The Ancestral Circadian Clock of Monarch Butterflies: Role in Time-compensated Sun Compass Orientation

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The circadian clock has a vital role in monarch butterfly (*Danaus plexippus*) migration by providing the timing component of time-compensated sun compass orientation, which contributes to navigation to the overwintering grounds. The location of circadian clock cells in monarch brain has been identified in the dorsolateral protocerebrum (pars lateralis); these cells express PERIOD, TIMELESS, and a *Drosophila*-like cryptochrome designated CRY1. Monarch butterflies, like all other nondrosophilid insects examined so far, express a second *cry* gene (designated insect CRY2) that encodes a vertebrate-like CRY that is also expressed in pars lateralis. An ancestral circadian clock mechanism has been defined in monarchs, in which CRY1 functions as a blue light photoreceptor for photic entrainment, whereas CRY2 functions within the clockwork as the major transcriptional repressor of an intracellular negative transcriptional feedback loop. A CRY1-staining neural pathway has been identified that may connect the circadian (navigational) clock to polarized light input important for sun compass navigation, and a CRY2-positive neural pathway has been discovered that may communicate circadian information directly from the circadian clock to the central complex, the likely site of the sun compass. The monarch butterfly may thus use the CRY proteins as components of the circadian mechanism and also as output molecules that connect the clock to various aspects of the sun compass apparatus.

INTRODUCTION

During their spectacular fall migration, eastern North American monarch butterflies (*Danaus plexippus*) use a time-compensated sun compass to help them navigate to their overwintering sites in central Mexico (Reppert 2006). Because this navigational capability is genetically determined, I propose that the monarch butterfly can be used as a model to study the molecular and cellular basis of time-compensated sun compass orientation. The ultimate goal of such studies is to understand the molecular and anatomical mechanisms for clock-compass interactions that enable migrants to maintain a set flight bearing as the sun moves across the sky each day.

In the course of these studies, a novel circadian clock mechanism has been discovered in monarch butterflies that had not been described before in any other animal. In addition, some butterfly clock proteins delineate neural pathways that may connect the clock to the sun compass (or its inputs). But many questions remain about how the clock and compass interact to give rise to appropriately oriented flight behavior.

A TIME-COMPENSATED SUN COMPASS

A fascinating function of circadian clocks is their use in time-compensated sun compass orientation, a phenomenon that was first described decades ago by Karl von Frisch (1967) in foraging honeybees and by Gustav Kramer (1957) in migratory birds. The amazing navigational abilities of monarch butterflies are part of a genetic program initiated in the migrants, as those that make the trip south are at least two generations removed from the previous generation of migrants (Brower 1996). Time-compensated sun compass orientation is believed to be an essential component of navigation required for successful migration.

Time compensation is provided by a circadian clock that allows the butterflies to continually correct their flight direction relative to skylight parameters to maintain a fixed flight bearing in the south/southwesterly direction as the sun moves across the sky during the day (Fig. 1). The ability to successfully navigate requires that this underlying program is constantly recalibrated by environmental factors. For example, the circadian clock is standardized to local time by dawn and dusk, whereas the sun compass may be calibrated by geomagnetic forces and/or visualizing certain landmarks. Barometric pressure and the prevailing wind direction also have a marked influence on proper navigation and the progression of the migration (Reppert 2006).

That monarch butterflies use a time-compensated sun compass to help them navigate has been most convincingly shown using a flight simulator that allows 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 (see below), abolishes the time-compensated component of flight orientation (Fig. 1) (Froy et al. 2003).

What are the skylight cues sensed by 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 utilized in a time-compensated manner (Hyatt 1993; Reppert et al. 2004). Moreover, polarized light relevant for proper orientation may be sensed through ultraviolet (UV) opsin-expressing photoreceptors in the dorsal rim area of the monarch eye, an

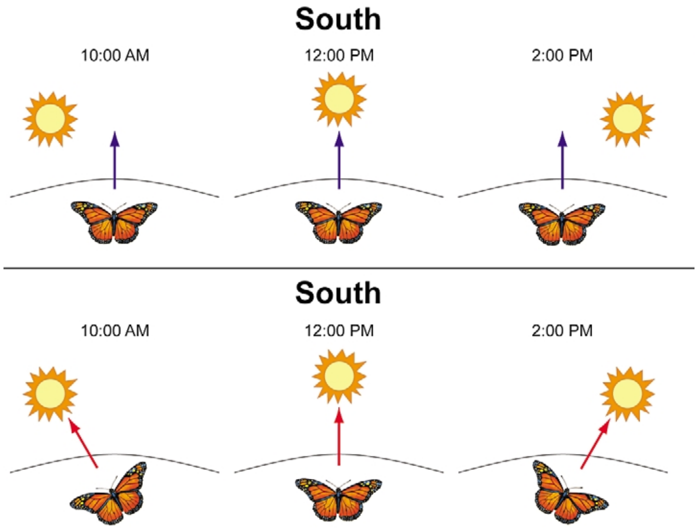

Figure 1. Lost without a clock. (*Upper panel*) Eastern North American monarch butterflies use a time-compensated sun compass to orient south during their fall migration. The circadian clock allows the butterflies to compensate for the movement of the sun. The migrants are thereby able to maintain a constant bearing in the southerly direction over the course of the day. (*Lower panel*) Monarch butterflies follow the sun without a functioning circadian clock. A broken circadian clock would disrupt migration south, and the butterflies would not be able to successfully travel to their overwintering grounds.

area in which photoreceptors are anatomically and physiologically specialized for polarized light detection (Sauman et al. 2005; Stalleicken et al. 2006).

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. 2005). 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 likely use the sun itself and/or color gradients to orient (Reppert et al. 2004; Stalleicken et al. 2005). Recent electrophysiological studies of single neurons in locusts have shown that nonpolarized chromatic gradients are used coordinately with polarized light to provide more explicit skylight cues for navigation (Pfeiffer and Homberg 2007). These results may be salient for helping determine the relative or combined importance of various celestial cues for proper navigation in monarchs.

LOCATION OF CELLULAR CLOCKS IN BUTTERFLY BRAIN

To fully understand how a circadian clock is involved in time-compensated sun compass orientation in the butterfly, it is important to determine where the cellular clock actually resides in brain, to understand the molecular mechanism of the clock itself, and to delineate neural pathways that connect the clock to the compass. In *Drosophila* and mammals, the intracellular clock mechanism involves transcriptional feedback loops that drive persistent rhythms in mRNA and protein levels of key clock components (Reppert and Weaver 2002; 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 heterodimerize and translocate back into the nucleus where PER inhibits CLK:CYC-mediated transcription. TIM appears to regulate PER protein stability and nuclear transport and is also necessary for photic responses that reset (entrain) the circadian clock. *Drosophila* cryptochrome (CRY), a flavoprotein, is colocalized with PER and TIM in clock cells and is a blue light photoreceptor involved in photic entrainment (Emery et al. 1998, 2000; Stanewsky et al. 1998).

Using a strategy that relied on the coexpression of PER, TIM, and a *Drosophila*-like CRY, designated CRY1, four cells in the dorsolateral region of monarch butterfly brain (the pars lateralis, PL) were identified as the putative location of a circadian clock (Fig. 2) (Sauman et al. 2005). Importantly, PER staining in the PL exhibits a robust 24-hour 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 (Fig. 2) (Sauman et al. 2005). Nonetheless, these PI cells may be part of a circadian network contributing to migratory behaviors.

THE DISCOVERY OF INSECT CRYPTOCHROME 2

In addition to CRY1, monarch butterflies also express a second *cry* gene, which encodes a light-insensitive protein, designated CRY2. CRY2 has potent repressive activity on CLK:CYC-mediated transcription, as the mouse CRY proteins do (Zhu et al. 2005). The finding of two functionally distinct *crys* in the butterfly, along with database searches, led to the recognition of the existence of *cry2* in every non-drosophilid insect so far examined (Yuan et al. 2007).

Drosophila expresses CRY1 only, whereas insects such as mosquitos and lepidopterans (butterflies and moths) express both CRY1 and CRY2. Surprisingly, the honeybee *Apis mellifera* and the beetle *Tribolium castaneum* genomes contain only CRY2 (as determined by BLAST searches of whole-genome sequences; Zhu et al. 2005; Rubin et al. 2006). This suggests that the core circadian oscillator has evolved throughout the insects such that at least three kinds of clocks exist: those containing both CRY1 and CRY2 (the ancestral state) as in monarch and mosquitoes; those containing only CRY1 as in *Drosophila*; and those containing CRY2 alone as in honeybee and beetle (Fig. 3) (Yuan et al. 2007).

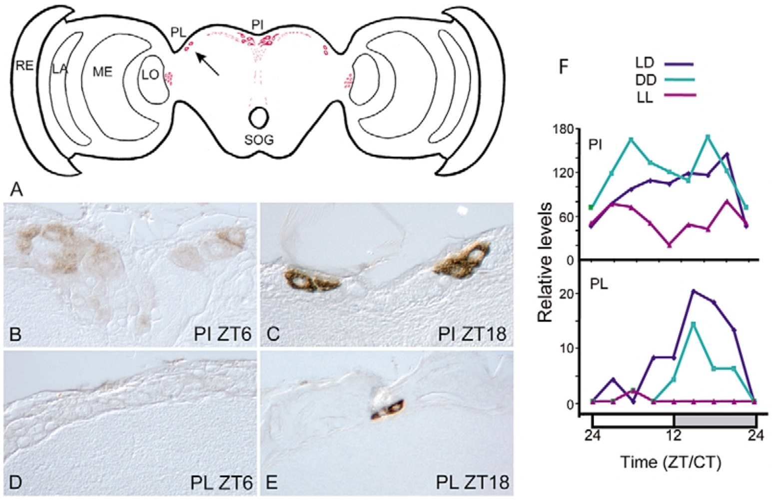

Figure 2. Cellular location of clock cells in monarch brain identified by PER immunoreactivity. (*A*) Schematic diagram of a frontal section illustrating the topography of PER-positive cells. (LA) Lamina; (ME) medulla; (LO) lobula; (PL) pars lateralis (*arrow*); (PI) pars intercerebralis; (SOG) suboesophageal ganglion; (RE) retina. (*B*,*C*) PER staining in a group of large neurosecretory cells in PI at zeitgeber time [ZT] 6 (B) and ZT 18 (*C*). (*D*,*E*) Daily osillation of PER immunoreactivity in two cells in PL at ZT 6 (*D*) and ZT 18 (*E*). (*F*) Semiquantitative assessment of PER immunostaining in PI or PL in a 12 hours light:12 hours dark (LD, *magenta*), the second day in constant darkness (DD, *green*), and the second day constant light (LL, *red*). Each value is the sum of intensity scores from three animals. (ZT) Zeitgeber time; (CT) circadian time. (Modified from Sauman et al. 2005.)

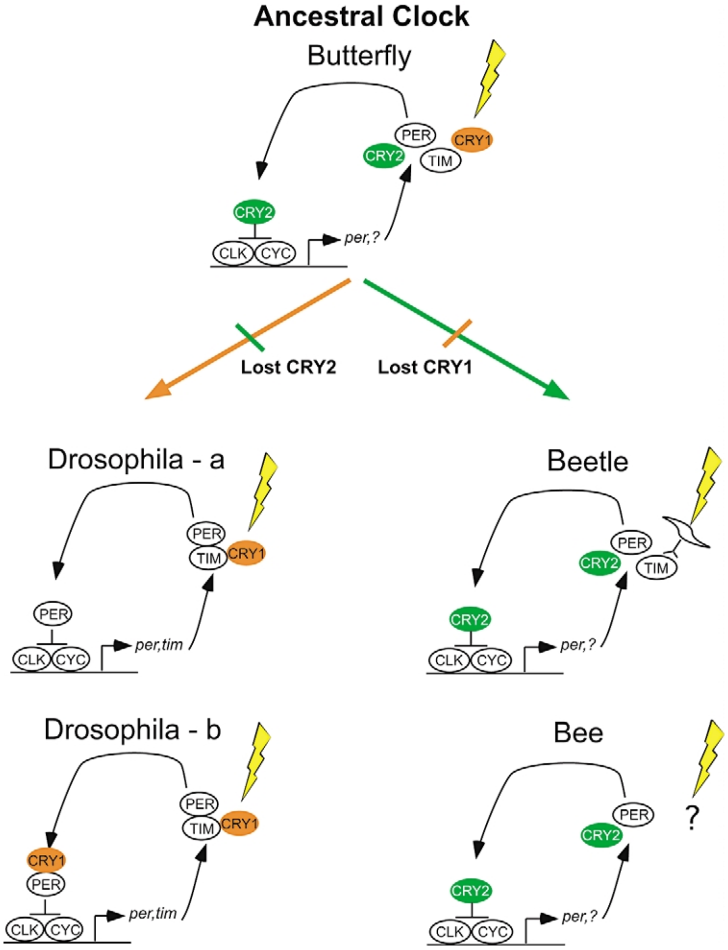

Figure 3. Insect clockwork models. Phylogenetic analyses show a least two rounds of gene duplication at the base of the metazoan radiation, as well as several losses, giving rise to the two *cry* gene families in insects (Yuan et al. 2007). With the existence of two functionally distinct CRYs in insects, three major types of clockwork models can be proposed: the ancestral clock (apparent in the monarch butterfly) in which both CRY1 (*orange oval*) and CRY2 (*green oval*) exist and function differentially within the clockwork; a derived clock (the *Drosophila* form, depicted below *orange arrow*) in which CRY2 has been lost and CRY1 only functions in the central brain clock as a circadian photoreceptor (*a*) (Emery et al. 1998) or in peripheral clocks as both a photoreceptor and central clock component (*b*) (Ivanchenko et al. 2001; Krishnan et al. 2001; Levine et al. 2002; Collins et al. 2006); and a derived clock (depicted below *green arrow*) in which CRY1 has been lost and only CRY2 exists and functions within the clockwork, as in beetles and bees. In beetles, CRY2 acts as a transcriptional repressor of the clockwork, and light input may be mediated through the degradation of TIM. In bees, which lack TIM (Rubin et al. 2006), CRY2 acts as a transcriptional repressor and novel light input pathways (?) are used to entrain the clock. (Modified from Yuan et al. 2007.)

All insect CRY2 proteins so far examined (including those of the bee and beetle) are potent repressors of CLK:CYC-mediated transcription in cell culture (Yuan et al. 2007). Importantly, the bee and beetle CRY2 proteins are not light-sensitive in culture, as assessed either by degradation of CRY2 or by derepression of inhibitory transcriptional activities. These results suggest that these species have novel light input pathways to their circadian clocks, perhaps opsin-based (see Spaethe and Briscoe 2005), as both lack CRY1 (Fig. 3) (Yuan et al. 2007).

THE ANCESTRAL CLOCK OF THE MONARCH BUTTERFLY

On the basis of the presence of two *cry* genes, the monarch butterfly utilizes an ancestral circadian clock, which has clockwork characteristics of both flies (through CRY1) and mice (through CRY2) (Fig. 4). In the butterfly, CRY1 functions primarily as a circadian photoreceptor (Song et al. 2007; Zhu et al. 2008b). CRY2, on the other hand, appears to function as a major transcriptional repressor of the core clock feedback loop in monarchs. In addition to being a potent repressor of CLK:CYC-mediated transcription in cell culture, monarch CRY2 is colocalized with the other clock proteins in the PL, and there it translocates to the nucleus at the appropriate time for transcriptional repression (Zhu et al. 2008b). Monarch PER does not inhibit CLK:CYC-mediate transcription, but it does stabilize CRY2 and may help translocate CRY2 to the nucleus (Zhu et al. 2008b). The ancestral circadian clockwork of monarch butterflies may be the prototype of a novel clock mechanism shared by those nondrosophilid invertebrates that express both *cry1* and *cry2*.

CLOCK-COMPASS NEURAL CONNECTIONS

The CRY-centric ancestral circadian clock defined in monarch butterflies may hold a key to understanding the regulation of time-compensated sun compass orientation. Indeed, a CRY1-staining neural pathway was found that could connect the circadian clock to polarized light input entering the brain (Fig. 5) (Sauman et al. 2005). 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 (Fig. 5). On the basis of studies in other insects, these photoreceptor axons would appear to communicate ultimately with the sun compass.

The site of the sun compass in insects now appears to be the central complex (Vitzthum et al. 2002; Heinze and Homberg 2007). The central complex is a midline structure consisting of the dorsally positioned protocerebral bridge and the more ventrally situated central body, which has upper and lower subdivisions. Recent studies in locusts and *Drosophila* have shown that the central complex is not only a control center for motor coordination, but also the actual site of the sun compass (for polarized skylight integration from both eyes and probably all skylight information) (Heinze and Homberg 2007), as well as being involved in visual pattern learning and recognition (Liu et al. 2006).

Our finding of a clock connection with the central complex in the monarch butterfly represents a major advance for beginning to understand its remarkable navigational capabilities. A dense arborization of CRY2 staining has been found in the central body of monarch butterflies, just

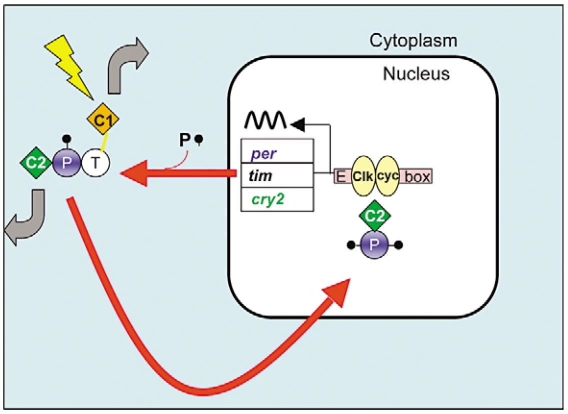

Figure 4. Proposed monarch butterfly circadian clock mechanism. The main gear of the clock mechanism in pars lateralis is an autoregulatory transcriptional feedback loop, in which CLK and CYC heterodimers drive the transcription of the *per*, *tim*, and *cry2* genes through E-box enhancer elements. TIM (T), PER (P), and CRY2 (C2) proteins form complexes in cytoplasm, and CRY2 is shuttled into the nucleus where it shuts down CLK:CYC-mediated transcription. PER is progressively phosphorylated and likely helps translocate CRY2 into nucleus. CRY1 (C1) is a circadian photoreceptor which, upon light exposure (*lightning bolt*) causes TIM degradation, allowing photic information to gain access to the central clock mechanism. (*Thick gray arrows*) Potential output functions for CRY1 and for CRY2. (Modified from Zhu et al. 2008b.)

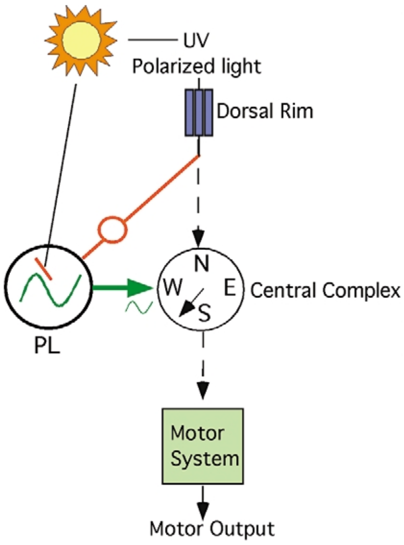

Figure 5. Proposed clock-compass pathways in monarch butterfly brain. A circadian clock in pars lateralis (PL) is entrained by light acting through CRY1 expressed in clock cells (*orange line*). A CRY1-positive fiber pathway (*orange*) connects the circadian clock to axons originating from polarized, UV-light-sensitive photoreceptors in the dorsal rim of the compound eye (Sauman et al. 2005). The circadian clock may also interact directly with the sun compass (in central complex) through a CRY2-positive fiber pathway (*green*) recently discovered. The sun compass ultimately controls motor output. (Modified from Zhu et al. 2008b.)

ventral to the protocerebral bridge (Zhu et al. 2008b). The CRY2 arbors in this area are under circadian control, with intense staining in the middle of the circadian night and little to no visible staining in middle of circadian day. Thus, the CRY2-positive neural pathway appears to be capable of providing circadian signals to the central complex (Fig. 5) (Zhu et al. 2008b). It is likely that the CRY2 fiber pathway to central complex originates from CRY2-positive cells in the PL, PI, or both, but this has not been definitively established. CRY2 may simply be marking the circadian pathway to the sun compass and/or the protein may be directly involved in rhythmic synaptic activity in that region.

MANY QUESTIONS REMAIN

As alluded to above, the central complex looms large as an area in need of focus for future studies of time-compensated sun compass orientation in monarchs. It will be important to define in more detail with confocal microscopy the anatomy of the central complex in monarch butterflies. Three-dimensional reconstruction can also be used to determine exactly where the CRY2 pathway to central complex arises. It will also be important to determine where the CRY2 pathway ends and whether it actually functionally communicates with the central complex.

Elegant electrophysiological studies in crickets and locust have begun to show how neurons in pertinent brain regions process skylight cues from both eyes relevant for proper orientation and subsequent navigational tasks. Studies in crickets have identified polarization-opponent interneurons in optic lobes, which process polarized light information from the orthogonally arranged photon-sensing microvilli in dorsal rim photoreceptors (Labhart and Meyer 2002). Studies in locusts have further shown that the columnar organization of cells in the protocerebral bridge of the central complex provides a topographical map of overhead polarized skylight patterns from both eyes (Vitzthum et al. 2002; Heinze and Homberg 2007). Moreover, interneurons in the posterior optic tubercle of the locust can process both polarization and color gradients for more precise skylight signals for navigation than could be obtained through polarized light signals alone (Pfeiffer and Homberg 2007). These studies provide a rich backdrop for potential electrophysiological correlates in monarch butterflies.

DNA microarray analysis could be used to determine genes important in central complex activity during different orientation states in tethered butterflies (Zhu et al. 2008a). Candidate genes could then be knocked down by RNAi to help determine function. The ultimate goal would be to selectively manipulate gene expression in the central complex (or other relevant brain regions) to understand the molecular logic behind the neuronal signaling patterns that have been identified and how they ultimately contribute to time-compensated flight orientation. Cracking the code of sun compass orientation will require a combination of molecular, anatomical, electrophysiological, and behavioral approaches in a single species. The monarch butterfly is ideally suited for such studies.

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