Chapter 2
Review of Protocols and Terminology to Enhance Understanding of Circadian-Based Literature

Vincent A. LaBarbera and Katherine M. Sharkey

The rhythms of life have intrigued humankind for millennia—since the first observations of the periodicity of parasitic fevers during the time of Hippocrates—through today, including the awarding of the 2017 Nobel Prize in Physiology or Medicine for the isolation and characterization of the clock gene "period" and its molecular regulation of rhythms in the fruit fly *Drosophila* [1, 2].

A biological rhythm can be defined as the recurrence of an event "within a biological system at more-or-less regular intervals" [3, 4]. The intervals may be on the order of one cycle per millisecond or per years and may occur at the level of the cell within an organism or even at the population level. Biological rhythms may be considered *exogenous*, meaning arising as a response to a periodic input coming from outside of the biological unit, or *endogenous*, that is those which arise from within.

The word *circadian* is derived from the Latin words "circa" and "diem," translating to "about a day." The term dates to 1959 and is attributed to Professor Franz Halberg, a leading circadian researcher from the University of Minnesota. Halberg also notably coined the term *chronobiology* or the study of time as it relates to biological processes. In addition to *circadian*, the description of other "circa" rhythms are attributed to Halberg: *circatidal* (in relation to the natural rhythm of the oceans' tides), *circalunar* (in relation to the approximately monthly rhythm of the moon's orbit around the Earth), and *circannual* (in relation to the yearly revolution of the Earth around our sun). *Circhoral* is used to describe an approximately hourly rhythm, the most well-studied being episodic hormone secretion [4].

V. A. LaBarbera
Department of Neurology, Rhode Island Hospital/Warren Alpert Medical School of Brown University, Providence, RI, USA

K. M. Sharkey (✉)
Department of Medicine and Psychiatry and Human Behavior, Alpert Medical School of Brown University, Providence, RI, USA
e-mail: katherine_sharkey@brown.edu

R. R. Auger (ed.), *Circadian Rhythm Sleep-Wake Disorders*,
https://doi.org/10.1007/978-3-030-43803-6_2

Circadian rhythms run independently of exogenous factors and are driven by an internal biological clock. Several examples of circadian rhythms have been documented throughout the plant and animal kingdoms. Notable examples in humans include the sleep-wake cycle, the rhythmicity of core body temperature, and hormonal cycling. For cycles with durations shorter and longer than 24 hours, the terms *ultradian* and *infradian*, respectively, are used. For instance, rapid-eye-movement and non-rapid-eye-movement (REM-NREM) sleep cycles that occur at ~90–120-minute intervals during sleep are examples of ultradian rhythms, whereas the human menstrual cycle, which lasts approximately 28 days, is an infradian rhythm.

The *suprachiasmatic nuclei* (SCN) of the anterior hypothalamus comprise the dominant clock in the brain and serve as the prime driver of circadian rhythms in mammals. Environmental lighting conditions are relayed to the SCN through the *retinohypothalamic tract* via unique receptors, the *intrinsically photosensitive retinal ganglion cells* (ipRGCs). Separate from the rods and cones that relay information to the visual cortex for image formation, the ipRGCs, along with input from the intergeniculate leaflet of the thalamus and the midbrain raphe nuclei, convey both photic and nonphotic information to the SCN circadian clock. The SCN, in turn, regulate peripheral clocks in cells throughout the brain and the rest of the body to produce downstream circadian rhythms in virtually every aspect of physiology and behavior, from circulation of immune cells to hormonal cycling to digestion and metabolism to cognition and mood regulation. Interestingly, there can be a hierarchical entrainment of multiple oscillations within an organism that follow an established sequence laid forth by the primary pacemaker. These secondary oscillations maintain an entrained cycle, but only due to the initial entrainment of the system pacemaker [3].

Circadian rhythms play an important role in our sleep-wake cycles. Sleep and wakefulness coordinate and counteract one another via two processes. One is the circadian process, which drives diurnal species to be behaviorally active during the solar day and to sleep in the dark at night. The other is the homeostatic process, which is dependent on the length of time that an individual has been awake, and aims to equilibrate the physiologic need for sleep with sleep initiation and maintenance. In other words, the longer an individual is awake, the greater the homeostatic drive for sleep; the converse is also true, in that if an individual has had recent sleep, the homeostatic drive for sleep is lessened [5].

The properties of circadian rhythms are analogous to the terminology describing harmonic oscillations in the field of physics. Circadian rhythms are represented schematically by the sine wave function (Fig. 2.1). In the case of the circadian rhythm of sleep propensity, the range from neutral sleepiness to the highest or lowest level of sleepiness, similar to the maximum displacement from equilibrium, is called the amplitude. Amplitude of sleep propensity can be mutable, depending on variables such as age, gender, or the individual's sleep state (i.e., NREM vs REM sleep). The duration of the rhythm from nadir to nadir, or peak to peak, is termed the period or tau (τ). This also may be thought of as the intrinsic duration of the internal clock in "free-running conditions," such as in no-light environments without time cues. In humans, period length is nearly 24 hours.

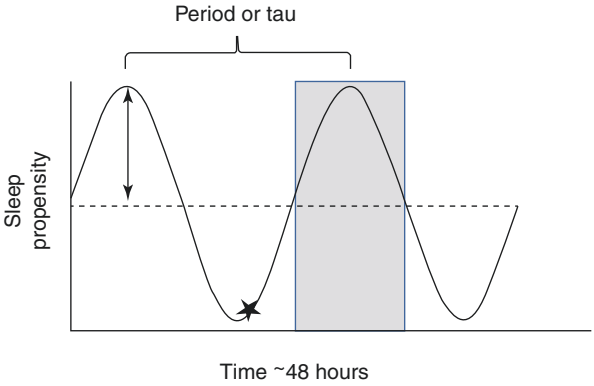

Fig. 2.1 This figure depicts an example of the circadian rhythm of sleep propensity across two cycles represented by a sine wave. The gray-shaded box indicates usual sleep time, the double-sided arrow depicts amplitude, and the black star notes a specific phase position, in this case, just after the nadir of sleepiness

To describe the current state of a particular circadian measurement, such as sleep, temperature, and melatonin secretion, among others, one uses the term "*phase*," which identifies the state at a specific instance in time. Commonly measured phase positions depend on the circadian marker of interest. For example, a conventional marker of temperature phase is the minimum, whereas for hormonal secretion the onset or peak is frequently used. The phase angle (ψ) describes the duration of time between two circadian rhythms, for example, an individual's sleep onset, midpoint, or offset in relation to the time of the temperature minimum, or the onset or peak of melatonin secretion.

Individuals can be catagorized by *chronotype*, i.e., a phenotype describing the person's tendency to adhere to a particular periodicity. "Larks" describe individuals with a tendency and/or preference to awaken early and retire early, whereas "owls" have and/or prefer later sleep patterns. Most individuals have a neutral chronotype landing somewhere in between owls and larks [6]. When an individual's chronotype is out of sync with their desired or required daily work, school, or social schedule, there are implications regarding the perpetuation of insomnia or the experience of daytime sleepiness.

Because most individuals' internal body clocks have a period length (tau) that is not precisely 24 hours in length, circadian rhythms must be synchronized or "entrained," daily. These small adjustments occur mostly in response to the natural light-dark cycle, which is the strongest "zeitgeber" or "time giver" to the biological clock. Entrainment allows the organism to align the internal clock with external time cues, including light-dark patterns. If the light-dark cycle changes, the circadian rhythms shift gradually to re-entrain with the new cycle. However, in the absence of time cues, these rhythms "free run," thereby only cycling based on endogenous periodicity. Since the endogenous tau is usually close, but not equal, to 24 hours, if entrainment is not achieved, the internal circadian rhythms may become uncoupled from external time cues. The most common cause of free-running circadian rhythms is a lack of photic stimulation of sufficient strength to entrain the SCN, such as may occur among people with blindness.

Circadian rhythms are also vulnerable to misalignment, wherein the phase angle between the endogenous propensity for sleep or wake and external time cues and schedules is not synchronized. Common causes of circadian misalignment are jet lag, daylight savings time, and night shift work. In these cases, the internal clock needs to readjust or "phase shift" to achieve realignment. An individual can "phase advance," which refers to circadian rhythms resetting to an earlier time, as is required for most eastward travel or "springing forward," or "phase delay," where the rhythms must entrain to later time cues. Since most individuals have a period length (tau) slightly longer than 24 hours, it is generally easier to phase delay than to phase advance. Exposure to the external light-dark cycle is the strongest zeitgeber to promote re-entrainment and correct misalignment. Night shift workers represent a special case of circadian misalignment because they continue to be exposed to a light-dark cycle that conflicts with re-entrainment.

Phase shifting to an earlier time, that is, phase advancing, is best accomplished with exposure to morning bright light, whereas exposure to evening bright light will facilitate a phase delay. The phase shifts produced by a stimulus (zeitgebers such as light exposure, exercise, or exogenous melatonin administration) at a specific circadian phase can be described using a phase response curve (PRC, See Fig. 2.2). A PRC is created by plotting the circadian phase shifts produced across multiple trials of zeitgeber exposure at different circadian phases. The resultant PRC can help researchers predict the magnitude and direction of a phase shift in response to zeitgeber exposure across the 24-hour circadian cycle. There is a robust literature on phase response to various stimuli, with several phase response curves documented to exogenous melatonin, light of variable intensity, duration, and wavelength, as well as physical activity [7–18]. One of the most potent zeitgebers is photic stimulation that does not necessarily come from natural light. For example, bright artificial lights and light of short wavelength (i.e., blue-green) have significant effects on the biological clock.

Protocols

Various protocols can be used to measure circadian rhythms. Oftentimes, research schema can measure circadian rhythms with more precision than can be done clinically. Two such protocols are the constant routine (CR) and the forced desynchrony (FD) [5].

Constant routine protocols aim to mitigate factors that impede the researcher's or clinician's attempt to measure endogenous circadian rhythms. Constant routines (CRs) minimize variables that influence the outcome of interest, literally by keeping the data collection conditions as constant as possible. There are many published CR protocols that impose strict limits on factors that can mask the output of the clock, including eating/feeding, movement, exercise, postural changes, light exposure, cognitive load, and knowledge of clock time e.g., [19, 20]. For example, the pattern of melatonin secretion is affected by light exposure and can be measured with more

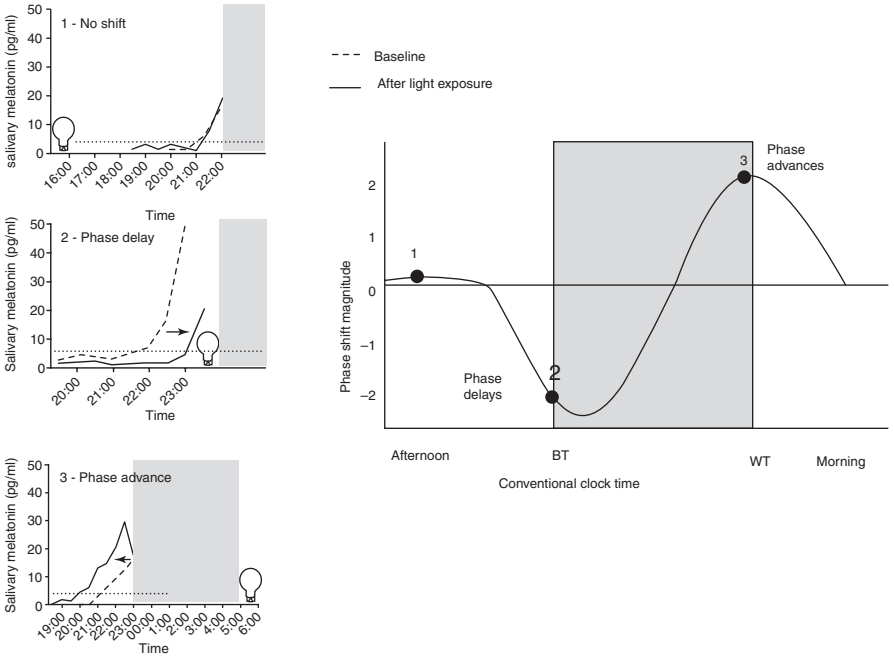

Fig. 2.2 An example phase response curve to light: This schematic illustrates how a phase response curve (PRC) is derived and indicates the expected circadian response to bright light exposure at various times of day. A PRC plots the results of multiple experimental trials; in each trial, the baseline phase position is measured and then the stimulus is presented at a specific time of day. A second phase position measure is obtained, and the difference between the two phase measures is calculated and plotted on the PRC with the stimulus time on the x-axis and the magnitude of the difference in the two phase measures on the y-axis. By convention, phase advances are plotted as positive and phase delays are plotted as negative, that is, below the 0 (no shift) line. The three small panels on the left show the expected phase shift in dim light melatonin onset (DLMO) at the three time-points plotted on the large PRC. No shift is expected when light is presented in the afternoon (point 1), a delay in DLMO is anticipated with bright light exposure in the late evening and early part of the subjective night (point 2), and a phase advance in DLMO is expected when light exposure occurs in the late part of the subjective night and early morning (point 3). There are many specific PRCs to light that detail the intensity and duration of light as well as other study parameters, including references [3, 8, 11–14]. (Graphic by Alexander Callahan)

fidelity by providing closely monitored uniform conditions in which samples for melatonin measurement are collected only in dim and/or long wavelength light. Many CR protocols require that participants maintain a wakeful state in a reclined position to attenuate the effects of sleep or activity on measurements of the circadian rhythm of body temperature. In other CR protocols, regimentation of food and liquid intake to regularly spaced meals, or even intravenous nutrition, have also been used. These protocols may require participants to stay in a laboratory setting for one or more circadian cycles to determine the endogenous circadian phase position, amplitude, etc.

Other less commonly used modifications of the constant routine protocol are the constant bed rest protocol and multiple nap protocol [5]. Constant bed rest allows patients to choose when and for how long they sleep. This protocol is oftentimes less burdensome on the patient, and circadian rhythms such as sleep propensity or REM sleep can be measured. The multiple nap protocol schedules longer naps across the day to suppress the homeostatic drive for sleep, thereby demonstrating rhythms that are normally hidden, such as subjective sleepiness in association with circadian cycles.

Forced desynchrony (FD) is another circadian rhythm measurement technique used mostly for research purposes. FD protocols aim to uncouple the two processes that control sleep and wakefulness: the circadian process and the homeostatic process. In a healthy, entrained individual, the endogenous circadian rhythm and homeostatic drive are highly coordinated, resulting in consolidated wakefulness during the day and sleep at night. This makes it impossible to distinguish whether an outcome of interest is related to the homeostatic drive, circadian phase, or both.

In an FD protocol, the sleep-wake schedule, and thus by definition the light-dark cycle, is altered such that the research participant's circadian cycle cannot entrain to the manipulation of their sleep timing. For example, a "20-hour day" FD protocol is comprised of 13.3 hours of wake and 6.7 hours of sleep opportunities, such that FD Day 2 starts after just 20 hours and when there are 4 hours remaining in the first calendar day. A 20-hour day is outside the human "range of entrainment." This means that the internal clock cannot adjust to a cycle that is so different from its endogenous cycle length. In these circumstances, the internal clock will continue its circadian rhythms on its endogenous tau and become desynchronized from the sleep-wake cycle imposed by the FD protocol. After several cycles, the circadian pacemaker and the homeostatic drive become uncoupled, so that they are running in parallel, each maintaining its own properties. Over time in an FD protocol, scheduled sleep and wakefulness will be allocated across all phases of the endogenous circadian rhythm, and variables of interest can be measured with respect to each parameter. In this manner, the homeostatic drive for sleep, which increases across wakefulness and diminishes with sleep, and the circadian drive for sleep, which is low during the typical "day" and increases when sleep occurs at night, become dissociated and are less confounding on the dependent variable being measured. Common dependent variables, such as sleepiness, metabolism, performance tasks and reaction time, mood, etc., can be measured upon awakening, thereby deducing the effect of circadian rhythm while controlling for the homeostatic drive, as ideally the drive to sleep would have dissipated during the preceding sleep opportunity.

Astute observers have utilized the concepts described in this chapter in their quest to characterize circadian behaviors and phenomena witnessed in the natural world for centuries. In recent decades, scientists and clinicians have applied these concepts to study the organization of fundamental physiological processes (e.g., eating/feeding, metabolism, hormonal regulation, growth, sleep, and wake) and have gained a deeper understanding of complex pathophysiology that is impacted by circadian rhythms.

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