Brandeis University on Clk

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Summary

Circadian rhythms of behavior, physiology, and gene
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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
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fruit fly, Drosophila melanogaster, circadian gene
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CLOCK Misexpression Results in Ectopic
Circadian timeless Expression

To characterize the consequences of Clk misexpression,
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Of note, cry expression is regulated in a similar manner

to Clk (Emery et al., 1998). The mechanism of Clk inhibi-
tion of Clk and cry gene expression has recently been pro-
posed to involve the clock genes vri and Pdp 1 (Cryan et al., 2003; Glossop et al., 2003). Interestingly, the ex-
pression of vri and Pdp 1, 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 cir-
cadian gene expression.

Although these intracellular feedback loops are pres-
ent in many tissues, they are not ubiquitous. In the adult
fly brain, clock gene expressing neurons are the excep-
tion 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 (Ka-
neko, 1998; Renn et al., 1999). The LNs can be subdi-
vided into a dorsal subgroup (LNd) and two ventral sub-
groups (LNv), consisting of small (LNvS) and large
neurons (LNvL). The LNv subgroup is distinguished by
its expression of the neuropeptide gene, pigment dis-
per (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 rhythmi-
cally expressed in the synaptic termini of the LNvS (Park
et al., 2000). Genetic disruption of Clk dramatically re-
duces pdf expression and alters the neuronal projec-
tions 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 misexpres-
sion, we utilized the GAL4/UAS system (Brand and Perrim-
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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) pdfGAL4 promoter drove GFP expression in the lateral neurons only (LNvL; LNvS).

(B) With the crypGAL4-13 promoter, GFP expression was only visible in the LNvL.

(C) crypGAL4-24 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, LNv, LNvS). Circadian neuronal projections are traced with a solid white line.

(D) timGAL4 promoter drove GFP expression in a broad distribution, but not in all the same cells showing tim RNA expression in crypGAL4-24 females.

(E) crypGAL4-16 brains expressed GFP in a pattern similar to but distinct from crypGAL4-24 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 (crypGAL4; 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 crypGAL4 (line 13; crypGAL4-13) 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 crypGAL4 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 DN5s and in the NLv 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, LNV5, and LN1d 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 UASClik 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, DN5s, as well as the three new locations (Figure 2B). Indeed, cycling is evident 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 antisense 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 null 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 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 antisense 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 UASClik. 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 UASClik, 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 (crypGAL4-13/UASClik) 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 (yw) 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...
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 an altered pacemaker amplitude but not on pacemaker phase under LD conditions. 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...
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 deficient 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 deficiency 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 crypGAL4-13/UASClk flies, consistent with their bimodal activity pattern (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 crypGAL4, 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 UASClk flies in combination with crypGAL4 as well as with the two crypGAL4 lines that induce ectopic clocks (Table 1). Given that GAL4 expression in the crypGAL4 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/UASClk are more clearly visible in confocal sections. (E and F) Ectopic cells in cry24 and MJ162a/UASClk 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/UASClk, 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), crypGAL4-13 (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/H11005 37 and crypGAL4-13/UASClk/H11005 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-pdf01, 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-pdf01 (13), and UASClk-pdf01 (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 (Al-lada 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,
activity. Interestingly, the MJ162a tor activity (Martin et al., 1999). As such, One of the prominent regions of crypGAL4 previously implicated in the higher order control of locomotor reveal potential cryptic clock cells are responsible for the altered LD behavior. is consistent with the notion that these cells are not fully expression is the ellipsoid body, a brain region pre- to compare expression of tim and pdf01 cry24 flies. In contrast, enhanced expression in the pacemaker lateral neurons with pdfGAL4 and crypGAL4-13 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 pdf01 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 (Heifrich-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 cyp 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 DN (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 cyp expression in these ectopic locations. If these cells were simply missing Clk, they should behave as Clk mutants and express high levels of cyp mRNA. However, we could not detect cyp expression in these brain regions, arguing against the hypothesis that these cells are largely pro-grammed 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 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-amplified using pSK(-) Clock cDNA and an oligonucleotide with HA epitope and XhoI site and an internal oligonucleotide. The amplified fragment was digested with Clal/XhoI and ligated to Clal/XhoI digested pSKClock vector to generated pSK(Clock)HA. ClockHA was subsequently ligated into pUAST (Eagl/XhoI) to generate pUAS-ClockHA. y w; Kp 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, crypGAL4, 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 crypGAL4 construct except that a Not/I-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, MJ290 (Joiner and Griffith, 1999), 201Y (Yang et al., 1995), and drfGAL4 (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 Hybriob (50% formalamide, 5 × SSOD, 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 Hybriob at −20°C until use. The hybridized RNA signals were detected using fluorescent tyramides (NEN LifeScience). Brains were mounted in Allada, R., White, N.E., So, W.V., Hall, J.C., and Rosbash, M. (1998). Aglycerol with 4% n-propyl gallate and examined with a Zeiss Axio-Imager, C. Three probe used are consisted of full 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 Hybriob at −20°C until use. The hybridized RNA signals were detected using fluorescent tyramides (NEN LifeScience).

**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 Trinketics 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 α = 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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