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Perfect timing: a Nobel Prize in Physiology or Medicine for circadian clocks

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On October 2, 2017, the Nobel Prize in Physiology or Medicine was awarded to Jeffrey Hall and Michael Rosbash of Brandeis University, and Michael Young of Rockefeller University, the three circadian biology trailblazers, for unraveling molecular genetic mechanisms of circadian rhythms. The three Nobel laureates completed their prize-winning works largely 20–30 years ago. Yet, this Noble Prize came with perfect timing: on the one hand, it can be rooted back to Chinese peculiar and shrewd observations and practices in ancient times as well as some groundbreaking experiments in the history of biology; on the other hand, it implicates far-reaching and pervasive impacts, particularly promising to offer potential therapeutic approaches for numerous dysrhythmia-based diseases or health problems derived from rapidly changing human lifestyles in the modern society. Further, the seminal discovery of the molecular genetic time-keeping mechanisms of life, pioneered by the trios and many others in the circadian biology field, stands as one of the most exciting, heroic and inspiring stories in the history of biology, arguably of modern sciences.

This Nobel Prize is certainly a pleasant surprise as the three laureates were all caught off guard during their Nobel interviews. As a matter of fact, for their contributions to elucidating molecular genetic mechanisms underlying circadian rhythms, the trios have been collecting international prizes and awards, including the Gruber Prize in Neuroscience in 2009, the Louisa Gross Horwitz Prize in 2011, the Massry Prize and the Canada Gairdner International Award in 2012, and the Shaw Prize (the "Nobel of East") in Life Science and Medicine and the Wiley Prize in Biomedical Sciences in 2013. Hence, this Nobel Prize in Physiology or Medicine is much-anticipated and well-deserved one for the trios, which should energize circadian biology and chronomedicine for the years to come.

1. Wondrous and ubiquitous daily oscillations of life processes and activities

Through long-term adaptation to the cyclic physical environment of the Earth, almost all living organisms have evolved time-keeping mechanisms operating with a period of approximately 24 h, so-called circadian clocks [1,2]. Franz Halberg (1919–2013), a Romanian American chronobiological pioneer, coined the word "circadian", meaning "about a day". The circadian clock allows for anticipation of external changes and coordination of the internal machinery and plays modulatory roles in various fundamental life processes and activities from molecular, biochemical, cellular, physiological, to behavioral levels [1,2]. Circadian misalignment leads to malfunctions of the body and numerous diseases [3,4].

"Time" has been an eternal and fascinating theme. In the ancient Chinese and Greek history, there have been numerous brilliant depictions of time, for instance, Confucius (551–479 BCE) once remarked, "Things flow away day and night." Aristotle (383–322 BCE) wrote, "It is well to be up before daybreak, for such habits contribute to health, wealth and wisdom" [5]. As for observations of daily variations, Androsthenes of Thasos, one of the ship captains following Alexander the great (356–323 BCE), described the daily movement of the leaves of the tamarind tree [5]. Amazingly, in an ancient Chinese medical text "*the Yellow Emperor's Inner Classic* (*Huangdi Neijing*)", completed as early as 475–221 BCE, a day was divided into 12 two-hour intervals, each of which was named as one of the 12 Earthly Branches; unbeknown to endogenous mechanisms, it was posited that activities of each internal organ such as the heart, the liver, the spleen, the lung, the kidney, the stomach, the intestine and the bladder wax and wane daily. The so-called *midnight-noon ebb-flow theory* (*Zi Wu Liu Zhu*) had successfully been practiced for the prevention, diagnosis, and treatment of many diseases since Chinese ancient times [6].

As for experiments on these wondrous phenomena of daily changes, French scientist Jean-Jacques d'Ortous de Mairan (1678–1771) placed a heliotrope plant *Mimosa pudica* in a dark cellar and observed that the plant still opened their leaves during subjective daytime and closed them during subjective night. In 1729, de Mairan presented his "botanical experiment" to the Academy of Sciences in Paris, suggesting that the heliotrope plant used an internal clock to control the opening and closing of its leaves [5]. De Mairan's finding was confirmed by many botanists, including Swiss plant taxonomist Augustin Pyramus de Candolle (1778–1841), who studied leaf movements of *Mimosa pudica* under constant light and observed that the period of "sleep-wake-like leaf movements was shorter than 24 h [5]. Hence, de Candolle also was credited with documenting an endogenous circadian rhythm.

Turning to the 20th century, German botanist Erwin Bünning (1906–1990) coined the word "endodiurnal" to describe the 24 h rhythm, which was later replaced by "circadian". Bünning studied the mechanisms underlying photoperiodism, and importantly

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crossed two bean strains with different periods and produced the beans with intermediate periods of their parents [5], being the first to suggest that the circadian clock has a genetic basis. German scientist Jürgen Walther Ludwig Aschoff (1913–1998) used a deep underground shelter to examine human circadian rhythms. Aschoff coined the word "zeitgeber", meaning "time giver" or "synchronizer". Franz Halberg also coined the word of "chronobiology", referring to a subdisciplinary of biology dealing with all rhythms and clocks. Halberg emphasized and promoted medical implication of circadian studies. British American Colin S. Pittendrigh (1918–1996) determined the timing and rhythm of eclosion (the process of emerging from the pupal case) in *Drosophila pseudoobscura*, and showed temperature compensation as one of the key characteristics of circadian clocks. Pittendrigh was the first circadian biologist elected to the National Academy of Sciences in 1963 at age of 45 years. Bünning, Aschoff, Pittendrigh, and Halberg have been regarded as founding fathers of modern circadian biology or chronobiology because of the significant contributions they each made to the field.

2. A groundbreaking study that started the whole story

The genetic studies of circadian rhythms were initiated by Ronald Konopka (1947–2015), a Ph.D. student at the Seymour Benzer's laboratory in California Institute of Technology, around 1968. Seymour Benzer (1921–2007) was a physicist by training but turned into a leading molecular biologist and a pioneering behavior geneticist. Around 1967s, Benzer started a research initiative employing fruit fly mutagenesis forward genetics to investigate the genetic basis of various behaviors such as phototaxis, courtship, and learning by screening individuals for interested phenotypes in EMS-induced mutated fly populations. After joining the Benzer group, Konopka started genetic dissection of the fruit fly circadian rhythms.

Konopka and Benzer wisely used a criterion of eclosion rhythms, as found by Pittendrigh earlier, to search for circadian mutants. Adult flies eclose around dawn. The Konopka-Benzer screen looked for mutated flies that eclose at different times of a day. They struck gold and found three mutant flies, a short-period mutant (approximately 19 h), a long-period mutant (approximately 28 h) and an arrhythmic mutant [7].

Konopka and Benzer then proposed that the three *period* mutants were essential for circadian rhythmicity [7]. First of all, the three mutants altered not only the eclosion rhythms but also locomotor activity rhythms, which also display a short period, a long period, or arrhythmicity. Secondly, genetic complement mapping showed that all three mutations occurred inside one single *period* gene resided in the fly X chromosome. Thirdly, these results indicated that this single protein was critical for circadian timing and determining the speed of the circadian clock, *i.e.*, the short-period mutant flies run faster than normal flies, while the long-period mutant flies run slower than normal flies. This study was groundbreaking because the fly *period* mutants represented the very first example of how mutations in one single gene affect behaviors and circadian clocks. The 1971 Konopka and Benzer's landmark PNAS paper ushered in the modern era of the circadian field. Yet, a race to isolate the fly *period* gene and elucidate its functions took an additional 15 years to unfold.

3. A race to isolate the fly *period* gene and elucidate its functions

While Konopka and Benzer's finding indicated genetic bases for behaviors and circadian clocks, the notion that behaviors are so complex that cannot be analyzed at the single gene level was still predominant in the field in the 1970s. In addition, the recombinant DNA technique and DNA sequencing methods were still in their early stages of development in the early 1970s. This all added up to the slowing pace of isolating this important gene. However, Konopka's remarkable study on circadian rhythms left indelible marks on the three laureates. Young was a graduate student at the University of Texas, Austin when Konopka and Benzer published the paper on *period* mutants. He was intrigued by the study because he was using classical complementation analysis to map the genes in the fly X chromosome. He was able to map the *period* locus between the *white* and the *zeste* loci in the X chromosome and to generate a translocation fly T (1: JC43) carrying the *period* mutation. After learning the DNA cloning technique from David Hogness at Stanford University, he set up his research group at Rockefeller University and decided to isolate the *period* gene around 1981. In the Hall-Rosbash team at Brandeis, Hall specialized in behavioral biology and *Drosophila* genetics, while Rosbash was an expert on molecular biology and biochemistry. Hall had a short overlapping stint with Konopka when he was a postdoc at the Benzer laboratory in the California Institute of Technology. Even though Hall did not study circadian rhythms back then, he was also fascinated by the genetic study of Konopka and Benzer. When Hall and Rosbash became faculty members at Brandeis University, they soon formed a strong friendship and often talked about the Konopka and Benzer study, and eventually decided to join forces to tackle the *period* gene around 1982. The breakthrough finally came in 1984 when the Young team at Rockefeller University and the Hall-Rosbash Team at Brandeis University independently used the rescue experiment to determine the correct *period* transcript, elegantly demonstrating that rhythms could be restored in arrhythmic per^0 mutants by microinjecting specific genomic fragments containing the locus [8,9].

The two teams then independently isolated the *period* gene and sequenced it, revealing that single-nucleotide substitutions underpinning the three *period* mutations. Specifically, missense mutations resulted from single-substitutions led to both the short period (per^s) and the long period (per^l) alleles, while a truncated peptide resulted from a premature stop codon introduced by a single-nucleotide substitution was responsible for the arrhythmic allele (per^0). Uncovering the mysterious molecular nature of the fly *period* mutations was another landmark achievement not only for circadian biology but also for the whole area of behavioral genetics.

The Young team and the Hall-Rosbash team started hunting for the *period* gene approximately the same time without knowing each other's efforts, which made the race extremely competitive and difficult. At that time, the isolated *period* was a pioneer gene without any information on its structure and function. Later, when the fly *single-minded* (*sim*) gene, and the vertebrate *aryl hydrocarbon receptor nuclear transporter* (*ARNT*) gene were isolated and sequenced, sequence comparison showed that fly Period (Per) and Single-minded (Sim) and vertebrate ARNT share a conserved domain, named as the Per-ARNT-Sim (PAS) domain. PAS was showed to be a protein-protein interaction domain, suggesting that the circadian protein Period would need a partner protein to function. All of a sudden, a chase for Period's partner was on in the circadian field.

4. A highly conserved transcription-translation feedback loop (TTFL)-based time-keeping mechanism

Approximately 20 years after Konopka did it, Amita Sehgal and Jeff Price, two postdocs back then in the Young group, conducted fly forward genetics mutagenesis screening again to hunt for additional circadian mutants. They were not as lucky as Konopka, who got the first *period* mutant after screening less than 200 flies.

Amazingly, they did persist and were able to find a circadian mutant after screening more than 7000 flies! This new fly circadian mutant was arrhythmic, reminiscent of the per^0 mutant, and was given a name of "*timeless* (*tim*)" [10]. Intriguingly, Leslie Vosshal, a graduate student back then in the Young laboratory, showed that the Per protein is accumulating in the cytoplasm in the *tim* mutant fly [11], implicating that Tim may be an interacting protein that was being searched for. *tim* was then cloned and sequenced. Yet, unexpectedly and to some degree of disappointment, the Tim protein does not possess a PAS domain [12]. Nevertheless, Tim indeed binds to Per, as showed by the yeast two-hybrid assay [13].

Paul Hardin, a postdoc back then at the Rosbash laboratory, examined expression of *per* in the fly head, found that *per* mRNAs oscillate, and the periodicity of *per* expression is shorter in the per^s mutant, just like the shorter period of its behavior; more importantly, *per* is up-regulated in the per^s mutant, implicating that the Per protein may feedback to inhibit its own transcription [14]. All of these findings suggest that circadian clocks may be controlled by a transcriptional mechanism. However, neither Period nor Timeless contains a DNA-binding motif. If transcription regulation acts in the circadian clocks, it must have circadian proteins harboring DNA-binding motifs. Hence the search for DNA-binding motifs-containing circadian factors was on.

A surprising breakthrough came from the Joseph Takahashi group back then at Northwestern University. Largely inspired by the success of fly chronogenetics pioneered by Konopka, Benzer, Young, Hall, and Young, Takahashi and his colleagues had courageously and heroically conducted mouse mutagenesis forward genetics screen to search for mammalian circadian clock genes. The Takahashi team was extremely lucky and found the first mammalian circadian mutant at 25th trial, which was named as Circadian Locomotor Output Cycle Kaput, abbreviated as *Clock* [15]. This mutant mice display a significantly lengthened period in the heterozygote (WT, 23.3–23.8 h; *Clock*+/–, 24.8 h) and becomes arrhythmic after exhibiting an extremely lengthened period (*Clock*–/–, 26–29 h) first in the homozygote under constant dark [15]. Isolating and sequencing *Clock* revealed that it contains two PAS domains, and more importantly a DNA-binding motif, basic Helix Loop Helix (bHLH), which recognizes and binds to E-Box (CACGTG) [16]. Thus CLOCK was one of the missing puzzle pieces being searched for. Soon, the fly homolog of the mammalian *Clock*, as well as its partner *Cycle*, was found by the Brandies team [17,18]. In 1998, approximately 30 years after Konopka and Benzer started genetic dissection of the circadian clock, with four key components being uncovered, a transcription/translation feedback loop (TTFL) was put forwarded by these laureates as follows: very simply, at the nucleus, CLOCK and CYCLE forms a heterodimer to activate transcription of *period* and *timeless* by binding to E-Boxes in their promoter regions; at the cytoplasm, PERIOD and TIMELESS then forms another heterodimer, which is translocated back to the nucleus, turn off their own transcription by binding to the CLOCK-CYCLE heterodimer and repressing its activities; thus forming a negative feedback loop, which is one of the most beautiful chapters in the history of biology, arguably of modern sciences.

The next question: is this amazing TTFL-based circadian clock just specific to flies or does it also act in other living organisms? Zhongsheng Sun, a postdoc back then at the Cheng Chi Lee laboratory at Baylor College of Medicine, identified the mouse *Period* gene [19] through cDNA library screening, which was named as "*Rigui*" after an ancient Chinese sundial, and a Japanese team isolated it independently via the elegant degenerate PCR approach [20]. Are these PERIOD genes functional in human? Louis Ptacek, in collaboration with physician Christopher Jones, demonstrated that a missense mutation in human *PERIOD2* results in a case of human familial advanced sleep phase syndrome (FASPS) in Utah, US [21]. These human PERIOD2 variants act like fly per^s mutant runs faster with a shorter period, implicating that human *PER* gene functions just like the fly *per*, and the TTFL-based clock machinery likely acts in humans as well [21]. This TTFL-based clock machinery not only functions in the animal kingdom, but also in *Neurospora crassa*, a fungus, and *Arabidopsis thaliana*, a plant, even though the clock components are different without clear homologs among fungi, plants, and animals [1], suggesting the TTFL-based clock machinery likely was originated multiple times during evolution. Thus, the transcription-translation feedback loop (TTFL)-based time-keeping mechanism is highly conserved among almost all forms of living organisms.

5. The post-Nobel Prize era – cornucopia of opportunities and challenges

Thanks to Hall, Rosbash and Young for winning the 2017 Nobel Prize in Physiology or Medicine, circadian biology just becomes one of the few prestigious Nobel Prize-winning fields in biology, and soon enters the post-Nobel Prize era, which definitively is not "the beginning of the end", rather, shall attract more peoples to the circadian field, inspire more circadian studies, and facilitate more discoveries and circadian applications in daily life and medical practices.

Elucidating the TTFL-based clock machinery is the crown jewel achievement of circadian biology in the past 50 years. Equally important was the discovery that circadian clocks act in each internal organ, just as the ancient Chinese described more than 2000 years ago, and even in each cell of the body. In the late 1990s, a series of studies demonstrated that circadian regulation is at work outside the brain: Hall, in collaboration with Steve Kay, reported that GFP (green fluorescent protein driven by the *per* promoter lights up almost all of the fly cells [22]; Ueli Schibler, at University of Geneva, Switzerland, demonstrated that both rat-1 fibroblasts and H35 hepatoma cells, after spiked by serum, display circadian rhythmicity [23]; David Whitmore and Nicholas Foulkes, two postdocs back then at Paolo Sassone-Corsi group in CNRS-INSERM-ULP, Strasbourg, France, showed that *clock* is rhythmically expressed in zebrafish organs, and remarkably in ex vivo cultured organs [24]. All these findings strengthened the notion of peripheral clocks distributed in the whole animal body.

Recently, John Hogenesch group used large-scale RNA-sequencing analysis to reveal that approximately 43% of genes are rhythmically expressed in mice [25]. A great proportion of these rhythmically expressed genes belong to disease genes and drug-target genes [25]. Many human diseases strike in specific time-of-day, and our abilities to absorb, distribute, metabolize, and excrete drugs vary daily, suggesting that circadian biology, circadian biology-based chronomedicine and chronopharmacology (chronopharmacodynamics and chronopharmacokinetics) should be indispensable components of personalized/precision medicine.

There are still many outstanding questions remained to be tackled in the circadian field, for instance, are there any new circadian clock genes? Are there new circadian regulatory mechanisms? With so many circadian clocks in the body, how are they reset and synchronized? Can the circadian study ever provide solutions to deal with jetlag and insomnia? Just like the case for sleep, the roles of the circadian clock often are ignored, first, because lack of sleep or circadian misalignment cannot kill a person right away; and second, because our body has the remarkable abilities to restore/recover sleep and clock. However, chronic dysrhythmia or chronic sleep deprivation has serious effects on human health. It will be a challenging task to popularize these circadian findings and convince regular folks to keep healthy by not messing up their body clocks. Some circadian studies, as well as many epidemiological investigations, indicate that circadian disruption leads to various diseases, including sleep disorders, cardiovascular diseases,

metabolic disorders, immune diseases and cancers, birth defects and reproductive problems, neurodegenerative and psychiatric diseases [3,4], however, mechanisms underlying how the circadian clock contributes to the pathogeneses of these diseases and disorders are far from certain. A lot of works lie ahead, and exciting discoveries will be made. The future of circadian biology will be brighter than before.

Conflict of interest

The authors declare that they have no conflict of interest.

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