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Drosophila Clock Can Generate Ectopic Circadian Clocks

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Summary

Circadian rhythms of behavior, physiology, and gene expression are present in diverse tissues and organisms. The function of the transcriptional activator, *Clock*, is necessary in both *Drosophila* and mammals for the expression of many core clock components. We demonstrate in *Drosophila* that *Clock* misexpression in naïve brain regions induces circadian gene expression. This includes major components of the pacemaker program, as *Clock* also activates the rhythmic expression of *cryptochrome*, a gene that CLOCK normally represses. Moreover, this ectopic clock expression has potent effects on behavior, radically altering locomotor activity patterns. We propose that *Clock* is uniquely able to induce and organize the core elements of interdependent feedback loops necessary for circadian rhythms.

Introduction

Circadian rhythms of gene expression and behavior are widespread in biology. These rhythms are the result of cell-autonomous intracellular clocks that are based in large part on the expression of transcriptional feedback loops (Allada et al., 2001; Panda et al., 2002b). In the fruit fly, *Drosophila melanogaster*, circadian gene expression is driven by circadian clocks present in many tissues and requires the function of the transcriptional activator *Clock* (*Clk*) (Allada et al., 1998; McDonald and Rosbash, 2001). CLK and its heterodimeric partner CYCLE (CYC) directly activate the transcription of the circadian rhythm genes, *period* (*per*) and *timeless* (*tim*) (Darlington et al., 1998; Rutila et al., 1998). PER and TIM feed back directly on CLK and CYC to inhibit their own synthesis (Darlington et al., 1998; Lee et al., 1998, 1999). *Clk* itself is also rhythmically expressed, but its expression is inhibited by CLK/CYC rather than activated (Bae et al., 1998; Darlington et al., 1998; Glossop et al., 1999). Synchronization of rhythmic gene expression to the daily cycle of sunlight involves the blue light photoreceptor, CRYPTOCHROME (CRY; Helfrich-Forster et al., 2001). Of note, *cry* expression is regulated in a similar manner to *Clk* (Emery et al., 1998). The mechanism of *Clk* inhibition of *Clk* and *cry* gene expression has recently been proposed to involve the clock genes *vri* and *Pdp1* (Cyran et al., 2003; Glossop et al., 2003). Interestingly, the expression of *vri* and *Pdp1*, like *per* and *tim*, is dependent on *Clk* (Blau and Young, 1999; McDonald and Rosbash, 2001). As CLK appears to be the limiting factor for CLK/CYC (Bae et al., 2000), it may be the critical factor that directs these interdependent loops and coordinates circadian gene expression.

Although these intracellular feedback loops are present in many tissues, they are not ubiquitous. In the adult fly brain, clock gene expressing neurons are the exception rather than the rule. These rhythmic neurons can be roughly divided into groups of dorsal neuron (DNs) and lateral neuron (LNs). Indeed, the most prominent circadian rhythm in fruit flies, the daily rhythm of rest and activity, appears to be mediated by the LNs (Kaneko, 1998; Renn et al., 1999). The LNs can be subdivided into a dorsal subgroup (LNd) and two ventral subgroups (LNv), consisting of small (LNvS) and large neurons (LNvL). The LNv subgroup is distinguished by its expression of the neuropeptide gene, *pigment dispersing factor* (*pdf*). Null mutants of *pdf* nearly abolish circadian rhythms, indicating that it is a key circadian component (Renn et al., 1999). Although there is no apparent circadian cycling of *pdf* RNA, PDF is rhythmically expressed in the synaptic termini of the LNvS (Park et al., 2000). Genetic disruption of *Clk* dramatically reduces *pdf* expression and alters the neuronal projections of the LNvS (Park et al., 2000). Given the vital role of *Clk* in a plethora of circadian functions, we hypothesized that it may be a decisive factor in determining circadian cell fate.

To investigate this potential role of *Clk*, we used the GAL4/UAS system to misexpress *Clk*. We show that *Clk* is capable of inducing rhythmic gene expression in brain cells that apparently do not express clock genes in wild-type flies. Furthermore, the appearance of these newly rhythmic neurons is correlated with striking effects on diurnal behavior, suggesting that some of these new clocks make functional connections with behavioral output programs. We propose that *Clk* acts as a critical switch to generate circadian rhythmicity.

Results

CLOCK Misexpression Results in Ectopic Circadian *timeless* Expression

To characterize the consequences of *Clk* misexpression, we utilized the GAL4/UAS system (Brand and Perrimon, 1993). Binding sites for the yeast transcription factor GAL4 (upstream activating sequence; UAS) were fused upstream of *Clk* cDNA (*UASClk*). The pattern of *UASClk* expression in transgenic flies is then determined by the spatial and temporal expression pattern of the GAL4 driver. In combination with numerous GAL4 drivers, *UASClk* resulted in developmental lethality (see Experimental Procedures). However, we were able to gen-

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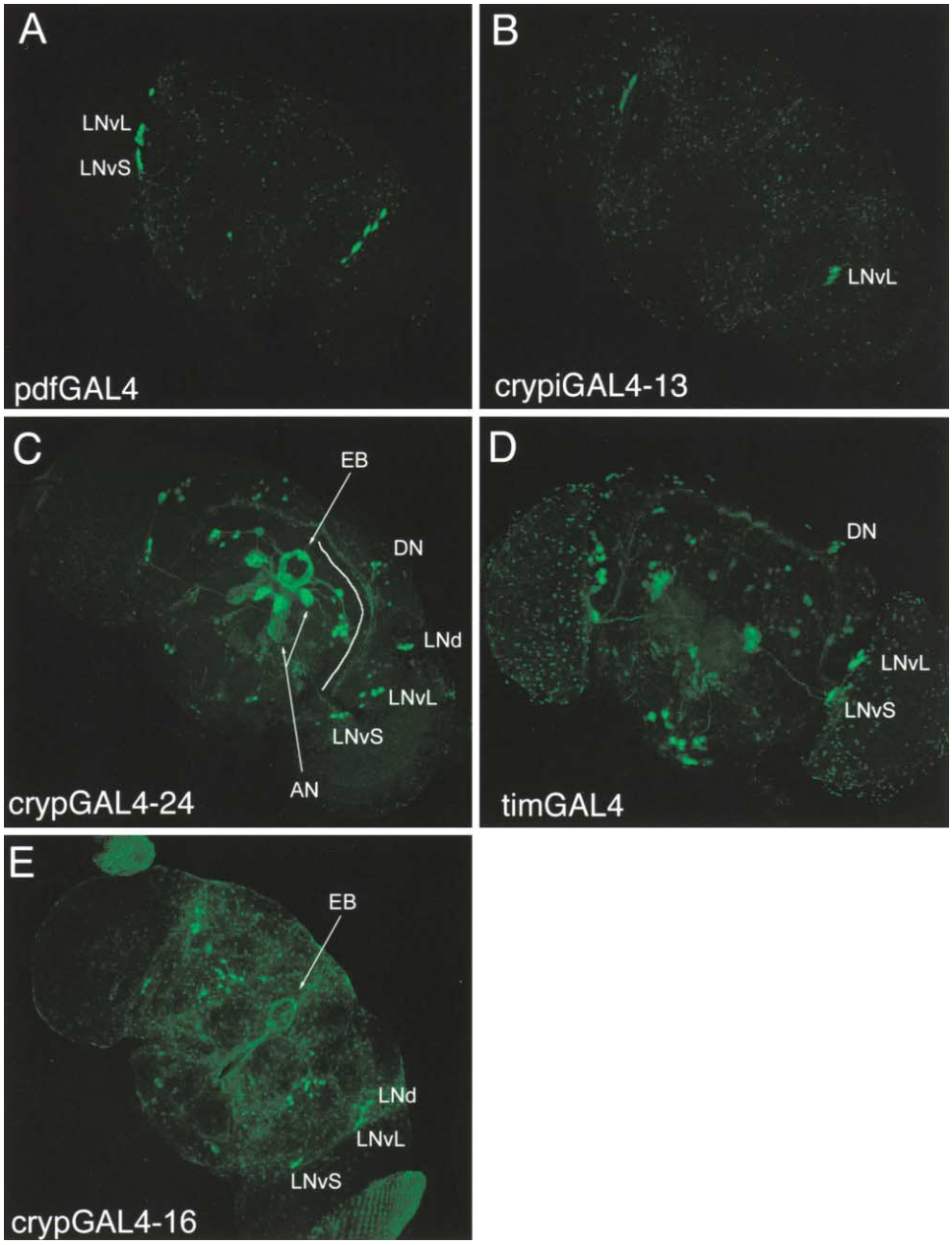

Figure 1. UASEGFP Expression Driven by Different Circadian Gene Promoters

Brains of female flies expressing UASEGFP under the indicated promoters were dissected, fixed, and imaged with confocal microscopy.

(A) *pdf*GAL4 promoter drove GFP expression in the lateral neurons only (LNvL; LNvS).

(B) With the *crypi*GAL4-13 promoter, GFP expression was only visible in the LNvL.

(C) *cryp*GAL4-24 females showed a broad distribution of GFP expression, including regions of novel expression (EB = ellipsoid body, AN = antennal neuropils) in addition to canonical circadian cells (DN, LNd, LNvL, LNvS) Circadian neuronal projections are traced with a solid white line.

(D) *tim*GAL4 promoter drove GFP expression in a broad distribution, but not in all the same cells showing *tim* RNA expression in *cryp*GAL4-24 females.

(E) *cryp*GAL4-16 brains expressed GFP in a pattern similar to but distinct from *cryp*GAL4-24 females. See text for details.

erate viable adult progeny with three clock-relevant drivers: the *pdf* promoter (*pdfGAL4*), a previously described *cry* promoter also containing the large *cry* first intron (*crypiGAL4; p = promoter; i = intron*) and a *cry* promoter without the first intron (*crypGAL4*; Emery et al., 2000; Park et al., 2000).

To characterize the GAL4 expression, these lines were crossed to a *UAS-EGFP* strain and the adult progeny assayed for brain GFP expression (Figure 1). Consistent with previous reports, *pdfGAL4* and *crypiGAL4* (*line 13*; *crypiGAL4-13*) expression is limited to a small number of adult neurons (Emery et al., 2000; Park et al., 2000). *pdfGAL4* expression is restricted to two cell groups whose morphology and position is consistent with the LNvS and LNvL (Figure 1A). The *crypiGAL4* appears to be expressed predominantly in the LNvL (Figure 1B). A previous report indicated that this driver is also expressed in the LNd and the LNvS in addition to the

LNvL (Emery et al., 2000). These three cell groups are a substantial subset of neuronal clock gene expression in the brain. The limited expression from these GAL4 lines contrasts markedly with the broader expression of two independent inserts of *crypGAL4*, *crypGAL4-24*, and *crypGAL4-16* (Figures 1C and 1E). In addition to the canonical circadian cells, expression is observed in other areas, such as the ellipsoid body (EB). Based on their characteristic morphology, many of these cells appear to be neuronal. Interestingly, we observed differences between the two inserts (Figures 1C and 1E). The most salient features of *crypGAL4-16* relative to *crypGAL4-24* were more prominent diffuse glial expression and much less (or absent) expression in the antennal neuropils (AN) as well as in the DNs and in the LNv axons (Figure 1 and data not shown). A third insert (*crypGAL4-17*) did not exhibit any detectable GAL4-driven GFP expression, further indicating that the *crypGAL4* expression pattern is dependent on insert location (data not shown). The ectopic expression patterns do not correspond with that of any known circadian gene and are distinct from that observed for *timeless* promoter-GAL4 (*timGAL4*; Figure 1D).

We then assayed the spatial and temporal expression of the direct *Clk* target gene, *tim*, in *crypGAL4-24/+; UASClk/+* (*cry24*) and wild-type (*y w*) females using fluorescent in situ hybridization (Figure 2). *tim* expression in wild-type flies showed expression restricted to the canonical clock-gene expressing cell groups (Figure 2A, right images). These are the three groups of lateral neurons, including the LNvL, LNvS, and LNd as well as the dorsal neurons (DN1 and DN2; Figure 2A). In contrast, many ectopic *tim*-expressing cells were observed in *cry24* females (Figure 2A, left images). In contrast to *tim*, *pdf* is not expressed ectopically in *cry24* flies (data not shown). To quantify ectopic *tim* expression, we arbitrarily defined three ectopic locations as New1, New2, and New3. The New1 cells in *cry24* flies do not correspond to cells in a similar area identified in *timGAL4* (Figure 1D; Kaneko and Hall, 2000). As both cell groups only appear in the context of GAL4-driven expression, this precludes simple double-labeling experiments. The results are consistent with *UASClk* activation of *tim* in the broad expression pattern of the *crypGAL4-24* driver.

We next determined whether ectopic *tim* mRNA is rhythmically expressed. Under 12 hr light: 12 hr dark (LD) conditions, *y w* flies show robust oscillations in *tim* RNA with a peak at ZT14 and a trough at ZT2 (Figure 2A, right images). The *cry24* flies also displayed robust oscillations, not only in the normal circadian neurons but also in all ectopic locations (Figure 2A, left images). Quantitative analysis indicates that the phase and amplitude of *cry24 tim* cycling is similar to those in wild-type flies in the LNs, DNs, as well as the three new locations (Figure 2B). Indeed, cycling is evident even in many scattered ectopic cells outside of these three groups. The comparable phase and amplitude is remarkable given that *tim* expression levels were substantially higher in *cry24* than in wild-type flies (Figure 2; at ZT8, ZT14, and ZT20). *tim* mRNA oscillations also persisted at least into the second day of constant darkness (DD; Figure 3), indicating that the ectopic oscillations are not purely light-driven. Interestingly, ectopic DD rhythms also occur with comparable phase despite the higher *tim* mRNA levels (Figure 3).

Clock Misexpression Induces Ectopic Rhythmic *cry* Expression

We hypothesized that if CLK is inducing the entire program of circadian gene expression then it should not only induce genes that it directly activates (e.g., *tim*) but also other rhythmically expressed genes that are indirectly regulated or even repressed by CLK. *cry* is rhythmically expressed with a peak and trough antiphase to those of *per*, *tim*, and *vri* (Emery et al., 1998). As opposed to these CLK-activated genes, levels of *cry* are high in a Clk^{Jrk} background (Emery et al., 1998). We therefore compared *cry* expression in *cry24* with wild-type flies and made two important observations (Figure 4). First, the locations in which we observed ectopic rhythmic *tim* expression do not express detectable *cry* in wild-type flies (Figure 4A). These cells therefore do not phenocopy a Clk^{Jrk} mutant (in which *cry* levels are elevated) and do not otherwise appear primed for circadian gene expression. Second, we clearly observe ectopic rhythmic *cry* expression (Figure 4B). Indeed, we observe significantly higher levels of *cry* at ZT 2 than at ZT 14, the opposite of that observed for *tim*. These data are consistent with *cry* cycling antiphase to that of *tim* in the ectopic cells. These observations suggest that *Clk* expression in certain cells is sufficient to create ectopic circadian clocks.

Induction of Ectopic Clocks Using an Independent GAL4 Driver

The GAL4 driver used to induce ectopic clocks was derived from the *cry* gene. Although we do not observe *cry* expression in the broad pattern of this driver in wild-type flies, the use of a clock-relevant promoter may still suggest that these ectopic cells already harbor some clock gene expression or properties. In testing numerous GAL4 lines, we found that one noncircadian line, *MJ162a*, was adult viable in combination with *UASClk*. This previously characterized line expresses GAL4 predominantly in the mushroom bodies and the antennal lobes, two regions that have not been previously associated with circadian gene expression (Joiner and Griffith, 1999). *MJ162a*, in combination with *UASClk*, gave rise to ectopic cycling *tim* expression (Figures 5C, 5D, and 5F). As expected, the patterns of ectopic rhythmic gene expression are distinct from *crypGAL4-24* induced clocks. This distinction was especially evident when we optically sectioned brains from flies collected at ZT14 and compared the two patterns (Figures 5E and 5F). The ectopic expression in *cry24* is clustered more ventrally, which is noticeable in the vicinity of the lateral neurons. In contrast, *MJ162a*-induced expression is primarily dorsal and very faint near the lateral neurons. These data suggest that the ability of *Clock* to ectopically induce rhythmic gene expression is not a peculiarity of the *crypGAL4* driver but reflects a more general function of this clock gene.

Clock Overexpression Dramatically Alters Behavior in Light-Dark Cycles

In LD cycles, wild-type flies exhibit a bimodal activity pattern, with a peak centered around lights-on (morning peak) and a second peak around lights-off (evening peak; Figure 6A). The *cry13* (*crypiGAL4-13/UASClk*) pattern is also bimodal, and the evening activity peak is

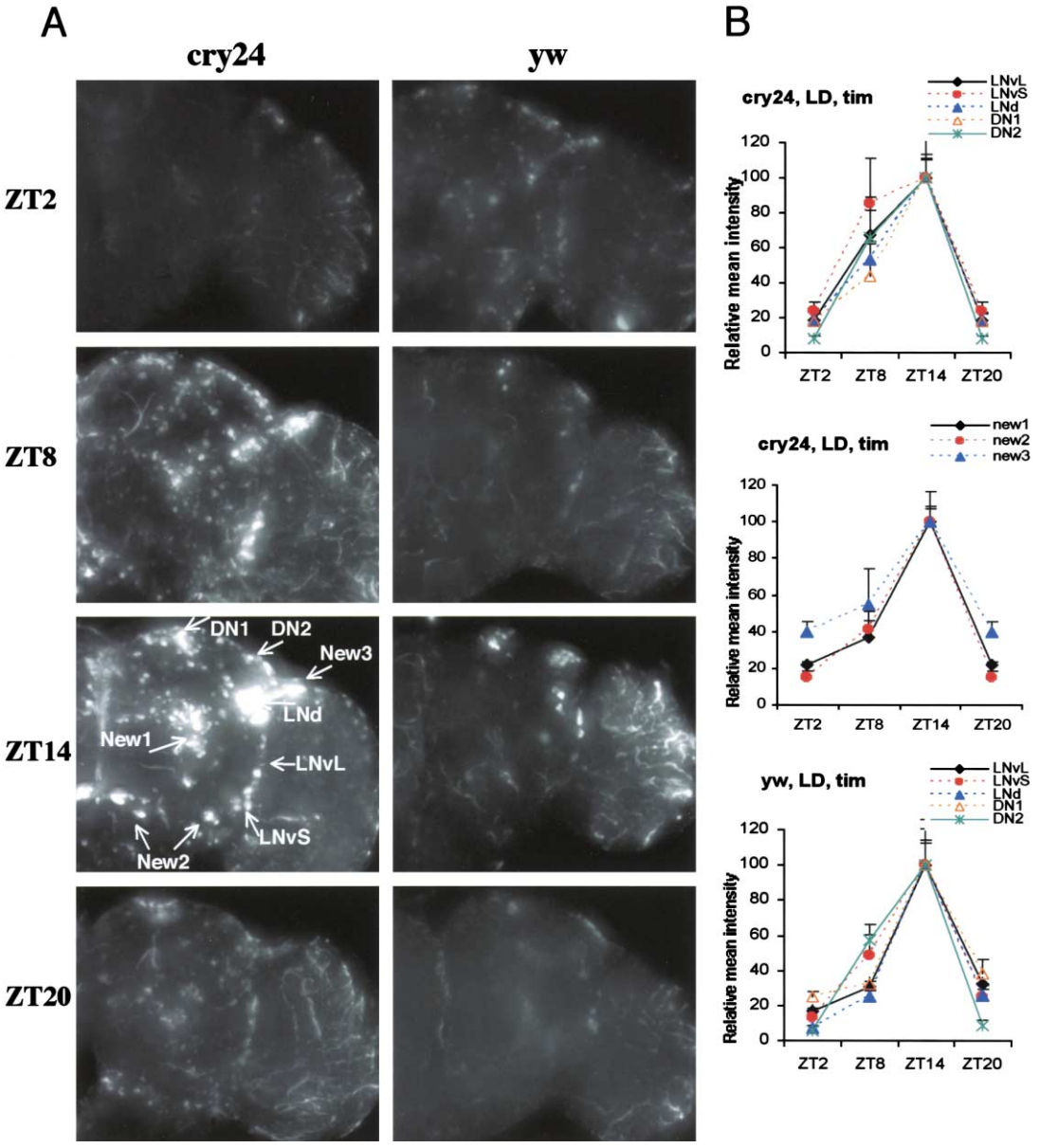

Figure 2. Cycling of *tim* RNA in *cry24* Flies During LD Cycles

Flies were entrained to LD cycles (12 hr light: 12 hr dark) for 3 days at 25°C and collected during the last day of LD at indicated Zeitgeber time (ZT, where ZT0 is lights-on, ZT12 is lights-off). In situ mRNA hybridization was performed on adult brain whole mounts from *cry24* female flies and *y w* female flies to detect *tim* expression.

(A) SenSys camera images of adult brain whole mounts in situ hybridization. Left images, brains from *crypGAL4-24/+;UASCLK/+* (*cry24*) female flies; right images, brains from wild-type (*y w*) female flies. Normal spatial *tim* expression seen in wild-type is characterized as *tim*-expressing cell groups: large and small ventral lateral neurons (LNvL; LNvS), lateral neuron dorsal group (LNd), and two dorsal neuron groups: DN1 and DN2. In *cry24* flies, in addition to the normal *tim*-expressing neurons, widespread novel *tim*-expressing cell groups were detected, as indicated in New1, New 2, and New3. Results show a representative of three experiments.

(B) Quantification of *tim* expression during the circadian cycle. The relative staining intensity of one experiment was quantified and plotted as mean ± SEM for each time point of 4–6 brains. Peak *tim* RNA levels of each cell group were set to 100. Five groups of normal *tim*-expressing neurons of *cry24* flies are plotted in upper image (*cry24*, LD, tim); the three ectopic new groups of cells of *cry24* flies are plotted in middle image (*cry24*, LD, tim); and the five groups of normal *tim*-expressing neurons of *y w* flies are plotted in lower image (yw, LD, tim).

phase advanced, consistent with the shortened period (Figure 6A; Table 1). Remarkably, *cry24* females have an LD activity pattern that is radically different from any previously described strain. Instead of the characteristic bimodal profile, the diurnal pattern has only a single peak dominating the light phase with little or no evening peak (Figure 6A). Interestingly, *cry24* males did not show this pattern (see below).

We considered the possibility that the evening activity peak in *cry24* females was so advanced (by >6 hr) that it merged with the morning peak. To infer the phase of the evening peak, we assayed the DD behavioral phase

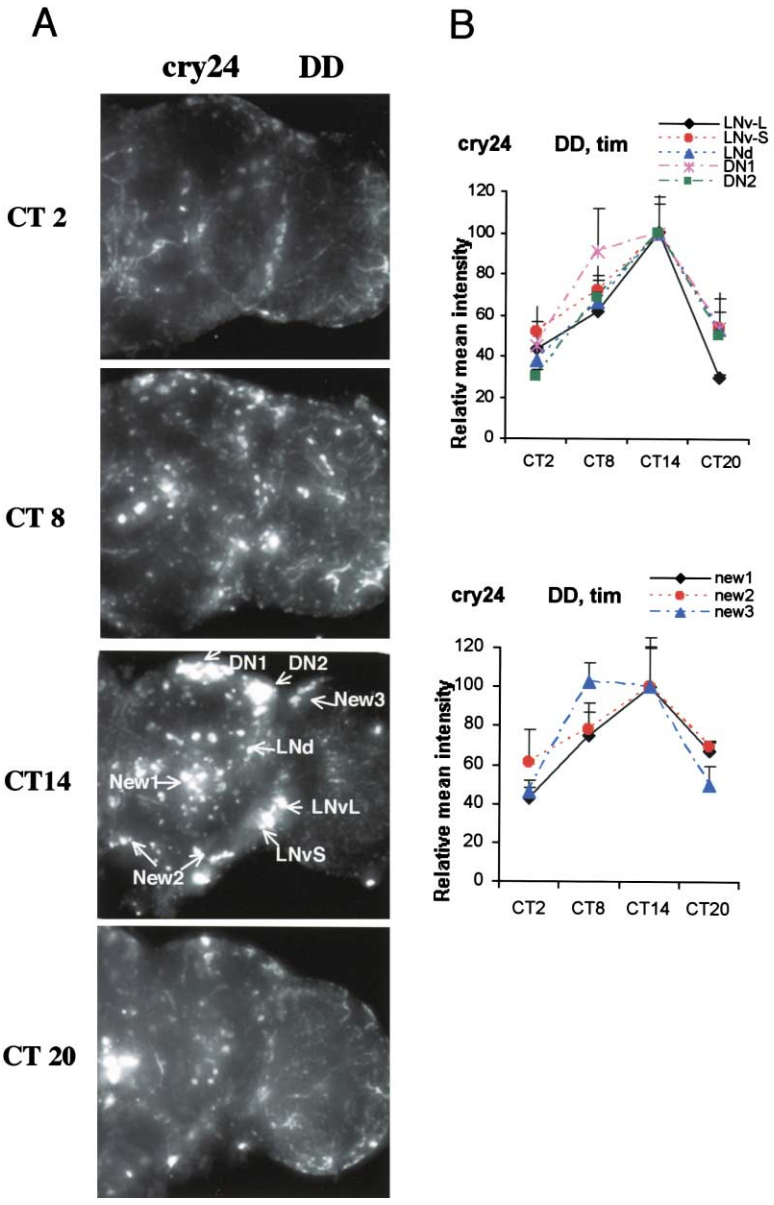

Figure 3. Cycling of *tim* RNA in *cry24* Flies Under DD Conditions

Flies were entrained to LD cycles for 3 days and then transferred to DD. They were collected at the indicated circadian time (CT) during the second day of DD and fly brains were subjected to in situ hybridization.

(A) SenSys camera images of brain whole mounts in situ hybridization from *cry24* female flies. Cyclic expression of *tim* in normal and ectopic cell groups persist in DD.

(B) Quantification of *tim* expression during the circadian cycle. The relative staining intensity was quantified and plotted as mean ± SEM for each time point of 4–6 brains. Peak *tim* RNA levels of each cell group were set to 100. Five groups of normal *tim*-expressing neurons of *cry24* flies are plotted in upper image and the three ectopic cell groups of *cry24* flies are plotted in lower image.

of *cry24* flies. As period also affects phase in DD, we compared the *cry24* and *cry13* lines that have almost identical periods (Table 1). The first four days of DD reveal little difference between the two genotypes, suggesting that an advanced evening activity peak cannot explain the altered diurnal behavior (Figure 6B). Given the progressive reduction in rhythmicity observed in these strains (Table 1), phase assessments beyond four days of DD were not informative. Interestingly, these data argue for a specific effect of *Clk* overexpression on LD behavior. As an independent measure of pacemaker phase under LD conditions, we performed anchored phase-response curves (PRCs; Figure 6C). In principle, PRCs define pacemaker phase by describing phase change in response to brief light pulses administered at different times of day. Both *cry24* and *cry13* were very similar with respect to phase, marginally advanced compared to the control *UASClk* strain (Figure 6C). We observed, however, that the overall amplitude of the *cry24* PRC is suppressed. The data are consistent with an inhibitory effect on circadian phototransduction or on altered pacemaker amplitude but not on pacemaker phase in LD. We also considered the possibility that ectopic *Clk* expression results in an exaggerated light response that swamps a mildly advanced evening activity peak. Indeed, it has been reported that CLK overexpression increases the locomotor activity response as a result of light exposure (Kim et al., 2002). However, *cry13* and *cry24* flies had similar levels of activity after lights-on (data not shown).

The strong effects of *Clk* overexpression on the LD behavior pattern are probably not due to alterations in pacemaker lateral neuron function. First, *cry24* flies have similarly shortened periods as *cry13* flies, in which only the LNs have excess *Clk* expression. Yet *cry13* flies have near normal LD profiles. Second, we did not observe

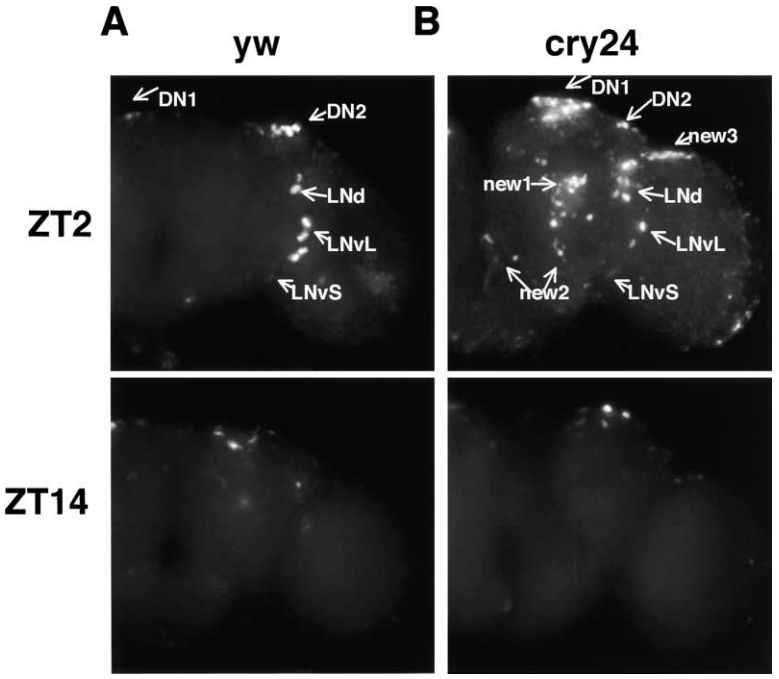

Figure 4. Cycling of *cry* RNA in Ectopic Locations in *cry24* Flies

Flies were entrained to LD cycles (12 hr light: 12 hr dark) for 3 days at 25°C and collected during the last day of LD at indicated Zeitgeber time (ZT, where ZT0 is lights-on, ZT12 is lights-off). Double-labeling in situ mRNA hybridization was performed on adult brain whole mounts from *cry24* and *y w* female flies to detect *cry* expression. SenSys camera images of adult brain whole mounts in situ hybridization were taken from wild-type (*y w*) (A) and *crypGAL4-24/+;UASCLK/+* (*cry24*) (B) flies. *cry* expression seen in wild-type is only observed in circadian cell groups: LNvL, LNvS, lateral neuron dorsal group (LNd), and two dorsal neuron groups: DN1 and DN2. In *cry24* flies, in addition to the normal *cry*-expressing neurons, widespread novel *cry*-expressing cell groups were detected, as indicated in New1, New 2, and New3. *cry* cycling is observed in canonical and ectopic cells antiphase to that observed for *tim*.

any significant LD behavioral effect of driving *Clock* only in the LNv with *pdfGAL4* (Figure 6D). Third, we assayed the behavior of *cry24* flies in a pdf^{01} background. PDF is thought to be the principal effector molecule of the LNv (Renn et al., 1999). However, we found no significant effect of pdf^{01} on the LD behavior of *cry24* flies (Figure 6D). Although we cannot exclude behavioral effects mediated by other circadian neurons, these data indicate that there is a ventral lateral neuron-independent behavioral effect of *Clk* overexpression.

The Presence of Ectopic Clocks Is Correlated With Altered Behavior in Light-Dark Cycles

To extend the correlation between ectopic *Clk* expression and the unusual diurnal behavioral pattern, we compared *cry24* males and females. In contrast to *cry24* females, *cry24* males have a wild-type bimodal pattern in LD (Figure 6A; Table 1). Importantly, ectopic *tim* expression was nearly absent from *cry24* males (Figure 7B). We also did not find any ectopic *tim* expression in *crypiGAL4-13/UASClk* flies, consistent with their bimodal activity patterns (Figure 6A; data not shown). Although the *crypGAL4-24* insert is on the X chromosome, there are no strong differences in GAL4 levels between males and females as monitored by GFP expression (data not shown). However, we did observe only a low fraction of *cry24* adult males, consistent with an upregulation of male transgenic GAL4 expression by dosage compensation. Given the male-specific lethality, we hypothesize that the GAL4-induced CLK toxicity selects against *cry24* males that express high levels of ectopic *Clk*. As a result, the surviving adult males express low levels of *Clk* and thus fail to exhibit ectopic clocks or behavioral phenotypes.

We also examined a second independent insert of the *crypGAL4* line, *crypGAL4-16*. Identical to *cry24*, we observed ectopic rhythmic gene expression and strongly altered diurnal behavior for *crypGAL4-16/UASClk* flies (*cry16*; Figures 7C and 7D): In *cry16* as well as *cry24* flies, there is a single peak of activity around the time of lights-on and little or no evening activity peak (Figures 6A, 7E, and 7F). However, there are behavioral differences between *cry16* and *cry24*, consistent with the anatomic differences in gene expression (Figures 1C and 1E; Figures 7A and 7C). For example, the single peak of activity present in *cry16* flies is more advanced than in *cry24*. In addition, ectopic gene expression and behavioral phenotypes are present in both *cry16* males and females (Figures 7C–7F; Table 1). Although we find subtle behavioral differences between *cry16* males and females, they are both clearly abnormal and these differences are much more subtle than the differences between *cry24* males and females. Considering both *crypGAL4* lines as well as *crypiGAL4*, there is an excellent correlation between ectopic *tim* expression and abnormal locomotor activity patterns. We favor the hypothesis that ectopic clocks are capable of making functional connections with the locomotor output program.

In contrast, the presence of *Clock*-induced changes in DD behavior does not require ectopic clock gene expression. *Clk* overexpression is associated with reductions in rhythmicity and shortened periods in *UAS-Clock* flies in combination with *crypiGAL4* as well as with the two *crypGAL4* lines that induce ectopic clocks (Table 1). Given that GAL4 expression in the *crypiGAL4* lines is restricted to canonical clock cells, it is likely that these shortened periods and reduced rhythmicity are the result of *Clk* overexpression in the lateral neurons. Loss of rhythmicity may also be related to the toxicity of *Clk* overexpression observed as developmental lethality. We propose that failure to observe period shortening effects of *UASClk* in combination with *pdfGAL4* may reflect insufficient expression levels in the pacemaker neurons.

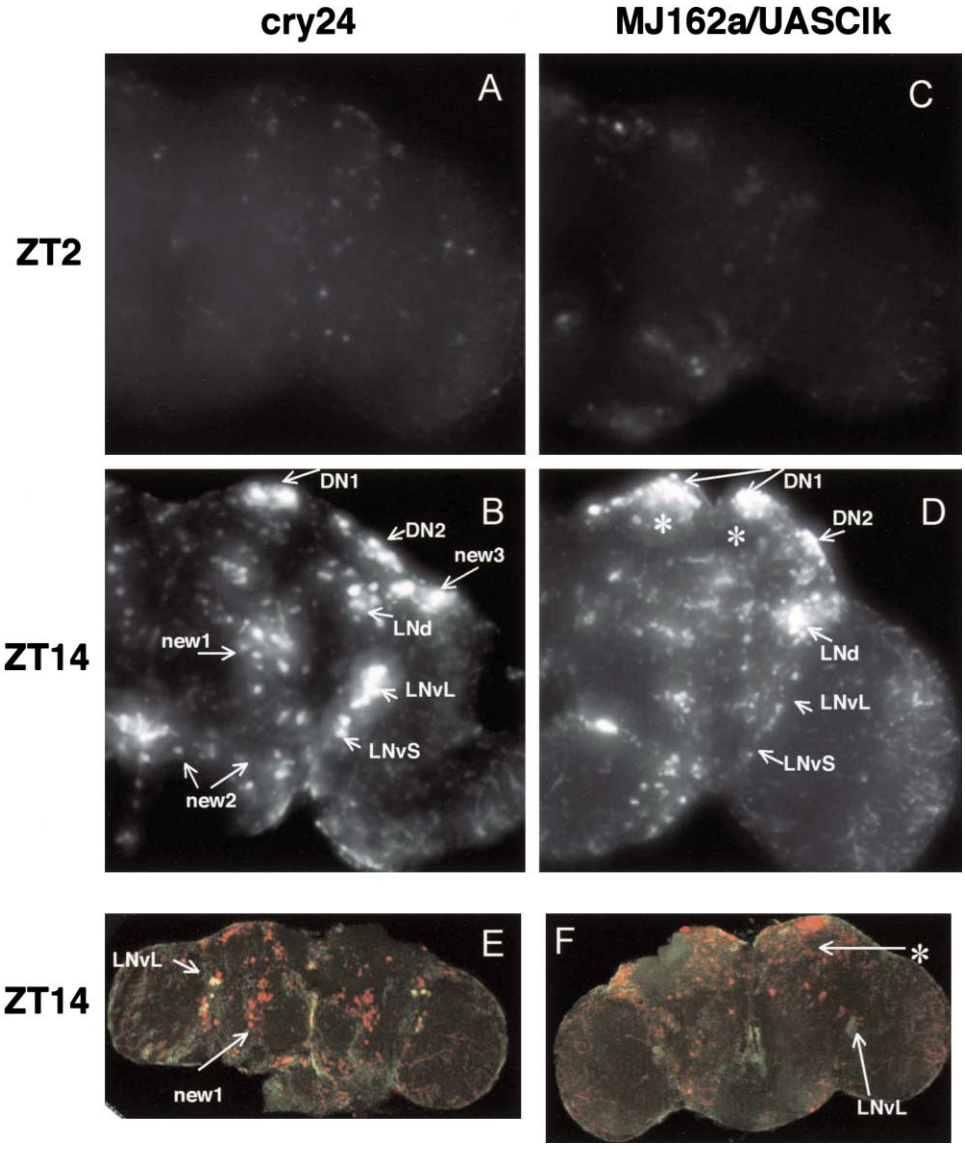

Figure 5. Induction of Ectopic *tim* Cycling with a Noncircadian GAL4 Line

Flies were entrained to LD cycles (12 hr light: 12 hr dark) for 3 days at 25°C and collected during the last day of LD at indicated Zeitgeber time (ZT, where ZT0 is lights-on, ZT12 is lights-off). In situ mRNA hybridization was performed on adult brain whole mounts from *crypGAL4-24/+;UASCLK/+* (*cry24*) female flies and *MJ162a/UASClk* female flies to detect *tim* expression. SenSys camera images of adult brain whole mount in situ hybridization. Left images (A and B) show brains from *cry24* female flies; right images (C and D) show brains from *MJ162a/UASClk* female flies. *tim* expression is seen in normal *tim*-expressing cell groups: large and small ventral lateral neurons (LNvL; LNvS), lateral neuron dorsal group (LNd), and two dorsal neuron groups: DN1 and DN2. In *cry24* flies (A and B) and *MJ162a/UASClk* flies (C and D) additional *tim*-expressing cell groups were also detected (see also Figure 2 and asterisks). Distinct ectopic cells in *MJ162a/UASClock* are more clearly visible in confocal sections. (E and F) Ectopic cells in *cry24* and *MJ162a/UASClock* are distinct. Double-labeling in situ mRNA hybridization was performed on adult brain whole mounts from *cry24* female flies and *MJ162a/UASClk* female flies to detect *tim* (red) and *pdf* (green) expression. Confocal sections containing the large ventral LNs (LNvL) are used to compare the two genotypes. In *MJ162a/UASClock*, prominent dorsal expression of *tim* is observed, while more ectopic expression is observed ventrally including in the vicinity of the lateral neurons in *cry24* flies.

Ectopic *period* Expression Does Not Induce Ectopic Clocks

Given that the expression of several key clock members is dependent on *Clk*, we reasoned that its ability to induce rhythmic gene expression would be unique among clock genes. To verify this experimentally, we induced ectopic *per* expression using the *crypGAL4-16* driver (Figure 7G). In these *per* overexpressing flies, we still observed evidence of central clock function (rhythmic *tim* expression and anticipation of LD transitions) but with reduced behavioral rhythmicity in DD (Figure 7G; data not shown). Consistent with our hypothesis, we observed neither induction nor cycling of ectopic *tim* expression in these flies. Thus, the ability to induce ectopic rhythmic gene expression appears to be specific to *Clk*.

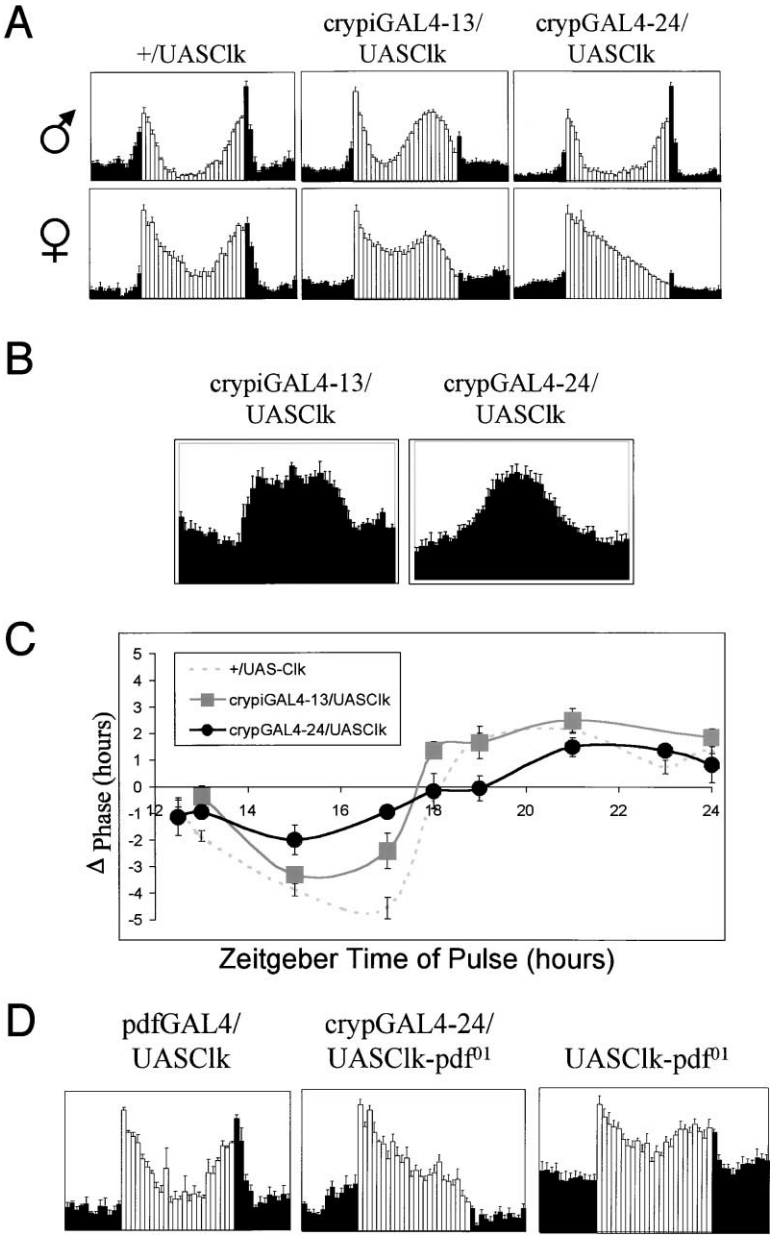

Figure 6. Assessment of Diurnal and Circadian Behavior in *Clock* Overexpressing Strains

(A) Altered diurnal behavior in *Clock* overexpressing strains. Activity profiles display average relative activity through four days of LD (12 hr light: 12 hr dark) for each indicated group of flies. Light bars display times of lights-on; dark bars times of lights-off. Genotypes are all heterozygous for the indicated transgenes except male *crypGAL4-24* that are hemizygous for this X-linked transgene. Error bars represent standard error for that bin. Number of animals for each line in combination with *UASClk* (males/females): + (16/47), crypiGAL4-13 (47/79), crypGAL4-24 (37/130). Detailed methods used to generate these profiles are outlined in Experimental Procedures.

(B) Phase of *Clock* overexpressing females is not significantly altered in constant darkness (DD). Each DD plot exhibits average activity through the first four period-length (22.5 hr) intervals of the DD record. Error bars represent the standard error across the four days. Number of animals tested is *crypGAL4-24/UASClk* = 37 and *crypiGAL4-13/UASClk* = 22. See Experimental Procedures for details.

(C) Phase-response curve suggests that induced *Clock* expression does not dramatically alter pacemaker phase. The phase-response curve indicates the change in phase of a population, with respect to a nonpulsed control, due to a brief light pulse administered at different times during LD. Genotypes are as indicated above; all flies tested were female. Error bars indicate the standard error for each pulse time point. Phase delays are seen in the early night (ZT12-17) and phase advances are observed in the late night (ZT21-24) for all strains indicating no large change in pacemaker phase.

(D) Altered diurnal behavior in *Clock* overexpressing strains is not due to expression in the ventral lateral neurons. Activity profiles display average relative activity through four days of LD (12 hr light: 12 hr dark) for each indicated group of flies. Light bars display times of lights-on; dark bars times of lights-off. Genotypes are all heterozygous for the indicated transgenes except *UASClk-pdf*^{01}, which is homozygous. Error bars represent standard error for that bin. Number of female animals analyzed for each line *pdfGAL4/UASClk* (32), *crypGAL4-24/UASClk-pdf*^{01} (13), and *UASClk-pdf*^{01} (13).

Discussion

We have demonstrated that *Clk* misexpression induces ectopic clocks. These ectopic clocks are evident by measurements of clock gene expression under light-dark and constant darkness conditions. The basic result is not dependent on a *cry*-derived GAL4 driver, as the independent noncircadian GAL4 line *MJ162a* induces ectopic clocks in distinct brain regions. Furthermore, it is likely that *Clk* is inducing major components of the clock gene program as it also induces rhythmic expression of *cry*, a gene that the CLK-CYC complex normally represses. The ectopic clocks appear to have potent effects on the LD behavioral program.

Several lines of evidence now place *Clk* at the top of a genetic hierarchy controlling circadian clock gene expression. Intact *Clk* is necessary for multiple aspects of the fly and mouse circadian phenotype. In both systems, there is strong genetic and biochemical evidence that CLK and its partner CYC (BMAL1 in mammals) form a heterodimeric complex and directly activate transcription of several important clock genes (reviewed in (Allada et al., 2001). In *Drosophila*, these genes, *per*, *tim*, *vri*, and *Pdp1* comprise the core elements of interdependent circadian feedback loops essential for rhythmic gene expression. Moreover, microarray analyses in both flies and mice indicate that all detectable rhythmic gene expression is dependent on *Clk* (McDonald and Rosbash,

Table 1. Circadian Behavior of Clock Overexpressing Strains

Males		
Genotype	Period ± SEM	% Rhythmic (n)
UASClk/+	23.3 ± 0.1	95 (48)
pdfGAL4/+;UASClk/+	23.3 ± 0.1	70 (58)
crypiGAL4-13/UASClk	22.4 ± 0.1	51 (63)
crypGAL4-24; UASClk/+	23.6 ± 0.1	100 (28)
crypGAL4-16/UASClk	22.2 ± 0.4	33 (47)
pdfGAL4/+	23.9 ± 0.1	75 (34)
crypiGAL4-13/+	23.5 ± 0.1	85 (26)
crypGAL4-24/+	24.2 ± 0.1	97 (34)
crypGAL4-16/+	24.7 ± 0.4	76 (18)
Females		
Genotype	Period ± SEM	% Rhythmic (n)
UASClk/+	23.3 ± 0.1	83 (48)
pdfGAL4/+;UASClk/+	24.1 ± 0.2	43 (58)
crypiGAL4-13/UASClk	22.7 ± 0.1	39 (96)
crypGAL4-24/+; UASClk/+	22.5 ± 0.1	50 (157)
crypGAL4-16/UASClk	AR	0 (80)
pdfGAL4/+	23.9 ± 0.1	73 (18)
crypiGAL4-13/+	23.4 ± 0.2	33 (19)
crypGAL4-24/+	24.3 ± 0.1	84 (34)
crypGAL4-16/+	24.0 ± 0.5	47 (18)

SEM is standard error of the mean; n = number of flies analyzed.

2001; Panda et al., 2002a). The abnormal pacemaker neuronal morphology in the fly mutant is consistent with an additional role in regulating circadian neuronal development (Park et al., 2000). All of these data suggest that the *Clk* gene may be necessary for many if not most aspects of clock cell specification as well as function.

The ectopic clocks appear to strongly influence diurnal behavior, implying that these new clocks make functional connections with locomotor output pathways. There is an excellent correlation between the altered diurnal behavior and ectopic *tim* expression, for example in male versus female *cry24* flies. In contrast, enhanced expression in the pacemaker lateral neurons with *pdfGAL4* and *crypiGAL4-13* has little or no effect on behavior under LD conditions. Consistent with this notion, the potent effects of *Clk* on LD behavior are not blocked in a pdf^{01} background.

Although we cannot completely exclude a role for increased expression in the lateral neurons or other known circadian cells, we favor the notion that new clock cells are responsible for the altered LD behavior. One of the prominent regions of *crypGAL4* driven gene expression is the ellipsoid body, a brain region previously implicated in the higher order control of locomotor activity (Martin et al., 1999). As such, *Clk*-driven expression here might be expected to influence locomotor activity. Interestingly, the *MJ162a* line does not drive detectable expression in the ellipsoid body (Joiner and Griffith, 1999), nor does it have prominent behavioral effects in combination with *UASClk* (data not shown). Differences between *cry16* and *cry24* flies further suggest that other neurons or even glia may mediate some of the ectopic *Clk* behavioral effects. One possibility is that the transgenic strains manifest a dramatically suppressed evening activity peak, which is normally tightly regulated by the circadian clock. This suggests that light and these new clocks may collaborate to antagonize positive factors (such as PDF), which are normally released by canonical clock cells in a temporally gated fashion (Helfrich-Forster et al., 2000). The failure of CLK to induce PDF in the ectopic locations is consistent with the view that other humoral factors or perhaps even new neural connections are involved in the behavioral changes. Alternatively, the ectopic clocks may alter the coupling between the central pacemaker and outputs under LD conditions.

To examine the mechanism of ectopic clock formation, we first considered that *Clk* might induce new clocks only in cells that are highly predisposed to expressing rhythmicity. In this case, *Clk* expression would induce one or only a few missing clock genes necessary for molecular oscillator properties. An analogous case from mammals may be that of cultured rat-1 fibroblasts, which mimic the behavior of peripheral clocks such as the liver. Exposure of the rat-1 cells to high concentrations of serum (serum shock) can induce rhythmicity in cells that otherwise exhibit no apparent rhythmicity (Balsalobre et al., 1998). The predisposition of these cells is reflected in their substantial level of clock gene expression. In contrast, we found that *tim* and *cry* expression is undetectable in the ectopic cells without *Clk* expression. This expression analysis is consistent with prior reports indicating that there is no detectable *per* and *tim* protein in adult brain neurons outside of the LNs and DNs (Stanewsky et al., 1998; Kaneko and Hall, 2000 and references within). We also considered the possibility that *tim* is expressed in these ectopic locations in wild-type flies but that *tim* mRNA levels are simply below the level of detection. Consistent with this possibility, *tim* promoter *gal4*-driven GFP can be visualized in neurons without detectable *tim* expression (Figure 1D; Kaneko and Hall, 2000). Similarly, broader expression of the *per* gene has been observed with artificial *lacZ* fusion proteins and in certain mutant backgrounds, suggesting some low level *per* expression in other brain regions (Kaneko et al., 1997; Price et al., 1998). The functional relevance of these transgene expression patterns without detectable *per* or *tim* expression remains unclear. Moreover, it is not even certain that the expression of these reporters is *Clk*-dependent. Nonetheless, a comparison of the ectopic rhythmic cells with the *timGAL4:UASEGFP* pattern indicates that they are two distinct cell populations (Figures 1D and 2A). The failure to express *tim* in the absence of *Clk* induction is consistent with the notion that these cells are not fully preprogrammed for rhythmicity. It will be of interest also to compare expression of *per-lacZ* fusion proteins that reveal potential cryptic *per* expression with the ectopic clock locations shown here.

Most compelling perhaps is the absence of *cry* expression in these ectopic locations. If these cells were simply missing *Clk*, they should behave as *Clk* mutants and express high levels of *cry* mRNA. However, we could not detect *cry* expression in these brain regions, arguing against the hypothesis that these cells are largely programmed for circadian rhythmicity. In addition, we were able to induce ectopic rhythms in distinct locations using a noncircadian GAL4 line, *MJ162a*.

Our analysis raises some intriguing parallels between *Clk* and *eyeless*, a gene involved in the induction of eye

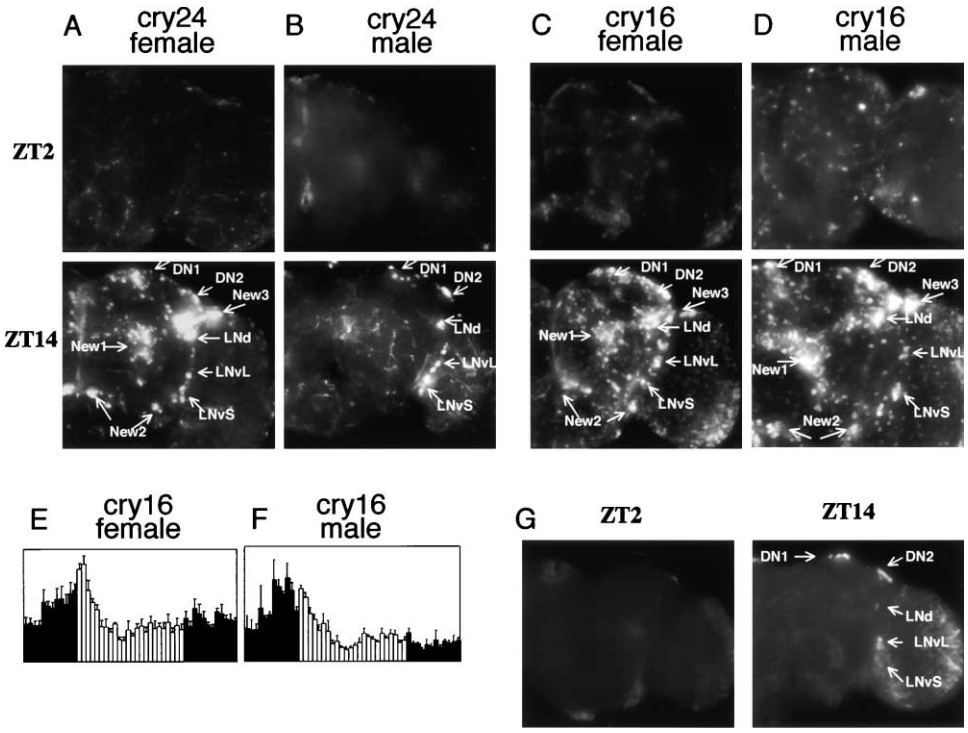

Figure 7. Differences in Ectopic Clocks Between Different Lines

All images are SenSys camera images of adult brain whole mounts in situ hybridization. Both *crypGAL4-24/+;UASClk/+* (*cry24*) and *crypGAL4-16;UASClk* (*cry16*) female and male flies were entrained to LD cycles for 3 days at 25°C and collected during the last day of LD at ZT2 and ZT14. Ectopic *tim* overexpression were observed *cry24* female flies and in both and female and male *cry16* flies. Ectopic cell groups are indicated by New1, 2, and 3, based on morphology and location. We cannot definitively determine if these three clusters are the same between *cry16* and *cry24* flies. No ectopic *tim* overexpression was found in male *cry24* flies. Figure shows a representative of two experiments.

(E and F) Diurnal behavior in *crypGAL4-16/UASClock* strains. Activity profiles display average relative activity through four days of LD (12 hr light: 12 hr dark) for each indicated group of flies. Light bars display times of lights-on; dark bars times of lights-off. Error bars represent standard error for that bin. Number of animals analyzed is 36 for *cry16* males and 23 for *cry16* females.

(G) *tim* expression in *UASper/+; crypGAL4-16/+* flies. *tim* expression analyzed as in Figures 7A–7D. No ectopic *tim* induction or cycling is observed.

morphogenesis. Like *Clk* for circadian rhythms, ectopic expression of *eyeless* can induce the formation of ectopic eyes (Halder et al., 1995). Both *eyeless* and *Clk* function in terminally differentiated neurons to control highly specialized gene expression: opsins in the case of *eyeless* and rhythm genes in the case of *Clk* (Sheng et al., 1997). It has been proposed that activation of a photoreceptor gene was the original function of *eyeless* and that its morphogenetic role was acquired much later in evolution (Sheng et al., 1997). Similarly, we propose that the original role of *Clk* was to activate expression of a clock gene ancestor, and its ability to direct the formation of temporally regulated feedback loops was a more recent acquisition.

Despite its reported function as a "master control gene," not all cells are substrates for *eyeless*-induced ectopic eye formation (Bonini et al., 1997). Similarly, we believe the presence of other rhythm factors, such as CYC, are likely required for *Clk* expression to induce functional clocks. Furthermore, we were unable to induce rhythmic gene expression with transfected *Clk* in CYC-expressing S2 cells, suggesting that still other factors might be necessary (data not shown). Future experiments assessing ectopic clock formation in different circadian mutant backgrounds and tissues should address this general issue. Despite the similarities between *eyeless* and *Clk*, we have reason to suspect that *Clk* may have more far reaching functions. For example, *eyeless*-induced ectopic eyes have never been shown to be functional, whereas we present substantial evidence that ectopic clocks can alter behavior. Taken together with the *Clk* mutant effects on LNv anatomy (Park et al., 2000), normal *Clk* expression may even contribute to pacemaker cell wiring properties. Given the similarities between the fly and mammalian clock systems, we suggest that the mammalian orthologs of *Clk* and *cyc* may play similar roles.

Experimental Procedures

Transgenic Flies

Clock was first tagged with hemagglutinin (HA) epitope by PCR cloning. Briefly, a C-terminal fragment of *Clock* was PCR-amplifed using pSK(−) *Clock* cDNA and an oligonucleotide with HA epitope and XhoI site and an internal oligonucleotide. The amplified fragment was digested with ClaI/XhoI and ligated to ClaI/XhoI digested pSKClock vector to generated pSK(−) ClockHA. ClockHA was subsequently ligated into pUAST (EagI/XhoI) to generate pUAS-ClockHA. *y w; Ki p^{p} (ry^{+} delta 2-3)/+* embryos were injected with pUAST-ClockHA. A single line (*UASClk*) was obtained as a third chromosome insert. HA epitope is not immunologically detectable (data not shown).

pdfGAL4, *crypiGAL4*, and *timGAL4* have been previously described (Emery et al., 2000; Kaneko and Hall, 2000; Park et al., 2000). *crypGAL4* was constructed similarly to the previously described *crypiGAL4* construct except that a NotI/NcoI fragment was cloned in front of the GAL4 coding region of pPTGAL4. As a result, only the promoter and a fraction of the first exon of the *cry* gene are present. The NcoI site is a natural site in the coding region of the *cry* gene. The 5′ end of the GAL4 coding region was thus modified by PCR to contain a 5′ NcoI site and to be in phase with the few *cry* codons present in the construct. The modified GAL4 region was sequenced to ensure that no PCR errors had occurred. Three independent lines were analyzed (16, 17, and 24). Line 17 did not show any expression or behavioral phenotypes.

Adult viability was not observed with *UASClk* in combination with the following brain GAL4 drivers: *hsGAL4* (even in the absence of heat shock), 7B, MJ94, MJ63, MJ126a, MJ250 (Joiner and Griffith, 1999), 201Y (Yang et al., 1995), and drl^{PGAL8} (Moreau-Fauvarque et al., 1998). Most lines were pupal lethal even when raised at 18°C. For adult viable lines, flies were crossed at 25°C for three days then transferred to 18°C to increase the number of healthy adult flies obtained. All molecular and behavioral analyses were conducted on flies entrained at 25°C.

GFP Expression Analysis

Adult flies expressing *UASEGFP* driven by GAL4 under various circadian promoters were dissected and the brains fixed in 3.7% formalin in PEM. After rinses in PBS + 0.3% Triton and PBS, brains were mounted in Fluoromount (company) and imaged on a Leica laser scanning confocal microscope. Optical sections were taken at 2–5 micron intervals and used to construct a maximum projection image for each brain.

In Situ mRNA Hybridization on Adult Brain Whole Mounts

Adult fly brains were dissected in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 30 min at room temperature. After prehybridization for minimum of two hours in Hybrix (50% formamide, 5 × SSD, 100 μg/ml tRNA, 100 μg/ml ssDNA, and 0.1% Tween 20) at 55°C, the brains were incubated with probes overnight at 55°C. Three *tim* probes were used in this study which correspond to nucleotides 405–1253, 1584–2580, and 2851–4193. These probes were used simultaneously. The *pdf* probe used corresponds to nucleotides 282–570. The *cry* probe used is generated from the full-length of the *cry* gene (nucleotide 1–1764). Antisense RNA probes were synthesized and labeled using digoxigenin (*tim, cry*) or biotin (*pdf*) RNA labeling kit from Boehringer Mannheim. The probes were hydrolyzed in sodium bicarbonate buffer and stored in Hybrix at −20°C until use. The hybridized RNA signals were detected using fluorescent tyramides (NEN LifeScience). Brains were mounted in glycerol with 4% n-propyl gallate and examined with a Zeiss Axiophot microscopy equipped with Photometrics SenSys CCD camera (Photometrics Ltd., Tucson, AZ). SenSys camera images were assembled in Adobe Photoshop and were used for quantification of fluorescence signals. For a given time course experiments, 4–6 individual brains from each time point were photographed in fluorescent field at 20× magnification. The intensities of the fluorescence signal were measured using Openlab software (Improvision).

Behavioral Analysis

Flies were monitored for 5 days in 12 hr light: 12 hr dark (LD) conditions, then 7–8 days in constant darkness (DD) using Trikinetics *Drosophila* Activity Monitors (Waltham, MA). Calculations of period and rhythmicity were performed using ClockLab analysis software package (Actimetrics, Evanston, IL) using chi-square periodograms with significance set to $\alpha = 0.025$. Flies with a chi-square statistic ≥10 over the significance line were scored as rhythmic. Selected data were confirmed by visual inspection of actograms.

To analyze behavior in LD, the average activity of a group was averaged across 4 days (days 2–5 of LD). Each fly's activity was normalized to its average activity. The normalized activity counts were then averaged together to create a single file, using Excel. This single file was segmented into single day intervals that were then averaged together to create a single averaged day of data. Each vertical column represents the average activity across 4 days for a single half-hour bin. Error bars indicate the standard error across the four single day intervals for each half-hour bin.

To determine phase in constant darkness (DD), analyses were performed as for LD analyses. Four days of data as defined by the period of the genotypes in DD were used (22.5 hr for *cry24* and *cry13*). Data from the four days were averaged together and plotted into a single day corresponding to this period. For determination of phase response curves, a 10 min long light pulse was administered during the dark period of the last full day of LD. After the pulse, flies are monitored in constant darkness for five days for assessment of phase. Data from populations of a given genotype or pulse time were pooled together. Phase was defined as the time at which the average fly activity was at 50% evening activity offset. We determined the time of the peak evening activity and the subsequent trough of evening activity for each day after the light pulse. The 50% evening activity offset was calculated as the (evening peak activity + post-evening trough)/2. The time at which activity is nearest this 50% evening activity offset defines the phase each day. Differences in the time of activity offset between pulsed and non-pulsed population were used to calculate phase changes on days 2–4 after the light pulse. The calculated phase changes for these three days were then averaged together to produce the average phase change for a given experiment. The final plot exhibits the averaged results for three experiments. We obtained results similar to published results for our wild-type control validating this methodology.

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