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Circadian clocks, brain function, and development

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Circadian clocks are temporal interfaces that organize biological systems and behavior to dynamic external environments. Components of the molecular clock are expressed throughout the brain and are centrally poised to play an important role in brain function. This paper focuses on key issues concerning the relationship among circadian clocks, brain function, and development, and discusses three topic areas: (1) sleep and its relationship to the circadian system; (2) systems development and psychopathology (spanning the prenatal period through late life); and (3) circadian factors and their application to neuropsychiatric disorders. We also explore circadian genetics and psychopathology and the selective pressures on the evolution of clocks. Last, a lively debate is presented on whether circadian factors are central to mood disorders. Emerging from research on circadian rhythms is a model of the interaction among genes, sleep, and the environment that converges on the circadian clock to influence susceptibility to developing psychopathology. This model may lend insight into effective treatments for mood disorders and inform development of new interventions.

Keywords: circadian rhythms; sleep; psychiatry; mood disorders; depression; bipolar disorder; clock; development

Introduction

The first International Scientific Group of Circadian Rhythm Experts (INSPIRE) conference, held March 14–16, 2013 in Viareggio, Italy, and attended by 30 senior scientists from around the world, covered the spectrum of research on circadian rhythms from basic circadian research through translational science to clinical studies, with each scientist accompanied by a junior colleague. The conference provided a rich opportunity for considering the role of circadian factors in health and disease, particularly mental disorders thought to be linked to circadian and sleep–wake regulation. This report summarizes the varied presentations and, in closing, attempts to capture a sense of the lively interchange that occurred among this varied group of scientists.

How circadian genetics might be linked to resilience versus psychopathology

Overview

In the keynote address, Joseph Takahashi (University of Texas Southwestern Medical Center) provided a thought-provoking opening plenary, discussing the role of circadian genetics in health and disease. As an initial caveat, Takahashi pointed out that, by strict standards, little evidence exists linking circadian genes to psychopathology. Thus, he redefined the question to "how circadian clocks contribute to resiliency and psychopathology." Following a brief overview of the history of circadian genetics, a review of new technologies and recent findings highlighted the rapid advances being made in our understanding of the dynamic mechanisms and gene network complexity that is at the core of the

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circadian clock system. Takahashi discussed how these findings hint at a system that is inherently complex yet flexible in its capacity to respond to diverse environmental and genetic perturbations. Such flexibility is a fundamental property of many complex biological systems and confers a degree of robustness/resilience to the system in the face of challenge.[1] Takahashi then discussed how the circadian system fulfills the criteria of a robust system. In doing so, he offered a tantalizing answer to the overarching question of the meeting: What makes a good clock?

New insights into circadian clock mechanisms

The expanding clock gene network. The mouse has provided an invaluable tool in the search for the genetic basis of the mammalian circadian clock. Prompted by the discovery of the *tau* mutation in hamsters that shortened circadian period,[2] the initial foray into the genetics of the mammalian clock began with the search for mutants rather than genes. Quite serendipitously, the first genetic screen yielded a mutant mouse that contained a semi-dominant mutation producing a longer circadian period. With the advent of the human genome project in the early 1990s and positional cloning technology, the gene containing this mutation was identified and mapped to the resulting protein aptly named CLOCK (circadian locomotor output cycles kaput).[3–5] This protein is a core component of the cell-autonomous and autoregulatory transcriptional feedback loop of the mammalian circadian clock (Fig. 1). CLOCK forms a transcription complex with brain and muscle ARNT-like protein 1 (BMAL1) and interacts

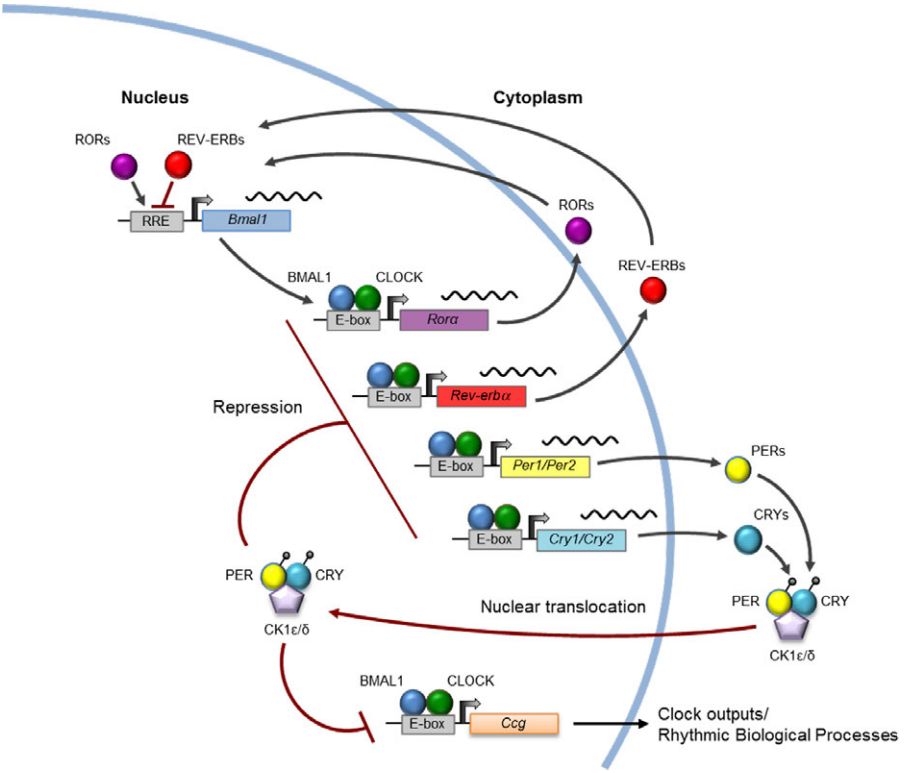

Figure 1. The expanding clock gene network. The mammalian circadian clock consists of a cell-autonomous and auto-regulatory transcription–translational feedback loop (TTFL), whereby the transcriptional complex, CLOCK:BMAL1, enhances *Period (Per)* and *Cryptochrome* (*Cry*) transcription. The resulting PER and CRY proteins accumulate during the day and translocate back to the nucleus in the evening to repress their own transcription through direct physical interaction with CLOCK and BMAL1—a process that takes approximately 24 h to complete. In addition to this core feedback loop, a second important feedback loop involving the nuclear receptors REV-ERBs and retinoic acid–related orphan receptors (RORs) have been discovered, which regulate BMAL1 transcription in an anti-phase cycle.[6] This new feedback loop plays a central role in the amplitude and robustness of the entire network. Ccg = clock-controlled genes; CK = casein kinase. Adapted from Mohawk *et al.*[139]

with enhancer box (E-box) regulatory elements in the *Period (Per)* and *Cryptochrome* (*Cry*) genes to activate their transcription. Upon production, the negative elements, PER and CRY proteins, accumulate during the day, dimerize, and translocate to the nucleus in the evening where they repress their own transcription through direct physical interaction with CLOCK and BMAL1. As the negative elements turn over late at night, repression of CLOCK and BMAL1 is removed, thus beginning a new cycle of transcription the following morning—a process that takes approximately 24 h to complete. In addition to this core feedback loop, a more recent view of the clock gene network includes a second important feedback loop involving the nuclear receptors REV-ERBs and retinoic acid–related orphan receptors (RORs), which activate and repress the transcription of BMAL1 in an anti-phase transcription cycle.[6] Recent work has suggested that double knockout of these two nuclear receptors in mice severely compromises the amplitude of the oscillation, shortens the period, and reduces stability of the rhythm. These findings highlight the central importance of this new feedback loop to amplitude and robustness of the entire network.

The circadian transcriptional architecture and landscape. Various physiological processes with vast clinical impact are under circadian control, yet how the molecular components of the clock translate their own inherent cycling to these downstream processes remains unknown.[7] With the advent of next-generation sequencing technology, Takahashi discussed the utility and power of chromatin-immunoprecipitation sequencing (ChIP-seq) and transcriptome sequencing (RNA-seq) technology in contributing to the progression of circadian biology research. Examples were drawn from a recent paper by Koike *et al.*[8] that elegantly demonstrated the use of these tools to uncover new mechanisms by which the mammalian circadian clock regulates genome-wide transcription (Fig. 2).

The circadian clock in health and disease

Robustness as a property of complex biological systems. From the expanded gene network to the dynamic landscape of the core circadian clock, these recent findings highlight the intricacy and genetic complexity inherent in the basic design of the circadian system. Such complexity is not unique to the circadian clock but is found in multiple biological systems that are considered robust (i.e., robustness can be defined as a relative insensitivity to perturbations whereby a system maintains function and is able to adapt to diverse external and internal changes).[9] Indeed, many of the design principles that have been described for other robust biological systems are present in the mammalian circadian system.[9] These general principles include (1) redundancy—genetic redundancy is apparent in the genetic orthologs that exist within the circadian gene network; (2) feedback control—negative feedback is the operating principle of the circadian clock and there exist multiple pathways within the gene network that are difficult to understand on the basis of a simple linear model; (3) modularity—the circadian gene network consists of a core clock in the middle of a larger network that interacts with other modules; and (4) hierarchy and protocols—the suprachiasmatic nucleus (SCN) is considered the master pacemaker, with protocols and rules for how the SCN communicates with peripheral oscillators.

How the SCN achieves robustness: coupling of neural activity. Although robustness is recognized as a fundamental property of complex living systems, it can be difficult to determine how this property is accomplished at the cellular and molecular level.[10] One way to study this is to use genetic perturbation on a single-cell level to determine its effects on molecular rhythms as detected by bioluminescence imaging of *Per2* gene expression.[11] Genetic knockout studies have revealed that mammalian SCN slice cultures maintain rhythmicity when *Per1* and *Cry1* are knocked out but that dissociated SCN neurons become completely arrhythmic under the same knockout conditions.[12] This suggests that the SCN compensates for single-cell genetic perturbations by acting as a coupled and collective network of oscillating cells rather than as a single cell-autonomous unit. Quite simply, SCN coupling can provide robustness against cell-autonomous genetic perturbation and is critical for synchronization of neural activity to maintain intercellular oscillations.[12]

What makes a good clock?

The fundamental network nature of genetic interactions is their capacity for flexibility.[1] Flexibility is woven into the network architecture of complex systems and confers resilience or robustness in the

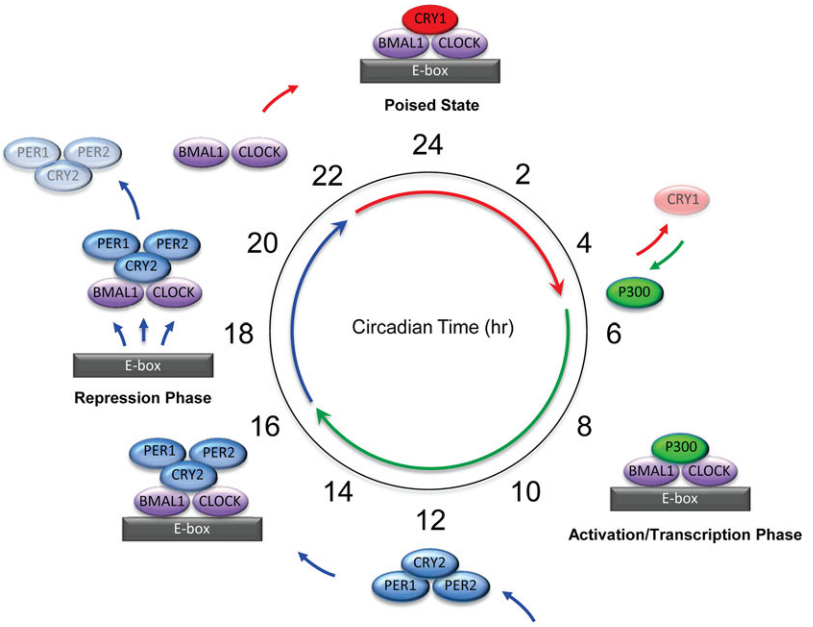

Figure 2. The circadian transcriptional architecture and landscape. New technologies are providing insight into how the circadian clock imparts oscillations in transcriptional activity on a genome-wide scale. A three-state model has been proposed for the circadian transcriptional cycle of the clock on the basis of the collective findings of Koike *et al.*,[8] using ChIP-seq and RNA-seq data: a poised state in which CLOCK and BMAL1 are bound but transcriptionally silent and repressed by CRY1 binding at the same regulatory sequences. A second state, where a transition to activation occurs as CRY1 levels diminish later in the morning and co-activator occupancy (P300) and marks of active chromatin increase. Transcription follows from this and peaks at CT15. This is followed by the third repressor phase where repressors (PER1, PER2, and CRY2) bind and transcription wanes, while CRY1 retains binding to repress the start of the next cycle. A major point to emphasize is that rhythms in gene expression are heavily generated by posttranscriptional regulatory mechanisms, rather than by RNA cycling per se.

face of challenge. As a simple example, perturbations such as genetic knockouts do not always yield an overt phenotype as a result of network compensation for this loss of function. Indeed, the circadian clock is inherently flexible in its ability to adapt to photoperiodic changes in the environment, and this flexibility constitutes one of the main design principles of the circadian clock. Being both robust yet flexible is a difficult feat for any system to achieve, and the circadian system must carefully navigate between achieving too little (elasticity) or too much (rigidity) robustness.

A good clock, therefore, is one that strikes a fine balance in the battle to achieve a "healthy" degree of robustness. The inability to achieve this balance in a dynamic and ever-changing environment has vast clinical implications for disease. If one considers that robustness is to biology what resilience is to psychiatry, then understanding robustness in the circadian system within a physiological context might inform our understanding of resilience in many other contexts. Ultimately, understanding the underlying mechanisms and properties that confer robustness on a cellular and systems level may lend insight into possible new therapeutic avenues for directed and targeted treatment of diseases linked to the circadian clock.

Selective pressures on the evolution of clocks

The first INSPIRE conference plenary lecture was given by Carl H. Johnson (Vanderbilt University) and covered selective pressures on the evolution of clocks. There are four fundamental properties of daily biological clocks. To be considered a true clock, the clock should (1) be self-sustained (i.e., keeps track of time even in constant conditions); (2) be entrainable (i.e., attains a period that equals that of the environment); (3) be temperature compensated (i.e., free-running period is the same at different constant temperatures); and (4) express conditionality (i.e., clock only runs under certain

environmental conditions). Together, these four properties have the adaptive values of creating a system that works as a "daily program," a continually consulted "clock," and a "stopwatch" that measures day/night length. For both program and clock-consultation features, the key element presumably selected by evolution is environment/season–appropriate phasing (phase angle) of biological processes.

Natural selection has affected each of these properties in different ways, which are discussed in the following paragraphs. The first property (self-sustained oscillatory function) was selected to be a sustained oscillator that persists for many cycles (one cycle is not sufficient to define an oscillator). Why was an oscillator selected? As described by Roenneberg and Merrow:[13] "Evolution has shaped circadian clocks in a cyclic world; temporal constancy of environmental qualities must have been an extremely rare exception. It is therefore the mechanism of entrainment that has evolved and not sustained rhythmicity in constant conditions. The latter is theoretically not even essential for a functional entrained circadian system. If free-running rhythms can be recorded under special constant conditions . . . this must reflect how the system evolved to function optimally under entrainment rather than being the object of the evolutionary process." In order to test whether selection of a sustainable oscillator improves fitness experimentally, Carl Johnson's laboratory used an assay of fitness with wild-type (WT) unicellular *Synechococcus elongatus* cyanobacteria, which were co-cultured (competing for resources) with mutant strains. The results indicated that mutants with either a damped or arrhythmic oscillator do not outcompete WT when the cultures were maintained in constant light; however, both mutant strains lost to WT when the cultures were maintained in a 12h:12h light:dark (LD) cycle.[14] Another study showed that who the winners were of a competition between WT and mutant strains with functional clocks of various period lengths (22 to 30 h) depended on the length of the environmental lighting cycle. For example, long-period mutants outcompeted WT strains when kept on a 30-h day (LD 15:15), and short-period mutants outcompeted WT strains when kept on a short 22-h day (LD 11:11).[15] Altogether, these results show that having a clock is beneficial in rhythmic environments because a clock with a free-running period that is resonant/consonant with that of the environmental cycle enhances fitness (i.e., a key factor is the phase angle).

The second property of a true clock is entrainment. Some studies have used a modeling approach to provide insight into the evolution of entrainment.[13,16] In 1965, Colin Pittendrigh proposed the "escape from light" hypothesis that circadian clocks evolved as a result of a selective pressure generated by daily cycles of light and darkness in which the light was deleterious to the optimal growth of the organism. The hypothesis postulates that light (both ultraviolet (UV) and visible light) can have deleterious effects on the genetics and biochemistry of cells and that organisms might respond to this photon bombardment by evolving (1) screening pigments, (2) colorless cellular components, and (3) a timing system to temporally segregate light-sensitive reactions to the nighttime when they will not be inhibited. The importance of environmental light to health is underscored by the observation that the growth of yeast and *Escherichia coli* is light sensitive. In fact, visible light (especially blue/green wavelengths) affects period and amplitude of the yeast respiratory ultradian oscillator (period is approximately 4–5 h; Robertson & Johnson, unpublished observations). Other organisms (e.g., chlamydomonas) are sensitive to UV light in a manner that is specific to the time of day.[17] The "escape from light" hypothesis would predict that organisms would have UV-sensitive processes confined to nighttime. Experimental evidence shows that UV light exposure at the end of the day and beginning of the night results in the fewest number of surviving colonies.[17]

Temperature compensation is a third requirement of a true circadian clock. It is important to note that compensation does not imply complete temperature independence because temperature can reset the clock. Rather, compensation means that the free-running period remains the same at different constant temperatures. Perhaps in the quest of phase angle conservation, there needs to be a counterbalancing of temperature effects between discrete/non-parametric/phase resetting and continuous/parametric/angular velocity. Consider, for example, if angular velocity (or free-running period) were *not* temperature compensated. In environments with a consistent daily temperature cycle, the clock phase relationship to the light–dark cycle

(phase angle) would remain consistent. However, in environments where the daily temperature fluctuates with warm and cooler temperature cycles, the phase angle would vary from day to day and within a day.[18] This biochemistry is difficult to explain when the free-running period at different constant temperatures is compensated but the change in phase angle in response to temperature changes is not. One possible explanation would be a system that contains a thermoreceptor (analogous to a photoreceptor), which is separate from the actual oscillator that senses temperature changes. However, this explanation fails to explain the temperature compensation of the simple *in vitro* KaiABC cyanobacterial clock that still shows phase-altering responses to temperature pulses.[19,20] It is amazing that the adaptive value of temperature compensation has never been rigorously demonstrated.

Finally, a true circadian clock must show conditionality in that the clock exhibits robust expression under certain conditions. Two examples are illumination and temperature. Specifically, constant darkness produces the most robust clock expression for nocturnal animals, while constant light produces the most robust free-running clock expression for diurnal animals and plants. In fact, constant light can cause the clock expression of nocturnal rodents to damp, ultimately becoming arrhythmic, and this damping effect includes the central clock's primary output signal of electrical activity.[21] This conditionality may be related to Aschoff's rule, which states that the free-running period in constant light is less than the free-running period in constant darkness for day-active animals while the reverse is true for night-active animals.

A second example of conditionality is temperature sensitivity to optimal growth conditions and temperature-induced resetting of the marine Gonyaulax clock. In particular, rhythmic bioluminescence of this species has greater amplitude at temperatures near that of the ocean (18 °C).[22] In addition, low temperatures are more likely to phase shift the Gonyaulax clock than higher temperatures.[23] More recently, Johnson's laboratory has shown that freshwater cyanobacteria (*S. elongatus*) exhibit enhanced physiological growth at temperatures near 30 °C and reduced growth at temperatures higher or lower than 30 °C despite an enhanced circadian amplitude in high temperatures that stifle growth (e.g., 38 °C).[24] One possible explanation is a mechanistic switching between circadian and non-circadian regulation of gene expression as an adaptive response to environmental conditions. For instance, the highly expressed cyanobacteria circadian clock genes *KaiB/C* are, surprisingly, not under selective pressure to exhibit conditional expression as most strongly expressed genes are. Johnson and colleagues have determined that although overexpression of Kai proteins leads to more robust rhythmicity at colder temperatures (18 °C and 23 °C), it also leads to slower growth. This suggests that in certain environments having robust rhythmic gene regulation is maladaptive.

At the conclusion of the presentation, Johnson revisited the four primary clock properties. First, the requirement of a self-sustained mechanism has been shown to be crucial and may relate to enhanced coupling among oscillators. Second, a clock must exhibit entrainment in order to conserve the proper phase angle of rhythmic expression to the environmental light–dark cycle, and this property is a primary target of selection. Third, temperature compensation appears to be necessary for phase angle conservation, but is difficult to explain biochemically (especially in the case of homeotherms). Finally, clocks must express conditionality, implying that turning the clock off may be beneficial under certain environmental conditions.

Sleep and synaptic plasticity: exploring the synaptic homeostasis hypothesis

We spend approximately a third of our life asleep, yet the essential function of sleep remains unclear. Giulio Tononi (University of Wisconsin) discussed recent advances in the understanding of sleep function, specifically focusing on the synaptic homeostasis hypothesis (SHY), which his laboratory has been studying for the past 15 years.

The rationale for SHY stems from the observation that most learning results in the strengthening of neural connections—an energy consuming process. Indeed, the brain is the most expensive organ of the body, accounting for almost 20% of the energy budget with more than two thirds of that budget spent supporting synaptic activity. Furthermore, a net strengthening of synapses is a major source of cellular stress due to the synthesis and delivery of cellular constituents, such as mitochondria, synaptic vesicles, proteins, and lipids. Thus, learning by synaptic strengthening is an unsustainable process

in the long run. According to SHY, a fundamental function of sleep is to downregulate synapses to sustainable levels both in relation to energy consumption and cellular stress. In other words, sleep is the price we pay for plasticity.

Tononi then summarized experiments from his and other laboratories that support SHY. For example, when fruit flies are given plenty of opportunities for interactions with other flies during the day, many regions of their brain contain more synaptic spines by evening. The following day, the number of spines returns to baseline but only if flies are allowed to sleep.[25] Another experiment documented a similar phenomenon in the cerebral cortex of adolescent mice, whereby the number of synaptic spines increased during wake and decreased during sleep.[26] In adult rodents, however, it is the strength of synapses rather than the absolute number that change, as indicated by changes in synaptic AMPA receptors following wake and sleep. For instance, electrical stimulation of neural fibers in the rodent cortex evokes larger responses in target neurons after a few hours of wake that are smaller after sleep.[27] A similar experiment in humans, using transcranial magnetic stimulation (TMS) and high-density electroencephalography (EEG), reached the same conclusion: the longer the subject was awake, the larger the cortical evoked responses, which decreased only after a night of sleep.[28]

Tononi concluded by discussing the implications of SHY for neural development. Childhood and adolescence are critical periods for the development of psychiatric disorders, as well as times of concentrated learning. The increase in synaptic number during early development is truly explosive and is bound to pose a great risk for synaptic overload. In this regard, sleep may represent a critical homeostatic process that acts to maintain both synaptic strength and number. Moreover, development is characterized by intense synaptic remodeling, with massive synaptogenesis accompanied by equally massive synaptic pruning. Thus, it is essential that down-selection occur in a fair and balanced manner. Sleep would be an ideal time for enforcing a fair competition: when the brain is off-line, neurons can sample inputs and try out both short- and long-range circuits, with little worry for the associated behavioral output. Tononi's laboratory is studying whether chronic sleep disruption during critical periods of rodent development impairs synaptic pruning, thus irreversibly interfering with the proper refinement of neural circuits.

Sleep and its complex relationship to the circadian system

Overview

This session focused on interactions between the sleep–wake and circadian regulatory systems. Derk-Jan Dijk (University of Surrey) opened the session, setting the stage for an exploration of how sleep may be related to the question, What makes a good clock? The last 30 years of research in human and animal systems have made it clear that sleep regulation is complex and not solely driven by the circadian system. The sleep homeostat, which itself is an oscillator, interacts with the circadian cycle, and these two oscillatory processes are commonly represented in models of sleep–wake regulation. The circadian regulator is located in the SCN; it is robust—even rigid—but the sleep homeostat is very sensitive and flexible. The interplay between the circadian and homeostatic oscillators regulates the sleep–wake cycle where the sleep–wake cycle feeds back on itself and the circadian cycle. As an example, the advent of artificial light has increased the impact that the sleep–wake cycle has on the light–dark cycle and provided an avenue for social factors to affect these cycles (e.g., jet lag, shift work).

Major themes addressed for this session included sleep changes that occur across adolescent development and its impact on circadian rhythms; the bidirectional nature of the circadian system as both an output of the clock and a self-regulator of behavior; and the impact of sleep on emotional reactivity and memory.

Sleep changes over adolescent development

Mary A. Carskadon (Brown University) discussed the interactions between sleep changes and circadian rhythms during adolescent development. Carskadon's laboratory has performed a series of studies exploring the delay of circadian rhythms over the course of development in human adolescents.[29] This work has been replicated and extended by Roenneberg and colleagues who showed a dramatic delay in chronotype across the second decade, followed by a gradual tendency toward earlier chronotype across the remainder of the adult lifespan.[30]

Manifestations of circadian clock development during adolescence are present in juvenile animals (rhesus monkeys, Octodon degus, Norwegian rats, laboratory mice, and the fat sand rat)[31] in addition to humans, suggesting that underlying biology plays an important role in this development. Indeed, laboratory studies have elucidated some of the underlying biological correlates behind the developmental circadian delay observed in adolescents. In one cross-sectional study of 27 human youngsters, salivary melatonin onset was significantly correlated with Tanner stage of pubertal development after controlling for sleep–wake schedule,[32] and a decrease in amplitude of the melatonin rhythm has been observed with advancing Tanner stage in humans.[33]

Several possible etiologies of circadian phase delay during adolescence have been considered, including altered light–dark exposure (reduced morning light and enhanced evening light) or a change in morning (reduced) or evening (enhanced) light sensitivity. A third possible mechanism is a lengthening of the internal circadian period (*tau*) across pubertal development that would tend to phase delay circadian rhythms and require a larger daily phase advance to maintain entrainment. Over nearly two decades, Carskadon has run approximately 120 young people aged 9–25 years through various forced desynchrony protocols, including 4-, 20-, and 28-h days. Her results show a significant shortening of period length with increasing age and Tanner stage (Carskadon, unpublished observations). Though these findings do not support the initial hypothesis that circadian period lengthens during adolescence and shortens in emerging adulthood, the studies reveal alterations in circadian biology during the second decade that likely interact with other biological and behavioral phenomenon to influence sleep and behavior.

How sleep/wake and other imposed activity patterns feed back on the clock

Tom de Boer (Leiden University Medical Center) discussed how the SCN plays a bidirectional role in sleep and circadian behavior. Light-phase response curves describe circadian-dependent light-induced behavioral phase shifts in wheel-running behavior in rodents, with evening/early night light producing circadian phase delays and early morning light producing phase advances.[34] These observed behavioral patterns reflect what is happening on a neural level, as light pulses during the night, when SCN firing is low, induce larger increases in SCN firing rates compared to light pulses during the day. Taken together, these data show that light perturbation can influence the phase of the circadian system.

Exercise also feeds back on the circadian clock at the levels of behavior (including circadian period and phase position) and neurophysiologic activity. Studies in humans have shown acute time-dependent changes in melatonin secretion following high-intensity exercise, with decreases in melatonin levels following morning exercise, no change after exercise in the afternoon, and increases in melatonin levels with high-intensity exercise at night.[35] Furthermore, changes in melatonin levels with exercise at night were associated with circadian phase delays of up to 30 minutes.[35–37] Studies of SCN firing rates in mice show neurophysiologic changes induced by locomotion. SCN neurons that normally have a high rate of firing during the subjective day in rodents (inactive phase) show firing suppression induced by spontaneous locomotor behavior.[38]

Furthermore, sleep state and restriction are known to have a profound impact on clock function. Deboer and colleagues observed that as rats go from a state of non–rapid eye movement (NREM) to REM or from NREM to waking, SCN neuronal activity increases, whereas a decrease in SCN firing is observed when rats make the transition from wake to NREM sleep. In conditions of sleep deprivation, however, SCN firing rates are attenuated in parallel with increases in slow-wave activity (SWA) in the rat.[39,40]

Another combined stimulus study examined the effects of sleep deprivation on light-induced phase shifts in hamsters and found that while 24 h of sleep deprivation did not induce a phase shift by itself, sleep deprivation did attenuate the phase delaying effects of light.[41] In humans, Burgess[42] showed that sleep restriction (4-h sleep opportunity) reduced phase advances produced by 3 days of morning bright light exposure. Most recently, work examining the underlying neurophysiology of attenuated phase shifting with altered vigilance states shows that in mice exposed to 6 additional hours of wakefulness, SCN firing rates in response to light stimuli are diminished (van Diepen, Meijer, and Deboer, unpublished observations). These studies, combining a light stimulus with increased sleep

Table 1. Associations between behavioral and environmental input with phase shift response and SCN firing rate

Input	Behavioral response	Neuronal activity response
Light	Phase shift (time-dependent) and interdaily stability	Increased SCN firing
Activity/exercise	Phase shift (time-dependent)	Mostly decreased SCN firing
Vigilance states	Phase shift unclear	State-dependent changes in SCN firing
Increased sleep pressure	Phase shift unclear	Decreased SCN firing
Sleep restriction/light	Decreased phase shift	Decreased SCN firing

pressure, indicate that sleep restriction affects clock function.

Table 1 summarizes the current knowledge regarding associations between behavioral and environmental input and SCN firing rate and phase shift response. These data suggest that SCN neuronal activity is an important marker and contributor in signaling and responding to environmental and behavioral information. More work is needed, however, to fully elucidate the bidirectional associations between zeitgeber input, SCN neuronal firing rates, and behavioral outcomes.

Sleep and emotional brain functions

The session on sleep and its relationship to the circadian system concluded with a presentation by Matthew Walker (University of California, Berkeley) on sleep and emotional regulation. Two main themes of the presentation were that (1) sleep deprivation and restriction can increase emotional reactivity and (2) sleep may serve a role in emotional regulation by depotentiating emotional memories.

Within the human experience, emotional lability can be related to poor sleep quality. Recent work in Walker's laboratory has focused on the interplay between REM sleep and emotional reactivity in healthy young adults.[43,44] In one experiment, 14 young adults who were awake for approximately 35 h were shown increasingly negative-valence photos from the International Affective Picture System,[45] while undergoing event-related functional magnetic resonance imaging (fMRI). Compared to a control group (who slept at their usual times the night before the experiment), sleep-deprived participants showed a 60% increase in amygdala response to negative photos.[43] Furthermore, sleep deprivation was associated with weaker functional connectivity between the amygdala and the medial prefrontal cortex (mPFC) and with stronger amygdala connectivity with the locus coeruleus and midbrain. Thus, the inhibitory top-down control of the amygdala by the mPFC is diminished with sleep deprivation, and the functional pathway between autonomic-activating brainstem centers and the amygdala are enhanced. A similar protocol using positive-valence photos demonstrated that people also display amplified neural reactivity to positive stimuli.[44] Taken together, this line of research suggests that sleep deprivation impacts our ability to form appropriate responses to next-day psychological challenges.

Further exploration of the interplay between sleep and emotional regulation has examined the role of REM sleep in emotional memory. Of note, REM sleep is under tight circadian control,[46] and may be deprived in individuals with delayed circadian phase who awaken before their biologically preferred time owing to social demands. Previous research supports a role for REM sleep in strengthening emotional memory.[47] Furthermore, recent work indicates that REM sleep may help diminish the emotional overtones of memories. Thus, according to the "sleep to forget and sleep to remember" model,[47] REM sleep not only reinforces recall of emotional memories, but also strips the memories of the strong affect that led to increased arousal and augmented the salience of the memory in the first place. This model proposes decreased adrenergic tone in the brain during REM sleep as the neurochemical mechanism for decreasing the emotional component of memory. Thus, individuals can remember an emotional event, but the memory itself is no longer emotional. These findings may have relevance and implications for posttraumatic stress disorder.

Thus, an intimate association exists between sleep and affective regulation. Experimental evidence in

healthy participants supports a role for sleep in relation to appropriate emotional reactivity and connectivity in the brain. Furthermore, REM sleep may play a particularly important role in modulating affect because of the unique neurobiological milieu of decreased adrenergic tone.

Systems development and psychopathology I: prenatal development through early childhood

Overview

This interesting and varied session focused on the development of the circadian clock through evolution and during fetal development, and how epigenetic information is passed between generations.

Circadian pacemaking at the single-cell level

In his presentation, John O'Neill (University of Cambridge) discussed circadian pacemaking at the single-cell level. Cyclical patterns in the environment have been internalized through evolution and have profound effects on biology at multiple organizational levels. In order to understand the molecular underpinnings of the circadian regulation of biology, it is important to understand circadian biology at its most fundamental level. The current paradigm of cellular oscillation is the transcription–translation feedback loop (TTFL) that is founded on the general principle that stable oscillations in any system can be generated by a simple negative feedback loop coupled to a positive feed-forward mechanism.[48] In the canonical mammalian clock, this takes the form of the *Per/Cry-Bmal/Clock* negative feedback loop. However, a simple circadian clock has been discovered in cyanobacteria—the KaiABC phosphorylation clock, which can be reconstituted without a transcriptional component.[49]

The key observation that hinted at non-TTFL components to circadian rhythms was the insensitivity of circadian phase to cordycepin, a potent transcriptional inhibitor, during the last two thirds of the circadian cycle (zeitgeber time (ZT)8–24). Similarly, when translation was inhibited by cycloheximide outside of an 8-h window (ZT4–12), the phase of the cycle was unaffected.[50] These overlapping, but not identical, windows of sensitivity are also observed in mouse SCN explants, which are not affected by cycloheximide until 18–24 h of treatment.[51] That transcription and translation are dispensable for the majority of the clock cycle implies that other factors such as post-translational regulation of gene expression are more important at these phases of the clock cycle.[52]

The mammalian red blood cell (RBC) is a potent model for studying non-TTFL rhythms. As an anucleate cell without organelles and no residual clock protein levels, any cellular rhythms detected are non-TTFL based. Critically, peroxiredoxin (Prx) proteins, a ubiquitous family of antioxidant enzymes, were found to have rhythmic oxidation states. The circadian rhythm of Prx oxidation was entrainable in RBCs by temperature cycling, and the period was temperature compensated, identifying the Prx rhythm as a bona-fide circadian marker.[50] The existence of a circadian oscillation in the RBC, which lacks canonical TTFL components, provides strong evidence that TTFLs are not necessary for circadian rhythms.[50] Interestingly, Prx rhythms are astonishingly well conserved and shown to exist in flies, fungi, mice, nematodes, cyanobacteria, and *Archea*,[53] suggesting that sensitivity to reactive oxygen species may have been a relevant selective pressure on the evolution of the circadian clock.

Circadian synchronization in utero

Next, Maria Seron-Ferre (University of Chile) discussed circadian synchronization in the fetus. In adults, the circadian clock is organized into a central clock located in the SCN with peripheral oscillators in various other tissues. Signaling between the SCN and peripheral tissues ensures synchrony between the clocks and maintains the temporal order of the organism. How this model of clock synchronization applies to the *in utero* fetus is unknown. It is also unknown whether mammalian fetuses express clock genes and how circadian cycles are organized hierarchically within the mothers.

Potential entrainment signals from the mother to the fetus include maternal rhythms in melatonin and temperature. As the fetal pineal gland does not produce melatonin, the only source is from the mother. In the capuchin monkey, experimentally induced suppression of maternal melatonin rhythms abolishes *Bmal1* and *Per2* expression rhythms in the fetal SCN.[54] Interestingly, adrenal tissue rhythms are more robust and do not change in response to melatonin depletion.[54] Furthermore,

temperature rhythms in newborn monkeys lose synchrony when the mother is exposed to constant light conditions but resynchronize upon rhythmic oral administration of melatonin. These data indicate that maternal melatonin is a chronobiotic signal that controls the circadian cycle of newborn temperature.[55]

The observation that SCN and adrenal oscillators are independent in the fetus has also been observed in rats. Clock gene expression in the fetal rat SCN does not oscillate whereas the adrenal gland does. Maternal melatonin has been shown to be a chronobiotic signal and homeostatic regulator of the fetal adrenal gland.[55,56] In effect, the maternal clock usurps the role of the SCN as master clock while an organism is *in utero*.

Together these observations provide strong evidence that maternal circadian patterns are important to early development. This could have important implications for human health if maternal melatonin rhythms are disrupted during pregnancy by, for example, artificial light or disruptive working patterns.

Epigenetic inheritance in mammals—environmental modulation of epigenetic states

In her presentation, Anne Ferguson-Smith (University of Cambridge) discussed the mechanisms of epigenetic inheritance. Epigenetic modifications are chemical marks on DNA or post-translational modifications (PTM) of histones, which affect chromatin packaging and accessibility and therefore gene expression. Epigenetic modulation of gene expression is highly dynamic during development and has the potential to be similarly important to circadian rhythms.[57]

Epigenetic inheritance is already well-established in plants and worms and considerable comparison of the implications of this and common mechanistic aspects have been considered between species.[58] Adaptive responses are thought to be stimulated, which prepare offspring for future environmental challenges. In mammals, this is suggested to lead to an increased risk of obesity, cardiovascular disease, and diabetes in both the F1 (offspring of environmentally challenged parents) and F2 generations (offspring of F1 generation).[59] In collaboration with Mary Elizabeth Patti at the Joslin Diabetes Center, a mouse model of epigenetic inheritance of diabetes risk was developed. Pregnant mice were subjected to 50% caloric restriction from gestation day (E) 12.5, causing low birth weights and an increased likelihood of developing markers of obesity and diabetes in the F1 generation. Strikingly, F2 mice were also more likely to develop obesity and diabetes markers even though their parents were never starved during their adult life.[44,60] Transcriptomic analysis of F1 livers at E16.5 revealed an upregulation of *Clock* and *Per2* expression in F1 fetal liver but this effect was not carried forward to the F2 generation.[60]

Another class of genes that are exquisitely epigenetically regulated are imprinted genes.[61] Despite their important role in growth and metabolism, these genes are not, as a class, significantly modulated in the undernutrition-induced diabetes model.[45] This group of mammalian genes is expressed according to their parental origin using a mechanism governed by epigenetic modifications that differ on the maternally and paternally inherited chromosomes resulting in expression from only the maternal or the paternal copy of the gene. Approximately 100–200 imprinted genes have been identified, for which a number of patterns of epigenetic control have been found. Ferguson-Smith noted that only a small number of imprinted genes have been shown to be under circadian regulation (Ferguson-Smith, unpublished observations), suggesting absence of a link between the epigenetic mechanisms regulating genomic imprinting and any dynamic epigenetic control associated with the circadian clock at these genes.

Systems development and psychopathology II: adrenarche through young adulthood

Overview

This session addressed those changes in the circadian and sleep–wake function that occur between preadolescent development and young adulthood and how these changes relate to resilience versus the development of psychopathology.

Clinical phenotypes in youth with emerging mood disorders

The post-pubertal period is a critical phase for the emergence of many psychiatric disorders. Ian Hickie (University of Sydney) opened this session with a presentation focused on disruptions in the

sleep–wake cycle and patterns of physical activity in young patients with emerging bipolar (BD) and unipolar mood disorders. Hickie emphasized the importance of focusing on clinical phenotypes such as fatigue (i.e., low activity, phase delay, later dim-light melatonin onset (DLMO)), rather than sleep, and activity rather than mood, as defining features of illness. These phenotypes present a challenge with reference to treatment development, as many diagnostics and classification manuals do not include the importance of circadian systems in medical, neurological, and psychiatry diseases.

Hickie and colleagues have proposed three predominate types of emerging mood disorders in youth: one dominated by early-onset anxiety (more common in females), one dominated by developmental abnormalities (more common in males), and the third based on circadian dysfunction. Twenty-five percent of referred depressed adolescents can be classified as having circadian-driven etiology (i.e., disruption of the 24-h sleep–wake and circadian systems as the fundamental biology). Indeed, the childhood phenotypes that precede adolescent-onset depressed mood and fatigue are those of disrupted and unstable sleep–wake cycles. As there is an ongoing debate in psychiatry regarding the use of selective serotonin reuptake inhibitors (SSRIs) in this group, an important question emerges as to the appropriate treatment. In this regard, treatments focused on the circadian system (i.e., melatonin analogues) may be a promising treatment option within this group, as may behavioral modifications focused on modifying sleep–wake cycles.

Hickie and colleagues have investigated circadian systems in two large clinical samples of young individuals with emerging mood disorders ($n = 300$, 30% bipolar spectrum; $n = 1700$, 15% bipolar spectrum). Young people with emerging BD exhibited significant phase delays in sleep onset (4–5 am) and offset (getting up late in the afternoon).[62] In addition, these patients slept longer, spent more time in bed, and had a higher wake-after-sleep onset, leading to reduced daytime activity.[63] There were also significant differences in the degree of desynchrony observed in bipolar patients. Likewise, these patients had later DLMO and melatonin curves. Focus on, and accurate identification of, these early subthreshold symptoms is an important step toward mitigating disease progression, with the ultimate goal of preventing full-blown mood disorders.

Sleep, cognitive, and emotional processing in children and adolescents

Avi Sadeh (Tel Aviv University) focused on the impact of sleep on children's behavioral, cognitive, and emotional information processing, with a special focus on the assessment of children within their natural environment.

Meta-analytic studies demonstrate that children are sleeping less over time. Matricciani *et al.*[64] reported a drop of 0.73 min/year over the last century, indicating an accumulation of sleep deprivation in young children. This becomes especially concerning given recent reviews reporting that sleep quality, duration, and sleepiness are associated with poorer academic and cognitive performance.[65–67] Interestingly, there exists individual variability in sleep duration and quality at each age, raising questions as to the meaning and impact of this natural variability. To address this question, Sadeh and colleagues conducted a series of studies looking at the correlates of children's sleep patterns.

Sleep and neurobehavioral functioning. In the first study by Sadeh *et al.*,[68] the relations between sleep and neurobehavioral functioning were examined in 144 healthy children from second-, fourth-, and sixth-grade classes. Children were divided into two groups according to sleep quality, with poor sleep defined as three night wakings (>5 min each) or a sleep efficiency lower than 90%. Poor sleepers were found to perform worse on complex tasks, such as the continuous performance test and the digit symbol substitution test—an effect that was more pronounced in younger children (second and fourth graders).

In the second study, Sadeh *et al.*[69] assessed the impact of minor sleep restriction/extension (+/− 1 h) on fourth and sixth graders. Sleep restriction/extension had a direct impact on the level of reported sleepiness/evening fatigue and on performance in a memory task that was equivalent to 2 years of maturation. These results highlight the potential detrimental effects of minimal sleep curtailment on neurobehavioral functioning in children.

Similar studies were performed in younger children. In a longitudinal study, the sleep of 12-month-old infants was assessed with actigraphy,

and neurobehavioral functioning was assessed at 3–4 years of age using the Posner paradigm for the orienting-of-attention task and the Stroop-like paradigm; both of which involve conflicting task demands. The results demonstrated significant concomitant correlations between sleep quality measures and attention measures. Interestingly, there were also significant predictive links between the number of infant night wakings at 12 months and poor task performance. The main conclusions of this study were (1) the features of natural sleep are concomitantly and longitudinally associated with neurobehavioral functioning in young children; (2) sleep fragmentation (night wakings) is a more significant predictor than sleep duration; and (3) complex or conflicting task demands increase sensitivity to sleep variations.

Bidirectional relationship between sleep and emotions in children. Loss of sleep is associated with altered brain functioning that includes changes in amygdala and frontal cortex activity that may affect the ability to regulate emotions. Sadeh and colleagues studied these issues during the transition to adolescence in a 3-year longitudinal study starting when children were 10 years of age.[70] Regarding sleep measures, a significant delay in sleep-onset time and a significant decrease in true sleep time were found. Emotional information processing was tested and it was found that lower sleep quality, as reflected by a higher number of night wakings, or lower sleep efficiency, predicted lower performance. Interestingly, sleep duration was not a significant factor, suggesting that emotional processing is more sensitive to sleep quality than quantity. On this note, it is important to re-emphasize the role of sleep fragmentation on performance. Therefore, sleep fragmentation experiments should be conducted in addition to the well-studied effects of sleep restriction.

Finally, it is important to remember that the links between sleep and emotions are bidirectional. Sleep patterns of children with nighttime fears are compromised on almost all parameters in comparison to controls.[71] When nighttime fears are addressed and ameliorated, their sleep quality significantly improves.[72] Therefore, as much as sleep can affect emotions and mood, emotions can affect sleep as well.

Systems development and psychopathology III: midlife through late life

Overview

The aim of this session was to examine the realm of circadian rhythms and sleep in mid- through late-life periods and its relationship to psychopathology.

Sleep fragmentation with aging: the clock and beyond

In the first presentation of this session, Eus Van Someren (Netherlands Institute for Neuroscience and VU University Medical Center) discussed the clock's role in sleep fragmentation in the elderly. Normal aging is associated with greater sleep fragmentation, poorly maintained sleep, and electroencephalography (EEG) slows oscillations of reduced amplitude.[73] Sleep and circadian rhythm changes, however, are even more strongly associated with problems related to aging, such as Alzheimer's disease (AD), where it is common for individuals to experience disrupted sleep.[74] Research has demonstrated that sleep–wake rhythm fragmentation is related to the expression of the peptide vasopressin in SCN neurons, which acts as a signature of SCN neural activity. Harper *et al.*[75] have shown that AD patients who experienced the most fragmented rhythms during life had a reduced number of vasopressin-expressing neurons in the SCN postmortem. Interestingly, these SCN neurons can be activated by intense light during the day.[76,77] As such, a long-term multicenter double-blind placebo-controlled randomized clinical trial assessed whether the use of light could reduce fragmentation and improve rhythms in elderly patients with dementia.[77] The results were impressive and demonstrated that light treatment improved circadian rhythms, mood, and cognition.

A follow-up study examined the association between fragmentation and cognitive function in non-demented elderly people.[78] Using actigraphy, periods of rest and activity were examined in order to quantify fragmentation, and neuropsychological assessments were conducted, including memory, mental speed, and executive functioning. Clear correlations emerged between fragmentation and neuropsychological performance. An experimental study extended the findings from the previous correlational study to suggest a causal contribution of fragmentation to cognitive functioning.[79] The

procedure involved intentionally fragmenting sleep in elderly individuals. Following both the normal and fragmented nights of sleep, individuals completed a picture-learning fMRI task that normally activates multiple cortical areas and the hippocampus. Results indicated that the hippocampus had reduced activation following the fragmented night compared to a normal night of sleep. In summary, this series of studies provides support for the proposal that biological clock deficiencies may result in sleep fragmentation during aging, leading to disrupted brain function. This raises the possibility that sleep continuity may be as or even more important than sleep duration.[80]

In the second part of Van Someren's presentation, individual differences (beyond the circadian clock) that also are likely contributors to disturbed sleep were discussed. For instance, an examination of grey matter in healthy individuals demonstrated that people with relatively little grey matter in a mid-posterior area of the left orbitofrontal cortex are more likely to report early morning awakenings.[81] This orbitofrontal area is involved in hedonic evaluation, including the sensing of thermal comfort.[82] Indeed, use of a thermosuit to slightly increase skin temperature by only 0.4 °C reduced the probability of early morning awakening in elderly individuals.[83] Insomnia has also been associated with reduced orbitofrontal cortex grey matter where reductions predict the severity of insomnia complaints.[84] Follow-up studies, however, suggest that different profiles, subtypes, or phenotypes of insomnia exist. Van Someren's group has created a website, www.sleepregistry.org, to collect data on a variety of facets, including personality traits and sensitivity to stress, in individuals with insomnia and good sleep.[85] The goal is to identify different behavioral profiles of insomnia through this sleep registry (14,000 participants to date) and apply a multimethod approach (e.g., MRI, high-density EEG, and TMS) to unravel the brain mechanisms involved among these different sleep profiles.

Circadian influences on sleep and cognitive performance in older adults

Jeanne Duffy (Harvard University) discussed circadian influences on sleep and cognitive performance in older compared to younger adults, beginning with an overview of older adult sleep followed by a review of the consequences of sleep deprivation in young adults.

Sleep in older adults is characterized by decreases in slow-wave sleep and increases in awakenings. In young adults, experimentally induced sleep deprivation (i.e., reduced duration or increased fragmentation) results in slower reaction times and poorer performance on cognitive tests. This raises the question of how older adults perform under such conditions, given their increased sleep fragmentation. This question—pertaining to the impact of acute sleep deprivation in older versus younger adults—was assessed using vigilance (i.e., reaction times) and sleepiness (i.e., slow eye movements) as outcome measures.[86] Surprisingly, results showed that younger adults were more affected by the sleep deprivation protocol than healthy older adults. These data were supported by two other studies demonstrating that older adults reported fewer accidental sleep episodes across a 36-h sleep deprivation protocol[87] and had fewer lapses of attention, more performance stability, and less subjective sleepiness during 40 h of sleep deprivation.[88] In summary, these studies suggest that while wake-dependent performance declines were experienced by both younger and older adults, the performance and alertness of healthy older adults is better preserved under acute sleep loss. Moreover, studies suggest that these age effects on performance are particularly pronounced during the night, raising the question of whether the effects are due to homeostatic or circadian processes.

In order to begin to answer this question, forced desynchrony protocols provide a method for separation of the circadian and sleep/wake–dependent influences. These protocols involve imposing a rest–activity schedule far from 24 h (e.g., a 28-h rest–activity cycle with scheduled sleep episodes occurring 4 h later each day[89]). Low light levels are used during wakefulness, such that the underlying circadian system cannot entrain. Humans thus develop a free-running rhythm under these circumstances, allowing either wakefulness or sleep to be observed with respect to the underlying circadian timing.

Duffy and colleagues used a protocol of 3 weeks of chronic sleep disruption with subjective alertness and objective and subjective measures of sleep as outcomes in older and younger adults. Older participants slept 22.3 h less across the three weeks, but nonetheless demonstrated greater subjective

alertness across all circadian phases during the final week of the study (Duffy *et al.*, unpublished observations). In regard to performance in the context of circadian disruption, another study with a forced desynchrony protocol with a 20-h "day" across 2–3 weeks added to the emerging evidence that older subjects can better tolerate circadian changes. In this study, older subjects reported lower subjective sleepiness and experienced fewer lapses of attention compared to younger adults.[90] In sum, the extant literature suggests that performance and alertness of healthy older adults is better preserved under various sleep loss (acute or chronic) conditions compared to younger adults.

Clock genes and Alzheimer's disease

Johannes Thome (Rostock University) began his presentation by focusing on how the circadian clock can be used to diagnose functional deficits in patients with Alzheimer's disease. A variety of biomarkers have been identified that have high reliability in predicting AD before symptom onset and include elevated cerebrospinal fluid (CSF) tau levels, decreased CSF amyloid levels, reduced glucose uptake (as measured by positron emission tomography (PET)), and brain atrophy (as measured by MRI). In addition, disturbances in diurnal rhythms are present in approximately 60% of patients and appear, on average, 10 months before a diagnosis is made,[91] suggesting that circadian disturbances may serve as a biomarker for Alzheimer's disease. These disturbances include fragmentation of the sleep–wake cycle, altered neuroendocrine function, neuropathological changes in the SCN, and altered rhythmic expression of the clock genes, *BMAL1, CRY1*, and *PER1* in the pineal gland.[92] As such, chronotherapeutics may prove useful in AD treatment and include long-term light therapy or social rhythm therapy. Whether pharmacological interventions, such as melatonin, could be useful has yet to be ascertained.

Thome concluded his presentation with a discussion of attention deficit hyperactivity disorder (ADHD); studies have suggested that clock gene variants could contribute to the condition. Thome's laboratory examined circadian rhythms in adult patients with ADHD at the molecular, behavioral, and endocrine levels. A first study using actimetry demonstrated that ADHD patients were much more active than controls during both the most active 10 h and the least active 5 h and also showed higher amplitudes. A key finding was that the period was significantly shortened in ADHD patients. More specifically, quantifying mRNA of clock genes (*PER2*) and melatonin indicated that the robust circadian pattern apparent in healthy controls did not exist in ADHD patients. A follow-up experiment demonstrated that a medication used in the treatment of ADHD, atomoxetine, affected both clock gene expression and activity in mice.[93] This result parallels preliminary data in humans that demonstrate agomelatine to be a useful alternative medication in individuals with ADHD and sleep problems.[94] Overall, the early data suggest that disturbances in the clock system may be fundamental pathophysiological factors in ADHD.

Circadian factors and applications in mood disorders

Overview

The concluding symposium of the first INSPIRE conference focused on the specific relationship of circadian factors to mood disorders and their treatment.

Gene expression in the brains of individuals with mood disorders is circadian

Huda Akil (University of Michigan) began her presentation by arguing that stress, developmental processes, genes, and environmental modifiers are among many factors affecting brain functions underlying mood disorders. Among these, influences of the circadian clock are of particular interest but are difficult to study because of the requisite repeated assessment of brain tissue over time. As such, few longitudinal gene expression studies of circadian rhythms in humans exist.

To circumvent this issue, postmortem brains from 34 patients with major depressive disorder (MDD) and 55 healthy controls were collected from individuals who died at different times across the 24-h circadian cycle. Samples from six brain regions (dorsolateral prefrontal cortex, anterior cingulate cortex, hippocampus, amygdala, nucleus accumbens (NAC), and cerebellum) were microdissected, and gene expression profiles from microarray studies were analyzed by time and for circadian rhythmicity.[95] Fitting the data to a 24-h curve provided good fits for hundreds of genes, suggesting many of them were rhythmic. Of the 50 most

rhythmic, 11 were identified as circadian clock genes by gene ontology analysis. *ARNTL* was the most reliably rhythmic transcript across brain regions. Interestingly, the phase relationships among expression profiles in the six brain regions studied were conserved for seven of the 11 clock genes identified, and the phase relationships among *PER1/2/3* were similar to those observed previously in animal studies.[96]

Importantly, there was significant loss of rhythmicity in the MDD brains, both within brain regions and across genes.[95] For example *PER2* and *PER3* were strongly rhythmic in controls, but mostly arrhythmic in MDD.[97] Similarly, the hippocampus and NAC expressed a number of rhythmic genes in controls, but were not rhythmic in MDD brains. The evidence presented suggests that specific brain regions may show differential vulnerability to circadian dysregulation in MDD. However, the mechanism of desynchronization in MDD is unclear. While it is unknown if rhythms underlie the primary pathology in MDD, impaired timing may play a role in illness onset, relapse, progression, and treatment resistance.

Nonpharmacologic circadian interventions for mood disorders

Allison Harvey (University of California, Berkeley) began her presentation by pointing out that in BD, sleep loss is common and is a marker of poor outcomes, including inadequate recovery, increased symptom burden,[98] and mood relapse.[99,100] Sleep and mood problems are correlated in BD, but a causal connection remains unclear. Sleep manipulation was discussed as a way to address the question of causality. Sleep is an open system that can be modified by many factors, including fatigue, social rhythms, light, and emotional state. Since there exists a bidirectional relationship between sleep and mood,[100] interventions that modify sleep by manipulating these factors may alter relapse risk and/or inter-episode mood function in BD. Because of the risk of inducing mood symptoms in BD through sleep deprivation,[99] manipulations that improve sleep are more desirable than sleep restriction. As clinical interventions, these manipulations have the benefit of being well tolerated and lead to enduring benefits.[101,102] Scientifically, these interventions may help determine which components of sleep are mechanistically related to mood changes.[103]

Testing the hypothesis that sleep interventions affect mood in BD, the researchers combined three mechanistically distinct sleep manipulations, collectively termed CBTI-BD, which combines cognitive behavior therapy for insomnia (CBT-I)—a therapy that aims to break associations between time in bed and wakefulness[102,104]—interpersonal rhythms therapy,[105] and exposure to dim light before bedtime and bright light on waking in the morning.[106] By intervening with multiple mechanisms it was hypothesized that more robust and broadly based improvements in illness outcomes would be realized.

A treatment trial of CBTI-BD was conducted to assess the effect on mood relapse and inter-episode functional outcomes in BD. In addition to regularly prescribed medications, subjects with BD received either the CBTI-BD multimodal sleep intervention or psychoeducation ($n = 26$/group). While both groups reported improvements in insomnia and overall disability, the effect sizes for both were approximately twice as high in the CBTI-BD group compared to the psychoeducation group. The CBTI-BD group also used less sleep medication and relapsed into mania or depression at a much lower rate than psychoeducation controls.

While these studies are promising, a number of questions remain. For example, to what extent were the effects on mood primarily due to influences on the sleep homeostat, the circadian systems, or both; and which factor(s) were most important among the CBTI-BD interventions? Therefore, while the question of causality remains to be fully answered, the CBTI-BD method suggests an effective strategy in managing mood symptoms and may address these important scientific issues.

Chronopharmacology for mood disorders

John Kelsoe (University of California, San Diego) discussed the utility of lithium and other medications that affect circadian function for the treatment of mood disorders. The symptoms of BD (e.g., sleep disturbances, seasonal mood variation) indicate that circadian rhythm dysfunction is an important feature of the illness.[107] Many different drugs are used to treat BD and it has been recognized for decades that some of these drugs have effects on the clock while manipulations of the clock have mood effects. Therefore, the relationship between the clock and mood is likely bidirectional but it remains unclear

if drug effects on circadian rhythms are universal among effective mood stabilizers, and if so, whether these actions are necessary for their therapeutic effects.

Lithium was discussed as the primary example. Lithium is one of the most effective drugs for treating BD, and has been shown to lengthen the circadian period in animals and humans.[108,109] Lithium works at multiple sites, including inhibition of inositol metabolism[110] and of glycogen synthase kinase 3β (GSK3β) activity. GSK3β is of particular interest as the enzyme that regulates circadian clock pathways[111] and is associated with clinical lithium response in pharmacogenetic studies,[112] possibly via interaction with the clock genes *NR1D1* and *CRY1*.[113] Data from large genome-wide association studies of lithium pharmacogenetics will further elucidate the relationships among the clock, mood, and lithium response. Two such trials are currently underway: ConLiGen, which has gathered genotypes and historical lithium response data from > 1,500 subjects,[114] and Pharmacogenomics of Bipolar Disorder (PGBD), an ongoing multicenter prospective pharmacogenetic trial of lithium monotherapy.

A second example of an antidepressant drug with circadian function is agomelatine, a dual-action melatonin receptor 1/2 (MT1/2) agonist and serotonin 2C (5HT2C) antagonist.[115] Agomelatine has antidepressant efficacy comparable to venlafaxine in animal models.[116] Preclinical studies revealed that the melatonin agonist action, and hence its circadian component, may be central as melatonin antagonists reverse the mood effects of agomelatine.[117] If so, this may be an important new mechanism for drug development.

In examining the clock–mood relationship from the other direction, manipulations of the clock were shown to be important in treating BD in a recent chronotherapy trial.[118] In addition to lithium, a combination of chronotherapuetic interventions was performed that included a night of total sleep deprivation and three consecutive nights of phase advance, followed by morning phototherapy. Despite the relatively short duration of the chronotherapeutic intervention, patients in the combined treatment arm showed a rapid improvement in mood, with a greater response compared to lithium treatment alone. Moreover, combination chronotherapy had enduring benefits over the duration of the study (7 weeks). Collectively, the data suggest that alterations in the circadian clock are directly involved in the mood-stabilizing response of treatments for bipolar disorder.

Debate: Are circadian factors central to mood disorders?

Overview

An additional and stimulating feature of the first INSPIRE conference was a debate on the question of the centrality of circadian factors to mood disorders. The debate was chaired by Guy Goodwin (Oxford University), with Daniel Buysse (University of Pittsburgh) arguing the affirmative and Philip Cowen (Oxford University) arguing the negative.

Pro: Circadian factors are central to mood disorders

Buysse's arguments were presented in a framework of multiple levels of analysis, asserting that evidence from animal, laboratory, clinical, and treatment studies all support a central role for circadian factors in mood disorders. He acknowledged a recent review[119] as an authoritative source on the topic, and noted that emerging data from the present meeting provided further cutting-edge evidence supporting the affirmative position.

Circadian genes are implicated in mood disorders. Despite a flurry of attention to circadian genetics in the past two decades, studies of their role in affective psychopathology are still in their infancy. For this reason, the failure of a recent prominent meta-analysis[120] to find any statistically significant associations between circadian genes and major depressive disorder should not close the door on further attempts to link clock genes to mood disorders. Studies based on single-nucleotide polymorphisms (SNPs) and Variable Number Tandem Repeats are currently limited to small samples, but they continue to provide encouraging findings. Indeed, recent data presented at the current meeting identified three SNPs (two in TIMELESS and one in RORA) with different allelic frequencies in bipolar patients relative to controls (Etain *et al.*, poster presented at INSPIRE 2013),[121] as well as evidence of altered circadian gene–gene interactions in patients with MDD relative to controls (Hagenauer *et al.*, poster presented at INSPIRE 2013).[122]

Critical molecules are implicated in mood disorders. Neurotransmitters relevant to mood disorders show circadian modulation. For example, dopamine, which drives reward circuits,[123] shows circadian variation[124,125] and is altered by clock gene variants.[126] Melatonin is not only the most reliable marker of the timing of the central clock,[127] but also is implicated in the pathophysiology and treatment of seasonal affective disorder. Specifically, altered timing of the DLMO relative to the timing of sleep correlates with the extent of depression in seasonal affective disorder (SAD) patients, and melatonin-induced corrective phase shifts correlate with the extent of improvement.[128]

Similarly, a variety of therapeutic molecules (i.e., antidepressants and mood stabilizers) have circadian effects. SSRIs may advance phase and/or shorten circadian period.[129,130] Lithium delays phase,[131] lengthens period,[132,133] and increases the amplitude of the *PER2* rhythm,[134] effects that may be mediated via GSK3β.[119] Valproic acid, another mood stabilizer commonly used for BD, induces phase shifts and increases amplitude of clock gene rhythms in the SCN.[135] Finally, a relatively new agent, agomelatine, acts on the 5HT2C and melatonin receptors (MT1/2),[136] with documented phase-shifting effects.[137,138] Whether any of these circadian effects are causally implicated in the mechanisms of action for mood regulation remains unknown.

Cells in the circadian system are implicated in mood disorders. Recent technological advances capitalize on the recognition that the molecular clock machinery exists throughout the periphery, and these peripheral clocks reflect the period of the organism.[139] These peripheral cell tissues, such as skin fibroblasts, show changes in amplitude with variance in circadian clock genes (reviewed in McCarthy and Welsh[119]) and provide a method for noninvasive, yet direct, measurement of human circadian parameters, including signaling pathways (Cuninkova *et al.*, 2013 INSPIRE).[140] Emerging data from the fibroblasts of patients with BD indicate differences in circadian period and an altered response to lithium (McCarthy *et al.*, 2013 INSPIRE).[141]

Circadian physiology is implicated in mood disorders. This level of analysis has received perhaps the most attention over the years, extending at least back to Wehr and Wirz-Justice's 1979 hypothesis that a phase advance of the circadian clock is depressogenic.[142] A variety of alterations in key markers of circadian physiology have been reported in mood disorders. These include reduced amplitude and advanced phase in the core body temperature rhythm,[143] an increased nadir and advanced phase in the cortisol rhythm,[143] and an altered phase in the melatonin rhythm, perhaps most convincingly in SAD patients.[128,144] Later circadian phase and late chronotypes are most consistently reported in mood disorder patients.[145–147] Implication at the level of circadian physiology is further supported by the efficacy of chronobiologically based interventions; in particular, the antidepressant effects of bright light in both seasonal and non-seasonal depression.[148,149] These findings suggest that there may be value in expanding how we think about affective disorders by phenotyping individuals according to circadian characteristics rather than symptoms alone.

Con: Circadian factors are central to mood disorders

Cowen began by asserting that major depression is a complex and multifactorial condition, and thus the contribution of a variety of causal mechanisms to the emergence of the disorder is a more likely scenario than a single mechanism purporting to be central to the disorder. Nevertheless, the phenomenology of the disorder clearly demands consideration of circadian rhythms.

The story of circadian disturbance in depression is an old one. Wehr argued in 1979[142] that some depressed patients are phase-advanced though currently the emphasis has shifted to phase delays. Indeed, both phase delays and advances are implicated in the pathophysiology of depression, as are reductions in amplitude, disruption, or desynchronization of circadian rhythm. This sheer number of potential abnormalities, however, makes it relatively easy to fit any particular dataset to some kind of circadian dysfunction and makes replication of specific defects difficult.

Studies using melatonin as a circadian marker in patients with non-seasonal depression have told a varied story. Some have reported decreases in melatonin production (based on area under the curve[150–152]), while others have reported increases in production[153,154] or no difference[155,156] relative to healthy controls. Evidence for differences in phase

is also mixed, with three of seven studies reporting delayed phase in depressed patients,[152,153,156] while the others reported no change. However, all of these studies have been limited by small samples and/or flawed methodology; for example, patients were taking medication, further challenging reliability of the findings.

Animal models have often been used to support circadian mechanisms in depression, but reported findings can be used to support a variety of mechanisms. In one recent and elegant study, the investigators reported that mice exposed to an aberrant light pattern displayed depression-like behaviors and elevated corticosterone levels.[157] Notably, however, the aberrant light schedule did not result in any apparent circadian disturbance. Corticosterone levels were elevated but still rhythmic, and there were no circadian alterations in core body temperature or *PER2* rhythms in the SCN. Importantly, the changes in depression-like behavior were reversed via antidepressant treatment without any concomitant changes in circadian rhythms.

In conclusion, the current state of the evidence demands an agnostic perspective on the role of circadian factors in depression. As with competing biological explanations of depression (e.g., the monoamine hypothesis), there is sufficient suggestive evidence to implicate circadian factors, but the evidence remains mixed and the picture remains puzzling. In contrast to reported circadian effects of antidepressants targeting monoamines, the rapid mood-lowering effects of monoamine depletion cannot be explained by circadian changes. Finally, there remains no clear evidence that circadian phase-shifting treatments are effective for major depression.

Selected responses from audience

Huda Akil: I would like to make a comment on the genetic argument. The trap of using the genetic argument—that no circadian genes have been shown to be positive for depression (in recent GWAS findings)—is that this argument, if extended to its logical conclusion, would lead to the determination that depression is not biologically based. This is because none of the hypothesized genetic mechanisms, whether stress genes, monoamine genes, growth factor genes, or neurogenesis genes, have emerged as statistically significant in GWAS studies. We need to take the genetic argument with a grain of salt.

Joe Takahashi: I would like to clarify the results of the recent 2012 meta-analysis of GWAS studies including 18,000 MDD patients,[120] in which no alleles reached statistical significance using *Nature's* GWAS criteria. To be clear, although the study is negative, this does not preclude the underlying genetics being causative. Rather, it says that the assumed genetic model in these GWAS studies—that MDD is a common allelic variant in the population—is unlikely to be correct. Notably, if there are many *de novo* mutations or rare mutations that are causative, GWAS is not a good way to identify those genes and the entire endeavor is likely to fail. A wave of technology has driven science to focus on GWAS . . . but that era is over. The future is to assemble complete genomic sequences that can be used to look at common and rare allelic variants and *de novo* mutant variants. It would seem that such an approach will be necessary for MDD, in contrast to BD and schizophrenia, which have more promising genetic findings.

John Kelsoe: Thus far, GWAS findings have been more supportive of circadian factors in bipolar disorder than unipolar depression. No one should be surprised that GWAS has not supported circadian genes in MDD given its heterogeneity. GWAS is testing a very specific hypothesis—a polygenic hypothesis—that many thousands of alleles combine with very small effect sizes to produce an effect. If that is not how clock genes operate to predispose to mood disorders, than GWAS is not the appropriate method. Given the highly interconnected nature of the clock, epistatic interaction effects are likely, but we have not been able to detect them yet. We will need larger samples for genetic studies given the small effect sizes. Notably, when GWAS analyses of schizophrenia moved from sample sizes of 25,000 to those of 50,000, suddenly we successfully identified 60 genes. Although we have some data to support the role of circadian genes in unipolar depression, they remain based on small samples. Sequencing all clock genes in large samples of mood-disordered patients will help.

David Kupfer: Returning to the phenotype issue, clinical depression is not going to be circadian-central across the board. The question is how to define an enriched phenotype to provide positive or negative answers. Do we start with bipolar disorder or bipolar spectrum disorders, focus on evening type, or do prospective studies on high-risk samples?

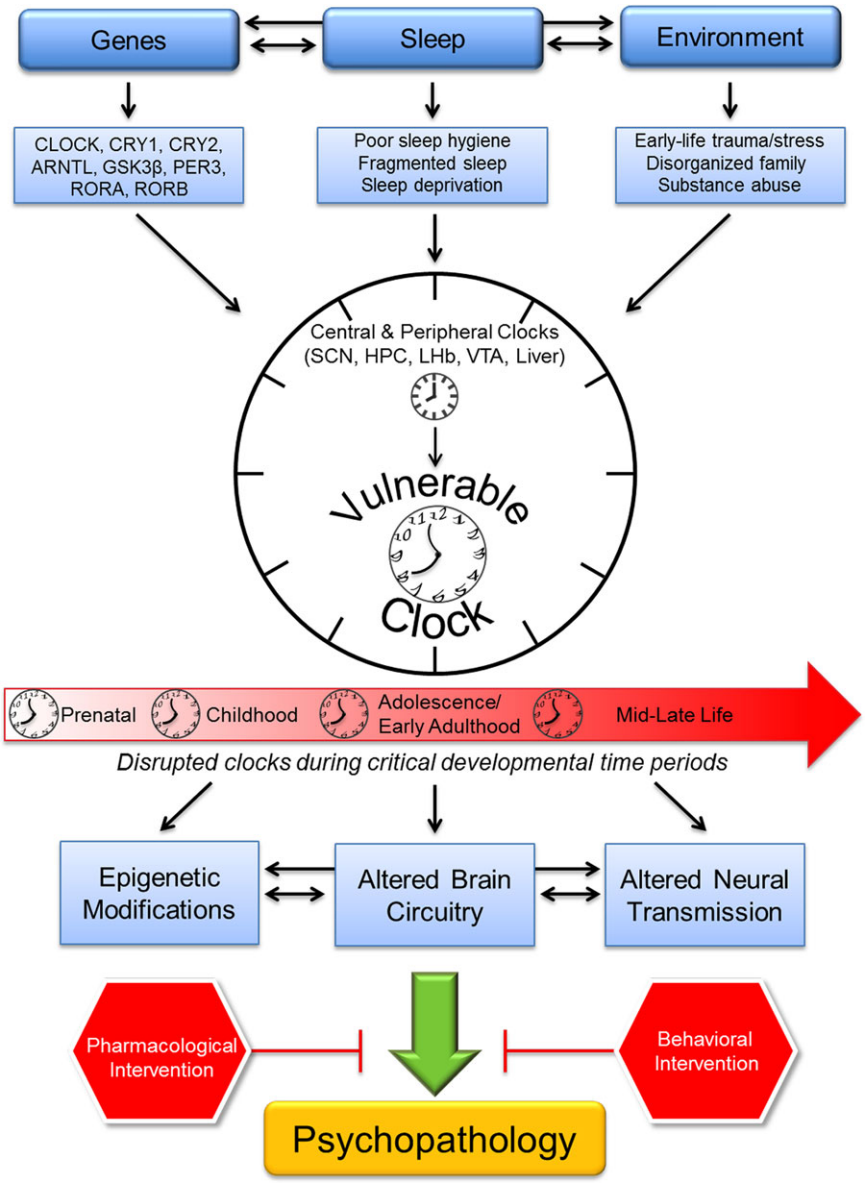

Figure 3. Circadian clocks, brain function and development. A simplified summary of the INSPIRE conference, depicting proposed relationships between the circadian clock and psychopathology. Genetic, sleep-related, and environmental factors can independently or synergistically converge on the circadian clock to increase susceptibility to developing psychopathology. Circadian disruption during critical developmental time windows may render the clock vulnerable to further insults along the developmental trajectory. Furthermore, as molecular components of the clock are expressed in key limbic regions implicated in mood regulation, altered clock function may influence mood either through immediate effects on brain circuitry or by altering the normal development and long-term function of relevant circuits. This may be achieved directly or indirectly through circadian disruption of epigenetic events and/or alterations to the observed daily rhythms in the expression levels of neurotransmitters and their receptors. Both pharmacological and behavioral interventions that affect the circadian system can influence disease progression and clinical outcome, suggesting a role for the circadian clock in the development and treatment of psychopathology. HPC = hippocampus; LHb = lateral habenula; VTA = ventral tegmental area; SCN = suprachiasmatic nucleus.

Daniel Buysse: This is the challenge, as defining phenotypes is a critical issue. One reason that bipolar disorder is a good place to start is because it has a more specific phenotype than MDD. Other potential phenotypes include seasonal variation, response to sleep deprivation or light, and different circadian activity patterns.

Michael Hastings: It is not all about one clock or the SCN, but about other clocks. The paper from LeGates and colleagues shows that the melanopsin pathway can affect depression independent of the SCN.[157] Melanopsin innervates other clocks besides the SCN, and indeed, the SCN might be irrelevant to this process. Have we been looking at the wrong circadian clock?

Ellen Frank: . . . neither speaker has said anything about ketamine, but could it be relevant to the topic?

Megan Hagenauer: The recent review by Bunney and Bunney[158] discussed evidence that ketamine may be acting in part via circadian mechanisms. I would like to note that . . . the animal literature actually provides clear evidence that circadian disruption produces depressive behavior . . . according to work by McClung and colleagues,[159] it is possible to directly modulate clock gene expression in the ventral tegmental area and produce mania. This again suggests that we may be looking in the wrong part of the brain.

Summary

Figure 3 shows the synthesis of the many ideas presented at the first INSPIRE conference. It depicts the complex interaction among genes, sleep, and the environment that converges on the circadian clock to increase an individual's susceptibility to developing psychopathology. Genetic and environmental factors can act synergistically, independently, or through possible influences on sleep quality, sleep duration, and chronotype to disrupt normal circadian clock function. This disruption may result in a vulnerable clock that is less robust to further perturbations across the lifespan and at critical time windows in development. As there is growing interest and attention being given to molecular clocks that reside outside the SCN, and in brain areas known to be involved in mood disorders, altered clock function may disrupt normal brain development and function leading to psychopathology. Nonpharmacological interventions that directly modify the circadian clock are a promising therapeutic alternative or add-on for psychiatric treatment. Furthermore, many currently prescribed psychopharmacological interventions have known actions on the circadian system that may be responsible, in part, for their therapeutic efficacy and mechanism of action. Collectively, the circadian clock acts as an important interface that synchronizes our biological systems to a dynamic external environment. Understanding how this is achieved at the molecular level will be an important step forward for the field and will provide much needed insight into how the circadian system affects brain function and development in both health and disease.

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Conflicts of interest

Dr. Frank is a member of advisory boards for Servier, has received honoraria from Servier and Lundbeck, and royalties from Guilford Press. She has a founder's interest in Psychiatric Assessments, Inc. Drs. Sidor, Gamble, Cirelli, Sharkey, Hoyle, Tikotzky, Talbot, McCarthy, and Hasler have no conflicts of interest to report.

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