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RESEARCH ARTICLE

The Evolution of Human Circadian Rhythms: An Ancient Defence Mechanism Impacting Modern Life-Styles

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ABSTRACT

Circadian rhythms are a feature of almost all living cells. When isolated from external stimuli, organisms exhibit self-sustaining cycles in behaviour, physiology and metabolism, with a period of approximately 24 hours. Sunlight is used to entrain this endogenously generated rhythmicity to the earth's rotation to provide a three-dimensional perception of the world plus the fourth external dimension, time.

I hypothetically trace the evolution of the system from blue-light photoreception by the earliest marine organisms to the rise of photosynthetic bacteria creating an aerobic environment. Species now had to contend with the exogenous threat of UV radiation plus the endogenous toxic by-products of oxidative phosphorylation. Mammalian systems incorporated tools from earlier systems but refined them to the present highly integrated system of control of physiology. Cryptochrome, the blue light photoreceptor, is incorporated but photoreception now the domain of opsins in the retina. The system having central control by the suprachiasmatic nucleus within the hypothalamus, well placed to receive light information from the retina but also communicating with other brain areas and the periphery through neural and hormonal links.

This system still has relevance to us as humans within our modern environment, since a de-synchronised circadian system can contribute to a number of diseases.

Introduction

It seems fundamental that human physiology and behaviour patterns should be coordinated with environmental circadian cycles. My interest is in skin cancer and more particularly melanoma. What is the connection? The requirements of skin function vary between different times of day and seasonally. The circadian system of endogenous timekeeping, entrained by exposure to changes of light at dawn and dusk coordinates DNA repair mechanisms to particular times of day within this system which has a relationship with exposure patterns and susceptibility to UV-induced skin damage. The cell cycle is interconnected with the circadian system to protect the more sensitive S phase of DNA synthesis from maximal UV exposure. Protection of the genome is of utmost importance but the influence of circadian rhythmicity, however, extends far beyond skin and DNA protective mechanisms to include patterns of metabolism, cyclic patterns of sleep and arousal and even reproductive drive. How are all these cycles and patterns maintained and integrated? How has the system developed over the history of living organisms and evolution?

THE transcription-translation feedback loop (TTFL) mechanism

The basic model of TTFL circadian clocks consists of positive and negative elements. Positive loops activate transcription, while negative elements inhibit the positive components in a cyclic manner. In mammalian clocks, BMAL1 and CLOCK proteins are the positive elements by forming a heterodimeric transcription factor complex that promotes the expression of *cryptochrome* and *period*. After entering the nucleus, the CRYPTOCHROME-PERIOD heterodimer inhibits their own transcription by repressing the activity of the BMAL1-CLOCK complex. A drop in the level of PERIOD and CRYPTOCHROME de-represses BMAL1-CLOCK activity to initiate a new cycle.

The traditionally held view has been that circadian rhythms are controlled by this TTFL of clock genes and proteins to create a ~24-hour circuit. This view is challenged by the fact that clock genes and proteins are not conserved across the domains, Bacteria, Archaea and Eukaryote. The cyanobacterial clock is modelled around three proteins: Kai A, B and C. In the fungus *Neurospora crassa*, the proteins FREQUENCY and the WHITE COLLAR complex drive cellular rhythms. The plant TTFL also involves a different set of elements. Furthermore, even though *Drosophila* and humans possess homologous components, their functions differ. So, across phylogenetic kingdoms clock components are not shared, suggesting independent evolution among different lineages[1]. It has now also been shown that red blood cells (RBCs), which do not contain a nucleus and cannot perform transcription or translation, exhibit redox rhythms through regulation of glucose metabolism which sustains daily redox oscillations[2]. Therefore, metabolic as well as genetic cycles can sustain circadian rhythmicity. Figure 1A.

Incorporation of cryptochrome into the circadian system

Another traditionally held view was that all photo-sensory response by the eye is opsin-based, but in addition to Vitamin A-based opsin, the eye contains a Vitamin B_2-based pigment, cryptochrome, that participates in the regulation of the circadian clock[3].

Evolutionarily conserved and structurally related to photolyase, a DNA repair enzyme still used by bacteria in UVR defence, but now no longer seen in more highly evolved species such as the vertebrates. This is, however, the basis of the strong link between UVR-related DNA damage response and repair mechanisms and the circadian patterning of behaviour.

The early evolutionary, pre-Cambrian environment was low in oxygen and derived ozone, offering less protection against the shorter, more damaging wavelengths of solar radiation, so protection from UV irradiation was of paramount importance. Within the aquatic environment UV irradiation could be avoided by descent to depths in the ocean in daylight providing a more protected setting and conducive for the evolution of living organisms. Photoreceptors were critical for sensing decreasing luminesce, signalling the coming of night with a return to the surface. Blue light photoreception preferentially evolved since only blue light can penetrate to substantial depths in water. The UV component of sunlight contributed to selective pressure for evolution of this specialised photoreceptor, cryptochrome from photolyases involved in DNA repair, in early aquatic metazoans[4]. This set the stage for the evolutionary relationship between blue-light photoreceptors and circadian rhythmicity. It is also no coincidence that blue-light sits at the shorter end of the wavelength spectrum of visible light, adjacent to the UV wavelengths, so blue light is well placed to determine potential UV exposure. Terrestrial organisms have evolved under predictable daily cycles related to the earth's rotation. Organisms that anticipate this cycle have a selective advantage driving the evolution of endogenous circadian rhythms that coordinate internal physiology to external conditions.

Bacteria evolved photolyases, blue-light activated flavoproteins mediating DNA repair in a light-dependent manner. Flavin adenine

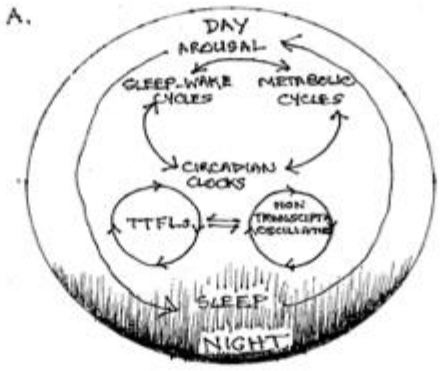
dinucleotide (FAD) as catalytic chromophore and menthenyltetrahydrofolate (MTHF) or 7,8-dimethyl-8-hydroxy-deazhariboflavin (8HDF) as the light harvesting chromophores. Light energy is harvested by the chromophore, the photolyase binding to the pyrimidine dimer and transfers an electron from the excited state of the flavin, which repairs the DNA by isomerisation to yield the two original pyrimidines[5].

Cryptochromes, blue light photoreceptor proteins present in both plants and animals, are closely related to photolyases belonging to the same family of proteins. They have FAD and MTHF as chromophores, having lost DNA repair function, but retaining the DNA binding property of photolyases[6,7]. Animal cryptochromes no longer participate in photoreception but have been co-opted as transcriptional repressors through their ability to bind to DNA allowing them to interact directly with CLOCK/BMAL1 on DNA. Cryptochromes also work outside the core clock transcription-translation loop to regulate transcription throughout the genome. They participate in additional signalling cascades forming feedback loops that initiates cross-talk between systems influencing metabolism, inflammation and DNA damage response to maintain cellular homeostasis. They possibly even function in magnetoreception[8]. The function of DNA repair has been taken over by nucleotide and base excision repair mechanisms that allow for repair of a wider range of DNA damage lesions[9,10,11].

Conservation of peroxiredoxin in circadian systems

It has, however, been found that peroxiredoxin (Prx) proteins are highly conserved and the oxidative state of these proteins exhibit circadian oscillations in cells from a range of organisms, including humans. Considering almost all organisms possess Prxs across all three phylogenetic domains, Archaea, Bacteria and Eukaryote, this metabolic marker may be a more ubiquitous timekeeper, reflecting an endogenous rhythm in the generation of reactive oxygen species (ROS)[12].

Prxs are thiol-dependent peroxidases, enzymes that break up peroxides. The substrate is typically hydrogen peroxide (H_2O_2). Thus, they catalyse the reduction of H_2O_2[13,14]. All Prxs contain a conserved cysteine (Cys) residue-peroxidatic Cys (C_p) in the NH-terminal region that serves as the site of oxidation by peroxides. 2Cys Prx contains an additional Cys residue-resolving Cys (C_r) in the COOH-terminal region of the protein. Prx's activity is dependent on the oxidation of the C_p. Cys contains a thiol side chain that participates in enzymatic reactions as a nucleophile i.e. it forms a bond with electrophiles by donating an electron pair. The catalytic Cys can become hyper-oxidised (Prx-SO_{2-3}) rendering the Prx catalytically inactive, but able to participate in ROS signalling[15,16]. Figure 1B.



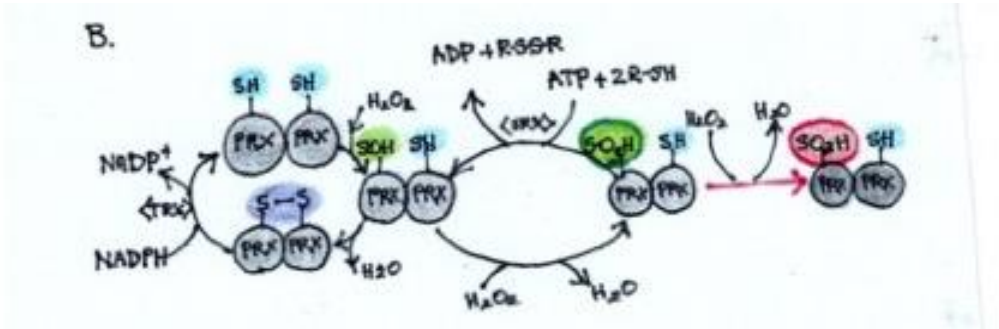


Figure 1. Eukaryotic time keeping mechanisms.
A. Cross-talk among circadian and metabolic oscillators are important components of daily time keeping mechanisms.
B. Redox cycles of Prx proteins are conserved markers of circadian clocks.
There are 2 interconnected cycles in the catalytic mechanism of 2cysteine Prx.
Peroxidation of catalytic Prx -> Cysteine-SOH (sulfenic acid) followed by disulphide bond (S-S) formation, recycling catalysed by Thioredoxin (TRX) and
further oxidation of sulfenic acid moiety to sulfinic acid (SO_2H). Sulfinic acid is recycled through a reduction reaction catalysed by sulfiredoxin (SRX) in an ATP dependent manner. The hyper-oxidation of sulfinic to sulfonic acid (Cyst-SO_3H) is irreversible.

Relationship between PRX cycles and TTFLs

A variety of chromatin re-modellers and epigenetic events are associated with the oscillatory nature of circadian transcription. This is linked to the cyclic changes in levels of specific metabolites, and thus, placing the clock at an interface between cellular metabolism and epigenetic control[17]. For example, Sirtuin 1 (SIRT1) is a NAD^+-dependent histone deacetylase (HDAC) that deacetylates the circadian transcription factors BMAL1 and PER 2, as well as histone H_3, thereby contributing to circadian gene expression. SIRT1 is also a metabolic sensor, requiring binding of its coenzyme NAD^+ for enzymatic activity. As the levels of NAD^+ oscillate over the circadian cycle the enzymatic activity of SIRT1 oscillates, which links the metabolic state of the cell with an epigenetic mechanism that depends on the circadian clock[18,19].

Studies on model organisms and their mutants suggest that PRXs are not required for oscillator function in systems that possess a TTFL and that cellular components that are required for rhythm outputs are not essential to rhythms in redox metabolism. In other words, both PRX and TTFL components of the circadian system are important, but potentially dispensable for circadian rhythms at the cellular level but cellular redox balance is important for robust clock function[20].

Evolution of clock function

About 2.5 billion years ago, photosynthetic bacteria acquired the capacity for photo-dissection of water, leading to a rapid accumulation of oxygen in the atmosphere with a catastrophic decline in anaerobic life[21]. Organisms that survived the transition to an aerobic environment were those that could metabolise oxygen and also deal with the inevitable toxic superoxide anion by-products of this metabolism. Superoxide dismutase, which converts superoxide to hydrogen peroxide, like Prx is ubiquitous in these organisms and is thought to have arisen at the same time[22]. This is about the era during which the most ancient known clock mechanism (the Kai oscillator) evolved and considering that the 24-hour cycle of Prx oxidation-reduction is represented in all domains of life, this suggests that cellular rhythms share this common molecular origin. The cellular role of Prxs is the removal of toxic metabolic by-products, namely ROS. Therefore, ability to gain a selective advantage in aerobic life depends on success in survival of episodic oxidative stress.

Rhythms of oxygen consumption and ROS generation are driven by the solar cycle and an interlinked endogenous circadian timekeeping system would confer further selective advantage with the anticipation of environmental change. Further cellular mechanisms seem to have been incorporated over time, such as post-translational modifications in eukaryotes. The most reasonable interpretation is co-evolution of the metabolic pathways and timekeeping systems.

Prx evolution

All aerobic organisms have evolved efficient defence against oxidative stress. Bacterial Prx is less sensitive to oxidative inactivation than in the eukaryote. This results in a eukaryotic Prx that is less robust but has gained a regulatory feature that facilitates peroxide signalling. The more sensitive eukaryotic 2 Cys Prxs not only act as antioxidants but also appear to regulate H_2O_2-mediated signal transduction. Though H_2O_2 is a source of oxidative stress it also acts as a secondary messenger in signal

transduction, in part, reacting with thiols on proteins involved in signalling. This adaptation allows the more evolved Prxs to act as a floodgate, keeping resting levels low, while permitting higher levels during signal transduction. So, the Prx is acting as a switch, determining whether H_2O_2 acts as a deleterious oxidant or as a beneficial signal.

In a resting state, 2cys-Prxs are present in large amounts and peroxide levels are low resulting in no signalling. A transient burst of peroxide inactivates sensitive 2 Cys-Prxs, and the peroxide is freed to act as a messenger by interacting with other proteins. At this stage the less abundant antioxidant enzymes, glutathione peroxidase and catalase mop-up peroxidase to prevent toxicity.

Wood et al were able to correlate structure with sensitivity to oxidative inactivation by demonstrating two possible conformations of PRX. A fully folded (FF) state where the C_p is active and the C_r is buried 14Å away or a locally unfolded state where the C-terminus and active site are unfolded allowing disulphide formation[15]. Figure 2.

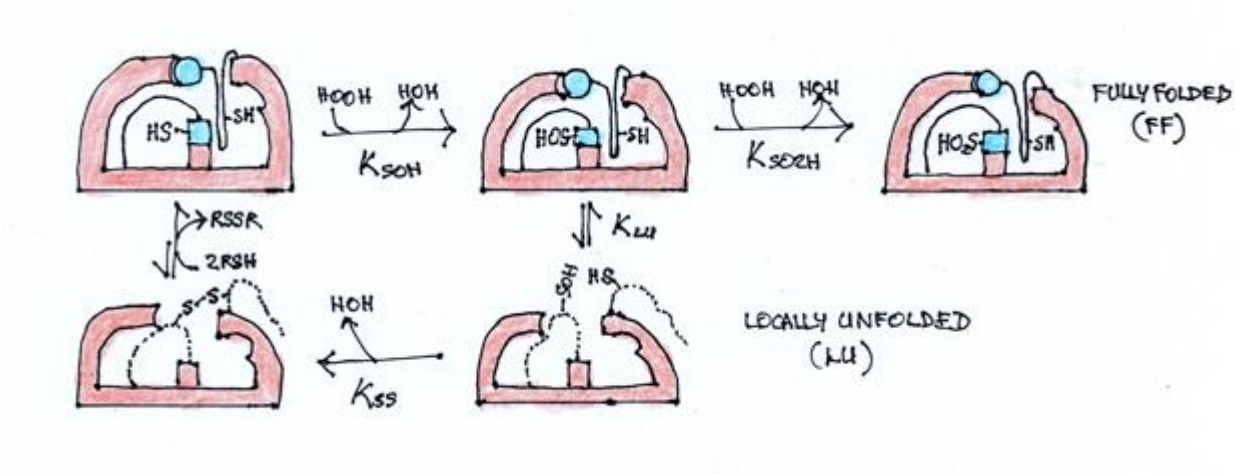


Figure 2. Model of correlation of structure and sensitivity in mammalian 2-Cys Prx.
The catalytic cycle is represented including local unfolding required for disulphide formation. Dotted lines represent chain segments in the more mobile, locally unfolded conformation. The C_p is shown in one of three possible redox states, as SH, SOH or SO_2H. The C-terminal helix present only in sensitive 2-Cys Prxs is shown as a blue circle in the FF conformation. (Modified from Wood et al 2003).

Localised regulation of H_2O_2 concentration

Cells produce H_2O_2 at various intracellular locations where it can serve as a signalling molecule. Given that peroxiredoxins are abundant and possess a structure that renders the Cys residue at the active site highly sensitive to oxidation by H_2O_2, the signalling function requires a highly localised regulation.

Receptor-mediated H_2O_2 production begins with the assembly of the NADPH oxidase (NOX) complex at discrete domains of the plasma membrane-lipid rafts-in the vicinity of the activated receptor[23]. Rhe and Kil found that Prx associated with the membrane at lipid rafts is phosphorylated at Tyr^{194} by sarcoma kinase (Src) and inactivated[24]. Active Prx is predominantly localised in the cytosol and only a small proportion is associated with the detergent resistant lipid raft[25].The localised inactivity of the raft associated Prx allows accumulation of H_2O_2 where signalling proteins are concentrated while insulating the rest of the cell from unwanted effects of the H_2O_2. Also present are activating Src kinases and inactivating Protein tyrosine phosphatases (PTPs) which promotes further phosphorylation and inactivation[26]. Amplification by these positive feedback loops allows sustained signalling by H_2O_2 necessary for regulation of biological processes[24]. Figure 3.

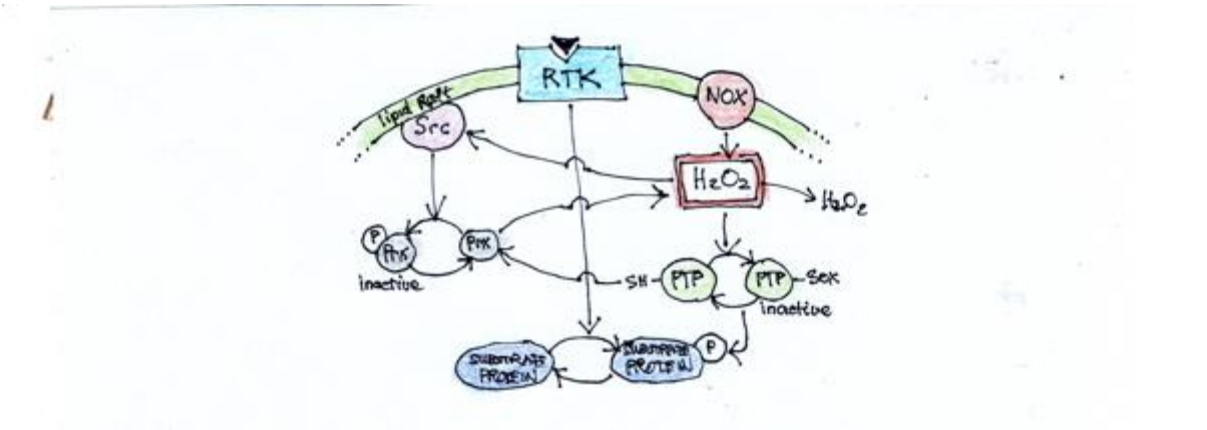


Figure 3. Model for accumulation of H_2O_2 around lipid rafts for signalling by RTK.
Accumulation of H_2O_2 for signalling by receptor tyrosine kinase (RTK) is dependent on Prx phosphorylation, protecting the H_2O_2 from reduction and thus providing a role in signalling by the RTKs. The activated RTK propagates signalling through multiple pathways. Oxidation of Cys residues on target proteins e.g. Src and PTPs, stimulation of NOX and tyrosine phosphorylation of substrate proteins. (Adapted from Rhee & Kil 2017).

Central and peripheral clocks

The Suprachiasmatic nucleus (SCN) receives photic information via the retinohypothalamic tract directly from the retina, and non-photic input from the intergeniculate leaflet and geniculo-hypothalamic tract. it acts as a central pacemaker to coordinate peripheral clocks. Light is traditionally considered the main *zeitgeber* but timing of food intake influences liver, kidney, heart and pancreatic clocks and scheduled exercise can influence skeletal muscle and lung clocks. Thus, a more complex system translates a range of signals with bidirectional communication between brain and peripheral organs adapting physiology to environment.

The SCN integrates with other brain regions. Most connections are within the medial hypothalamus- pre-optic, para-ventricular areas and dorsomedial hypothalamus, organising hormone release and autonomic control[27,28]. The SCN also innervates and receives feedback from the arcuate nucleus and lateral hypothalamus[29], and the Pineal gland. Figure 4.

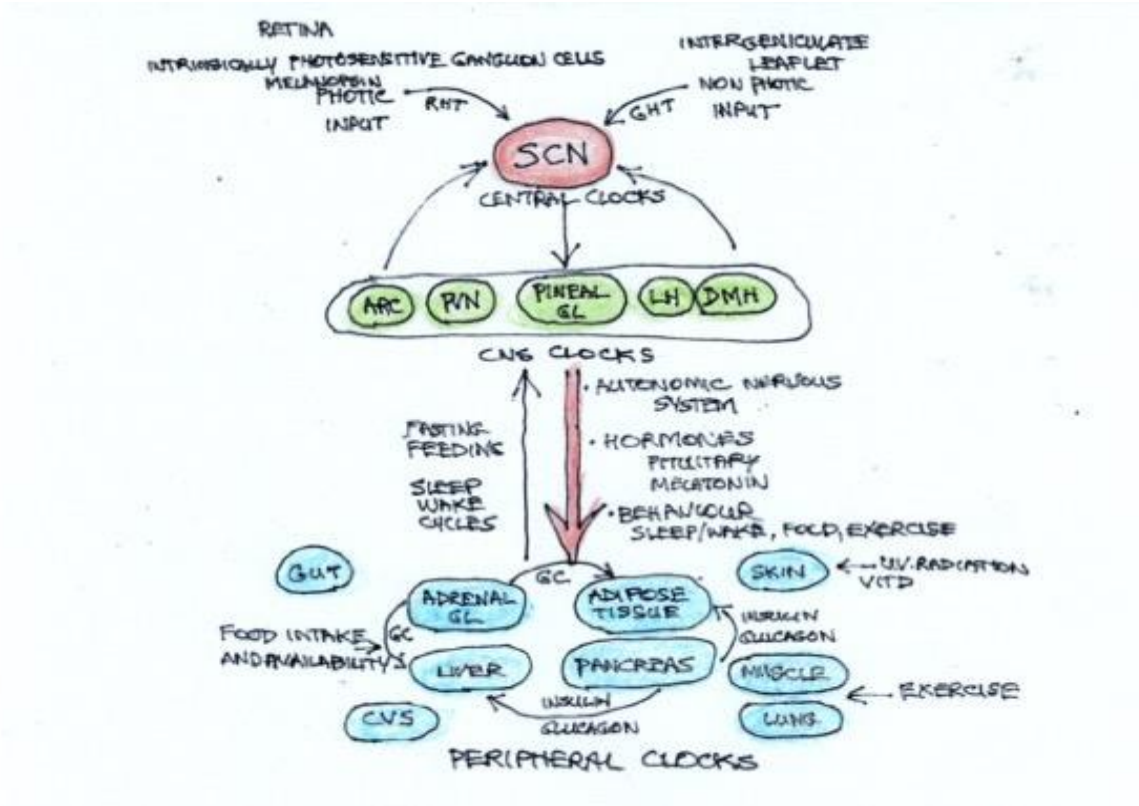


Figure 4. Cerebral anatomy and clock-clock communication.
Pathways and signals involved in clock-clock communication responsible for integration and stabilisation of biological rhythms at central and peripheral levels. TheSCN receives photic and non-photic input.The neurons in the

SCN are organised as a coupled network of neuronal connections through neurotransmitters. The temporal information is then conveyed via Vasopressin, gamma-aminobutyic acid (GABA), and diffusible signals to other clocks in the brain, such as; Arcuate nucleus (ARC), Paraventricular nucleus (PVN), Lateral hypothalamus (LH), dorsomedial hypothalamus (DMH),and Pineal gland, among others.

An integrated response is translated from the brain, through neural projections via the autonomic nervous system (ANS) and humoral signals, to peripheral tissues. Peripheral clocks receive the time information, communicate with each other, and release signals that feedback to the clock in the brain. This cooperative network between central and peripheral clocks results in the stabilisation of the rhythms that regulate tissue physiology in synchrony with external time stimuli. Glucocorticoid (GS).

Intracellular regulation of the clock

The intracellular redox state can modulate DNA binding of transcription factors to E-box promoter sequences to regulate core clock protein synthesis. For example, $NAD(P)^+/NAD(P)$ redox ratio modulating BMAL1/CLOCK dimer binding[30]. Oscillatory intracellular NAD^+ levels additionally control gene expression by activation of the deacetylase SIRT1, promoting necessary chromatin remodelling[31]. The rhythmic carbohydrate metabolism and mitochondrial oxidative phosphorylation contribute to oscillation of ROS levels with its effect on response to oxidative stress and role in signalling[32]. A key transcription factor Nuclear factor (erythroid-derived 2)-related factor 2 (NRF2) is under transcriptional control by BMAL1/CLOCK contributing to the integration of the cellular rhythmicity to the cellular redox state[33]. Other cellular metabolites, e.g., AMP/ATP ratio, have been identified as sensors of cellular metabolic status with AMP kinase leading to phosphorylation and destabilisation of CRY1. Thus, AMPK activation alters the clock phase contributing the energetic state to the local clock machinery[34]. Figure 5.

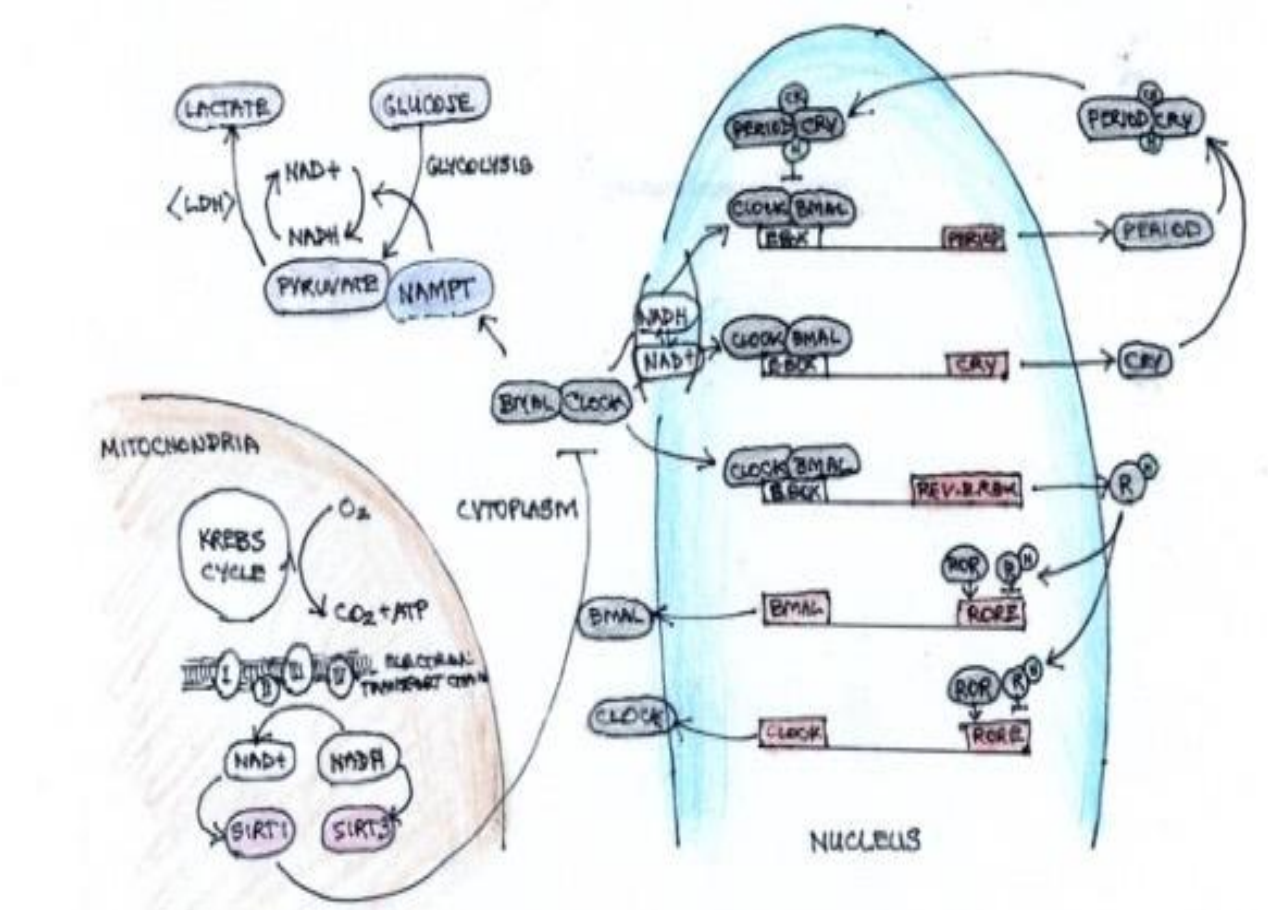


Figure 5. Cross talk between circadian and metabolic clocks.

Clock genes and proteins interact to create the transcription-translation feedback loop (TTFL) oscillator. Heterodimers of positive transcription factors CLOCK/BMAL (or CLOCK/NPAS2 in some brain regions) bind to E-box motifs in the promoters of *period, cryptochrome* (Cry) and *Rev-Erbα,*activating transcription. The protein products can undergo various post-translational modifications in the cytoplasm. PERIOD and CRY heterodimerise, translocate to the nucleus and repress the transcriptional activity by CLOCK/BMAL. An additional feedback loop involves REV-ERBα (R, nuclear heme receptor, a negative regulator) and ROR (a positive regulator), which compete for binding to and regulation of ROREs within the *bmal* promoter. It can also bind to ROREs in the *period* and *Cry* promoters regulating transcription. Elements within molecular loops are sensitive to redox state which can modulate both binding and transcriptional activity. DNA binding affinity of BMAL and CLOCK are controlled by the

NAD+/NADH ratio. NAMPT acts as the rate limiting enzyme in mammalian NAD+ biosynthesis and its expression is regulated by core clock elements[35]. NAD+ and heme (H) denote redox-sensitive nodes on clock proteins. NAD+, nicotinamide adenine dinucleotide; NMAPT, nicotinamide phosphoribosyl-transferase; NPAS2, neuronal PAS domain protein 2; ROR, retinoic acid-related orphan receptor: RORE, retinoic acid-related orphan receptor response element; SIRT1, sirtuin 1.

Clocks and disease

An intimate relationship between circadian rhythms, metabolic cascades and sleep/wake cycles and their significance in daily biological timekeeping mechanisms has been demonstrated. Misalignments between their phasing can lead to diseases, most notably metabolic disturbance but including a wide range of other disorders, including cardiovascular, psychiatric, neurodegenerative and cancers, mediated through changes in gene and protein expression, hormone levels, and neuronal firing thresholds. I have previously examined the influence of the circadian patterns on skin cancer[36] but now would like to focus on sleep patterns and metabolic disturbances. Acute in jet lag, and chronic in shift work.

Early investigation with induction of bilateral SCN lesions in rodents resulted in complete loss of rhythmic locomotor activity, drinking behaviour, food consumption, hormone release and body temperature[37,38]. Although light has traditionally been considered the main *zeitgeber*for the circadian system, timing of food intake has an effect on liver, kidney, pancreatic and cardiovascular clocks[39]. Scheduled exercise can also induce phase shifts in skeletal muscle and lung clocks[40].

Night shift workers are characterised by significantly greater postprandial glucose, insulin and triglyceride responses[41]. And several studies indicate that shift work is associated with increased incidence of metabolic syndrome, obesity and diabetes[42-44]. According to a poll by the National Sleep Foundation, the mean sleep duration of American adults was 6hrs 40 min in 2008 compared with 8 hrs 30 min in 1960[45]. Another study induced sleep deprivation, with and without circadian misalignment, the misalignment group increased insulin sensitivity twofold[46]. Circadian disruption, as seen in shift workers, negatively impacts health due to impaired glucose and lipid homeostasis, reverse melatonin and cortisol rhythms and loss of clock gene rhythmicity[47].

Conclusion

Mechanisms underlying communication within the mammalian circadian clock system are complex, highly interconnected and present at all levels, intra- and intercellular and systemic. A system that has evolved over time to allow adaption to environmental change providing the hallmark of the history's most successfully adaptive species. Homo sapiens. Modern lifestyles, however, appear to have now overridden the innate intelligence of cellular homeostatic systems, with changed patterns of behaviour associated with urban indoor living. Sunlight is replaced by artificial lighting, changing patterns of arousal and sleep cycles. Traditional 24-hour cycles of behaviour now disconnected from exposure to daylight at dawn and dusk. This has been combined with continuous access to high energy and calorific foods, potentially obtainable without any energy expenditure or any regular pattern of physical activity. It now becomes obvious why there is an explosive increase in obesity, diabetes and metabolic syndrome. Modern medicine and technology have been brought to bear to right the balance but maybe there are simpler, more basic solutions to our modern health dilemmas. Rising at dawn, eating wholesome food at regular intervals and finding some time for some regular exercise through the day. This medical challenge goes beyond metabolic disorders to include psychiatric and neurodegenerative disorders, some malignancies and even longevity.

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