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MECHANISM OF CIRCADIAN CLOCK. THE 2017 NOBEL PRIZE IN PHYSIOLOGY OR MEDICINE

Most animals lead rhythmic lives; some are active at night and sleep during the day, while others are diurnal, being active during daytime. Although these rhythms have a period of 24 hours matching the solar day, they are not merely a response to a daylight or darkness at night. When animals are placed in constant conditions such as constant darkness and constant temperature, they do not lose a sense of time but maintain rhythmicity of rest and activity. However, in these conditions, the period between two sequential activity onsets is not exactly 24 hours, but rather it is about or "circa" 24 hours; therefore, these cycles are called circadian rhythms. Even humans isolated from a solar day and left in artificial light to schedule their own activities, maintain a clear circadian rhythm of sleep and wakefulness. These types of experiments demonstrate that organisms have evolved their own internal sense of time, which is synchronized daily to a 24h solar cycle but in constant conditions displays its endogenous, free-running nature. The internal sense of time allows anticipation of cyclical life events. For example, before we wake up, our internal clock orchestrates an increase in blood pressure and in the levels of hormone cortisol to prepare us for activities of the day.

The question of how animals and humans can measure time has intrigued scientists for many decades. Chronobiogists named the internal mechanism the "circadian clock" and depicted it as a black box when drawing models. Experiments in different species suggested that the clock may have genetic basis, because the period of free-running rhythms could be inherited, or modulated by long term selection. Yet, the mechanism of circadian clocks remained a total mystery until researchers working with fruit flies, *Drosophila melanogaster*, decided to test experimentally whether genes are involved in the clock function. Fruit flies have been used as a genetic model for over a hundred years; in the early 20th century, US biologist Thomas Morgan used fruit flies to confirm that genes are located on chromosomes like beads on a string, and established genetics as a modern science. Flies have a high reproduction rate, short life cycle of 10 days from egg to adult, and there are well-established methods to induce mutations and map them on fly chromosomes.

Working at the California Institute of Technology (CalTech), Dr. Seymour Benzer and his graduate student Ron Konopka decided to use rhythm of emergence of adult flies from their pupal cases to probe the mystery of the clock. Individual adult flies tend to emerge in the morning while no emergence takes place in the afternoon, and a free-running rhythm of adult emergence persists in constant darkness. The experimental approach was to mutate hundreds of flies in hope of finding a few that would emerge at the "wrong" time. Indeed, the authors of this study isolated several of such flies and by analyzing their progeny they discovered that a single genomic locus, which they named *period* (*per*) carried three different mutations (KONOPKA and BEN-

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ZER 1971). One mutant completely lost the emergence rhythm (*per*^{0}), another mutation shortened the free-running rhythm from circa 24h to 19h (*per*^{short}), and the third mutation produced long-period rhythms of 29h (*per*^{long}) of adult emergence. Excitingly, the same mutations caused corresponding changes in the period of the free-running rhythm of locomotor activity in individual flies, indicating that the *period* gene is part of the clock controlling different behavioral rhythms.

This 1971 discovery of the gene *period* was the first milestone on the way to understanding biological clocks. However, the sequence and function of *period* remained unknown until the mid-80s, when three Americans, Drs. Jeffrey Hall and Michael Rosbash at Brandeis University and Michael Young at Rockefeller University, used newly developed genetic and molecular tools to sequence *period* DNA. The Brandeis and Rockefeller teams independently demonstrated that the introduction of *period* genomic fragments into an arrhythmic *per*^{01} mutant caused rescue of both adult emergence rhythm and locomotor activity rhythm (BARGIELLO and YOUNG 1984, REDDY *et al.* 1984). Further studies in the labs of J. Hall and M. Rosbash showed that PER protein (SIWICKI *et al.* 1988) and *per* mRNA (HARDIN *et al.* 1990) undergo daily oscillations and suggested that clock may consist of a negative feedback loop with the PER protein acting as a repressor of transcription (HARDIN *et al.* 1990). Meanwhile, another mutant that abolished circadian rhythms in flies was uncovered in the lab of M. Young (SEHGAL *et al.* 1994). This second clock gene was named *timeless* (*tim*) and the TIM protein turned out to be a partner of PER, necessary for its stability and nuclear entry (GEKAKIS *et al.* 1995, VOSSHALL *et al.* 1994)

Although it was evident that PER and TIM proteins somehow affected transcription of their own genes, the mechanism was not clear owing to the lack of DNA-binding domains in both proteins. Fortunately, a search for more arrhythmic mutants in the labs of J. Hall and M. Rosbash revealed two genes *Clock* (*Clk*) and *cycle* (*cyc*) encoding transcription factors (ALLADA *et al.* 1998, RUTILA *et al.* 1998) that activate *per* and *tim* mRNA transcription. Interestingly, the *Clock* gene was first identified as part of the mammalian timing mechanism (VITATERNA *et al.* 1994), and communication between fly and mouse researchers greatly facilitated the progress in the understanding of the circadian clock mechanism.

By the turn of the century, it was clear that the transcription-translation negative

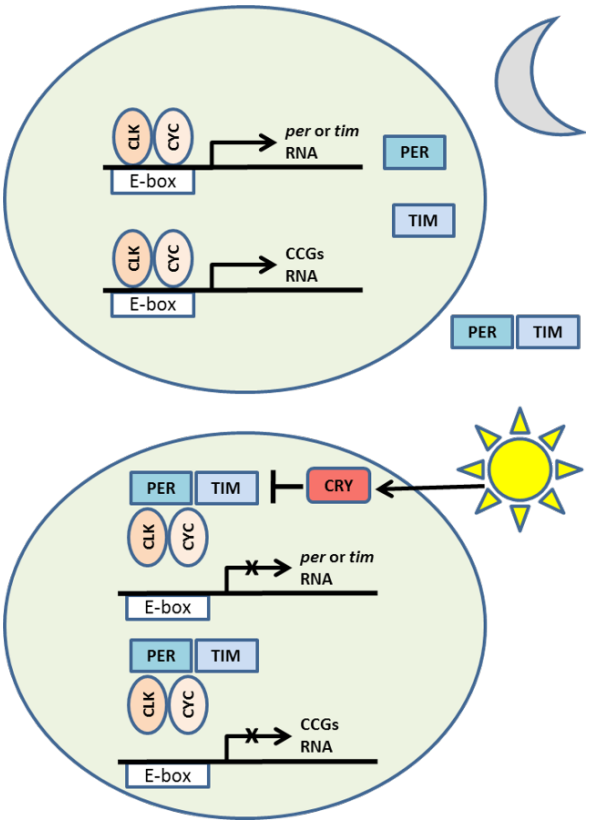

Fig. 1. Schematic depiction of the negative feedback loop that forms the core mechanism of the *Drosophila* clock.

At night (upper panel) the CLK/CYC heterodimers bind to E-box sequences in *per* and *tim* promoters and activate transcription of these genes. Resulting PER and TIM proteins form heterodimers, enter the nucleus and bind to CLK/CYC repressing further transcription of *per* and *tim*. Morning light activates the CRY protein (lower panel) which binds to TIM causing its degradation. PER, which is stabilized by TIM, also degrades, ending the repressive phase of the clock and allowing positive arm of the clock to restart. Many clock-controlled genes (CCGs) also contain E-boxes in their promoters and their transcription is directly stimulated by CLK/CYC. Some of these CCGs encode transcription factors, which indirectly generate rhythmic transcription of additional CCGs (modified from GIEBULTOWICZ 2017).

feedback loop is at the core of the circadian clock in both flies and mammals. A model of the core feedback loop in *Drosophila* was reviewed recently (GIEBULTOWICZ 2017) and is shown in Fig. 1. Two transcription factors encoded by the genes *Clock* (*Clk*) and *cycle* (*cyc*) act as the positive clock factors, whereby CLK and CYC proteins form complexes, which bind to the E-box sequences in the promoters of *per* and *tim* genes, stimulating their transcription in the early night. After translation, PER and TIM proteins act as the negative limb of the clock when they accumulate in the cell nuclei

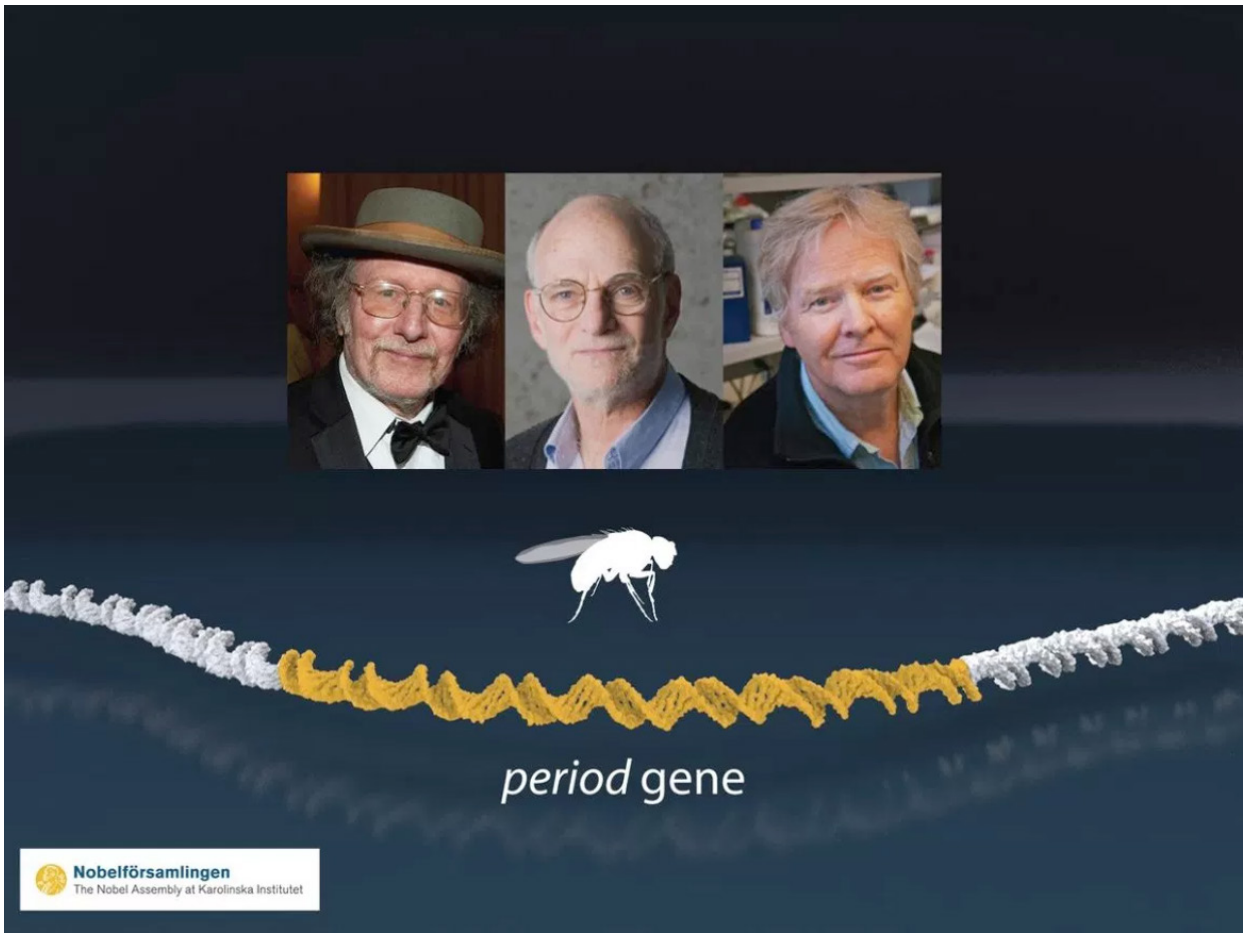

Fig. 2. The poster depicting Nobel Prize winners, from left to right: Jeffrey C. Hall, Michael Rosbash and Michael W. Young. Copyright © The Nobel Assembly at Karolinska Institutet, source, https://www.nobelprize.org/.

late at night and repress CLK-CYC activity. This results in the suppression of *per* and *tim* transcription until the repressive PER and TIM are degraded. Degradation of TIM is initiated by light via the photoreceptive CRY protein encoded by the *cryptochrome* (*cry*) gene characterized in *Drosophila* by J. Hall and M. Rosbash (EMERY *et al.* 1998, STANEWSKY *et al.* 1998). Upon activation by light, CRY binds to TIM protein leading to its degradation. Because TIM stabilizes PER, the latter is also degraded within few hours of lights-on. Mammalian clocks operate by the same mechanisms and contain mostly homologous genes as *Drosophila* clocks. A major difference between fly and mammalian clocks is the use of CRY, rather than TIM, as the PER binding partner. Mammalian CRY lost light sensitivity and gained a function as the circadian repressor.

The research that led to the understanding of the circadian clock mechanism earned their discoverers the 2017 Nobel Prize in Physiology or Medicine. The Prize was awarded jointly to (Fig. 2): Jeffrey C. Hall, Michael Rosbash and Michael W. Young, all three of them doing basic research in *Drosophila melanogaster*. It was not the first time that the tiny fruit fly was "honored" in this way. At least five other groups have received Nobel Prize for their work using fruit flies to decipher the secrets of human physiology and disease. Sequencing of human and *Drosophila* genomes revealed that about 75% of known human disease genes have a functional match in fruit flies, including genes involved in Down's syndrome, Alzheimer's disease, autism, diabetes, cancer and others.

Based on early observations of behavioral rhythms in sleep/activity, feeding, and cognitive functions, it was assumed that the clock would reside in specialized neurons. Indeed, the circadian clocks regulating behavioral functions are located in specific brain neurons of mammals and insects; this was investigated using perturbation of locomotor activity rhythms as a readout of clock function. However, it is now well established that animals possess multi-oscilla-

tory circadian systems. In addition to central or master clocks residing in the central nervous system, there are peripheral clocks in cells forming most other tissues, and their molecular mechanism is very similar. The existence of peripheral clocks that can function independently of the brain was first demonstrated in moths (GIEBULTOWICZ *et al.* 1989), then in *Drosophila* (HEGE *et al.* 1997) and finally in mammals (BALSALOBRE *et al.* 1998). Clocks that exist in cells making up most body organs in flies and mammals provide the temporal framework to organize activity of different tissues, allowing synchronization of compatible and separation of incompatible metabolic processes. The molecular rhythms generated by the tissue-specific clocks contribute to rhythmic physiology such as daily fluctuations in the levels of hormones, enzymes, and various metabolites. In fact, nearly all aspects of metabolism vary with time of day, at both cellular and systemic levels (BROWN 2016). These rhythms are synchronized with daily cycles of food intake, digestion, motor, and cognitive activities which are followed by a period of sleep, which is associated with fasting and cellular repair. Increasingly, modern humans tend to disrupt these cycles by shift work, irregular eating habits, prolonged exposure to artificial light, and travel across time zones and these disruptions increase risk of several diseases.

Studies in model organisms including *Drosophila* were first to suggest that disruption of circadian rhythms may have pathological consequences. Laboratory mammals with genetically engineered defects in their circadian clocks show many pathologies including obesity, diabetes, steatosis, cardiomyopathy, and atherosclerosis (BROWN 2016). There is also accumulating evidence that age-related disruptions of normal circadian rhythms and sleep cycles can affect neuronal health and contribute to pathogenesis of neurodegenerative diseases, such as Alzheimer's disease (MUSIEK and HOLTZMAN 2016). Chronobiologists hope that the Nobel Prize for the discovery of the circadian clock mechanism will increase the awareness that humans should maintain regular "circadian hygiene" to stay healthy. Eating, working and sleeping at the right time of the solar day supports human health and well-being, while disrupting these natural rhythms may be associated with a host of pathological problems.

The discovery of the circadian clock was driven by the curiosity of scientists coming from different fields of study and collaborating by putting together their respective expertise. Such interdisciplinary approach is always evident at the meetings of the Society for Research on Biological Rhythms, which brings together researchers working on clocks in bacteria, plants, and animals as well as medical doctors dealing with timing disorders in humans. They can learn from each other because most molecular pathways are conserved in evolution and human cells function and divide by the same mechanisms as in flies. The Nobel Prize for the three fly scientists highlights the unity of fundamental life processes and underscores the value of basic research on simple model organisms for the understanding of our own physiology and for making progress in preventing and treating various human diseases.

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Summary

Since 1901, the Nobel Prize has been awarded to scientists who have made the most important discoveries for the benefit of humanity. The 2017 Nobel Prize in Physiology or Medicine was awarded jointly to Jeffrey C. Hall, Michael Rosbash and Michael W. Young "for their discoveries of molecular mechanisms controlling the circadian rhythm." It may be surprising to learn that those three scientists dedicated their entire careers to research on the fruit fly, *Drosophila melanogaster*. However, as their studies progressed, it became increasingly clear that the mechanism of the biological clock that they discovered in *Drosophila* is very similar to a timekeeping mechanism present in mammals, including humans. Through interdisciplinary work between scientists performing basic research on model organisms and medical doctors, we have learned over time that daily rhythms support human health while disruption of these rhythms is associated with a range of pathological disorders such as cardiovascular problems, metabolic, neurological, and many other diseases. This short review highlights critical milestones on the way to understanding biological clocks, focusing on the roles played by the three Nobel Prize winners.

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MECHANIZM ZEGARA BIOLOGICZNEGO. NAGRODA NOBLA 2017 W DZIEDZINIE FIZJOLOGII LUB MEDYCYNY

Streszczenie

Od roku 1901 Nagroda Nobla jest przyznawana naukowcom za najważniejsze odkrycia służące dobru ludzkosci. Nagrodę Nobla w dziedzinie fizjologii lub medycyny w 2017 roku otrzymali trzej amerykańscy uczeni Jeffrey C. Hall, Michael Rosbash i Michael W. Young „za odkrycie mechanizmu molekularnego, który kontroluje rytmy okołodobowe". Może się to wydać zaskakujące, ale ci trzej nobliści poświęcili swoje kariery naukowe badaniom nad muszką owocową, *Drosophila melanogaster*. Jednak w miarę postępu ich badań stawało się coraz bardziej oczywiste, że mechanizm zegara biologicznego, odkryty u muszki *Drosophila,* jest bardzo podobny do zegara, który posiadają ssaki, łącznie z człowiekiem. Interdyscyplinarna współpraca między naukowcami prowadzącymi badania podstawowe na organizmach modelowych i lekarzami prowadzącymi badania kliniczne ujawniła istotną rolę rytmów dobowych w utrzymaniu zdrowia człowieka. Długotrwałe zakłócenie tych rytmów stanowi czynnik ryzyka wielu patologii, takich jak choroby serca, cukrzyca, otyłość czy choroby układu nerwowego. Artykuł krótko podsumowuje odkrycia, stanowiące kamienie milowe na drodze poznania mechanizmu zegara biologicznego, ze szczególnym uwzględnieniem roli trzech noblistów 2017 w tym procesie.

Słowa kluczowe: *Drosophila melanogaster*, geny zegarowe, nagroda Nobla, rytmy okołodobowe, zegar biologiczny