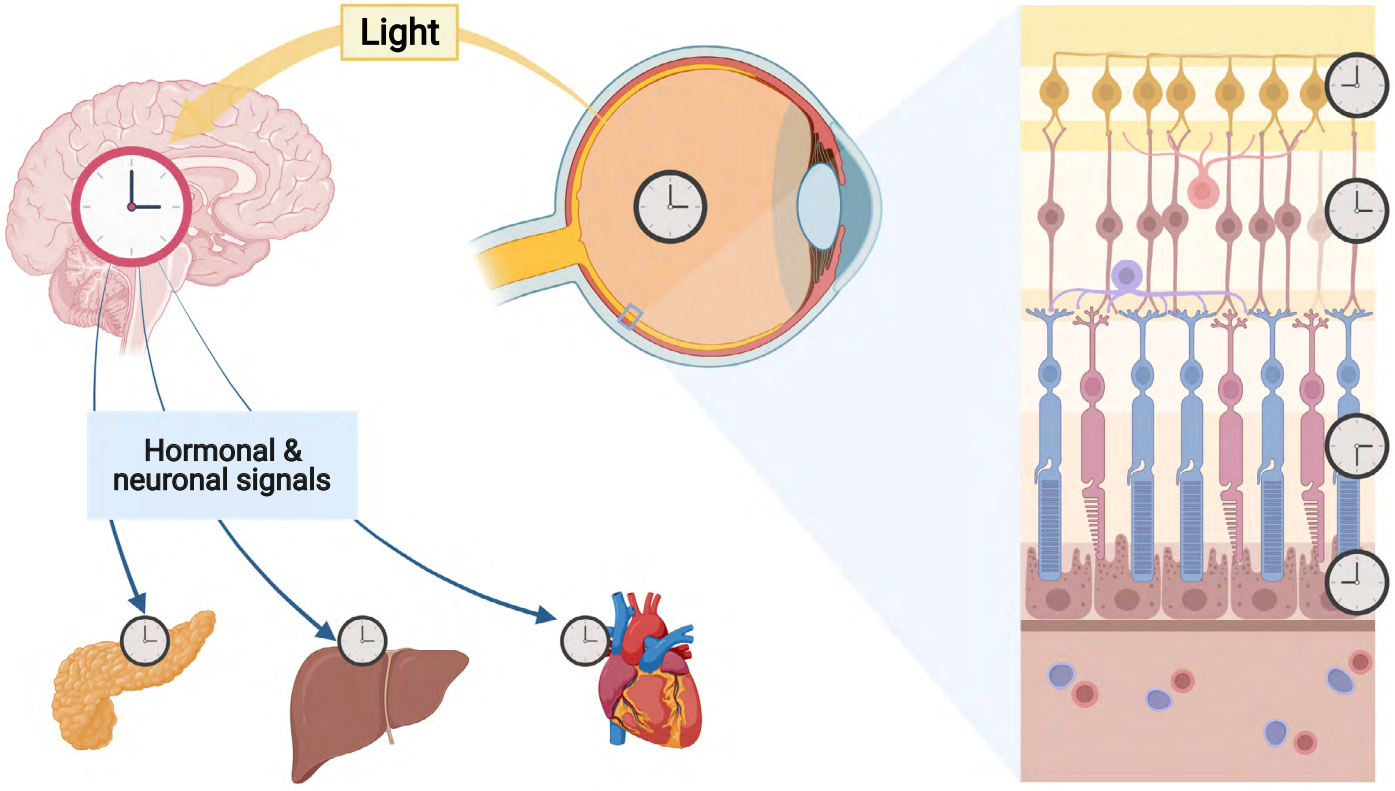

Figure 1. The master clock in the hypothalamus of the brain is the topmost layer of the circadian system, synchronizing all the peripheral clocks that exist in organs around the body so that they act in concert with each other. The most important timing cue for the circadian system is light signaling, which comes via non-visual photoreceptor cells in the retina called intrinsically photosensitive retina ganglion cells.[3] One of the reasons that the retina clock is unique is that it is made up of many different clocks on the different retinal layers that oscillate in different phases. Created by the authors using BioRender.com.

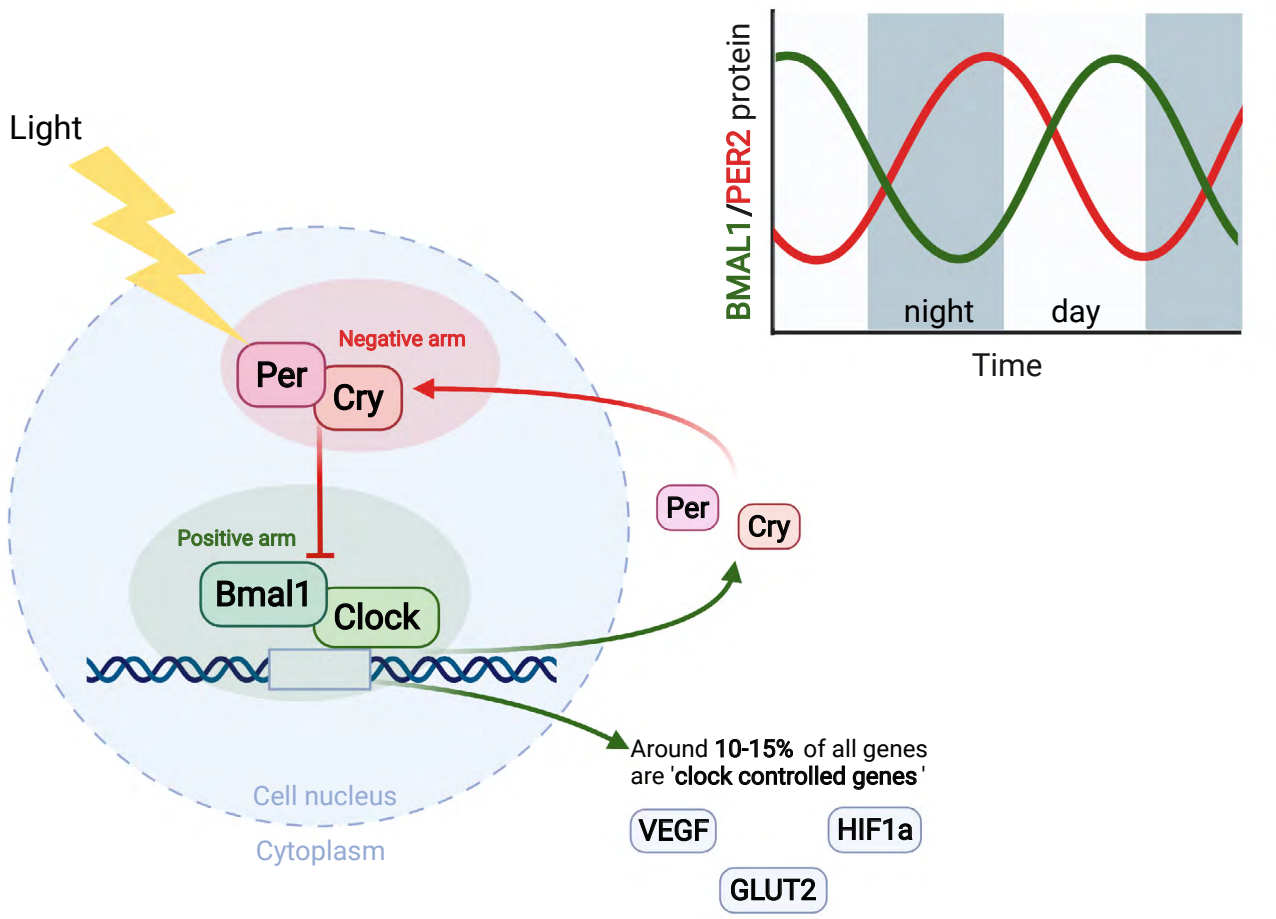

*Figure 2. The main loop of the molecular circadian clock completes a cycle once approximately every 24 hours. The positive arm of the clock is made up of two positive transcription factors, BMAL1 and CLOCK. Over the circadian day, BMAL1 and CLOCK drive the transcription of PER and CRY proteins. When PER and CRY reach a high enough concentration in the cytoplasm, they move back into the nucleus to inhibit any further action of the positive arm, including their own transcription. PER and CRY are degraded overnight until the cycle can start again the next morning. This loop is the pathway by which environmental light cues are integrated into the circadian system. Light causes induction of the PER proteins to change the molecular clock each day and keep it in line with the external environment.[6] Positive arm proteins = BMAL1 and CLOCK; negative arm proteins = PER and CRY; clock-controlled gene examples = vascular endothelial growth factor (*VEGF*), hypoxia-inducible factor 1-alpha (*HIF1a*), and glucose transporter 2 (*GLUT2*). Created with BioRender.com.*

of a multitude of 'clock-controlled genes,' and so the effect of the clock ripples each day to affect diverse processes in tissue-specific ways. In fact, between 10 and 15% of all gene transcription is rhythmic, with as many as 50% of genes in mammals predicted to be rhythmically expressed in at least one tissue.[5]

Circadian rhythms were first described by the French astronomer Jean-Jacques d'Ortous de Mairan in the mid-1700s, based on his observations of how the mimosa plant continued to move its leaves up and down each day, even in constant darkness. However, disagreements as to whether circadian rhythms were truly self-sustained and endogenous and not due to some external cue persisted until at least the 1950s. This was addressed in a seminal Cold Spring Harbor symposium in 1960, where Pittendrigh and Aschoff laid out their 'generalizations'[7] and 'rules.'[8] These comprehensive lists, collating the work in the field to date, placed them among a list of founding 'fathers of chronobiology.' The master clock in the mammalian hypothalamus and the retinohypothamic tract that leads to it from the eye became the subject of intense study upon their discovery in 1972,[9] but it was the retina that was the first tissue outside of the central circadian pacemaker to be described as having a self-contained circadian function.[10] Shortly after, in the 1980s, the molecular basis of the clock was explicated by various groups, with the discovery of the negative clock arm gene *period* by Hall, Rosbash, and Young leading to a shift of the field from a behavioral to a molecular basis.[11] A study decades later, using new opportunities afforded by genetic knockout models, revealed the extent of rhythmicity in the retina. This study used a novel retina-only genetic knockout of the clock positive arm transcription factor BMAL1 to reveal that hundreds of genes in the mammalian eye are controlled by the circadian clock[12] (**Figure 3**).

In the eye specifically, counts of rhythmicity in gene expression range from just over 5% in primate retinas[13] to almost 7% in whole eyes of mice.[12] More recently, the Beli lab has detected just over 9% rhythmicity in the mouse retina. Perhaps surprisingly, slightly more genes appear to be expressed rhythmically in the diabetic retina. Among these are genes involved in processes under the direct control of the clock, including angiogenesis in the retina,[14] the hypoxia response,[15] and inflammation.[16] The potential relevance of the clock to DR is, therefore, immediately obvious. Rhythmicity in diverse gene expression allows the function of the retina to change dramatically between day and night, and this change even manifests in the ways that we clinically assess the retina, such as those to investigate vessel permeability, retina thickness,[17] or retina function with electroretinography, depending on the time of measurement. In mice, electroretinograms change from day to night since the sensitivity of the retina to light is under circadian control,[12] showing that at least some of the retina's functions

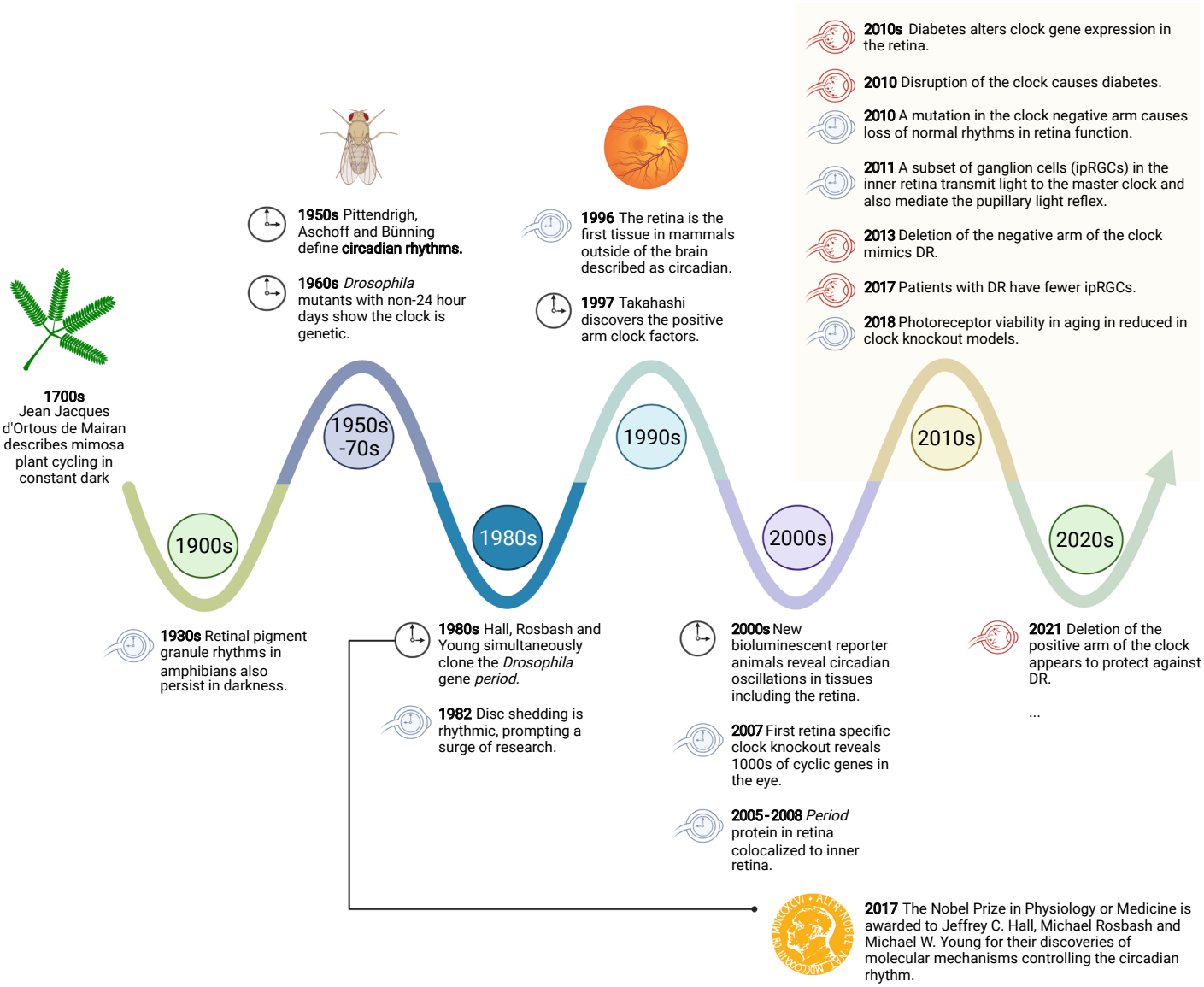

Figure 3. The first description of circadian rhythms appeared in the 1700s. Since then, the field of chronobiology has expanded rapidly with a string of technological and molecular discoveries in the 1900s. Epidemiological studies first implicated the circadian clock as a factor in human disease in 1996, specifically in breast cancer incidence in shift workers,[18] and since then, the list of diseases linked to circadian disruption has grown, including diabetes. Since 2009,[19] the evidence surrounding the role of circadian disruption specifically in DR has grown, promising compelling new opportunities for understanding DR progression and in the future, perhaps even targets for intervention and treatment. Created with BioRender.com.

that can be detected in the clinic are also under the control of the circadian clock. We are at the forefront of discovering functions of the retina controlled by the clock that could be translated to clinical settings as this research is still in its infancy.

The molecular clock in diabetes

In animal models of diabetes,[19,20] clock gene expression is altered in the peripheral organ clocks such as the liver and kidney, including the retina clock.[21] Endothelial cells have a robust circadian clock with rhythms in processes like the expression of adhesion molecules.[22] Thus, we asked whether diabetes alters the expression of circadian genes in endothelial cells. In endothelial cells derived from induced pluripotent stem-cell[23] of diabetic patients and healthy controls, we observed changes in clock gene expression.

Our next question was what processes in diabetes were responsible for these changes. To answer this, we measured the expression of the core clock genes in primary human retinal endothelial cells, mimicking specific elements of the diabetic microenvironment to see which drive the largest clock changes. Hypoxia, rather than hyperglycemia, has so far proven to stimulate more changes in the circadian clock in these cells, at least more acutely, as presented at the EASDec 2021 meeting.

Hypoxia is a major underlying driver for the pathogenesis of DR; thus, understanding how it disrupts the clock may give better insight into the role of the clock in disease progression. Early reductions of oxygen in the diabetic retina are suggested in patient studies, where inhalation of pure oxygen improves visual functions in early diabetes,[24] and supported by various animal model studies.[25] Early hypoxia in diabetic retinopathy appears to be caused by blood-flow abnormalities in the diabetic vasculature that reduce tissue oxygen in the retina. Later in the disease, progressive vascular degeneration leads to areas of non-perfusion (ischaemia) that results in more profound hypoxia and end-stage complications.[25] Later, cells respond to this early hypoxia by increasing their expression of angiogenic factors such as VEGF in an attempt to increase oxygen, which in turn

results in neovascularization that typifies the proliferative disease. Retinal hypoxia in DR is thought to be exacerbated at night by the normal rise in oxygen demand of the photoreceptor cells when dark-adapted. Increased photoreceptor oxygen consumption, therefore, limits the amount of oxygen reaching the inner retina from the choroid at this time (**Figure 4**).[26] It is this hypothesis that has provided the rationale for studies seeking to modify nightly dark current activity.[27] An example is the CLEOPATRA trial in the UK, which asked patients to wear light masks overnight to reduce dark adaptation and, by extension, hypoxia. In this case, the result appeared not to be therapeutically beneficial.[28] Therapies using light have also been explored for reasons related directly to the clock, although not concerning DR.

Hypoxia and the circadian clock

While the impact of hypoxia on the clock in the diabetic retina is not yet clear, the emerging picture in the wider literature is of a dichotomy in how the positive and negative clock arms steer the hypoxic response and vice versa. It is already clear that there is a strong interaction between these two processes (**Figure 5**). For example, the magnitude and type of response induced by hypoxia are controlled by the molecular clock, meaning that hypoxia at different times of day creates different outcomes in different tissues.[30] The transcription factors that make up the positive arm of the clock and that drive the transcription of all of the clock target genes share a very close structural similarity with the major proteins in the hypoxia response. Models where the positive clock arm is knocked out have lower expression of hypoxia outputs,[15] and the clock positive arm has even been shown to target the same gene promoter regions that stimulate the hypoxic response.[15]

How the negative arm of the clock, which represses the positive clock transcription factors, responds in hypoxia is more complex. Cells manipulated to remove the negative arm of the clock have much stronger reactions to hypoxia, suffering more cell death.[30] As with the positive arm, though, the negative arm gene *PER2* and a central hypoxia gene share a similar structure and belong to the same superfamily of signal sensors, allowing the clock to sense light and the hypoxia pathway to sense oxygen. In ischemia, the clock gene *PER2* even stabilizes hypoxia drivers[31] but protects against injury by enhancing glycolytic capacity.[31] In the heart, this *PER2*- mediated protection comes from the endothelial cells.[32]

Because of these interactions, using light therapeutically appears promising for reducing hypoxia damage, such as in myocardial ischemia.[30,32] However, light treatment in DR has proven more complicated. As mentioned above, a study asking patients to wear light masks overnight to reduce dark adaptation, and

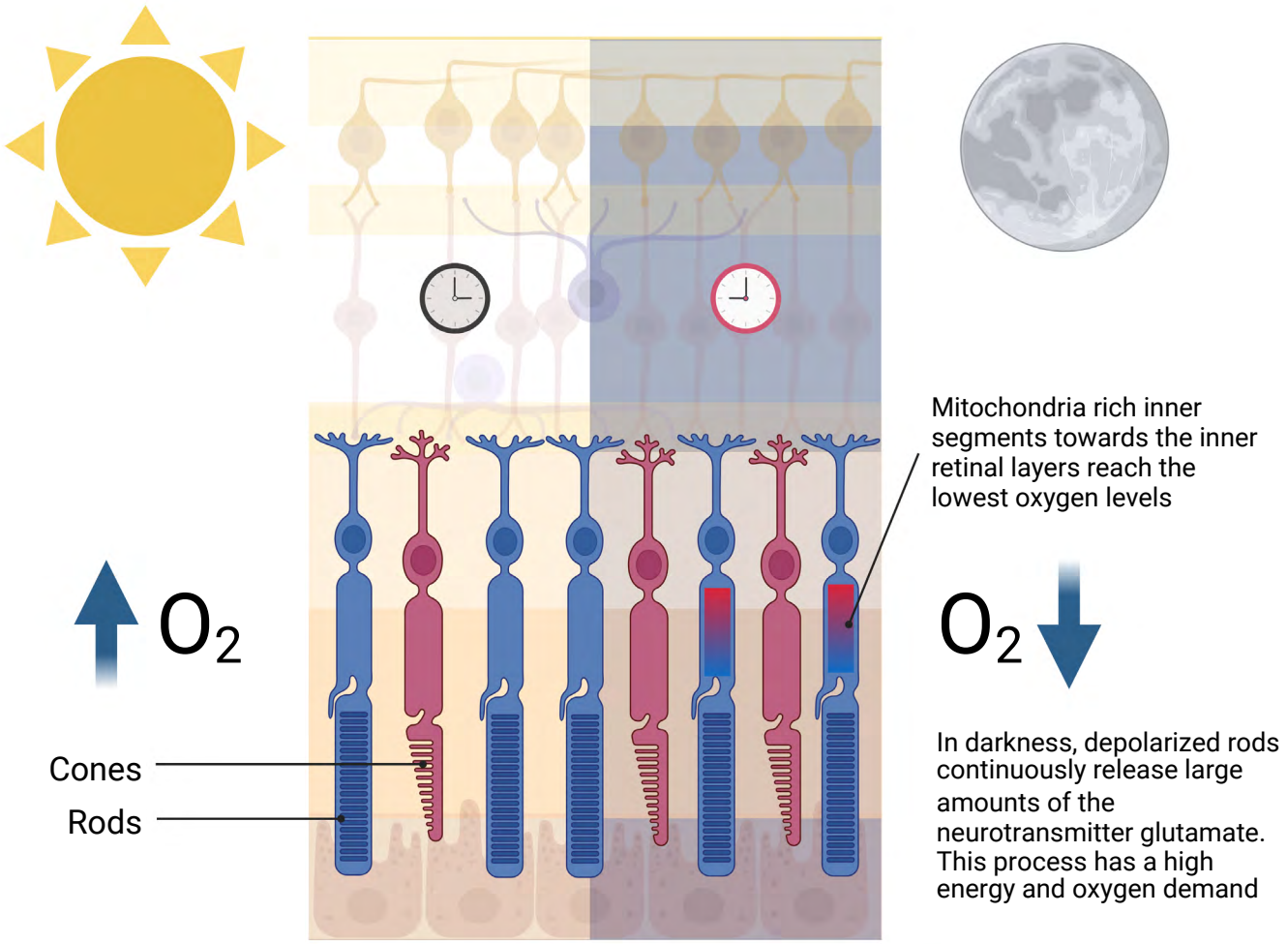

Figure 4. Nightly dark adaptation of the photoreceptors creates an energy and oxygen demand that cannot be met in the diabetic retina. This results in nightly low oxygen that triggers hypoxia response in the cells and possibly also alters the circadian molecular clock. Since this hypoxia is not experienced equally across the retinal layers,[29] desynchrony between the layers might also be triggered. Created with BioRender.com.

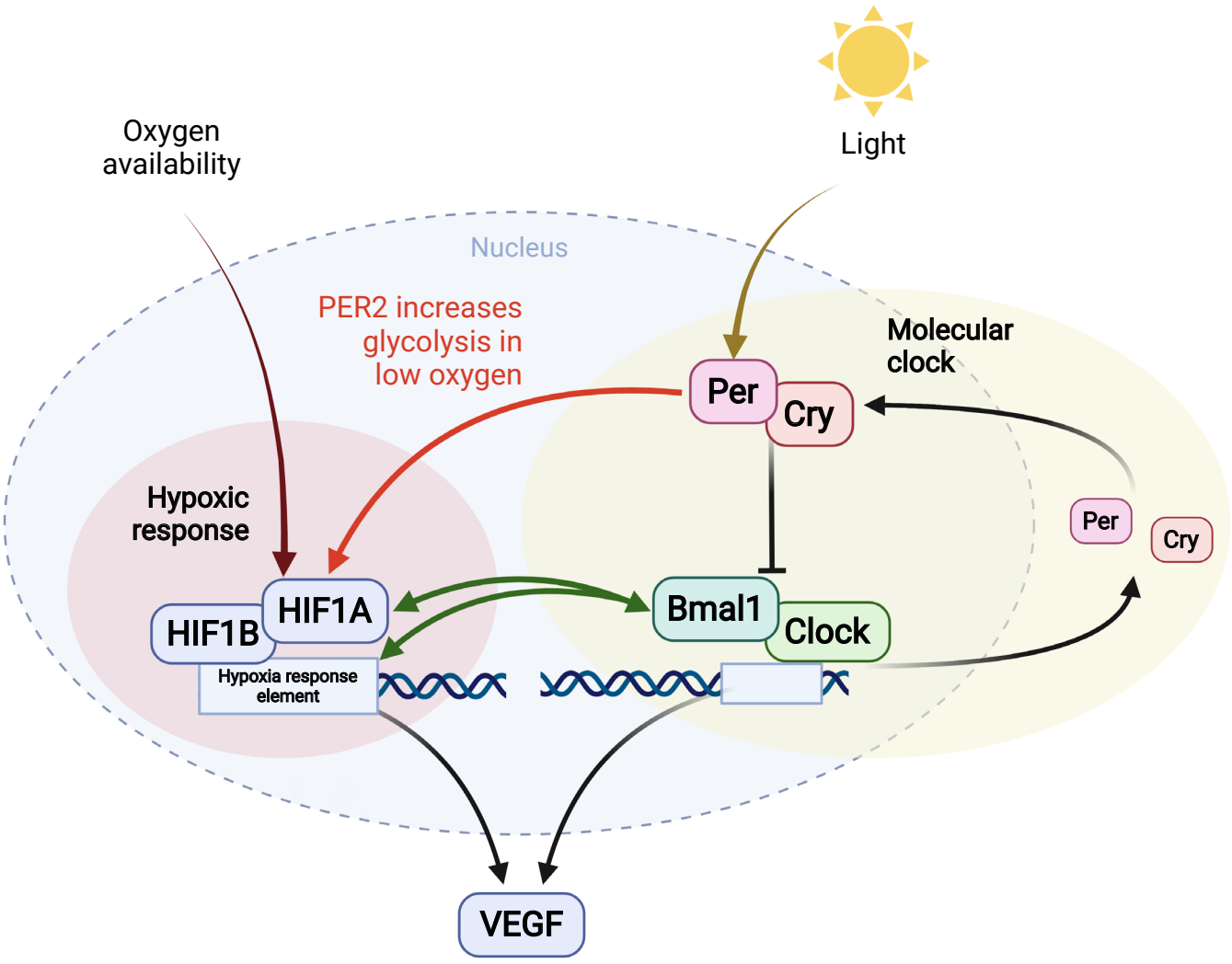

Figure 5. There are several parallels and links between the molecular clock and the hypoxia response pathway. Negative clock factor PER2 and key hypoxia effector gene HIF1A even belong to the same family of signal sensor proteins, the first sensing light and the second oxygen. Positive arm factor BMAL1 also interacts with the same HIF1A gene. In health, these interactions should result in an appropriate and time-dependent response to environmental changes, but, in disease, these measured responses are often lost. Positive arm molecular clock proteins = BMAL1 and CLOCK; negative arm molecular clock proteins = PER and CRY; hypoxia response proteins = HIF1A and HIF1B.

therefore hypoxia, found no therapeutic benefit. Conversely and counter-intuitively, a study in diabetic mice found that light deprivation was actually protective against DR.[33] Since the retina is critical to providing light timing to the overall clock system, these approaches must be considered carefully. Although the CLEOPATRA trial used light levels lower than those known to cause melatonin changes in humans, constant light has the effect of weakening circadian rhythm strength across the circadian system.[34] An intervention designed to target the molecular clock rather than just reduce hypoxia might, instead, use an intense but short light application, which has the effect of strengthening the circadian system. If hypoxia, such as that created by the photoreceptor cells during the night, can change the molecular clock in the retina as suggested by our observations in retinal endothelial cells, an intense application of light during the day may aid in retrieving normal clock timing by restoring the negative arm of the clock.

The changes that we observe in retinal endothelial cells suggest that hypoxia bolsters the positive arm of the clock by increasing its expression while its regulator, PER2, is reduced at all time points (**Figure 6**). The peak expression for these genes comes earlier after synchronization than in cells maintained at normal oxygen levels. While the period of a single oscillation of the positive arm is reduced in hypoxia, the opposite happens for the negative arm. These changes together suggest that hypoxia causes a desynchrony of this central clock in the diabetic retina. Targeting the clock might be a tool to fine-tune the hypoxic response.

Conclusion

Hypoxia, which is widespread in the diabetic retina, causes dysregulation of the cellular circadian machinery in retinal endothelial cells by favoring the positive arm so that its transcription factors are more abundant in the endothelium. What might the impact of this be for diabetic retinopathy? Ramifications are potentially as far-reaching as the targets of these positive arm transcription factors, which notably include VEGF,[14] a major growth factor implicated in DR. The next steps will be to see how outcomes like metabolism and barrier function are impacted as a result of this manipulation of the circadian clock. As we start to build a clearer picture of the circadian clock in the diabetic retina, we may even be able to target the clock itself to manage the pathologies that underlie diabetic retinopathy.

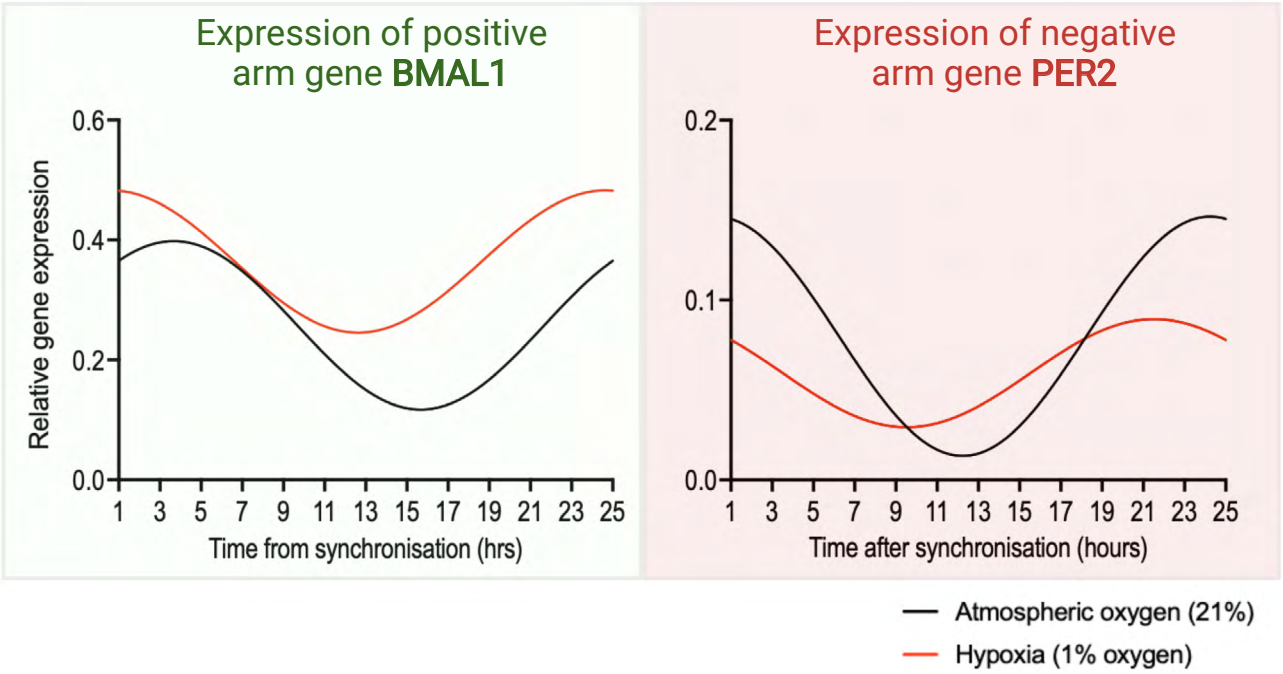

Figure 5. Expression of core clock genes BMAL1 and PER2 in endothelial cells in the hours after the cells were synchronized, cultured in either atmospheric oxygen or hypoxia (1% oxygen). The cell cultures were synchronized by treating them with a high serum content (50%) media for two hours before starting to collect samples. This ensures that all cells within the culture are at the same phase of expression. In hypoxia, BMAL1 expression is increased, and the phase shifted. Its negative regulator PER2 is downregulated. Peak transcription happens earlier for both. Created with BioRender.com.

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References

1. Felder-Schmittbuhl, M. P. et al. Ocular Clocks: Adapting Mechanisms for Eye Functions and Health. Investigative Ophthalmology & Visual Science 59, 4856-4870, doi:10.1167/iovs.18-24957 (2018).
2. DeVera, C., Baba, K. & Tosini, G. Retinal circadian clocks are major players in the modulation of retinal functions and photoreceptor viability. The Yale Journal of Biology and Medicine 92 (2019).
3. SK, C., TC, B. & S, H. Photoentrainment and pupillary light reflex are mediated by distinct populations of ipRGCs. Nature 476, doi:10.1038/nature10206 (2011).
4. Buhr, E. D. & Takahashi, J. S. Molecular components of the Mammalian circadian clock. Handbook of Experimental Pharmacology, 3-27, doi:10.1007/978-3-642-25950-0_1 (2013).
5. Zhang, R., Lahens, N. F., Ballance, H. I., Hughes, M. E. & Hogenesch, J. B. 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, doi:10.1073/pnas.1408886111 (2014).
6. Shigeyoshi, Y. et al. Light-induced resetting of a mammalian circadian clock is associated with rapid induction of the mPer1 transcript. Cell 91, 1043-1053, doi:10.1016/s0092-8674(00)80494-8 (1997).
7. Pittendrigh, C. S. Circadian rhythms and the circadian organization of living systems. Cold Spring Harbor Symposia on Quantitative Biology 25, 159-184, doi:10.1101/sqb.1960.025.01.015 (1960).
8. Aschoff, J. Exogenous and endogenous components in circadian rhythms. Cold Spring Harbor Symposia on Quantitative Biology 25, 11-28, doi:10.1101/sqb.1960.025.01.004 (1960).
9. Moore, R. Y. & Eichler, V. B. Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. Brain Research 42, doi:10.1016/0006-8993(72)90054-6 (1972).
10. Tosini, G. & Menaker, M. Circadian rhythms in cultured mammalian retina. Science 272, doi:10.1126/science.272.5260.419 (1996).
11. Huang, R. C. The discoveries of molecular mechanisms for the circadian rhythm: The 2017 Nobel Prize in Physiology or Medicine. Biomedical Journal 41, doi:10.1016/j.bj.2018.02.003 (2018).
12. Storch, K. F. et al. Intrinsic circadian clock of the mammalian retina: importance for retinal processing of visual information. Cell 130, 730-741, doi:10.1016/j.cell.2007.06.045 (2007).
13. Mure, L. S. et al. Diurnal transcriptome atlas of a primate across major neural and peripheral tissues. Science (New York, N.Y.) 359, doi:10.1126/science.aao0318 (2018).
14. Jensen, L. D. et al. Opposing effects of circadian clock genes bmal1 and period2 in regulation of VEGF-dependent angiogenesis in developing zebrafish. Cell Reports 2, 231-241, doi:10.1016/j.celrep.2012.07.005 (2012).
15. Peek, C. B. et al. Circadian Clock Interaction with HIF1α Mediates Oxygenic Metabolism and Anaerobic Glycolysis in Skeletal Muscle. Cell Metabolism 25, 86-92, doi:10.1016/j.cmet.2016.09.010 (2017).
16. Wang, Q. et al. Regulation of retinal inflammation by rhythmic expression of MiR-146a in diabetic retina. Investigative Ophthalmology & Visual Science 55, 3986-3994, doi:10.1167/iovs.13-13076 (2014).
17. Kotsidis, S. T., Lake, S. S., Alexandridis, A. D., Ziakas, N. G. & Ekonomidis, P. K. 24-Hour variation of optical coherence tomography-measured retinal thickness in diabetic macular edema. European Journal of Ophthalmology 22, doi:10.5301/ejo.5000119 (2012).
18. Roenneberg, T. & Merrow, M. The circadian clock and human health. Current Biology 26, doi:10.1016/j.cub.2016.04.011 (2016).
19. Busik, J. V. et al. Diabetic retinopathy is associated with bone marrow neuropathy and a depressed peripheral clock. The Journal of Experimental Medicine 206, doi:10.1084/jem.20090889 (2009).
20. Hou, T., Su, W., Guo, Z. & Gong, M. C. A novel diabetic mouse model for real-time monitoring of clock gene oscillation and blood pressure circadian rhythm. Journal of Biological Rhythms 34, 51-68, doi:10.1177/0748730418803719 (2019).
21. Lahouaoui, H., Coutanson, C., Cooper, H. M., Bennis, M. & Dkhissi-Benyahya, O. Diabetic retinopathy alters light-induced clock gene expression and dopamine levels in the mouse retina. Molecular Vision 5, 959-969 (2016).
22. He, W. et al. Circadian expression of migratory factors establishes lineage-specific signatures that guide the homing of leukocyte subsets to tissues. Immunity 49, 1175-1190, doi:10.1016/j.immuni.2018.10.007 (2018).
23. Vilà-González, M. et al. Enhanced function of induced pluripotent stem cell-derived endothelial cells through ESM1 signaling. Stem Cells 37, 226-239, doi:10.1002/stem.2936 (2019).
24. Harris, A. et al. Hyperoxia improves contrast sensitivity in early diabetic retinopathy. The British Journal of Ophthalmology 80, 209-213, doi:10.1136/bjo.80.3.209 (1996).
25. Curtis, T. M., Gardiner, T. A. & Stitt, A. W. Microvascular lesions of diabetic retinopathy: clues towards understanding pathogenesis? Eye (London, England) 23, 1496-1508, doi:10.1038/eye.2009.108 (2009).
26. Majidi, S. P. & Rajagopal, R. Photoreceptor responses to light in the pathogenesis of diabetic retinopathy. Visual Neuroscience 37, doi:10.1017/S0952523820000061 (2020).
27. Ramsey, D. J. & Arden, G. B. Hypoxia and dark adaptation in diabetic retinopathy: Interactions, consequences, and therapy. Current Diabetes Reports 15, doi:10.1007/s11892-015-0686-2 (2015).
28. Sivaprasad, S. et al. Clinical efficacy and safety of a light mask for prevention of dark adaptation in treating and preventing progression of early diabetic macular oedema at 24 months (CLEOPATRA): a multicentre, phase 3, randomised controlled trial. The Lancet. Diabetes & Endocrinology 6, doi:10.1016/S2213-8587(18)30036-6 (2018).
29. Yu, D. Y. & Cringle, S. J. Outer retinal anoxia during dark adaptation is not a general property of mammalian retinas. Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology 132, doi:10.1016/s1095-6433(01)00528-1 (2002).
30. Wu, Y. et al. Reciprocal regulation between the circadian clock and hypoxia signaling at the genome level in mammals. Cell Metabolism 25, 73-85, doi:10.1016/j.cmet.2016.09.009 (2017).
31. Eckle, T. et al. Adora2b-elicited Per2 stabilization promotes a HIF-dependent metabolic switch crucial for myocardial adaptation to ischemia. Nature medicine 18, 774-782, doi:10.1038/nm.2728 (2012).
32. Oyama, Y. et al. Intense light-mediated circadian cardioprotection via transcriptional reprogramming of the endothelium. Cell reports 28, 1471-1484, doi:10.1016/j.celrep.2019.07.020 (2019).
33. Thebeau, C. et al. Light deprivation reduces the severity of experimental diabetic retinopathy. Neurobiology of Disease 137, doi:10.1016/j.nbd.2020.104754 (2020).
34. Blume, C., Garbazza, C. & Spitschan, M. Effects of light on human circadian rhythms, sleep and mood. Somnologie : Schlafforschung und Schlafmedizin 23, doi:10.1007/s11818-019-00215-x (2019).