# 4th Annual Center for Chronobiology Symposium: From Cells to Clinic, February 13-15, 2013

# Poster Abstracts

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**A transposon insertion allele of a novel gene, *crm*, blocks rhythmic output of the circadian clock in *Synechococcus elongatus* PCC 7942**

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The SasA-RpaA two-component system constitutes the major output pathway of the cyanobacterial Kai circadian oscillator.[1] RpaA is required for global transcription rhythms and appears to lie at the convergence of multiple outputs from the circadian clock.[2] To date *rpaA* is the only gene, other than *kaiA, kaiB*, and *kaiC* which encode the oscillator itself, whose mutation causes completely arrhythmic gene expression. We have identified a novel transposon insertion allele in a small ORF, named *crm* (circadian rhythmicity modulator), located immediately upstream of *rpaA* in *Synechococcus elongatus* PCC 7942 which results in arrhythmic promoter activity but does not affect steady-state levels of RpaA. Translation of the *crm* ORF, expressed in *trans*, is necessary for complementation of the defect, indicating that *crm* encodes a small protein. KaiC retains low-amplitude cyclic phosphorylation rhythms in the absence of RpaA, whereas cycling is not detectable in the *crm* insertion mutant. Taken together, these results are consistent with the hypothesis that the Crm polypeptide modulates a circadian-specific activity of RpaA. In comparison to wild-type, *in vivo* levels of phosphorylated RpaA measured over time are significantly altered in the *crm* insertion mutant and in the absence of KaiC. In addition, phosphorylated RpaA is present *in vivo* in the absence of SasA and that the wild-type RpaA phosphorylation cycle displays differential oscillation in constant light vs. light:dark conditions, suggesting a regulation of RpaA activity distinct from the central oscillator. Our results are consistent with a network model of the clock wherein parallel input and time signals are integrated via the dynamic multiprotein clock complex.

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## Molecular inheritance of the cyanobacterial circadian clock

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The timing of cell division is coordinated with other cyclic events of other periodicities in the lives of cells. From bacteria to algae to regenerating liver cells of mammals, the circadian biological clock controls the time of day during which cell division can occur[1, 2]. The mechanism and function of this time restriction of cell division, referred to as gating, and is poorly understood in any system. The circadian control of cell division in cyanobacteria provides an opportunity to assess both how and why these processes are interlocked. The model organism *Synechococcus elongatus* is a unicellular cyanobacterium for which genetic manipulation is simple, circadian rhythms of gene expression are readily measured, and extensive genetic tools are available. Although *S. elongatus* can divide once or more during a single circadian cycle, the fidelity of the clock is remarkably stable and inherited with perfect phase from mother to daughter cell[3]. This project aims to understand how and why the circadian clock regulates cell division and how the central oscillator components, encoded by the *kaiA*, *kaiB* and *kaiC* genes, are inherited by daughter cells during cell division. We present evidence for the identification of gate-bypass mutants, capable of dividing despite an inhibitory signal from the clock, and address the specific localization of the Kai proteins during the circadian and cell cycles. Through the use of functional fluorescent fusion proteins, we find that KaiA and KaiC localize as discrete foci at or near the poles of cells. Specifically, we find that KaiA focus formation is dependent on *kaiC*$^+$ and co-localizes with KaiC. Additionally, KaiC focus formation is both dynamic and temporally regulated throughout the day. While KaiC focus formation does not appear to be dependent on known clock genes, we find that it is influenced by components of the input pathway. Taken together, these data suggest that spatial regulation of the Kai oscillator may be important for clock function. Ongoing investigations related to the consequences of bypassing the cell division gate and the segregation of the Kai proteins during cell division will enlighten our understanding of the relationship between the cell and circadian cycles within the three-dimensional architecture of intact cells.

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## Time in the cell: a plausible role for the plasma membrane

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All cells must keep time to consistently perform vital biological functions. To that end, the coupling and interrelatedness of diverse subsecond events in the complex cellular environment, such as protein folding or translation rates, cannot simply result from the chance convergence of the inherent chemical properties of these phenomena, but may instead be synchronized through a cell-wide pacemaking mechanism. The possibility of picosecond vibrations of lipid membranes playing a role in such a mechanism will be discussed.

**A new chronobiological approach to discriminate between acute and chronic depression using peripheral temperature, rest-activity, and light exposure parameters**

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**ABSTRACT**

**Background:**

Circadian theories for major depressive disorder have suggested that the rhythm of the circadian pacemaker is misaligned. Stable phase relationships between internal rhythms, such as temperature and rest/activity, and the external day-night cycle, are considered to be crucial for adapting to life in the external environmental. Therefore, the relationship and possible alterations among (i) light exposure, (ii) activity rhythm, and (iii) temperature rhythm could be important factors in clinical depression. This study aimed to investigate the rhythmic alterations in depression and evaluate the ability of chronobiological parameters to discriminate between healthy subjects and depressed patients.

**Methods:**

Thirty female subjects, including healthy subjects, depressed patients in the first episode, and major recurrent depression patients. Symptoms were assessed using scales. (Hamilton Depression Scale; Beck Depression Inventory; Montgomery-Äsberg Scale) Motor activity, temperature, and light values were determined for 7 days by actigraph, and circadian rhythms were calculated.

**Results:**

Depressed groups showed a lower amplitude in the circadian rhythm of activity and light exposure, but a higher amplitude in the rhythm of peripheral temperature. The correlation between temperature and activity values was different in the day and night among the control and depressed groups. For the same level of activity, depressed patients had lowest temperature values during the day. The amplitudes of temperature and activity were the highest discriminant parameters.

**Conclusions:**

These results indicate that the study of rhythms is useful for diagnosis and therapy for depressive mood disorders.

**Key Words:** depression, temperature, activity, light, circadian rhythm

**References**

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MPH received financial support from CNPq and GD received financial support from CAPES/PNPD. TC and ADN received financial support from the Ministerio de Educación y Ciencia (Spain) (project: BFU2008-00199).

# Circadian Clock Gene Expression Rhythms in Cells from Patients with Bipolar Disorder Differ by Genotype and Clinical Features in Response to Lithium

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Background: Bipolar disorder (BD) is associated with abnormal circadian rhythms, leading to the hypothesis that variation in genetic components of the circadian clock ("clock genes") may contribute to some of the pathological features of BD. In responsive patients, lithium (Li) corrects illness related changes in mood and activity, and reduces the risk for suicide. Li also affects circadian rhythms and the expression of clock genes. Variants in clock genes have also been associated with the clinical response to Li, but the extent to which mood and rhythms share common biological mechanisms is unknown, and to date there have been few direct measurements of clock gene function in samples from BD patients. Hence the role of clock genes in BD and Li treatment remains unclear. Methods: Skin fibroblasts obtained from subjects with BD (N=19) or healthy controls (N=19) were cultured to confluence. DNA from the cells was genotyped for 13 SNPs in clock genes with prior evidence of association to mood disorders. Cells were infected with lentiviral Per2::luc, a bioluminescent reporter gene that is expressed rhythmically under the control of the circadian clock. Gene expression rhythms were measured in a luminometer for 5 days in cells that were either untreated or treated with Li (1 mM, 10 mM) continuously for 7 days. For all cell lines, rhythm parameters (period, amplitude, and goodness of fit) were established under Li treated and untreated conditions. A genetic analysis was conducted to evaluate how rhythm parameters vary in cases and controls among subjects harboring clock gene variants. A case-case analysis was conducted in BD subjects to identify rhythm differences associated with clinical features (dysphoric mania, substance use, family history, and suicide history). Results: In untreated cells, BD cases had slightly longer periods than controls. In all cell lines, Li affected Per2::luc expression rhythms, but the magnitude of change in rhythm parameters after Li differed between BD and controls. At 1 mM, Li increased amplitude in controls, but on average, failed to do so in BD cases. At 10 mM, Li reduced rhythm amplitude equally in cases and controls, and lengthened rhythm periods. The magnitude of period lengthening was greater in controls than in BD cases. Genetic variants in GSK3 Beta, RORA, and PER3 were associated with rhythm differences. Case-case analyses revealed that BD subjects with a history of suicide were more sensitive to the effects of Li 1mM.

# Simultaneous imaging of intracellular $Ca^{2+}$ and PER2 expression in dissociated SCN neurons

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Daily oscillations in mammalian physiology are controlled by the suprachiasmatic nucleus (SCN). In the SCN, various components of cell physiology are rhythmic, including clock gene expression, neuronal firing rate, intracellular $Ca^{2+}$, cAMP, and neuropeptide release[1,2]. Recent work indicated that an intracellular $Ca^{2+}$ oscillation wave spreads across the SCN slice culture with a topographical pattern similar to that seen in clock gene expression[3]. To delineate the causal relationship between circadian clock gene expression and intracellular $Ca^{2+}$ rhythms, we imaged these parameters simultaneously in dissociated SCN neurons. For simultaneous imaging, a genetically encoded fluorescent $Ca^{2+}$ indicator, GCaMP3[4], was introduced by viral vector into dissociated SCN neurons obtained from PER2::LUC knockin mice[5]. Of 93 neurons observed, we found that 82% had rhythmic PER2 expression and 30% had rhythmic $Ca^{2+}$ levels. All neurons with rhythmic $Ca^{2+}$ also had rhythmic PER2 expression. Peak $Ca^{2+}$ level preceded peak PER2 expression by an average of 7.2 h, with a large range of 0 to 11 hours. No SCN astrocytes had rhythmic $Ca^{2+}$ levels. Application of tetrodotoxin, a voltage-gated sodium channel blocker, did not significantly affect either PER2 expression or $Ca^{2+}$ rhythms. Application of cycloheximide, a protein synthesis inhibitor, abolished both PER2 expression and $Ca^{2+}$ rhythms. These results suggest that the $Ca^{2+}$ rhythm in SCN neurons is dependent on translation-transcriptional feedback loops of clock genes.

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## The Active Output State of the *Synechococcus* Circadian Oscillator

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Circadian oscillators are ubiquitous systems that evolved to anticipate daily environmental changes (1,2). In cyanobacteria, the oscillator is composed of KaiA, KaiB, and KaiC, proteins whose structural and biochemical features have been described in detail. The timekeeping aspect of this oscillator involves a progression of phosphorylation states at Thr432 and Ser431 of KaiC. Subsequently, the oscillator output activity (*OOA*) transmits this information to modulate expression for the majority of cyanobacterial genes (3). To better elucidate these distinct activities, we developed a simple metric that does not require additional knowledge of the number of output components, the structure of their network, or their regulatory effects. The expression of reporter strains that carry wild-type or phosphomimetic-encoding alleles of KaiC was compared to that in strains lacking a functional clock, and the difference between them was used to calculate a value of *OOA*. In the absence of the clock, expression of the class I paradigm promoter $P_{kaiBC}$ was locked at its maximal level; conversely, that of the class II paradigm promoter $P_{purF}$ was locked at its trough level. For both classes of promoters, peak *OOA* in wild-type strains coincided late in the circadian cycle near dawn (CT= 24 hours), at which time Ser431 is phosphorylated and Thr432 is not (KaiC-pST). Analogously, peak *OOA* was detected specifically for the phosphomimetic allele of KaiC-pST (KaiC-ET) for both classes of promoters. Thus, we conclude that KaiC-pST is the critical phosphorylation substate of KaiC that drives *OOA*.

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## A slow conformational switch regulates BMAL1 activity

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Mammals have a cell-autonomous molecular clock that synchronizes diverse physiological processes into rhythms that coincide with the solar day, providing enhanced evolutionary fitness by coordinating timing of integrated processes and optimizing energy utilization. Disruption of the clock leads to metabolic syndromes, cancer, and premature aging by eliminating the temporal coordination of physiology with our behavior and the external environment. By developing a structural and mechanistic understanding of our molecular clock, we hope to identify new and innovative strategies to treat a broad spectrum of human diseases. This project aims to identify the structural basis for transcriptional regulation by the primary circadian transcription factor, CLOCK:BMAL1, which drives dynamic transcriptional regulation of the genome on a daily basis. A recent crystal structure of the DNA binding and tandem PAS (Per-Arnt-Sim) domains of the CLOCK:BMAL1 heterodimer[1] illustrates how it assembles into a functional dimer, but lacks the partially structured regions downstream that dynamically regulate its activity. Using solution NMR spectroscopy, we identified a slow conformational switch in the BMAL1 C-terminal transactivation domain mediated by *cis*/*trans* isomerization of a conserved Trp-Pro backbone imide bond. Here we demonstrate how this slow timescale conformational switch potentially contributes to regulation of the CLOCK:BMAL1 complex by controlling binding of activators and repressors. By identifying factors that influence the temporal balance of activator and repressor recruitment to CLOCK:BMAL1, we will contribute to our understanding of how the intrinsic 24-hour period of the mammalian circadian clock is generated.

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## Estimating Confidence Intervals in Predicted Responses for Circadian Models

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Deterministic models of circadian rhythms often consist of a set of ordinary differential equations, describing a network of biochemical reactions, and unknown kinetic parameters, chosen such that the model best captures experimental data[1]. Such models are often used to predict the response of the circadian clock to various inputs, validating the model and providing mechanistic insight. However, since a model's parameter values are uncertain, and since input sensitivities are highly parameter-dependent, it is difficult to assess the confidence associated with these *in silico* predictions[2]. In particular, circadian models must be fit with computationally expensive global optimization routines, and cannot take advantage of existing measures of identifiability[3]. In this study, we employ an efficient parameter estimation technique to enable a bootstrap uncertainty analysis for limit cycle models. In the method, we use a multiple shooting approach to convert the highly discontinuous global optimization problem into one that can be solved via local methods, such as nonlinear programming. With the aid of increasingly available time-series data on mRNA and protein activity, a suitable initial guess can be found that greatly speeds convergence.

In a bootstrap method, parameter optimizations are performed on multiple sets of experimental data to estimate errors in parameter values. Since the amount of data needed for a bootstrap analysis is impractical to collect for biological systems, we expand experimental data into 2000 independent data sets, using confidence intervals in experimental measurements. Since the primary role of circadian models is the insight they provide on responses to rate perturbations, we extend our uncertainty analysis to include first order sensitivity coefficients. By examining the resulting distributions, it is possible to determine which predictions have a practically identifiable direction.

Using a literature model of circadian rhythms, we show how predictive precision is degraded with decreasing sample points and increasing relative error[4]. Additionally, we show how this method can be used for model discrimination by comparing the output identifiability of two candidate model structures to published literature data[5]. Our method permits modelers of circadian rhythms to confidently show that a model's dynamic characteristics follow directly from experimental data and model structure, relaxing assumptions on the particular parameters chosen. Ultimately, this work highlights the importance of continued collection of high-resolution data on gene and protein activity levels, as they allow the development of predictive mathematical models.

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## Stochastic Approximations in Computational Modeling of *Cry1* Knockout SCN Neurons

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A novel role of the CRY1 and CRY2 isoforms in the core clock mechanism was recently uncovered. Each isoform is part of its own negative feedback loop, with nuclear import being rate-limited by the amount of PER available for heterodimerization[1]. Knocking out one isoform while leaving the other functional leads to period shortening in CRY1 knockouts, and period lengthening in CRY2 knockouts, respectively.

Additionally, single uncoupled *Cry1*$^{-/-}$ SCN neurons tend to oscillate arrhythmically, while *Cry2*$^{-/-}$ neurons stay rhythmic[2]. We evaluate if this may simply be an effect of lower nuclear molecule numbers resulting from knocking out CRY1, and wild type CRY1 abundance being higher than CRY2 abundance. To this end, a recent single-cell ODE model of the dual CRY loops[1] is extended into a stochastic version and fit to experimental data. The rhythmicity of single SCN neurons with CRY1 knocked out can however be restored when allowing intercellular coupling[3]. We attempt to reproduce this effect in the stochastic model by implementing a simple version of mean-field coupling.

Finally, on the theoretical side, we expand the mathematical foundations for stochastic model reduction. The Michaelis-Menten approximation from enzyme kinetics is widely used for reducing deterministic ODE model complexity. When developing stochastic models it was commonly assumed to hold under the same conditions, this assumption was however only recently formally proven[4]. The same research also noted that introducing the Michaelis-Menten approximation would overestimate the variance of stochastic noise in a system. We employ the recently-developed ssLNA[5] in conjunction with the classic LNA to give an upper bound to variance overestimation at steady state as well. Creation of reduced stochastic models that use less states and parameters (and thus data) but still capture the system dynamics of interest is still a “subtle business”[6]. This highlights the need for further development of stochastic model reduction techniques.

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