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A Colorful Model of the Circadian Clock

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The migration of the colorful monarch butterfly provides biologists with a unique model system with which to study the cellular and molecular mechanisms underlying a sophisticated circadian clock. The monarch circadian clock is involved in the induction of the migratory state and navigation over long distances, using the sun as a compass.

The yearly migration of eastern North American monarch butterflies (*Danaus plexippus*) is a visually and biologically breathtaking event (Urquhart, 1960). During the fall migration, it is not uncommon to witness a landscape filled with hundreds of colorful butterflies flying in a southerly direction. Indeed, millions of butterflies from the US and southern Canada make the annual journey to winter in warmer climes in just a few restricted areas of central Mexico, some having traveled distances approaching 4000 km.

The migratory state is characterized by reproductive diapause, a condition in which the butterflies suspend mating behavior and exhibit arrested reproductive development, as migrants need to conserve energy for the long journey (Brower, 1996). Migrants also have increased cold tolerance and abdominal fat stores, a marked increase in longevity, and an overwhelming urge to fly south. Diapause persists at the wintering sites until the early spring, when the butterflies reproduce and take wing, flying northward to lay fertilized eggs on newly emerged milkweed plants (genus *Asclepias*) in the southern United States. Another two to three generations of reproductively competent, short-lived "summer" butterflies follow the progressive, northward emergence of milkweed to reestablish, by late summer, the most northerly reaches of the eastern population of monarch butterflies. In the fall, decreasing daylength triggers the migratory generation and, once again, the long journey south begins.

The seemingly mystical qualities of monarch butterfly migration and the precarious state of the wintering grounds have stymied rigorous research into tractable aspects of the migratory process. I propose that monarch butterfly migration provides biologists with a unique system in which to study the cellular and molecular mechanisms underlying a circadian clock and its involvement in two unconventional outputs—the induction of the migratory state and navigation over long distances using the sun as a compass.

A Cellular Clock's Location in the Brain

To fully understand how a circadian clock controls vital migratory activities in the butterfly, it is essential to understand the molecular machinery that runs the circadian clock itself and to determine where the cellular clock actually resides in the brain. In *Drosophila* and mammals, the intracellular clock mechanism involves transcriptional feedback loops that drive self-sustaining rhythms in mRNA and protein levels of key clock components (Stanewsky, 2003). The negative transcriptional feedback loop is essential for clockwork function and in *Drosophila* involves the transcription factors CLOCK (CLK) and CYCLE (CYC), which drive the expression of the *period* (*per*) and *timeless* (*tim*) genes. The resultant PER and TIM proteins form heterodimers and move to the nucleus where PER inhibits transcription mediated by CLK:CYC heterodimers. TIM appears to regulate stability and nuclear transport of the PER protein and is also necessary for photic responses that reset (entrain) the circadian clock. The *Drosophila* cryptochrome (CRY), a flavoprotein, colocalizes with PER and TIM in clock cells and

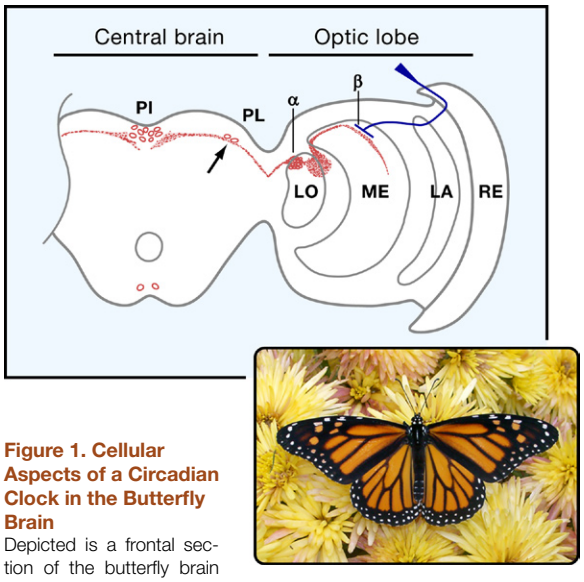

Figure 1. Cellular Aspects of a Circadian Clock in the Butterfly Brain
Depicted is a frontal section of the butterfly brain (central brain and one of the two optic lobes) illustrating the location of a putative cellular clock (arrow). CRY1-positive cells and fibers are highlighted in red. Also shown is the location of CRY1-positive cells in the optic lobe (α), CRY1-positive fibers in the dorsolateral medulla (β), and a dye-labeled projection (blue) from injected photoreceptor cells in the dorsal rim. LA, lamina; ME, medulla; LO, lobula; PL, pars lateralis; PI, pars intercerebralis; RE, retina. (Modified from Sauman et al., 2005.) (Inset) A monarch butterfly.

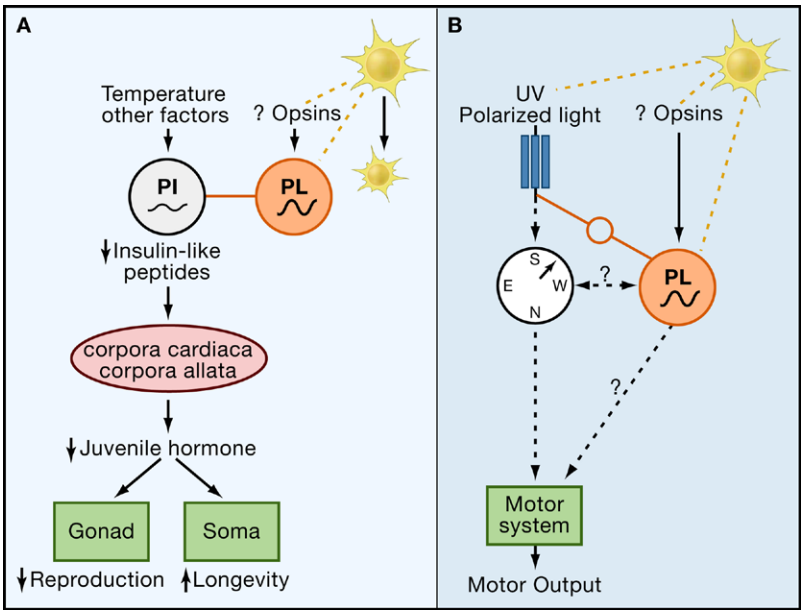

Figure 2. Circadian Clock Involvement in Butterfly Migration

(A) Model of a circadian clock pathway for the induction of migration. Changing daylength (indicated by decreasing sun size) impinges on the circadian clock in the pars lateralis (PL) of the butterfly brain. A CRY1-positive pathway (orange) communicates relevant daylength information to neurosecretory cells in the pars intercerebralis (PI) where insulin-like peptides decrease, reducing synthesis of juvenile hormone in the corpora cardiaca-corpora allata complex. Juvenile hormone deficiency, in turn, leads to reproductive quiescence and an increase in life span.

(B) Model of clock-compass interactions in the monarch butterfly brain. A circadian clock in the pars lateralis (PL) is entrained by light acting through CRY1 expressed in clock cells and perhaps through opsins in the eye and stemmata. A CRY1-positive fiber pathway (orange) connects the circadian clock to axons from polarized UV light-sensing photoreceptors in the dorsal rim area (blue rectangles). The circadian clock may also interact with the sun compass apparatus through interactions with the sun compass itself (which may be located in the central complex, based on studies in locusts; see Labhart and Meyer, 2002) or with the interface between the sun compass and the motor system.

is a blue-light photoreceptor involved in photic entrainment in the fly.

Using a strategy that relied on the coexpression of PER, TIM, and a *Drosophila*-like CRY, designated CRY1 in the butterfly (see below), four cells in the dorsolateral region of the monarch butterfly brain (the pars lateralis, PL) were recently identified as the putative location of a circadian clock (Sauman et al., 2005) (Figure 1). Importantly, PER staining in the PL exhibits a robust 24 hr rhythm that is under circadian control and that is abolished by constant light. PER, TIM, and CRY1 are also colocalized in the central brain in large neurosecretory cells in the pars intercerebralis (PI), but the circadian control of PER levels is less apparent there (Sauman et al., 2005). Nonetheless, these PI cells may be part of a circadian network controlling migratory behaviors. As described below, CRY1-positive neural pathways connect the circadian clock cells in the PL to other brain areas involved in migratory activities (Figure 1).

Migration Triggered by a Circadian Clock

It is likely that a circadian clock is involved in the induction of butterfly migration (Figure 2A), as migration is initiated in the fall by decreasing daylength (Goehring and Oberhauser, 2002), similar to the photoperiodic induction of seasonal events in other insects. As in *Drosophila*, juvenile hormone is a key regulator of adult reproductive activity and longevity in monarch butterflies (Herman and Tatar, 2001). In migratory monarchs, juvenile hormone levels are significantly reduced, reproductive development is curtailed, and longevity is increased—from a life span of a few weeks in summer to several months during migration. Moreover, experimental manipulation of juvenile hormone levels in adult butterflies causes predictable changes in reproductive activity and longevity. Thus, reproductive diapause and increased longevity, phenotypic markers of the migratory state, are induced by juvenile hormone deficiency (Herman and Tatar, 2001). Synthesis of juvenile hormone is likely to be regulated by insulin-like peptides produced by neurosecretory cells in the PI. A CRY1-positive neural pathway connects the circadian clock in the dorsolateral region (PL) of the monarch butterfly brain to the neurosecretory cells in the PI that may be critical for the photoperiodic regulation of reproductive diapause (Sauman et al., 2005) (Figure 1).

Transcriptional profiling to compare gene expression in the brains of migratory versus summer butterflies is now possible with the availability of a brain-expressed sequence tag (EST) library (Zhu et al., 2005). These studies could elucidate gene expression patterns in the brain that reveal transcriptional networks that might be characteristic of the migratory state. Is it possible that there is a "migratory gene"? An increased understanding of the mechanisms behind environmentally induced changes in monarch longevity may offer new insights into fundamental principles that apply to the aging process in general. Results from such studies would provide an interesting comparison to mechanisms of increased life span in the nematode, fly, and mouse. Understanding how the induction of diapause occurs could also lead to methods to produce migratory butterflies for study in the laboratory at any time of the year.

A Time-Compensated Sun Compass

Migratory butterflies provide a model for circadian biologists interested in the molecular logic behind the biological integration of information about time and space, which is an integral component of navigation for a variety of animals. The remarkable navigational abilities of monarch butterflies are part of a genetic program that is

initiated in migrants—it is not learned, as the butterflies that are migrating are always on their maiden voyage, and those that make the trip south are at least two generations removed from the previous generation of migrants (Brower, 1996). The centerpiece of the navigational process is a time-compensated sun compass. Time compensation is provided by a circadian clock that allows the butterflies to continually correct their flight direction relative to changing skylight parameters to maintain a fixed flight bearing in the south/southwesterly direction as the sun moves across the sky during the day. The ability to successfully navigate requires that this underlying program is constantly being recalibrated by environmental factors. For example, the circadian clock is standardized to local time by dawn and dusk, whereas the sun compass may be modulated by geomagnetic forces or by the visualization of certain landmarks. Barometric pressure and the prevailing wind direction also have a marked influence on the progression of migration.

That monarch butterflies use a time-compensated sun compass to help them to navigate has been most convincingly shown using a flight simulator that allows the study of flight trajectories from tethered butterflies during a sustained period of flight (Mouritsen and Frost, 2002). The importance of the circadian clock in regulating the time-compensated component of flight orientation has been shown in two ways. First, clock-shift experiments, in which the timing of the daily light-dark cycle is either advanced or delayed, cause predictable alterations in the direction the butterflies fly (Perez et al., 1997; Mouritsen and Frost, 2002; Froy et al., 2003). Second, constant light, which disrupts the molecular clock mechanism, abolishes the time-compensated component of flight orientation (Froy et al., 2003).

But how does time-compensation information communicate with the sun compass? Some studies suggest that monarch butterflies can use the skylight pattern of polarized light as a sun compass cue and that this information is used in a time-compensated manner (Hyatt, 1993; Reppert et al., 2004). Moreover, polarized light that is important for proper orientation may be sensed through ultraviolet photoreceptors in the dorsal rim area of the monarch eye, an area in which photoreceptors are anatomically specialized for polarized light detection (Sauman et al., 2005; Stalleicken et al., 2005a). In addition, a CRY1-staining neural pathway was found that could connect the circadian clock to polarized light input entering the brain (Sauman et al., 2005) (Figure 2B). The CRY1-positive fiber pathway ends in the posterior dorsolateral region of the medulla of the optic lobe, in the same location where the axons from dorsal rim photoreceptors terminate (Figure 1, β). Electrophysiological studies of polarization-sensitive photoreceptors in the dorsal rim and of polarization-sensitive neurons in the optic lobe in the butterfly brain will help to clarify whether the CRY1-containing pathway provides the relevant time-compensation information (Labhart and Meyer, 2002). It is quite possible that there are several pathways that connect the circadian clock to different aspects of the sun compass system.

Dorsal rim-sensed polarized light is not necessary for proper flight orientation in the flight simulator, as long as the sun can be seen (Stalleicken et al., 2005b). This is consistent with the primary role of polarized light orientation occurring during cloudy days with some blue sky visible. In addition to polarized light, monarch butterflies are likely to use the sun itself or spectral gradients to orient themselves (Stalleicken et al., 2005b; Reppert et al., 2004). The relative importance of various celestial cues in the daylight sky for proper navigation needs to be evaluated in free-flying butterflies during the actual migratory period.

A "Radical" Hypothesis

The widespread distribution of CRY1-positive cells and fibers in the monarch butterfly brain suggests that CRY1 has pleiotropic activities in the butterfly. As CRY1 is light sensitive (Zhu et al., 2005), it is likely to act as a blue-light photoreceptor for the circadian clock of monarch butterflies, as CRY does in *Drosophila* (Stanewsky, 2003). In fly brain, CRY and PER are always found together, whereas in monarch butterfly brain, the CRY1-positive cells in the lobula region of the optic lobe do not contain detectable PER (Figure 1, α).

So what is the function of CRY1 in optic lobe cells and in the projections emanating from that structure? One interesting possibility is that the CRY1-positive optic lobe cells "mark" the location of an inclination magnetic compass, with the CRY1-staining projections acting as a magnetoreceptor. It has been proposed that light-sensitive CRY proteins, which use flavin-dependent redox reactions, could generate magnetosensitive radical pairs that would provide a photoinduced electron transfer reaction for the detection of magnetic fields (see Ritz et al., 2002). To sense geomagnetic field direction, this radical pair mechanism requires a geometrically fixed arrangement of radical pair-generating photopigments—the axonal alignment of CRY1 molecules in the optic lobes may fulfill this requirement. Although the use of a magnetic compass for navigation by monarch butterflies during migration has not been demonstrated (Mouritsen and Frost, 2002), a possible molecular substrate for such sensing now exists. Fibers in the medulla containing CRY1 therefore could provide a focus of information integration between a time-compensated sun compass and a magnetic compass. The migratory monarch butterfly may be an ideal model for studying complex sensory integration, leading to adaptive physiological and behavioral responses.

A Tractable Molecular Clock Mechanism

Search of the butterfly EST library led to the recent discovery of a second *cry* gene in monarch butterflies that encodes a protein designated CRY2 (Zhu et al., 2005). In contrast to CRY1, CRY2 is light insensitive and has potent repressive activity on CLK:CYC-mediated transcription. The finding of two functionally distinct *cry* genes in the butterfly led

to the recognition of the existence of two *cry* genes in several nondrosophilid insects; in contrast, only the previously characterized photoreceptive CRY is found in *Drosophila*. The finding of a second *cry* gene in insects has redefined our view of the evolution of animal CRYs, as the transcriptionally active insect CRY2 shares a common ancestor with the two mammalian CRYs (Zhu et al., 2005).

As in *Drosophila*, transcription of the *per* gene is under circadian control in monarch butterflies (Froy et al., 2003), presumably via the activity of CLK:CYC heterodimers (Zhu et al., 2005). However, PER is not detected in the nucleus of clock cells in the monarch brain (nor in the brains of several other nondrosophilid species) (Sauman et al., 2005). It is therefore possible that the newly discovered CRY2 is an important transcriptional repressor for the central clockwork of monarch butterflies, as well as in other nondrosophilid insects, similar to CRY function in mammals (Stanewsky, 2003). Thus, details of the monarch clock mechanism may well differ from those in *Drosophila* and aid our understanding of clock mechanisms in more complex animals.

Which available genetic and molecular tools are likely to expedite studies of molecular mechanisms in monarch butterflies? The EST library has already proven its usefulness. Genetic transformation of monarchs, although a potential way of manipulating gene expression, requires that difficult animal husbandry issues be overcome. RNA interference (RNAi) strategies currently hold the greatest promise. If RNAi can be exploited as a consistent way to disrupt gene expression in monarchs in vivo (with disrupting *cry2* expression the obvious priority), butterflies will become a powerful nondrosophilid model in which to study circadian clock mechanisms and outputs. Work on the monarch clock mechanism has been facilitated by the availability of a cultured cell line that endogenously expresses several clock proteins.

Even as the molecular toolbox begins to fill, the extraordinary biology of the monarch migration continues to cry out for more study. An increased understanding of the fundamental mechanisms behind the migration of these colorful insects may well aid in the preservation of their migration, currently threatened by the loss of their mountainous habitat in central Mexico.

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