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The *Drosophila* Circadian Network Is a Seasonal Timer

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SUMMARY

Previous work in *Drosophila* has defined two populations of circadian brain neurons, morning cells (M-cells) and evening cells (E-cells), both of which keep circadian time and regulate morning and evening activity, respectively. It has long been speculated that a multiple oscillator circadian network in animals underlies the behavioral and physiological pattern variability caused by seasonal fluctuations of photoperiod. We have manipulated separately the circadian photoentrainment pathway within E- and M-cells and show that E-cells process light information and function as master clocks in the presence of light. M-cells in contrast need darkness to cycle autonomously and dominate the network. The results indicate that the network switches control between these two centers as a function of photoperiod. Together with the different entraining properties of the two clock centers, the results suggest that the functional organization of the network underlies the behavioral adjustment to variations in daylength and season.

INTRODUCTION

Circadian clocks drive daily oscillations of biochemical, physiological, and behavioral parameters in diverse organisms from bacteria to human. Free-running circadian pacemakers maintain internal order, of cellular metabolism for example (Rutter et al., 2002), but entrainment is required to create and maintain a stable phase relationship between these internal oscillations and the daily fluctuations of the external world. Although the natural 24 hr light-dark cycle is a stable zeitgeber (temporal cue), photoperiod (daylength) varies dramatically with season in many locations throughout the world. As a consequence, temporal niches also vary with the photoperiod, exerting a vital pressure on the animal capacity of temporal adjustment and anticipation. The seasonal changes in photoperiod may also be fundamental in animal extraction of information on calendar time, maintenance of circannual cycles in physiology and behavior, and timely preparation of critical phenomena such as hibernation, migration, diapause, and reproduction. How animal clocks organize the adaptation to seasonal changes in photoperiod is largely unknown.

In contrast, a good understanding of clock entrainment to a fixed photoperiod has emerged in recent years. In *Drosophila* in particular, multiple photosensitive pathways converge on clock-gene-expressing neurons in the brain (Hall, 2003; Helfrich-Forster et al., 2001). Nonetheless, the *Drosophila* brain itself appears to be the principal light-sensitive entity, as circadian rhythms adjust to shifts in light regimes even when external visual inputs are absent (Hall, 2003; Helfrich-Forster et al., 2001). A large body of data now implicates the photoreceptor *cryptochrome* (*cry*) as the key intracellular mediator between light information and the core circadian machinery within *Drosophila* brain neurons (reviewed by Hall, 2003). For example, CRY is degraded in light, and the action spectrum of CRY degradation matches closely the behavioral action spectrum for a phase response curve (PRC), i.e., the characteristic phase delays and phase advances caused by light pulses in the early night and late night, respectively (Busza et al., 2004; Suri et al., 1998). CRY forms a light-dependent complex with the clock proteins TIMELESS (TIM) and PERIOD (PER) (Busza et al., 2004; Ceriani et al., 1999; Rosato et al., 2001), and there is substantial evidence that CRY-mediated TIM degradation is important for light-mediated phase shifts (Koh et al., 2006; Lin et al., 2001; Naidoo et al., 1999). This is indicated by the almost complete elimination of light-mediated TIM degradation and behavioral activity phase shifts in the strong loss-of-function mutant cry^b (Lin et al., 2001; Stanewsky et al., 1998). This mutant retains robust locomotor activity rhythms in constant darkness (DD), suggesting that CRY is not necessary for core circadian oscillator function. However, transgenic rescue of CRY function only within brain

clock cells can restore phase responses to light in cry^b flies (Emery et al., 2000b), indicating that CRY photoreception within clock neurons is a key feature of entrainment. The importance of CRY to light regulation is underscored by an additional phenotype of cry^b flies: they have lost the ability to respond normally to constant light (LL). This condition normally causes arrhythmia in wild-type *Drosophila* and many other diurnal species; cry^b flies, in contrast, maintain robust free-running rhythms in LL (Emery et al., 2000a).

Under standard 12 hr light: 12 hr dark (standard LD) conditions, CRY activation by lights-on (laboratory surrogate of dawn) leads to PER and TIM degradation and the start of a new cycle (Lin et al., 2001). Lights off (dusk-surrogate) terminates the CRY signal and leads to the nighttime accumulation of PER and TIM (e.g., Qiu and Hardin, 1996). This daily cycle of degradation and subsequent accumulation is tightly coupled to circadian oscillator function. Indeed, negative feedback by PER-TIM on *per* and *tim* transcription is integrated with PER and TIM posttranscriptional regulation to generate the characteristic circadian oscillations of PER and TIM levels.

The relevance of PER-TIM posttranscriptional regulation has been enhanced by the discovery of clock-gene kinases that modify PER and TIM. These enzymes probably regulate PER-TIM half-life, the timing of their nuclear entry, and their activity as transcriptional regulators. PER kinases include *doubletime* (CKIe) (Kloss et al., 1998; Price et al., 1998) and *timekeeper* (CKII) (Akten et al., 2003; Lin et al., 2002), which are relevant clock kinases in other circadian systems, including mammals (Lowrey et al., 2000), plants (Sugano et al., 1999), and Neurospora (He et al., 2006). *Shaggy* (*sgg*) is the *Drosophila* ortholog of mammalian GSK3 and a putative TIM kinase. Overexpression or mutant versions of *sgg* dramatically alter circadian period (Martinek et al., 2001). This is typically measured in constant darkness (DD), which indicates that these PER and TIM kinases are important for the circadian program even in the absence of light and CRY activation. However, the posttranscriptional regulation of PER-TIM in darkness is likely integrated with the CRY program and its potent destabilizing effect on PER-TIM in light (Lin et al., 2001).

Our previous work on cellular aspects of free-running rhythms (Stoleru et al., 2004) led us to this interface between CRY, PER-TIM posttranscriptional regulation, and the LL arrhythmic phenotype of wild-type *Drosophila*. We had previously defined two populations of circadian brain neurons, morning cells (M-cells) and evening cells (E-cells), connected to morning and evening locomotor activity, respectively (Grima et al., 2004; Stoleru et al., 2004). Interactions between the two oscillator populations were studied by selectively overexpressing *sgg* to speed up the clock in only one cell population or the other (Stoleru et al., 2005). In this study, we found that *sgg* overexpression gives rise to LL rhythmicity, which led to a search for the cellular substrates of entrainment. The rhythmicity is predominantly due to *sgg* overexpression in E-cells, which suggested that this subset of the clock network is particularly important in the light and that SGG affects the biochemical pathway through which light impacts clock molecules and adjusts phase to the correct time of day. Indeed, we present strong evidence that SGG modulates CRY function, which affects in turn the core clock proteins PER and TIM. The separate manipulation of the SGG/CRY pathway within E- and M-cells also reveals that the E-clocks drive the behavioral rhythm in light, with prominent PER oscillations of nuclear localization. This light dependence of E-cells contrasts with M-cells, which need darkness to cycle autonomously and dominate the activity output pathway. This distinction suggests a simple dual-oscillator model for how the clock adjusts to photoperiod changes, and support for this seasonal model was obtained by examining E- and M-cell cooperation under different photoperiods.

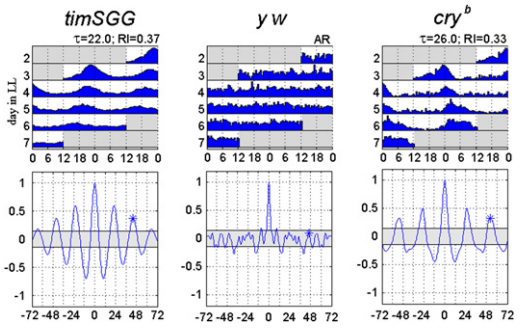

Figure 1. Overexpression of *sgg* in Clock Cells Inhibits LL Arrhythmicity

Average locomotor activity profiles of three genotypes maintained in LL: *timSGG* (left panels; n = 40); *y w* (middle; n = 32); cry^b (right; n = 32). (Upper panels) Double-plotted actograms of LL activity. (Lower panels) Autocorrelation plot reflecting rhythm strength. Periods (τ) and rhythm strength indices (RI) are shown above each graph (Levine et al., 2002b). Four days of activity are compared, starting with day 2 (LL2–LL5).

RESULTS

SGG Overexpression in Clock Neurons Causes LL Rhythmicity

In a previous study addressing circadian oscillator interactions, we selectively overexpressed the clock gene *sgg* to increase the speed of circadian oscillations. SGG is the *Drosophila* ortholog of mammalian GSK3, and its overexpression shortens circadian period (Martinek et al., 2001). We discovered by chance that a large fraction (~50%) of SGG-overexpressing flies (*timGAL4/UAS-sgg*, or *timSGG* for simplicity) is rhythmic in LL (Figure 1). Although cry^b mutant flies have stronger rhythms and are 100% rhythmic in LL, *timSGG* flies are dramatically different from wild-type-like flies (*y w* in Figure 1), which are uniformly arrhythmic under these conditions (Emery et al., 2000a).

cry^b flies are probably LL-rhythmic because TIM degradation is light-insensitive (Lin et al., 2001). Because SGG is a putative TIM kinase (Martinek et al., 2001), we

hypothesized that TIM hyperphosphorylation by SGG also inhibits light-mediated degradation. However, the shorter period in *timSGG* (Martinek et al., 2001) and the notion that early TIM degradation precedes every new cycle rather suggested that hyperphosphorylated TIM is more sensitive to degradation, at least in darkness. We therefore considered that SGG might act indirectly on TIM by modulating CRY light sensitivity or activity. We examined this hypothesis initially in cell culture.

SGG Interacts with and Stabilizes CRY

CRY western blots revealed that *Drosophila* S2 cells contain abundant endogenous CRY (as suggested; Lin et al., 2001), which is degraded after light exposure (Figures 2A and 2B). To our surprise, SGG overexpression dramatically increased CRY stability. The most striking effect was seen in the light, a condition in which CRY is normally undetectable. An opposite response occurred with inactivation of endogenous SGG (*sgg* dsRNA); this led to a decrease in endogenous CRY levels in darkness. As endogenous TIM is undetectable in S2 cells, TIM probably does not contribute to the SGG effect on CRY levels. (The CRY response to SGG expression levels was insensitive to TIM coexpression; Figure 2B and see below). The effect of SGG overexpression on CRY was verified in flies. Western blots of *timSGG* fly heads revealed increased CRY levels in darkness as well as in light (Figure 2C and data not shown).

We then determined that the CRY-SGG interaction was direct both in transgenic flies and in S2 cells. First, SGG-V5, CRY-His6, or both were overexpressed in S2 cells, immunoprecipitated with anti-V5 antibody, Nickel beads, or both, and probed for CRY, SGG, or both, respectively (Figure 2D and data not shown). To confirm this interaction, we used a previously described MYC-tagged *cry* construct in flies (*tim-GAL4/UAS-myc-cry*, or *TMC*; Busza et al., 2004). Immunoprecipitation of CRY from fly head extracts with anti-MYC antibody followed by western blot with anti-SGG antibody validated the CRY-SGG interaction (Figure 2E) and indicated that SGG and CRY interact directly or exist together in a complex. In contrast, we were unable to detect a comparable interaction between SGG and TIM, a difficulty also encountered by others (M. Young, personal communication).

SGG Affects TIM Stability in a CRY-Dependent Manner

Nonetheless, SGG overexpression affects TIM levels as well as CRY levels. This is because SGG downregulation (*sgg* dsRNA) led to a marked reduction of TIM levels in S2 cells (Figure 2F). SGG overexpression also increased TIM levels, but only marginally (Figures 2F and 2G). The effect of SGG on TIM levels was confirmed in brains and heads of SGG-expressing flies. In both dark (Figure 2H and data not shown) and light (Figure 2H, Figure 4A, and data not shown) conditions, TIM staining was notably more intense in the SGG overexpression genotype than in wild-type.

Because our immunoprecipitation experiments could not demonstrate a SGG-TIM interaction, and because CRY is important for TIM degradation (Lin et al., 2001), we examined if CRY mediates the effect of SGG on TIM, by reducing endogenous CRY levels of S2 cells with *cry* dsRNA; indeed, this partially prevented TIM degradation by *sgg* dsRNA (Figure 2G). The results indicate that the SGG effects on TIM stability are at least partly CRY-dependent and suggest that there should be a substantial difference in circadian period between *timSGG* and *timSGG/cry^b* flies in DD. Indeed, *timSGG* flies have a 20.3 hr period as previously reported (Figure 2I; Martinek et al., 2001), whereas *timSGG/cry^b* flies have a period markedly longer and closer to that of wild-type (τ = 22.6 hr). Since *cry^b* alone does not lengthen period in DD, the results are consistent with the notion that the SGG effect on the clock machinery is mediated at least in part by CRY.

These previous results indicate that SGG may modulate the biochemical cascade of light entrainment. To test this hypothesis, we examined the light-mediated disappearance of TIM signal from circadian neurons at Zeitgeber Time 1 (ZT1), 1 hr after lights-on (Figure 3). In wild-type flies, signal is gone from all brain clock cells at ZT1. In *cry^b* flies, TIM is still strongly present at ZT1 and maintains a predominantly nuclear localization (~90% of the examined brains), the expected result for flies missing a key component of the light-signaling pathway. A similar result was observed in *timSGG* (in ~40% of the brains), confirming that SGG overexpression strongly affects clock entrainment by light. When SGG is overexpressed only in non-pigment-dispersing-factor (PDF) circadian cells (E-cells; *timSGG/PdfGAL80*), the TIM signal disappears only in PDF cells while remaining detectable in other clock-gene-expressing neurons at ZT1 (data not shown), indicating that SGG overexpression acts cell autonomously.

E-Cells Are the Primary Source of Photoentrainment

The cell autonomy of SGG action allowed us to ask which circadian neurons contribute to SGG-mediated LL rhythmicity. Because the small ventral lateral neurons (s-LN$_v$s, or M-cells) express the neuropeptide PDF and had been shown to be dominant pacemaker cells in DD (Stoleru et al., 2005), a *Pdf-GAL4* driver was used to restrict SGG overexpression to these cells (*Pdf-GAL4/UAS-sgg*, or *PdfSGG*). Surprisingly, *PdfSGG* flies were fully arrhythmic in LL (Figure 4A), identical to wild-type flies. In contrast, SGG overexpression in the E-cell groups (LN$_d$s, DN1s, and DN3s; *timSGG/PdfGAL80*) (Stoleru et al., 2005) gave rise to LL rhythmicity (Figure 4A). Moreover, these flies were as LL rhythmic as *cry^b* (Figure 1) and more rhythmic than flies overexpressing SGG in the entire cell network (>90% versus 50% for *timSGG*). An additional genotype verified that SGG overexpression in E-cells (CRY$^+$PDF$^-$) is crucial: *timSGG/cry-GAL80* flies exclude SGG expression from M- and E-cells (Stoleru et al., 2005) and are fully arrhythmic in a way indistinguishable from that of wild-type flies (Figure 4A).

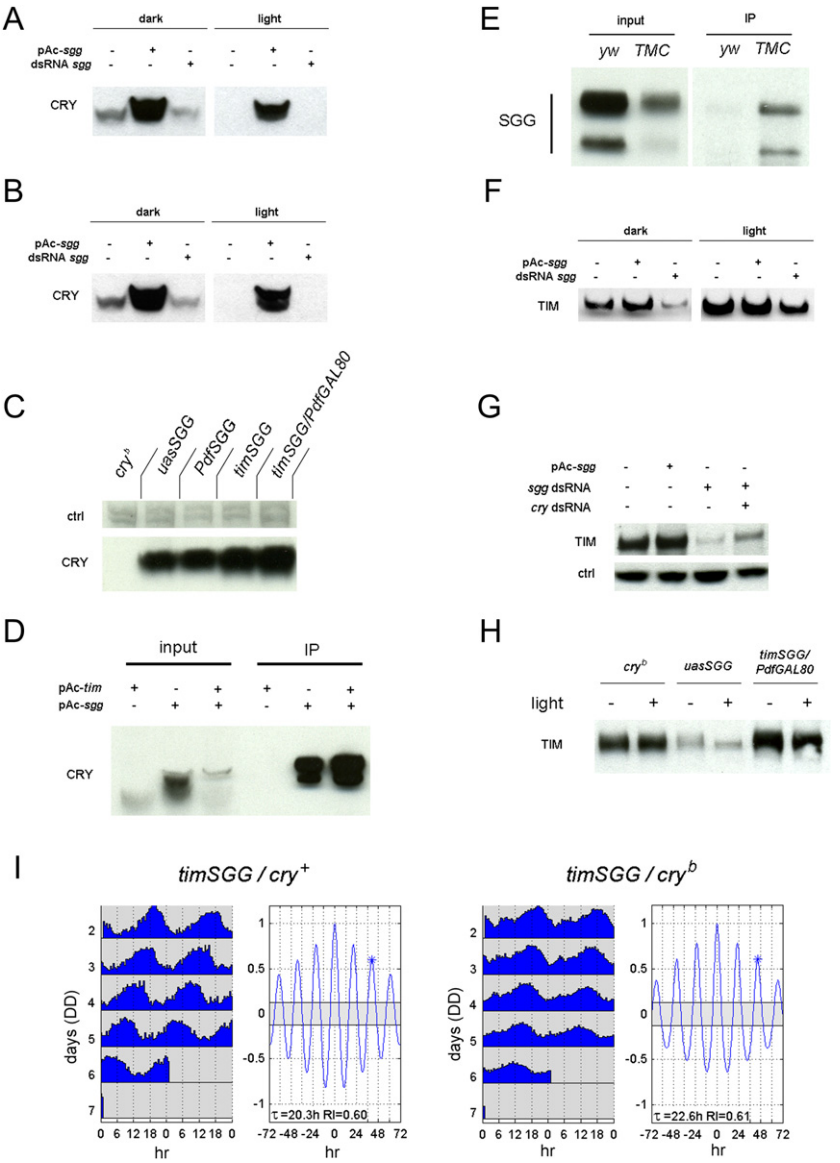

Figure 2. SGG Interacts Directly with CRY and Modulates the Stability of TIM and CRY

(A and B) SGG expression levels affect CRY stability independent of TIM in S2 cells. SGG was overexpressed by pAc-*sgg*-V5 or inactivated by *sgg* dsRNA, in dark (left panel) or light (right) conditions; CRY levels were assayed by western blot. TIM was cotransfected in (A), but not in (B).

(C) Overexpression of SGG increases CRY stability in flies. CRY western blots from fly heads of different SGG genotypes maintained in DD (CT22) are shown. CT is the circadian time within a free-run experiment, with hour CT0 corresponding to the beginning of a new cycle.

(D and E) SGG interacts with CRY in S2 cells and in flies. SGG immunoprecipitation (IP) from transfected S2 cells, with anti-V5 antibody followed by CRY western blot (D, left lanes: input; right lanes: IP). IP of CRY from *y w* and *TMC* fly heads with anti-MYC beads followed by SGG western blot (E, left panel: input; right panel: IP) is shown.

(F and G) SGG expression level affects TIM stability in a CRY-dependent manner in S2 cells. TIM was detected by western blot; SGG was expressed with pAc-*sgg*-V5 or inactivated with *sgg* dsRNA. In (G), CRY was also inactivated by *cry* dsRNA (last lane).

(H) Overexpression of SGG increases TIM stability in flies. CRY western blots from fly heads of different genotypes exposed to either DD (CT22) or LL (CT22) are shown.

(I) Average locomotor activity of flies overexpressing SGG in clock cells in wild-type background (left panels) and in cry^b mutant background (right). Behavioral periods and rhythm indices are shown under the plots.

To verify the major role of E-cells in circadian light perception, we rescued the circadian-blind cry^b genotype only in this subset of the circadian network (*tim-GAL4/UAS-myc-cry/PdfGAL80/*cry^b). LL arrhythmicity was completely restored, similar to that of wild-type flies and flies in which CRY function was rescued in all clock cells (*tim-GAL4/UAS-myc-cry/*cry^b, or *TMC/*cry^b). Rescue in PDF^+ cells only was much more limited, as more than 50% of

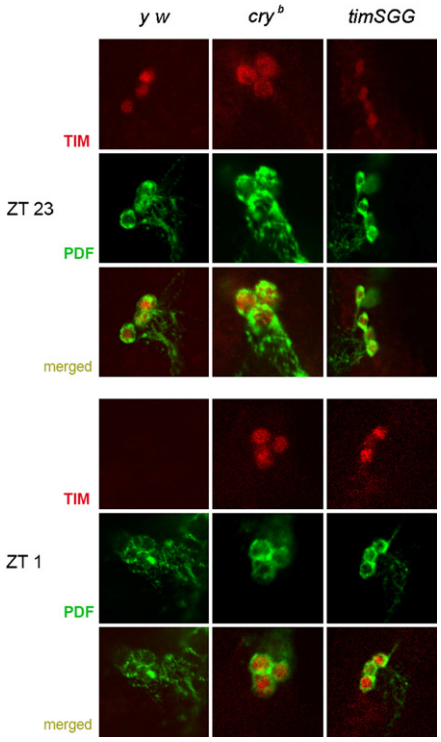

Figure 3. SGG Overexpression Disrupts the Normal Light-Mediated Degradation of TIM

The effect of light onset on clock proteins in s-LN_vs, at the transition between the third and fourth day in LD. Comparison of TIM expression levels (red), 1 hr before lights-on (ZT23, top half panels) and 1 hr after lights-on (ZT1, bottom) in three genotypes (*y w*, left; *cry*^{b}, middle; *timSGG*, right) is shown. PDF stainings (green) confirm the identity of LN_v cells.

these flies are still rhythmic (*Pdf-GAL4/UAS-cry/cry*^{b}) (Emery et al., 2000b; data not shown). This indicates that the E-cell CRY is a major contributor to circadian photoreception.

We then used these CRY-rescued flies to characterize the E-clock contribution to entrainment by analyzing their PRCs; this is a robust assay for circadian light responsiveness (Figure 4B). In wild-type flies, light pulses cause phase delays in the early night and phase advances in the late night. These both require CRY, because there is little or no phase response in the *cry*^{b} mutant (Stanewsky et al., 1998). The PRC of *TMC/PdfGAL80/cry*^{b} was indistinguishable from that of *TMC/cry*^{b} flies (Figure 4B), which is very similar to that of wild-type flies (Figure 4C, black curve). This is consistent with the rescue of LL arrhythmicity shown above and indicates that E-cell rescue alone provides robust light entrainment. In contrast, *cry* rescue restricted to M-cells alone was much more limited and had a severely attenuated PRC in the early night-delay zone (Emery et al., 2000b; data not shown).

We compared these results with PRCs from flies in which molecular entrainment was disrupted by SGG expression in specific clock cells (Figure 4C). The *PdfSGG* PRC was indistinguishable from that of wild-type-like flies (Figure 4C; *UAS-sgg* control), whereas the *timSGG/PdfGAL80* PRC was very similar to that of *timSGG*, which is indistinguishable from the originally described PRC of this genotype (Martinek et al., 2001). These two transgenic strains have attenuated delay zone amplitudes and earlier transitions (breakpoints) between the delay and advance zones (Figure 4C). These unusual PRCs require SGG overexpression in the E-cells, as *timSGG/cryGAL80* flies suppress SGG overexpression in E-cells and have a PRC more similar to that of wild-type flies (Figure 4C). Taken together with the CRY rescue data shown above, these results reinforce the importance of the E-cells in the light entrainment system. More importantly, the shifted (early) PRC breakpoints suggest that E-cells are driving the clock under LD conditions (Rutila et al., 1998). This is because the intrinsic E-cell clock oscillates faster in these two genotypes (*timSGG* and *timSGG/PdfGAL80*) (Stoleru et al., 2005). Based on this consideration, the PDF pacemaker cells make no detectable contribution to clock speed in this light paradigm.

A Subset of E-Cells Drives the Rhythm in Presence of Light

To verify the hypothesis that E-cells make an important contribution to clock pace in light, we performed PER-TIM double staining of brains from *timSGG/PdfGAL80* flies incubated in LL (Figure 5A and Figure S4 in the Supplemental Data). The data show that the DN1s (a subset of E-cells) and DN2s undergo robust cycling of PER nuclear localization in LL. The phase of PER nuclear localization was similar to that observed in DD in circadian neurons of flies with similar behavioral period (ca. 24 hr), i.e., PER nuclear accumulation reached a peak toward the end of the cycle (Shafer et al., 2002). It is intriguing that no significant PER signal intensity oscillations were visible. Moreover, all time points showed no nuclear TIM signal and the same constant cytoplasmic TIM levels in these dorsal cells, despite the oscillations in PER nuclear localization. Finally, there was no detectable cycling of any kind in PDF cells (Figure 5B and data not shown). As LL-arrhythmic wild-type brains exhibited no cycling in any clock cells, the data indicate that E-cells run the rhythmic program in light.

To further confirm this surprising conclusion, we turned to additional LL-rhythmic genotypes. We incidentally found that PER overexpression in E-cells also gives rise to LL rhythmicity (*tim-GAL4/UAS-per/PdfGAL80*). We expressed three different *UAS-per* transgenes (Kaneko et al., 2000; Yang and Sehgal, 2001), alone and in combination, and all strains gave rise to robust LL rhythmicity (Figure 6A).

Overexpression presumably mitigates PER degradation by LL exposure, an interpretation further supported by LL behavioral rhythmicity when TIM expression is driven in E-cells (*tim-GAL4/UAS-tim/PdfGAL80*; Figure 6B). Because TIM and PER degradation is downstream of the CRY signaling pathway, these results may mimic the

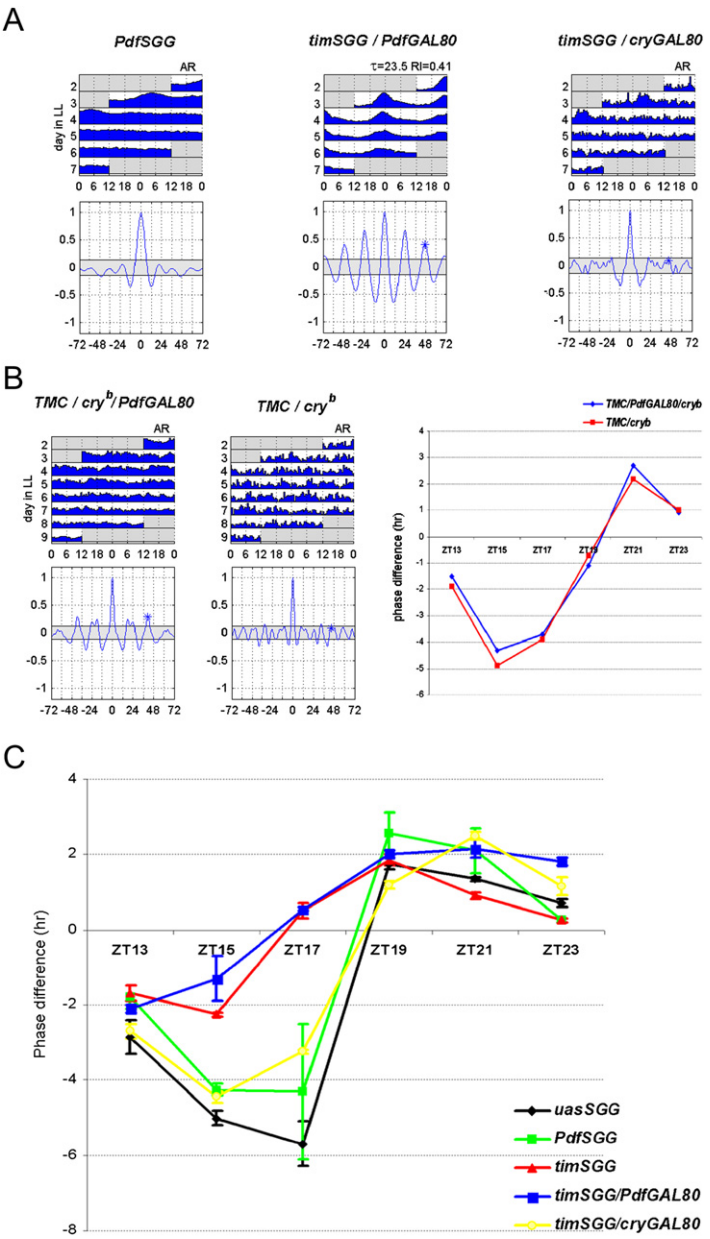

Figure 4. Light Entrainment Derives from the E-Cells

(A) Overexpression of SGG in E-cells only causes LL rhythmicity. Average activity profiles in LL2–LL5: *PdfSGG* (left panels; n = 64); *timSGG/PdfGAL80* (middle; n = 64); *timSGG/cryGAL80* (right; n = 64). The panels are analogous to those in Figure 1.

(B) CRY rescue in E-cells only restores the normal light response. Average activity profiles of *cry* rescue genotypes: *TMC/PdfGAL80/cry*^{b} (left panel) and *TMC/cry*^{b} (middle, see text for details). The right panel represents the PRC of *cry* rescue genotypes. The time onset of the photic stimuli was plotted on the x axis in hr (ZT). The phase response was plotted on y axis as the difference (hr) from the phase of nontreated flies.

(C) Overexpression of SGG in E-cells disrupts the PRC and entrainment. PRCs of genotypes expressing SGG in different cell groups are shown. The phase response was plotted as above. The values represent averages of three independent experiments, with error bars indicating SEM.

upstream effect of SGG overexpression on CRY stability. In addition, there is an interesting deterministic relationship between PER expression levels (Yang and Sehgal, 2001) in E-cells and LL period (Figure 6A), consistent with the notion that the E-cell clock determines period in LL. Emery and colleagues have independently come to the same conclusion based on PER overexpression (Murad et al., 2007). In contrast to the importance of E-cells in LL, PDF cells make no apparent contribution to behavioral clock pace under these conditions. This is because the DD period, and therefore M-cell clock speed in darkness, was ca. 24 hr in all these strains with different E-cell PER expression levels and different LL periods (Figure S3). This conclusion is also consistent with the failure to observe molecular oscillations in PDF cells of the rhythmic *timSGG/PdfGAL80* flies.

There are indications, however, that PDF^{+} cells influence the circadian program even in LL. This is because protecting them from either SGG or PER overexpression (with *PdfGAL80*) dramatically improves behavioral rhythmicity (Figure 1 versus Figure 4A for SGG; Figure 6A for PER). In the most striking example, *timSGG/PdfGAL80* flies differ from *timSGG* flies not only in rhythm strength, but also in the coherence of the LL phenotype. Although the average period was not significantly affected, the periods of individual *timSGG* flies ranged from 19.8 hr to 27.3 hr (Figures S1 and S2), whereas *timSGG/PdfGAL80* periods were strongly coherent around 23.5 hr (Figure S2). Because

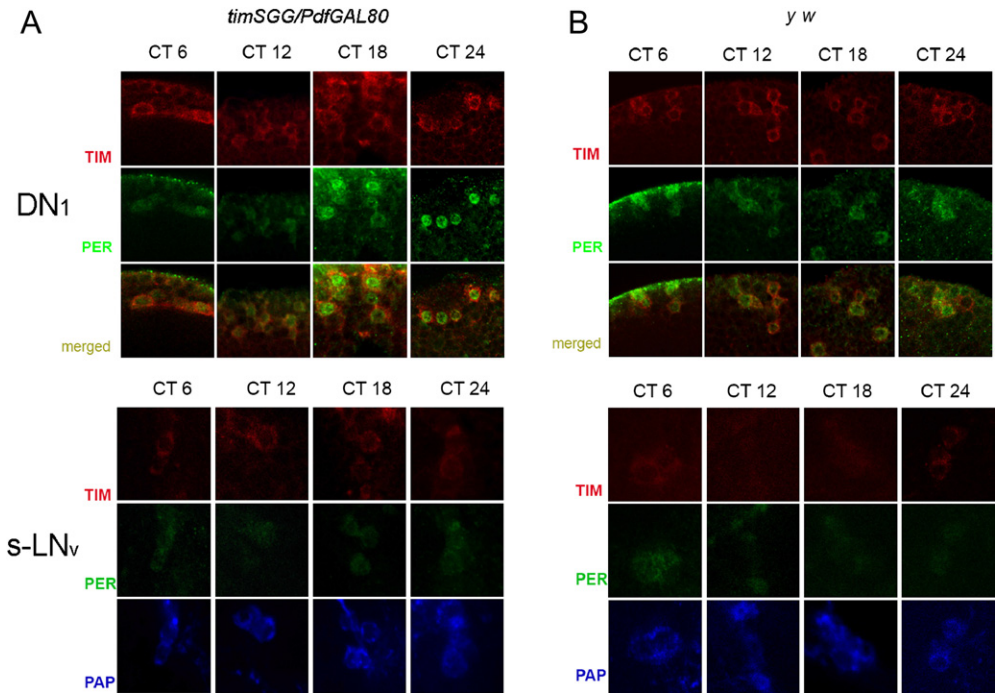

Figure 5. A Subset of E-Cells of LL Rhythmic Flies Maintains Molecular Cycling in Constant Light

Comparison of TIM and PER expression levels in LL in two genotypes: (A) an LL behaviorally rhythmic one (*timSGG/PdfGAL80*) and (B) an LL arrhythmic control (*y w*). Double staining of TIM (red) and PER (green) is shown for DN1 cells (top panels) and s-LN$_v$s (bottom). s-LN$_v$s are identified with PAP staining (blue). The experiment was done after 2 days of exposure to LL. Time points are indicated above each panel.

we suspected that the PDF$^+$ cells might provide some internal synchronization function (Rieger et al., 2006; Stoleru et al., 2005), we also assayed *timSGG* in a *Pdf* null mutant background (*timSGG/Pdf*^{01}) in LL; these flies were fully arrhythmic (Figure S5). The data indicate that M-cells, perhaps through PDF, affect system rhythmicity in LL and suggest that E-cells may not be fully autonomous oscillators under constant illumination conditions.

E-Cells and M-Cells Alternate the Role of Setting the Phase of Rhythmic Locomotor Activity According to the Photoperiod

This effect of M-cells in light reminded us that functional cooperation is a likely foundation of normal oscillations under more physiologically relevant LD conditions (Stoleru et al., 2004). Moreover, M-cells synchronize the network and are master clocks at night (Stoleru et al., 2005), complementing the function of E-cells in the light. These concepts suggested that the master clock role may switch between E- and M-cells within the same LD cycle, with E-cells timing events during day and M-cells timing events at night. This predicts that E-clocks should have no effect on morning activity phase and M-clocks no effect on evening phase, which we tested by increasing the pace of the two oscillators separately (E-cell advance, *timSGG/PdfGAL80*; M-cell advance, *PdfSGG* flies). The phase of the two outputs was measured in these genotypes by calculating the anticipation indexes (AI; an LD activity phase surrogate) (Stoleru et al., 2004) for both morning and evening activity under standard LD conditions.

Contrary to the prediction, morning anticipation was robustly advanced by a fast E-clock (as well as by M-cell acceleration; Figures 7A and 7B), indicating that the E- clock not only influences the direct evening activity output, but also the phase of morning activity, and therefore the M-cell oscillator, in standard LD conditions. The results confirmed our previous suggestion that E-cells can drive the morning output in the presence of light (Stoleru et al., 2004). Although fast M-cells had no significant effect on the phase of evening output under these standard LD conditions, the dominant role of M-cells on the pace of evening oscillations in DD (Stoleru et al., 2004, 2005) hinted that M-cells might influence evening activity phase under different LD conditions, i.e., those with longer nights (Figure 8), a notion that suggests a different explanation for the alternating master/slave relationship, namely, a seasonal dominance of one oscillator or the other.

To test this possibility, we exposed the same two strains with the two oscillators running at different speeds (*timSGG/PdfGAL80* and *PdfSGG*) to different photoperiods. With the short photoperiod entrainment regime (winter-like; L:D 10:14), the fast M-cell strain (*PdfSGG*) advanced evening as well as morning activity, whereas fast E-cells (*timSGG/PdfGAL80*) were now ineffective in advancing morning activity (Figures 8A and 8C and Figure S6). The opposite result was obtained when flies

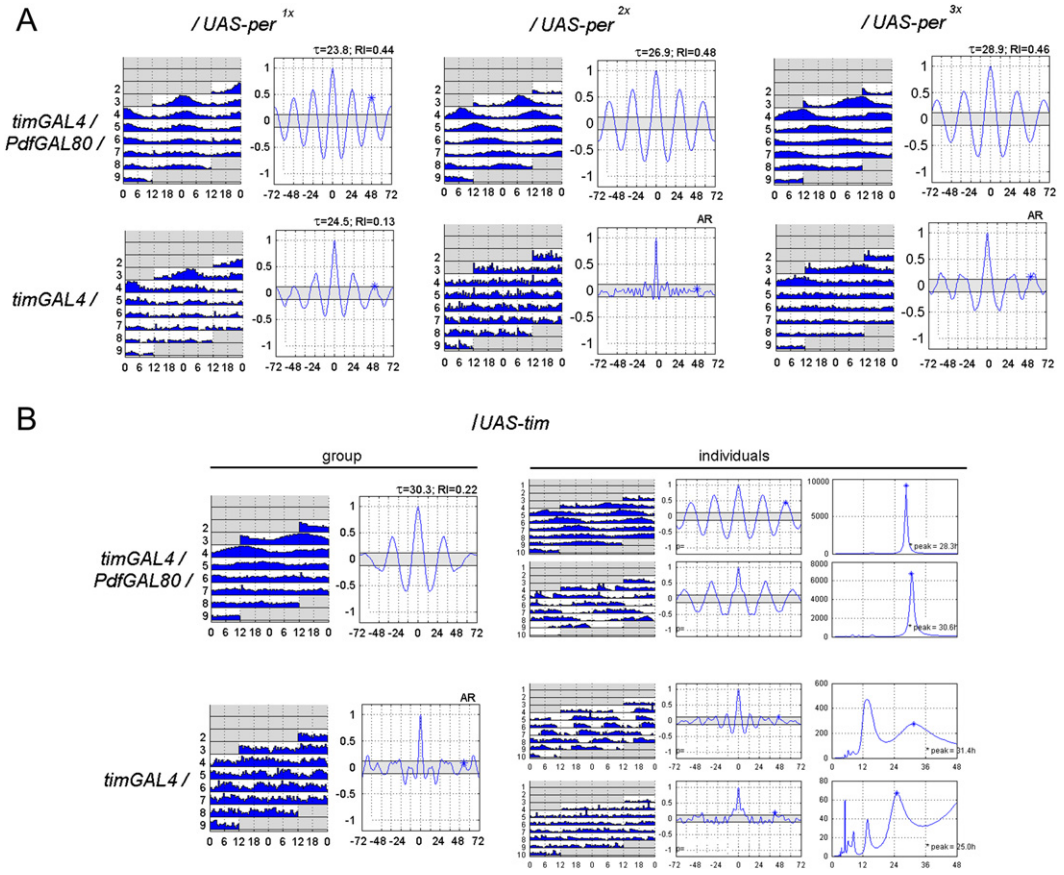

Figure 6. E-Clocks Drive Behavioral Rhythms in LL

LL2–LL7 activity profiles of groups of flies that overexpress PER or TIM in either E-cells (top panels: with a *timGAL4/PdfGAL80* driver) or in all clock cells (bottom: *timGAL4*). (A) PER overexpression. Three different combinations of *UAS-per* transgenes are shown: *UAS-per2.4* (1x copy of *per* transgene; left); *UAS-per10* (2x copies of *per*; middle); *UAS-per2.4/+;UAS-per3.1/UAS-per3.1* (3x copies of *per*; right).

(B) TIM overexpression with a *UAS-tim* transgene. The right panels represent individual behavioral plots typical for each genotype, analyzed on the left as a group (from left to right: double-plotted actogram/autocorrelation/MESA spectral analysis [Levine et al., 2002b]); n = 64 flies were used in all experiments shown in (A) and (B).

were exposed to extended photoperiod (summer-like; L:D 14:10; Figures 8B and 8D and Figure S7); i.e., E-clocks controlled the phase of both evening and morning activity, whereas M-cells affected principally their own direct output (the morning behavior). The results indeed reflect a winter/darkness-dominance of M-cells and a summer/light-dominance of E-cells (as indicated graphically in Figures 8A and 8B).

To verify these conclusions in different genotypes, we entrained flies carrying nonfunctional M-clocks (*per^0*/*elav-GAL4/uas-per24/PdfGAL80*) to different photoperiods. We previously showed that this genotype behaves similarly to wild-type flies in standard LD conditions (Stoleru et al., 2004). The phase of both morning and evening behavior adjusted well to summer-like extended photoperiod (Figure S8), but morning anticipation failed to adjust properly under winter-like short photoperiods (Figure S9) in the clockless M-cell flies. We suggest that the differential circadian photoentrainment features of E-cells and M-cells (Figure 4) together with interoscillator communication (Stoleru et al., 2004, 2005) underlies circadian adjustment to changes in photoperiod, or seasonal adaptation (Figure 8 and Figures S8 and S9).

DISCUSSION

The free-running pacemaker and entrainment are two important and increasingly understood aspects of circadian rhythms. In contrast, little information exists about seasonal adjustment, namely, how a constant ~24-hr timekeeper accommodates dramatically different photoperiods. We show here that the previously defined dual oscillator system in *Drosophila*, M-cells and E-cells, creates different rhythmic patterns by alternating master clock roles. This understanding emerged from restricting SGG overexpression to E-cells, which allowed the E-oscillator to function and render flies rhythmic in LL. SGG probably modulates CRY activity and, when overexpressed,

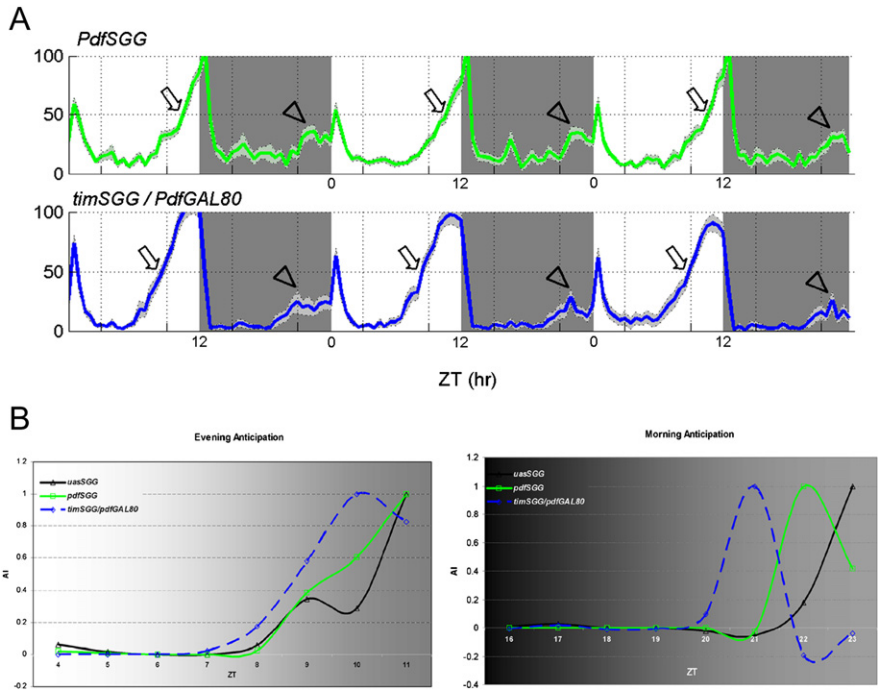

Figure 7. In LD, E-Clocks Set the Phase of both Evening and Morning Activity

(A) Phase comparison of locomotor activity of flies overexpressing SGG in either M-cells (*PdfSGG*, fast M-clock; green) or E-cells (*timSGG/PdfGAL80*, fast E-clock; blue [Levine et al., 2002a]). Three standard LD days are shown, with timing indicated by alternating white and gray background areas: white represents the illuminated interval of LD (ZT0–ZT12), whereas gray is the dark period (ZT12–ZT24). The arrows indicate the phase of evening anticipation, whereas the arrowheads point toward the morning anticipations (n > 100 flies for each experiment).

(B) Normalized anticipation index (AI) (Stoleru et al., 2004) plotted as function of time (1 hr bin), for an interval of 8 hr before light transitions (ZT4–ZT11 for L/D transition, left panel; ZT16– ZT23 for D/L transition, right panel). The plots were generated using data from the same experiments for which results are shown in Figure 7A. AI of wild-type control flies is shown in black. The plot background left-right gradients represent the quality of the light transition: black/gray gradient reflects the D/L transitions (mornings), whereas the white/gray gradient suggests the L/D transitions of evenings. No gradient in light intensity has been applied during either dark or light intervals of these typical standard LD experiments.

provides sufficient PER and TIM to allow E-oscillator function under constant illumination conditions. The E-clocks therefore manifest free-running properties and function as the master pacemakers in LL, analogous to our previous finding that the M-oscillator is the master in DD (Stoleru et al., 2005). Nonetheless, these constant conditions, and even the perfect standard LD cycles commonly used in the laboratory, are poor approximations of the changing LD environments found in nature. Circadian oscillators and their entrainment mechanisms have adapted to the dramatic seasonal changes in photoperiod. Our previous strategy of using oscillators with different speeds, combined with different photoperiods, has led to a model of alternating control between the M-oscillator and E-oscillator.

SGG appears to attenuate, rather than inactivate, CRY activity in E-cells. This is because the LL period of *timSGG/PdfGAL80* (~23.5 hr) is longer than the intrinsic period of SGG-expressing E-clocks in DD (~21 hr) (Stoleru et al., 2005). A longer period in light is compatible with attenuated light perception under our high light intensity conditions (1600 lx, which renders wild-type flies completely arrhythmic) and the application of Aschoff's rule to insects (Aschoff, 1979). As there is also a prominent effect on CRY stability, SGG may be the regulator previously predicted to bind to the CRY C terminus (Busza et al., 2004; Dissel et al., 2004). Although we favor CRY as the major circadian substrate of SGG, there may be others, e.g., the serotonin receptor (Yuan et al., 2005). Biochemical support for GSK3 involvement in mammalian rhythms has recently been obtained (Yin et al., 2006). As GSK3 is a proposed therapeutic target of lithium (Stambolic et al., 1996), the relationship between SGG and CRY reported here recalls the intriguing relationship between mood disorders, light sensitivity, and circadian rhythms (Magnusson and Boivin, 2003).

The cry^b genotype markedly affects DD period in some of the rhythmic genotypes described here. Although CRY is probably unnecessary for M-cell rhythmicity (Stanewsky et al., 1998), this could reflect some redundancy or assay insensitivity. Moreover, the DD period of cry^b is slightly shorter than that of wild-type (23.7 versus 24.4) (Stanewsky et al., 1998), suggesting that "dark CRY" makes some contribution to pacemaker function in M-cells as well as E-cells. For these reasons, we suggest that *Drosophila* CRY is closer to the central pacemaker

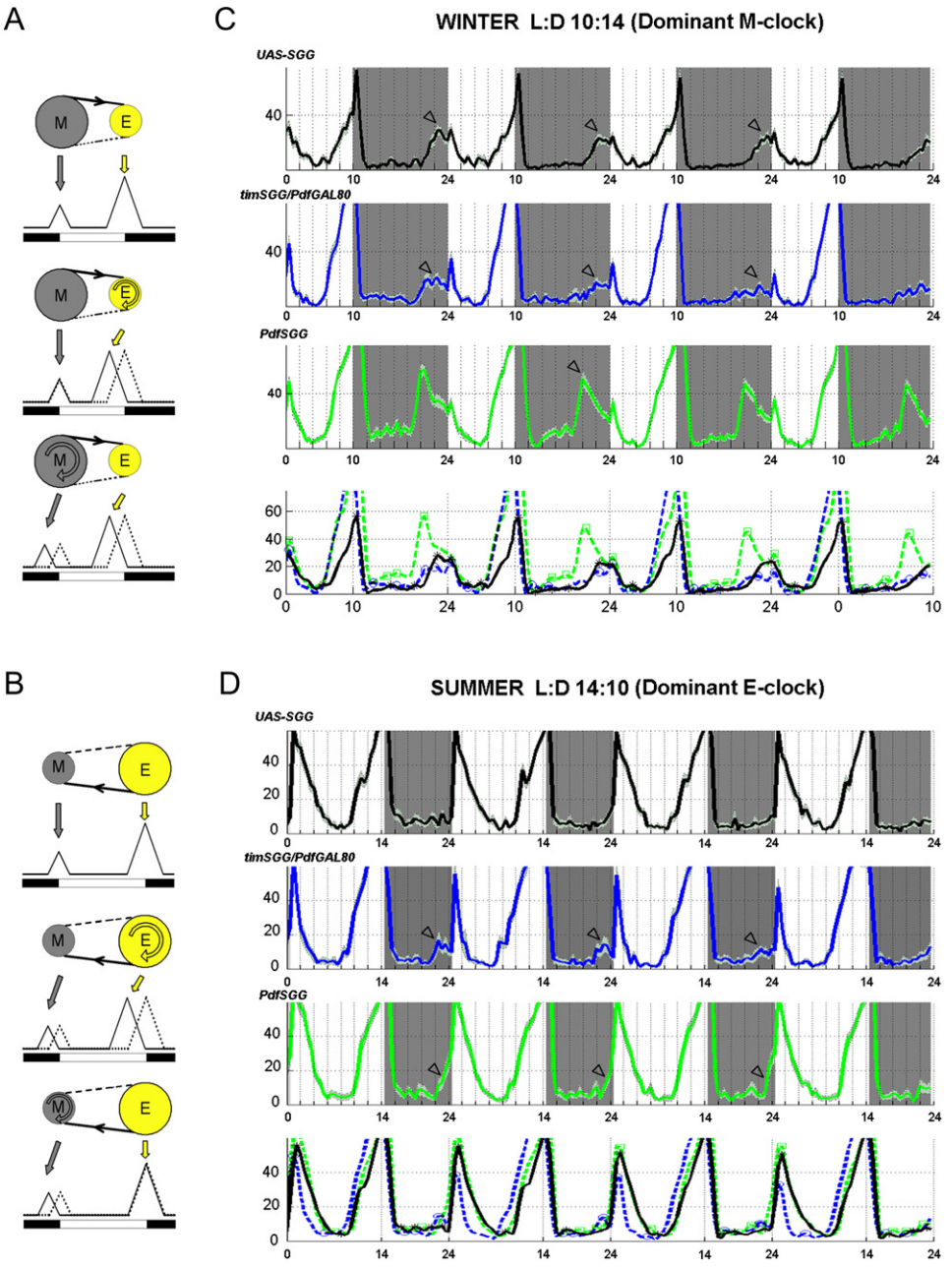

Figure 8. The Circadian Neuronal Network Adjusts Behavior to Seasonal Changes by Alternating the Master Clock Role between M- and E-Cells

(A and B) Hypothetical model of neuronal-network-based seasonal adaptation. Photoperiod determines the master clock identity. The long nights of winter-like photoperiods cause M-clocks to run the entire circadian cellular network and set the timing (phase) of both M- and E-cell output. The long days of summer prevent the scotophilic M-cells from developing robust clock protein expression, whereas the photophilic E-cells undergo clock protein cycling and function as master clocks in this condition. Changing the pace of the master clock changes the phase of both M- and E-cell output, whereas manipulating the pace of the slave only affects the direct output of this oscillator. (See Discussion for details).

(C and D) Model predictions tested. Phase comparison of flies expressing SGG in either E-cells (fast E-clocks, blue) or M-cells (fast M-clocks, green), exposed to different photoperiods. In (C), winter-like conditions are simulated by an L:D pattern of 10:14 hr; in (D), summer is simulated by an alternation of 14:10 hr of L:D. The light and dark periods are indicated as in Figure 7A. Top panels represent group phase analysis results for the indicated genotypes, as in Figure 7A. The bottom panels represent superpositions of the same results, after a smoothing filter was applied for identification of the peaks (Levine et al., 2002a).

than previously believed, and therefore closer to the level of importance of its mammalian paralogues in influencing free-running pacemaker activity. Unlike mammalian CRY, however, *Drosophila* CRY still appears to function predominantly at a posttranslational level. Indeed, the effects of cry^b on SGG overexpression in DD suggest that the proposed effect of SGG on TIM stability is really an effect of SGG on CRY followed by an altered CRY-TIM interaction. We note that there is a recent proposal that *Drosophila* CRY, like mammalian CRY, also functions as a transcription factor in peripheral clocks (Collins et al., 2006).

The importance of E-cells in LL rhythmicity is underscored by the staining results of *timSGG/PdfGAL80* brains (Figure 5A). Only some E-cells and DN2s manifest robust cycling. We had suspected that E-cells were important in light because they could rescue the output of arrhythmic M-cells in LD, but not in DD (Stoleru et al., 2004). Indeed, all of these observations make it attractive to view E-cells as autonomous pacemakers. There is, however, evidence that M-cells may not be completely dispensable (Figure 4, Figure 6, and Figure S2). Moreover, a synchronizing or stabilization function is compatible with previous observations under different conditions (Lin et al., 2004; Peng et al., 2003).

In the *timSGG/PdfGAL80* genotype, only PER nuclear localization changes were detectable near the end of LL cycle (Figure 5). The nature of the assay makes it hard to conclude that there were no differences in total PER staining intensity, i.e., no oscillations in PER levels, so the unique nature of the PER nuclear localization cycling is a tentative conclusion. The same caveat applies to the absence of TIM oscillations and nuclear staining, i.e., negative results cannot exclude low-amplitude oscillations; we note, however, that TIM cytoplasmic sequestration has been previously observed in cry^b flies after several days in LL (Rieger et al., 2006). Furthermore, the circadian nuclear accumulation of TIM has been shown to respond differently than that of PER to changes in photoperiod (Shafer et al., 2004). Nonetheless, TIM could be shuttling with a predominant steady-state cytoplasmic localization, nuclear TIM could be rapidly degraded to create a low nuclear pool, or both.

The importance of E-cells in entrainment is strongly supported by the potent effect of restricted CRY rescue of cry^b: E-cell rescue is much more impressive than M-cell rescue. Moreover, the differences between the two rescued PRCs are striking; E-cell rescue is virtually complete (Figure 4D), whereas the M-cell rescue is notably deficient in the delay zone (Emery et al., 2000b). In addition, flies with SGG overexpression in E-cells show altered PRCs, whereas flies with SGG overexpression in M-cells respond normally to light. The results are strikingly different in darkness, as M-cell-restricted expression causes the typical short period determined by SGG overexpression, whereas E-cell overexpression has no systemic effect.

The PRC delay zone is the region impacted most strongly by E-cell SGG overexpression (Figure 4C), indicating that the lights-off early night region is most important to E-cell function and light entrainment. Exposure to light in this interval should mimic long days (summer), which we speculate will delay phase by many hours so that "evening" output of the following day will coincide with the objective evening of the environment. Even the short nights of summer are probably enough time for E-clocks to accumulate sufficient TIM and PER, shuttle them into the nucleus, and reconstitute the rhythmic substrate observed in the SGG-overexpressing brains in LL. In contrast, M-cells need darkness to cycle robustly. They will become the master clocks and drive the system whenever lights fail to turn on more than 12 hr past lights-off, i.e., during the long nights of winter that mimic the beginning of a DD cycle. Since the intrinsic pacemaker program of M-cells in darkness relies on the changing nature of clock proteins during the night, we hypothesize that the activity phases under long nights (winter) are locked to lights-off. This suggestion is supported by preliminary data (not shown) and previous observations showing that *per* transcription remains locked to lights-off under different entrainment regimes (Qiu and Hardin, 1996). M-cells are also capable of fully entraining the system in the PRC interval that determines a phase advance (late night) (Emery et al., 2000b). This is consistent with their predicted role in generating an advanced evening output, coincident with the early evenings typical of winter. Otherwise put, long summer days should underlie light primacy as well as long and prominent evening delay zones; both suggest E-cell dominance. Night primacy and M-cells should dominate under winter conditions. This concept endows E- and M-cells with the properties originally envisioned by the Pittendrigh and Daan dual-oscillator model of entrainment (Pittendrigh and Daan, 1976).

EXPERIMENTAL PROCEDURES

Fly Strains and Behavioral Assays

Fly lines used were all previously described. Locomotor activity was monitored and analyzed as previously described (Hamblen et al., 1986; Levine et al., 2002b; Stoleru et al., 2004). For all LL experiments, light intensity was 1600 ± 200 lx. Analysis of LL behavior did not include data from the first day of LL (after lights were left permanently on), as arrhythmicity occurs only after one full day of LL in wild-type flies.

PRC experiments were performed as previously described (Stanewsky et al., 1998). Briefly, flies were exposed to a single 15 min light pulse of 1600 (± 200) lx at each time point. The phase calculation was performed as described elsewhere (Stoleru et al., 2005) (by using algorithms described in Levine et al., 2002a).

For the photoperiod experiments, flies were entrained to the respective photoperiod for 3 days prior to any data recording. The AI for a certain bin of activity has been previously described (Stoleru et al., 2004). In the experiment shown in Figure 7, AI was calculated and plotted for individual bins before the light transitions, using the group histogram data. Almost all experiments have been repeated more than three times with similar results. The only exceptions are the *cry* rescue PRC experiments shown in Figure 4B, and the per^0 rescue photoperiod experiments (Figures S8 and S9); they have been performed twice.

Fly Brain Immunocytochemistry

The immunostaining, anti-TIM, anti-PER, anti-PDF, and anti-PAP antibodies and secondary antibodies have been described previously

(Shafer et al., 2002). For immunostaining in LD (Figure 3), flies were entrained for at least 4 days in standard LD conditions before collection and dissection. For LL immunostaining (Figure 5 and Figure S4), flies were entrained for 4 days in standard LD and then exposed to LL. Flies were collected and dissected during the second day in LL. At least 15 fly brains were observed for each time point.

S2 Cell Experiments

S2 cell transfections were performed as described (Nawathean et al., 2005). In experiments requiring light exposure, cells were kept in continuous light (1500 lx) for 12 hr before harvesting, whereas plates were wrapped with two layers of aluminum foil for the dark experiments.

Plasmids

TIM, CRY, and SGG were cloned under control of the actin promoter, as described previously (Nawathean and Rosbash, 2004).

Western Blot and Immunoprecipitation

Fly head and S2 cell western blots, as well as immunoprecipitation, have been previously described (Edery et al., 1994; Nawathean and Rosbash, 2004). Other fly strains, antibodies, plasmids, and general techniques used (Renn et al., 1999; Rorth, 1996; Zeng et al., 1996; Rush et al., 2006; Papadopoulou et al., 2004; Ruel et al., 1999; Worby et al., 2001) are more extensively described in the Supplemental Data.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.cell.com/cgi/content/full/129/1/207/DC1/.

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