Celebrating clocks and flies – 2017 Nobel Prize for Physiology or Medicine

At a time when most 'Nobel watchers' expected the much coveted prize in physiology or medicine to go to CRISPR technology or even to immunotherapy, the announcement to award discoveries made using fruit flies, in deciphering the molecular underpinnings of the fundamental biological process of timekeeping, is reassuring to scientists engaged in basic research across the world.

The humble fly has had its share of recognition from the Nobel Committee in the past; the earliest with Thomas Hunt Morgan for the fundamental idea of the Chromosomal Theory of Inheritance (1933) followed by his student Hermann J. Muller for his discovery of mutagenesis by radiation (1946). Developmental biologists Edward B. Lewis, Christiane Nüsslein-Volhard and Eric F. Wieschaus were recognized in 1995, Linda Buck and Richard Axel for their discoveries of olfactory receptors (2004) were followed by Jules Hoffman for his discoveries in innate immunity (2011).

In the early 1980s, the three awardees for this year's Nobel Prize ventured forth in an attempt to uncover genes that may play central roles in circadian (~24 h periodicity) rhythm generation, maintenance and synchronization to the geophysical day and night, at a time when giants in the field of chronobiology or the study of biological rhythms, were skeptical of such an approach. However, another discovery made more than a decade before, by Seymour Benzer and Ronald Konopka[1] was pivotal in steering the efforts of the trio. Konopka and Benzer showed that a locus on the X-chromosome of the fly is critical for rhythmic behaviours such as adult emergence from pupal cases in populations of flies and the locomotor activity of individual flies. Allelic mutations at this locus either rendered flies arrhythmic in both behaviours or altered the periodicity of the rhythms in opposite directions (either lesser than or greater than 24 h), thus prompting them to name it *period*. This landmark study took the chronobiology community by storm, partly due to the remarkably clear and robust phenotype obtained by a random mutagenesis screen. At that time, the field was dominated by scientists who did not think that single gene mutations could yield significantly large effects on rhythmic behaviour. Understandably, clocks were expected to be a more complex entity with built-in redundant machinery that was unlikely to be laid bare by mutations in single genes.

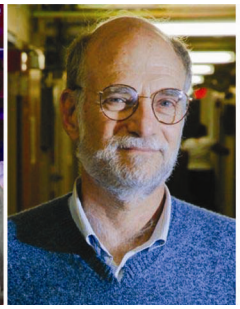
Michael W. Young, one of the awardees, was a graduate student at the University of Texas in 1971, when *period* mutants were described and happened to be studying the *Drosophila* genome using classical cytogenetic methods. He had fly lines with chromosomal rearrangements on the X chromosome, including one with a break in the region predicted by the Benzer study, which he would use only later. While a post-doc at Stanford, Young recalls having heard a highly animated and persuasive talk by Seymour Benzer who likened his genetic dissections of behaviour to those of surgical lesions that had been used previously to localize the sites of brain functions. Incidentally, the suprachiasmatic nucleus, a structure in the mammalian brain had been localized to be the site of the circadian pacemaker in 1972 using surgical lesions. In 1978 when Young had a faculty position at the Rockefeller Centre in New York City he was able to pursue the idea of locating and mapping the *period* gene using a chromosomal walk (a method of positional cloning). With Thaddeaus Bargiello, a post-doctoral fellow at his new laboratory, the precise location and size of *period* was described, followed by the demonstration that P-element mediated transformation of mutant arrhythmic flies with the wildtype copy of the gene can restore both adult emergence as well as activity rhythms.

Around the same time, two adjacent laboratories in nearby New England were engaged in a similar quest to unravel the mysteries of *period*. Piqued by the finding that the *period* gene which has an effect on daily rhythms also impacts high frequency rhythms in courtship songs, the foundations of a highly fruitful collaboration between two professors, Jeffrey C. Hall and his friend and neighbour at the Biology Department in Brandeis University, Michael Rosbash were laid. Soon the duo was harnessing the power of molecular genetics, that had just become available, to isolate *period* gene and elucidate its properties. Nearly simultaneously as the group from Rockefeller, the Brandeis labs published their first collaborative paper on the molecular analysis of the *period* locus, and showed evidence of the rescue of a null mutation by P-element mediated transformation.

These studies can be considered as the official start of a race that was sometimes bitter, but in hindsight may have been the catalyst for the speed with which studies progressed in the direction of understanding the biochemical and cell biological basis for self-sustained, near-24 h rhythm generation. Thus, within a decade of isolating and mapping the *period* gene, all the other molecular players involved in circadian rhythm generation were identified and characterized. Today we celebrate what circadian biologists now agree was a rivalry that put forth a highly conserved mechanism that has since been found in the vast majority of organisms examined.

In the early 1980s the question remained as to how a single gene could bring about rhythmic phenomenon? The first breakthrough came in the form of a

Jeffrey C. Hall



Michael Rosbash

Michael W. Young

discovery by Paul Hardin, a post-doctoral fellow with Rosbash who showed that *period* mRNA from fly heads exhibited a circadian oscillation and that the oscillation was affected by mutations in the gene. Subsequently the same group showed that the precursor and mature forms of period mRNA have similar amplitude and are in-phase and that the *period* gene promoter can produce oscillation even to heterologous mRNA. Most importantly, they found that the period protein oscillation is delayed in phase by 6 hours from the period mRNA oscillation. Around that time, it became known that PERIOD shuttles between the cytoplasm and the nucleus[2] in a temporally restricted manner in brain neurons (the proposed location of the fly clock) and that its amino acid sequence showed similarity with two other proposed transcriptional regulators (SINGLE MINDED and ARNT). This led to the hypothesis that the lag in PERIOD accumulation may be due to negative regulation of itself. For the first time ever, there was reasonable evidence to propose that *period* mRNA and its protein product influence each other[3], through a negative feedback mechanism that had been proposed as the basis for self-sustained oscillators. Although it seems obvious today that this may well be the case for a bonafide circadian clock gene, many coincident factors were critical to establishing this idea. Amongst them were, the previous discovery from the Hall lab in 1988, that PERIOD protein also oscillates with a day–night rhythm, the fact that the Rosbash lab was at the fore-front of molecular techniques to handle RNA samples, and the reasoning of Paul Hardin to assay mRNA in fly heads rather than whole bodies.

The race that was alluded to previously, was following another parallel path in the Young lab at Rockefeller. Given the proposition that PERIOD is regulating its own transcription, it required PERIOD to have a DNA-binding domain, yet its sequence did not reveal any. This led to the idea that perhaps PERIOD may interact with other proteins bearing a DNA-binding domain. In this pursuit, a post-doctoral fellow, Amita Sehgal along with her colleagues Jeffery Price and Bernice Man took on the task of repeating the heroic approach of Konopka and Benzer to uncover other mutations on autosomes, this time by a genetic method of mutagenesis using transposable elements. In a primary screen of about 7000 fly lines, a candidate was found on the second chromosome that did not show the normal preference for dawn adult emergence and instead emerged more-or-less equally throughout the day and night, which they named *timeless*[4]. Like the *period* null mutant, these flies also showed arrhythmicity in locomotion. Strikingly, this mutation also affected the oscillation of *period* mRNA, such that it was no longer rhythmic, suggesting an interaction between the *period* and *timeless* loci. Leslie Vosshall and colleagues in the same lab showed that indeed the *timeless* mutant has profound effects on the PERIOD protein, the latter's ability to enter the nucleus at a certain specific time of day was contingent upon *timeless* function, leading to the hypothesis that *timeless* and *period* are both part of one intracellular mechanism that generates a self-sustained oscillation. Subsequently, *timeless* mRNA was also shown to cycle, and PERIOD and TIMELESS proteins were shown to interact in order to enter the nucleus.

In a flurry of reports that followed, yet other mutants that affect the nature of the activity/rest rhythm via defects in the underlying TTFL were isolated by the Young and the Hall-Rosbash laboratories. Jeffery Price, Brian Kloss and colleagues at the Young Lab demonstrated that a protein DOUBLETIME affects the stability of PERIOD due to its role as a kinase in phosphorylation of PERIOD protein[5]. The phosphorylated form of the monomeric PERIOD protein being unstable ensures that only the heteromeric and stable PERIOD–TIMELESS complexes can enter the nucleus, thus building in a time delay between peaks of *period/timeless* mRNA and protein levels. These studies and others from the three laboratories provided evidence for the idea of a transcription–translation feedback loop (TTFL) as a molecular/biochemical basis for generation of self-sustained rhythms.

The discovery of two more new genes *clock*[6] and *cycle*[7] brought an essential missing piece, namely transcriptional activators (positive elements) into the feedback loop. Interestingly for the first time, the identification of a fly circadian gene was done by working backwards from a mouse mutagenesis screen that gave the first mammalian circadian gene named Circadian locomotor output cycles kaput (*Clock*)[8], *Drosophila* CLOCK and CYCLE were shown to physically interact with one another and bind to E-Box elements on the *period* and *timeless* genes, thus providing evidence of being members of the core-clock. Further studies showed that in fact CLOCK–CYCLE activity was inhibited by PERIOD and TIMELESS. In later years a second feedback loop comprising of *vrille-pdp1* as transcriptional regulators was proposed by the laboratory of Justin Blau[9]. In the same year, a more complex model of interlocked feed-back loops incorporating both the loops (PERIOD–TIMELESS and VRILLE-PDP1) bound by a common requirement of the CLOCK-CYCLE complex for transcriptional activation, was put forth by the studies of Paul Hardin's laboratory[10].

While the above studies explained how a self-sustained clock machinery with a near 24 h period might be generated in the absence of external time cues, it was also necessary to explain how such clocks might synchronize with the external environment – the phenomenon of entrainment. Previously, the behavioural attributes of entrainment had been the subject of intense study mostly using mammalian systems of a wide hue (series of papers by Pittendrigh and Daan, 1976). Light, a major entraining agent ('zeitgeber' or time-giver in German) was shown to impact the fly circadian clock via TIMELESS degradation. The finding of a dedicated photoreceptor – CRYPTOCHROME for the clock in flies came once again from the Rosbash-Hall laboratories in 1999. Subsequently, studies from various laboratories showed that light via CRYPTOCHROME modifies TIMELESS to enable its proteasomal degradation and thus reset the circadian clock[11]. Integral to the ability of TTFLs to function as the core-clock mechanism are several other processes that have been discovered over the past two decades. Among them are epigenetic modifications that can change chromatin architecture, RNA-mediated post-transcriptional modifications and micro-RNA induced alternative splicing mediated regulation of transcription. As also post-translational modifications such as SUMOylation and O-GlcNAcylation, and ubiquitination, in addition to phosphorylation have been demonstrated to alter clock output across mice and flies. So how prevalent and integral are the TTFLs in sustaining rhythms across life forms? Analogous systems have been found

across phyla – cyanobacteria, *Neurospora*, plants and mammals, have TTFLs governing their rhythms; however, the lack of homology among the components of the TTFL suggests that such a mechanism has arisen independently and more than once along evolutionary timescales.

More recently, non-TTFL based clocks have also been discovered. Prominently, a protein-phosphorylation dependent system has been found in cyanobacteria, where a self-sustained, near-24 h rhythm can be generated in the absence of transcriptional machinery by a series of sequential phosphorylation steps[12,13]. Another non-TTFL based clock has been discovered, first in anucleate cells such as erythrocytes from many organisms, including humans. Here the ratio of over- and hyperoxidized form of peroxiredoxin was found to show a free running circadian rhythm along with the ability to entrain to temperature cycles as well as robust temperature compensation, all of which are considered as characteristic features of endogenous circadian clocks[14]. Remarkably such a mechanism has hence been discovered in distantly related organisms such as the alga *Ostreococcus tauri*, the archaeon *Halobacterium salinarum*, cyanobacterium *Synechoccus* and yeast *Saccharomyces cerevisae*. Not withstanding the fact that the last decade or so has revealed many non-TTFL based clocks, one cannot but acknowledge the fact that widespread interest was fuelled by the early breakthroughs achieved via elucidating the TTFLs, in what some may go so far as to say, excruciating detail.

Coming back to flies, it is important to note here that while the intracellular mechanisms were being investigated, there were also studies asking the question of where in the brain these clocks lay. In 1998, in parallel with the 'clockwork explosion' in molecular terms, Charlotte Helfrich-Forster from Tubingen, Germany continuing on a long tradition of neurobiologists interested in circadian clocks showed by very elegant means that in fact, a set of about 8 cells in the fly's brain are sufficient for the self-sustained rhythm in locomotion. These cells were shown to be integral also for generation of the morning bout of activity in the presence of light/dark cycles earning the sobriquet 'M' cells. However, flies exhibit a second peak of activity around the lights-off transition, and these cells also influenced the phase of the evening bout of activity via the production of a neuropeptide, Pigment Dispersing Factor that was proposed to actively delay the onset of evening activity. Subsequently, other subsets among the approximately 150 neurons that express TIMELESS/PERIOD have been shown to be critical for various aspects of rhythmic behaviour including the evening activity under light cycles, or temperature cycles. The wealth of genetic tools in flies along with the somewhat distributed anatomical localization of the pacemakers have enabled fly circadian biologists to dissect the neuronal circuit to an unprecedented level, possibly more than for any other behavioural phenotype. Now we know that both in flies and in mammals, clocks exist in a variety of tissues throughout the body. So-called peripheral clocks are located outside the brain and even in non-neural tissues, notably in the liver, kidneys, adipocytes and stomach in mammals; in the fat-body, Malpighian tubules and even antennae in flies. It is well accepted that proper synchrony among these peripheral clocks is integral to well being, based on empirical evidence from multiple experimental models and paradigms.

Indeed, the field is ripe with questions about how circadian clocks and timing impinge upon metabolism and physiology. Ironically, rhythm research has come a full circle as mutations in genes that were first identified as core clock members, have also now been found to be linked with several sleep/wake disorders in humans such as familial advanced sleep-phase syndrome (*Per2*), delayed sleep-phase syndrome (*Per3*), bipolar disorder (*Timeless* and *Per3*) and seasonal affective disorder (*Npas2*). Additionally, sleep disturbances occurring due to shiftwork and jetlag result in metabolic and hormonal imbalances, thereby giving an impetus to chronotherapeutic treatments of lifestyle and related disorders. Chronotherapy has proceeded to an extent of appropriately timed drug delivery and post-surgical treatments for optimum recovery. The impact of clocks on health and well-being are being recognized by medical practitioners, personnel management boards of business establishments, defence organizations, as well as policy makers across the globe. While chronotherapeutics might still be in its infancy, a great deal of basic research akin to the one conducted by the 2017 Nobel laureates and several giants before them remains to be conducted. It is not difficult to imagine that once again the fruit fly will be at the forefront of this endeavour. The Nobel Prize this year is a definite shot-in-the arm for clockwatchers and fly biologists alike!

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