Circadian Rhythms and the Transcriptional Feedback Loop

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INTRODUCTION

Circadian clocks undoubtedly arose in response to the daily changes in illumination that are due to the rotation of the earth. Indeed, sunrise and sunset are very consistent external events, if we ignore the slight change in timing that usually occurs every day. Although this change can be substantial at some times and in some locations, in March near the north and south poles for example, life and biological clocks probably evolved in more temperate zones.

Clocks function to allow organisms to anticipate daily changes in their environment. When something happens every day at the same time, organisms "learn" that the event will occur. This anticipation, preparing for what is going to happen, is a superior strategy to merely reacting to that change. Animals use their clocks to maximize or minimize their encounters with what I like to call the big 3: finding food, finding mates, and avoiding predators.

Clocks may also serve to provide order to internal processes. The temporal separation of different biochemical reactions is an example and may

provide an additional fitness advantage. These inexorable rhythms, due to the persistent ticking of a biological clock in our brains and in many if not most other tissues, serve to coordinate myriad features of our behavior, metabolism and physiology – even our sleep-wake cycle (Hardin and Panda, 2013).

Circadian rhythms are present in most if not all animals, plants, and some species of fungi like *Neurospora*. Even some photosynthetic bacterial species like cyanobacteria have well-studied circadian clocks (Kondo et al., 1993). Indeed, the first circadian clock may have belonged to photosynthetic cyanobacteria, which are probably responsible for oxygenation of the atmosphere. These cyanobacterial clocks as well as plant clocks are very different from those of animals, with no credible homology between the different clock proteins. Therefore circadian rhythms probably emerged multiple times in evolution, which underscores their importance (Rosbash, 2009).

The rotation of the earth on its axis is a source of daily light and temperature cycles in most locations on earth. These daily cycles are considered the oldest and most continuous environmental features to which life was originally exposed. Even the self-replicating molecules of the original RNA world that preceded cellular life probably arose in the presence of light-dark and temperature cycles, well before the atmosphere was anything like it is today. The rotation of the earth was also perhaps 20 percent shorter four billion years ago than it is today, which may help explain why circadian clocks are not precise twenty-four-hour timekeepers (Rosbash, 2009).

The fact that endogenous circadian rhythms have periods distinct from precisely 24hr is a key piece of evidence that the rhythms are entrained rather than driven by the solar cycle. It is generally acknowledged that the first person to recognize that circadian rhythms are not driven was an eighteenth-century French astronomer named de Mairan. He had been musing about the movements of plants, which famously extend their leaves during the daytime and retract them at night. It was assumed that this daily leaf movement rhythm is light-driven, but de Mairan tested this hypothesis in 1729. To perform this first "free-running" circadian experiment in the absence of a light-dark cycle or entraining cues, he took a plant down to the basement, probably his wine cellar. In principle, it had constant conditions, i.e., no daily variations in light. Miraculously, the leaf movements continued unabated on a circa twenty-four-hour schedule, indicating that they were driven by an endogenous circadian clock rather than by light (Daan, 2010).

Importantly however, de Mairan did not carefully measure the free-running period of the leaf movements, nor did other scientists addressing this problem for decades thereafter. Although there were no light fluctuations in the basement during the experiment, other oscillating

stimuli were not measured or considered, e.g., humidity, temperature etc. Because the world lives on a 24hr schedule, any human interference could provoke an environmental oscillation of 24hr and drive the plant rhythms. Neither de Mairan nor other plant scientists who followed him considered that the key was not only the absence of light but also a careful measurement of non-24hr period length. Although a 24hr period could reflect an endogenous timekeeper with that precise period, it could also reflect a driven oscillator, one that requires external 24hr oscillations of some kind. A non-24hr period however must reflect an endogenous timekeeper. (I am taking a bit of license in making this statement so strongly.) For some reason, it was a very long time before this distinction was appreciated and leaf movement period measurements made with much more precision. Eventually, however, they were done and shown to be non-24hr, indicating unambiguously that leaf movements were not driven by a feature of the external world but reflected endogenous circadian timekeepers (Daan, 2010).

The circadian period of other organisms is similarly almost always measured under constant or "free-running" conditions, which usually means many days, weeks or even months in darkness; humans are commonly measured in another more elaborate way because of ethical issues with a constant darkness protocol. Endogenous periods usually vary between 22 and 26 hours. Mice for example have periods of about 23.5 hours and rats about 24.5 hours. Importantly, there are even differences between inbred strains of mice, indicating that quantitative features of the circadian system are under genetic control; I will have much more to say about genetics and mutants below. Importantly, these circadian or not exactly 24hr rhythms are normally reset or "entrained" by the light-dark cycle. This is how humans, rodents and other organisms have a cycle that is identical from one day to the next: a somewhat slow or fast clock is advanced or delayed every day by the 24hr light cycle (Daan, 2010).

In the early-mid 20th century, a similar endogenous clock assay was carried out for *Drosophila*. This organism shows a rhythm of eclosion, the emergence of adult flies from their pupal cases. This is a population assay as any one fly experiences this event only once. However, the population emerges in a circadian manner with a major peak after dawn. In other words, adult emergence is gated by the circadian clock; animals that have not emerged by late morning must wait until the following morning. This is presumably to reduce exposure of newly emerged adults to mid-day and late afternoon heat. As expected of a circadian phenomenon, this circadian gating of eclosion continues in constant darkness. Endogenous periods of *Drosophila* were measured under these conditions; although they vary somewhat from species to species and strain to strain, they are very close to 24hr (Bunning, 1935; Kalmus, 1935, 1940a, b).

The circadian pioneer Colin Pittendrigh introduced and championed many important aspects of circadian biology. He was among the first if not the first to appreciate the importance and universality of the following phenomenon: if clocks were to function properly, especially in poikilotherms like *Drosophila*, then there must be a mechanism to keep their timing system effectively insensitive to temperature changes (Pittendrigh, 1954). When the ambient temperature drops or increases substantially, say by 10°C, the insect still must be able to tell time, e.g., to properly anticipate dawn and dusk. Pittendrigh showed that the endogenous period of *Drosophila pseudoobscura* does not change much with temperature as measured with the eclosion assay in constant darkness (Pittendrigh, 1954), and the importance of temperature compensation was also appreciated by others (Hastings and Sweeney, 1957). The eclosion assay was used in the landmark 1971 paper by Konopka and Benzer, which arguably initiated the modern era of circadian molecular genetics and certainly initiated my circadian work (Konopka and Benzer, 1971).

KONOPKA AND BENZER

Seymour Benzer is an American hero, a one-of-a-kind scientist (Weiner, 1999). As a solid-state physics Ph.D. student at Purdue University in the 1940s, he came close to discovering the transistor, a discovery that won William Shockley, John Bardeen, and Walter Brattain of Bell Labs the 1956 Nobel Prize in Physics. He transferred his interests to molecular genetics and studied the fine structure of the gene as a Purdue faculty member. For this work during the ’50s and early ’60s, he shared many prizes including the 1971 Lasker Award with Sydney Brenner and Charles Yanofsky. Many people, including me, often wondered why Benzer did not win a Nobel Prize for this groundbreaking work. (Brenner won a Nobel Prize in 2002 for his later work on the worm, *C. elegans*.)

Benzer moved to Caltech in the mid-’60s and changed his research focus, from a study of phage genes to the foundations of behavior using fly genetics. Caltech had been a major center of *Drosophila* genetic work since T.H. Morgan moved there from Columbia in 1928. (Morgan won the 1933 Nobel Prize in Physiology or Medicine; this was the first American Nobel Prize in Physiology or Medicine and the first *Drosophila* Nobel Prize.) Benzer’s strategy followed what had been done in other areas of fly biology, namely associate single mutations and their underlying genes with a phenotype. The strategy was simple: the phenotypes in this case would be behavioral and the identified genes would therefore be – by definition – behavioral genes.

Ron Konopka was Benzer’s first student (Fig. 1). Importantly, Konopka had become interested in circadian rhythms in college and brought the

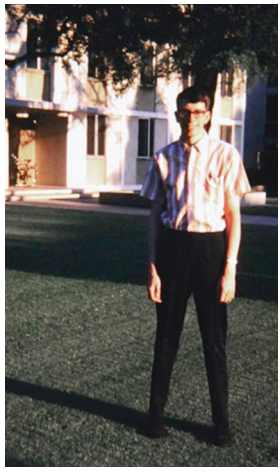

Figure 1. Ronald J. Konopka, around the time he began graduate school at Caltech (Rosbash, 2015).

circadian problem to Benzer, contrary to what one might imagine from first principles. Konopka also designed as well as carried out the first eclosion screen to search for credible circadian mutants (Rosbash, 2015).

Konopka exploited the remarkable genetics toolkit of *Drosophila* to simplify the eclosion screen. He used the classic attached X strategy so that the X chromosome of a mutagenized male would be inherited by his male progeny; like in humans, the Y chromosome is normally inherited by males from a male parent. However in *Drosophila* and now unlike in mammals, it is the X:autosome ratio that determines phenotypic sex; the Y chromosome predominantly contributes to sperm motility. With this attached X strategy therefore, the phenotypes of mutated genes on the X could be assayed in males of the first (F1) generation with no need for

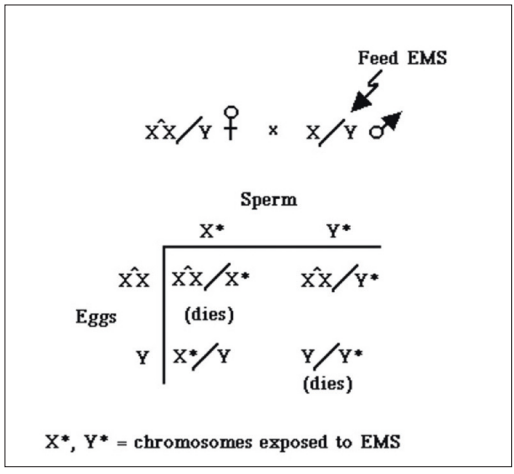

Figure 2. Identification of X-linked mutations in D. melanogaster. EMS-mutagenized sperm is used to fertilize eggs containing either an attached X-chromosome or a Y chromosome. Recessive mutations on the X-chromosome from mutagenized sperm are phenotypically visible in the F1 male offspring (Baker and Woodard, 2007).

backcrossing. As there is only a single mutagenized X chromosome in these F1 males, even recessive mutations would be visible. The disadvantage was that only about 20% of fly genes are present on the X chromosome, so potentially interesting genes present on the autosomes would be invisible (Fig. 2).

The screen was remarkably successful, and the Konopka-Benzer paper identified 3 mutant strains, each with a very different circadian phenotype from the normal wild-type fly with a ca. 24hr period. One mutant had a short period, about 19–20 hours, the second had a long period, about 29 hours, and the third had no apparent rhythm at all; it appeared arrhythmic. Remarkably, the phenotypes were not only assayed with the traditional fly eclosion assay (Fig. 3) but also with a locomotor activity assay (Fig. 4). Even more remarkably, the mutations would not complement, i.e., they appeared to be alleles of the same gene, which they named *period*. More extensive genetic analyses in the paper were consistent with this single gene conclusion. In addition, the phenotypes were semi-dominant, a result that has been extended to many circadian genes in different systems in the subsequent literature. These properties – including the mind-boggling fact that a single gene could apparently mutate to speed

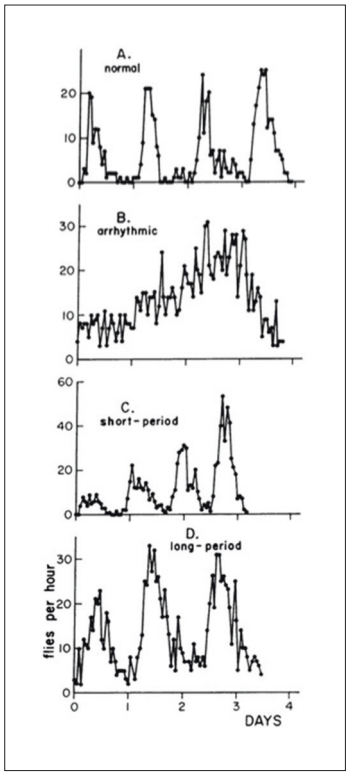

Figure 3. Eclosion rhythms of wild-type and Konopka and Benzer's period mutants (Konopka and Benzer, 1971).

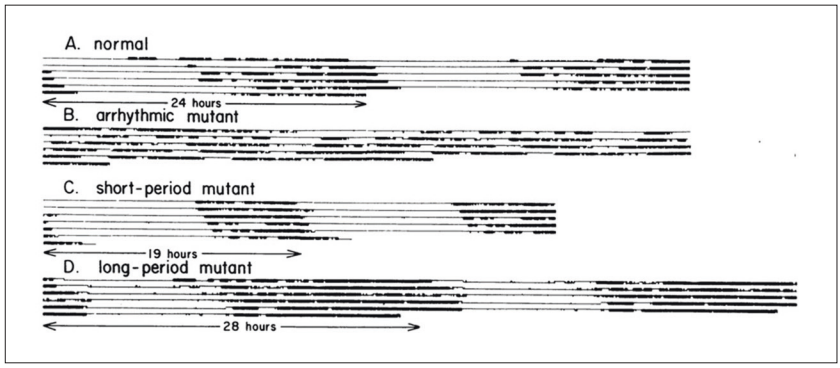

Figure 4. Locomotor activity patterns of wild-type and period mutants (Konopka and Benzer, 1971).

up, slow down or stop the clock – suggested that the rhythm-generating machinery reads out *period* gene expression in some semi-quantitative manner to influence circadian time-keeping in multiple directions (Konopka and Benzer, 1971).

The field made very little progress for the next decade or so. Although I am not aware of another laboratory other than Konopka's that was working on *Drosophila melanogaster* circadian rhythms in the early-mid '70s, I suspect that the major reason for the lack of progress was not the lack of manpower but the lack of viable approaches other than genetics. The insect was too small for most physiological approaches, and the strictly genetic approach, or phenogenetic approach as it is sometimes called, was inherently limited.

BRANDEIS AND RECOMBINANT DNA

One new lab that did enter the field of *Drosophila* circadian biology in the '70s was that of my friend and Brandeis colleague Jeff Hall. He also trained with Seymour Benzer but as a post-doc. Jeff knew Ron Konopka well from their time together at Caltech; Ron was doing his Ph.D. in the Benzer lab and then returned to Caltech as an assistant professor after a brief post-doc with Colin Pittendrigh at Stanford. Jeff's research interests in the late '70s were focused on neurogenetics writ large and also more narrowly on fruit fly courtship. His post-doc Bambos Kyriacou made a finding in the late '70s that merged the field of courtship with that of circadian rhythms. He discovered that the courtship song that the male fly sings to a female has about a 1 min rhythm. Moreover, the *period* mutants affect this ultradian rhythm similar to the way that these mutants affect circadian rhythms, i.e., shorter, longer and no rhythm at all (Kyriacou and

Hall, 1980). Although this finding lacks any mechanistic underpinning and has recently become quite controversial (Kyriacou et al., 2017; Stern, 2014; Stern et al., 2017), the reason for mentioning it here is historical: it enhanced Jeff's interest in *Drosophila* circadian biology and by extension my own.

Despite all this fascinating phenomenology, it was my contention in the early '80s that one could not strike off in a new direction, with new methods and ideas, without being able to get at the molecules – the RNAs and proteins – that were participating in timekeeping; they included the *period* RNAs and proteins. I must confess that this view also reflected a certain subjective bias; I was not comfortable with strictly genetic arguments, regardless of their strength in hindsight. This means that I was still far from certain that *period* and its gene products played a central role in circadian timing.

Enter recombinant DNA technology into the story in the late '70s-early '80s; it had transformed genetics, including my own yeast work. We were actively cloning yeast genes and using recombinant DNA methods to study their expression. It goes almost without saying that this new approach also solved my "comfort problem"; one could finally imagine cloning and sequencing enigmatic genes like *Drosophila period*. The application of recombinant DNA technology would therefore not only enable an entirely new generation of experiments but also might provide some insight into the function of this gene and by extension the molecular underpinnings of circadian rhythms.

Jeff and I discussed the possibility of collaborating in this new direction for at least a year but were unable to begin for a number of reasons, including most importantly the lack of personnel. This obstacle was overcome when Pranitha Reddy, a Brandeis Biochemistry graduate student, transferred into my lab. Her young thesis advisor Vivian Ernst had tragically passed away, and Pranitha needed a new home. I offered her this new project, the cloning of the *period* gene, and she enthusiastically accepted. The loose collaboration plan was that Jeff's lab would provide the genetics and behavior expertise, whereas Pranitha and my lab would do the cloning and provide the recombinant DNA expertise.

Importantly, this technology was making rapid progress in ways that were highly relevant to the identification of period. It became feasible by the early '80s to pursue the cloning of *Drosophila period* by chromosome walking, a strategy pioneered by the Hogness lab at Stanford (Bender et al., 1983a; Bender et al., 1983b; Spierer et al., 1983). Moreover, gene identification could then be done by gene rescue, which had just been accomplished by Rubin and Spradling (Rubin and Spradling, 1982; Spradling and Rubin, 1982). Our dream was to rescue the per^{o} strain and its arrhythmic phenotype with a piece of cloned DNA containing the wild-type (nor-

mal) *period* gene. Although chromosome walking and gene identification by rescue were not relevant to the yeast recombinant DNA research ongoing in my lab, we were deep into other recombinant DNA studies as mentioned above. The gap between what was ongoing in my lab and these new genetic strategies was therefore not big.

Pranitha led the successful Brandeis effort to clone this DNA, and she was aided by Will Zehring and Dave Wheller from Jeff's lab (Reddy et al., 1984). We had collaborative help in the important initial phases of this work from Christopher Hadfield and Vince Pirrotta then at the EMBO labs in Heidelberg, Germany. They had expertise in making phage libraries from DNA microdissected from *Drosophila* giant salivary glands. We knew from the genetic mapping work of Konopka that the *Drosophila* period gene was located at position 3B 1-2, between the white and zeste genes on the X chromosome (Konopka and Benzer, 1971). Our European collaborators made a DNA library from a piece of the salivary gland X chromosome surrounding region 3B 1-2, which reduced the amount of chromosome walking we would have to undertake.

Our rescue experiments were spearheaded by Zehring and Wheeler in the Hall lab with help from Reddy as well as Ron Konopka and Bambos Kyriacou (Zehring et al., 1984). Bambos had by this time returned to a faculty position in the UK but remained a frequent collaborator. He was responsible for the courtship song rhythm assay, which was also measured in this first gene rescue publication. Konopka had also moved, from Caltech to Clarkson College in upstate New York. Jeff was in frequent

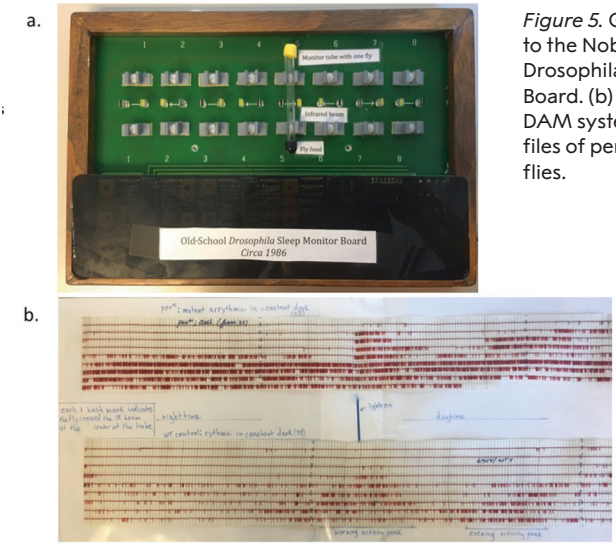

Figure 5. Gift of Hall and Rosbash to the Nobel Museum. (a) Original Drosophila Activity Monitor (DAM) Board. (b) Recording traces from DAM system showing activity profiles of per01 mutant and wild-type flies.

communication with him, and Ron helped us set up our initial Brandeis locomotor activity monitoring system. It became our gift, from me and Jeff, to the Nobel Museum (Fig. 5). Konopka also acted as an advisor, to make sure that we were carrying out and interpreting these locomotor activity rhythm assays – effectively sleep-wake assays – properly. This was our principal assay (Fig. 6); we only rarely assayed eclosion. My graduate student Qiang Yu also helped with these experiments, with cloning and especially with DNA injections into embryos. Qiang had spent several years in the Chinese countryside during the cultural revolution. Some of that time was spent repairing watches, which made him an expert at fine motor movements under magnification. These were precisely the skills required for the embryo injections, which generated the transgenic fly strains.

These two first papers, the cloning and characterization of the putative period DNA as well as the gene rescue experiments, relied for identification on classical genetic rearrangements used by Konopka in his characterization of the *period* gene (Konopka and Benzer, 1971). They dated from an earlier characterization of the white-zeste region of the fly genome within which period maps. This region and its chromosomal rearrangements were studied in detail by Bert Judd and his collaborators at the University of Texas. It is not coincidental that they included his

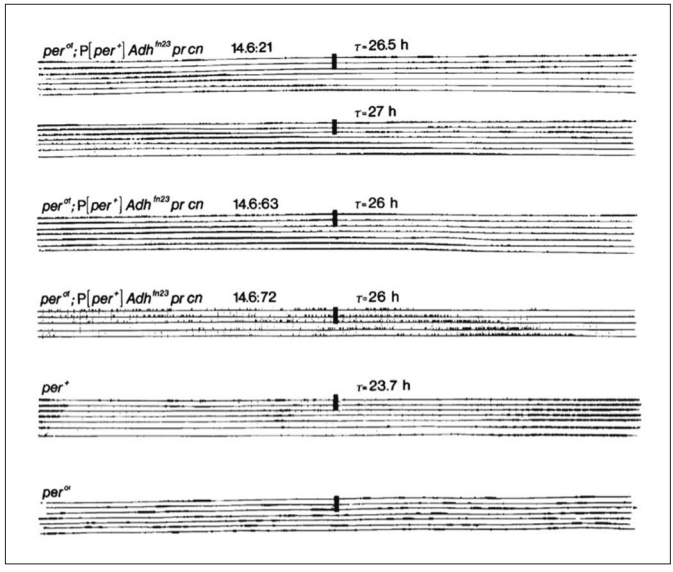

Figure 6. Activity grams showing rescue of per01 arrythmicity by segments of DNA from the per region. Each line represents a subjective day, while the midline mark in each actogram denotes the timing of lights turning on (Zehring et al., 1984).

Ph.D. student Mike Young, with whom Jeff Hall and I have shared this Nobel Prize (Young and Judd, 1978). Importantly in the context of our initial cloning and gene rescue efforts, our combined group at Brandeis did not know that the Young lab at Rockefeller University was engaged in very similar experiments (Bargiello et al., 1984; Bargiello and Young, 1984). Both groups soon learned about the other one, and we were locked in an intense battle for primacy during the first few years of this work. Importantly, the cloning and rescue of *period* was done independently and at the same time in both places. Although unpleasant at the time, the competition and focus probably contributed to some of these early successes. I note that the functional identification of a behavioral gene by transgenic rescue by both groups, Rosbash-Hall and Young, was accomplished 13 years before the same goal was achieved in mouse with the *Clock* gene by Takahashi and colleagues. Admittedly, this was a much more challenging task given the size of the mouse genome and other issues.

Mike Young and his colleagues deserve high marks for these early accomplishments. Although many of the conclusions were similar to ours, the early Young papers did a better job than we did of focusing in on a 4.5 kb transcript as the correct *period* mRNA. We were distracted by a 0.9 kb transcript, which was encoded just downstream of period and underwent apparent circadian oscillations in level (Reddy et al., 1984)) This transcript turned out to be a red herring: it is strongly expressed during pupation, just prior to eclosion. The daily synchronized emergence of young adults combined with the rapid decay of the transcript after eclosion gave rise to "apparent" circadian oscillations during adulthood (Lorenz et al., 1989).

SEARCHING FOR *PERIOD* PROTEIN FUNCTION

The next big question was, what is the function of the *period* protein or PER? This was approached in the obvious way, i.e., by DNA sequencing and done once again in parallel by the Young group and our Brandeis group (Baylies et al., 1987; Citri et al., 1987; Jackson et al., 1986; Yu et al., 1987b). The single nucleotide changes of the 3 mutations handsomely conformed to the simplest interpretation of the Konopka and Benzer data, but we were disappointed that the protein sequence itself was not instantly illuminating. These were, however, the early days of sequencing with rather few proteins in the database. This explains why the PER sequence did not link to its now known family of proteins; it was the first member of this family, i.e., a "pioneer protein." However, PER did link to proteoglycans, which often have repeated sequences that resemble the glycine-threonine (GT) repeat sequences of PER (Jackson et al., 1986; Reddy et al., 1986). The serines of proteoglycan GS repeats are sites of O-linked glycosylation.

We pursued this relationship and published a paper indicating that PER is indeed a proteoglycan (Reddy et al., 1986). In hindsight, the biochemical characterization of PER was simply too superficial to draw such a strong conclusion. The Young lab competition issue (I feared – correctly it turned out – that they too were working on this proteoglycan hypothesis) and a lack of prudence led me to push forward and publish these modest biochemical experiments. I also thought that the GT repeat sequence had to be significant and made the classical error of letting a potential exciting conclusion erase what should have been vigilant skepticism about my own data. I still don't know to this day if those experiments were in error or just their interpretation. I suspect the latter, for which I am 100% responsible, i.e., the results were probably correct but simply due to protein aggregation. In any case, the "PER is a proteoglycan" hypothesis stood tall in 1986 with no other proposed functions for PER. Moreover, this hypothesis soon stood even taller because of a high profile paper published in 1987 through a collaborative effort from the Young laboratory and that of David Spray at Albert Einstein (Bargiello et al., 1987). This paper argued that the per mutations affect intercellular communication in *Drosophila* larval salivary glands. PER would be resident in the extracellular matrix and quantitatively affect gap junction function, a perfect role for a proteoglycan. Importantly, there were no strong data to the contrary nor even another competing hypothesis for PER function in 1987.

The first hint that this proteoglycan hypothesis might be on the wrong track was also published in 1987 and came from my outstanding graduate student Qiang Yu (Yu et al., 1987a). In one of the first of many behavior-transformation papers in which per DNA was manipulated in vitro and then assayed functionally in vivo, Qiang cleanly excised the GT repeat region from the PER coding region and assayed the function of this deleted gene and protein. We assayed as usual two behaviors, circadian locomotor activity rhythms and the courtship song rhythms. The deletion had a strong effect on the more enigmatic courtship rhythms, but the canonical ca. 24hr locomotor activity rhythm assay was unaffected. Although the paper title featured the positive result, we recognized that the lack of an effect on the circadian assay was important. If the GT repeat region is the cardinal feature that defines proteoglycan properties, e.g., O-linked glycosylation, might this mean that the protein was not a proteoglycan? Might the biochemistry and the salivary gland gap junction results be misleading?

The results of a study we published in the following year was also relevant to the proteoglycan hypothesis (Colot et al., 1988). We cloned and sequenced period from D. *pseudoobscura* and D. *virilis*. The coding sequences of both genes are missing the GT repeat region but can rescue

behavioral rhythms of *per*^{o} *D. melanogaster* flies, consistent with the deletion experiment described above. Because the coding regions of the two genes still contain several pairs of TG and SG amino acids and because single SG dipeptides can serve as sites for the O-linked attachment of glycosaminoglycan side chains, the data could not eliminate the proteoglycan possibility. They did, however, add to a growing sense of doubt.

Unrelated to proteoglycans, the assay of behavior with manipulated *period* DNA was used in other papers published at about the same time. We characterized an unusual collection of spliced transcripts encoded by *per* and showed that two of them have biological activity in transformation-rescue experiments (Citri et al., 1987). The first author, Yoav Citri, became fascinated with fly rhythms and began these experiments when he was a post-doc in the Baltimore lab at MIT; he then moved over to my lab to continue the work. He left Brandeis to become an assistant professor at the Weizmann Institute around 1989 and died tragically in 1995 in a traffic accident.

We also used this sequence and rescue strategy to address the molecular identity of the famous Konopka mutations, with Qiang Yu once again principally responsible for these experiments in my laboratory (Yu et al., 1987b). Our sequence data largely agreed with that published the previous year from the Young group (Jackson et al., 1986), but our per gene contained an extra exon and was also missing an intron present in the Young publication, which interrupted a long contiguous coding exon. As a consequence, our predicted sequence was 97 amino acids longer than the one published by Young. We identified the *per*^{01} and *per*^{S} mutations, and in both cases identified only a single nucleotide change from wild-type per DNA. The work did not just rely on these sequence differences but also used chimeric DNA fragments to show that altered biological activity tracked with the mutation. Remarkably and consistent with the original hypothesis of Konopka that *per*^{01} is a null mutant with no biological activity, this mutation creates a stop codon; there is therefore no wild-type PER encoded and perhaps no protein at all. The *per*^{S} nucleotide change creates a serine to asparagine missense mutation at position 589. Both changes were in locations that underscored the fact that the 4.5 kb transcript encodes PER as originally suggested by Young and colleagues.

Although our original sequencing data made it almost certain that the mutation at position 589 is responsible for the *per*^{S} short period phenotype, we recapitulated this change in a subsequent study in which position 589 of wild-type DNA was changed to encode a number of different amino acids including asparagine (Rutila et al., 1992). The results not only verified that the serine to asparagine change was indeed responsible for the short period phenotype but also indicated that short period is likely to be the default phenotype of most amino acids at position 589, i.e., only

serine and threonine have a ca. 24 hr period. The data in this paper also indicated that the *per*^{S} protein is a hypomorph (has decreased function) rather than a hypermorph, with an increase in gene/protein activity as originally postulated by Konopka and Benzer (Konopka and Benzer, 1971). The Young lab identified all 3 Konopka and Benzer mutations and also showed that more copies of *period* transgenes led to shorter periods (Baylies et al., 1987).

At the risk of making a slight tangent to discuss much more recent data, we now have a quite sophisticated view of at least part of the PER molecular cycle, and these data underscore the hypomorphic or loss-of-function character of the *per*^{S} protein. PER amino acid 589 is phosphorylated by the kinase Nemo, which probably delays subsequent PER phosphorylation events by the important circadian kinase doubletime (DBT); they lead to the degradation of PER, which happens too rapidly in the absence of PER 589 phosphorylation (Chiu et al., 2011). This rapid degradation of PERS occurs after the disappearance of TIM in the late night or early day, a scenario that is also consistent with more recent data on the effects of the *perS* mutation (Li et al., 2014; Li and Rosbash, 2013). In contrast to our understanding of the PERS effect, there is still no good mechanistic understanding of the PERL amino acid change and why it slows

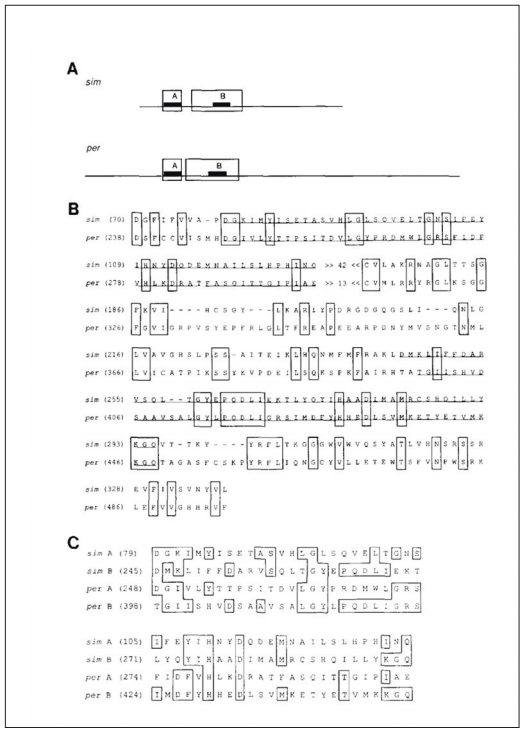

Figure 7. Comparison of SIM and PER sequences (Crews et al., 1998).

the circadian cycle (Kidd et al., 2015). Although PERL may interact more weakly with TIM (Gekakis et al., 1995), it is still uncertain which feature(s) of PER activity is specifically affected by PERL.

The 1987 mechanistic speculations about *perS* protein function were difficult to reconcile with a proteoglycan function. Moreover, we soon learned of an impending publication that further eroded my confidence in this hypothesis. Steve Crews, working in Corey Goodman's lab had cloned and sequenced a *Drosophila* gene called *single-minded* with protein coding homology to PER. Their 1988 paper showed that Single-minded (SIM) is a nuclear protein and strongly suggested it was a transcription factor (Crews et al., 1988). Especially difficult to square with proteoglycan theory was SIM nuclear localization. We now know that the homology reflects a protein-protein interaction domain called PAS and present in a family of transcription factors; the three founding members are PER, ARNT and SIM; ARNT is a mammalian protein, the aryl hydrocarbon receptor nuclear translocator (Huang et al., 1993). Although the homology was quite modest and could have reflected a motif shared by proteins with completely different functions (Fig. 7), it offered an important competing possibility: perhaps *period* encodes a transcription factor.

BACK ON TRACK: GENE EXPRESSION AND THE FEEDBACK LOOP

The possibility of a gene expression function was dramatically enhanced by the first relevant data from my post-doc Paul Hardin, who was examining *period* mRNA from fly heads at different circadian times. The paper that was ultimately published made two findings (Hardin et al., 1990). The first is that these mRNA levels undergo robust circadian oscillations in fly heads. The second is that the phase of RNA cycling was affected by the *per* missense mutations identically to their effects on behavioral phase (Fig. 8). Paul gets full credit for making these seminal findings, including the important idea to use fly heads rather than whole flies for the source of RNA. The lack of cycling in certain body tissues diluted the cycling signal and therefore reduced the cycling amplitude in RNA from whole flies so that it was not reliable.

Paul's experimental success was helped by his local environment, namely, the fact that a good fraction of his colleagues in my lab studied yeast RNA and were facile with state-of-the-art RNA methods. As PCR did not exist at this time, Paul had to use RNase protection to assay specific per mRNA levels. This method was not terribly difficult but also not trivial, so this aspect of the Brandeis circadian work benefited from being adjacent to – in effect embedded within – a nucleic acid-centric research program. Circadian labs elsewhere had a difficult time establishing these kinds of nucleic acid-centric approaches (personal communication).

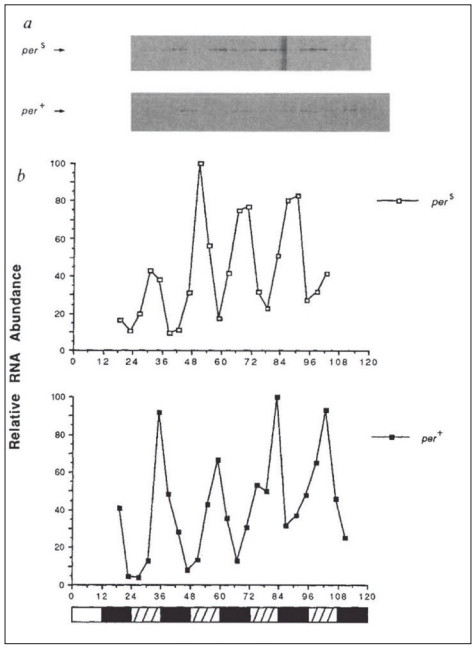

Figure 8. Both *Per*$^{+}$ and *per*^{s} mRNA levels undergo circadian oscillations. pers mRNA oscillations correspond to the shortened period exhibited by these mutant flies (Hardin et al., 1990).

We now know that the feedback effects of the *per* missense mutations on *per* RNA cycling operate predominantly at the transcriptional level and are upstream of their effects on behavior, i.e., the protein directly affects its own transcription as part of the timekeeping mechanism. (I should note that this is still inferred rather than directly shown, because we do not have a *per* mutation that is known only to impair the feedback effect on its own transcription with a period effect as measured behaviorally.) At the time of this first paper, however (Hardin et al., 1990), there were many possibilities, and we were very conservative in interpreting these results. For example, there were no transcriptional or pseudo-transcriptional assays, so feedback could have operated at a post-transcriptional level. We also had no evidence that PER functions directly to affect its own gene expression, for example by binding to chromatin or to pre-mRNAs, and our model considered the possible role of intermediate effector proteins. We even considered that circadian behavior would be required to generate oscillating mRNA levels, i.e., the feedback effects on gene expression might then be downstream of a PER effect on behavior. In this case, PER would be affecting circadian behavior and perhaps the circadian clock independent of its feedback effect on its own gene expression (Fig. 9). Nonetheless, the Hardin et al. data taken together with a simple and positive interpretation of the modest homology to the Single-minded transcription factor suggested a simple parsimonious hypothesis: PER

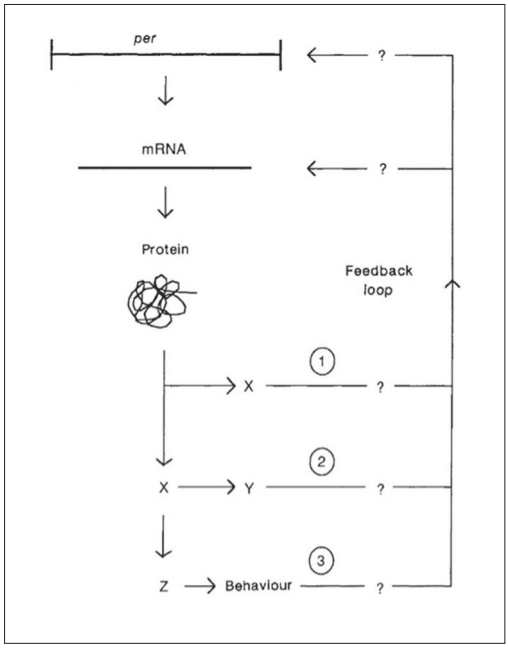

Figure 9. Original feedback loop model showing broadly how per might regulate its own expression (Hardin et al., 1990).

was engaged in a negative feedback loop that affects its own expression and that this loop was "the clock" or at least central to timekeeping. The negative feedback loop would therefore be upstream of the behavioral rhythms that are usually measured by circadian biologists.

This new working model of how PER functions, namely at the level of gene expression regulation, was very attractive, and my lab pushed hard to support and extend this new view of timekeeping. First was to address transcriptional vs. post-transcriptional regulation. To this end, Paul Hardin showed that feedback regulation almost certainly occurred at the transcriptional level (Hardin et al., 1992). This paper had two principal experiments. i) It showed that pre-mRNA oscillations were similar if not identical to mRNA oscillations. ii) It showed that the feedback could be recapitulated with *per* 5' flanking sequences, i.e., they would drive cycling of a reporter gene. (Fig. 10).

To my surprise, we had serious trouble publishing this important paper. Perhaps this was because transcriptional regulation was taken for granted by the scientific community; post-transcriptional regulation, splicing for example, was not yet broadly appreciated as a viable regulatory mechanism.

The second paper addressed PER subcellular localization. Is it a nuclear protein like SIM or is it cytoplasmic? The latter would favor an indirect effect on transcription and possibly even other functions. To

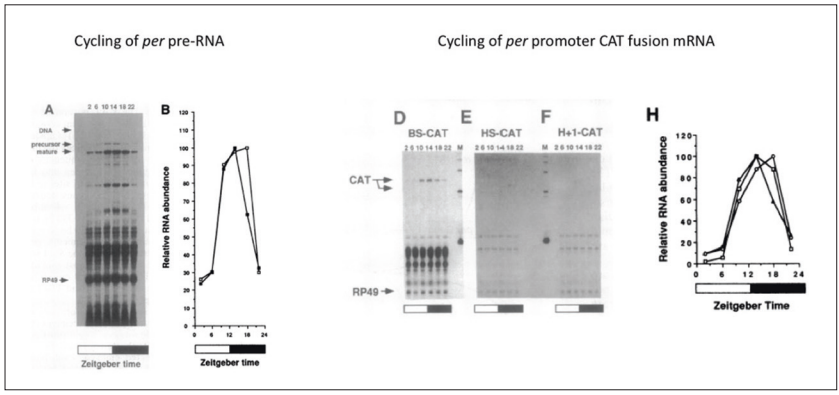

Figure 10. per mRNA oscillations are transcriptionally regulated (Hardin et al., 1992).

address this issue, my graduate student Xin Liu went to Seymour Benzer's lab for a few months to do immunoelectron microscopy. (Xin recently retired after many years as a UCLA professor.) PER was a nuclear protein (Liu et al., 1992). This was consistent with a more direct role on transcription and made the proteoglycan hypothesis even less likely.

The third paper was carried out by an excellent graduate student, Honkui Zeng, who addressed whether PER functioned in a direct, cell-autonomous manner. (Honkui has been with the Allen Brain Institute for more than a decade and is currently the Executive Director of its Structured Science Division.) There was at this time no evidence that PER inhibited transcription within the same cells in which it was expressed. To this end, Honkui showed that the photoreceptor cells of the adult fly eye contain a circadian oscillator system similar to the brain. She also showed that constitutively overexpressing PER in these cells prevented endogenous *per* RNA cycling and caused low *per* RNA levels with no effects on locomotor activity rhythms, consistent with cell-autonomous negative feedback (Zeng et al., 1994). A similar strategy was subsequently applied to brain circadian neurons with similar results (Yang and Sehgal, 2001).

The fourth paper came from yet another outstanding graduate student, Josh Huang. (Josh is current a Professor of Neuroscience at the Cold Spring Harbor Laboratory). He addressed the role of the PAS domain, the protein sequence link between PER and known transcription factors (Huang et al., 1993). At the time of this work in 1994, there were 3 known PAS-containing (PAS-bHLH, basic-helix-loop-helix) transcription factors: In addition to PER there were Single-minded as mentioned above and the two subunits of the aryl hydrocarbon (AH) receptor. Josh's work showed that PAS could function as a novel protein dimerization motif

and mediate associations between different members of the PAS protein family. As PER had and still has no known DNA interaction motifs, we proposed that it regulates circadian transcription by interacting with the PAS domain of an important positively-acting circadian transcription factor. This prediction turned out correct with the identification of the PAS-containing positive circadian transcription factors, the heterodimeric Clock and BMAL1 in mammals and their orthologs Clock and Cycle in flies (see below).

Taken together with the original Hardin feedback loop paper, these four subsequent papers laid the foundation of a coherent transcription-centric view of circadian timekeeping; it seemed incompatible with gap junctions. Indeed, I already had doubts about this hypothesis in 1989; at this time the work for the first Hardin feedback loop was largely done (Hardin et al., 1990), and we had shown that the GT region – the linchpin of the proteoglycan relationship – is not conserved in related *Drosophila* species (Colot et al., 1988) and that this region is also not necessary for PER circadian function (Yu et al., 1987a). Although I had no idea how to advance or challenge our biochemistry paper (Reddy et al., 1986) in an efficient way, I thought we should repeat the simpler Young-Spray salivary gland-gap junction paper to see if it was correct (Bargiello et al., 1987). Also, for this experiment, I really had no idea what to do; it was not only completely beyond my expertise but also outside that of my circle of friends and colleagues. Nonetheless I was quite obsessed and for the next year or two would ask scientific strangers, "Do you know anything about gap junctions?" "Can you help us out with this experiment?" One day at a Brandeis cocktail party I was introduced to Dan Goodenough. I asked him naively, "Where do you work and what do you do?" He said, "Harvard and on gap junctions." I latched onto his ankle: "Can you PLEASE help us repeat a gap junction experiment in Drosophila larval salivary glands?" He said sure, and we were on our way. I had no available personnel, but Jeff had a technician with time to spare. So, Kimberly Flint spent several months hosted by the Goodenough lab and showed that those key gap junction experiments would not repeat. Dan Goodenough was a prince, not only to host Kimberly and let her work in his lab but also because he refused to coauthor any paper that might emerge from the work. We communicated our failure to Young before publication, and Jeff also told his former post-doc Kathy Siwicki about our results. She was at the Marine Biological Laboratory (MBL) at Woods Hole at the time and was apparently doing the same experiments in collaboration with David Spray, the senior author of the Bargiello et al. paper who was also there during that summer of 1992. They wrote a short paper for the *Biological Bulletin*, the local MBL journal. It was published super rapidly, in October, and they included Kimberly, Jeff and me as coauthors (Siwicki et al., 1992). Young,

Spray and all coauthors then acted properly and retracted their *Nature* paper in December of 1992 (Saez et al., 1992), and we published an independent and properly documented paper the following year in the *Journal of Membrane Biology* (Flint et al., 1993). This was the death knell for the proteoglycan hypothesis, and we forged ahead with gene expression.

THE LINK TO MAMMALS

The identification of mammalian PER in 1997 was incredibly important. This is because for the first time it seemed likely that what we had learned in flies would be applicable to mammals including humans. This finding, that mammals contain one or more *period* genes, was independently accomplished in two different laboratories and done before the existence of the human genome sequence, meaning one could not just search the data base for a mammalian protein sequence with a credible relationship to PER (Sun et al., 1997; Tei et al., 1997). It was done by PCR and required a judicious selection of primer sequences. My laboratory and others also tried to do this but unsuccessfully (data not shown; personal communication).

NEW CIRCADIAN MUTANTS

The Young laboratory also made a connection to mammals, with their modern (post-Konopka and Benzer) efforts to identify *Drosophila* circadian mutants. They discovered two very important fly clock genes, one of which linked in an important and simple way to mammals. That gene was *doubletime*, which encodes the *Drosophila* version of the important mammalian kinase CKI (Kloss et al., 1998; Price et al., 1998). It modifies PER and also CLK during the circadian cycle. Well after the publications on *doubletime* from the Young lab, we also identified and studied two novel mutant versions of *doubletime*, which emerged from our own efforts to isolated new circadian mutants (Suri et al., 2000). CKI not only plays an important role in the mammalian clock but a mutation in this gene also underlies the phenotype of the famous 20hr *tau* hamster (Lowrey et al., 2000).

The other Young laboratory clock gene from this early period is *timeless* (Gekakis et al., 1995; Myers et al., 1995; Price et al., 1995; Sehgal et al., 1994; Sehgal et al., 1995). Although its relationship to mammals was not immediately apparent and is considerably weaker even today than that of *period* and *doubletime* (Gotter, 2006), *timeless* played an incredibly important role in the fly circadian system. In addition, its identification and cloning in 1995 showed that *period* was not unique and that scientists other than Konopka and Benzer could identify clock mutants. This fear

was not entirely unfounded, as almost 25 years had passed between the publication of Konopka and Benzer on *period* and these Young lab publications on the second *Drosophila* clock gene *timeless* (*tim*).

Importantly, the characterization of *tim* connected back to PER and to the feedback loop, i.e., *tim* mRNA undergoes RNA cycling like *per* mRNA, the *tim* mutants affect *per* as well as cycling of their own mRNA, i.e., *tim* mRNA (Sehgal et al., 1995) and the *timeless* protein (TIM) interacts with PER (Gekakis et al., 1995). In fact, TIM and PER associate together in the cytoplasm and are probably transported together into the nucleus (Jang et al., 2015; Meyer et al., 2006; Vosshall et al., 1994). However, the regulatory role of this transport event – the extent to which it contributes to circadian timing (Meyer et al., 2006) – is less certain in my view. All proteins are synthesized on cytoplasmic ribosomes. So nuclear proteins must be transported from the cytoplasm to the nucleus, but this transport event need not be under clock control. Temporal control of nuclear and cytoplasmic localization (Shafer et al., 2002) could equally well reflect regulation of cytoplasmic or nuclear retention rather than nuclear transport per se. Indeed, we have some evidence that PER-TIM retention on chromatin is temporally regulated (data not shown).

Our own principal contribution to the *tim* story came from biochemistry (Zeng et al., 1996). My graduate student Hongkui Zeng was trying to identify PER partner proteins, by characterizing proteins associated with PER in fly head extracts with mass spectometry. Because we realized that there might be different partners at different times of day, Hongkui began by characterizing the size of the protein complex within which PER was located at different times. PER appeared to be monomeric in the morning during its declining/degrading phase but in a larger complex later in the day during its increasing/accumulating phase. Moreover, that complex appeared to have one additional major protein, with an apparent molecular weight somewhat greater than PER (Zeng et al., 1996). That complex and the additional protein disappeared in the late night or rapidly after a light pulse. We were gearing up to identify that seemingly light-sensitive protein by mass spectrometry when we heard about the Young lab success in identifying TIM. Because the TIM molecular weight was somewhat greater than that of PER and the two proteins associate in a yeast two hybrid assay (Gekakis et al., 1995), TIM was an excellent candidate for the PER partner protein we had identified in fly head extracts. The Young laboratory was very gracious in providing us with an anti-TIM antibody with which we were able to definitively identify it as the higher molecular weight PER partner identified in our gels (Zeng et al., 1996).

The rapid degradation of TIM at the end of the night or in response to a phase-shifting pulse of light was independently observed and proposed to be important for light-mediated phase-shifting and entrainment in 3

labs at about the same time (Hunter-Ensor et al., 1996; Myers et al., 1996; Zeng et al., 1996). We also provided evidence that TIM degradation and phase-shifting do not just occur in parallel. This study (Suri et al., 1998) made use of a new *tim* allele. In an attempt to shed light on the enigmatic *perL* mutation, we had screened for allele-specific suppressors or enhancers of that mutation, namely, additional mutations elsewhere in the fly genome that would have a different phenotypic effect on that *perL* background than on a wild-type background. It was to my knowledge the first genetic screen of its kind, a behavioral suppressor-enhancer screen. It identified a new *tim* allele, which we named *tim suppressor of long* or *timSL* (Rutila et al., 1996). In this context of TIM degradation and light, *timSL* enhanced the light-sensitivity of TIM degradation and phase-shifting in parallel (Suri et al., 1998), indicating that TIM destabilization indeed connects the core central clock to entrainment by the light-dark cycle, the principal zeitgeiber (temporal cue) of the environment in which we live.

What is the molecular connection between light and TIM degradation? Patrick Emery in my lab and Ralf Stanewsky in the Hall lab worked closely together and identified Cryptochrome as a major circadian photoreceptor; they also cloned the gene and identified a mutant strain that was significantly impaired in circadian light perception (Emery et al., 1998; Stanewsky et al., 1998). The work also relied on important previous work from mammals and constituted another strong link between the fly and mammalian circadian systems.

The story begins with the important DNA repair enzyme photolyase, which is present from E. coli to man. This enzyme had been studied for a long time by the biochemist Aziz Sancar, who shared the 2015 Nobel Prize in chemistry for his outstanding work. The plant blue light photoreceptor cryptochrome is a close structural relative of photolyase. There were mammalian gene sequences that were related to cryptochrome, and Sancar was convinced that their encoded proteins, cryptochrome 1 and 2 (CRY1 and CRY2) must function in circadian photoreception. Indeed, the initial properties of these gene and proteins, including phenotypes of mouse mutants missing these cryptochromes, was consistent with a circadian photoreceptor function for cryptochromes in mammals (Vitaterna et al., 1999). Moreover, there was strong evidence that there was an unknown photoreceptor in the mammalian eye that accounts for persistent circadian photoreception in rodless-coneless mice (Lucas et al., 1999); this is in addition to the known photoreceptor function of cryptochromes in plants. Importantly, melanopsin was soon discovered as that missing eye photoreceptor (Provencio et al., 1998) and mammalian cryptochromes currently have no function in circadian photoreception.

Nonetheless, these Sancar ideas about a circadian photoreceptor function for cryptochrome and the known mammalian cryptochrome genes

resonated with us at Brandeis. The fly circadian system was known to be maximally sensitive to blue light, and there was no known eye pigment or other identified photoreceptor that might play this role. So, Patrick Emery in my lab set out to clone and characterize *Drosophila cryptochrome* (*cry*; the sequence information indicated there was only gene as is commonly the case in flies). The work showed that its protein product CRY was light-sensitive, i.e., degraded in response to light exposure, and that CRY overexpression enhanced circadian light sensitivity (Emery et al., 1998).

At the same time, Ralf Stanewsky in the Hall lab had identified a new circadian mutant that was light-insensitive. It had very interesting properties, which were consistent with the absence of a circadian photoreceptor. With Emery's map position and the identification of a deletion that uncovered the locus, Stanewsky could quickly assign the mutation to the *cry* gene. Characterization of the mutant strain, called *cryb*, indicated that the gene *cry* indeed encoded a circadian photoreceptor (Stanewsky et al., 1998) as was also indicated by the Emery et al. paper described directly above (Emery et al., 1998).

Subsequent work over the next couple of years, predominantly from Emery, extended this initial work by showing quite remarkably that *cryb* flies remain rhythmic in constant light (Emery et al., 2000a). This is unlike wild-type flies as constant light destroys their rhythms – and those of many organisms. This simple observation indicates that CRY acts as the only principal circadian photoreceptor in *Drosophila*.

Emery and colleagues also showed at about the same time that expressing CRY only in a small number of key circadian neurons in the brain could rescue the circadian deficits of the *cryb* mutant background. This indicates that CRY functions within the brain and is therefore a deep brain photoreceptor (Emery et al., 2000b).

How does CRY work, i.e., how does it interface with the circadian clockworks? CRY and TIM interact in a light-dependent fashion, even in yeast (Ceriani et al., 1999). Amita Sehgal and colleagues then discovered that the F-box protein Jetlag interacts with CRY and TIM after light exposure and helps deliver them both to the proteosome. As predicted, phase-shifts are reduced in jetlag mutants (Koh et al., 2006).

This CRY-TIM mechanism is not the only way that the *Drosophila* clock system can achieve phase shifts. Clock neuron firing, specifically PDF neuron firing, can also elicit phase shifts and in a CRY-independent manner (Guo et al., 2014). This mode of firing also results in TIM degradation and presumably reflects in part the connections of the fly eye to the PDF neurons and/or the role of other clock neurons (Fig. 11).

The common feature of these two modes of phase-shifting, light-CRY and light-firing, is TIM degradation or post-transcriptional regulation. This contrasts with the enhanced clock gene transcription that is the pri-

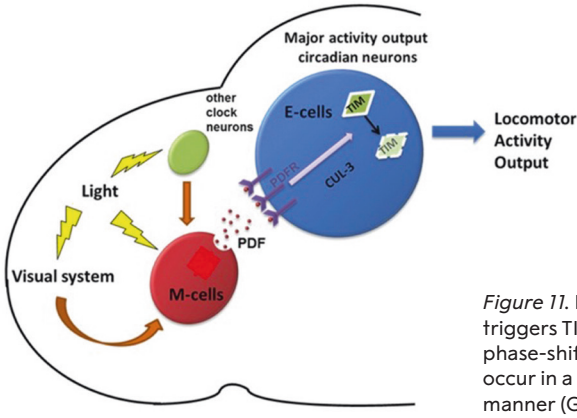

Figure 11. Firing of PDF cells triggers TIM degradation and a phase-shift, both of which can occur in a CRY-independent manner (Guo et al., 2014).

mary means of achieving light-mediated phase-shifts in mammalian systems (Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al., 1997). Nonetheless, it remains uncertain whether this difference will remain so striking, i.e., whether future results will reveal some important light-mediated post-transcriptional regulation of clock gene expression in mammals or some light-mediated clock gene transcriptional regulation in flies.

However, one strong argument in favor of this difference between flies and mammals is the different roles of cryptochrome in the two systems. In contrast to its photoreceptor role in flies, cryptochrome is the major transcriptional repressor in mammals (Reppert and Weaver, 2002). These two roles reflect two major features of the cryptochrome ancestor photolyase, namely, photon capture and interaction with DNA (Emery et al., 1998; Gehring and Rosbash, 2003). It is striking that butterflies have two cryptochromes: one that resembles more closely *Drosophila* cryptochrome and functions as a photoreceptor; and another that resembles more closely mammalian cryptochrome and functions as a transcriptional repressor (Zhu et al., 2005). The butterfly situation appears to be the insect "rule," with the *Drosophila* situation the "exception" (Yuan et al., 2007).

The role of cryptochrome in transcriptional repression was preceded by the discovery of CLK-BMAL1 in mammals and their orthologs CLK-CYC in flies. Joe Takahashi and colleagues spearheaded the cloning of CLK through a forward genetic screen in mice. A robust behavioral phenotype of the CLKD17 allele was first identified by behavioral screening (Vitaterna et al., 1994). The gene was identified three years later as a member of the bHLH-PAS-containing protein family (Antoch et al., 1997; King et al., 1997). The bHLH domain suggested DNA binding and other bHLH-PAS family members had been identified as bona fide transcription factors, making this function an excellent candidate for CLK. Moreover,

given the presence of a PAS domain in PER and the circadian behavioral phenotype of the mutant, CLK was probably also a major circadian transcription factor as described above. Importantly however, the two 1997 papers did not connect CLK to the transcription of PER (Antoch et al., 1997; King et al., 1997), i.e., they did not clarify whether CLK was the positive transcription factor that drives the synthesis of the negative regulator PER. This was done a year later by Takahashi and coworkers, in papers that also identified the CLK partner BMAL1 (Darlington et al., 1998; Gekakis et al., 1998).

We came to the identical conclusion at almost the same time, by identifying the CLK-BMAL1 orthologs Clock-cycle or CLK-CYC (Allada et al., 1998; Rutila et al., 1998). This was the major result of a genetic screen we had been carrying out over several years. It had the goal of finding the positive transcription factor that drives the transcription of *per* and *tim*. To this end, we decided to focus on a large number of mutant strains, which were totally arrhythmic. The logic was that a loss of function mutation in this positive transcription factor would not be able to synthesize PER and TIM and therefore should be arrhythmic. We also adopted this strategy to do something different than what we knew was well underway in the Young laboratory, i.e., competently searching for mutant strains with altered circadian periods. Some of these inevitably emerged from our screen as well.

It turned out to be much easier to find arrhythmic strains than strains with altered periods, presumably because functional rhythms rely on many different functions and pathways, e.g., output pathways from circadian neurons to motor neurons, in addition to the core timekeeping mechanism. A subscreen was therefore required to avoid these output pathways and focus in on that subset of the arrhythmic strains that might be candidates for encoding the positive transcription factor(s). So about 50 arrhythmic strains were assayed for cycling of *per* and *tim* mRNA. The vast majority of these mutant strains still manifested robust cycling of *per* and *tim* mRNAs, consistent with the notion that they did not impact core circadian timekeeping. (As a byproduct, these results indicated that robust behavioral oscillations are not necessary for molecular feedback, e.g., for the effect of PER on the cycling of its own mRNA (Fig. 9).) However, 3 mutant strains were exceptional and manifested non-cycling, low levels of *per* mRNA even in a light-dark cycle. We then identified and cloned the 3 mutant genes. Remarkably, two of them were alleles of *cycle*, and one was an allele of *Clock*, exactly the phenotype one might expect from a mutant in the key positive circadian transcription (Allada et al., 1998; Rutila et al., 1998). Given the results of Takahashi and colleagues (Darlington et al., 1998; Gekakis et al., 1998), the field was really on its way by 1998 with powerful, parallel systems in flies and mammals.

The final coda to the feedback loop chapter was the demonstration that PER interacts directly with CLK-CYC in flies (Lee et al., 1998; Menet et al., 2010) and with CLK-BMAL1 in mammals (Lee et al., 2001).

A FAMILY STORY RESULTING IN TRIKINETICS LOCOMOTOR ACTIVITY MONITORS

I went on sabbatical to Paris with my family in 1988–1989. In the spring of 1989, shortly before returning to Brandeis, I received a phone call in Paris from Max Cowan, the Chief Scientific Officer of the Howard Hughes Medical Institute (HHMI). He informed me that I would be appointed as an Investigator of HHMI as of July 1, 1989. He asked me what I wanted for "set-up funds." I told him I had no idea because I already had a functional lab. He then suggested $100,000.00, and I said "sure." As there were no pressing needs, I began to consider what I might do with those funds.

Shortly after returning to Brandeis in late June, I went to a family (wife's family) barbecue in Newton, Massachusetts where I live. My wife is Chilean, and her brother-in-law was visiting with us. His first cousin, Sergio Simunovic, is also Chilean by birth and lives in Newton, had gone to MIT, and was the host of the barbecue. I asked Sergio what he did for a living. He answered that he was an engineer and owned a small robotics company in Waltham, Massachusetts. Waltham is next to Newton and also where Brandeis University is situated. I immediately asked Sergio if he might be interested in designing and building *Drosophila* locomotor activity monitors for us. He introduced me to his partner Mark Spencer, and we decided to begin a collaboration. It used up half my HHMI set-up funds, i.e., I paid $50,000.00 to TriKinetics to fund the R&D costs of the *Drosophila* locomotor activity monitors. (Perhaps some monitors were included in this initial cost; I can't recall.) For several months, Mark worked almost exclusively with my post-doc/research assistant Joan Rutila, the talented individual in my lab who supervised our genetic screening efforts, to design functional activity monitors. The challenge was to adjust the detection sensitivity so beam crossing detection was reliable – with minimal false positives and false negatives; this obstacle was eventually surmounted by Mark and Joan. In summary, success was achieved through a 3-way collaboration: my HHMI funds and initiative, Mark, and Joan. These same monitors are still sold today (Fig. 12).

I never imagined that these monitors would be used all over the world, i.e., I had assumed that they would be used by our two labs at Brandeis, perhaps also the Young lab at Rockefeller and then eventually by a few of our students who would establish independent labs. One would expect that I or my lab received some money or discount from TriKinetics but this was not the case.

Figure 12. Drosophila Activity Monitors.

EVOLUTIONARY CONSIDERATIONS

Somewhat after the first transcriptional feedback loop papers appeared from Brandeis, the Dunlaps at Dartmouth published an outstanding mechanistic paper describing a rather similar feedback loop scheme for *Neurospora* circadian rhythms (Aronson et al., 1994). This story started with Jerry Feldman, who was at UC Santa Cruz. Feldman and his colleagues isolated *Neurospora* circadian mutants not too long after the Konopka story was published (Feldman and Hoyle, 1973; Feldman and Hoyle, 1976). These studies identified the *frequency* gene, which had genetic properties remarkably similar to those described by Konopka and Benzer for *Drosophila* and its *period* gene (Gardner and Feldman, 1980; Loros and Feldman, 1986; Loros et al., 1986). Jay Dunlap was a post-doc in the Feldman lab at Santa Cruz and moved along with his now wife Jennifer Loros to Dartmouth and began a molecular attack on the circadian problem in *Neurospora*. Over the subsequent almost 25 years since the first molecular paper (Aronson et al., 1994), the *Neurospora* circadian clock has developed into a wonderful scientific tale, which has striking similarities to the fly and mammalian clock (Dunlap and Loros, 2017). Dunlap and Loros have even argued that these clocks have a single origin (Lee et al., 2000). However, I find these data less than totally convincing, i.e., the similarities either reflect convergent evolution or the evolutionary relationship between animal and *Neurospora* rhythms has not yet been clarified.

From a more general perspective and as mentioned near the beginning of this manuscript, circadian clocks almost certainly arose multiple times in evolution; there is essentially no molecular bridge between the cyanobacterial clock and eukaryotic clocks, and only the most speculative connection between plant clocks and the mammalian-fly clock (Rosbash, 2009). Fly cryptochrome does suggest, however, that this particular branch of the circadian world arose in response to the DNA damage caused by sunlight. Moreover, this "flight from light" hypothesis also explains the blue light sensitivity of cryptochrome. This is the wavelength that penetrates substantial depths in the ocean, perhaps reflecting the origin of life in the oceans as well as the diel migration that still occurs there today (Emery et al., 1998; Gehring and Rosbash, 2003; Pittendrigh, 1993; Rosbash, 2009). Responding to light would have preceded the origin of clocks, e.g., the ability to anticipate the daily appearance of light at dawn. In this view, the circadian kinases and other molecules that participate in the DNA damage response might even bridge plant and animal clocks (Rosbash, 2009). There is also another competing hypothesis to "flight from light" for the origins of clocks, namely the temporal organization of metabolism (Tu and McKnight, 2006).

WHAT REMAINS INTERESTING TO STUDY IN CIRCADIAN BIOLOGY?

Metabolism segues conveniently to medicine and future practical advances: metabolism is one of the myriad aspects of human physiology that are under circadian control. However, this Nobel Prize was given predominantly for our contributions to physiology rather than to medicine. (The Prize is after all for Physiology OR Medicine.). We now know about the dangers of shift work, the benefits of good circadian and sleep hygiene as well as the possible benefits of restricted feeding (Melkani and Panda, 2017), but major medical applications remain a future goal. Although this sentiment is similar to the one expressed by T.H. Morgan about genetics and medicine in his Nobel manuscript (The Nobel Lecture 1934), I am more optimistic about future medical advances from circadian biology than he was about medical advances from genetics in 1933. For understandable reasons, Morgan could not foresee the remarkable future that lay ahead for genetics, a practical as well as a conceptual future. The future is now more tangible in the case of clocks, because so much of the mammalian genome and therefore so much of physiology is known to be under circadian control (Zhang et al., 2014). There are also a number of possible drug targets implicated in the regulation of circadian physiology.

Additional future challenges are the precise timing mechanisms of animal circadian clocks and temperature compensation. What is really keeping time, i.e., what are the rate-limiting steps and why is there such little

change with temperature? Although some progress has been made in our understanding of temperature compensation (Mehra et al., 2009), I suspect there is more to learn.

Another frontier is the relationship between neuroscience, clocks and sleep. Circadian clocks have been known for a long time to impact sleep (Borbely, 1982; Borbely et al., 2016; Daan et al., 1984). Moreover, sleep remains a major mystery. There are many hypotheses about how and why we sleep, but in my opinion these issues – especially why we sleep – remain major mysteries. Moreover, the neuronal circuitry that underlies the "how" of sleep and perhaps also the "why" is quite complex in mammals (Saper and Fuller, 2017). I am optimistic that the rather simple organization of the fly brain and especially its circadian circuitry will provide an entrée into this fascinating topic; my own current research is beginning to focus in this direction (Guo et al., 2016). I look forward to a future that will reveal the mysteries of sleep comparable to the progress I have witnessed in circadian biology since I began in this field 35 years ago.

FINAL THOUGHTS

What began in 1982 as a foray into cloning a clock gene became a grand adventure. Much to my surprise, a remarkable landscape was eventually revealed. I say this not because I was particularly pessimistic or uninterested in circadian biology 35 years ago but because most research problems don't work out in such remarkable fashion.

I have been asked by numerous students since October 2 something like, "How can I do something important and significant like you have done?" The answer is, "I have no idea, not only for you but also for me: I don't know how I arrived at this place today. This answer is a bit tongue in cheek of course, so here's a somewhat more detailed answer.

Luck played a huge role in my success, indeed in anyone's success. A substantial fraction of this luck was just being in the right place at the right time. Another fraction, perhaps not unrelated to the "right place-right time" comment, was knowing about nucleic acids and macromolecular metabolism when this turned out to be a key to thinking about the problem. Having an operating yeast RNA lab interspersed among my circadian researchers was an enormous advantage when it was time to put in place these methods.

It is also important to stress that almost all aspects of luck are just "one-off" and cannot be repeated. Like the pinball that seemingly traces the same path, it doesn't come out exactly the same the second time 'round. For this reason, trying to do something great – shooting for prizes and fame let's call it – is a fool's errand. It is good to pick an important

problem, but even more important is to love your work, the craft of being a scientist. My partner in crime Jeff Hall adored the craft of fly genetics; he was damn good at it too. A second piece of advice is to enjoy the people. Life science research is truly a social activity – or at least it was during my more than 50 years since I began graduate school at MIT. I have made remarkable friends through science, people I truly admire and in some cases love. I am also fond of almost all the fantastic people who joined my circadian lab at key points in time. It goes without saying that I wouldn't be writing this document without their efforts, intellectual as well as physical. There is also family to thank, wife as well as children. They have been long-suffering and an unending source of support and joy.

I will close by saying that I never saw myself in historical context when work was ongoing (I still don't) but as the scientific equivalent of a working class guy; I was always trying to put one foot in front of the other so that my research was moving forward more often than it was moving backwards – at least some of the time. Like almost every other active scientist, I was trying to maintain my funding, make sure my students and post-docs had decent papers and fulfill my obligations to Brandeis and to HHMI. Even when we published the feedback loop paper (Hardin et al., 1990), there was no dramatic sense of accomplishment. We simply moved on to the next stage of the work, the next stage of worry I might call it: Is the paper correct? How could we resolve the ambiguities in the final figure/model? How does PER work? This is truly how I see myself; I am just a regular guy, a scientist who got incredibly lucky.

REFERENCES

Albrecht, U., Sun, Z.S., Eichele, G., and Lee, C.C. (1997). A differential response of two putative mammalian circadian regulators, *mper1* and *mper2*, to light. *Cell* **91**, 1055–1064.

Allada, R., White, N.E., So, W.V., Hall, J.C., and Rosbash, M. (1998). A mutant Drosophila homolog of mammalian Clock disrupts circadian rhythms and transcription of period and timeless. *Cell* **93**, 791–804.

Antoch, M.P., Song, E.-J., Chang, A.-M., Vitaterna, M.H., Zhao, Y., Wilsbacher, L.D., Sangoram, A.M., King, D.P., Pinto, L.H., and Takahashi, J.S. (1997). Functional identification of the mouse circadian *clock* gene by transgenic BAC rescue. *Cell* **89**, 655–667.

Aronson, B.D., Johnson, K.A., Loros, J.J., and Dunlap, J.C. (1994). Negative feedback defines a circadian clock: Autoregulation of the clock gene *frequency*. *Science* **263**, 1578–1584.

Bargiello, T.A., Jackson, F.R., and Young, M.W. (1984). Restoration of circadian behavioural rhythms by gene transfer in *Drosophila*. *Nature* **312**, 752–754.

Bargiello, T.A., Saez, L., Baylies, M.K., Gasic, G., Young, M.W., and Spray, D.C. (1987). The *Drosophila* clock gene per affects intercellular junctional communication. *Nature* **328**, 686–691.

Bargiello, T.A., and Young, M.W. (1984). Molecular genetics of a biological clock in *Drosophila*. *Proc Natl Acad Sci USA* **81**, 2142–2146.

Baylies, M.K., Bargiello, T.A., Jackson, F.R., and Young, M.W. (1987). Changes in abundance or structure of the per gene product can alter periodicity of the *Drosophila* clock. *Nature* **326**, 390–392.

Bender, W., Akam, M., Karch, F., Beachy, P.A., Peifer, M., Spierer, P., Lewis, E.B., and Hogness, D.S. (1983a). Molecular Genetics of the Bithorax Complex in *Drosophila melanogaster*. *Science* **221**, 23–29.

Bender, W., Spierer, P., and Hogness, D.S. (1983b). Chromosomal walking and jumping to isolate DNA from the Ace and rosy loci and the bithorax complex in *Drosophila melanogaster*. *J Mol Biol* **168**, 17–33.

Borbely, A.A. (1982). A two process model of sleep regulation. *Hum Neurobiol* **1**, 195–204.

Borbely, A.A., Daan, S., Wirz-Justice, A., and Deboer, T. (2016). The two-process model of sleep regulation: a reappraisal. *J Sleep Res* **25**, 131–143.

Bunning, E. (1935). Zur Kenntniss der endergonen Tagesrhythmik bei Insekten und Pflanzen. *Ber Deut botan Ges* **53**, 594–623.

Ceriani, M.F., Darlington, T.K., Staknis, D., Mas, P., Petti, A.A., Weitz, C.J., and Kay, S.A. (1999). Light-dependent sequestration of TIMELESS by CRYPTOCHROME. *Science* **285**, 553–556.

Chiu, J.C., Ko, H.W., and Edery, I. (2011). NEMO/NLK phosphorylates PERIOD to initiate a time-delay phosphorylation circuit that sets circadian clock speed. *Cell* **145**, 357–370.

Citri, Y., Colot, H.V., Jacquier, A.C., Yu, Q., Hall, J.C., Baltimore, D., and Rosbash, M. (1987). A family of unusually spliced biologically active transcripts encoded by a *Drosophila* clock gene. *Nature* **326**, 42–47.

Colot, H.V., Hall, J.C., and Rosbash, M. (1988). Interspecific comparison of the period gene of *Drosophila* reveals large blocks of non-conserved coding DNA. *EMBO J* **7**, 3929–3937.

Crews, S.T., Thomas, J.B., and Goodman, C.S. (1988). The *Drosophila single-minded* gene encodes a nuclear protein with sequence similarity to the *per* gene product. *Cell* **52**, 143–152.

Daan, S. (2010). *A History of Chronobiological Concepts: In The Circadian Clock*, U. Albrecht, ed. (New York, NY: Springer), pp. 1–35.

Daan, S., Beersma, D.G., and Borbely, A.A. (1984). Timing of human sleep: recovery process gated by a circadian pacemaker. *Am J Physiol* **246**, R161–183.

Darlington, T.K., Wager-Smith, K., Ceriani, M.F., Staknis, D., Gekakis, N., Steeves, T.D.L., Weitz, C.J., Takahashi, J.S., and Kay, S.A. (1998). Closing the Circadian Loop: CLOCK-induced Transcription of Its Own Inhibitors *per* and *tim*. *Science* **280**, 1599–1603.

Dunlap, J.C., and Loros, J.J. (2017). Making Time: Conservation of Biological Clocks from Fungi to Animals. *Microbiol Spectr* **5**.

Emery, P., So, W.V., Kaneko, M., Hall, J.C., and Rosbash, M. (1998). CRY, a *Drosophila* clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. *Cell* **95**, 669–679.

Emery, P., Stanewsky, R., Hall, J.C., and Rosbash, M. (2000a). A unique circadian-rhythm photoreceptor. *Nature* **404**, 456–457.

Emery, P., Stanewsky, R., Helfrich-Forster, C., Emery-Le, M., Hall, J.C., and Rosbash, M. (2000b). Drosophila CRY is a deep brain circadian photoreceptor. *Neuron* **26**, 493–504.

Feldman, J.F., and Hoyle, M. (1973). Isolation of circadian clock mutants of *Neurospora crassa. Genetics* **755**, 605–613.
Feldman, J.F., and Hoyle, M.N. (1976). Complementation analysis of linked circadian clock mutants of *Neurospora crassa. Genetics* **82**, 9–17.
Flint, K.K., Rosbash, M., and Hall, J.C. (1993). Transfer of dye among salivary gland cells is not affected by genetic variations of the period clock gene in *Drosophila* melanogaster. *JMembrane Biol* **136**, 333–342.
Gardner, G.F., and Feldman, J.F. (1980). The frq locus in Neurospora crassa: A key element in circadian clock organization. *Genetics* **96**, 877–886.
Gehring, W., and Rosbash, M. (2003). The coevolution of blue-light photoreception and circadian rhythms. *J Mol Evol* **57** Suppl 1, S286–289.
Gekakis, N., Saez, L., Delahaye-Brown, A.-M., Myers, M.P., Sehgal, A., Young, M.W., and Weitz, C.J. (1995). Isolation of timeless by PER protein interactions: Defective interaction between timeless protein and long-period mutant PERL. *Science* **270**, 811–815.
Gekakis, N., Staknis, D., Nguyen, H.B., Davis, C.F., Wilsbacher, L.D., King, D.P., Takahashi, J.S., and Weitz, C.J. (1998). Role of the CLOCK protein in the mammalian circadian mechanism. *Science* **280**, 1564–1569.
Gotter, A.L. (2006). A Timeless debate: resolving TIM's noncircadian roles with possible clock function. *Neuroreport* **17**, 1229–1233.
Guo, F., Cerullo, I., Chen, X., and Rosbash, M. (2014). PDF neuron firing phase-shifts key circadian activity neurons in *Drosophila. Elife* **3**.
Guo, F., Yu, J., Jung, H.J., Abruzzi, K.C., Luo, W., Griffith, L.C., and Rosbash, M. (2016). Circadian neuron feedback controls the *Drosophila* sleep–activity profile. *Nature* **536**, 292–297.
Hardin, P.E., Hall, J.C., and Rosbash, M. (1990). Feedback of the *Drosophila* period gene product on circadian cycling of its messenger RNA levels. *Nature* **343**, 536–540.
Hardin, P.E., Hall, J.C., and Rosbash, M. (1992). Circadian oscillations in period gene mRNA levels are transcriptionally regulated. *Proc Natl Acad Sci USA* **89**, 11711–11715.
Hardin, P.E., and Panda, S. (2013). Circadian timekeeping and output mechanisms in animals. *Curr Opin Neurobiol* **23,** 724–731.
Hastings, J.W., and Sweeney, B.M. (1957). On the mechanism of temperature independence in a biological clock. *Proc Natl Acad Sci USA* **43**, 804–811.
Huang, Z.J., Edery, I., and Rosbash, M. (1993). PAS is a dimerization domain common to *Drosophila* Period and several transcription factors. *Nature* **364**, 259–262.
Hunter-Ensor, M., Ousley, A., and Sehgal, A. (1996). Regulation of the *Drosophila* protein timeless suggests a mechanism for resetting the circadian clock by light. *Cell* **84**, 677–685.
Jackson, R.F., Bargiello, T.A., Yun, S.-H., and Young, M.W. (1986). Product of the per locus of *Drosophila* shares homology with proteoglycans. *Nature* **320**, 185–188.
Jang, A.R., Moravcevic, K., Saez, L., Young, M.W., and Sehgal, A. (2015). *Drosophila* TIM binds importin alpha1, and acts as an adapter to transport PER to the nucleus. *PLoS Genet* **11**, e1004974.
Kalmus, H. (1935). Periodizität und Autochronie (= Ideochronie) als zeitregelnde Eigenschaften des Organismus. *Biologia Generalis* **11**, 93–114.
Kalmus, H. (1940a). Diurnal Rhythms in the Axolotl Larva and in *Drosophila. Nature* **145**, 72–73.

Kalmus, H. (1940b). New research in the diurnal periodicity of animals. *Acta Med Scand*, suppl **108**, 227–233.

Kidd, P.B., Young, M.W., and Siggia, E.D. (2015). Temperature compensation and temperature sensation in the circadian clock. *Proc Natl Acad Sci USA* **112**, E6284–6292.

King, D.P., Zhao, Y., Sangoram, A.M., Wilsbacher, L.D., Tanaka, M., Antoch, M.P., Steeves, T.D.L., Vitaterna, M.H., Kornhauser, J.M., Lowrey, P.L., et al. (1997). Positional Cloning of the mouse circadian clock gene. *Cell* **89**, 641–653.

Kloss, B., Price, J.L., Saez, L., Blau, J., Rothenfluh, A., Wesley, C.S., and Young, M.W. (1998). The *Drosophila* clock gene double-time encodes a protein closely related to human casein kinase Iepsilon. *Cell* **94**, 97–107.

Koh, K., Zheng, X., and Sehgal, A. (2006). JETLAG resets the *Drosophila* circadian clock by promoting light-induced degradation of TIMELESS. *Science* **312**, 1809–1812.

Kondo, T., Strayer, C.A., Kulkarni, R.D., Taylor, W., Ishiura, M., Golden, S.S., and Johnson, C.H. (1993). Circadian rhythms in prokaryotes: Luciferase as a reporter of circadian gene expression in cyanobacteria. *Proc Natl Acad Sci USA* **90,** 5672–5676.

Konopka, R.J., and Benzer, S. (1971). Clock mutants of *Drosophila melanogaster. Proc Natl Acad Sci USA* **68**, 2112–2116.

Kyriacou, C.P., Green, E.W., Piffer, A., and Dowse, H.B. (2017). Failure to reproduce period-dependent song cycles in *Drosophila* is due to poor automated pulse-detection and low-intensity courtship. *Proc Natl Acad Sci USA*.

Kyriacou, C.P., and Hall, J.C. (1980). Circadian rhythm mutations in *Drosophila melanogaster* affect short-term fluctuatons in the male's courtship song. *Proc Natl Acad Sci USA* **77**, 6729–6733.

Lee, C., Bae, K., and Edery, I. (1998). The Drosophila CLOCK protein undergoes daily rhythms in abundance, phosphorylation and interactions with the PER-TIM complex. *Neuron* **4**, 857–867.

Lee, C., Etchegaray, J.P., Cagampang, F.R., Loudon, A.S., and Reppert, S.M. (2001). Posttranslational mechanisms regulate the mammalian circadian clock. *Cell* **107**, 855–867.

Lee, K., Loros, J.J., and Dunlap, J.C. (2000). Interconnected feedback loops in the Neurospora circadian system. *Science* **289**, 107–110.

Li, Y., Guo, F., Shen, J., and Rosbash, M. (2014). PDF and cAMP enhance PER stability in *Drosophila* clock neurons. *Proc Natl Acad Sci USA* **111**, E1284–1290.

Li, Y., and Rosbash, M. (2013). Accelerated degradation of perS protein provides insight into light-mediated phase shifting. *J Biol Rhythms* **28**, 171–182.

Liu, X., Zwiebel, L.J., Hinton, D., Benzer, S., Hall, J.C., and Rosbash, M. (1992). The period gene encodes a predominantly nuclear protein in adult *Drosophila. J Neurosci* **12**, 2735–2744.

Lorenz, L.J., Hall, J.C., and Rosbash, M. (1989). Expression of a *Drosophila* mRNA is under circadian clock control during pupation. *Development* **107**, 869–880.

Loros, J.J., and Feldman, J.F. (1986). Loss of temperature compensation of circadian period length in the frq-9 mutant of Neurospora crassa. *J Biol Rhythms* **1**, 18767–18198.

Loros, J.J., Richman, A., and Feldman, J.F. (1986). A recessive circadian clock mutant at the frq locus in Neurospora crassa. *Genetics* **114**, 1095–1110.

Lowrey, P.L., Shimomura, K., Antoch, M.P., Yamazaki, S., Zemenides, P.D., Ralph, M.R., Menaker, M., and Takahashi, J.S. (2000). Positional syntenic cloning and functional characterization of the mammalian circadian mutation tau. *Science* **288**, 483–492.

Lucas, R.J., Freedman, M.S., Munoz, M., Garcia-Fernandez, J.M., and Foster, R.G. (1999). Regulation of the mammalian pineal by non-rod, non-cone, ocular photoreceptors. *Science* **284**, 505–507.

Mehra, A., Shi, M., Baker, C.L., Colot, H.V., Loros, J.J., and Dunlap, J.C. (2009). A role for casein kinase 2 in the mechanism underlying circadian temperature compensation. *Cell* **137**, 749–760.

Melkani, G.C., and Panda, S. (2017). Time-restricted feeding for prevention and treatment of cardiometabolic disorders. *J Physiol* **595**, 3691–3700.

Menet, J.S., Abruzzi, K.C., Desrochers, J., Rodriguez, J., and Rosbash, M. (2010). Dynamic PER repression mechanisms in the Drosophila circadian clock: from on-DNA to off-DNA. *Genes Dev* **24**, 358–367.

Meyer, P., Saez, L., and Young, M.W. (2006). PER-TIM interactions in living *Drosophila* cells: an interval timer for the circadian clock. *Science* **311**, 226–229.

Myers, M.P., Wager-Smith, K., Rothenfluh-Hilfiker, A., and Young, M.W. (1996). Light-induced degradation of TIMELESS and entrainment of the *Drosophila* circadian clock. *Science* **271**, 1736–1740.

Myers, M.P., Wager-Smith, K., Wesley, C.S., Young, M.W., and Sehgal, A. (1995). Positional cloning and sequence analysis of the *Drosophila* clock gene timeless. *Science* **270**, 805–808.

Pittendrigh, C.S. (1954). On temperature independence in the clock-system controlling emergence time in *Drosophila. Proc Natl Acad Sci USA* **40**, 1018–1029.

Pittendrigh, C.S. (1993). Temporal organization: Reflections of a Darwinian clock-watcher. *Annu Rev Physiol* **55**, 17–54.

Price, J.L., Blau, J., Rothenfluh, A., Abodeely, M., Kloss, B., and Young, M.W. (1998). double-time is a novel *Drosophila* clock gene that regulates PERIOD protein accumulation. *Cell* **94**, 83–95.

Price, J.L., Dembinska, M.E., Young, M.W., and Rosbash, M. (1995). Suppression of PERIOD protein abundance and circadian cycling by the *Drosophila* clock mutation timeless. *EMBO J* **14**, 4044–4049.

Provencio, I., Jiang, G., De Grip, W.J., Hayes, W.P., and Rollag, M.D. (1998). Melanopsin: An opsin in melanophores, brain, and eye. *Proc Natl Acad Sci USA* **95**, 340–345.

Reddy, P., Jacquier, A.C., Abovich, N., Petersen, G., and Rosbash, M. (1986). The period clock locus of *D. melanogaster* codes for a proteoglycan. *Cell* **46**, 53–61.

Reddy, P., Zehring, W.A., Wheeler, D.A., Pirrotta, V., Hadfield, C., Hall, J.C., and Rosbash, M. (1984). Molecular analysis of the period locus in *Drosophila melanogaster* and identification of a transcript involved in biological rhythms. Cell **38**, 701–710.

Reppert, S.M., and Weaver, D.R. (2002). Coordination of circadian timing in mammals. *Nature* **418**, 935–941.

Rosbash, M. (2009). The implications of multiple circadian clock origins. *PLoS Biol* **7**, e62.

Rosbash, M. (2015). Ronald J. Konopka (1947–2015). *Cell* **161**, 187–188.

Rubin, G.M., and Spradling, A.C. (1982). Genetic transformation of *Drosophila* with transposable element vectors. *Science* **218**, 348–353.

Rutila, J.E., Edery, I., Hall, J.C., and Rosbash, M. (1992). The analysis of new short-period circadian rhythm mutants suggests features of D. *melanogaster* period gene function. *J Neurogenet* **8**, 101–113.

Rutila, J.E., Suri, V., Le, M., So, W.V., Rosbash, M., and Hall, J.C. (1998). CYCLE Is a Second bHLH-PAS Clock Protein Essential for Circadian Rhythmicity and Transcription of *Drosophila* period and timeless. *Cell* 93, 805–814.

Rutila, J.E., Zeng, H., Le, M., Curtin, K.D., Hall, J.C., and Rosbash, M. (1996). The timSL mutant of the *Drosophila* rhythm gene timeless manifests allele-specific interactions with period gene mutants. *Neuron* **17**, 921–929.

Saez, L., Young, M.W., Baylies, M.K., Gasic, G., Bargiello, T.A., and Spray, D.C. (1992). Per – no link to gap junctions. *Nature* **360**, 542–542.

Saper, C.B., and Fuller, P.M. (2017). Wake-sleep circuitry: An overview. *Curr Opin Neurobiol* **44**, 186–192.

Sehgal, A., Price, J., Man, B., and Young, M. (1994). Loss of circadian behavioral rhythms and per RNA oscillations in the *Drosophila* mutant timeless. *Science* **263**, 1603–1606.

Sehgal, A., Rothenfluh-Hilfiker, A., Hunter-Ensor, M., Chen, Y., Myers, M., and Young, M.W. (1995). Rhythmic expression of timeless: A basis for promoting circadian cycles in period gene autoregulation. *Science* **270**, 808–810.

Shafer, O.T., Rosbash, M., and Truman, J.W. (2002). Sequential Nuclear Accumulation of the Clock Proteins Period and Timeless in the Pacemaker Neurons of *Drosophila melanogaster. J Neurosci* 22, 5946–5954.

Shearman, L.P., Zylka, M.J., Weaver, D.R., Kolakowski, L.F.J., and Reppert, S.M. (1997). Two period homologs: circadian expression and photic regulation in the suprachiasmatic nuclei. *Neuron* **19**, 1261–1269.

Shigeyoshi, Y., Taguchi, K., Yamamoto, S., Takekida, S., Yan, L., Tei, H., Moriya, T., Shibata, S., Loros, J.J., Dunlap, J.C., et al. (1997). Light-induced resetting of a mammalian circadian clock is associated with rapid induction of the mPer1 transcript. *Cell* **91**, 1043–1053.

Siwicki, K.K., Flint, K.F., Hall, J.C., Rosbash, M., and Spray, D.C. (1992). The *Drosophila* period gene and dye coupling in larval salivary glands: a re-evaluation. *Biol Bull* **183**, 340–341.

Spierer, P., Spierer, A., Bender, W., and Hogness, D.S. (1983). Molecular mapping of genetic and chromomeric units in *Drosophila melanogaster. J Mol Biol* **168**, 35–50.

Spradling, A.C., and Rubin, G.M. (1982). Transposition of cloned P elements into *Drosophila* germ line chromosomes. *Science* **218**, 341–347.

Stanewsky, R., Kaneko, M., Emery, P., Beretta, M., Wager-Smith, K., Kay, S.A., Rosbash, M., and Hall, J.C. (1998). The cryb mutation identifies cryptochrome as a circadian photoreceptor in *Drosophila. Cell* **95**, 681–692.

Stern, D.L. (2014). Reported *Drosophila* courtship song rhythms are artifacts of data analysis. *BMC Biol* **12**, 38.

Stern, D.L., Clemens, J., Coen, P., Calhoun, A.J., Hogenesch, J.B., Arthur, B.J., and Murthy, M. (2017). Experimental and statistical reevaluation provides no evidence for *Drosophila* courtship song rhythms. *Proc Natl Acad Sci USA* **114**, 9978–9983.

Sun, Z.S., Albrecht, U., Zhuchenko, O., Bailey, J., Eichele, G., and Lee, C.C. (1997). RIGUI, a putative mammalian ortholog of the *Drosophila* period gene. *Cell* **90**, 1003–1011.

Suri, V., Hall, J.C., and Rosbash, M. (2000). Two novel doubletime mutants alter circadian properties and eliminate the delay between RNA and protein in *Drosophila. J Neurosci* **20**, 7547–7555.

Suri, V., Qian, Z., Hall, J.C., and Rosbash, M. (1998). Evidence that the TIM light response is relevant to light-induced phase shifts in *Drosophila melanogaster. Neuron* **21**, 225–234.

Tei, H., Okamura, H., Shigeyoshi, Y., Fukuhara, C., Ozawa, R., Hirose, M., and Sakaki, Y. (1997). Circadian oscillation of a mammalian homologue of the *Drosophila* period gene. *Nature* **389**, 512–516.

Tu, B.P., and McKnight, S.L. (2006). Metabolic cycles as an underlying basis of biological oscillations. *Nat Rev Mol Cell Biol* **7**, 696–701.

Vitaterna, M.H., King, D.P., Chang, A.-M., Kornhauser, J.M., Lowrey, P.L., McDonald, J.D., Dove, W.F., Pinto, L.H., Turek, F.W., and Takahashi, J.S. (1994). Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior. *Science* **264**, 719–725.

Vitaterna, M.H., Selby, C.P., Todo, T., Niwa, H., Thompson, C., Fruechte, E.M., Hitomi, K., Thresher, R.J., Ishikawa, T., Myazaki, J., et al. (1999). Differential regulation of mammalian period genes and circadian rhythmicity by cryptochromes 1 and 2. *Proc Natl Acad Sci USA* **96**, 12114–12119.

Vosshall, L.B., Price, J.L., Sehgal, A., Saez, L., and Young, M.W. (1994). Block in nuclear localization of period protein by a second clock mutation, timeless. *Science* **263**, 1606–1609.

Weiner, J. (1999). *Time, love, memory: A great biologist and his quest for the origins of behavior,* 1st ed. (New York: New York: Knopf).

Yang, Z., and Sehgal, A. (2001). Role of molecular oscillations in generating behavioral rhythms in *Drosophila*. *Neuron* **29**, 453–467.

Young, M.W., and Judd, B.H. (1978). Nonessential Sequences, Genes, and the Polytene Chromosome Bands of DROSOPHILA MELANOGASTER. *Genetics* **88**, 723–742.

Yu, Q., Colot, H.V., Kyriacou, C.P., Hall, J.C., and Rosbash, M. (1987a). Behaviour modification by *in vitro* mutagenesis of a variable region within the period gene of *Drosophila*. *Nature* **326**, 765–769.

Yu, Q., Jacquier, A.C., Citri, Y., Hamblen, M., Hall, J.C., and Rosbash, M. (1987b). Molecular mapping of point mutations in the period gene that stop or speed up biological clocks in *Drosophila melanogaster*. *Proc Natl Acad Sci USA* **84**, 784–788.

Yuan, Q., Metterville, D., Briscoe, A.D., and Reppert, S.M. (2007). Insect cryptochromes: Gene duplication and loss define diverse ways to construct insect circadian clocks. *Mol Biol Evol* **24**, 948–955.

Zehring, W.A., Wheeler, D.A., Reddy, P., Konopka, R.J., Kyriacou, C.P., Rosbash, M., and Hall, J.C. (1984). P-element transformation with period locus DNA restores rhythmicity to mutant, arrhythmic *Drosophila melanogaster*. *Cell* **39**, 369–376.

Zeng, H., Hardin, P.E., and Rosbash, M. (1994). Constitutive overexpression of the *Drosophila* period protein inhibits period mRNA cycling. *EMBO J* **13**, 3590–3598.

Zeng, H., Qian, Z., Myers, M.P., and Rosbash, M. (1996). A light-entrainment mechanism for the *Drosophila* circadian clock. *Nature* **380**, 129–135.

Zhang, R., Lahens, N.F., Ballance, H.I., Hughes, M.E., and Hogenesch, J.B. (2014). A circadian gene expression atlas in mammals: Implications for biology and medicine. *Proc Natl Acad Sci USA* **111**, 16219–16224.

Zhu, H., Yuan, Q., Briscoe, A.D., Froy, O., Casselman, A., and Reppert, S.M. (2005). The two CRYs of the butterfly. *Curr Biol* **15**, R953–954.