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Application of an *ex vivo* cellular model of circadian variation for bipolar disorder research: a proof of concept study

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Abstract

Objectives—Disruption of circadian function has been observed in several human disorders, including bipolar disorder (BD). Research into these disorders can be facilitated by human cellular models that evaluate external factors (zeitgebers) that impact circadian pacemaker activity. Incorporating a firefly luciferase reporter system into human fibroblasts provides a facile, bioluminescent readout that estimates circadian phase, while leaving the cells intact. We evaluated whether this system can be adapted to clinical BD research and whether it can incorporate zeitgeber *challenge paradigms*.

Methods—Fibroblasts from patients with bipolar I disorder (BD-I) (n = 13) and controls (n = 12) were infected *ex vivo* with a lentiviral reporter incorporating the promoter sequences for *Bmal1*, a circadian gene to drive expression of the firefly Luciferase gene. Following synchronization, the bioluminescence was used to estimate period length. Phase response curves (PRC) were also generated following forskolin challenge and the phase response patterns characterized.

Results—Period length and PRCs could be estimated reliably from the constructs. There were no significant case–control differences in period length, with a nonsignificant trend for differences in PRCs following the phase setting experiments.

Conclusions—An *ex vivo* cellular fibroblast-based model can be used to investigate circadian function in BD-I. It can be generated from specific individuals and this could usefully complement ongoing circadian clinical research.

Keywords

biorythm; bipolar disorder; *Bmal1*; circadian; fibroblast

Circadian rhythms are defined as inherently autonomous variations in physiological and behavioral processes that have a period of approximately 24 hours. They are generated by

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‘clock’ genes with self-sustaining oscillations resulting from regulatory transcriptional/translational feedback loops (1). The molecular basis of circadian rhythms was first elucidated using model organisms. Genes mediating circadian variation were first identified in *Drosophila* (2–4). Several genes including *period* (*per*), *timeless* (*tim*), *double-time* (*dbt*/casein kinase 1), *dClock* (*dClk*), and *cycle* (*cyc*) constitute a transcription-translation feedback loop with posttranscriptional controls. Together with *Cryptochrome* (*Cry*) these “clock” genes produce proteins that interact to regulate the progression and timing of a single intracellular circadian oscillator. Mammalian orthologs of these genes participate in similar rhythms (5, 6). In the mammalian model, based mainly on studies of the mouse, two transcription factors called Bmal1 and Clock form heterodimers that accelerate the transcription of the genes *mPer1* and *mPer2* by binding to their E-box sequences (7). The proteins *mPer1* and *mPer2* are phosphorylated by casein kinase 1 delta (CK1δ) in the cytoplasm and then shuttle into the nucleus where they complex with *mCry1*, *mCry2* and inhibit the transcription of *mPer1* and *mPer2* by binding to Bmal1/Clock. The negative feedback ceases when *mPer1* and *mPer2* turn over (through ubiquitination and proteasome pathways), thus permitting the recurrent action of Bmal1/Clock to increase transcription of *mPer1* and *mPer2* again (8–11). Dynamic regulation in the spatial (i.e., nuclear/cytoplasmic) and temporal (production/degradation) domains thus produces a basic self sustaining feedback loop at the core of the synchronizable circadian clock (7, 12). Other processes supplement the feedback loop, e.g., post-translational modification and sumoylation of different components (13, 14). The net effect is a network of intergenic and intragenic loops (15). Though the molecular components and mechanisms are virtually identical in several cell types (16), the mammalian peripheral clocks are synchronized by the suprachiasmatic nucleus (17–19).

Molecular circadian rhythms must be able to respond to external stimuli (zeitgebers) to adjust to an organism’s environment. In *Drosophila*, environmental light/dark cycles regulate the circadian phase by affecting accumulation of the TIM protein (6, 20). *Drosophila* maintained in constant dark continues to show rhythmic behavior with a phase dictated by their prior experience in a light/dark cycle. Pulses of light reset the phase of these *free-running* rhythms. Pulses given between subjective dusk and midnight lead to phase delays, while advances are produced by pulses between subjective midnight and dawn (6). In mammals, light or melatonin can serve as zeitgebers. In cultured cells, chemical agents can reset the clock. The response to resetting agents is measured by the amount of time the clock is offset from its original phase. This change in phase (measured in hours) can be plotted against the original clock time; the resulting graph is called a Phase response curve (PRC). An extremely potent phase resetting agent would reset the clock to the same point regardless of the original phase; this is called a type 0 PRC. A weak resetting agent elicits more modest changes in clock phase; this is known as a type 1 PRC.

Dysfunction in circadian rhythms is the hallmark of diseases such as Familial Advanced Sleep Phase Syndrome (FASPS) (21) and Delayed Sleep Phase Syndrome (DSPS) (22, 23). Circadian dysfunction can also impact directly or indirectly on several aspects of human health, e.g., sleep disorders, metabolic disorders, heart disease, rheumatoid arthritis and depression (24–26). The inherent cyclical nature of BD also invites analogies to circadian variation (27). Patients experiencing acute episodes of BD (both episodes of mania and episodes of depression) manifest marked changes in diurnal activities such as sleep, activity, appetite, as well as the diurnal secretion of hormones (28–33). Persons with BD are at increased risk of a manic episode after significant life stress, particularly when the stress involves a challenge to the usual timing of their daily activities (34), or phase changes such as those due to time zone changes; sleep loss often immediately precedes the beginning of a manic episode (35–37). Normalization of sleep-related symptoms usually accompanies clinical stabilization and mood stabilizing drugs are known to modulate circadian rhythms

(38–40). Finally, treatment paradigms that directly affect the sleep-wake cycle (and probably circadian rhythms) such as bright light treatment, dark treatment and sleep deprivation are frequently effective in the treatment of mania and bipolar depression (41). Hence circadian abnormalities may be critically involved in the pathogenesis of BD, especially rapid cycling BD (42, 43).

Clinical research into circadian dysfunction relies on intensive sleep laboratory based studies, as well as evaluation of core body temperature and hormonal secretion (particularly cortisol), measures of behavioral diurnal rhythms using pen and paper tests, actigraphy and Ecological Momentary Assessment. Cellular models can usefully complement such studies if they can also reflect individual variation. Such models can potentially also be used to mimic the impact of external zeitgebers. Recently, there has been much interest in fibroblasts, as primary fibroblast cultures show robust cyclic expression of circadian genes *in vitro* (44, 45). Cultured fibroblasts can provide convenient cellular models as they are easy to obtain from minimally invasive skin biopsies and can be grown easily *in vitro*. A survey of transgenic mice with mutant *Per* and *Cry* alleles has shown the molecular oscillations of primary fibroblast cells recapitulates the behavioral oscillations, in most cases with an exaggerated difference in period length (45).

In the present study, we investigated a modified fibroblast system to model human circadian function, namely phase length and phase response to external stimuli. This assay allows continuous measurement of molecular circadian rhythms by using the promoter region of a circadian gene (*Bmal1*) to drive expression of the firefly Luciferase gene. Simply measuring the bioluminescence from synchronized cells containing this construct enables an assessment of molecular circadian cycling without sacrificing the cells (45).

Methods

Clinical studies

Following written informed consent, individuals with bipolar I disorder (BD-I) and control individuals were assessed at the University of Pittsburgh (Pittsburgh, PA, USA) as described (46). Briefly, consenting participants were interviewed using the Diagnostic Interview for Genetic Studies, a semi-structured interview schedule (47). This information was synthesized with available medical notes to assign consensus diagnoses. Skin biopsy samples were collected via 4-mm full thickness punch biopsies under local anesthesia. Data from additional control individuals were obtained as part of a study of Delayed Sleep Phase Disorder at Cornell University. These individuals were screened for psychiatric symptoms or diagnosis before undergoing an intensive sleep study and an identical biopsy technique was used to collect skin samples. The samples were collected at variable times during the day.

Laboratory studies

The biopsy samples were digested with Collagenase and primary culture initiated in T25 flasks. The cells were passaged three times before being plated in 6 well plates for the assay.

Each fibroblast cell line was infected with lentivirus expressing a *Bmal1*-luciferase reporter (45) and then split into individual 35-mm cell culture dishes. The cells were grown to confluence in each dish. These cultures were simultaneously synchronized by exposing cells to 1 μ m Dexamethasone for 30 min, washed and placed in Luciferin medium. Bioluminescence was measured for a minimum of five days. In most cases, the sample was infected with lentivirus at least twice and assayed with least three technical replicates for each individual. The initial response to synchronization with dexamethasone was discarded and four days of data were used in the circadian evaluation. The data for period length (time

between peaks), amplitude (height of peaks), and damping (rate of signal decay) was obtained by plotting luminescence for sequential time points (Fig. 1).

PRCs were also generated (Fig. 2). Each transfected fibroblast sample was split into eight dishes. These dishes were simultaneously synchronized with Dexamethasone (as above) and placed in Luciferin medium. Bioluminescence was measured continuously. After one peak of Luciferase expression, one dish was serially resynchronized every four hours across a 24-hour day by adding forskolin (0.3 μ M final concentration) with one dish serving as an unchanged control. Bioluminescence was measured throughout the resynchronization and for three more days afterwards. The time shift in peak Luciferase expression after forskolin stimulation was compared with the control well and a 24-hour PRC generated for each sample. The serial stimulation procedure was repeated with two separately infected cell lines derived from the skin biopsy of each individual. This assay was used to detect a difference in phase resetting ability of morning and evening chronotypes by (48).

The studies were approved by the Institutional Review Boards at the University of Pittsburgh, Rockefeller University and Cornell University.

Data analysis

Data editing was standardized; the first 12 hours of data were ignored due to the initial effects of resynchronization; and the quality of the subsequent data evaluated to determine the optimal number of days to use for analysis (estimate 3–6 days). This editing was identical for all samples, blind to clinical status. For each sample, 2–3 infections were assayed three times and the period length for an individual was estimated as the average of all these values. Luciferase traces for each sample were analyzed with the Clocklab program from Actimetrics (actimetrics.com) to calculate period length. PRCs were generated using the *Find Peak* function to identify the peak of Luciferase expression 24–48 hours after forskolin stimulation. The time of peak expression was subtracted from the peak expression in the control well for that sample, i.e., the well not incubated with forskolin. The change in peak expression was plotted against the time between the first peak of Luciferase expression and the time of forskolin stimulation to minimize the differences in the response of each individual to the initial Dexamethasone synchronization. PRCs are designated as either a weak (type 1) or strong (type 0), as illustrated in Figure 2. Individuals were categorized as having a type 1 PRC if all points on the PRC were < 8 and some points regressed towards zero at the crossover point between phase advance and phase delay. Individuals were categorized as having a type 0 PRC if the curve contained phase shifts > 8 hours with increasing values at the crossover point. Most samples could be placed in one of these categories, however some contained features of both types of PRCs and were categorized as *intermediate*.

Results

The demographic features of the cases and controls are listed in Table 1. Though there were no significant differences with regard to age, there were proportionately more women among the cases. There were no significant differences between BD-I cases and controls with regard to period length. The type 0 PRC phenotype was demonstrated by 6 out of 13 cases and 4 out of 12 controls, but there were no significant case–control differences (chi square = 4.7, 2 degrees of freedom, $p = 0.095$, see Table 1).

Discussion

We have demonstrated the feasibility of using a luciferase reporter based *ex vivo* cellular model to investigate circadian variation in BD-I. We successfully established fibroblast

reporter models from all tissue samples. It was feasible to estimate circadian period length and allied variables from the modified cell lines. Others have previously estimated period length among BD-I patients by assaying circadian gene transcripts from fibroblasts cell lines throughout a 24 hour period (49). The major advantage with our system is that cells need not be sacrificed; thus, estimates are based on the *same* cells sampled throughout a one week period. By estimating bioluminescence, the expense of repeated gene expression assays is also obviated. Fibroblast cells in culture have the added advantage of being disassociated from the transient biological confounds that can effect behavior, such as medications. Thus, fibroblasts provide a reliable model to measure the output of a particular individual molecular circadian clock. They allow for minimally invasive analyses, eliminate the need for extended and expensive stays in sleep laboratories and importantly, make it possible to isolate a specific behavioral aspect (circadian function) from several other symptoms and comorbidities. All the participants consented to the skin biopsy procedures, suggesting the viability of our approach for future studies. Thus, our model can usefully complement other methods to investigate circadian abnormalities in the clinic, such as self-report of behavioral circadian rhythms related to the timing with which various social, work, feeding and rest-related activities occur, actigraphic recording. Many of these variables have been utilized as proxies for estimating circadian rhythms. They suffer from one or more disadvantages: lack of accurate reflection of the central circadian clock, confounds related to lifestyle or other common factors, cost or inconvenience to participants. Even methods such as sampling of salivary cortisol or melatonin are subject to a variety of confounds and potential for missing data unless done in the laboratory, which itself represents a kind of confound relative to sampling in an individual's natural environment. Our cellular assay overcomes many of these hurdles and therefore marks a significant innovation for human circadian research.

The estimate of period length reported here is a cumulative output of all the components of circadian rhythm in that cell line. For example, the estimated period length is a 'read out' of the molecular components of the cell as well as the cell culture environment (temperature, media ingredients). Previous studies have shown that the relative difference in behavioral period length is preserved in cellular estimations of period length (45, 48). Through *in vitro* modifications (e.g., gene knockdown experiments), this system can enable evaluation of the functions of specific genes and thus provide estimates of important characteristics of the circadian clock. They can also enable an understanding of the factors that regulate the expression of such genes in humans.

The PRC experiments were designed to estimate the impact of quantifiable stressors and thereby could be used to investigate the effect of zeitgebers such as light. Using this model, it should be possible to test the hypothesis that the circadian rhythm in BD-I patients is more susceptible to external perturbations than control individuals. We observed that in cultured fibroblast cells from patients with BD-I, a type 0 (strong) PRC was somewhat more frequent than in normal controls. If replicated, the increased sensitivity of the molecular circadian clock to phase resetting stimuli may partially explain the altered sleep events at the molecular/cellular levels in individuals with bipolar disorder. Indeed, a circadian clock more sensitive to external zeitgebers could create a neural instability contributing to other aspects of bipolar disorder, such as changes in interest, energy, mood and sleep. Thus, individuals with BD, once exposed to 'incorrect' zeitgebers (such as light at the wrong time of day, or a meal or cognitive stimulation at an inappropriate circadian time) could encounter difficulties in resetting not only their sleep/wake cycle, but also the circadian rhythms of energy, interest and mood. We acknowledge that the circadian clock of individual subjects in their *milieu interior* cannot be modeled completely using our assays, but it does provide important insights. Our method also helps to analyze circadian phase setting responses to external stimuli for individual participants, without requiring expensive and time consuming sleep laboratory evaluations that, in the end, do not really replicate what the individual's response

might be in his or her natural environment. Our model circumvents confounding factors like medication effects. Further studies are required to evaluate correlations between estimates of free running period length from sleep laboratory studies and the current cell-based estimates. If present, the normative data from the control individuals will also be useful for research in other diseases.

In conclusion, we have adapted a luciferase reporter based system in human fibroblasts to model circadian function in relation to BD-I research. It provides several advantages over current estimates of circadian function, but is limited by the impact of *in vitro* conditions, as with any cellular assay. Further studies to rigorously evaluate this model system are justified.

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Fig. 1.

Representative circadian variation patterns. A typical pattern for circadian variation is shown. The luminescence was plotted against time. From these curves, several significant variables can be derived, including period length (time between peaks), amplitude (height of peaks), and damping (rate of signal decay)

Fig. 2.

Representative phase response curves (PRC). In (A), the strength of the pulse, or biological response to the pulse, is weak, and cannot ‘push’ the system across the singularity to the opposite phases of the cycle. Thus, the new phase is similar to old phase and the resetting is type 1. In (B), the limit cycle is the same as that shown in A; however, the strength of the input, or response to the stimulus, is stronger and, therefore, can push the system across the singularity to the opposite side of the limit cycle, which results in very large phase shifts or type 0 resetting.

Table 1

Demographic features and circadian period lengths for cases and controls

	N	Male/female	Age (years)	Period length (hours)	PRC type (0/1/2)
Cases	13	5/8	36.5 ± 10.5	26.3 ± 1.2	6/4/3
Controls	12	8/4	35.6 ± 11.5	25.6 ± 0.6	4/8/0

Quantitative variables presented as mean ± standard deviations. PRC = phase response curve, classified as *strong* (type 0), *weak* (type 1), or *intermediate* (2) (see Fig. 2).