Circadian rhythms in a nutshell

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Edery, Isaac. Circadian rhythms in a nutshell. Physiol Genomics 3: 59–74, 2000.—Living organisms on this planet have adapted to the daily rotation of the earth on its axis. By means of endogenous circadian clocks that can be synchronized to the daily and seasonal changes in external time cues, most notably light and temperature, life forms anticipate environmental transitions, perform activities at biologically advantageous times during the day, and undergo characteristic seasonal responses. The effects of transmeridian flight and shift work are stark reminders that although modern technologies can create “cities that never sleep” we cannot escape the recalcitrance of endogenous clocks that regulate much of our physiology and behavior. Moreover, malfunctions in the human circadian timing system are implicated in several disorders, including chronic sleep disorders in the elderly, manic-depression, and seasonal affective disorders (SAD or winter depression). Recent progress in understanding the molecular mechanisms underlying circadian rhythms has been remarkable. In its most basic form, circadian clocks are comprised of a set of proteins that, by virtue of the design principles involved, generate a self-sustaining transcriptional-translational feedback loop with a free-running period of about 24 h. One or more of the clock components is acutely sensitive to light, resulting in an oscillator that can be synchronized to local time. This review provides an overview of the roles circadian clocks play in nature, how they might have arisen, human health concerns related to clock dysfunction, and mainly focuses on the clockworks found in Drosophila and mice, the two best studied animal model systems for understanding the biochemical and cellular bases of circadian rhythms.

clocks; Drosophila; mammalian; PER

WHY THE RECENT FASCINATION WITH CIRCADIAN CLOCKS?

The concept of time has always perplexed and fascinated people. What is the nature of this mysterious thing that influences our lives so much? Although the ancient Greek philosophers believed that time was infinite, they perceived it as following endless cycles where the universe is born and dies, with an exact recurrence of everything in each cycle. The Newtonian view held that time is an immutable entity flowing on an infinite linear scale operating independent of nature’s forces. Einstein’s theory of relativity radically changed this conventional wisdom by revealing that time is actually embedded in the very fabric of the physical universe giving rise to a reality that is more accurately expressed as a four-dimensional space-time continuum. It follows from the big bang theory that time and our physical universe were jointly created at a singular event that, according to current estimates, occurred ~12–15 billion years ago, at least from our frame of reference. Interestingly, Kabbalists had intimate knowledge of old oral traditions that discussed the deep relationship between time, corporeal entities, and their creation. For example, almost 800 years ago a famous rabbi known as Nachmanidies, in his commentary to Genesis, wrote, “with this primeval creation, which was like a very small point having no
substance, everything in the heavens and the earth was formed, and when the heavens and the earth came forth from nothing into existence, time came into being and from the moment some substance came into existence time was already part of it” (for an authoritative English translation of the original text see, Ref. 20).

Despite our changing understanding of the nature of time, one thing has remained constant, the human obsession with harnessing this elusive entity. From sundials to calendars to cesium clocks, the quest to capture the essence of time and measure its passage has significantly influenced human history.

But on what basis do we rationalize the units we use to measure time? Essentially, this is a tale of spheres and cycles. The passage of time has been recorded (at least historically) by observing the rhythms of “heavenly bodies”, most notably the daily rotation of the Earth on its axis, the monthly cycle of the moon around the Earth, and the yearly journey of our planet around the Sun. Considering the average human lifespan and spatial distribution on our tilted planet, the broad frequencies encompassed by these reliable celestial rhythms (day, month, and year) provide quite relevant and useful units for recording time. These predictably recurring physical events inspired humans to design timing devices that could measure the passage of a known amount of time (e.g., sand-filled hourglasses), identify a specific phase in a cycle (e.g., sundials, Stonehenge?), or both (e.g., modern clocks).

The most influential physical oscillation that reminds us of our inescapable rhythmic relationship with time is the day-night cycle. Although we intuitively know that time proceeds in a unidirectional flight into the future, our lives are largely organized into a 24-h schedule dominated by periods of wakefulness and sleep. Thus we perceive time as being spiral in its geometry; there is ever forward progression into the future (e.g., counting years) coupled with time coordinates that are revisited in a cyclical manner (e.g., measuring time of day).

But is our cyclical concept of daily time and its technological expression in the design of clocks just a convenient invention of the human intellect that allow us to better cope with the waxing and waning of day and night?

In 1729, a French astronomer named Jean Jacques d’Ortous de Marian took plants that displayed daily leaf movements and put them in the dark for several days. He noted that the leaves of the plants continued to open during the day and close at night despite the absence of sunlight. Based on this seminal experiment, he concluded that the observed rhythm was not passively driven by a cyclic environment but was an innate property of the plant. From these humble beginnings, the formal study of circadian (endogenously driven biological rhythms with periods of ≈24 h) biology can be traced. It is now quite clear that living organisms have been tracking the passage of daily time long before we ever invented clocks.

However, the contention that life forms have internal timekeeping devices was only widely accepted about 60 years ago. For some it seemed too incredulous that organic material had properties similar to clocks fashioned by human hands (e.g., 12). After all, it was claimed that although daily pacemakers keep on ticking with a reasonably constant period in the absence of environmental cues (zeitgebers), in natural conditions they are accurately synchronized to local time. Adding to the dismay was a peculiar attribute of circadian rhythms; the length of the period is invariant over a wide range of temperatures (63, 128). This still mysterious property, known as temperature compensation, was viewed largely enigmatic from a chemical or biochemical perspective because most chemical reactions speed up about two- to threefold for every 10°C increase in temperature (or Q₁₀). Moreover, the prevailing thought at the turn of the previous century was that physiology is governed by the principles of homeostasis, effectively dismissing any observed oscillatory behavior as nothing more than random fluctuations of little or no significance.

With the eventual realization that endogenously driven daily rhythms are “real” and widespread, occurring in virtually all organisms, much interest was placed on elucidating the nature of the underlying pacemaker or clock. What are the molecular equivalents of the gears and springs in a watch? Unfortunately, trying to unmask the circadian clock was more like probing a black hole than a black box. While the tools of genetics and molecular biology were reaping great benefits in many areas, as recently as 1994 only two “clock” genes, period (per) in Drosophila (8, 89, 135, 206) and frequency (frq) in Neurospora (42, 108), were molecularly characterized.

Then, almost overnight, there are now a bunch of clock genes identified in humans, rodents, fish, frogs, insects, plants, and even cyanobacteria. This gold mine led to important paradigm shifts in how the organization of the circadian timing system is viewed. From a molecular perspective, the basic message from these recent studies is that circadian clocks use the same design principles; namely, the period, amplitude, and phase of a circadian clock are determined by a specialized set of interconnected proteins, many of which undergo daily rhythms in one or more character traits, most notably abundance (reviewed in Ref. 31). From a theoretical point of view, this framework for understanding the molecular underpinnings governing circadian rhythms is very satisfying, because regular change is the basis for timekeeping devices. However, although the RNA and protein products from many genes display cyclical behavior (e.g., 9, 55, 100, 102, 109, 115, 141, 183, 186), those that define the clock operate within molecular loops that by virtue of their intrinsic design principles generate their own rhythms. The emerging picture is that at least two interconnected transcriptional and translational feedback loops, one mainly functioning in a positive manner and the other in a negative capacity, provide synchronized pulls and pushes that generate an oscillator with a stable period of around 24 h (51, 156). Indeed, cells displaying a synthetic oscillatory network were engi-
neered based on the introduction of a simplified negative transcriptional-translational feedback loop (37). Synchronization of circadian oscillators with the outside world is achieved because light (or other external temporal cues) has acute effects on the levels of one or more of a clock’s components, the consequences of which have ripple effects that are experienced throughout the interconnected molecular loops, leading to a stable phase realignment of the endogenous rhythm generator and the external entraining conditions. Yet, despite striking similarities, there are interesting differences that reveal incredible flexibility in how molecules can assemble to build circadian timekeeping devices.

With this preamble, we can begin to appreciate why research on circadian rhythms has generated so much interest lately, being recognized in 1998 by the American Association for the Advancement of Science (AAAS) as the first runner-up in their list of breakthrough of the year. Clocks were also on the AAAS “top 10 list” in 1997. Given the enormous recent progress in our understanding of the molecular bases of circadian clocks, many excellent reviews have been written just in the last 2 years detailing these discoveries (31, 34, 54, 58, 61, 64, 72, 73, 75, 76, 81, 87, 110, 137, 148, 152, 153, 163, 165, 195, 197, 204, 205). This review is mostly structured as an overview of research on circadian rhythms, from human health issues to mechanisms. More detailed discussions on any particular aspect can be found in the cited references. As far as mechanisms are concerned, focus is placed on the circadian clocks of Drosophila and mice; the best-studied animal model systems.

Overview of the Circadian Timing System

Circadian rhythms are operationally defined as biological rhythms that exhibit the following three properties: 1) persist (or free-run) with a period of \( \approx 24 \) h in the absence of external time cues (or zeitgebers); 2) reset by changes in environmental conditions, most notably the daily light-dark and temperature cycles; and 3) have an invariant period length over a wide range of physiologically relevant temperatures (temperature compensation, see above) (for a comprehensive treatise of circadian rhythms see Refs. 62 and 114).

It is not clear why circadian clocks are designed with the capacity to keep on ticking for long periods of time in the absence of a cyclical environment (first property), a situation not normally faced in nature. A free-running oscillator might enable animals to maintain synchrony even during days when adverse weather conditions or other unfavorable settings force them to seek shelter in places that receive little or no light. Alternatively, this self-sustaining property might reflect some peculiarity of the design principles required to build these oscillators and not an adaptation with a particular advantage. The ability to reset a circadian clock (second property) allows it to maintain temporal alignment with local time. The last property (i.e., temperature compensation) makes biological sense, because regardless of whether it is a cold day or a warm day, it still lasts 24 h. A mechanism that can offset the effects of temperature on the periodicity of an oscillator is likely to be absolutely necessary in non-homeotherms, if they are to maintain accurate timekeeping. Indeed, early versions of man-made clocks were not very accurate, because increases in temperature lengthened the pendulum causing the clock to slow down.

The circadian timing system is usually depicted as being composed of three interconnected parts: 1) input pathways that can receive and transmit environmental cues, such as light and temperature, to a 2) clock or pacemaker, connected to 3) downstream effector pathways that manifest overt rhythms. Although the input to clock to output paradigm is usually depicted as moving from left to right, there are examples where the flow of information occurs in the opposite direction. These findings support a recent model whereby photic input and circadian clock are viewed in a more fluid relationship being composed of overlapping molecular loops (140). The demonstration that even in metazoans isolated cells can manifest bona fide circadian rhythms (e.g., 112, 192) indicates that an intracellular network can describe the molecular events beginning with input and ending with rhythmic output (e.g., 41).

What Need for a Circadian Clock?

Why do we have internal clocks that can record the passage of a day? Knowing how long circadian rhythms have been around could suggest a primordial role(s). Although we can only speculate, some 15 years ago it was shown that cyanobacteria manifest bona fide circadian rhythms, dispelling a long-held view that only nucleated creatures danced to the rhythm of endogenous daily pacemakers (59 and references therein). Thus circadian clocks might have been present in the earliest life forms. Early clocks could have endowed organisms lacking spatial barriers with the means to separate incompatible biochemical reactions (e.g., reduction/oxidation or photosynthesis and nitrogen fixation) in time. Another possibility that has been suggested is that because the first appearance of life might have occurred while the earth lacked an extensive ozone layer, having a synchronizable clock could enable an organism to, for example, synthesize DNA at night when the ionization radiation from the Sun is minimal. (For more detailed discussions of these and other speculations see Refs. 129 and 198.)

But how did clock mechanisms arise? Again we can only speculate. A hint may be offered by the conserved molecular logic underlying circadian oscillators. As noted above, compelling evidence strongly suggests that all circadian clocks are based on periodic oscillations in contrast to a more hourglass-type of mechanism that has to be turned over every day. It is possible that primitive cells exhibited “spontaneous” oscillations in the levels of macromolecules, perhaps driven by changing rates of synthesis and destruction. These oscillations with widely differing periods could have
enhanced the adaptive capabilities of cells by providing them with the means to routinely sample large concentration gradients. It has been speculated that one or more “random” biochemical oscillations with an intrinsic period close to 24 h and likely containing a photosensitive entity was widely adopted by cells because of the selective advantage conferred in tracking daily time (e.g., 14). Relevant to this discussion is the observation that PAS or PAS-like domains (named after the three founding members of this superfamily: Drosophila PER, mammalian ARNT, and Drosophila SIM) are protein motifs commonly found in several eukaryotic clock-relevant proteins (4, 6, 24, 28, 48, 67, 79, 82, 119, 143, 162, 166, 175, 194, 196). Although the PAS domain was originally shown to mediate protein-protein interactions (70), in some cases it functions as an interface for ligand or cofactor binding (15). It is possible that the more canonical PAS domain found in clock proteins from animals evolved from the PAS-like LOV domain (light, oxygen, voltage) that plays a role in a growing number of diverse signaling pathways (reviewed in Ref. 174). Incorporating a motif that can sense environmental conditions into an oscillatory feedback loop with an endogenous period of about a day could have been the start of entrainable circadian clocks (24, 79).

Whatever the early driving force(s), from our present vantage point it appears that the most critical property of circadian clocks under natural conditions is that they can be reset by external time cues. This property was not merely selected so that we could avoid perpetual jet lag following transmeridian flight. Rather, the ability to anticipate environmental changes enables organisms to organize their physiology and behavior such that they occur at biologically advantageous times during the day. In addition, a second function that is widely regarded as important is that these endogenous timekeeping devices also serve to impose internal alignments between different biochemical and physiological oscillations.

With this in mind we can appreciate why circadian rhythms are observed at all levels of cellular organization. There are daily oscillations in the levels of enzymes and hormones that affect the timing of cell function, division, and growth. Physiological parameters such as body temperature, immune responses, digestion, susceptibility to anesthesia, and dental pain threshold (the best time to go to a dentist is in the afternoon; Ref. 198) all undergo cyclic changes peaking at fixed times during the day. Our visual and mental acuity fluctuate during the day, affecting complex behaviors.

In addition to circadian rhythms that are manifested by and within individuals, there are also group or population rhythms. Some of these rhythms occur multiple times during the lifetime of the organism. For example, in many Diptera, males and females have the same peak time for activity during a daily cycle, increasing the chances of productive encounters between the sexes. In this regard it is interesting to note that because related species of insects have varying daily distributions of activity (e.g., 127), the circadian clock might have contributed to insect speciation by establishing temporal barriers limiting the mating opportunities of individuals sharing the same spatial constraints (182). Other population rhythms involve events that occur once in a lifetime. A well-studied example is the eclosion (emergence from pupal cases) rhythm in Drosophila, which is only apparent in a group of individuals comprising mixed developmental stages. The circadian clock gates the timing of eclosion such that it happens in the early morning when the relative humidity in the air is high (128). This is important because upon emerging from its pupal case the fruit fly is susceptible to desiccation, and its wings do not readily expand at low humidities. An interesting example of a population rhythm that is composed of many synchronized once-in-a-lifetime events is the daily oscillation in luminescence displayed by the cyanobacterium Synechocystis sp. (reviewed in Refs. 75 and 87). It is interesting to note that this rhythm is somehow transmitted from mother to daughter in mid-stride without missing a beat, as the replication cycle is shorter than 24 h (88).

Stable population rhythms are not restricted to individuals of the same species. The classic tango between bees and plants is a case in point. Different flowering plants have characteristic times during the day when they open and close their petals, making nectar available only at restricted times. The presence of an endogenous and synchronizable clock maximizes the feeding success of bees by enabling them to return to the same plants at times in the day when their nectar is available.

These rhythms also highlight the fact that the “adaptive value” of a circadian rhythm might only be understood within the framework of the dynamic interactions occurring in particular habitats. On a more global perspective, it is important to consider that organisms do not adapt to a static environment but one that undergoes daily changes. Oscillations in physical parameters (e.g., intensity of visible light, water and air temperature, relative humidity) will pervade natural habitats and their occupants, adding a strong daily component to the intricate relationships that govern ecosystems. Whether physical or biotic factors play primary or secondary roles in influencing the daily activity patterns of animals is likely to be largely dependent on the species in question. It has been suggested that the high rate of water loss in dry air might be the main driving force for the nocturnal activities of some small animals (22). On the other hand, biotic factors such as predation are relatively more significant in determining the daily activity patterns of larger animals. In any case, it is almost certain that many behaviors involved in mating, reproduction, seeking shelter, hunting for food, and avoiding predators evolved to take advantage of temporal niches. A recent study showed that the ability of Drosophila to smell odors is under circadian regulation (90), suggesting that many cyclical behaviors are ultimately “hard-wired” into clocks that regulate physiological changes.
in the ability to sense, interpret, and respond to various cues in the environment.

Two points of caution should be made at this stage. First, given the “chicken-and-egg” problem (which came first?) inherent in the complex and highly interdependent interactions between life forms and their physical environments, it is not possible to determine with a high degree of certainty the cause and effect in the dynamic relationships between individuals. Second, although light is the primary synchronizer of circadian clocks in nature, other physical or biotic factors might be the primary force(s) governing the adaptive function of a circadian rhythm.

Circadian clocks are not limited to timing daily events, but also play a role in adapting to seasonal changes in day length (photoperiod). By distinguishing between the long days (or short nights) of summer/spring and the short days (or long nights) of autumn/winter, organisms that live in temperate latitudes can anticipate and respond to seasonal changes in external conditions by controlling appropriate developmental, physiological, and behavioral switches. For example, certain species of insects enter diapause, a period of growth arrestment that is induced by short photoperiods or cold temperatures (reviewed in Refs. 149 and 187). The Siberian hamster typically breeds only in the spring and summer months, a seasonal adaptation that is partly regulated by regression of the gonads induced by the expanded nocturnal release of melatonin (203). Photoperiodism has been extensively studied in plants, where floral initiation can be experimentally controlled by altering day length. Recent genetic evidence in the flowering plant Arabidopsis clearly indicates that common elements participate in circadian clock function and in eliciting photoperiodic responses (119, 146, 162).

In addition to day length, circadian rhythms are modulated by seasonal changes in average daily temperatures. Diurnal animals typically respond to colder temperatures by displaying a greater proportion of their activity during daytime hours, whereas nighttime activity predominates at warmer temperatures. This directional response has a clear adaptive value, ensuring that the activity of an organism is maximal at a time of day when the temperature would be expected to be optimal for activity (169). Direct evidence for circadian clock function in temperature-induced alterations in the timing of the daily distribution of activity has been shown in Drosophila melanogaster, where a thermosensitive splicing event in per RNA contributes to preferential daytime activity on cold days (107).

A less classic example in which circadian clocks are used is during long distance navigation of birds, insects, and other animals to predetermined target areas using the azimuth of the sun as a compass. By artificially resetting the circadian clock, the animal misrepresents the position of the sun leading to a predictable change in the direction of navigation (e.g., 19, 93, 123, 126, 151).

Despite the widespread manifestation of circadian rhythms by living forms, ablating central pacemaker tissues or abolishing the activities of key clock components does not appear to affect viability. An exception is the D. melanogaster “double-time” (DBT) protein, a kinase that regulates the phosphorylation and stability of PER (85, 131). A presumptive null or strong hypomorphic allele called dbt<sup>-</sup> is associated with pupal lethality. However, it is almost certain that this lethal phenotype is unrelated to clock-specific roles for DBT and is a consequence of developmental defects (210). Despite the (presumably) nonessential nature of circadian oscillators, there are examples where longevity, ability to avoid predators, and reproductive fitness can be reduced in mutant strains that are either arrhythmic or manifest behavioral rhythms with significantly abnormal periods (29, 83, 124). Part of the problem in trying to address the presumed selective advantage of circadian oscillators is that, for the most part, organisms that lack or have altered clocks have been assessed under laboratory conditions in which individuals are housed under favorable environmental conditions in the absence of predators or competition from their wild-type counterparts for food acquisition or partners to mate.

**Human Health Issues**

The medical implications of circadian rhythms are immense and can be broadly classified into the following three groups (based on Ref. 114).

**Effects imposed by external conditions on otherwise healthy individuals.** This group can be further divided into symptoms that arise from acute changes in external time cues, such as transmeridian flight (jet lag), and those that result from continual changes in light-dark cycles, most notably arising from shift work. Technological advances, from the invention of the light bulb to dramatic increases in the speed of air travel to highly integrated world-wide communication systems, have permitted individuals and societies to escape the temporal constraints otherwise imposed by the natural environment. However, this “escapement” has a price tag, because human physiology has not undergone comparative changes and remains firmly interconnected with the internal pacemakers we all carry. Performing tasks during times in the day when psychomotor capabilities are suboptimal is associated with many serious consequences. For example, nurses on a repetitive shift work schedule are two- to threefold more likely to misdiagnose and wrongly treat patients than their daytime counterparts (53). More extreme examples of accidents related to the ill effects of “unnatural” work schedules include the Chernobyl nuclear plant in 1986, the chemical explosion of the Union Carbide plant in Bhopal, India, in 1984 and the grounding of the oil tanker Exxon Valdez in 1989 (92).

The effects of transmeridian flight and shift work on the human circadian timing system likely occur at two levels. For many years it was believed that the primary circadian pacemaker in mammals is located in the suprachiasmatic nucleus (SCN) in the brain (reviewed in Ref. 191). Although this idea remains relatively
intact, more recent studies have shown that similar to *Drosophila* (49, 50, 60, 130), independent circadian pacemakers are present in many tissues in vertebrates such as zebrafish (193, 194) and mammals (7, 180). The emerging picture is that in intact mammals, much of the photic input to the circadian timing system is transduced via the retinohypothalamic tract (RHT) to the SCN (reviewed in Refs. 44 and 104), which in turn conveys time-of-day information to peripheral clocks that have tissue-specific regulatory features (e.g., 157, 212). Desynchronization not only occurs between the external environment and the SCN rhythm generator but also affects phase alignments between the different peripheral clocks (201). Different rates of resynchronization amongst the cellular clocks in the SCN and those found in the various tissues likely contribute to the dysfunction associated with jet lag and other abrupt changes in light-dark cycles (201).

Melatonin, a naturally produced hormone that is under circadian regulation, has been used to alleviate disorders associated with jet lag and shift work (reviewed in Ref. 13). Numerous lines of evidence suggest that the administration of melatonin can elicit phase shifts (e.g., 99), although other roles for this “wonder drug”, such as beneficial effects on longevity, combating cancer, and mounting immune responses remain controversial (138). Another successful approach for treating jet lag and shift work has been the use of phototherapy (11, 32, 33). The rationale for this non-invasive treatment is based on earlier work in model organisms showing that depending on when during the night a short pulse of light is administered, it can evoke either a delay or advance in the phase of the clock. Ideally, by correctly timing the phototherapeutic treatment, the rate of resynchronization to local time can be accelerated.

It is estimated that more than 20% of the U.S. workforce is subjected to shifting work schedules (201). This includes a wide variety of occupations where suboptimal psychomotor capabilities could have disastrous consequences for many people, such as medical personnel, pilots (16), air traffic controllers and other systems administrators (26, 106), security and military personnel (52), and commercial truck drivers (57). More attention needs to be placed on the physiological, behavioral, social, and economic consequences of maintaining societies that are active round the clock. Indeed, a growing number of private and public entities have emerged that use circadian principles to recommend ways of minimizing the malaise associated with abrupt changes in light-dark schedules.

**Issues related to diagnosis and treatment.** Many physiological and behavioral variables change in a rhythmic manner over the course of a day. Whether the lack of accounting for circadian variations in medically relevant variables has had a significant negative impact on diagnosis and treatment plans is not clear. Sampling at different times of day and knowing the natural rhythm of the variable in question would enable physicians a more precise account of the status of the patient. However, in addition to the inherent problem of feasibility in round-the-clock sampling, other factors such as exposure to “unnatural” light conditions or patients with malfunctions in their circadian timing system might lead to rhythms that are altered, rendering the variable unreliable as a diagnostic indicator.

For many years it has been known that the efficacy of certain drugs is dependent on time of delivery. Some well-studied examples are in the treatment of cancer (reviewed in Ref. 97). It is possible to increase the therapeutic potential and minimize toxic side effects by optimizing schedules for administering drugs. Many drugs used in chemotherapy affect the function and replication of normal and malignant cells. By targeting times when normal cells are less likely to be undergoing DNA synthesis, higher levels of chemotherapeutic drugs can be tolerated, increasing the effectiveness of the treatment (84, 160). In general, it is not surprising that there is circadian variation in the efficacy of certain drugs or agents. Rates of absorption, metabolism, target susceptibility, and excretion vary throughout the day, contributing to time-of-day differences in the beneficial and toxic effects of drugs.

**Disorders or disease states that appear to be causally linked to malfunctions in the circadian timing system.** Malfunctions in the circadian timing system are associated with several disorders such as chronic sleep disturbances, manic-depression and seasonal affective disorders (SAD, or winter depression) (reviewed in Ref. 23). This is a very active area of research. The extent to which circadian disturbances are causally linked to the manifestation of the disorder or are secondary downstream events of the diseased state are not clear. Nonetheless, many of the symptoms associated with certain chronic sleep problems and affective disorders can be alleviated by alterations in light-dark schedules (e.g., 176). With recent advances in molecular genetics, it will be possible to determine whether polymorphisms in clock genes are causally linked to disorders that show a strong circadian component. Whether this line of investigation will also explain the basis for “night owls” and “early birds” remains to be seen (78). The recent demonstration that rest in *D. melanogaster* has physiological and behavioral correlates with sleep in mammals should provide interesting insights into understanding the role of circadian factors on regulating sleep and its substrates (66, 155).

**The Fly and Mammalian Circadian Clocks**

Progress in understanding the molecular underpinnings governing circadian rhythms has been remarkable in the last 3 years. This has included the isolation, characterization, and manipulation of clock gene products and their interconnections in cyanobacteria, *Neurospora*, plants, *Drosophila*, zebrafish, amphibians and mammals. The discussion that follows is limited in scope to a bird’s-eye view of the clockworks operating in *Drosophila* and mice, the two best-studied animal model systems for understanding neural circadian pacemakers. Both organisms show remarkable similarities in clock components and overall molecular functions.
logic. Indeed, due to extensive similarities, much “cross-fertilization” has occurred in studies using these two model organisms, and the story of one is not complete without the other. Despite numerous apparent similarities, it is also clear that striking differences exist.

Drosophila. At least seven circadian-relevant clock/light-input genes have been characterized in D. melanogaster (to avoid confusion with structural homologs in other species, all the genes and protein products corresponding to those from D. melanogaster will be preceded by the suffix “dm”). These are dmclock (dmClk) (4, 6, 28), dmcycle (dmcyc/dmBmal1) (28, 143), dmperiod (dmper) (8, 21, 89, 135, 206), dmtimeless (dmtim) (47, 117, 154), doubletime (dmdbt) (85, 131), dmcryptochrome (dmcry) (36, 39, 164) and dmvrille (dmvri) (10). In addition, at least one rhythmically expressed factor that functions downstream of the clock in effector pathways has been identified, namely, pigment dispersing factor (dmpdf) (65, 125, 136). How do the RNA and protein products of these genes interact to assemble an entrainable self-sustaining oscillator that can control overt rhythms? Although the picture is not complete, it is clear that the core mechanism comprises at least two interconnected positive and negative transcriptional-translational feedback loops that take 24 h to complete a cycle (51) (Fig. 1). Entrainment of these molecular loops by light-dark cycles is achieved because photic cues stimulate the rapid degradation of dmTIM (71, 116, 208). Because most of the core clock proteins regulate clock gene expression, daily oscillations in their activities likely drive rhythmic expression of target genes involved in output effector pathways (125, 141, 186), although a direct molecular link has not been shown in Drosophila.

dmCLK and dmCYC are members of the basic helix-loop-helix (bHLH)/PAS (PER-ARNT-SIM) superfamily of transcription factors that heterodimerize to activate dmper and dmtim by binding E-box elements (4, 6, 28, 95, 143) and act to repress dmClk transcription (51).

Fig. 1. Model of circadian clock in Drosophila melanogaster showing photic input pathway (light; CRY) and two downstream effector pathways (ccgs, clock-controlled genes; PDF, pigment-dispersing factor). Note that the mechanism shown is a combination of intracellular pathways occurring in two distinct cells in the adult head, the photoreceptors in the eyes (R1-R8) and a small group of pdf-expressing brain cells called the small ventrolateral neurons (s-LNv.s). The light-induced CRY-mediated degradation of TIM might not occur in the s-LNv.s (164; however, see Ref. 40), whereas the regulation of pdf RNA abundance by dCLK:CYC and the posttranscriptional control of PDF neuropeptide levels by PER and TIM appears to be restricted to the s-LNv.s and not other pdf-expressing cells (125). Other steps shown are likely to be common to all clock cells in the adult head. During the late day/early night, the levels of PER (indicated by large P) and TIM (indicated by T) reach critical concentrations that favor dimerization, an event that stabilizes PER and stimulates the nuclear entry of the PER-TIM complex. The enhanced degradation of monomeric PER in the cytoplasm as a result of DBT-mediated phosphorylation events and the light-induced degradation of TIM, contribute to a delay in the nuclear accumulation of PER and TIM. In the nucleus, PER, TIM, or both interact with dCLK:CYC, blocking its ability to stimulate transcription of per, tim, vri, and possibly ccgs and 2) by a mechanism that is not clear, upregulate expression of dClk and cry. Not shown is the degradation of highly phosphorylated PER and TIM in the nucleus, which relieves the block on dCLK:CYC-mediated transcription and leads to the downregulation of dClk and cry expression. Green lines, pathways leading to upregulation; red lines, pathways leading to downregulation; dashed lines, uncertain pathways. Small black boxes indicate E-box elements; small P, phosphorylation; ub, ubiquitin.

http://physiolgenomics.physiology.org
The biochemical activities of \textit{dmPER} and \textit{dmTIM} are less well understood than those of \textit{dmCLK} and \textit{dmCYC}, but current evidence indicates that they interact with \textit{dmCLK:dmCYC} blocking its activity (5, 94, 95), an event that leads to downregulation of \textit{dmper} and \textit{dmtim} transcription (28, 95, 161, 207) and activation of \textit{dmClk} expression (6, 51, 94).

Transcription of \textit{dmper} and \textit{dmtim} begins in the early-to-midday resulting in peak RNA levels being reached in the early night. However, the temporal profiles of \textit{dmPER} and \textit{dmTIM} protein accumulation are delayed by several hours relative to their RNA cycles, with maximal levels attained in the middle of the night (207). Several posttranscriptional regulatory features have been identified that contribute to this lag (reviewed in Ref. 34). Newly synthesized monomeric \textit{dmPER} in the cytoplasm is phosphorylated in the presence of \textit{dmDBT}, a kinase with homology to casein kinases, targeting \textit{dmPER} for rapid degradation (85, 131). In addition, light stimulates the rapid degradation of \textit{dmTIM} by the proteasome (118). So how do \textit{dmPER} and \textit{dmTIM} levels accumulate in the cytoplasm? As noted above, the RNA levels of \textit{dmper} and \textit{dmtim} peak during the early night. In the presence of high levels of template RNA and the absence of light, \textit{dmTIM} accumulates rapidly during the early night. Furthermore, the interaction of \textit{dmTIM} with monomeric \textit{dmPER} in the cytoplasm protects \textit{dmPER} against \textit{dmDBT}-induced degradation by forming a stable \textit{dmPER-dmTIM} complex (85, 131, 132, 190). The life cycle of \textit{dmPER} is highly dependent on \textit{dmTIM}, and the daily upswing in \textit{dmper} RNA levels is likely only important in timing when critical concentrations of \textit{dmPER} that favor binding \textit{dmTIM} are reached (107, 167).

Numerous lines of evidence indicate that the interaction of \textit{dmPER} with \textit{dmTIM} stimulates nuclear entry of the \textit{dmPER-dmTIM} complex (116, 145, 190), which occurs during a restricted gate in the middle of the night (27). The interaction of \textit{dmPER} with \textit{dmTIM} overrides cytoplasmic localization determinants (CLDs) that enable the functioning of their respective nuclear localization signals (NLSs) (145). Once in the nucleus, \textit{dmPER}, \textit{dmTIM}, or both interact with \textit{dmCLK:dmCYC} blocking its activity (5, 94, 95). At the same time, \textit{dmPER} and \textit{dmTIM} inhibit the action of an unidentified negative regulator(s), leading to stimulation of \textit{dClyk} (and likely \textit{dmcry}) expression (51). The slow accumulation of monomeric \textit{dmPER} and \textit{dmTIM} in the cytoplasm presumably introduces the necessary time delay to enable the daily upswing in the levels of \textit{dmper} and \textit{dmtim} RNAs. In the absence of de novo synthesis, nuclear localized \textit{dmPER} and \textit{dmTIM} undergo progressive phosphorylation events (35, 208). The \textit{PRQ} protein in the \textit{Neurospora} clock also undergoes qualitatively similar time-of-day-specific phosphorylation events that have been proposed to function as “biochemical time constraints.” Essentially, the progressive phosphorylation of clock proteins that participate in autoinhibition might lengthen their lifespans, endowing them with the means to function over extended periods of time in the absence of de novo synthesis, an attribute that helps to stretch the periodicity of transcriptional feedback loops to ~24 h (46, 111). It is believed that once \textit{dmPER} and \textit{dmTIM} reach critical phosphorylated states, these act as molecular switches targeting these hyperphosphorylated isoforms for rapid degradation. Dramatic reductions in the concentrations of \textit{dmPER} and \textit{dmTIM} in the nucleus relieves autoinhibition of \textit{dmCLK:dmCYC}, beginning another round of \textit{dmper/dmtim} expression and concomitant repression of \textit{dmClk} transcription. These opposite effects of \textit{dmPER} and \textit{dmTIM} on their own expression and that of \textit{dmClk} likely explain the antiphase cycling of \textit{dmCLK} RNA levels compared with those of \textit{dmper} and \textit{dmtim} (6).

The role of \textit{dmvri} in the oscillator is more enigmatic. It was isolated in a screen for transcripts that were differentially expressed in wild-type flies and the arrhythmic \textit{per\textsuperscript{101}} mutant (10). Sequencing of one such candidate revealed that it was \textit{dmvri}, a previously identified gene encoding a basic zipper (bZIP) transcription factor with an essential role during development. The \textit{dmvri} RNA levels cycle in phase with those of \textit{dmper} and \textit{dmtim} and are regulated by \textit{dmCLK:dmCYC} binding to an E-box element. Constitutive misexpression of \textit{dmvri} in \textit{dmtim}-expressing cells suppresses \textit{dmper} and \textit{dmtim} transcript levels. In addition, continuos \textit{dmvri} expression decreases the levels of \textit{dmPDF} via a posttranscriptional regulatory mechanism. Reduced levels of \textit{dmPDF} might account for the long period and increased arrhythmic phenotypes found in flies where \textit{dmvri} levels are manipulated to remain constant. Regulation of \textit{dmPDF}, a clock-controlled neuropeptide that acts as a circadian effector in the manifestation of rhythmic locomotor activity in \textit{Drosophila} (65, 136), is very complex and tissue specific (125). Numerous studies indicate that a small number of neurons in the brain called the ventrolateral neurons (LN\textsubscript{vs}) are critical pacemaker cells underlying daily locomotor activity (reviewed in Ref. 77). In a subset of these neurons called the small LN\textsubscript{vs} (s-LN\textsubscript{vs}), \textit{dmPDF} levels cycle, but those of its RNA are constant (125). Mutations in \textit{dmCLK:dmCYC} activity are associated with low levels of \textit{dmPDF} RNA and protein, whereas in the absence of \textit{dmPER} and \textit{dmTIM}, the posttranscriptional rhythm of \textit{dmPDF} levels is eliminated with no apparent changes in \textit{dmPDF} RNA levels (125). How \textit{dmPDF} functions as a circadian transmitter is not clear, but recent evidence indicates that \textit{dmCRY}, \textit{dmPER}, \textit{dmTIM}, and \textit{dmPDF} are all expressed in the LN\textsubscript{vs}, showing that elements associated with input, clock, and output can be present in the same cell (41).

As mentioned above, the two most important zeitgebers in nature are the daily and seasonal changes in visible light and ambient temperature. The primary clock-specific photoresponse in \textit{Drosophila} is the enhanced degradation of \textit{dmTIM}, which initiates a chain of events that ultimately result in the (re)synchronization of the clock to the entraining light-dark cycles (71, 96, 116, 168, 202, 208). For example, the light-induced

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degradation of dm TIM during its accumulation phase delays progression of the cycle, whereas the opposite is true during its declining phase. This simple model can account for the observation that light pulses administered in the early night evoke phase delays in behavioral rhythms, whereas similar treatments in the late night elicit phase advances (98, 116, 150). It appears that in most, if not all, pacemaker cells the photoreceptor dm CRY is required for the light-induced degradation of dm TIM and to mediate all circadian photoprocesses (40, 41, 164). Presumably, in the presence of light, activated dm CRY interacts with dm TIM (17), followed by phosphorylation of dm TIM by a tyrosine kinase that targets it for ubiquitination and rapid destruction by the proteasome (118).

Although temperature likely influences the circadian oscillator at many levels, a thermosensitive splicing event in the 3′-untranslated region (3′-UTR) of dm per RNA contributes to an earlier rise in the levels of its RNA (107). This phase advance enables flies to maintain daytime activity during seasonally cold days when day length is shortened. In this situation, the effects of temperature on the rate of dm PER accumulation and those of light on the metabolism of dm TIM are integrated, because dm PER and dm TIM engage in a functional interaction that appears necessary for progression of the cycle.

Mice and a hamster. The identified circadian clock-relevant genes in mice are remarkably similar in sequence to those of Drosophila except that in some cases multiple homologs exist. The clock or clock-candidate genes in the mouse are m Per (3 homologs designated m Per1, m Per2, and m Per3) (3, 157, 166, 171, 173, 175, 209, 212), m Clock (82, 188), m BMAL1 (48, 67, 170), and m Cry1 and m Cry2 (56, 91, 122, 177, 184, 189). The role of a putative homolog of dm tim, m Tim, in the mammalian circadian oscillator is presently not clear (43, 86, 147, 172, 178, 211). Work in hamsters has also significantly contributed to the emerging picture in mammals. A spontaneous mutation in a Syrian hamster with a short period phenotype was identified in a daily accumulation of m Per1, m Per2, m Per3, m Cry1, and m BMAL1 (48, 74, 91, 121). Unlike the scenario for Drosophila, m BMAL1 undergoes daily oscillations in its RNA levels (68, 120, 121), whereas m Clock is constitutively expressed (166, 175). Despite this difference, similar to dm Clk in Drosophila (6), m BMAL1 RNA levels cycle in antiphase to those of the m Per1 and m Cry1, which appear to have synchronous oscillations (91, 113, 121, 156, 212). m PER2 functions as a positive regulator of the BMAL1 loop (perhaps in a similar fashion to the stimulation of dm Clk expression by dm PER in Drosophila) (6, 51, 156). The most radical departure from the situation in Drosophila is that m CR Y1 and m CR Y2 are not required for light perception by the circadian pacemakers (122, 189) but have essential roles in the core oscillator by inhibiting the activity of m CLOCK:m BMAL1-mediated transcription (56, 91, 122, 156, 184, 189).

From what is known so far, it appears that there are at least two pathways for the nuclear entry of the m PERs. In one case, m CR Y1 and m CR Y2 pair up with each of the m PERs and transllocate them to the nucleus (43, 91). More recent findings indicate the existence of m PERs.
a mCRY1/mCRY2-independent pathway whereby the nuclear translocation of mPER1 and mPER2 is stimulated by physical interactions with mPER3 (200). It appears that the mPER1:mPER3 and mPER2:mPER3 interactions lead to masking of the mPER3 CLD sequence with concomitant activation of its NLS (200). The interplay between CLD and NLS sequences in targeting mPERs to the nucleus in mammals is similar to that proposed for regulating the nuclear entry of dmPER and dmTIM in Drosophila (145). Once in the nucleus, there is a division of labor whereby the mCRYs downregulate expression of the mPER and mCry cycles. On the other hand, mPER2 acts as a positive regulator of the BMAL1 loop (156) and is required for circadian oscillations in the expression of mPer1 and mPer2 (209). Also, despite the mid-to-high constitutive levels of mPer2 RNA levels in the SCN of mCry-deficient mice (122, 189), little or no staining of mPER2 immunoreactivity is observed, suggesting that the mCRYs either directly or indirectly stabilize mPER2 (156). A role for the mCRYs in the nuclear translocation and stabilization of mPER2 is very reminiscent of the effects of dmTIM on dmPER metabolism in Drosophila (145, 190). Another parallel is that the stability (80) and subcellular localization of mPER1 (and possibly the other mPERs; 187a) are also regulated by CKIε-mediated phosphorylation much like the effects of dmDBT on dmPER (85, 131).

Further experiments are still required to sort out the individual roles of the mPERS and mCRYs (and perhaps mTIM) in the circadian feedback loops. Given that in the SCN the period and cryptochrome oscillations are relatively synchronous and the various encoded protein products appear to colocalize in circadian-relevant cells it suggests the possible formation of numerous qualitatively different complexes with different or competing functions. Indeed, in brain homogenates or in cultured cells engineered to produce the relevant protein products appear to colocalize in circadian-relevant cells it suggests the possible formation of numerous qualitatively different complexes with different or competing functions. Indeed, in brain homogenates or in cultured cells engineered to produce the relevant proteins, intra- and interfamily interactions can be observed between the mPERS, mCRYs, and mTIM, but perhaps no mPER/mTIM interactions (43). Whether the various mCRY:mPER and/or mPER:mPER interactions have supplanted the role that dmTIM plays in regulating the stability and nuclear entry of dmPER in Drosophila is not fully understood (91, 200, 211). Clearly, the clock-relevant functions of the individual
members from each family are not redundant. For example, mice that are homozygous for a mutation that inactivates either mCry1 or mCry2 manifest free-running activity rhythms with short and long periods, respectively (177, 184, 189). How this antagonistic effect on circadian periodicity is accomplished is not known. Furthermore, in different mutant backgrounds, the RNA and protein products from mPer1 and mPer2 are differentially regulated despite their relatively synchronous cycles and acute responses to brief light stimulation (156).

There is some suggestive evidence that light-induction of mPer1 and/or mPer2 might be the primary clock-specific photoresponse in the photic entrainment of circadian pacemakers in the SCN (2, 3, 157, 158, 212; however, see 178). As mentioned above this is very different from the scenario in Drosophila where photic cues evoke rapid decreases in the levels of dmTIM (71, 116, 208) and dmCRY (39) (but not dmPER) via a posttranscriptional mechanism that involves the proteasome degradation machinery (118). The induction of mPer1 and mPer2 is reminiscent of the light-induced increases in the levels of fqr transcripts in Neurospora (25). It has been speculated that a more transcriptional-based response in mice and fungi might reflect the fact that the circadian clocks in these two species are referred to as daytime oscillators, where the primary photoresponsive clock element reaches peak values during the day (30, 158). The idea that upregulation of a light-sensitive clock component might be better achieved by the rapid induction of template RNAs, whereas posttranscriptional modes of regulation might be better suited for effecting rapid decreases in light-sensitive clock components.

Another difference in photic entrainment pathways between Drosophila and mammals is that, whereas circadian pacemakers in Drosophila appear to be independently photoresponsive (38, 49, 130) and dmCRY functions as a deep brain photoreceptor (41), clocks in the SCN do not appear to be sensitive to light. Rather, the eyes are largely responsible for providing visible light information to the circadian pacemakers in the SCN (reviewed in Ref. 44). Although the nature of the circadian-relevant photoreceptor(s) that functions in the mammalian eye is not known, retinal cones or rods are not required for photic entrainment (45, 105). The fact that the eyes are so critical for the synchronization of circadian clocks in the SCN most likely explains why blind people display various forms of circadian dysfunctions (144). With regard to temperature, it is not clear whether this modality plays a significant role in the regulation of circadian rhythms in homeotherms. Nonetheless, although the pacemaker temperature of homeothermic mammals in vivo is homeostatically controlled within narrow limits, circadian rhythms recorded from isolated tissues are temperature compensated and phase shifted by heat pulses (142, 181). These results suggest that temperature compensation of period length is a basic property of the intracellular molecular loops that define circadian oscillators.

Although studies on the clockworks operating in peripheral tissues have not been as extensive as those in the SCN, there are tissue-specific differences in the cycling profiles of some of the clock RNAs (74, 91, 157, 212). These differences might be important in maintaining optimal internal phase alignments. Work in zebrafish also makes it clear that the circadian feedback loops will differ amongst vertebrates. For example, the RNA products from the putative orthologs of mammalian Clock in zebrafish (zfClock) and BMAL1 (two homologs zfBMAL1 and zfBMAL12) both display circadian oscillations in abundance (18, 194).

Conclusions

Incredible progress has been made in understanding the molecular and cellular bases of circadian rhythms, and this is sure to continue at a rapid pace. The further we peer into the clockworks of a growing number of model organisms, the more we are struck by two seemingly opposing viewpoints; namely, within the apparent constraints of striking similarities are embedded radical differences. Whether this reflects divergent or convergent evolution (or something else) is a discussion for another time. However, understanding the molecular logic underlying the differences in these oscillatory networks will give us insights into how different species adapted to life on our rotating planet.

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