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Understanding Circadian Mechanisms of Sudden Cardiac Death – A Report from the National Heart, Lung, and Blood Institute Workshop, Part 1: Basic and Translational Aspects

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Abstract

Sudden cardiac death (SCD), the unexpected death due to acquired or genetic cardiovascular disease, follows distinct 24-hour patterns in occurrence. These 24-hour patterns likely reflect daily changes in arrhythmogenic triggers and/or the myocardial substrate caused by day/night rhythms in behavior, the environment, and/or endogenous circadian mechanisms. To better address fundamental questions regarding the circadian mechanisms, the National Heart, Lung, and Blood Institute convened a workshop, “Understanding Circadian Mechanisms of Sudden Cardiac Death.” We present a two-part report of findings from this workshop. Part 1 summarizes the workshop and serves to identify research gaps and opportunities in the areas of basic and translational research. Among the gaps noted was a lack of standardization in animal studies for reporting environmental conditions (e.g., timing of experiments relative to the light dark cycle or animal housing temperatures) that can impair rigor and reproducibility. Workshop participants also pointed to uncertainty regarding the importance of maintaining normal circadian rhythmic synchrony and the potential pathological impact of desynchrony on SCD risk. One related question raised was whether circadian mechanisms can be targeted to reduce SCD risk. Finally, the experts underscored the need for studies aimed at determining the physiological importance of circadian clocks in the many different cell types important to normal heart function and SCD. Addressing these gaps could lead to new therapeutic approaches/molecular targets that can mitigate the risk of SCD not only at certain times but over the entire 24-hour period.

Keywords

Circadian Rhythm; Sudden Cardiac Death; Basic Science; Translational Science; NHLBI; Workshop; Chronobiology

Subject terms:

Arrhythmias; Electrophysiology; Basic Science Research; Translational Studies

Introduction

The National Heart, Lung, and Blood Institute (NHLBI) convened a workshop, *“Understanding Circadian Mechanisms of Sudden Cardiac Death,”* in June 2019. The participants included experts in basic, translational, and clinical research in cardiovascular physiology, sleep disorders, chronobiology and neuroscience, and they represented academic

institutions, as well as federal and non-federal agencies. Their charge was to identify high priority research gaps and future opportunities for delineating circadian mechanisms contributing to sudden cardiac death (SCD).

Objectives:

1. Determine knowledge gaps and barriers in basic and translational research that, if overcome, would expedite understanding of the role of the circadian system and its regulation/dysregulation in the pathophysiology of SCD, in order reveal potential targets to lessen the occurrence of SCD.
2. Identify basic science findings that are ready for translation into clinical research.
3. Provide opportunities to augment advancement of basic and translational research that facilitate the development of novel and effective therapies to prevent SCD.

SCD is sudden, unexpected death due to abrupt loss of heart function that occurs in the setting of genetic and acquired cardiovascular diseases, including coronary vascular disease, congenital heart disease and ion channelopathies.[1, 2] SCD and arrhythmogenic events can exhibit a 24-hour pattern of occurrence in cohorts of people with acquired or genetic forms of heart disease.[3–9] The 24-hour pattern suggests that the likelihood of SCD and life-threatening events at specific times of day could be influenced by aspects of usual day/night rhythms (Table) in behaviors, environmental changes, and internal circadian changes (e.g., circadian rhythms) (Figure 1).[10]

The overall risk for SCD is understood by looking at the complex interaction between arrhythmogenic triggers and changes in the myocardial substrate. Triggers include ischemia, neurohumoral signaling, electrolyte imbalance, medications, behaviors (e.g., physical activity or sleep), and factors affecting the autonomic nervous system. Pro-arrhythmic changes in the myocardial substrate are thought to arise from genetic or acquired cardiovascular disease that results in impaired cardiac and coronary artery blood flow, ion channel function, Ca^{2+} handling, and cell-to-cell coupling, as well as increases in heart size, myocardial fibrosis, scarring and/or myocardial inflammation.

Day/night behavioral, environmental and circadian rhythms generate variations in arrhythmogenic triggers across the 24-hour cycle. Theoretically, in healthy individuals, daily spans of increased vulnerability are offset by corresponding protective changes in cardiac responsivity to prevent sustained arrhythmias. However, stressors could increase the risk for arrhythmias at specific spans during the 24-hour cycle if they increase the relative amplitude in arrhythmogenic triggers, alter the cardiac substrate to cause a loss of protection or desynchronize/misalign (Table) trigger and protection cycles (Figure 2).[10]

Many fundamental questions remain unanswered regarding which circadian mechanisms impact cardiovascular health and disease. Organisms evolved circadian rhythms to prepare the body in anticipation of predictable changes in the environment (e.g., daily cycles of light and darkness, and resultant temperature cycles, as well as predictable rhythms in predator, prey, mate, nutrient, and UV radiation cycles). These endogenous rhythms are generated

by autonomous circadian clocks, present in nearly every cell in the body, that resonate with a periodicity of ~24 hours.[11] Circadian clocks are formed by interlocking transcription-translation feedback loops that drive rhythmic changes in clock gene and protein expression (Figure 3).[12, 13] Although ubiquitous, clock systems are organized hierarchically with a specialized role for the neurons in the central pacemaker, the suprachiasmatic nucleus (SCN) in the hypothalamus. Thus, the SCN regulates the daily alignment between rhythms in physiology, behavior and the environment. Its function is considered important for synchronizing the circadian clocks in all the cells, tissues, and systems throughout the body.[14, 15]

Daily oscillations have been characterized in the expression of clock genes in cells of the cardiovascular system in animal models and in humans.[16–19] *In vitro* studies demonstrate that circadian clocks drive biological time-dependent oscillations in circadian clock components as well as “clock output genes” (Figure 3). In doing so, circadian clocks modulate fundamental biological processes in the cardiovascular system, including cardiac metabolism,[20] inflammation,[21] and electrophysiology,[4, 22, 23] as well as the responsiveness of the cells/tissues to stimuli and insult. The circadian clocks also modulate the severity of myocardial damage in response to ischemia.[24]

The workshop participants developed a two-part report that presents findings organized in two categories: gaps and opportunities in basic and translational research (Part 1) and gaps and opportunities in population and clinical research (Part 2).

Basic and Translational Gaps

Recent studies demonstrate that the circadian clocks in cardiomyocytes can regulate critical processes that alter arrhythmogenic triggers and the myocardial substrate to impact arrhythmia susceptibility. These processes include excitation-contraction coupling, Ca^{2+} handling, ion channel expression/activity, signal transduction cascades, metabolic status, and chromatin dynamics.[25] A clearer picture is required to understand the function of circadian clocks in cells critical for normal heart physiology, including different types of cardiomyocytes, endothelial cells, fibroblasts, immune cells, platelets, and neurons (central, peripheral, and intrinsic cardiac neurons). Specific areas of focus should include studies to determine the importance of normal circadian synchrony/alignment and the potentially pathologic impact of circadian disruption at the cellular, tissue, organ, and organismal levels. Several challenges to exploring these questions are rooted in the limitations of both animal and cellular models. To address these challenges, we identified three basic/translational science gaps that could be targeted to expedite advances in our understanding of the circadian system and promote translation into therapeutic strategies to prevent SCD.

Gap 1: The lack of standardization of animal models and approaches in considering day/night rhythms in experimental measures or design can impair rigor and reproducibility.

Opportunity: Consider time of day as a biological variable in such studies.

Rigorous consideration and reporting of chronobiology variables (e.g., biological time of day (Table) and temperature) in cardiovascular studies offers the promise of enhancing

reproducibility across a wide range of studies and uncovering heretofore unknown levels of regulation important in cardiovascular health, including cardiovascular disease states that predispose to SCD.

The biological time of day refers to the timing of experiments relative to the light/dark cycle, which is a key, but often unreported, component needed to ensure rigor and reproducibility. Most studies using nocturnal rodent models perform experiments (e.g., functional testing, monitoring, tissue harvesting, etc.) during the subjective daytime because it is convenient. However, the subjective daytime corresponds to the biological rest phase in nocturnal rodents. As such, extrapolating findings from these studies to diurnal species may be especially limited given that many physiological parameters, such as autonomic tone, are strongly influenced by the sleep-wake cycle.[10] Similarly, endogenous regulation of many physiological processes, ranging from gene transcription to posttranslational signaling, are under control of endogenous circadian clocks.[12, 13, 26] Thus, when sampling is limited to the biological rest phase, what remains unknown is whether key variables exhibit different properties when measured during the biological active phase. For example, we know that mitochondrial respiration exhibits strong rhythmic variation under control of the cell autonomous clock.[27] Thus, any analyses of bioenergetic processes are incomplete without observation and measurements across the entire circadian or 24-hour light-dark cycle. Another important variable for rigor and reproducibility that impacts autonomic balance is the temperature at which animals are housed. Studies show cardiac sympathetic tone dominates the control of heart rate in mice housed at room temperature (21–23ºC). However, these temperatures are well below their thermoneutral zone (~30ºC), and in the absence of cold stress, the vagal tone is an important determinant of mouse heart rate control.[28, 29]

> Opportunity: Establish guidelines and best practices for reporting and controlling day/night variables in animal studies of SCD and cardiac arrhythmias.

Mice are ideal for genetic manipulation of critical clock genes, which makes it possible to study the impact of altering the endogenous circadian clock. They are also useful for determining how the clock interacts with environmental/behavioral rhythms (e.g., manipulations in the light cycle, activity, and feeding behavior). Determining the impact that genetic and behavioral manipulations have on key physiological variables that promote vulnerability to SCD, especially according to circadian time, is valuable.

In standard laboratory animal housing conditions, animals are typically maintained in a controlled environment with 24-hour light-dark cycles (e.g., 12-hour span of light and 12-hour span of darkness) to approximate the animals' intrinsic rest-activity cycle. The animals entrain to the imposed 24-hour cycle. However, in order to determine whether an endogenous behavioral, physiological or molecular process displays circadian properties and/or whether a given experimental manipulation alters circadian rhythms, formal testing must be performed under free-running conditions (conditions of constant darkness). For mice, this requirement means completing assays under constant darkness for at least two days. Under free-running conditions, the endogenous period (duration) of the central clock cycle in the SCN can be easily detected through analysis of daily rhythms in behavior, core body temperature, hormone levels, and other measurements.[26] The endogenous cycle length

of the SCN in the mouse is somewhat shorter than 24 hours[12, 13] and displays an advanced phase of the behavioral/physiological outputs. The standard representation of circadian timing involves plotting daily wheel running activity (or other behavioral/physiological/molecular rhythms), often with each day double-plotted on the abscissa and stacked along the ordinate to produce a temporal raster plot or "actogram" (Figure 4).

Clocks in peripheral tissues or cell culture can similarly be monitored directly, *ex vivo*, using genetic reporters to interrogate endogenous molecular rhythms (Figure 4).[26, 30] At the cellular and tissue levels, there are several stimuli that can entrain cells/tissues *in vitro* (e.g., the use of serum shock, glucocorticoids, temperature pulses, or forskolin). These stimuli enable the rigorous interrogation of discrete processes, such as tissue responses to hormones, drugs, or other bioactive substances at distinct phases of the 24-hour circadian cycle.[31] A further strategy involves analyses of animal models in which components of the canonical molecular clock activator limb and/or the repressor limb are selectively abrogated through conditional mutagenesis. The results from a number of such models clearly illustrate the potential of these strategies and are discussed below. Researchers fully recognize, however, that care must be taken in interpreting results obtained using engineered cells/tissues/animals to distinguish phenotypes. It is critical that efforts be made to discern potential similarities and differences in the impact of manipulating circadian cycles across different cells/tissues. A clear example of the positive impact of careful cross-tissue analyses was revealed with the demonstration that the selective ablation of clock activators within pancreatic beta cells leads to profound hypoinsulinemic diabetes. In contrast, ablation of the same clock activators in the liver causes fasting-induced hypoglycemia, reflecting tissue-specific actions of the circadian regulated anabolic and catabolic pathways.[32]

> Gap 2: There is uncertainty regarding the importance of maintaining normal circadian rhythmic synchrony and the potential pathological impact of desynchrony in terms of SCD risk. Can circadian biology be targeted to modify the risk of SCD?
>
> Opportunity: Determine how perturbations in circadian rhythms can impact SCD risk in animal models.

Circadian clocks influence a number of cellular processes through both transcriptional and post-transcriptional means. Unbiased transcriptomic approaches in animal models of genetic disruption of the cardiomyocyte circadian clock suggest that this mechanism temporally governs numerous biological processes critical for normal function, and possibly SCD in vulnerable populations.[33, 34] These processes include transcription, translation, signal transduction, metabolism, and transport (substrates, ions, etc.). Consistent with the diverse array of fundamentally important processes regulated by circadian clocks, it is not surprising that genetic disruption of circadian clock factors results in a severe dilated cardiomyopathy, reduced lifespan, and/or electrophysiologic abnormalities.[23, 34–36]

Due to its high level of redundancy (e.g., multiple isoforms exist for many circadian clock components), there are only limited examples of single genetic interventions that result in complete circadian clock disruption. Targeting the canonical molecular clock activator limb BMAL1:CLOCK heterodimer is a commonly employed strategy, either through deletion of BMAL1 or mutation of CLOCK (e.g., overexpressing a dominant negative CLOCK mutant)

(Figure 3), which can result in ‘temporal suspension’ with the clock-controlled processes often being ‘stuck’ at the beginning of the rest phase.[37, 38] The cardiomyocyte-specific BMAL1 knockout and/or CLOCK mutant mice has revealed clock control over cardiac signaling, metabolism, and electrophysiology.

Signaling.

Posttranslational modifications of specific proteins is often utilized as a marker of activation status of distinct signaling cascades, and many signaling components exhibit 24-hour rhythms in the heart. Examples include phosphorylation of AMPK, GSK3β, mTOR, ERK1/2, PKB, and PKA.[39] PKA activity appears augmented in the heart at the beginning of the active phase, while calcineurin (a Ca^{2+}-activated phosphatase, which reverses several PKA actions) is more active at the end of the active phase.[40] Oscillations in the activity of many of these signaling components are attenuated/abolished following genetic circadian clock disruption. However, a complex interplay between environmental/extracellular factors and cell autonomous clocks is apparent. Notably, circadian clocks have the capability to modulate sensitivity of the heart to extracellular stimuli (e.g., epinephrine, insulin, fatty acids) and stresses (e.g., ischemia/reperfusion) over the course of the 24-hour period.[41]

Metabolism.

Given the dramatic fluctuations in energetic demand and substrate supply associated with regular sleep/wake, rest/activity, and fasting/feeding cycles, it is not surprising that cardiac cellular metabolism is subject to circadian control. This control is observed at the levels of carbohydrate, lipid, and protein metabolism, as reviewed previously.[42] For instance, the beginning of the active period is associated with increased oxidative metabolism capacity (e.g., glucose uptake and subsequent oxidation is augmented at this time). During the latter half of the active phase, cardiac glycogen and triglyceride synthesis both increase (thereby replenishing fuel stores prior to the fast necessitated by the sleep episode). The beginning of the sleep episode is a time of cellular consistent turnover, exemplified by simultaneous activation of protein synthesis and degradation. Oscillations lasting 24 hours in cardiac cellular metabolism are attenuated/abolished following disruption of the cardiomyocyte circadian clock, indicative of governance by this endogenous timekeeping mechanism.

Electrophysiology.

Selective disruption of the circadian clock mechanism in adult mouse cardiomyocytes generates an electrophysiological phenotype (prolongation in the RR- and QT-intervals of the electrocardiogram) and increases arrhythmia propensity in the absence of any obvious structural remodeling.[23, 36] This increased propensity likely reflects changes in ion channel activity that is influenced by both post-translational modifications and the energetic status of the cell.[43] Moreover, the cardiomyocyte circadian clock mechanism directly and indirectly regulates the circadian and steady-state expression of several key ion channel transcripts. Disruption of the cardiomyocyte clock mechanism causes a selective downregulation in several ion channel transcripts and corresponding ionic currents that are known to be important for mouse and human cardiac electrophysiology.[44, 45]

Opportunity: Investigate the impact of timing for feeding, exercise, and drug administration on alleviating SCD risk.

Transgenic studies have helped to dissect the role of the circadian clocks on cardiac physiology and pathophysiology. However, in real life, underlying circadian rhythms should be considered in relation to their interactions with the ongoing behavioral and environmental cycles, and how these factors culminate to impact the susceptibility to SCD across the 24-hour span. The behavioral and environmental cycles include changes in timing of light exposure,[46–49] feeding behavior,[50, 51] activity and exercise,[52, 53] as well as medication administration. Light is the most well-recognized environmental time-keeping cue, but non-photic zeitgebers, such as timing of eating and activity (exercise and sleep), can also impact the phase and amplitude of circadian clocks, especially in peripheral tissues including the heart. Studies imposing desynchrony/misalignment of the light cycle or limiting the time of eating activity show profound impacts on metabolism, cardiac function, as well as the phase in the 24-hour rhythms in heart rate, the QT interval, and blood pressure.[46, 51, 54] New studies should interrogate how changes in behavior, or the biological timing of behaviors, as well as the timing of pharmacotherapy, hold promise for identifying new strategies to improve outcomes in patients at risk for SCD.

Gap 3: There is a lack of understanding regarding the contributions of circadian clocks in cells critical for normal heart function, and uncertainty regarding the molecular and physiologic rhythms at the cellular, tissue, and whole organism levels that are most relevant to SCD.

Opportunity: Determine the importance of circadian synchronization in central and peripheral clock signaling among cells and tissues relevant to SCD (including neuronal and cardiac cells).

Impaired autonomic control of the heart has been implicated in arrhythmogenesis and SCD in many acquired and genetic cardiac pathologies; yet scientists do not know whether autonomic and/or cardiac clock disruption is involved. Autonomic activity and circulating catecholamine levels vary substantially over the 24-hour period,[49] as do myocardial adrenergic receptor expression and responsiveness.[33, 55, 56] Presumably under normal conditions, adrenergic receptor density and sensitivity are optimized to respond to predicted oscillations in autonomic tone. Disruption in either neuronal or cardiac cells could lead to a misalignment in autonomic sensitivity and resulting cardiac responses. Augmented adrenergic responses in the heart are known to cause intracellular Ca^{2+} overload, as well as action potential heterogeneity, setting up the trigger and substrate for lethal arrhythmias. Further dissection of the circadian clocks in neurons and other cell types important for cardiac function (e.g., blood cells and platelets, adipocytes, endothelial cells, fibroblasts, resident cardiac stem cells) is an important future research step.

Additional studies to determine how selective disruption of circadian clocks in subsets of cardiomyocytes (e.g., sino-atrial node, atrio-ventricular node, Purkinje fibers, atrial cardiomyocytes and endocardial vs. epicardial ventricular myocytes) are also needed. Each cell type has distinct structural, contractile and electrophysiological properties, which are all at least partially influenced by clock-controlled genes, including those encoding extracellular matrix proteins, signaling components, Ca^{2+} handling proteins, and ion

channels. The potentially different roles of the circadian clock and the modulation of clock-controlled genes among different cardiomyocyte populations, both in normal and pathological settings, remain unknown. Likewise, whether there is a role for circadian signaling in establishing or maintaining cellular heterogeneity within the heart has yet to be explored.

> Opportunity: Apply contemporary genomic, biochemical, and molecular tools to interrogate circadian signaling (including transcription, translation, and post-translational modification) in cardiovascular cells and tissues relevant to SCD.

Thus far, most work in the field has focused on transcriptional control of a subset of genes regulating a specific function by specific core clock factors. However, scientists know that the expression of a large number of genes changes in a predictable pattern throughout the course of each 24-hour cycle in order to coordinate cellular and organismal physiology. The existence of this pattern raises fundamental questions as to *how* core clock transcription factors and/or tissue-specific circadian factors control the temporal dynamics of transcription. Recent technical advances have led to the development of biochemical and genomic tools that allow for detecting long-range chromatin interactions.[57–59] These studies reveal that rhythmic gene expression occurs within enhancer regions that often reside at great distances from the transcriptional start sites of oscillating genes. There are numerous important questions for the field in general, and cardiovascular biology in particular. How do core clock transcription factors and/or tissue specific clock-controlled genes regulate higher order genome structure to achieve temporal and spatial control of gene expression and attendant biological responses? Studies of cardiovascular cells are needed to characterize chromatin dynamics, epigenetic modifications, chromatin looping and spatial positioning across the circadian cycle. Furthermore, do external inputs impact chromatin epigenetics and dynamics and thereby alter circadian rhythms? Specifically, do hormonal, inflammatory, metabolic signals, and/or stresses alter clock-controlled chromatin dynamics in cells of the cardiovascular system *in vitro* and *in vivo*? Does manipulation of core clock factors and tissue specific circadian factors impact chromatin dynamics and physiology? Are key principles uncovered through such studies conserved in human tissue or inducible pluripotent stem cell derived cardiovascular cells? Can such insights be leveraged for therapeutic purposes?

The genomic, biochemical, and molecular approaches described above will be vital for subsequent investigations into circadian processes among and between cell types important for heart function. It will be important to determine whether different populations of cardiomyocytes maintain their circadian clock function in disease states such as myocardial infarction, hypertrophy, and heart failure, which are known to increase risk for SCD. Scientists still do not know whether pathological remodeling due to myocardial infarction or heart failure, for example, has the potential to cause circadian clock disruption among regional populations of cardiomyocytes. Causes for circadian disruption among populations of cardiomyocytes include changes in neurohumoral factors that elicit cell type specific responses, in blood flow and endocrine factor accessibility in ischemic heart disease, and in local autocrine/paracrine factor levels (e.g., during increased inflammation). Given the large number of circadian-regulated ion channels (reviewed in[25]), such clock disruption between

cardiomyocytes could contribute to repolarization heterogeneity and increased arrhythmia risk.

Conclusion

We know several day/night rhythms, including circadian, regulate critical processes that impact arrhythmogenic triggers and the vulnerability of the myocardial substrate. Many fundamental questions regarding the mechanisms by which 24-hour day/night rhythms and circadian clocks impact cardiovascular processes remain to be explored. We outlined several critical research gaps and opportunities that, when addressed, could lead to advancements that translate into improved treatment of people at risk for SCD. A clearer picture of the functional role(s) of circadian clocks in cells critical for normal heart function requires new investigations with an emphasis on natural circadian rhythmic synchrony and alignment, and the potentially pathologic impact of disruptions at the cellular, tissue, organ and organismal levels. The challenges to including chronobiology as part of basic and translational cardiovascular research are real, but addressing these challenges will help in advancing rigor and reproducibility, as well as our understanding of health, disease, and patient outcomes.

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Non-Standard Abbreviations and Acronyms

SCD	Sudden cardiac death
SCN	Suprachiasmatic nucleus
CLOCK	Circadian locomotor output cycles kaput protein
BMAL1	Brain and muscle ARNT-Like 1 protein
AMP	Adenosine monophosphate
AMPK	5’ AMP-activated protein kinase
GSK3β	Glycogen synthase kinase 3 beta
mTOR	Mammalian target of rapamycin
ERK1/2	Extracellular-signal-regulated kinase
PKB	Protein kinase B
PKA	Protein kinase A

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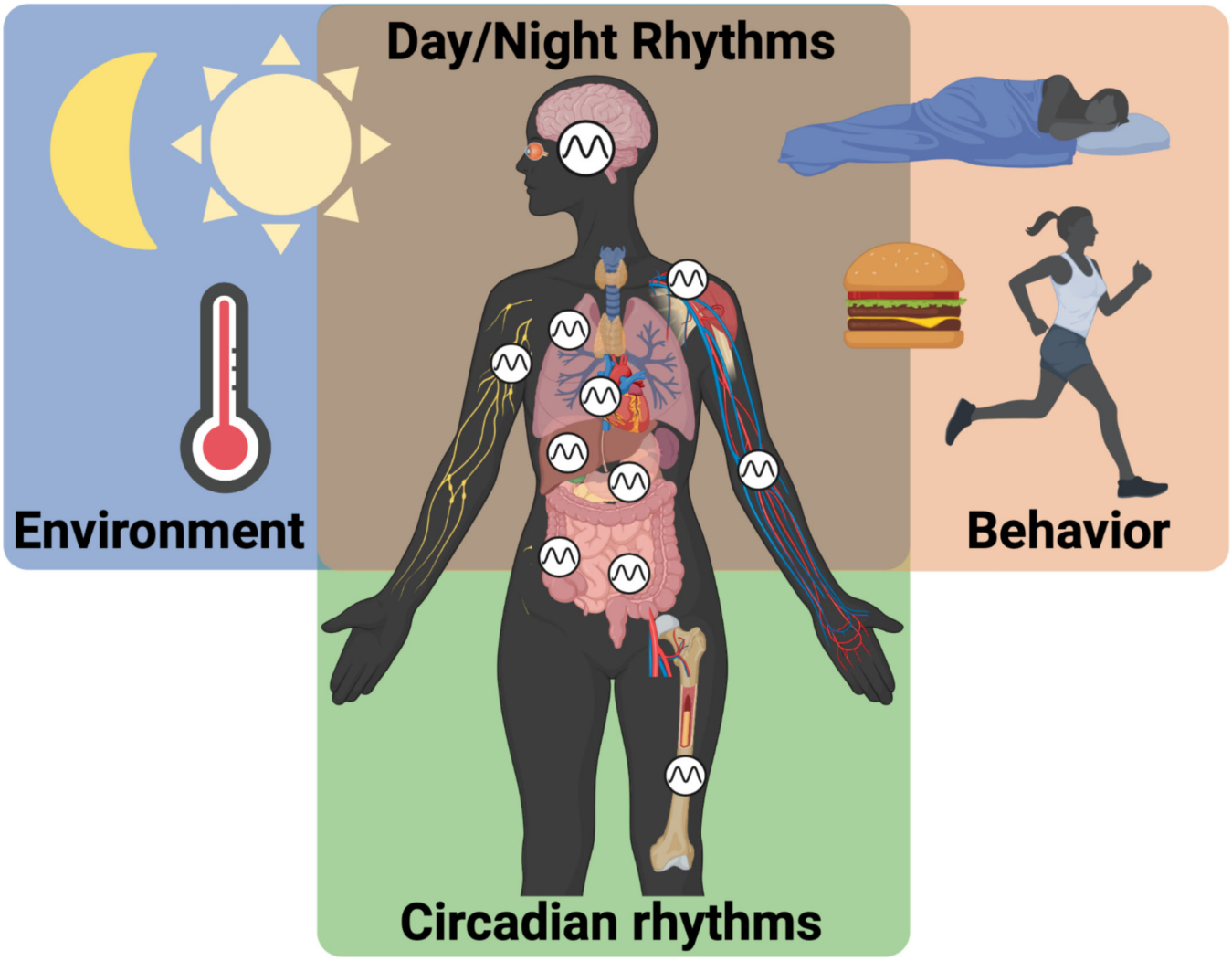

Figure 1. SCD and life-threatening triggers for arrhythmias can exhibit a 24-hour pattern of occurrence.

This 24-hour pattern suggests that the aspects of usual day/night rhythms, including 24-hour environmental changes (daily cycles in light, temperature and ambient air composition), the 24-hour behaviors (e.g., postural change and increased activity upon awakening, daily sleep/wake cycle, daily fasting/feeding cycle), or 24-hour internal body clock changes (endogenous circadian rhythms) could, alone or in combination, lead to a day/night rhythm in the risk for SCD. Circadian rhythms are generated by cell autonomous circadian clocks, present in nearly every cell/tissue in the body and resonate with a periodicity of ~24 hours.

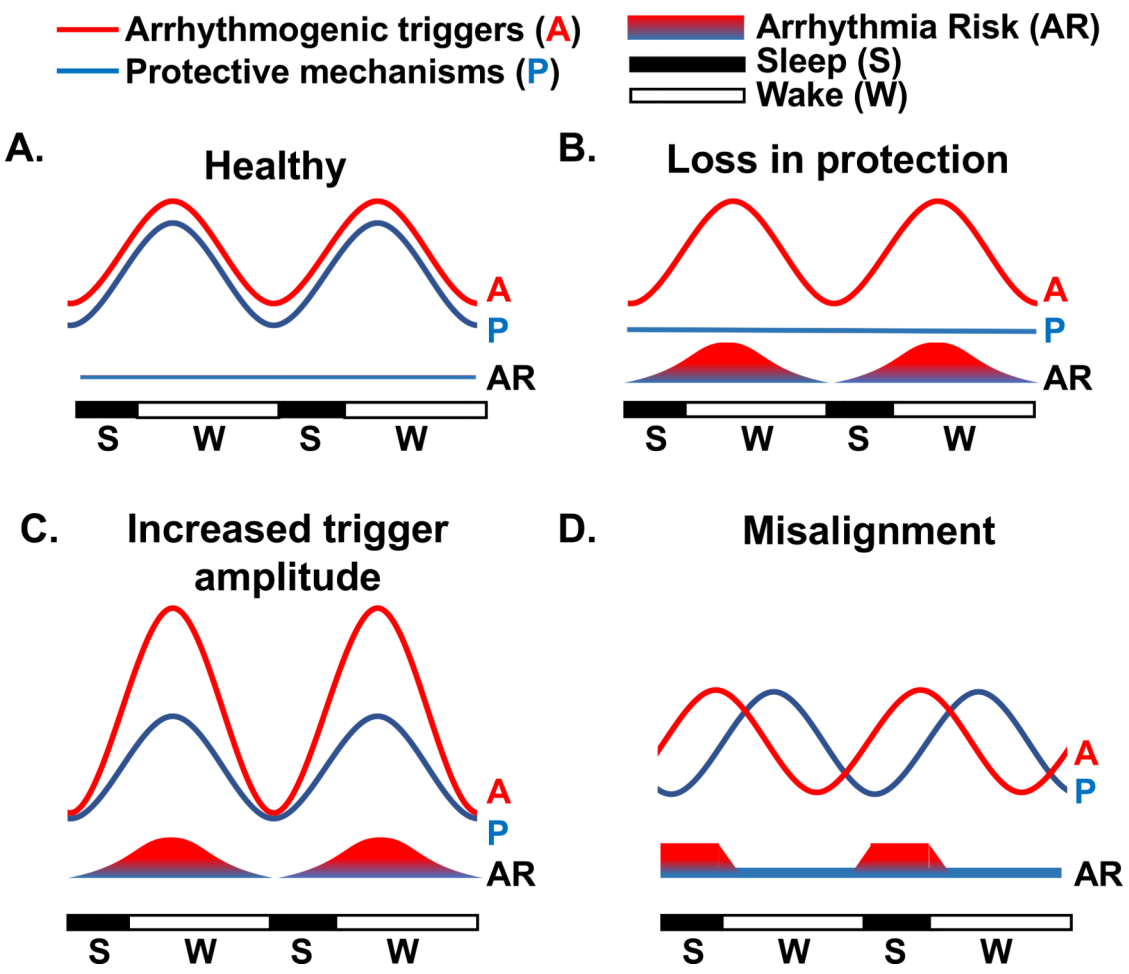

Figure 2. Theoretical framework for generating day/night patterns in arrhythmias.

There are day/night rhythms in both arrhythmogenic triggers (red line, A) and anti-arrhythmic or protective mechanisms (blue line, P). A disruption in these rhythms could impact arrhythmia risk (AR, blue = low risk and red = higher risk) during the sleep (S) wake (W) cycle. **A.** In a healthy individual these patterns may synchronize to produce an overall low arrhythmia risk throughout the sleep-wake cycle. Increase in arrhythmia susceptibility at certain times during the sleep-wake cycle may be caused by **B,** disease-states that alter the myocardial substrate to cause a loss in protection; **C,** stressors that increase the relative amplitude of the arrhythmogenic triggers; or **D,** disruptions that result in a misalignment of arrhythmogenic and protection rhythms.

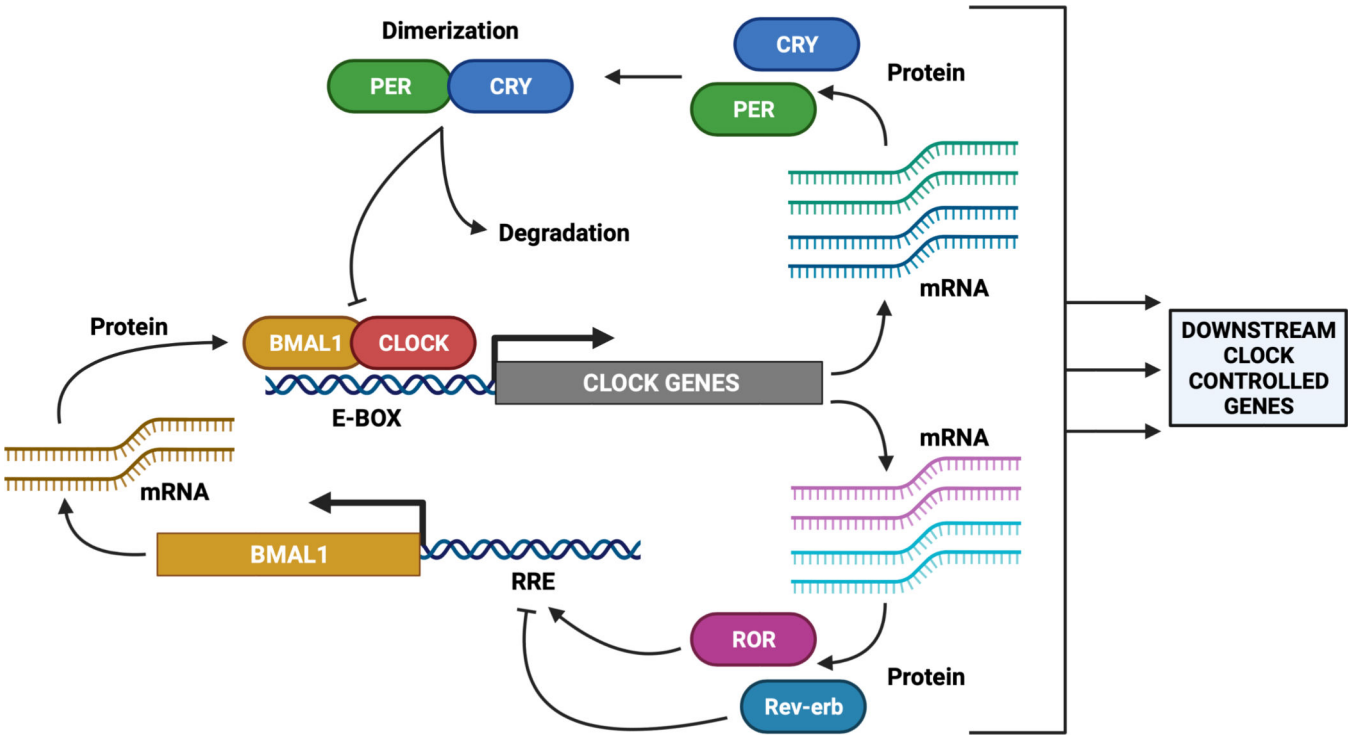

Figure 3. Schematic of the circadian clock, a transcription/translation feedback loop that drives cellular circadian rhythms.

Heterodimerized BMAL1 and CLOCK bind E-box (or E-box related) elements to activate transcription of core clock genes including Period (PER), Cryptochrome (CRY), Rev-erb, and ROR. Top loop: PER and CRY proteins dimerize and negatively feedback on BMAL1 and CLOCK activity. Bottom loop: ROR and Rev-erb proteins directly increase and decrease, respectively, the transcription of BMAL1. Box: The circadian clock mechanism also regulates the cell and tissue-specific expression of “clock controlled genes” that, although outside the timekeeping network, are important in the regulation of physiology and behavior.

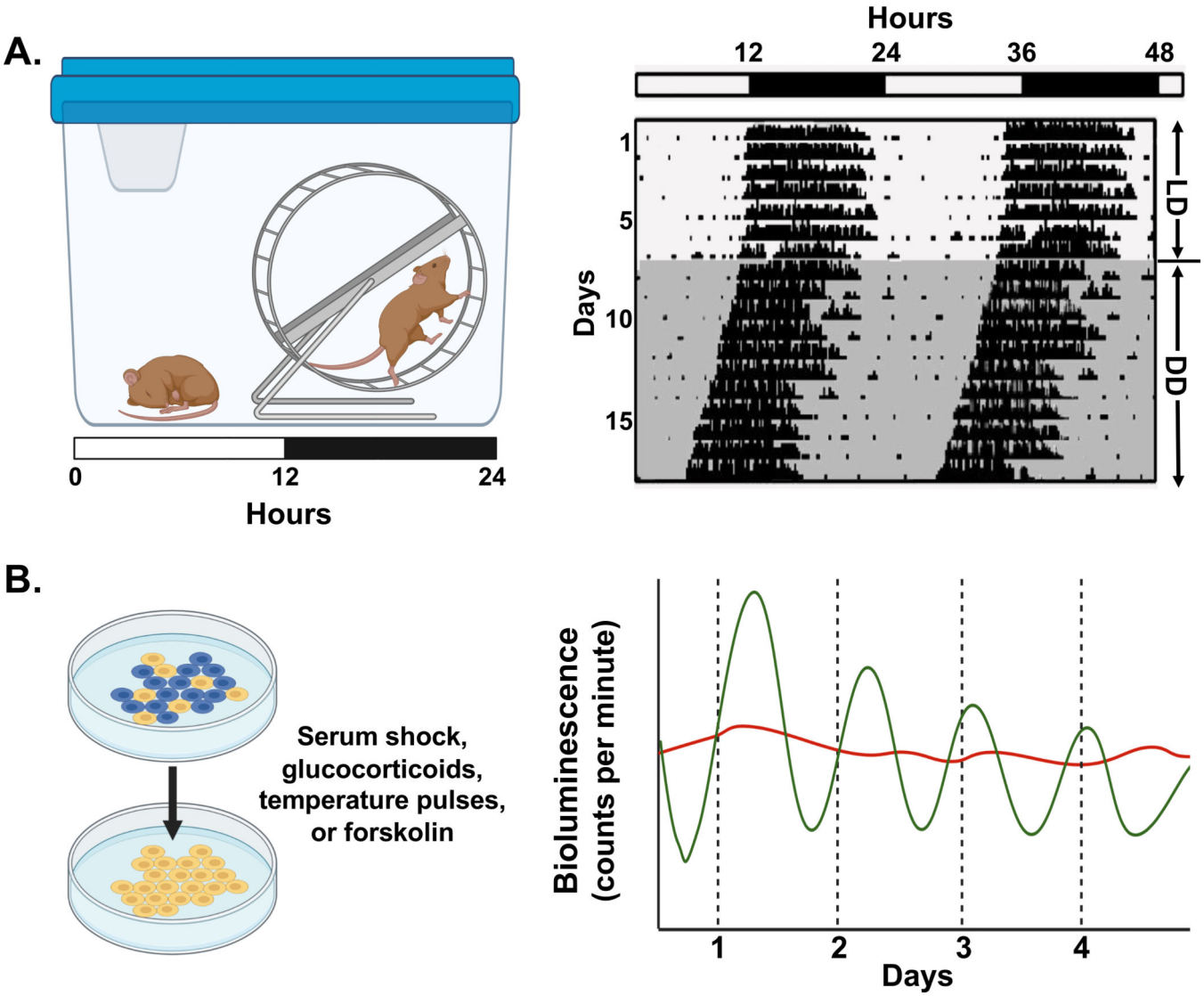

Figure 4. Quantifying circadian rhythms *in vivo* and *in vitro*.

The standard representation of circadian timing involves plotting variables across numerous consecutive days. **A**, Mouse rhythms (eg, behavioral or physiological) are often shown as each day double-plotted on the abscissa and stacked along the ordinate to produce a temporal raster plot or "actogram." Mice maintained in the standard laboratory 12- hour light (open bar):12-hour dark cycle (solid bar) display outputs that align to the light dark (LD) cycle. Shown is an example of nocturnal wheel running activity. Switching to constant darkness (DD, shaded region) causes mice to display a consistently advancing phase in outputs because the endogenous cycle length of the suprachiasmatic nucleus (SCN) is shorter than 24-hours. **B**, Measuring circadian clock/molecular rhythms in tissues/cells requires genetic reporters that typically use luciferase (due to the short half-life of the protein). Cultured cells/tissues can be entrained by the use of several tissue culture techniques that synchronize/align circadian clocks (yellow cells). Bioluminescence activity can then be quantified in living cells/tissue over the course of several days to clearly

delineate reporters that generate robust molecular rhythms (green) from those that do not (red).

Table

The Identification of Knowledge Gaps and Opportunities for Understanding Circadian Mechanisms Contributing to SCD Clearly Requires Some Definitions as a Framework. This table provides operational definitions to several of the terms used throughout this paper. We recognize different disciplines sometimes use the same term to describe different aspects of chronobiology (herein also different terms to describe similar concepts), and although an interdisciplinary consensus for definitions is warranted, this task is beyond the scope of this report.

Day/night rhythms: ~24-hour rhythms caused by a combination of behavioral rhythms (e.g., postural change and increased activity upon awakening, daily sleep/wake cycle, daily fasting/feeding cycle), environmental rhythms (daily cycles in light, temperature and ambient air composition) and ~24-hour internal circadian rhythms.
Circadian rhythm: Throughout this article, we refer to “circadian” as being driven by the internal body clock (ie, endogenous) with a period of about 24 h. Circadian rhythms persist even in the absence of environmental or behavior-related time cues that ordinarily entrain them. Until researchers begin to use the term consistently, it is advisable to read the protocols of all studies in this area for appropriate data interpretation to match the timing of other inputs, such as a repetitive physiological trigger or rhythmic signal.
Entrainment: The synchronization of a self-sustaining oscillation (such as a circadian rhythm).
Synchronization: The action of causing two or more processes to proceed at the same frequency, i.e., express the same period (cycle length).
Circadian synchrony: State of the circadian system when two or more rhythmic variables exhibit periodicity with the same frequency or with frequencies that are integer multiples or submultiples of one another.
Circadian alignment: The state of 2 or more processes to start at the same time (or have a consistent phase relationship) or to occur at a normal or optimal phase angle.
Circadian disruption: The action of acutely or chronically altering one or more circadian rhythms from having a normal period, phase and/or amplitude (circadian system disruption, clock disruption, perturbations in circadian rhythms, genetic circadian clock disruption, circadian desynchronization, circadian misalignment).
Biological/circadian time of day: The timing of experiments relative to the internal body clock.