Generalization and Discovery by Assuming Conserved Mechanisms: Cross Species Research on Circadian Oscillators

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Abstract

In many domains of biology, explanation takes the form of characterizing the mechanism responsible for a particular phenomenon. Mechanisms are often discovered in specific biological systems, and this raises the question of how explanations generalize. One way scientists achieve generalization is to recognize conservation of mechanisms through evolutionary descent. In the case discussed in this paper, the central mechanism for circadian rhythms in animals was first identified in *Drosophila* and then extended to mammals. Scientists' working assumption that the clock mechanisms would be conserved indeed yielded important generalizations. Equally important, though, this assumption also served as a heuristic for discovery: the insect clock provided a provisional account of the mammalian clock, enabling researchers to focus on identifying important variations. This made research more efficient, and yielded an elegant account in which conserved aspects of the mechanism provided context for understanding variations.

1. Introduction

How do scientific explanations generalize? When explanation is viewed as the application of scientific laws to specific cases, generalization is relatively straightforward: even if discovered in a particular instance, a regularity is rendered into a law by quantifying it universally. It then applies to any condition in which the antecedent is satisfied. But biologists, especially in domains such as cell and molecular biology, seldom invoke laws in their explanations. Instead, they seek to explain a phenomenon by uncovering and describing the biological mechanism that appears to be responsible for it. Several philosophical accounts of mechanistic explanation in biology have been advanced recently. Although the terminology varies somewhat across authors, the key elements of a mechanistic explanation are the identification of the relevant parts of the mechanism, the determination of the operations they perform, and an account of how the parts and operations are organized such that, under specific contextual conditions, the mechanism realizes the phenomenon of interest (Bechtel & Richardson, 1993; Bechtel & Abrahamsen, 2005; Bechtel, 2006; Craver, 2007; Darden, 2006; Machamer, Darden, & Craver, 2000). The challenge for understanding generalization is that a mechanistic account is highly particularized: researchers develop it for a model organism, study mechanisms in model organisms and it is anticipated that there will be important differences—involving parts, operations, and organization—between already-studied organisms and those to which a scientist wishes to generalize a mechanistic explanation.

A first part of the answer to the question of how biologists generalize mechanistic explanations generalize is that, as a result from descent from a common ancestor, biologists expect similarities among the mechanisms responsible for the same or similar phenomena in related organisms. Thus, biologists provisionally assume that the parts, operations, and organization of a mechanism in an ancestral species are largely conserved in descendant species. However, evolutionary processes also introduce variation; hence researchers must probe for differences in parts, operations, and organization. This appeal to conservation is particularly productive when the mechanism is found to be largely conserved, as posited, but exhibits interesting variations. An

altered operation may have resulted in other changes, for example, and the original account can guide researchers towards the most likely loci of such changes.

In this paper I will illustrate how the assumption of conservation served both generalization and discovery in a particular case: research on circadian oscillators within animals. Circadian rhythms, the approximately 24 hour rhythms that are endogenously maintained, are widespread in living organisms and affect such aspects as body temperature, metabolism, endocrine function, locomotion, sexual activity, and mental function. Since circadian rhythms are found not only in organisms with a central nervous system but also in single-cell organisms, researchers generally assumed that the internal clock (oscillator) employed intracellular processes even in higher organisms. In *Drosophila*, researchers focused on lateral neurons whereas in mammals they concentrated on a structure in the hypothalamus above the optic chiasm known as the *suprachiasmatic nucleus* (SCN). The challenge was to explain how concentrations of certain proteins in these structures could oscillate with a period of approximately 24 hours. In the next sections I consider how researchers first addressed this challenge using *Drosophila* as the model organism and how the assumption that the mechanism was conserved then led to a productive interaction with researchers studying mammals.

2. The *Drosophila* Model System for Studying Circadian Oscillators

Before the quest to identify the responsible mechanism, *Drosophila* had provided a model for studying circadian rhythms at the behavioral level due to the fact that fruit flies emerge from the pupa (eclose) only around dawn. Whatever time of day a fruit fly completes its development, it delays eclosing until the subsequent dawn. Even if the pupa are kept in total darkness, they eclode at what would have been dawn (Pittendrigh, 1954). Fruit flies had also served as a model species for genetic research, so it is not surprising that Konopka and Benzer (1971) employed them to begin identifying genes affecting circadian behavior. Applying mutagens targeted to the X-chromosome of *Drosophila*, they generated mutant flies that exhibited shortened, lengthened, and null oscillations in timing of eclosion. They traced all three mutations to a common locus and named the affected gene *period* (*per*).

The cloning of *per* in the mid-1980s by Michael Rosbash and his colleagues made it possible to fill in additional components of the molecular mechanism responsible for circadian rhythms. The expression of *per* results in increased concentrations of *per* mRNA and, in turn, of the protein PERIOD (PER) that is synthesized from *per* mRNA in the cytoplasm. (Protein names are standardly capitalized while the names of genes are written in italics. In *Drosophila* the names of genes are entirely in lowercase, whereas in mammals the first letter is capitalized.) Hardin, Hall, and Rosbash (1990) determined that the concentrations of both *per* mRNA and PER exhibited circadian oscillations, with the peaks and valleys in PER concentrations following those of *per* mRNA by approximately 8 hours. Moreover, these cycles were shortened, lengthened, or absent in the previously discovered *per* mutants. Since PER is found not only in the cytoplasm, but also in the nucleus, Hardin et al. proposed a feedback mechanism in which PER could inhibit the transcription of its own gene, *per*, as illustrated in Figure 1. When there is a low concentration of PER in the nucleus, the synthesis of additional molecules of PER proceeds normally in the cytoplasm. As they accumulate they get transported back into the nucleus, where they inhibit further synthesis. But they break down over time and, failing to be replenished, no longer inhibit

synthesis. The rate of synthesis returns to normal, beginning a new cycle. Appropriate timing of these operations results in a regular oscillation in PER concentrations, with the rise and fall (one period) occupying about 24 hours.
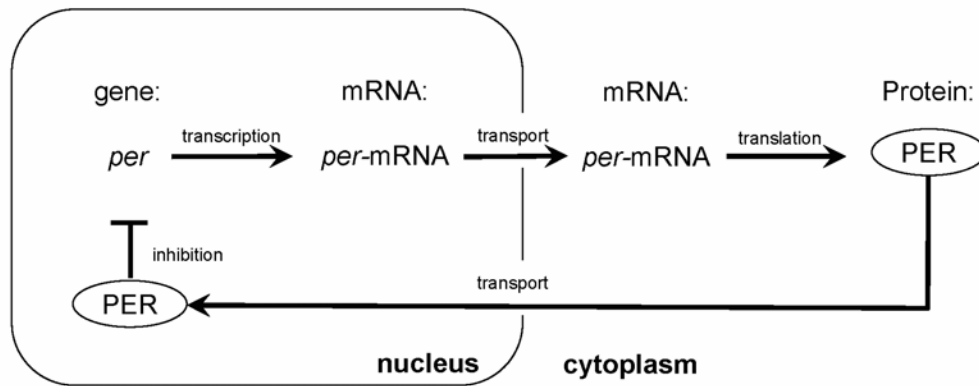

Figure 1. Proposed feedback mechanism for generating circadian oscillations.

This proposal, though, raised a number of questions about exactly how the feedback loop might operate. Particularly important were the questions of what delayed PER's transport back into the nucleus and how PER could inhibit *per* transcription. Researchers realized PER could not do this directly as it lacked a site where it could bind to DNA. This led researchers to search for other component parts in the clock mechanism.

A second component part the mechanism was discovered by Seghal, Price, Man, and Young (1994). They followed essentially the same approach as Konopka, generating a large number of mutations involving the second and third chromosomes in *Drosophila*. One such mutation eliminated rhythmic eclosion or locomotion. In flies with this mutation, moreover, *per* mRNA concentrations ceased to oscillate. Seghal et al. took this to indicate an interaction between *per* and the new gene, which they named *timeless* (*tim*). A second study (Vosshall, Price, Sehgal, Saez, & Young, 1994) indicated that *tim* is required for PER to be transported from the cytoplasm, where it is synthesized, back into the nucleus, where it can inhibit transcription of its own gene and proposed that "PER contains sequences that somehow inhibit PER nuclear localization in the absence of *tim*" (p. 1607). These researchers soon determined that PER and TIM form a dimer (a compound of two similar units) before both are transported back into the nucleus and that a region found on both PER and TIM, which they named the *cytoplasmic localization domain* (CLD), was responsible for preventing either alone from migrating into the nucleus (Gekakis et al., 1995). This region was presumably masked in the dimer, allowing the dimer to be transported into the nucleus (Saez & Young, 1996).

The discovery of *tim* helped explain the translocation of PER into the nucleus, but it did not explain how PER (or TIM) inhibited its own transcription since TIM, like PER, lacked a DNA binding site. Investigators next explored the possibility that PER bound with a *per* activating factor, and when it did so, blocked the activator from activating *per* transcription. Support for the existence of such a factor was provided by the identification of an E-box (CACGTG) promoter upstream of *per* that is a target for a basic-helix-loop-helix (bHLH) DNA binding site on an

activator protein, and by determination that this E-box was required for robust cycling (Hao, Allen, & Hardin, 1997). The activating protein, however, was first discovered not in *Drosophila* but in mammals.

3. Linking the clocks in *Drosophila* and mice

In the early 1990s the search for circadian mutants was also proceeding for mammals, especially mice. Vitaterna et al. (1994) identified a mutation that yielded increased period length and, in a homozygotic form, loss of rhythms after two weeks. They labeled the mutant gene *Clock* (for "circadian locomotor output cycles kaput"), localized it to chromosome 5, and determined that its protein oscillated in a circadian fashion. Three years later the same laboratory (King et al., 1997) cloned *Clock* and predicted "that this candidate gene encodes a novel member of the bHLH–PAS domain family of transcription factors" (p. 645). Noting the assumption of *Drosophila* researchers that unknown transcription factors interact with PER, King et al. proposed "The mouse *Clock* gene could be the mammalian ortholog of such a gene" (p. 649). In addition, Gekakis et al. (1998) predicted that CLOCK must dimerize with a partner, and identified BMAL1, a protein whose function was then unknown, as a protein whose concentrations oscillated in a similar as CLOCK. They also demonstrated that the mammalian CLOCK-BMAL1 dimer would bind with *per*'s E-box in *Drosophila* and proposed processes by which PER might interfere with the action of CLOCK-BMAL1 to inhibit *per* expression.

The discovery of *Clock* in mice led *Drosophila* researchers to seek a *Drosophila* homolog, and they soon found a gene that possessed very similar bHLH and PAS domains (Darlington et al., 1998). Moreover, they determined that its protein, dCLOCK, was a specific activator of the *per* and *tim* promoters. They also found evidence for a *Drosophila* homolog of *Bmal1* and concluded "It is tempting to speculate that the *Drosophila* four-component transcriptional feedback loop described here is sufficient to generate a rudimentary circadian rhythm" (p. 1602).

As this work was proceeding, other researchers followed up on an indication of a mammalian homolog to *per* and in 1997 identified such a homolog in mice and in humans. Examining the proteins, researches found the mouse protein to be approximately 44% identical to the fly protein, with many of the differences involving neutral amino acid substitutions (Sun et al., 1997; Tei et al., 1997). Soon after it was recognized that in fact there are multiple mammalian homologues of *per*, designated *mPer1*, *mPer2* (Albrecht, Sun, Eichele, & Lee, 1997), and *mPer3* (Zylka, Shearman, Weaver, & Reppert, 1998). They reside on different chromosomes and differ in such respects as their responses to light pulses. One of the first noted consequences of the differentiation of three *Per* genes in mammals is increased robustness: deletion of just one *Per* gene does not eliminate circadian cycling, whereas deletion of both *Per1* and *Per2* does.

4. Just How Conserved is the Mammalian Clock?

At this point there seemed to be a high degree of conservation between the *Drosophila* and mammalian clock mechanism. Mammalian homologues had been found for *per*, *clock*, and *bmal1*; the only major change was that *per* had differentiated into three mammalian genes. This supported a straightforward generalization of the mechanistic account achieved for *Drosophila* to mammals. But the story soon became more complex as researchers sought a mammalian

homolog for the fourth component of the *Drosophila* clock, *tim*. They found such a gene: its protein occurs in the SCN and can dimerize with mPER1 or mPER2 as well as with dPER. In *Drosophila* cells the dimers are transported back into the nucleus and inhibit *per* transcription; in mouse cells the TIM:mPER1 inhibited activation of the *mPer1* promoter (Sangoram et al., 1998). So far the parallels held up. But there were significant differences: neither the concentrations of *mTim* mRNA nor its protein, mTIM, oscillate under constant light or darkness, and in response light mTIM levels increase whereas dTIM levels decrease.

These seemingly minor differences took on greater significance in the context of a major feature of circadian rhythms, their entrainment (resetting) by exposure to light, especially around subjective dawn or subjective dusk. This process is essential for organisms to adjust to seasonal differences or for us to adjust after travel to different time zones. The discovery of photoreceptors in *Drosophila* that are conserved from blue-light photoreceptors (known as *cryptochromes*) in plants provided a critical clue to the entrainment mechanism. Searching for mammalian homologues to *cry*, Todo et al. (1996) found two each in mice and humans. Unlike in plants, concentrations of cryptochromes in *Drosophila* and in mammals were found to undergo circadian oscillations, indicating they might have significant clock functions. In *Drosophila*, Emery et al. (1998) determined that this oscillation was due to light exposure by showing that when flies were maintained in continuous darkness, CRY levels did not oscillate but rather continued to increase. They inferred "CRY is a major photoreceptor for *Drosophila* locomotor activity rhythms" (p. 674). Since TIM concentrations are responsive to CRY levels in wildtype flies, but not in *cry^b* mutants (Stanewsky et al., 1998), and CRY light regulation is not affected by TIM or other clock molecules, investigators concluded that CRY served an entrainment function by affecting concentrations of TIM.

Assuming conservation, researchers at first thought that CRY figured in the input pathway in mammals as well, and the initial evidence lent support. Miyamato and Sancar (1998) found that CRY1 and CRY2 occur in retinal ganglion cells as well as the inner nuclear layer of the mouse retina and that CRY1 concentrations oscillate in a circadian manner in the SCN itself. They interpreted this evidence as indicating a photoreceptor role for both CRY proteins. Thresher et al. (1998) offered further support for this view by showing that CRY2 deficient mutants failed to entrain to light. Thresher et al. also observed, however, that CRY2 deficient mutants showed elongated periods in total darkness, a finding that would not be expected if CRY2 figured only in entrainment. Whereas Thresher et al. attempted to explain away this finding, other evidence soon pointed to a different conclusion—that mammalian CRY homologues were not serving the entrainment function and were actually part of the central clock mechanism. One piece of evidence was the finding that loss of both CRY1 and CRY2 eliminated circadian rhythms in constant darkness and loss of just one resulted in shortened (CRY1) or lengthened (CRY2) periods of oscillation (van der Horst et al., 1999). Using an assay in cultured cells in which CLOCK-BMAL1 drives a luciferase reporter gene from the *mPer1* gene E-box, Griffin, Staknis, and Weitz (1999) determined that in humans both CRY1 and CRY2 produced a specific inhibition of CLOCK-BMAL1 activity. This indicated that the "role for CRYs in the mammalian circadian clock is to inhibit *Per1* gene expression" (p. 769). Citing as well evidence that CRY no longer performed an entrainment function, these researchers concluded that CRY1 and CRY2 had supplanted TIM in the mammalian clock. (Figure 2 shows this change as well as the role of each as a dimerization partner with PER.)

Subjective Nighttime

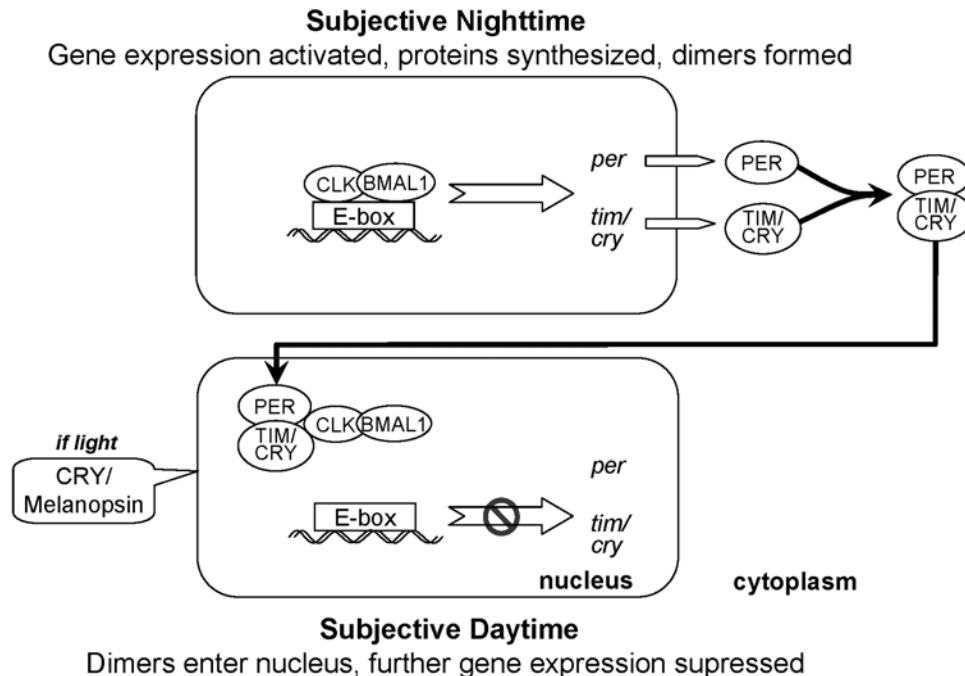Gene expression activated, proteins synthesized, dimers formed

Figure 2. Comparison of *Drosophila* and mammalian clocks. CRY replaces TIM as the dimerization partner of PER. PER/TIM and PER/CRY dimers interact with the BMAL1/CLOCK dimer, removing them as activators of *per/Per* and *tim/Cry* genes. Melanopsin replaces CRY in entraining the clock.

The discovery that, although conserved, mCRYs were no longer operative in entrainment in mammals generated a new research question: What performs the entrainment operation in mammals? The clue was again provided by conservation. Melanopsin, a member of the opsin family of photopigments, was discovered in melanophores (melanin pigment containing cells) of the frog *Xenopus laevis* (Provencio, Jiang, De Grip, Hayes, & Rollag, 1998). Subsequently, Provencio et al. (2000) reported finding melanopsin in the mammalian inner retina. Linking melanopsin to circadian entrainment required demonstrating its presence in the retinohypothalamic tract that had long been known to provide the input to the SCN. Hannibal had previously identified pituitary adenylate cyclase activating peptide (PACAP) as the neurotransmitter active in the retinohypothalamic tract during subjective day, and he now showed that both melanopsin mRNA and protein are found in the same cells as PACAP (Hannibal, 2002). Although knockout of melanopsin alone does not eliminate entrainment, it does reduce it (Ruby et al., 2002), and when the knockout is combined with loss of the rods and cones, entrainment is eliminated (Hattar et al., 2003). Together this provided strong evidence that melanopsin had assumed the entrainment function of CRY in mammals.

With melanopsin replacing CRY in entrainment, and CRY replacing TIM in the central oscillator, a natural question is: what happened to TIM? It is still expressed in mammals, but many researchers initially concluded it ceased to perform any clock function (Albrecht, 2002). Griffin et al., in the study that suggested CRY had supplanted TIM, had described an antagonistic interaction between either CRY1 or CRY2 and TIM which they took to "suggest cross-regulation among the proteins inhibiting CLOCK-BMAL1 activity within the circadian

clock feedback loop" (p. 470). Since deletion of *mTim* is lethal in embryonic development, the role of TIM could not be settled through a knock-out experiment. The development of the gene knockdown technique in which a reagent complementary to an active gene (antisense oligodeoxynucleotides, αODN) binds to the site and suppresses its expression, allowed for a more specific examination of the role of *mTim* in slice preparations. When a αODN targeted to the sequence surrounding the start codon of *mTim* was applied for prolonged periods, full-length mTIM (mTIM-fl) expression was suppressed. Importantly, so were circadian rhythms (Barnes et al., 2003). When the αODN was only administered for two hours, it resulted in a phase shift if applied during subjective day (advances when applied early, delays when applied late), but not if administered during subjective night. It was also demonstrated that mTIM-fl interacts with the three mPERs as well as both mCRYs. In the knockdown preparation, levels of the mPERs decreased, a finding that corresponds well with the low levels of dPER in *dtim* mutatants and, according to Barnes et al., shows that "mTIM-fl is a functional homolog of dTIM". Relying on additional evidence, Barnes et al. propose locating "*mTim* on the negative arm of the molecular feedback loop in the SCN, as it is in the *Drosophila* clock" (p. 441). In particular, they hypothesized "that mPER2 may be the physiologically preferred partner of mTIM-fl and that this heterodimer could be the functional mammalian counterpart of dTIM:dPER. Addition of *mTim* to the mammalian clockwork completes a core having each functional homolog of the *Drosophila* clockwork and emphasizes the highly conserved nature of the biological timekeeping mechanism" (p. 441). At present the question of the role of *Tim* remains undecided.

5. Conservation as a Strategy for Both Generalization and Discovery

The initial success in developing a core mechanism for circadian oscillations in *Drosophila* invited the heuristic of seeking mammalian homologues for the *Drosophila* genes so as to generalize the explanation to mammals. This proved successful in the case of *per* where three mammalian homologues were identified, at least two of which play comparable roles in the mammalian clock. Independent work on the mammalian clock resulted in the identification of two additional components of the clock mechanism, *Clock* and *Bmal1*, and this guided the search for homologues in *Drosophila*. The assumption of conservation and the search for homologues thus serves as a heuristic for generalizing accounts of mechanisms across species.

Conservation provides the basis for an even more powerful discovery heuristic when the mechanism in the model organism does not align perfectly with the one in the species of interest. The quest for the mammalian homolog of *cry*, which figures centrally in the entrainment mechanism in *Drosophila*, led to the discovery that in mammals *Cry* appears not to figure in entrainment but rather to have usurped the role of *tim*. Initially this discovery was viewed as showing that *Tim* had no clock function in mammals, but the fact that *Tim* continues to be expressed motivated more detailed studies which have suggested that it might retain a clock function by serving as the preferred dimerization partner of one of the mammalian PER homologues. Discovering a change in the operation performed by CRY, however, also prompted a search for what filled the role that CRY played in *Drosophila*. This yielded the discovery of a different photopigment, melanopsin, and an investigation into whether it might figure in entrainment in mammals.

Scientific discovery was once viewed as an intractable problem for philosophers. Several philosophers who have addressed mechanistic explanation in biology have noted that the search for mechanisms is often guided by discovery heuristics (Bechtel & Richardson, 1993; Darden & Craver, 2002). The conservation of mechanisms further advances this project. Mechanisms are typically investigated in specific model organisms chosen because of their accessibility for study. These model organisms are often not the primary systems of interest. The assumption of conservation of component parts, operations, and organization undergirds a heuristic of seeking conserved components and generalizing from studied mechanisms to those of principal interest. Moreover, when conservation turns out not to be perfect, it gives rise to other discovery heuristics. When a component is preserved but appears to be performing a different operation, researchers are prompted to investigate what performs its old operation and what happens to the component that had performed the new operation.

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