Chapter 1
Biological Timekeeping: Scientific Background

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Introduction

The timekeeper, often underappreciated, is an individual or device in competition, experimentation, and daily life that records and regulates the initiation and termination of key processes. In physiology, the timekeeper or pacemaker is crucial for maintaining delicate temporal organization of molecular and physiological events that dictate the survival of an organism. Throughout the human body, there are a myriad of biological rhythms that oscillate at rates as short as 1–2 seconds (i.e., sinoatrial node of the heart) and as long as weeks to months (i.e., estrous cycle) that are essential to life. A circadian rhythm is the term for a process that is under the control of our internal pacemakers with a period of approximately 24 hours [1]. Rhythms shorter than a day are referred to as ultradian rhythms, while rhythms longer than a day are called infradian rhythms [2]. The word circadian is derived from the Latin words *circa* ("approximately") and *dies* ("day") [1]. As such, circadian rhythms adapt to, follow, and oscillate with a period nearly matching the Earth's 24-hour rotation with respect to the sun.

It is becoming clear that nearly all fundamental physiological processes are under the control of the circadian system [3]. From the autonomic control of blood pressure to the coordination of hormonal secretion, investigators are starting to

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unravel the importance of the circadian system for anticipating and optimizing the timing of various physiological functions [4]. In concert with these findings, recent clinical and preclinical research have begun to shed light on the detrimental consequences associated with chronic disruption of circadian rhythms [5–9]. The information technology revolution of the twenty-first century has inadvertently created a "24/7" environment that does not abide the natural rhythms of the Earth. In 2018, Nielsen estimated American adults spent an average of 11 hours per day exposed to artificial light emitted from electronic screens, an increase of 25% over the past 5 years [10]. This time does not even include the time that is spent using these devices at work or school which are becoming ever more digital. Additionally, shift work is becoming increasingly common in our connected world. It is estimated by the International Agency for Research on Cancer that 15–30% of the Western population is exposed to rotating shift work [11]. Taken together, modern humans are exposed to stressors that negatively regulate the circadian system. Due to its fundamental role in essential physiological processes, disruption of one's circadian rhythm has been linked to an increasing risk of developing cancer [12], type 2 diabetes [4, 13], neurological disorders [14, 15], and cardiovascular disease [16, 17], among other adverse health consequences. Therefore, it is critical to further our understanding of the mechanisms underlying the circadian system to develop new therapies and strategies for maintenance of the circadian clock in our twenty-first-century environment.

History of Chronobiology

The concept of timekeeping has permeated civilization for millennia; however, endogenous biological clocks were not truly appreciated until the eighteenth century [18]. Although ancient physicians such as *Galen* and *Hippocrates* noted that conditions like fevers exhibited periodic 24-hour rhythms, they unlikely appreciated that these rhythms were controlled, in part, by an endogenous timekeeper which persists independent of environmental cues [19]. In 1729, French astronomer Jean Jacques d'Ortous de Mairan was the first to realize that daily rhythms were intrinsically controlled and were not simply a response to the rhythmic light cycle of the Earth [18]. He observed that *Mimosa* leaves moved with a 24-hour cycle, even when placed in constant darkness, and therefore concluded that the movements could not have been affected by the light-dark cycle. Following de Mairan's work, many confirmed his findings and demonstrated that the circadian rhythms in plants were independent of other rhythmic external stimuli. However, it was still unclear exactly what internal factors drove the maintenance of the circadian rhythm. With the explosion of Mendelian genetics in the early 1900s, Dr. Erwin Bunning, a German botanist, crossed bean plants with various period lengths and demonstrated that the period length of the offspring was an intermediate of the previous generation [20]. As a result, Bunning inferred that endogenous circadian clocks were "heritable" from the genetic code. Bunning's pioneering work unraveling the interaction

between intrinsic clocks and light sensitivity in plants set the stage for an explosion in the understanding of circadian rhythms throughout the rest of the twentieth century.

The modern field of chronobiology is an ever-growing discipline which traverses a broad range of basic and clinical research [21]. Nevertheless, the diversity in the field today mirrors its composition upon its founding in the early 1960s [22]. At the time, the field was led by Dr. Colin Pittendrigh and Dr. Jurgen Aschoff, often considered the fathers of chronobiology. Both pioneered early studies defining the properties and characteristics of circadian rhythms [23]. Most notably, Pittendrigh developed a parametric model describing how circadian rhythms can be reset or disrupted by a singular pulse of light [24]. Aschoff, meanwhile, showed that humans have intrinsic clocks and that these clocks could be synchronized by external cues. He coined the term *zeitgeber*, a German word meaning time-giver, to describe the environmental stimuli that can synchronize internal clocks [25]. While Aschoff, Pittendrigh, and others were unraveling the underpinnings of the biological clock, there was a push to apply these principles to improve human health. Dr. Franz Halberg, a physician at the University of Minnesota, led this charge at the time by pioneering novel preclinical and clinical studies that actively considered circadian time as a biological variable [1]. In one of his early studies, Halberg measured the circadian variability in the temperature of oral tumors, as a metric of their circadian metabolism, in order to optimize radiation therapy treatment [26]. He observed that patients who received treatment at peak tumor temperature were twice as likely to be cancer-free 2 years following radiation. Despite their diverse approaches and methods, these individuals clearly understood the importance in maintaining robust circadian rhythms and provided the guiding principles being applied today to improve human health.

Measurement and Assessment of Circadian Rhythms

Circadian rhythms, like any other biological or physical rhythm, consist of predictable patterns of oscillations over a finite element of time [22]. In order to describe and compare how circadian patterns of motor activity, temperature, food intake, and other rhythmic behaviors vary between individuals or organisms, one must appreciate the distinct features of a rhythm. To identify whether a process is truly a circadian rhythm, the period or frequency must be assessed. The period is the time to complete one cycle, while the frequency, also known as the inverse period, is the number of cycles completed over a unit of time. In many scientific disciplines, Fourier analysis, pioneered by Joseph Fourier in the late eighteenth century, is used to quantify the various frequency components of a signal [27]. Simply put, Fourier analysis transforms a discrete or continuous rhythmic signal into a sum of sinusoidal functions with varying frequencies. The relative contribution of each frequency to the signal can then be visualized by calculating their spectral densities, otherwise known as a periodogram. In circadian biology, modified periodogram analysis such as the chi-squared periodogram, which utilizes least squares regression, is used to measure the

dominant period of physiological rhythms [28]. Periodogram analysis also reveals the strength or robustness of the rhythm. Essentially, periods that are "neater" and more consistent will have a larger power because the period has a greater contribution to the overall sinusoidal signal. The power is different than the amplitude of the signal as the latter only quantifies the relative deviation from the mean to the peak value, while the former is not affected by signal deviation and relies solely on the contribution of the various frequency components of the rhythm. Although the rhythm's period may describe whether processes occur under the control of the circadian system, it does not explain why they occur and why they are optimized to occur at different times of the day. The secretion of cortisol and melatonin, for example, both occur with a robust 24-hour period; however, their peak secretion occurs at different times of the circadian cycle [29]. Typically, plasma cortisol levels rise throughout the solar day and begin to fall just as melatonin levels begin to rise in anticipation of the biological night. The relationship of the opposing rhythms can be quantified by the phase, which describes the initial time (or angle) at the signal's origin. The lag in the phase of the melatonin rhythm relative to the cortisol rhythm is referred to as the phase difference.

The use of melatonin and cortisol secretion as markers of circadian timekeeping exemplifies how the field of chronobiology still mainly relies on indirect, noninvasive methods such as hormonal secretion, internal temperature, and voluntary locomotor activity to "keep time" *in vivo* [30, 31]. With the development of mobile, noninvasive devices for monitoring of voluntary locomotor activity, assessment of circadian activity rhythms is most commonly used to indirectly measure circadian pacemaking in humans [32]. Activity rhythms are typically visualized by means of an actogram, which vertically organizes each day of behavior allowing for the efficient visual analysis of the period and phase of this circadian output [33]. Nevertheless, novel methods to directly measure circadian pacemaking are currently being explored. Recent preclinical animal studies visualizing fluorescently tagged circadian genes have started to allow for the longitudinal and direct measurement of circadian rhythms in pacemakers of mice; however, it is unclear whether such techniques will become applicable to humans [34]. Advances in imaging technology such as BOLD fMRI show promise, as they may be able to directly quantify biological timekeeping activity via blood flow to the pacemaking region [35]. Moreover, novel computational methods using integrated transcriptomic data are also being explored in humans to estimate the internal circadian time from a single blood or tissue sample [31, 36, 37]. Taken together, these metrics and tools will be essential to allow for the direct assessment of circadian rhythms in humans under various physiological and pathophysiological conditions.

Properties of Circadian Rhythms

Over the past 300 years, endogenous circadian rhythms have been assessed in a diverse group of plants, insects, birds, fish, amphibians, and mammals. Although the underlying molecular mechanisms that encode circadian processes may not be

completely conserved between species, the essential properties of their circadian rhythms remain. The three key properties that define a circadian rhythm are (1) persistence under constant conditions with a free-running period of approximately 24 hours, (2) ability to entrain to a 24-hour cycle via rhythmic environmental stimuli, and (3) free-running periods which are temperature compensated such that an organism maintains an approximately 24-hour circadian period regardless of temperature fluctuations [22].

Circadian Rhythms Persist Under Constant Conditions

Jean Jacques d'Ortous de Mairan's initial observation that the *Mimosa* plant maintained a 24-hour period in constant darkness suggested that circadian rhythms persist with a free-running period (FRP) of ~24 hours. The FRP is the period of activity in constant conditions and is also referred to as tau (t) or the inverse/frequency of the FRP [38]. An intrinsic FRP in organisms is critical for biological timekeeping because it ensures that the "clock keeps ticking" regardless of external stimuli. Nevertheless, it was unclear for many years whether this was conserved in higher mammals such as humans. In order to test this hypothesis, subjects were placed in an underground bunker devoid of any timing cues or potential zeitgebers [39] and were asked to maintain a "regular" life for 3–4 weeks. The light intensity and internal temperature were carefully controlled from the outside. Upon recording activity, temperature, and urine rhythms, Aschoff's team calculated an average free-running period of ~25 hours in the subjects. Future studies led to uncertainty in the study's findings [40, 41], and researchers further attempted to refine measurements by desynchronizing subjects' sleep-wake cycles from their circadian pacemakers. Nathaniel Kleitman pioneered a technique whereby subjects are exposed to a 28-hour "day", such that the zeitgebers related to the sleep-wake schedule are equally distributed throughout the circadian cycle over the duration of the experiment [30]. Indirect measurement of the periods of activity, core temperature, plasma melatonin, and plasma cortisol all revealed an intrinsic period of 24.18 hours with 90% of measurements ranging between 24.00 and 24.35 hours. This study demonstrated that the intrinsic human pacemaker was precise and truly circadian.

Rhythmic Environmental Stimuli Can Entrain Circadian Rhythms

Although the internal timekeeper maintains an approximately 24-hour rhythm, environmental cues are required to continually tune the internal timekeeper to maintain a precise period and phase. Consider an intrinsic period of 24.18 hours described above, which is only 12 minutes longer than the Earth's 24-hour solar cycle. After only 1 week, individuals would be subjected to an ~1-hour phase shift which would

progressively push one to a nocturnal state after a few months. Without tuning, our internal pacemakers would create an asynchronous and inefficient world that would overthrow our modern societal norms. Most typically, the Earth's light-dark cycle is considered the foremost entrainment agent; however, other agents such as food availability and external temperature have been also shown to act as zeitgebers for internal clocks [25, 42].

The paradigm describing how light tunes the period and phase of the biological clock was championed by Pittendrigh, leading to the development of a nonparametric, discrete model of synchronization [43]. The model suggests that the circadian pacemaker maintains equilibrium by shifting its phase in response to a stimulus of light outside of its current circadian phase. In order to visualize how pulses of light affect the phase of the pacemaker, phase response curves (PRCs) can be constructed describing whether the light pulse will advance, delay, or have little effect [44]. Intuitively, the effect of a light pulse during the active phase of a diurnal mammal will be minimal, while exposure to light during the night will significantly phase shift the FRP. This concept has been translated and repeatedly demonstrated in humans by exposing subjects to a 3–6-hour light stimulus at various times throughout the circadian cycle and subsequently measuring physiological rhythms in the absence of entrainment cues [45–48]. Researchers demonstrated that there is variability in response to the pulses of light; however, humans generally experience a phase delay when exposed to light early in sleep and a phase advance as the sleep phase approaches dawn [49]. Practically, PRCs have been a valuable tool in assessing the efficacy of chronotherapies for individuals exposed to circadian disruptions [50, 51].

Free-Running Periods Are Temperature Compensated

The light-dark cycle plays a dominant role in resetting and entraining the circadian clock; however, the biological pacemaker encodes these environmental cues through a series of biochemical reactions that are directly affected by the temperature of the reaction environment. Dr. Hans Kalmus first demonstrated that the rate of the circadian cycle in drosophila increased threefold with every 10 °C increase in temperature [52, 53]. Intuitively, a temperature-dependent clock would present significant challenges as the clock would run faster on a hot, summer day and slower on a cold, winter day, resulting in unreliable period measurements from 1 day or one season to the next. As such, further investigations on bees, mice, drosophila, and cultured human cells have refuted this initial finding [54–56]. In the presence of increasing ambient temperature, the FRP has been shown to remain relatively constant and, therefore, temperature compensated. Despite this finding, *in vitro* studies of chick pineal cells demonstrated that a heat pulse, similar to light pulses described above, could cause a phase shift of the circadian clock as measured by melatonin secretion [57]. Recently, *Buhr* et al. demonstrated that *ex vivo* tissue, absent of photic cues, can also be entrained to a new phase by a heat pulse while still maintaining an approximately 24-hour period [58]. Taken together, these findings revealed that an organism's innate circadian period is insensitive to temperature; however, it also

demonstrates that other rhythmic environmental stimuli, such as the daily circadian rhythm in body temperature, play a role in setting the phase of the circadian pacemaker.

Organization and Entrainment of the Circadian System

Provided the knowledge that the circadian pacemaker can be tuned by external cues, it is clear that it is actively engaged with the surrounding environment rather than passively counting time [59]. In turn, the circadian system can be directly modeled by simple oscillator networks. An oscillator is a rhythmic signal that provides inputs into a system to initiate change (i.e., response to light pulse), records and measures feedback from the system, and institutes a delay in response to feedback from a system not at equilibrium. Early models of the circadian oscillator system considered various configurations including a single, master pacemaker that drives systemic circadian outputs, coupled master oscillators that drive dependent or independent peripheral outputs, and a hierarchical oscillator system that couples a master timekeeper to peripheral oscillators that control local circadian outputs [60, 61].

The discovery of the suprachiasmatic nuclei (SCN) of the hypothalamus as the master pacemaker in mammals by two independent teams in 1972 began to eliminate the concept of a coupled oscillator model [62, 63]. Lesioning of the SCN resulted in loss of circadian regulation of locomotor activity, drinking behavior, and cortisol rhythms. Electrical recordings of SCN neurons *in vivo* and *in vitro* demonstrated that the SCN functions as a self-sustained oscillator as the neurons have the ability to intrinsically maintain a rhythm in electrical activity regardless of external input [64, 65]. Additionally, transplantation studies that implanted the SCN into an arrhythmic animal were successful in restoring the circadian rhythmicity of the donor animals [66, 67]. Although the SCN was demonstrated as the master circadian pacemaker, peripheral cells and tissues were also found to have intrinsic circadian oscillations [68, 69]. In the absence of SCN input, *ex vivo* examination of the mammalian liver, lung, and skeletal muscle revealed that these tissues displayed a robust circadian oscillation in gene expression. Recent studies using a luciferase (light) reporter as a real-time marker of circadian rhythms in mice confirmed the existence of self-sustainable peripheral oscillators in nearly all peripheral tissues [70, 71]. The growing experimental evidence suggests that the circadian system exhibits a hierarchical oscillator structure whereby a master oscillator provides inputs and tunes a network of peripheral oscillators that control downstream physiological outputs.

Light Is Encoded at the Master Timekeeper by a Specialized Detection Mechanism

Light, as the primary zeitgeber, requires an efficient communication system to relay changes in the light-dark cycle to the SCN. Intrinsically photosensitive retinal ganglion cells (ipRGCs) contain specialized melanopsin receptors which are present in

only ~1% of RGCs [72, 73]. Melanopsin is a photopigment sensitive to short wavelengths of light with a peak absorption at ~480 nm corresponding to blue/green light [74]. Activation of melanopsin leads to an intracellular signaling cascade causing ipRGCs to depolarize, initiating an action potential that travels down the retinohypothalamic tract (RHT) and directly innervates the SCN [75]. Lesioning the RHT causes animals to free-run, despite a rhythmic light-dark cycle, exemplifying its role in entrainment [76]. Simply put, the fundamental role of the RHT is to indicate the start and end of the day in order to entrain the master oscillator to a consistent phase response.

The symbiotic relationship between the SCN and RHT is the key to maintaining precise timing and is best illustrated by the monosynaptic connection between them [77]. Increase in the activity of the RHT at dawn, for instance, is directly received by SCN neurons. Inappropriate exposure to light stimuli due to jetlag or shift work can induce spiking activity of the RHT which results in a phase shift of SCN neuronal activity, as described by the PRC previously discussed. Synaptic communication between the RHT and SCN occurs via presynaptic release of the excitatory neurotransmitter glutamate [78]. The released glutamate is received postsynaptically by N-methyl-D-aspartate (NMDA) receptors, causing an influx of intracellular Ca^{2+} into SCN neurons and a cyclic adenosine monophosphate (cAMP)-dependent signaling cascade that resets and entrains the pacemaker to the light input [79]. Thus, a light stimulus can be quickly detected and communicated by ipRGCs to SCN neurons via an elegant and efficient signal transduction system.

Organization of the Suprachiasmatic Nucleus (SCN): The Master Timekeeper

The SCN is located in the ventral periventricular zone of the hypothalamus, dorsal to the optic chiasm, and is composed of approximately 20,000 neurons that have a circadian pattern of electrical activity [80]. Division of SCN neurons by amplitude of electrical activity, neuropeptide expression, and afferent inputs reveals two distinct subpopulations [81]. The core SCN neurons receive the majority of photic input, fire with low-amplitude rhythms that can be easily reset by environmental stimuli, and express a variety of neuropeptides including vasoactive intestinal peptide (VIP) and gastrin-releasing peptide (GRP) [82, 83]. Moreover, light-induced rhythmic oscillations occur exclusively in core neurons, suggesting that the core is primarily responsible for sensory processing of environmental cues and entrainment of the circadian system to the light-dark cycle [84, 85]. In contrast, shell SCN neurons primarily express arginine vasopressin (AVP), experience high-amplitude, self-sustaining oscillations, and receive little photic input [83]. These features highlight the shell's role in relaying SCN outputs to modulate the clock in peripheral tissue oscillators [86]. Despite these differences, communication between the core and shell SCN neurons is critical for maintaining the central pacemaker's 24-hour circadian rhythm. In an *ex vivo* SCN preparation, separation of the shell and core neurons

caused the shell SCN to desynchronize [87]. Elimination of VIP in the SCN, a key marker of the core, abolished behavioral rhythms and highlighted the downstream role the substance plays in entraining SCN outputs to light stimuli [88]. In combination with studies tracing neuronal circuits [83], it is clear that signaling from the core to the shell is necessary for the entrainment of the master circadian pacemaker.

SCN-Dependent Outputs Entrain Peripheral Oscillators

The hierarchical oscillator model of the circadian system suggests that outputs from the master pacemaker can modulate peripheral circadian oscillators. The SCN directly and indirectly entrains peripheral clocks through a combination of synaptic and neuroendocrine outputs [89]. Projections from SCN neurons mainly innervate the hypothalamic paraventricular nucleus (PVN). The PVN serves as the hypothalamic hub for hormonal and autonomic control and thus effectively relays SCN outputs for the circadian control of physiological outputs. Pre-autonomic neurons in the PVN are directly innervated by the SCN and subsequently project to sympathetic and parasympathetic motor nuclei controlling several organs including the heart, pancreas, and liver [90–92]. Lesioning the connection between the SCN and PVN was effective in eliminating the circadian rhythmicity in heart rate suggesting that the SCN plays a direct role in autonomic regulation of cardiac, as well as other peripheral rhythms [91].

Additionally, the PVN relays SCN outputs to the pineal gland for the circadian regulation of melatonin secretion. The pineal gland is indirectly controlled through a multisynaptic connection. SCN outputs travel from the PVN to the intermediolateral cell column and finally to the superior cervical ganglion (SCG) which innervates the pineal gland [93]. During the day, the SCN provides an inhibitory signal (via γ-aminobutyric acid [GABA]) to this pathway in order to suppress melatonin secretion [94]. These inhibitory signals suppress the expression and activity of the enzymes responsible for synthesizing melatonin from serotonin, arylalkylamine N-acetyltransferase (AANAT) and methionine adenosyltransferase 2a (MAT2A) [95]. Conversely, glutamatergic output from the SCN to the PVN has been demonstrated to be responsible for enhanced melatonin synthesis and secretion during the night in mammals [96]. Importantly, the nightly increase in melatonin secretion has been shown to functionally impact a wide range of tissues and processes and is considered a potential zeitgeber for peripheral tissue oscillators [97–100].

The hypothalamic-pituitary-adrenal (HPA) axis also receives SCN output. In a series of experiments by Kalsbeek and Buijis, they demonstrated that the SCN mediates its effect on the HPA by secretion of the neuropeptide vasopressin during an organism's inactive cycle. In turn, this inhibits the secretion of corticotrophin-releasing hormone (CRH) and vasopressin from PVN neurons [101–104]. During an organism's active cycle when the SCN ceases to secrete vasopressin, CRH and vasopressin subsequently induce the secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary, which can then act directly on the adrenal

cortex, modulating the release of glucocorticoids, catecholamines, and mineralocorticoids [105–107]. Similar to the role of melatonin, the daytime increase in stress hormone (i.e., corticosterone) secretion from the adrenal cortex has been proposed as an SCN-dependent mechanism of peripheral oscillator entrainment [108]. Through local control of its synaptic output, the SCN exerts its role as master timekeeper by controlling downstream autonomic and endocrine outputs to entrain peripheral clocks.

Non-photic Entrainment of Peripheral Oscillators

Although the SCN is fundamentally required to entrain peripheral tissues to the light-dark cycle via direct and indirect pathways, peripheral circadian rhythms are thought to also be modulated and entrained by SCN-independent mechanisms. The timing of a variety of rhythmic behaviors such as food intake and exercise has been demonstrated to phase shift peripheral oscillators while leaving SCN rhythms unaffected [42, 109, 110]. One of the strongest non-photic entrainment agents is food, specifically the timing of feeding patterns, and is likely due to feeding's role in mammalian survival [111]. Precise timing of feeding patterns or time-restricted feeding (tRF) can act as a zeitgeber for peripheral food entrainable oscillators (FEO). The concept that FEOs are independent of the SCN was confirmed in a seminal study where tRF during the inactive cycle of a mouse shifted the phase of the liver, pancreas, heart, and kidney while leaving the phase of the SCN unchanged [42]. Current research is building on this finding to identify the signals that mediate feeding-dependent phase responses. Centrally, FEOs seem to depend in part on orexin, a neuropeptide regulating appetite, because ablation of orexin-expressing neurons effectively prohibited food anticipatory activity in response to tRF [112]. Dopamine and ghrelin have also been implicated as responsible for food anticipation in response to tRF [113, 114].

In peripheral tissues, nutritional signals have been thoroughly investigated as the potential zeitgeber for FEOs. By feeding nutritionally homogeneous food to mice during their inactive phase, it was discovered that a combination of glucose and protein (casein) resulted in a significant phase advance in the liver that was not observed with mono-nutrient diets, suggesting that a balanced diet is required for entrainment of peripheral oscillators [115]. Parallel studies also demonstrated that glucose controls peripheral clock oscillations via an AMP-activated protein kinase (AMPK) signaling cascade [116, 117]. Hormones responsible for the systemic response to feeding and fasting such as insulin, incretins, and glucagon exhibit a robust circadian rhythm and thus have been suggested as potential entrainment agents for FEOs [118–120].

In addition to feeding, timing of exercise has also been implicated in entraining peripheral tissue oscillators. In humans, exposure to an acute bout of high-intensity exercise during the evening caused a significant phase shift in melatonin onset relative to the baseline melatonin onset [121]. Additional studies exposing subjects to

various exercise routines have confirmed the potential role of exercise in non-photic entrainment [122]. In animal studies, wheel-running during the inactive phase caused a phase advance in the liver and kidney clocks without affecting SCN rhythms [123]. The exercise-induced entrainment was likely coupled to an SCN-independent (exercise-dependent) secretion of corticosterone and/or catecholamines. Nevertheless, other exercise-induced signals such as local hypoxia and acute inflammation have been demonstrated to modulate the peripheral circadian clock [124, 125]. Overall, non-photic cues clearly play an important role in entraining peripheral tissues to the rhythmic environment for the optimization of physiological outputs.

Molecular Basis for Circadian Rhythms

Thus far, we have considered central and peripheral circadian rhythms as cellular and physiological outputs in response to various environmental cues. This is an overly simplified view of the circadian system which ignores the intracellular mechanisms that transduce an external stimulus into a physiological response. The circadian system is centrally and peripherally encoded by a transcriptional-translational negative feedback loop (TTFL) that was first proposed by Hall, Robash, and Young [126]. Their work deciphering the molecular clock in drosophila was recognized with the Nobel Prize in Physiology or Medicine in 2017. The TTFL is an elegant and efficient negative feedback loop that encodes and maintains an organism's circadian rhythm [127]. In short, the TTFL comprises an integrated circuit whereby the positive limb initiates the transcription of a negative limb that, upon translation, translocates back into the nucleus to negatively regulate its own transcription. The discovery of this approximately 24-hour oscillator network effectively described Bunning's initial observation that the inherited FRP is derived from the period of the molecular clock and encodes the circadian rhythm.

Organization of the Mammalian Molecular Clock

The components and structure of the mammalian molecular clock are highly conserved, allowing for the translatable study of this framework in various model organisms [128]. The positive limb of the mammalian TTFL is comprised of the core circadian transcription factors circadian locomotor output cycles kaput (CLOCK) and its heterodimer partner brain and muscle aryl hydrocarbon receptor nuclear translocator-like 1 (BMAL1), encoded by the aryl hydrocarbon receptor nuclear translocator-like (*ARNTL)* gene [127]. Genetic deletion of BMAL1 leads to loss of behavioral and physiological circadian rhythms because BMAL1 is the only mammalian circadian clock gene that does not have a compensatory paralogue [129]. In contrast, loss of CLOCK expression leads to mixed phenotypes because

neuronal period-aryl hydrocarbon receptor nuclear translocator protein-single-minded protein (PAS) domain protein 2 (NPAS2) can compensate since it is a paralogue to CLOCK [130]. The CLOCK-BMAL1 heterodimer promotes transcription of genes *period 1–3* (*PER1, PER2, PER3*) and *cryptochrome 1–2* (*CRY1, CRY2*), comprising the negative limb of the TTFL [131, 132]. PER and CRY proteins, along with the stabilizing casein kinases 1δ and 1ε (CK1δ, CK1ε), join together upon translation and translocate into the nucleus where they interact with CLOCK-BMAL1 to inhibit their own transcription [133]. The TTFL is further stabilized by the nuclear receptor subfamily 1, group D, member 1 and 2 (REV-ERBα/β) and nuclear receptor subfamily 1, group F, member 1 (RORα/β) that, respectively, repress and activate the transcription of *ARNTL* as part of secondary regulatory loops [134]. Overall, the molecular oscillator ensures the generation of robust circadian rhythms by encoding a 24-hour period into the TTFL.

Outside of its role as the master molecular timekeeper, the CLOCK-BMAL1 heterodimer activates transcription of target genes by binding to E-box regions in deoxyribonucleic acid (DNA), which are conserved promoters coded by a palindromic CACGTG sequence [135]. The binding of the heterodimer also includes recruitment and interaction with a variety of epigenetic factors such as histone acetyltransferases (i.e., p300), the cAMP response element binding protein (CREB), and cell-specific enhancers (i.e., pancreatic and duodenal homeobox 1) to increase expression of target genes [135–138]. A seminal study by *Koike* et al. demonstrated that CLOCK and BMAL1 bind to more than 10,000 combined sites regulating ~3000 unique genes in the mammalian liver [139]. Highlighting the critical role of the circadian architecture, these genes were significantly enriched for pathways regulating liver metabolism, cancer development, and insulin signaling. The negative repressors, PER1/PER2 and CRY1/CRY2, were demonstrated to bind to more than 12,000 and 25,000 liver sites, respectively. Consistent with this study, CRY1–CRY2 are thought to regulate nuclear receptors such as the glucocorticoid receptor, which are critical for non-photic entrainment [140]. PER1/PER2, meanwhile, have been shown to directly control cell proliferation and lipid metabolism; however, additional work is needed to fully investigate their downstream role in cellular physiology [141, 142]. Overall, the circadian control of molecular processes effectively transduces environmental cues into precisely timed physiological outputs.

Entrainment of the Molecular Clock

The entrainment of central and peripheral pacemakers is mediated by both photic and non-photic cues. These zeitgebers cause a distinct phase response by modulating the molecular clock through intricate intracellular signaling cascades. The phase shifting effects of light on SCN neurons described previously can be directly attributed to the plasticity of the system's molecular framework. Glutamatergic signaling induced by a light stimulus elicits an influx of Ca^{2+} and activation of cAMP in SCN core neurons [143]. The transcription of *PER1* and *PER2* is directly

stimulated at the core because the *PER1/PER2* promoter region contains a cAMP/Ca^{2+} response element (CRE), in addition to the E-box element [144]. Communication from the core to the shell is mainly mediated by VIP, a potent adenylate cyclase activator, which leads to an activation of cAMP and, in turn, *PER1/PER2* expression within a few hours [145]. This allows the circadian system to quickly adapt to inappropriate light exposure (i.e., jet lag) because the expression of *PER1/PER2* will reset the TTFL to a new phase as it interacts with and inhibits the BMAL1-CLOCK heterodimer. Relative to the PRC, light exposure toward dawn while endogenous *PER1/PER2* expression is low will accelerate and advance the cycle, while light during the day has essentially no impact on *PER1/PER2*. Non-photic entrainment, meanwhile, has been shown to utilize both CRE-dependent and CRE-independent mechanisms to modulate the phase of peripheral oscillators [116]. Time-restricted feeding, for instance, has been demonstrated to effectively modulate the phase of CREB, resulting in a resetting of the hepatic clock [146]. Nevertheless, parallel studies have demonstrated that inhibition of mitogen-activated protein kinases and phosphoinositide 3-kinases prevents the entrainment of the liver oscillator by insulin, suggesting that these kinases are likely involved in the signaling cascade responsible for resetting peripheral clocks [119]. Moreover, the nutrient regulated AMP-activated protein kinase has also been demonstrated to directly regulate the stability of CRY1 *in vitro*, providing additional evidence that CRE-independent pathways may be required for entrainment of peripheral clocks [116]. Finally, the heat shock signaling pathway mediated by heat shock factor 1 has been shown to be necessary for the peripheral entrainment by both heat and feeding [58, 147]. Taken together, the phase response of circadian oscillators to entrainment cues is transduced via intracellular signaling cascades that allow the circadian system to quickly adapt to its environment, the details of which are only beginning to be fully appreciated.

Conclusion

In summary, the circadian system is an endogenous feature of organisms that precisely maintains an approximately 24-hour period, driven by a molecular negative feedback loop. The tight and stable control of intrinsic circadian rhythms allows for the anticipation of external stimuli such that relevant physiological outputs can be optimized for efficient and effective responses. This chapter highlights the extensive investigations over the last 300 years that delineate the physiological and molecular mechanisms which form the framework of the circadian system. Recent clinical and preclinical studies have confirmed that disruption of these mechanisms through genetic or environmental stressors produces a state of circadian misalignment which is associated with the development of a variety of diseases. As such, further investigation defining the cellular and molecular mechanisms controlling the circadian machinery is needed to identify potential therapeutic targets and to restore the body's temporal balance in our 24/7 world.

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