Physiology Flies with Time

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The 2017 Nobel Prize in Medicine or Physiology has been awarded to Jeffrey Hall, Michael Rosbash, and Michael Young for elucidating molecular mechanisms of the circadian clock. From studies beginning in fruit flies, we now know that circadian regulation pervades most biological processes and has strong ties to human health and disease.

We live in a cyclic environment, and adaptation to this, not surprisingly, appears to have favored rhythms in physiology in almost all organisms. Persistence of daily rhythms in the absence of cyclic environmental cues (such as light) was noted as early as 1729, by the French astronomer de Mairan, but the idea that physiological rhythms are generated by clocks within organisms was not accepted until the 20th century. We now know that organismal clocks drive rhythms in most physiological processes and behaviors, and disruption of circadian regulation, which includes desynchrony between endogenous clocks and the environment, has deleterious health consequences. The importance of circadian rhythms is recognized this year by the Nobel Assembly at the Karolinska Institute, which has awarded the 2017 Nobel Prize in Medicine or Physiology to three pioneering scientists for their work on elucidating the molecular mechanisms underlying these rhythms: Jeffrey Hall, Michael Rosbash, and Michael Young.

Control of daily rhythms by endogenous clocks was supported by the finding that periodicity of the internal daily rhythm varies slightly from species to species and often does not match the period of the external environment. This gave rise to the term "circadian" from "circa" (about) and "dies" (day). Synchrony to the 24 hr environmental cycle is an important feature of circadian clocks, though, and they synchronize in a manner determined by their intrinsic period. For instance, an organism with an endogenous circadian period <24 hr might become active before dawn in anticipation of the daily dark–light transition.

The heritable nature of circadian periodicity was demonstrated by Erwin Bunning in the 1930s through experiments in which he crossed bean plants with different periods of leaf-movement rhythms and found that the next generation displayed intermediate period lengths. Efforts to address relevant genetic factors came to fruition when Ron Konopka, at the time a graduate student in the laboratory of Seymour Benzer at Caltech, undertook a genetic screen for mutants affecting rhythms in *Drosophila*. Konopka screened flies for aberrant timing of eclosion, which is the emergence of adult flies from their pupal cases and typically occurs at dawn under the control of a circadian clock. Although eclosion occurs only once in a single fly, it can be monitored as a rhythm in a population, where peaks of emerging flies are evident in the early daytime hours. Konopka's screen identified mutants that showed unusually high levels of eclosion at night and turned out to also exhibit altered patterns of locomotor activity (Figure 1). In this historic screen, Konopka identified a mutant line with a short period (19 hr), a second line with a long period (29 hr), and yet another line that lacked rhythms altogether. Mapping of the three mutations and complementation tests indicated that they localize to the same gene, which Konopka named *period* (*per*) (Konopka and Benzer, 1971). Although the *per* mutants were reported in 1971, the gene was not isolated until the 80s, when Jeffrey Hall and Michael Rosbash, at Brandeis University, and Michael Young, at Rockefeller University, applied newly developed genetic and molecular tools for this purpose.

Each of the Laureates came from a different background. Hall brought a behavioral biology and *Drosophila* genetics perspective to the collaboration with Rosbash, who had expertise in molecular biology and biochemistry. Young trained as a *Drosophila* geneticist/cytogeneticist and was focused on the analysis of the fly X chromosome region that houses *per*. Both groups pinpointed the correct *per* transcript through rescue experiments, showing that rhythms were restored in arrhythmic per^{01} mutants by introduction of specific genomic fragments from the locus (Bargiello et al., 1984; Zehring et al., 1984). Konopka, who had retained his interest in circadian rhythms and in *per*, was a collaborator on the work from Brandeis.

Cloning *per* was a landmark, not only in the circadian field but also more broadly in the area of behavioral genetics. Cloning and sequencing of mutant *per* alleles was carried out independently by the two groups and revealed single-nucleotide changes. In the case of the short period (per^s) and the long period (per^l) alleles, the substitutions resulted in missense mutations, while in the arrhythmic allele (per^0), it introduced a premature stop codon, thereby truncating the reading frame, explaining the functionally null nature of the mutation. Phenotypes of all the mutants were very robust and penetrant, providing one of the earliest examples of the influence of small genetic changes on behavior. Follow-up studies from both groups revealed the essential nature of the residue mutated in the per^s allele, with the Young laboratory defining a per^s domain in which any of several amino acids could be mutated to produce a short period (Baylies et al., 1992; Rutila et al., 1992). *per* became a paradigm for a behavior-regulating gene, although we now know that its effects also extend to many other aspects of physiology.

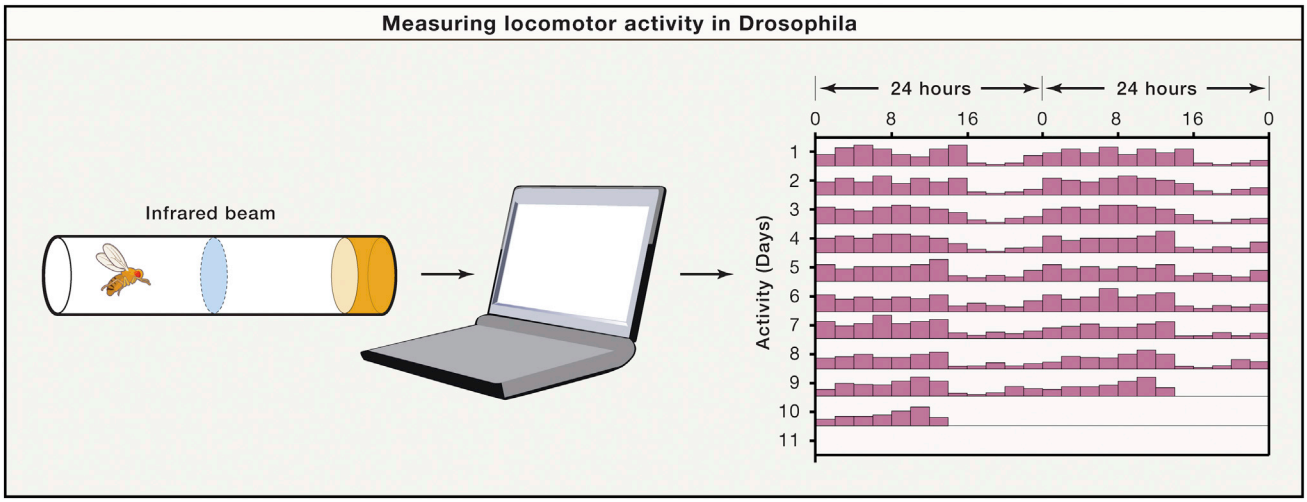

Figure 1. Assay for Locomotor Activity Rhythms in *Drosophila*

Individual flies are placed in glass tubes. Movement of the fly across the tube breaks an infrared beam (invisible to the fly), which generates an event recorded on a computer. The record depicts locomotor behavior over an ~10 day experiment with the shaded areas corresponding to activity. The data are double plotted such that the left side of each line replots the data shown on the right side of the line above. This particular fly was monitored in constant darkness to assess free-running rhythms and displays a period slightly less than 24 hr (note the leftward drift in the active phase each successive day).

Despite the clear importance of *per*, the molecular mechanisms underlying the circadian clock remained a mystery for several years after its isolation. PER's predicted protein sequence was not informative with respect to its biochemical function as the only distinguishing feature was a stretch of glycines and threonines. Ultimately, analysis of the expression levels of PER over a daily cycle provided clues to its role as a clock molecule. Hall and Rosbash found that levels of PER protein (Siwicki et al., 1988) and *per* mRNA (Hardin et al., 1990) vary over the course of the day with an ~6 hr delay in protein expression relative to the mRNA. *per* mRNA oscillations were altered or eliminated in period-altering or arrhythmic mutants of *per*, respectively, suggesting a role for the protein in the cycling of the mRNA. In support of a role for the protein in RNA cycling, short period (20 hr) cycles of mRNA expression were observed in per^{s} mutants in constant darkness (when rhythms are driven only by the endogenous clock), and transgenic expression of wild-type PER was found to drive cycling of the per^{0} transcript. Thus, the data indicated a feedback loop, which was proposed as part of the central clock mechanism (Hardin et al., 1990).

The identification of the second circadian mutant, *timeless* (*tim*), by the Young group added a new dimension to the proposed feedback loop mechanism (Sehgal et al., 1994). *tim* came out of a genetic screen very similar to the one conducted by Konopka >20 years earlier. While Konopka focused on the X chromosome, this screen was designed to identify recessive mutations on the second and third chromosomes (the two major autosomes in *Drosophila*). A single arrhythmic mutant was isolated, *tim*, which, like the *per* mutants, exhibited disrupted eclosion and locomotor activity rhythms. In addition, the *tim* mutant (eventually termed tim^{01} after additional alleles were discovered) was found to alter regulation of *per*, such that oscillations of *per* mRNA were abrogated and the localization of a PER-β−galactosidase fusion protein was shifted from the nucleus to the cytoplasm (Vosshall et al., 1994). Overall stability of PER was also reduced in tim^{01} mutants. As was the case for *per*, cloning of the *tim* gene did not provide a biochemical function, but it led to the finding that *tim* is expressed cyclically with a pattern similar to that of *per* (Sehgal et al., 1995). Elimination of *tim* mRNA cycling in tim^{0} mutants and additionally in per^{0} mutants indicated that *tim* also functioned in an autoregulatory loop and suggested that the two genes display mutual regulation. Based on these findings, Young and colleagues proposed a clock model whereby the *tim* and *per* genes are co-regulated through rhythmic feedback by the two proteins and TIM is critical for ensuring timely expression and nuclear localization of PER (Sehgal et al., 1995; Vosshall et al., 1994). They suggested that the delay separating peak expression times of *per* RNA and protein arises from temporal regulation of TIM expression in a daily cycle. A delay in negative feedback by the protein is thought to be critical for maintaining a cycle as it separates the phase of mRNA synthesis from the phase of repression, thereby preventing the opposing effects on transcription from reaching an equilibrium.

Remarkably, the predictions of the model proposed by the Hall, Rosbash and Young were borne out by subsequent experiments. As predicted, TIM was found to cycle and stabilize PER in a timely fashion. Through continued genetic screening for circadian mutants, the Young laboratory discovered a gene they termed *doubletime* (*dbt*) (Price et al., 1998), that encodes casein kinase 1ε. DBT phosphorylates and destabilizes PER, and TIM counteracts DBT to stabilize PER at a specific time of day. The *per-tim* feedback loop is important not only for the timekeeping mechanism, but also for entrainment of the clock to light. Exposure to light degrades the TIM protein, which affects the stability of PER and sets the internal clock to a specific

time of day. Light signals are transmitted to TIM primarily by a blue light photoreceptor, Cryptochrome (CRY), discovered in *Drosophila* by Hall and Rosbash (Emery et al., 1998; Stanewsky et al., 1998).

While PER and TIM appeared to regulate their own transcription, the mechanism by which they did so was debated. Lack of a DNA binding domain in either protein led to the idea that they repress transcription by inhibiting activity of transcriptional activators. Insights into the type of transcription factor that might be involved came from identification of a unique domain that PER shares with the *Drosophila* single-minded protein (SIM) and the mammalian aryl hydrocarbon receptor nuclear translocator (ARNT). This domain, which was discovered by Steve Crews and termed PAS (for PER, ARNT, and SIM), consists of two repeats (A and B) of ~50 amino acids each, separated by an ~150 amino acid spacer, and was shown, by the Rosbash laboratory, to mediate protein-protein interactions (Huang et al., 1995). Given that SIM and ARNT belong to the basic-helix-loop-helix (bHLH) family of transcription factors, the model then was that PER inhibits transcription by binding to a bHLH factor through its PAS domain. The first such transcription factor was found in mice in the Takahashi laboratory and termed *Clock* (Vitaterna et al., 1994). Shortly thereafter, the *Drosophila* homolog of CLOCK and a second bHLH protein, CYCLE, which heterodimerizes with CLOCK, were identified, and the CLOCK partner was also found in mammals.

The transcription-translation feedback loop (TTFL) became the dogma for the clock mechanism across eukaryotes. Other than flies, one of the earliest models for dissecting molecular mechanisms of the clock was the bread mold, *Neurospora crassa*. Jay Dunlap cloned the first Neurospora clock gene, *frequency (frq)*, and went on to show that it functions in an autoregulatory loop similar to that reported for *per* (Aronson et al., 1994). Indeed, induction of constitutive *frq* expression did not support rhythms in either wild-type or *frq* mutant fungi, indicating the importance of rhythmic feedback, and step changes in *frq* could specify the phase of the conidiation rhythm in Neurospora. Interestingly, neither Neurospora *frq* nor plant clock genes identified later (Nohales and Kay, 2016) are orthologs of fly clock genes or of each other. Nevertheless, the mechanisms they use to generate a clock are similar, suggesting that the TTFL mechanism evolved more than once. Within the animal kingdom, however, the genes are also largely conserved.

We now know that mammalian clocks comprise the same mechanisms and most of the same molecules as *Drosophila* clocks. A major difference is the use of CRY, rather than TIM, as the PER partner. Other aspects of regulation, including the enzymes responsible for critical post-translational modifications of clock proteins, such as phosphorylation, are also conserved. Thus, CK1ε phosphorylates mammalian PER, and the first human mutation linked to a circadian disorder, Advanced Sleep Phase Syndrome, was found to affect a CK1ε binding region in the *hPer2* gene (Toh et al., 2001). As the name suggests, this syndrome is characterized by very early sleep and wake-up times and likely results from a clock that runs with a shorter period and so adjusts to a 24 hr cycle with an advanced phase. At the other end of the spectrum, the Young laboratory recently showed that delayed sleep phase disorder is associated with a longer period of the endogenous clock (Patke et al., 2017). Similar advanced and delayed phases are seen in *Drosophila* short- and long-period mutants, respectively, when they are maintained in a 24 hour light–dark cycle.

Identification of the molecular clock mechanism was clearly important in and of itself, but the impact of this prize-winning work went considerably beyond that. Several laboratories, including the Rosbash laboratory, mapped effects of *Drosophila* clocks on behavior to discrete neurons in the fly brain, attributed different aspects of the behavior to specific subsets of clock neurons, and identified interactions between those neurons, altogether providing a comprehensive account of the clock circuits that underlie circadian behavior (e.g., see Stoleru et al., 2005). *per* expression, however, is not restricted to the brain; early experiments following its cloning showed that it is widely distributed throughout the body. This pattern turned out to also be true for *tim* and other clock genes in flies and mammals, leading to the discovery of clocks in almost all body organs and revealing that they control physiology to a larger extent than previously appreciated. Identification of functional clocks in cultured mammalian fibroblasts has allowed study of clocks in culture models (Balsalobre et al., 1998), greatly facilitating research in the area.

Transcriptional control constitutes a major mechanism by which molecular clocks drive rhythms of physiology. Within each clock-containing tissue, as many as 15%–20% of genes may be expressed with a circadian rhythm. Thus, rhythmic feedback by clock proteins does not just confer rhythmic expression to their own mRNAs but also promotes cycling of many downstream genes. Some of the downstream genes are transcription factors that can, in turn, contribute to the circadian transcriptome. For the most part, different tissues use the same clock proteins, but the downstream genes expressed cyclically vary greatly, reflecting the diverse functions represented in these tissues. Transcriptional regulation of circadian outputs is conserved across species and even seen in prokaryotes. While the molecular clock in cyanobacteria can be reconstituted *in vitro* as a phosphorylation–dephosphorylation cycle involving key clock proteins (Nakajima et al., 2005), transcriptional control drives cycling of many cyanobacteria output genes. In mammals, mechanistic studies of circadian transcription have revealed the involvement of multiple factors and epigenetic modifications that help to establish different patterns of cyclic gene expression. Over the years, the clock model has also expanded to include additional feedback loops that interlock with the core loop described here.

Given that circadian regulation is an integral component of normal physiology, it is perhaps to be expected that disrupted rhythms are associated with a wide array of pathological problems, which include metabolic diseases, cardiovascular dysfunction, and neuropsychiatric disorders. Studies have also revealed a close link between aging and altered circadian rhythms, which could potentially impact diagnosis and treatment of age-related pathology. Nonetheless, circadian disruption is commonplace in the modern world, resulting from travel

across time zones, excessive exposure to light at night, shift work, and even aberrant eating habits. Future investigation will likely continue to address the extent to which this disruption contributes to the onset or progression of disease. Recognition of the field by the Nobel Assembly will hopefully help stress the importance of circadian rhythms for maintaining health and fitness. At the same time, the awarding of this prize highlights the importance of basic, fundamental research using simple model organisms. It underscores the idea that advances in medicine can come from the pursuit of questions based solely on scientific curiosity and rigorous experimentation.

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