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REVIEW ARTICLE

For whom the bells toll: Networked circadian clocks

G. Mark FREEMAN Jr, Alexis B. WEBB, Sungwon AN and Erik D. HERZOG
Department of Biology, Washington University, St Louis, Missouri, USA

Abstract

Circadian cycles are robust and precise biological rhythms common in unicellular and multicellular organisms. Single cells have been shown to sustain autonomous near 24-h rhythms, however, many cells and tissues appear to require cell–cell interactions to maintain periodicity. This review highlights the mechanisms and benefits of coupling circadian oscillators. We focus on how populations of circadian oscillators synchronize in a variety of biological systems and describe recent efforts to model mathematically coupling and synchrony in the mammalian suprachiasmatic nucleus. We conclude by discussing the effects of disrupted circadian coupling on health and behavior.

Key words: computational model, Period gene, suprachiasmatic nucleus, *Synechococcus,* vasoactive intestinal polypeptide.

Circadian rhythmicity, or the display of near-24 h oscillations, may be either inherent to certain cell types or may emerge through intercellular communication ("circadian coupling"). Studies in unicellular and multicellular organisms have demonstrated that some, but not all, cells are capable of self-sustained, autonomous rhythmicity. This has led to the suggestion that some cells are specialized circadian pacemakers and a much broader class of cells can be driven to express circadian oscillations under the correct conditions.

SINGLE CELLS CAN BE COMPETENT CIRCADIAN OSCILLATORS

Among prokaryotes, unicellular cyanobacteria *Synechococcus elongatus* show circadian controlled expression of most of their genome. Real-time bioluminescence recordings show that cyanobacteria can exhibit stable gene expression rhythms (Fig. 1a[1,2]). Using a bacterial luciferase reporter and a cooled CCD camera with high quantum efficiency, Mihalcescu and colleagues were able to detect light from a single cyanobacterium on the order of 10–20 photons/minute/ cell. The results indicate that the clocks in cyanobacteria are quite stable and are impervious to perturbations including cell division or the activity of neighboring cells. Remarkably, the period and cycle-to-cycle precision of the cyanobacterial oscillator can be reconstituted in a test tube by incubating three proteins (KaiA, KaiB and KaiC) with adenosine triphosphate. Under these conditions the phosphorylation state of KaiC exhibits persistent near-24 h rhythms (Fig. 1b[3]).

In multicellular organisms the strongest evidence for single-cell circadian pacemakers lies in recordings made from the retina of a marine snail, *Bulla gouldiana.*[4] Basal retinal neurons (BRN) cultured alone in a microtiter well transition to a lower membrane conductance around dawn (Fig. 1c; predawn, black trace; post-dawn, gray trace). Because single BRN do not live for many hours during this procedure, a daily rhythm in conductance was revealed by sampling different cells around the clock.

Potential pacemakers have also been identified in more complex organisms. In vertebrates rhythms in melatonin release persist in small pieces of the avian

Correspondence: Dr Erik D Herzog, Washington University, Biology Department, Box 1137, St Louis, MO 63130, USA. Email: herzog@wustl.edu

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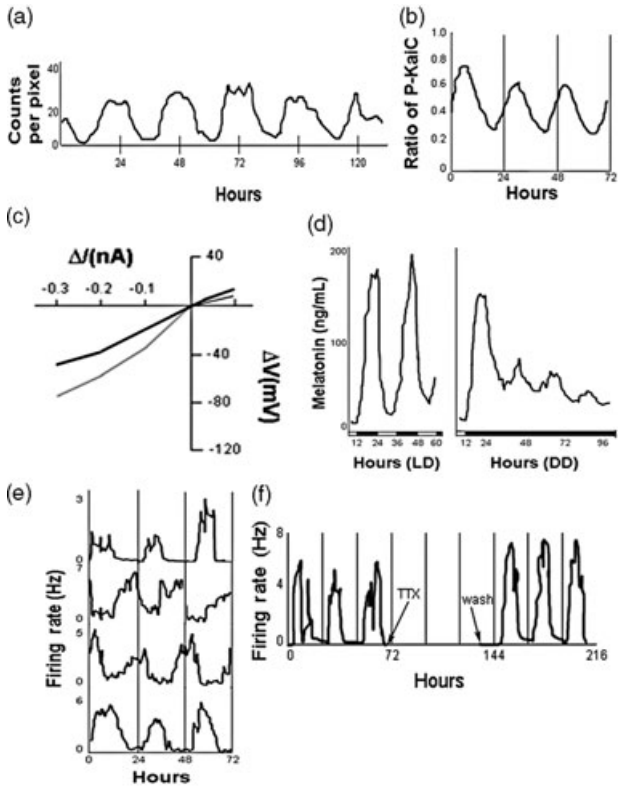

Figure 1 Examples of autonomous oscillators across phyla. Schematic trace of bioluminescence from a single cyanobacterium (a) as a function of time shows rhythmicity over several days (modified from Mihalcescu *et al.*[1]). (b) When essential protein components from the oscillator in cynobacteria (KaiA, KaiB, and KaiC) are added in a dish with ATP, the rhythm in phosphorylated KaiC (P-KaIC) compared to non-phosphorylated KaiC persists (modified from Nakajima *et al.*[3]). (c) Membrane conductance in a basal retinal neuron from the *Bulla* eye is higher predawn (black trace) than post-dawn (gray trace) as seen in the current-voltage curves (modified from Michel *et al.*[4]). (d) The dissociated avian pineal also displays rhythms in melatonin release measured at 90 min intervals from cultures perfused in a light/dark cycle (LD) or constant dim red light (DD) (modified from Takahashi *et al.*[7]). (e) Mammalian SCN neurons plated at low densities in the same culture show rhythms in firing rate with independent phases and periods (modified from Welsh *et al.*[6]). (f) In the presence of tetrodotoxin (TTX) no action potentials are recorded, though it appears after TTX is removed the oscillations continued unperturbed (modified from Welsh *et al.*[6]).

pineal.[7] The perfusate was collected every 90 min from isolated chicken pineal glands in a sterile flow-through culture apparatus. The melatonin levels in the perfusate samples were measured using radioimmunoassay with the concentration of melatonin peaking during the subjective night. The melatonin rhythms continued for at least 5 days in constant conditions, suggesting that cells in the pineal are autonomous oscillators (Fig. 1d). It is not clear which, or if all, cells are responsible for pacemaking in the pineal.

Data from mammalian cells also suggest, but do not prove, their circadian pacemaking ability. It is known that the suprachiasmatic nucleus (SCN) of the anterior hypothalamus functions as the master clock for all other peripheral oscillators in the body, coordinating daily rhythms in behavior and physiology.[8] The best evidence that SCN neurons function as cell-autonomous oscillators comes from work monitoring electrical activity from neonatal rat SCN cells dissociated on multi-electrode arrays.[6] The firing rate rhythms recorded from individual neurons plated at low density continue in constant conditions with a range of phases and periods (Fig. 1e). Furthermore, an addition of the sodium-channel blocker tetrodotoxin (TTX) for several days blocked firing and presumably cell–cell communication but it did not appear to stop the timekeeping of individual neurons (Fig. 1f). It has not been tested whether or which SCN neurons would remain rhythmic when they are completely isolated from all other cells.

FROM SINGLE OSCILLATORS TO GROUP SYNCHRONY

While it may be beneficial for individual cells to express autonomous circadian rhythms it can be advantageous for these individual oscillators to synchronize and produce a coherent population rhythm. How this synchrony occurs and is maintained within a cellular population depends upon the organism in question.

Intrinsically oscillating cells of *Arabidopsis thaliana* exhibit very weak coupling to nearby neighbors through plasmadesmata and to longer range targets via vascular bundles. Period length in constant conditions can vary greatly in different organs, ranging from 24 h in a rosette center to 35 h in a distant leaf,[9] suggesting that no coupling exists between organs. Cells in a single leaf, however, are very weakly coupled through an intact vasculature. If different regions of a leaf are phase-inverted, coincident spatiotemporal waves of gene activation will initiate in all regions of a leaf and lead to a slow resynchronization of the population.

In *S. elongans* the ability to maintain population synchrony appears to require no intercellular communication.[1] One way of showing this involved mixing two populations of bacteria that had different initial

circadian phases, and subsequently noting the lack of change in the periods or phases of the constituent cells. It would seem that, in the lab, cyanobacteria remain synchronized because each cell's clock is similar in period and precision to the rest.[2]

In contrast, the circadian clocks of many animal cells appear to be noisier.[10] Variations in period have been well documented among neurons dispersed from the rodent SCN.[6,11,12] When the periods of mouse wheel running behavior are compared to Period 1 gene expression in SCN explants and firing rates in dispersed SCN cultures, the cycle-to-cycle period variability is significantly greater in cultures of dissociated neurons than it is in intact SCN explants or behaving animals.[13] These data lead to two striking hypotheses: first, individual cells that utilize an autonomous transcription-translation rhythm generator may develop less stable rhythms when disconnected from a greater network; second, populations of cells may be able to compensate for this inherent noise by coupling to one another and may thereby produce an aggregate rhythm that is significantly less variable and potentially more robust than the rhythms of its constituent parts.

CIRCADIAN COUPLING: MECHANISMS ACROSS PHYLA

Although circadian oscillators utilize a variety of mechanisms to maintain coupling, the end result of this coupling is often the same: a more accurate and robust circadian output. Indeed, several examples, which will be discussed below highlight the interesting observation that without coordination between the oscillators, arrhythmicity in animal behavior may ensue even when all the genetic components of the molecular clock remain intact.

The intrinsically oscillatory BRN of the snail eye electrically couple to one another through gap junctions. By virtue of this mechanism, the retinal cells are able to coordinate their output to the brain and furthermore, to send a coherent signal that synchronizes daily rhythms in neurons of the contralateral retina.[14]

In the fruit fly *Drosophila melanogaster*, a circadian pacemaker in a network of approximately 100 neurons in the lateral brain drives rhythms in locomotion and photophobicity. Disrupting Period gene expression in this network disrupts the normal bouts of morning and evening locomotion.[15–18] When a small subset of lateral neurons were genetically altered to oscillate at a different circadian period, the unmanipulated neurons also changed their periods.[16,17,19] A coupling factor that coordinates some of these pacemakers is the neuropeptide, pigment dispersing factor (PDF).[20,21] Circadian neurons desynchronize from one another in the absence of PDF[18] and overexpression or loss of PDF leads to arrhythmic locomotor behavior in constant darkness.[22,23]

Coupling in the mammalian SCN is also required for stable, coherent SCN output. Yamaguchi *et al.*[24] showed that by blocking action potential-dependent activity with TTX, the Period1::luciferase rhythms of individual SCN neurons desynchronized and the ensemble rhythm damped. Critically, the rhythm amplitudes of the desynchronized cells were also dramatically lower than in the intact SCN, suggesting that coupling may regulate the amplitude as well as the phase of gene expression rhythms. Four signaling pathways have been implicated in circadian synchrony in the SCN.

Vasoactive intestinal polypeptide (VIP)

VIP may be one of the most potent coupling agents discovered in the SCN.[25] It is expressed predominately in ventrolateral (VL) neurons which account for approximately 15% of the 20 000 neurons in the SCN.[26] Several reports confirm that VIPergic neurons send dense projections throughout the SCN[26–29] and its release has been shown to be rhythmic in rat SCN slice preparations.[30] The VIP receptor (VPAC2) is expressed in approximately 60% of SCN neurons. Many of these neurons express arginine vasopressin or VIP,[27] implying that intercellular VIP signaling may be important for modulating subsequent neuropeptide release. Importantly, mice which lack VIP or its receptor exhibit disrupted locomotor activity with multiple circadian bouts of activity in constant darkness.[25,31–34] At the cellular level, deletion of the VIP receptor or VIP results in the low or arrhythmic expression of the canonical clock genes mPer1, mPer2, mCry1 and mBmal1 when measured at the level of the SCN tissue,[28,31,35] as well as a loss of circadian synchrony in firing rate[25,34] and Per1 expression among individual SCN cells.[35] Intriguingly, loss of synchrony was again associated with fewer rhythmic cells. These last studies support the hypothesis that intercellular VIP signaling is crucial to maintaining rhythmicity in single neurons as well as synchrony among rhythmic SCN populations. It remains unknown how VIP mediates circadian synchrony and changes gene expression in the SCN.

Electrical synapses

Electrical coupling through gap junctions may also promote neuronal synchronization independent of chemical synaptic transmission and voltage gated sodium channels.[36,37] Various studies utilized the intracellular diffusion of specific dyes to suggest that SCN neurons are coupled via electrical synapses.[38–40] Using whole cell recording techniques, Long and his colleagues reported electrical coupling between pairs of SCN neurons in rat and mouse SCN slices.[41] They also showed that spike-for-spike synchrony between cells disappeared in the SCN of mice which lack connexin 36, a molecule necessary to form certain types of gap junctions. It is unclear, however, if gap junctions are prevalent or important to circadian synchrony. Recent immunogold labeling reported that connexin 36 is present, but is not associated with strong electrical coupling in the SCN.[42] The authors concluded that limited SCN neurons may express connexin 36 to form miniature gap junctions that produce weak coupling and little spike-for-spike synchrony. It would be interesting to know if the circadian synchrony of the firing rate or Period gene expression is disrupted in the connexin 36 knockout mice.

GABA

Given that most SCN neurons express the inhibitory neurotransmitter GABA, GABA signaling has long been expected to be a potent coupling agent in the SCN. Two GABA receptors, $GABA_A$ and $GABA_B$, are expressed in the SCN. A single exogenous GABA application to *in vitro* slice preparations phase shifts firing rhythms of single SCN neurons, and a daily application synchronizes circadian firing among SCN neurons.[43] Albus *et al.*[44] found that delaying light onset by 6 h in a light–dark cycle causes the rhythms of the ventral and dorsal SCN to acutely dissociate. This bimodal pattern in firing can be maintained by either physically separating the ventral and dorsal SCN or by treating the intact SCN with bicuculline, a $GABA_A$ receptor blocker. GABA thus appears necessary to resynchronize the ventral and dorsal SCN after a phase shift,[44] either through a direct mechanism or by modulating the effects of VIP. Evidence for this latter hypothesis comes from two observations: VIP modulates GABA-induced inhibitory currents in the SCN and is required for the circadian rhythm in their frequency,[45] and VIP synchronizes SCN neurons even during the blockade of GABA signaling.[46] Future studies are required to fully dissociate the effects of GABA and VIP. A first step should be to determine whether the phase-resetting effects of GABA are mediated by VIP.

PSA-NCAM

Polysialic acid (PSA)-attached neural cell adhesion molecule (NCAM) negatively regulates cellular interactions.[47] PSA is rhythmically expressed in cultured SCN[48] and in the SCN *in vivo* during light–dark cycles and constant darkness.[49] The genetic deletion of NCAM leads to a gradual loss of locomotor rhythmicity in constant darkness. One possible role of the PSA–NCAM complex in the coupling of SCN neurons is that it alters synaptic or neuronal-glial connections in the SCN. An analysis of the bioluminescence signal of single cells in the PSA–NCAM null mice crossed with PER2::LUC transgenic mice would help to clarify the precise role of this extracellular polymer.

COMPUTATIONAL MODELING HIGHLIGHTS GAPS IN OUR UNDERSTANDING OF SCN COUPLING

The mechanisms by which heterogeneous SCN neurons couple and synchronize have become a topic of great interest to computational modelers. Modeling offers the ability to quantify abstract parameters such as coupling and synchrony, to monitor changes in clock gene transcription and translation in single cells and to render testable hypotheses for experimental investigation. For instance, Gonze *et al.*[50] investigated critical factors related to generating synchrony among neurons with different periods (Fig. 2a). They generated a model system of 10 000 globally connected oscillators that had periods ranging from 20 to 27 h. Each cell released a coupling agent whose production was tied to clock gene transcription. Because all the cells were universally coupled, the total concentration of the coupling agent was equal to the sum of each individual cell's production. In this way, a synchronously oscillating population produced a high amplitude oscillation in the coupling agent. Likewise, when the population became perfectly desynchronized, the mean concentration of this agent damped to a steady state level. Based on this model, the authors found that the coupled population could synchronize rapidly and each individual cell would begin to oscillate with a period of 26.5 h. The authors subsequently modeled the effects of a light–dark cycle and noted that the entire population entrained and

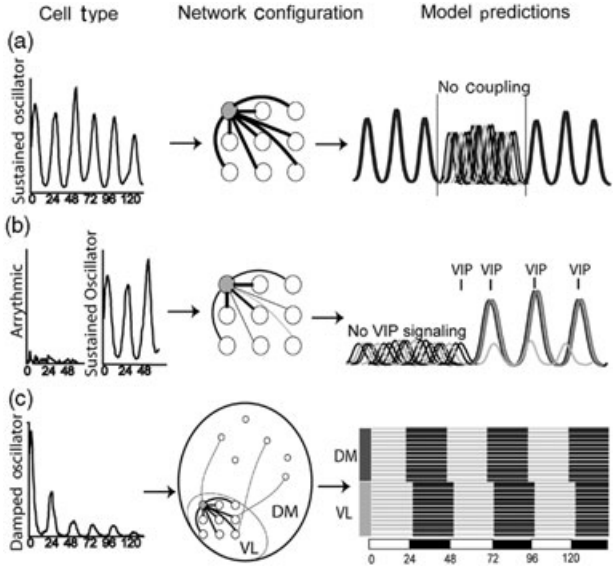

Figure 2 Computational models of the suprachiasmatic nucleus (SCN). Gonze *et al.*[50] (a) modeled a population of sustained oscillators which exhibited 'all-to-all' coupling. The synchrony of the population's oscillations was dependent on a mean-field coupling mechanism. To *et al.*[51] (b) modeled the SCN as a population of sustained oscillators and a population of arrhythmic cells that all produce and respond to vasoactive intestinal polypeptide (VIP). VIP signaling between model neurons decreased as a function of distance. The model predicts that daily VIP release is necessary and sufficient to induce rhythmicity and synchrony in a population of SCN neurons. Bernard *et al*[52] (c) assumed that the SCN comprises a population of damped oscillators which exhibit nearest neighbor coupling in the ventrolateral (VL) SCN, but also random coupling between oscillators in the VL SCN and dorsomedial (DM) SCN. The simulation predicted that coupling leads to synchrony in a light–dark cycle, with the DM cells exhibiting rhythms that are slightly phase-advanced with respect to the VL oscillators.

synchronized to a period of exactly 24 h. Based on this modeling, they found that synchrony is driven by the mean field oscillation of the entire population. They further predicted that individual cells must act as damped oscillators in the presence of high levels of coupling in order to achieve synchrony. While this study did not explicitly identify a biological coupling factor that would exhibit these properties, another recent study has modeled the effects of VIP on coupling and gene expression in the SCN.[51]

The VIP-coupling model created a population of 400 cells whose strength of coupling to their nearest neighbors declined with distance (Fig. 2b[51]). Each cell was able to rhythmically produce the coupling factor VIP and respond to VIP through constitutive VPAC2 expression. VPAC2 receptor activation initiated a signaling cascade leading to increased Per transcription. Notably, the authors incorporated cellular heterogeneity, reflecting experimental findings that only 30% of SCN neurons are intrinsically rhythmic in the absence of VIP signaling.[25] Forty percent of neurons were modeled as sustained oscillators while 60% were initially arrhythmic. The onset of VIP coupling synchronized the population and caused 90% of the neurons to rhythmically express Per within 3 days. Interestingly, *in silico* experiments showed that a three-hour pulse of a VPAC2 agonist increased the amplitude of single cells as well as the overall synchrony of the population in VIP-/– neurons, while constant agonist application increased Period gene expression in single oscillators but desynchronized the population. The authors hypothesized that synchrony depends on both stochastic (cell-to-cell variability) and deterministic factors such as network architecture.

A third recent article has attempted to model SCN network architecture more realistically and combines aspects of the two previous models.[52] Specifically, Bernard *et al.* created a compartmentalized model of damped oscillators where neurons in the putative VL region globally coupled, but also sent random projections into the dorsomedial (DM) SCN to entrain DM cells (Fig. 2c). The initial period distribution of the cells ranged from 20 to 28 h; however, the periods of DM cells were set slightly shorter than those of the VL neurons to be consistent with experimental results.[53] The results from this model indicated that coupling is necessary for both robust population synchrony and individual cell rhythmicity. Furthermore, the authors showed that in this model of damped oscillators individual neurons will damp and cease to oscillate if the phase of their oscillations diverges significantly from the phase of the overall population. In a 12 h : 12 h light–dark paradigm, the model interestingly predicted that coupled cells will synchronize and entrain to the light–dark schedule and that neurons in the DM compartment will oscillate with a slightly advanced phase compared to VL neurons. Additionally, VL neurons will resynchronize in only two days after a 12 h phase shift while DM neurons require 10 days to fully resynchronize.

Although each model begins with different initial conditions and network architectures they all confirm two observations: first, coupling significantly reduces the period variability across cellular oscillators and

second, achieving synchrony is dependent on the intrinsic properties of individual cells in addition to intercellular signaling. Additionally, the models make contradictory predictions: To *et al*. predict that constant release of a coupling agent will desynchronize a population but increase Period amplitudes in individual cells, whereas Gonze *et al*. predict that constant, high neurotransmitter release dampens the amplitudes of individual oscillators. This conundrum would be testable by monitoring single cell Period::luciferase rhythms during chronic VIP administration.

SYNCHRONIZING A SYSTEM: CIRCADIAN COUPLING AT THE ORGANISMAL LEVEL

Arousal, body temperature and blood levels of glucose and almost all hormones exhibit diurnal rhythms in mammals. Since ablation of the SCN abolishes their circadian rhythmicity,[8,54,55] these rhythms depend on the proper functioning of the SCN. Interestingly, at least some of these peripheral rhythms may feed back on the SCN. In humans it has been proposed that a 'symphony of oscillators' interact with the SCN to govern sleep, wakefulness, body temperature and cognitive performance.[56,57] This concept of peripheral modulation of SCN function becomes even more intriguing given the exciting discovery that clock genes are rhythmically expressed not only in the master circadian clock, but also throughout the body.

In *Drosophila,* studies have recently shown that the period gene is expressed in tissues including the antennae, cardia, gut, wings and reproductive organs.[58] Likewise, experiments in mice have elucidated an array of tissues and cell types including the liver, spleen, skeletal muscle and sperm that express the three period genes.[59–61] Some peripheral oscillators such as the olfactory bulb will continue to oscillate synchronously when decoupled from the SCN,[62] and they are even able to feed back onto the SCN and alter its gene expression.[63] In other tissues such as the liver, synchronous rhythmicity will cease in the absence of SCN coupling, even though individual hepatocytes will continue to oscillate in a state of desynchrony.[64,65]

DESYNCHRONY AND ITS EFFECTS ON HUMAN HEALTH

Desynchrony at the level of the cell and the organism has become a topic of special interest in recent years, given findings that relate circadian dysfunction to human disease. For example, mice with a mutation in the *Clock* gene not only exhibit altered behavior in running wheels, but are also susceptible to obesity, metabolic syndrome and mania-like behavior.[66,67] In humans circadian rhythm disorders have been linked to manic-depressive behavior, and forced bed-rest has been used to treat patients with this disorder who prove refractory to standard pharmacological therapy.[68–70]

Interestingly, the International Agency for Research on Cancer, an arm of the World Health Organization, has recently categorized 'shift work that involves circadian disruption' as a probable human carcinogen.[71] This specific announcement came after a review of several epidemiological studies, including three reports that noted that nurses who engaged in shift work at night had a modestly increased risk of breast and endometrial cancer, compared with those who did not engage in night shift work.[72–74] The report also highlighted evidence for the link between circadian dysfunction and disorders of cell proliferation. Specifically, inactivation of the circadian *Period2* gene promotes tumor development in mice, and expression of the *Period* genes is inhibited in human breast and endometrial tumors.[75,76]

It is now clear that dysfunction of the circadian system can have potent effects on human health and can be strongly linked to ailments as diverse as mental disease and tumorigenesis. The future challenge to circadian biologists and medical researchers will be to understand how the circadian system modulates these disease processes and how other aspects of human health and disease are affected by the circadian clock.

FINAL THOUGHTS: THE IMPORTANCE OF COUPLED CLOCKS

Circadian rhythmicity is robust and common across phyla. At the microscopic level autonomously rhythmic bacteria oscillate in unison, coupling critical functions such as metabolism and cell division to the daily rhythms of the outside world. In higher organisms synchrony among circadian oscillators allows sun compass navigation, photoperiodic responses and daily sequencing of physiological functions. The importance of coupling between oscillators becomes especially apparent when the mechanisms of coupling break down. Disruptions in coupling by mechanisms as diverse as genetic perturbation or trans-meridian airline travel can have striking effects on sleep, wakefulness, memory, metabolism and behavior. In the future it will be the task of

scientists and physicians to better understand how complex networks of cells and organs interact on a circadian timescale to promote synchrony and homeostasis in the organism.

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