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Vasculature on the clock: Circadian rhythm and vascular dysfunction

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ABSTRACT

The master mammalian circadian clock (*i.e.* central clock), located in the suprachiasmatic nucleus of the hypothalamus, orchestrates the synchronization of the daily behavioural and physiological rhythms to better adapt the organism to the external environment in an anticipatory manner. This central clock is entrained by a variety of signals, the best established being light and food. However, circadian cycles are not simply the consequences of these two cues but are generated by endogenous circadian clocks. Indeed, clock machinery is found in mainly all tissues and cell types, including cells of the vascular system such as endothelial cells, fibroblasts, smooth muscle cells and stem cells. This machinery physiologically contributes to modulate the daily vascular function, and its disturbance therefore plays a major role in the pathophysiology of vascular dysfunction. Therapies targeting the circadian rhythm may therefore be of benefit against vascular disease.

1. Introduction

Ubiquitous amongst almost all life on Earth, internal clocks allow organisms to anticipate and prepare for changes in their environment brought by daily solar cycle. Light, as well as its absence, directly influences the functioning of the clock and presents the main environmental cue for synchronizing internal rhythms according to Earth's rotation, a 24-hour period [1]. Circadian rhythms (derived from the Latin words *Circa* (=around) and *dies* (=day)) are therefore physical, mental and behavioural changes that follow a daily cycle. The discovery of the existence of circadian rhythm in living species goes back to the 18th century but the physiological mechanisms responsible for this circadian adaptation have only been elucidated in the 20th century, with the first major discoveries by Young, Hall and Rosbash, Laureates of the 2017 Nobel Prize in Medicine.

Multiple physiological functions can be modulated by the circadian rhythm and the vascular function system is no exception [2–4]. A better understanding of the machinery behind circadian rhythms in the pathophysiology of the vascular function, from the role of the suprachiasmatic nucleus (SCN) and a clear evidence for the involvement of peripheral clocks within major cell types of the vascular system, including endothelial cells, smooth muscle cells, fibroblast and stem or progenitor cells in humans, is crucial for targeting the circadian rhythm as a potential therapy in vascular disease.

2. Machinery behind circadian rhythms

If mechanisms of circadian rhythms in mammals were to be placed in a hierarchical manner, the top of the pyramid would be represented by the SCN, comprised of approximately 20,000 neurons in the anterior hypothalamic region of the brain [5]. Photoreceptors in the retina receive light which is then transmitted to the SCN, regulating the release of melatonin (*N*-acetyl-5-methoxytryptamine) from the pineal gland which synchronizes the peripheral clocks to the central oscillator by endocrine and autonomous mechanisms [6,7]. Aside from light, external synchronizers, *i.e.* Zeitgebers, include social behaviour, food regime and exercise [1,8,9]. Rather than in the SCN, their effect takes place in the peripheral clocks which have now been identified in almost every cell in the mammalian body [10–13]. While remaining their autonomy, peripheral clocks function in synchrony with the SCN.

It is worth emphasizing the self-sustainability of internal rhythms; while influenced by external inputs, they persist in their absence, as has been shown in the studies where animals were kept in the complete darkness and isolated cells in culture [14]. Molecular machinery that governs and ensures robustness of these rhythms is comprised of so called clock genes, and its detailed overview has been given in many review articles up to date [15–18]. Overall, intertwined positive and negative molecular feedback loops regulate the rhythmic expression of clock-controlled genes (CCG), including *Bmal1/2* (Brain and Muscle ARNT-like 1/2), *Clock* (Circadian Locomotor Output Cycles Kaput),

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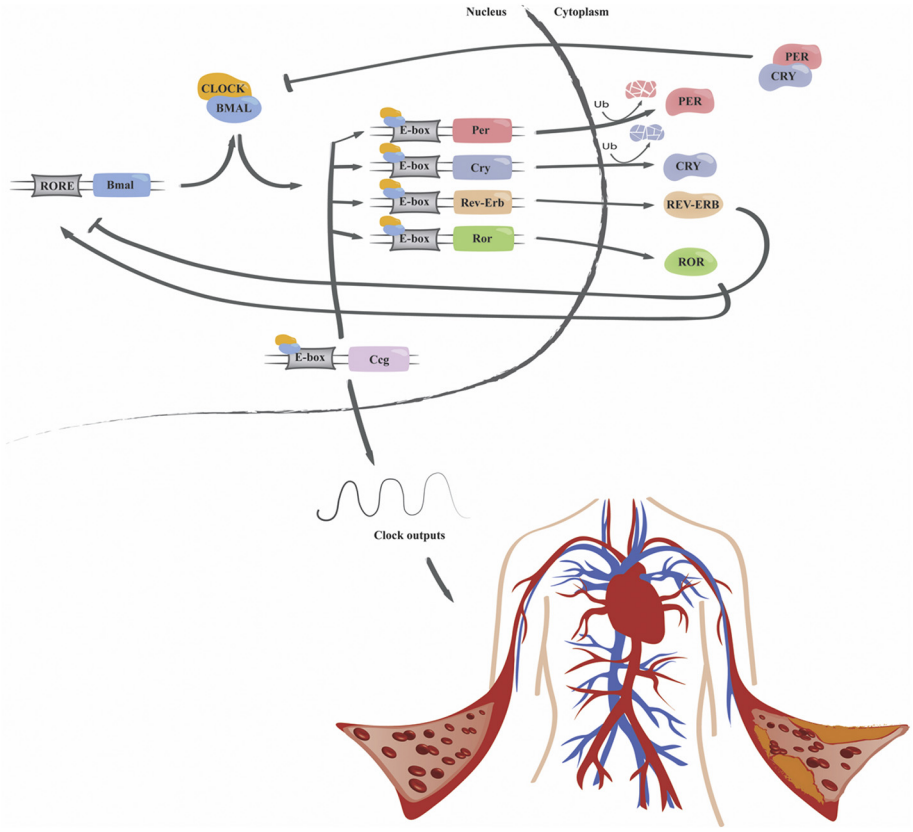

Fig. 1. Core clock mechanism with implications in vascular health and disease. BMAL1 and CLOCK form a heterodimer which, upon binding to *E*-boxes of several clock genes, induces the transcription of *Per* and *Cry*. Consequently, PER and CRY accumulate in the cytoplasm, form a PER: CRY dimer and translocate into the nucleus thereby inhibiting the BMAL1: CLOCK heterodimer and forming a negative feedback loop. Additionally, ROR and REV-ERB, whose transcription is activated in the same manner as described above, compete for RORE binding site in the promotor region of *Bmal1,* leading to either activation or inhibition of its transcription. As a result, clock controlled genes (CCGs) are rhythmically activated, leading to oscillations in functions of different vascular cells, both in physiological and pathophysiological states.

Cry1/2 (Crypto chrome 1/2) and *Per1/2/3* (Period 1/2/3), consequently leading to oscillations in cell functions [19] as depicted in Fig. 1. Briefly, BMAL1 and CLOCK proteins form a heterodimer [20], which binds to the enhancer box elements (*E*-boxes) in the promoter region of other clock genes, such as *Per* and *Cry*, therefore inducing their transcription. Upon accumulation of PER and CRY proteins in the cytoplasm, a PER: CRY dimer is formed and translocated into the nucleus [21] where it binds to the NuRD (nucleosome remodelling deacetylases) transcriptional repressor complex and directs it to the BMAL1: CLOCK dimer [22]. Thus, transcription driven by the BMAL1: CLOCK complex is inhibited and the negative feedback loop formed in which PER: CRY inhibit their own transcription by blocking BMAL1: CLOCK activity. This repression of the BMAL1: CLOCK complex is lifted with ubiquitin- mediated degradation of PER: CRY, leading to the activation of a new transcriptional cycle [23,24].

Although the process described above is the main feedback loop, there are additional layers of activation and regulation of clock genes, including the orphan nuclear receptors RORα/β and REV-ERBα/β [25,26]. Similarly, BMAL1: CLOCK heterodimers bind to *E*-boxes of *RORα/β* and *Rev-Erbα/β*, initiating their transcription and second feedback loop. The *Bmal1* promoter region contains a binding site for both RORα/β and REV-ERBα/β, termed retinoic acid-related orphan receptor response element (RORE) binding sites and Rev-Erb response element (RRE), respectively. Depending on which competing molecule binds to *Bmal1*, RORα/β or REV-ERBα/β, it will lead to either activation or inhibition of *Bmal1* transcription, respectively [27]. As a result, translation of CCGs is rhythmically activated within approximately 24 h, which is the duration of one described cycle.

Aside from transcriptional/translational feedback loops, many other finesses take place in regulating and fine-tuning circadian rhythmicity, including microRNAs and chromatin remodelling [28–30]. For details, the reader is referred to recent reviews on the complex matter of additional layers of circadian regulation [18].

3. Peripheral clocks: circadian gene expression in the vasculature

As described in the previous chapter, circadian clocks can be divided into central clocks, located in the suprachiasmatic nucleus, and peripheral clocks. The latter can be found in almost every tissue in the body, including cardiovascular tissues. Davidson et al. [31] proved the existence of circadian rhythmicity in cardiovascular tissue explants by recording rhythmic expression of luciferase activity in veins, arteries and hearts obtained from transgenic *Per1*-luciferase rats. Other evidence confirming the existence of the clock in the vasculature is summarized in various review articles [2–4,32,33].

Even though the connection between cardiovascular diseases and circadian rhythms has been recognized, the exact biological relevance of internal peripheral clocks remains to be elucidated. In this article, current understanding of intrinsic biological rhythm within endothelial cells, vascular smooth muscle cells, fibroblasts and stem cells will be discussed, as they present major cell types within vasculature.

3.1. Endothelial cells

Endothelial cells, located at the luminal vessel wall, regulate the exchange between bloodstream and the surrounding tissues [34]. Existence of intrinsic circadian rhythms within vascular endothelial cells has been confirmed *in vitro* by synchronizing hemangioendothelioma and human umbilical vein endothelial cells (HUVECs) with serum shock [35]. The same study further investigated clock-controlled genes in HUVECs using microarray technology. Interestingly, thrombomodulin, an integral membrane glycoprotein that regulates intravascular coagulation, was found to be regulated by the circadian clock in vascular endothelial cells [35]. Furthermore, circadian oscillations were found in plasminogen activator inhibitor-1 production of endothelial cells [36].

Various roles of the circadian clock, or lack of it, in atherogenesis are summed in the review article by McAlpine and Swirski [32], where

the collected data of circadian influence in endothelial cells spreads from coagulation cascade components, expression of chemokines (Cxcl5, Ccl20, and Ccl8), endothelial cell expression of the adhesion molecules ICAM and VCAM (intercellular adhesion molecule-1 and vascular cell adhesion molecule-1), to above mentioned thrombomodulin, emphasizing the importance of the intrinsic vascular clock.

3.2. Vascular smooth muscle cells

Vascular smooth muscle cells (VSMCs) represent the middle layer of blood vessels. They are responsible for constriction and dilation of the vessel, the tone of which is regulated by the sympathetic nervous system, circulating mediators and endothelial cells [37]. VSMCs express circadian oscillations, as shown *in vitro* following serum shock of VSMCs derived from healthy arteries, with robust peaks and troughs in *Clock*, *Bmal1*, *Per2*, *Cry1*, and *Rev-Erbα* expression [38]. A study by Nonaka et al. demonstrated that molecular oscillators exist in the aorta *in vivo* and in cultured VSMCs. In addition, circadian expression of clock genes can be induced by angiotensin II, a molecule regulating cardiovascular function [39]. Aside from the clock genes, circadian patterns were found in tissue inhibitor of metalloproteinase 1 and 3 (*timp1/3*), collagen 3a1 (*col3a1*), transgelin 1 (*sm22alpha*), and calponin 1 (*cnn1*), using the immortalized vascular smooth muscle cell line Movas-1 [40].

That understanding circadian rhythms can have a translational significance was nicely shown by a study in which expression differences of clock genes between normal human carotid VSMCs and human plaque-derived VSMCs was found [38]. Rhythms in human plaque-derived VSMCs were altered and significantly attenuated, which may be involved in atherosclerosis and plaque rupture.

3.3. Fibroblasts

Lastly, fibroblasts present the outer, connective tissue layer of the vessel. Together with endothelial cells, they play a crucial role in angiogenesis [41]. It has long been recognized that circadian clock components of cultured fibroblasts oscillate in a circadian manner. Balsalobre et al. demonstrated this for rat fibroblasts when they exposed the cells to 50% serum as a phase synchronizer [10]. Further studies confirmed this finding on the single cell level by using bioluminescence imaging of rat fibroblasts transfected with a *Bmal1*:luc plasmid, along with the primary fibroblasts obtained from *mPer2* Luciferase-SV40 knockin mice [12]. The main findings of the study were the robust and independent, self-sustained oscillations in individual fibroblasts. Subsequently, aside from serum shock, different studies showed an array of signals with the ability to synchronize the rhythms in fibroblasts, one of which being the vasocontracting peptide endothelin-1 [42–44].

3.4. Stem- or progenitor cells

Stem cells are defined as a type of cells with the ability to self-renew and differentiate into different cell types. They can also be found in adult tissues, including cardiovascular, where they play a role both in homeostasis processes and disease progression [45]. Bone marrow-derived endothelial progenitor cells (EPCs) are an example of this, although controversy exists on their nomenclature [46]. Considering cited references in this review, we use the (historical) term EPC for the sake of clarity.

The role of core clock gene *Per2* was investigated in EPCs, which circulate in the blood and contribute to re-endothelialisation and neoangiogenesis upon injury. In *Per2* mutant mice endothelial dysfunction was observed, partly mediated by impaired endothelial EPC function [47]. Since the influence of physiological circadian rhythms on stem cell function has already been shown, disruption of one of the circadian genes, *Per2* in this case, is likely to disturb the function of bone marrow-derived cells, specifically endothelial progenitor cells [18,45,48–50]. Further studies investigated the role of *Per2*, forming the conclusion that this circadian clock gene may be a key player in maintaining *in vitro* EPC function, as well as *in vivo* therapeutic angiogenesis [51].

4. Clinical and experimental evidence for a role of circadian rhythms in vascular pathophysiology

4.1. Role of circadian rhythm in vascular function

The most obvious circadian rhythm in humans is the sleep/wakefulness cycle. However, accumulating evidence also suggests that the cardiovascular system behaves rhythmically over the course of a day. This is particularly true for blood pressure (BP) and heart rate. In healthy humans, it has been known for decades that BP rises before awaking, reaches its highest level in the midmorning and then decreases throughout the day to reach a nadir at around 3:00 AM [52]. These time-dependent effects are not just the consequences of the sleep/awake or rest-exercise changes, but are also linked to significant fluctuation of the intrinsic properties of blood vessels.

In the nineties, surgery was used to demonstrate the potential role of the master clock in vascular physiology. Hence, ablation of suprachiasmatic nuclei disrupted the circadian variations in BP, along with behavioural and endocrine rhythms [53]. Although necessary, this rudimentary approach did not afford direct molecular insight into an interaction between the circadian/biological clock and BP. Another strategy was to test the sensitivity of rat aorta to vasoactive agents in regards to the time of the day the tissues were collected [54]. The authors showed a clear time-dependent effect on aortic vaso-reactivity, suggesting cyclic changes in underlying molecular properties of the vessels. These findings are in line with a more recent study by Zhang et al. reporting that a significant proportion (around 5%) of protein coding genes display circadian cycle in transcription in mice aorta [55].

A definite role for circadian control of vascular physiology has emerged with genetically modified mice in which genes controlling peripheral clocks were altered [56–58]. *Clock* mutants provided genetic evidence linking circadian rhythmicity and vascular dysfunction, vascular remodelling and injury. The first description of circadian expression of clock gene in mouse vasculature (aorta) was that of McNamara in 2001 [59]. Afterwards, a flurry of studies has confirmed the involvement of these clock genes in the vascular physiology. For instance, genetic loss of *Bmal1* abolishes circadian variability of BP and reduces production of catecholamines [60]. Interestingly, transplanted aortas from *Bmal1* knockout mice into wild-type mice still develop severe atherosclerosis without altering hemodynamic, signifying also an important role for peripheral clocks in the generation of this phenotype [61]. It has also been shown that genetic loss of other genes of the circadian clock (*e.g. Per*) impairs endothelium-dependent relaxation from acetylcholine [62]. More recently, Anea et al. demonstrated in mice lacking Bmal1 or the Period isoforms that circadian clock dysfunction contributes to hardening of the vasculature, which involves impaired control of the extracellular matrix composition [63].

4.2. Role of circadian rhythm in vascular dysfunction

Circadian rhythm disturbances are relevant to humans. For example, hypertension remains a major public health problem. Development of ambulatory BP monitoring raised questions about chronobiology of the vasculature. As described above, the circadian rhythm of BP is characterized roughly by high pressures at daytime and low pressure at night time. These time-dependent variations also occur at the molecular level including changes in gene expression in blood vessel. In recent years, disruption of the circadian rhythm of BP, particularly the absence of nocturnal dipping, has been identified as a major cardiovascular risk factor [64]. Accordingly, the 2017 ACC/AHA guidelines recommend the use of ambulatory BP monitoring which allows to determine mean BP during the entire circadian cycle, including

mean BP during night time and the day-to-night time ratio, thus allowing to assess the extent of nocturnal dipping and the early morning surge pattern [65]. Unfortunately, international recommendations still largely ignore the impact of circadian rhythm in the development, diagnosis and therapy of hypertension [65,66].

Thirty years ago, it was believed that cardiovascular events occurred randomly. Since then, a series of epidemiological studies demonstrated that almost all acute cardiovascular events, such as unstable angina, myocardial infarction, sudden cardiac death or stroke, displayed a circadian rhythm, with a peak of frequency in the early morning [67]. These events coincide with morning BP surge, decreased vasodilatation capacity, enhanced platelet aggregability and altered repolarization [34]. In the same way, early morning exaggeration in basal tone of coronary arteries in patients with endothelial dysfunction may contribute to the morning peak frequency of the onset of acute myocardial infarction [68]. More recently, it has been shown in large cohorts that infarct size varies according to the time of symptom onset, and that this relationship follows a circadian cycle, supporting the hypothesis that the myocardium's vulnerability to ischemia/reperfusion is subject to a 24-h cycle [69,70]. Importantly, these results were independent of ischemic time and quality of care, which can both vary quite significantly between day and night.

4.3. Targeting the circadian rhythm as a potential therapy for vascular disease

Several new lines of evidence reveal translational application of vascular circadian rhythmicity to clinical medicine (Fig. 2). Because circadian rhythms are significantly involved in almost all cardiovascular events, these characteristics should be taken into account when making decisions about treatment and prevention. The treatment of patients with cardiovascular disease should consider not only correcting the symptoms and/or organic lesions but also restoring/resynchronizing circadian rhythm, such as BP dipping at night. Chronotherapy, which involves timing therapies, may also improve therapeutic efficacy while limiting toxicity.

4.4. Dark/night

Modern life exposes more and more to disruption of normal sleep rhythms due to shift work, flights across different time zones, prolonged nocturnal activities with exposure to artificial light, and social activities. Night shift work, undertaken by 15% of the workforce, is a risk factor for diabetes, elevated BP, hypertension and cardiovascular disease, even after controlling for other risk factors [71]. By nature, night shift work provokes desynchronization between the endogenous circadian system and environmental/behavioural cycles. However, epidemiological studies could not demonstrate a direct link between shift work-induced circadian rhythm disruption and cardiovascular disease. Indeed, many other factors such as dietary habits, workload, and sleep deprivation, may bias the conclusions of these studies. In a highly controlled study in healthy individuals, Morris et al. demonstrated that circadian misalignment, as observed in shift workers, not only increases BP but also markers of inflammation [72]. Future studies are warranted to identify preventive measures for this population at greater risk of cardiovascular disease. Shift workers are not the only ones to be exposed to circadian cycle disruption. The simple exposure to light at night including that from light-emitting diodes in television, computer screens or light from eReaders negatively affects sleep, circadian timing and next-morning alertness [73]. Observational studies in cardiac intensive care units have even shown an association between room illuminance during night and mortality [74]. Conversely, exposure to bright light in the early morning has a potent effect on circadian rhythms that could be used in acute cardiovascular disease. This is especially so because bright light seems to influence in a positive way circadian microRNA expression after myocardial infarction [75].

4.5. Sleep disorders

A large proportion of the world's population does not achieve the recommended 7–8 h per hours per night. This sleep deficit is associated with an increased risk of cardiovascular disease [76]. Sleep debt and fatigue are often associated with caffeine consumption, which can further exacerbate circadian disruption. For example, it has been shown that chronic caffeine consumption strengthens the period of locomotor activity in rodents and alters clock gene expression under constant light/dark conditions [77]. However, clinical evidence indicates that coffee and tea drinkers are less prone to develop stroke or acute myocardial infarction [78]. One explanation for this finding is that antioxidants and other protective bioactive compounds contained in coffee and tea overwhelm the potential deleterious stimulant effects of these drinks.

Quality of sleep is often overlooked in cardiovascular medicine, especially for patients who are hospitalized [79]. However, recent experimental evidence suggest that short-term disruption of circadian rhythm caused by sleep deprivation after acute myocardial infarction, might increase infarct size and impair remodelling [80]. There is therefore some convincing scientific rationale to maintain the biological cycles of critically ill patients to help healing through a promising non-pharmacological approach [81].

Obstructive sleep apnea is a common disorder that affects one in five adults in western countries. It is responsible for disruption of circadian rhythms (including at the molecular level), which can lead to the worsening of cardiovascular disease [82]. Nocturnal continuous positive airway pressure represents a very effective therapeutic strategy to improve outcomes. Recent data suggest that this treatment resynchronizes circadian clocks in humans, as shown by the analysis of *Per1* mRNA expression in peripheral blood cells in patients treated with or without non-invasive ventilation [83]. Interestingly, a form of familial sleep syndrome is associated with a mutation of *Per2* [57].

4.6. Pharmacological approach

Modifying circadian rhythmicity with a therapeutic goal is known as chronotherapy. It comprises non-pharmacological approaches such as exposure to bright light in the morning and consolidated dark periods at night or continuous positive pressure for sleep apnoea, and pharmacological treatments, melatonin being the most studied [84]. Chronotherapy also seeks to improve the efficacy and to limit toxicity of treatments by determining the most appropriate time of the day for their administration. For example, nocturnal haemodialysis, as compared with daytime renal replacement therapy, is associated with regression of myocardial hypertrophy in patients with cardio-renal syndrome [85]. Aspirin may decrease the morning peak of platelet reactivity in healthy volunteers and lower BP, but only when given at night. One can take advantage of these effects to further reduce the risk of acute coronary syndrome during the peak morning hours [86]. With regards to hypertension, antihypertensive medications (whatever the drug class) ingested at bedtime have a significant impact on circadian BP rhythms and improve outcomes [87]. Given that the activation of the renin angiotensin aldosterone system clearly displays a circadian rhythm, this schedule of dosing may be even more significant for both angiotensin-converting enzyme inhibitors and angiotensin II receptors blockers [88].

Decreased melatonin production was found in patients with essential hypertension, coronary artery disease and acute coronary syndrome, opening new opportunities for the treatment or prevention of cardiovascular disease from a circadian perspective. However, although there is no doubt that melatonin restores circadian rhythm, there is a paucity of data demonstrating clinical benefits in the field of cardiovascular disease [7]. In a double-blind trial, Sheer et al. reported that repeated bedtime melatonin (2.5 mg controlled release) intake reduced BP by about 5 mmHg in male patients with untreated hypertension as

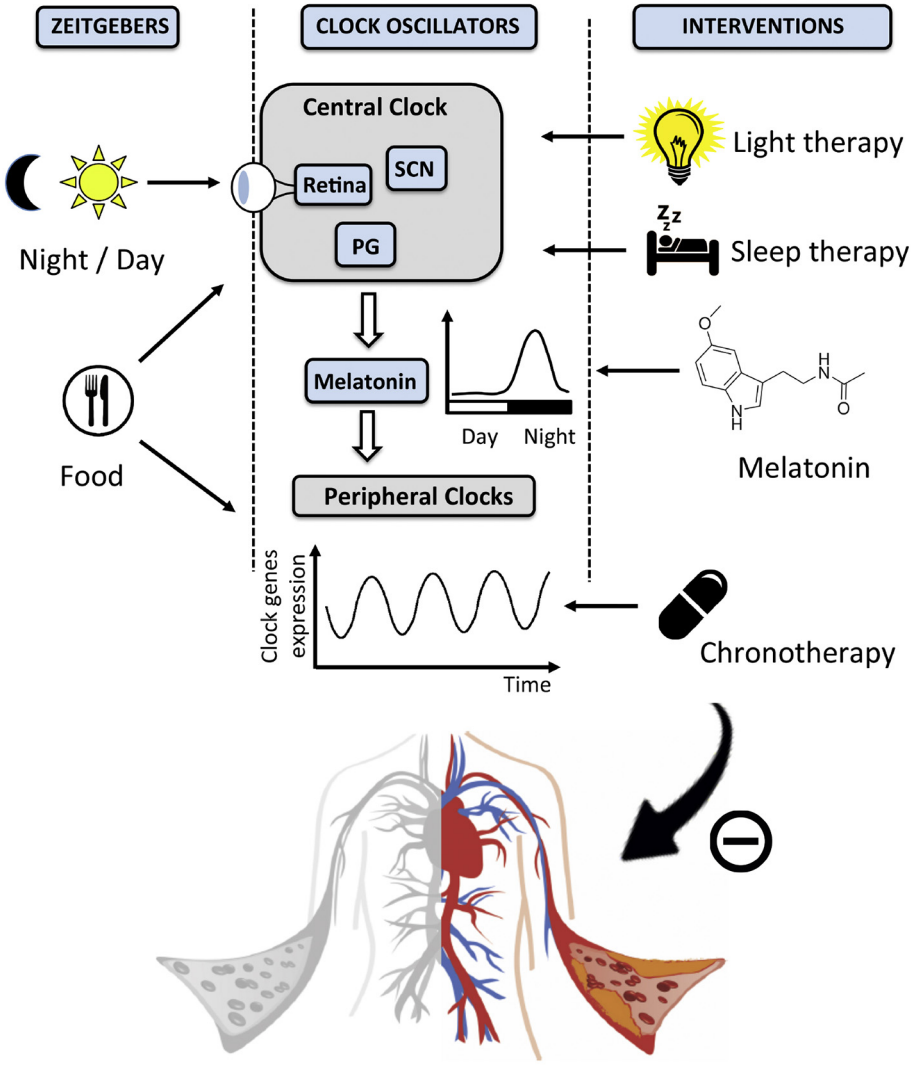

Fig. 2. Potential interventions targeting the circadian rhythm in vascular disease.
The dark/light cycle is the most important external cue (or zeitgebers) for the entrainment of circadian clocks through the stimulation/inhibition of the suprachiasmatic nucleus (SCN) which controls the production of melatonin by the pineal gland (PG). Several treatments (including light therapy, correction of sleep disorders, exogenous melatonin) targeting the clock's machinery at different levels may prevent vascular diseases by limiting disruption of molecular circadian rhythms. Taking into account chronobiology may also improve efficacy and safety of cardiovascular drugs.

compared with placebo [89]. However, long-term beneficial effects were not demonstrated. Moreover, another study showed that a combination of melatonin (2 mg) given at midnight and calcium blocker nifedipine worsened hypertension, through an unknown mechanism [90]. The most recent meta-analysis of 7 controlled clinical trials, including 221 patients, suggests that only add-on controlled release melatonin to hypertensive therapy might ameliorate nocturnal hypertension [91]. However, melatonin is still not recommended to treat hypertension and, to date no melatonin formulation is authorized by the Food and Drug Administration in the US. Only one trial in the field of cardiovascular disease is registered in ClinicalTrials. It will examine the effect of melatonin on metabolic risk, including 24-hour BP and change in endothelial dependent vasodilation (NCT02681887).

Better knowledge in the pathophysiology of the circadian rhythm lead to the discovery of inhibitors/mediators of the main component of the peripheral clocks. Thus, new drugs targeting REV-ERB and ROR nuclear receptors or CRY have been developed with potential clinical benefits [92]. For example, a recent experimental study demonstrated that SR8278, a synthetic antagonist of the nuclear heme receptor REV-ERB, increased tolerance to myocardial ischemia-reperfusion injury at the time of sleep to awake transition [93]. Such a pharmacological approach might herald a new era for the treatment or prevention of vascular diseases.

5. Conclusion

The vascular system is particularly subject to circadian influences because of its sensitivity to centrally-mediated (neurohumoral) input as well as the existence of strong peripheral clocks in most relevant cell types of the vasculature. This implies that novel therapeutic interventions can be directed at either the central clock, the peripheral clock, or a combination of both [94]. Nevertheless, many clinical observations with regard to circadian oscillations remain unexplained thus far. While physiological day-night variations in various organisms have been recognized for centuries, the exact impact of the circadian clock on human health and disease is just beginning to be discovered. Most of the physiological studies are descriptive in nature due to the inherent difficulty of consecutive sampling in a single organism and it is critical to take into account the impact of circadian rhythms into the design of experimental research [95]. However, with new molecular techniques becoming available, many discoveries will undoubtedly follow in the decades ahead of us especially from a mechanistic perspective. Novel platforms for studying the circadian clock in relevant cell types should be developed for vascular cells just as have been realized for cardiomyocytes [96,97]. Eventually, a bedside to benchside and back to bedside approach will likely result in the development of pharmacological and environmental interventions based on this exciting new topic of vascular chronotherapy.

Conflict of interest

None.

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