Disruption of Synaptic Transmission or Clock-Gene-Product Oscillations in Circadian Pacemaker Cells of *Drosophila* Cause Abnormal Behavioral Rhythms

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ABSTRACT: To study the function of clock-gene–expressing neurons, the tetanus-toxin light chain (TeTxLC), which blocks chemical synaptic transmission, was expressed under the control of promoters of the clock genes *period* (*per*) and *timeless* (*tim*), each fused to GAL4-encoding sequences. Although TeTxLC did not affect cycling of a clock-gene product at the gross level, it disrupted the rhythmic behavior of adult *Drosophila.* In constant darkness, the proportion of rhythmic flies was reduced in flies expressing active TeTxLC compared to controls, including those expressing inactive toxin. The behavior of TeTxLC-expressing flies was less synchronized to light:dark cycles than that of controls. To determine which neurons are responsible for these effects on behavior, the toxin was also expressed in restricted subsets of *per/tim*-expressing, laterally located pacemaker neurons by expressing TeTxLC under the control of a driver in which GAL4-encoding sequences are fused to the promoter of the *pigment dispersing factor* (*pdf*) gene. *pdf-gal4*–driven TeTxLC expression had relatively little effect on behavioral rhythms, implying that *per/tim* neurons other than *pdf*-expressing lateral neurons participate in the generation of rhythmic behavior. In another set of experiments, *period* gene products were expressed under the control of *per-gal4* or *tim-gal4.* This resulted in an increased level of PER protein in many brain cells and reduction of bioluminescence cycling reported by a *per-luciferase* transgene, especially in the case of *per* expression affected by *tim-gal4.* This indicates a disruption of the transcriptional feedback loop that is a part of the oscillatory mechanism underlying *Drosophila*'s circadian rhythms. Consistent with this molecular defect, the proportion of rhythmic individuals in constant darkness was subnormal in flies expressing PER under the control of *tim-gal4,* and their behavior in light:dark cycles was abnormal. © 2000 John Wiley & Sons, Inc. J Neurobiol 43: 207–233, 2000

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In *Drosophila,* many tissues throughout the body express the clock genes *period* (*per*) and *timeless* (*tim*) (Liu et al., 1988; Siwicki et al., 1988; Saez and Young, 1988; Plautz et al., 1997a; Kaneko and Hall, 2000). These spatial patterns include the brain, within which certain *per/tim* cells have been implicated in the control of the adult's behavioral rhythmicity (Ewer et al., 1992; Frisch et al., 1994; Helfrich-Förster, 1998). There are six clusters of brain neurons that contain PER and TIM proteins. Three of these clusters are located in the dorsal protocerebrum; the other three are in the anterior lateral cortex, near the inner margin of the medulla of the optic lobes. The dorsally located neuronal clusters, dorsal neurons 1, 2, and 3 (DN1, 2, and 3) (Helfrich-Förster, 1996; Kaneko, 1998; Kaneko and Hall, 2000) contain approximately 55 pairs of neurons expressing PER and TIM in these

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brain regions. The other PER/TIM cells are in the lateral brain: a relatively dorsal cluster, consisting of ca. six neurons and called LN_d; and ventrally located lateral neurons (LN_vs), which are further classified into two clusters by the size of their somata, large LN_vs (four to five cells) and small LN_vs (four to five cells).

Among these putative circadian-pacemaker neurons, four small LN_vs and four large ones express a neuropeptide called pigment-dispersing factor (PDF; reviewed by Helfrich-Förster et al., 1998). The PDF-containing small LN_vs, which project dorsally to the superior protocerebrum, seem to be necessary for robust circadian rhythmicity of adult behavior, in terms of the structures themselves and clock functions operating within the cells (Zerr et al., 1990; Frisch et al., 1994; Helfrich-Förster, 1998; Stanewsky et al., 1998). The biological, let alone chronobiological, significance of the remaining DNs and LNs (>90% of *per/tim* neurons) is largely unknown (Kaneko, 1998). One purpose of the current experiments was to disrupt the function of these cells and assess the behavioral consequences.

Cell ablation provides a way to study the functions of a particular cell or cell type. Physical cell ablations have been applied in developmental studies of large insects (e.g., Kuwada and Goodman, 1985; Smith, 1989). The small size of *Drosophila* precludes killing specific cell types by physical ablation, especially in the adult brain. Alternative methods available in *Drosophila* involve chemical and genetic ablations, which have been used in developmental and behavioral studies (e.g., Kalb et al., 1993; de Belle and Heisenberg, 1994; Hidalgo et al., 1995; White et al., 1996; Ito et al., 1997; Stocker et al., 1997; Hiesinger et al., 1999). Certain such disruptions have been effected by application of the GAL4 transcription-control system (Kaiser, 1993). This is a binary system in which the expression of various types of factors that affect cells of interest can be brought under the control of a given promoter-*gal4* fusion or enhancer trap. Sequences encoding the factors are fused to the GAL4-responsive element UAS and such factors include cell-death agents (e.g., McNabb et al., 1997; Renn et al., 1999) and tetanus toxin (e.g., Reddy et al., 1997; Martin et al., 1998, 1999; Tissot et al., 1998; Heimbeck et al., 1999).

The form of this toxin that has been brought under the control of GAL4 is the Tetanus-Toxin Light Chain (TeTxLC), which blocks chemical synaptic transmission by cleaving the neuronally expressed synaptobrevin protein (Sweeny et al., 1995). Therefore, TeTxLC can be applied to affect only neuronal cells (e.g., Allen et al., 1999; Baines et al., 1999). We applied a UAS-*tetxlc* construct in order to study the function of clock-gene–expressing neurons as they are hypothesized to regulate locomotor activity rhythms. Usage of this toxin, rather than general ones or cell-death factors, was necessary, because *per-gal4*– and *tim-gal4*–mediated expression of reporter gene has been found in numerous cell and tissue types throughout the body of flies, many of which are nonneuronal (Hall, 1995). Widespread cell ablation would kill the animals, but if they were to survive and be behaviorally testable, the results would be uninterpretable: On the one hand, the survival of such flies would mean that several cells were not killed (and coexpression of two factors involved in the apoptosis can be necessary for induction of cell death in some embryonic neuronal cells; Zhou et al., 1997); this would imply that certain behaviorally relevant brain neurons survived as well, and such subtleties might not be detectable histologically. On the other hand, a live fly expressing a cell killer under the control of *per* or *tim* might have suffered certain general-tissue losses (other than neuronal ones) that could impinge on its ability to behave for nonchronobiological reasons. Therefore, TeTxLC should be a sharper cell-disruptive tool for behavioral experiments involving genes like clock ones that are pleiotropically expressed.

The *per*- and *tim-gal4* fusions allowed for another kind of disruption of clock-neuron functioning. For this, we aimed to overexpress the *period* gene (sequences that were fused to UAS) in transgenic flies carrying *per-gal4* or *tim-gal4.* Analogous overexpression approaches have been taken in certain developmental studies (Johnson et al., 1995; Morimura et al., 1996). In rhythm-related experiments, overexpression of *per* could well occur, because GAL4 tends to amplify expression levels compared to those mediated by the relevant promoter region as connected to the sequences it normally drives. Also, the *tim* promoter, which is regulated such that this clock gene is largely coexpressed with *per* (Kaneko et al., 1997; Kaneko and Hall, 2000), seems to be stronger than the *per* promoter (Stanewsky et al., 1998; Kaneko and Hall, 1999).

Effects of high, constitutive levels of PER have been studied in the retina by the *rhodopsin-1* promoter/*per* fusion gene (Zeng et al., 1994). A high level of PER in the eye of these transgenic flies resulted in reduction of endogenous *per* transcript in the eye (Zeng et al., 1994). This result and other molecular studies implied that a transcriptional feedback loop comprises part of the circadian pacemaker mechanism, in which PER negatively regulates its own transcription (reviewed by Hardin and Siwicki, 1995). Subsequent gene discoveries and manipulations of their products led to an expansion of this model: PER and TIM cooperate to inhibit both *per* and *tim* tran-

scription, via negative regulation of known transcription factors (reviewed by Hardin, 1998; Young, 1998). This kind of pacemaking mechanism also functions in the circadian clock of other organisms (reviewed by Whitmore et al., 1998; Wilsbacher and Takahashi, 1998; Dunlap, 1999; Dunlap et al., 1999).

An issue that has not been addressed in conjunction with the emergence of these models is the biological significance of negatively acting factors (such as PER) insofar as overt *Drosophila* rhythms are concerned. Thus, PER overexpression in the eye affects *per* expression in that tissue, in which circadian biological rhythms are unknown. Therefore, we wanted to determine whether PER overexpression in CNS cells that express *per* and *tim* would lead to abnormal behavioral rhythmicity. Analogous experiments have been performed by manipulation of the *Neurospora* clock gene, *frequency* (*frq*): constitutive expression of *frq* mediated by a heterologous, inducible promoter reduced the level of frq^+-encoded transcripts and abolished the fungus's circadian rhythm of conidiation (Aronson et al., 1994).

Our experiments showed that PER overexpression, as well as the introduction of TeTxLC into pacemaker neurons, causes abnormal behavioral rhythms. These phenotypes are different from those caused by clock-gene mutations or by *disconnected* mutation that eliminates the LN from the brain (Dushay et al., 1989; Zerr et al., 1990; Hardin et al., 1992; Wheeler et al., 1993; Helfrich-Förster, 1998). Furthermore, expression of TeTxLC only in the LN_vs under the control of *pdf*-promoter-*gal4* fusion gene (Park and Hall, 1998; Park et al., 2000) had only a mild effect on behavioral rhythmicity. This suggests that clock neurons other than the LN_vs are significant components of the neural substrates underlying rest–activity cycles in *Drosophila.*

MATERIALS AND METHODS

Fly Strains

Cultures of *D. melanogaster* were reared on a medium containing agar, yeast, corn meal, dextrose, and a mold inhibitor (Lexgard), in 12-h : 12-h light : dark (LD) cycles, at 25°C, unless otherwise stated below.

As GAL4-driver strains, we used transgenic strains carrying *per-gal4, tim-gal4,* or *pdf-gal4* fusion genes. Constructions of the transgenes *per-gal4* (containing a 4.2-kb DNA fragment, flanking *per*'s transcription unit at its 5′ end) and *tim-gal4* (containing a 6-kb 5′-flanking region of this clock gene) have been described, as have the GAL4-controlled tissue expressions revealed by UAS-fused markers (Plautz et al., 1997a; Emery et al., 1998; Kaneko and Hall, 2000). Construction of *pdf-gal4,* involving a 2.4-kb upstream regulatory sequence of the *pdf* gene is presented elsewhere, as are results revealing marker expression controlled by *pdf-gal4* to be the same as the spatial pattern of endogenous *pdf* (Park et al., 2000).

We used two transgenic lines containing upstream activating sequences (UAS) fused to those encoding tetanus-toxin light chain (*tetxlc*); each carry a transgene encoding active toxin (TNT-G and TNT-E). We also applied one line carrying a control transgene that encodes a mutated, inactive toxin (IMPTNT-V). These TNT transgenics were originally reported by Sweeny et al. (1995). The UAS-*per* line was generated by digesting a full-length *per* cDNA (pSP65ATper; Citri et al., 1987) with *Spe*I (which is 46 bp upsteam of the translation-initiation site ATG), blunting the end with the Klenow fragment of *Escherichia coli* DNA polymerase, adding an *Xho*I linker, removing a 3.9 kb *per* cDNA fragment by *Xho*I and *Xba*I digestion, and ligating the *per* cDNA fragment into the Xho I and Xba I sites of pUAST (Brand and Perrimon, 1993). This *per* cDNA, which terminates 212 bp downstream of the translation stop codon, contains the entire 89-bp alternative intron indicative of a type A transcript (Cheng et al., 1998). The UAS-*per* cDNA construct was transformed into the germline by standard methods, resulting in four independently isolated strains. A double-insert line was generated by recombining UAS-*per* transgenes at two different locations on chromosome *3.* A further derivative of this line was generated in which the *X* chromosomal per^+ allele was replaced by the per^{01} null mutation, permitting the generation of flies in which the only functional *per* genes are those controlled by GAL4.

Three different lines of *per-gal4* (1b, 2, and 3), each with the transgene inserted on chromosome *2,* and five lines for *tim-gal4* (26, 27, 62, 82, and 86), also on chromosome *2,* were crossed separately to UAS-*tetxlc* for behavioral assays. The three *per-gal4* lines exhibit similar patterns of GAL4-driven marker expression in adults, but *per-gal4*-1b leads to marker-gene expression in ectopic locations in the larvae, that is, in additional locations beyond those observed in the other two lines (Kaneko and Hall, 2000). Four of the five *tim-gal4* lines (27, 62, 82, and 86) exhibit similar marker-expression patterns in adults, but in larvae of lines 27 and 82 signals are found in ectopic locations in addition to those observed in the other three lines (Kaneko and Hall, 2000). The current histological tests, involving anti-TIM histochemistry (see below), applied the progeny of one of these *tim-gal4* strains (27), crossed to UAS-*tetxlc.* One line of *pdf-gal4* in which the transgene is inserted on chromosome *2* (used previously by Renn et al., 1999) was crossed to UAS-*tetxlc* for behavioral tests. Crosses involving UAS-*tetxlc* were carried out at 18°C in order to maximize the survival of the progenies by minimizing GAL4-mediated TeTxLC expression at this relatively low temperature (cf. Morimura et al., 1996). One exception involved crosses between UAS-*tetxlc* and *pdf-gal4,* which were carried out at 25°C. No apparent influence on viability by TeTxLC expression under the control of *pdf-gal4* was detected.

To bring control of the *period* under the influence of its own promoter or that of the *timeless* gene, males from the three *per-gal4* lines and four of the five *tim-gal4* ones

(27, 62, 82, and 86) were crossed separately to a UAS-*per* line. The progeny were subjected to behavioral tests. Histological assays involved one line each of *per-gal4* (2) and *tim-gal4* (27).

In order to assay for *per*- or *tim*-driven *luciferase* (*luc*) expression in flies expressing PER under the control of *per-gal4* or *tim-gal4,* several true-breeding strains were established, each carrying a *luc*-containing and a *gal4*-containing transgene. The former were an *X*-chromosomal *per* (BG)-*luc* (in which the *per* promoter plus sequences encoding N-terminal two thirds of PER are fused to *luc;* Stanewsky et al., 1997b) and an *X*-chromosomal *tim-luc* (in which the *tim* promoter is fused to *luc;* Stanewsky et al., 1998). These two fusion genes were, in turn, combined with 2nd-chromosomal *per-gal4* (lines 1b, 2, and 3) and *tim-gal4* (lines 27, 62, 82, and 86). Females from such double-transgene lines were crossed to the UAS-*per* strain (with double inserts on chromosome *3*) to combine three transgene types in a given progeny. As controls, flies carrying BG-*luc* or *tim-luc* and *per-gal4* or *tim-gal4* (without UAS-*per*), those carrying BG-*luc* or *tim-luc* and UAS-*per* (without a GAL4-driver transgene), and those carrying only BG-*luc* or *tim-luc* were assayed for bioluminescence.

A clock-normal strain carrying the *white* eye-color mutation as well as a Canton-S wild-type strain were used as positive controls for immunohistochemical studies. As their negative controls, per^{01} and tim^{01} flies were used.

Viability and Longevity Tests of Flies Carrying *per*- or *tim-gal4* and UAS-*tetxlc* Transgenes

All the GAL4-driver transgenes on chromosome *2* were balanced by introducing a dominantly marked, multiply inverted 2nd chromosome, *In(2LR)0, Cy* (*CyO*). Those on chromosome *3* were balanced by introducing *In(3LR)-TM6,D* (*TM6*). Males from each GAL4-driver line were subsequently crossed (at 18°C) to females homozygous for a given UAS-*tetxlc* type. For each cross, more than 100 offspring were counted for their genotypes. Ratios (%) of progeny carrying a given GAL4-driver transgene (those without a balancer marker for autosomal lines and females for the *X*-chromosomal line) to those without GAL4 (those with a balancer chromosome for autosomal lines and males for the *X*-chromosomal line) were computed as a viability score for each combination of GAL4-driver and UAS-*tetxlc* transgene. Offspring containing one copy each of a GAL4-driver and a UAS-*tetxlc* (those without a balancer chromosome) were transferred to a fresh vial within 1 day of eclosion and were kept at 18°C. Longevity indices were computed as percentages of flies surviving at 2 weeks of adult age.

Behavioral Testing and Analysis

Locomotor activity rhythms were monitored as described in Hamblen et al. (1986) and Hamblen-Coyle et al. (1992) for 3 or 6 days in LD cycles, then 7 to 9 days in constant darkness (DD). For flies monitored for 3 days in LD, only the data for DD are shown. Flies expressing TeTxLC under the control of *per-, tim-,* or *pdf-gal4* were tested at 25°C. Flies expressing PER under the control of either *per*- or *tim-gal4* were tested at 29°C.

Data were analyzed according to Hamblen et al. (1986) and Hamblen-Coyle et al. (1992). LD activity records shorter than 288 0.5-h bins and DD records shorter than 300 0.5-h bins were discarded unless otherwise stated. Whether a given fly behaved rhythmically, and if so the estimate of its period, were determined mainly by χ^2 periodogram (Sokolove and Bushnell, 1978). Flies with a "power" value (height of the χ^2 periodogram peak above the 5% significance line, in arbitrary units) greater or equal to 10 and a "width" (number of period values in 0.5-h increments above that line) greater or equal to 2 were considered to be rhythmic. These criteria for significant rhythmicity were chosen because per^{01} flies frequently yield locomotor-activity records whose corresponding periodograms contain "spikes" that cross the significance line but do not extend far above it and correspond to only one time bin on the abscissa (Hamblen-Coyle et al., 1986). Certain previous studies have used rhythmic cutoffs with power and width values greater than 20 and 2, respectively (e.g., Hamblen et al., 1998; Kaneko et al., 2000); we reduced the former cutoff value to 10, because many flies with $10 \leq$ power ≤ 20 and width ≥ 2 seemed almost certainly to have behaved rhythmically by eye inspection of their actograms (72 of 96 such records, involving flies of various genotypes, were rhythmic by eye inspection of the actogram; data not shown). It was also clear from visual inspection of actograms that several flies were weakly but unambiguously rhythmic even if the periodogram contained a peak with width $= 1$, but the power ≥ 40 (all seven of such records were rhythmic by eye inspection of the actogram; data not shown); therefore, those flies with power ≥ 40 and width $= 1$ were also categorized as rhythmic.

Average-activity plots for a given fly's LD behavior (histograms in which each bar displays the mean activity per day) and for all flies of a given genotype (histograms in which each bar displays a mean of a mean, i.e., the average activity per day per fly) were generated according to Hamblen-Coyle et al. (1992). In addition, average-activity levels (mean number of locomotor events per 0.5-h bin per day) were computed for the LD records, separately for the light and the dark phases (cf. Hamblen-Coyle et al., 1989).

Statistics

Comparison of average activity counts among flies of different genotypes was carried out by the Wilcoxin/Kruskal-Wallis nonparametric test (Sokal and Rohlf, 1995) using the Jump program (SAS Institute, Cary, NC; Macintosh version 3.1). For the comparisons of period lengths for flies expressing TeTxLC under the control of *pdf-gal4,* Mann-Whitney *U* tests (Sokal and Rohlf, 1995) were performed using the Instat program (GraphPad Software; Macintosh version 2.0). Comparison of peak phases for bioluminescence cycling was performed by a nonparametric test for common mean direction by Watson (1983; cf. Fisher, 1993).

Immunohistochemistry

Immunohistochemistry on frozen sections was performed as described in Stanewsky et al., (1997a). Polyclonal anti-PER from rabbit (Stanewsky et al., 1997a) was used at 1:15,000; polyclonal anti-PER from rat (Kaneko et al., 1997) at 1:2000; and polyclonal anti-TIM from rat (Stanewsky et al., 1998) at 1:4000. Sections stained after application of the appropriate secondary antibodies and colorimetric methods were observed under a Zeiss Axioskop microscope using Nomarski optics. Quantification of staining intensities was effected subjectively but blindly as in Kaneko et al. (1997).

As negative controls *per*^{01} flies were stained for anti-PER and *tim*^{01} flies for anti-TIM. In both cases, background staining was observed in the gut, Malpighian tubules, and fat body.

Luciferase Monitoring and Analysis

Time-based automated assays of luciferase activity in individually monitored, *luc*-containing flies were performed essentially as described in Stanewsky et al., (1997b). Data were analyzed for phase, period, and amplitude according to Stanewsky et al., (1997b) using an interactive, coupled Fourier-transform/nonlinear, least squares multicomponent analysis (Plautz et al., 1997b).

RESULTS

Locomotor-Activity Rhythms Disrupted by *per* or *tim* Control of a *tetanus-toxin* Gene

To study the function of clock-gene–expressing neurons, the *tetanus-toxin-light-chain* (*tetxlc*) gene (Sweeny et al., 1995) was expressed under the control of *per-gal4* or *tim-gal4*. The clock-gene-promoter-*gal4* transgenics were combined with each of two transgenes in which the target of GAL4 (UAS) is fused to *tetxlc* (TNT-G and TNT-E). As a control, an inactive mutant form of the *tetxlc* (IMPTNT-V) fused to UAS (Sweeny et al., 1995) was combined (separately) with *per-gal4* and *tim-gal4*.

Before flies of these types were tested for adult behavior, *per-gal4* lines and *tim-gal4* lines were tested for viability and longevity in combination with each of these UAS-*tetxlc* lines. The scores for these two indicators of general health (see Materials and Methods) varied among different *per-gal4* and *tim-gal4* strains—involving different chromosomal insertion sites of the transgenes—when they were combined with UAS-active *tetxlc* (data not shown). The *tim-gal4* lines 62 and 86 were found to be especially impaired: 0–4% flies survived after 2 weeks. Therefore, these *tim-gal4* lines were behaviorally studied only in combination with UAS-inactive *tetxlc* (data not shown). When viability and longevity scores were compared among flies carrying a given GAL4-driver transgene and UAS-*tetxlc* (active) at two different chromosomal locations (TNT-E and TNT-G), flies carrying the TNT-E insert in general survived better than those carrying the TNT-G insert (data not shown). For example, the short-lived *tim-gal4* flies derived from lines 62 and 86 (see above) had ca. 64–67% viability scores (relative adult-emergence probabilities) when driving TNT-E but only 2–4% with TNT-G. This suggests that TNT-G gives a higher level of UAS-promoted toxin than does TNT-E.

Flies carrying one copy each of UAS-*tetxlc* and *gal4* were tested for locomotor-activity rhythms in light : dark cycles (LD) and constant darkness (DD) (Fig. 1; Table 1). In LD, the proportions of individually tested adults that synchronized to the environmental cycles (i.e., that entrained) were reduced in those carrying *per-gal4* or *tim-gal4* and UAS-*tetxlc* (active TNT-G or TNT-E) compared to those carrying only the GAL4 driver or UAS-*tetxlc*. Approximately 100% of these single-transgene controls entrained to the LD cycles. The proportions of entrained flies were smaller when *per-gal4* or *tim-gal4* was combined with TNT-G compared to the TNT-E combinations (Table 1), consistent with the viability and longevity results. A companion phenotype to the toxin-induced reduction of entrainability was that period values slightly diverged from 24 h in flies expressing active TeTxLC under the control of *per-gal4* or *tim-gal4;* whereas almost all unimpaired *Drosophila* exhibit 24.0-h periodic behavior in 12-h : 12-h LD (e.g., Hamblen-Coyle et al., 1992). Flies carrying *tim-gal4* or *per-gal4* and IMPTNT-V entrained better than did those that expressed active toxin under the control of *per-gal4* or *tim-gal4*. However, there was a slight decrement in the proportion of entrained flies when certain of the GAL4-driver lines, such as *per-gal4*-2, were combined with IMPTNT-V, compared to the behavior of flies carrying only a GAL4 driver or IMPTNT-V (Table 1).

These idiosyncrasies notwithstanding, the average-activity plots in Figure 1 show that the LD behavioral patterns of *per-gal4*/IMPTNT-V and *tim-gal4*/IMPTNT-V were indistinguishable from those of control flies carrying only a IMPTNT-V or a GAL4-driver transgene. For instance, flies expressing inactive TeTxLC showed the conventional activity peaks at dawn and dusk (cf. Hamblen-Coyle et al., 1992); such behavioral maxima were observed in control flies carrying only a GAL4-driver or a IMPTNT-V transgene. In contrast, flies expressing active toxins under the control of *per-gal4* or *tim-gal4* tended to be uniformly active during the day and night and exhibited

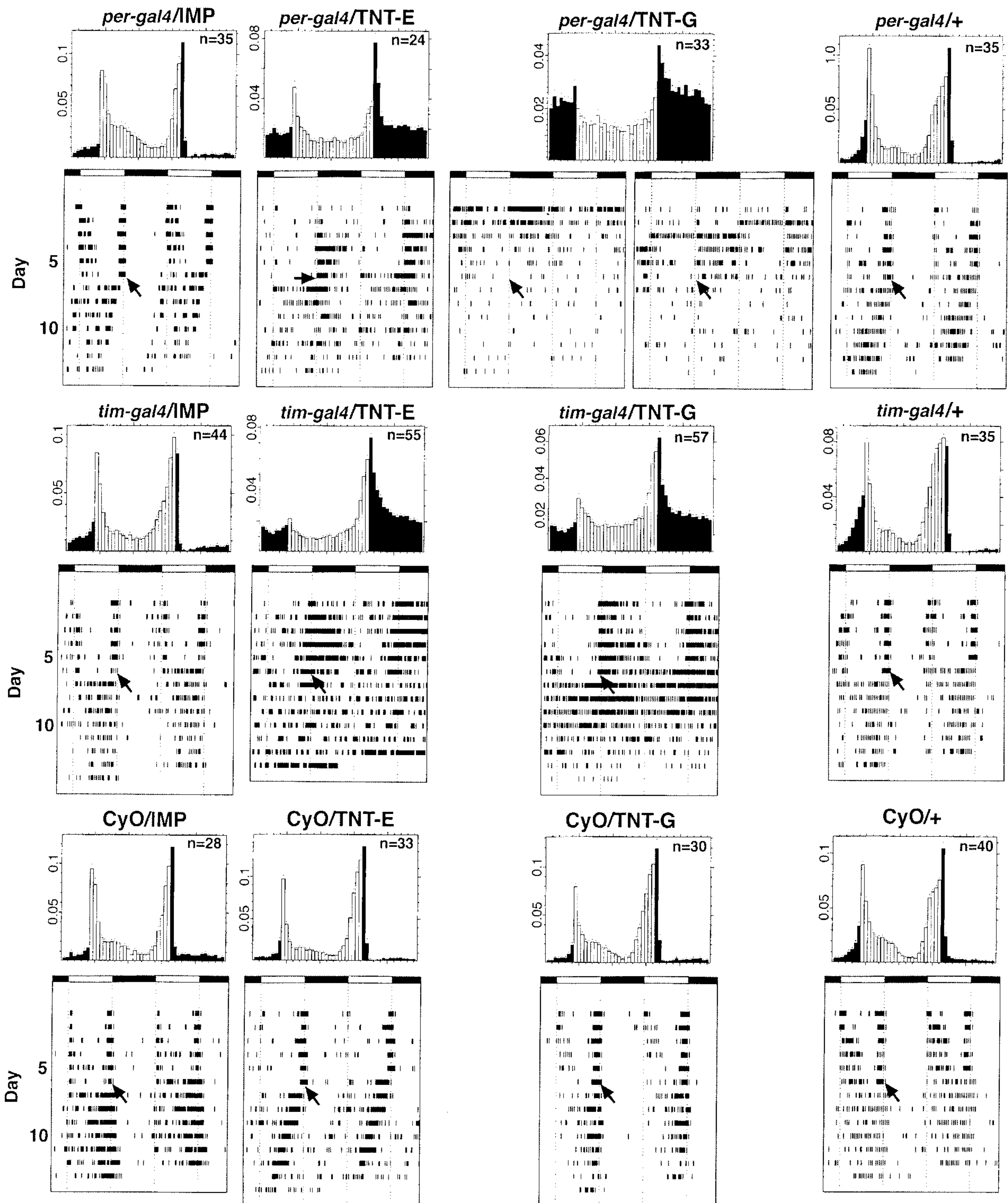

Figure 1 Average-activity plots and actograms for flies expressing TeTxLC under the control of *per-gal4* or *tim-gal4*. In the former (presented as histograms, each with 48 0.5-h time bins), a per-day average of the LD behavior of a given fly was generated; then the average behavior of all flies of that genotype was produced; the averaging procedure involves normalizing the activity levels of the separate flies (as in Hamblen-Coyle et al., 1989) so the ordinates are dimensionless; dots above the black or white bars (12 h worth of nighttime and of daytime bins, respectively) indicate S.E.M.'s for the (normalized) per-fly activity average for the separate time bins. The actograms (presented below each average-activity plot) display locomotor activity as "tick marks,"

relatively small increases in locomotion after lights-on.

Wild-type *Drosophila* in LD exhibit a substantial locomotor-activity rise during the second half of the day (e.g., Hamblen-Coyle et al., 1992). Flies expressing active TeTxLC under the control of *per-gal4* or *tim-gal4* tended to behave in this manner (i.e., before lights-off), as reflected in the average-activity plots (Fig. 1). However, eye-inspection of these double-transgenics' average-activity plots revealed that clear anticipations of lights-off occurred in only 15–80% of the animals tested for effects of the TNT-G insert, depending on the GAL4 line used to drive TeTxLC. (The plots are histograms of per-day means for individual flies, as opposed to those of per-fly means for the entire population of flies of a given genotype; the latter are exemplified in Fig. 1). In contrast, anticipation of lights-off (by a steady increase in locomotion) was observed for all the control individuals, those expressing inactive toxin and those carrying only a GAL4-driver or only the TNT-G insert.

Is the relatively uniform day and nighttime activity caused by *per* or *tim*-driven toxin a matter of anomalously increased locomotion during the night or a decrease in activity during the day? The answer cannot leap out by inspection of the per-fly average-activity plots, because peak locomotion values are normalized among flies (Hamblen-Coyle et al., 1992). Thus, average-activity counts were compared among different genotypes for the light and for the dark phases [Table 2(A)]. During the day, TeTxLC (active) causes mild decrements in locomotion, although such an activity-reducing effect of the toxin was significant only when the TNT-G (not TNT-E) insert was used to drive strong expression of active TeTxLC under the control of GAL4-driver lines (except for *per-gal4*-1b) [Table 2(A)]. This modest reduction of daytime activity in the (experimental) double-transgenic types (*gal4* plus TNT-G) seems to be mostly due to a reduction of the activity peak at dawn (Fig. 1).

During the nighttime, flies expressing active toxins under the control of *per-gal4* or *tim-gal4*—compared to those expressing inactive toxin, a GAL4 driver, or a UAS-*tetxlc* transgene [Table 2(A)] alone—exhibited higher than normal activity. Such increased levels of locomotion in flies carrying TNT-G in combination with a *tim-gal4* transgene were small compared to those observed in flies carrying TNT-E (along with the self-same GAL4 driver). In contrast, flies carrying TNT-G showed more severe defects in viability, longevity, and basic LD entrainability than did those carrying TNT-E. In a *per-gal4* background, increased nighttime activity was again observed and to the same extent in flies carrying TNT-G or TNT-E. In spite of the mild anomalies associated with these data (TNT-G vs. -E; *tim-* vs. *per-gal4*), one of the main points is that the behavioral abnormalities involve high nighttime activity: hence, *per-gal4* or *tim-gal4,* driving active *tetxlc,* do not lead to general sluggishness in LD, which could occur if the function of motor neurons or generic motor centers were impaired.

In constant darkness (DD), abnormally high degrees of behavioral arrhythmicity occurred when GAL4-driver transgenes were combined with UAS-active toxins, compared to the behavior of controls, for example, those carrying only GAL4 driver or UAS-*tetxlc* (Table 1). Expression of inactive toxin under the control of *tim-gal4* reduced the number of rhythmic flies, but *per-gal4*-driven expression of this mutant form of the toxin resulted in no decrease of rhythmicity.

Importantly, active TeTxLC expression under

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with respect to 6 days of LD behavior (top part of each actogram), then 7–8 days of free-running behavior in DD; each tick mark is equivalent to 25 activity events; the black (night) and white (day) boxes above each actogram indicate that these records are double plotted: 2 successive days of behavior are displayed horizontally and vertically (days 1 and 2 on the top line, 2 and 3 on the second line, etc); arrows point to the time of the last lights-off, i.e., before the flies were shifted to DD. Each actogram represents the typical pattern of activity for flies of a given genotype. Two actograms are shown for flies behaving under the influence of *per-gal4* and TNT-G. Number of flies that were averaged for each average-activity plot is shown on the top right corner of each plot. Control flies carrying no GAL4 transgenes were progeny carrying the dominantly marked *CyO* balancer chromosome from crosses between GAL4-driver strains (in which the transgene is balanced by *CyO*) and homozygous UAS-*tetxlc* lines. For *per-gal4,* plots for line 2 are shown here. The effect of active toxins on the pattern of activity in LD conditions—best revealed in the average-activity plots—was strongest under the influence of *per-gal4* from this line (among the three such lines used); qualitatively similar effects of active toxin were observed when UAS-*tetxlc* was combined with this GAL4 driver from the other lines. For *tim-gal4,* plots of UAS-*tetxlc*/GAL4-driver flies—the latter transgene derived from line 27—are shown; similar patterns of activity were found in the flies with the driver derived from other two lines of this type, tested in combination with UAS-active toxin as well as with inactive toxin.

Table 1 Rhythmicity and Periods of Locomotor Activity for *Drosophila* Expressing the *tetxlc* Gene, Controlled by *per-gal4* or *tim-gal4,* in Light–Dark Cycling or Environmentally Constant Conditions

		LD			DD		
UAS-*tetxlc* transgenes	GAL4 driver lines	*n*	Entrained (%)	Behavioral cycle* (h)	*n*	Rhythmic (%)	Behavioral cycle* (h)
IMPTNT-V	*per-gal4*-1b	35	97	24.0 ± 0.0	35	100	23.7 ± 0.1
	per-gal4-2	35	86	24.0 ± 0.0	38	97	23.4 ± 0.1
	per-gal4-3	36	97	24.0 ± 0.0	41	100	23.4 ± 0.1
	tim-gal4-26	36	94	24.0 ± 0.0	26	85	24.2 ± 0.1
	tim-gal4-27	44	100	24.0 ± 0.0	28	93	24.3 ± 0.1
	tim-gal4-82	40	100	24.0 ± 0.0	38	84	24.5 ± 0.1
	None	28	96	23.6 ± 0.4	35	94	23.7 ± 0.1
TNT-E	*per-gal4*-1b	37	100	23.8 ± 0.3	34	88	23.7 ± 0.1
	per-gal4-2	24	67	24.0 ± 0.1	22	27	23.5 ± 0.2
	per-gal4-3	33	82	24.0 ± 0.1	33	73	23.7 ± 0.1
	tim-gal4-26	39	69	24.1 ± 0.1	26	35	24.6 ± 0.4
	tim-gal4-27	55	100	24.3 ± 0.1	35	34	24.1 ± 0.2
	tim-gal4-82	36	53	24.1 ± 0.1	19	53	24.5 ± 0.2
	none	33	100	24.0 ± 0.0	44	100	23.2 ± 0.1
TNT-G	*per-gal4*-1b	30	87	23.8 ± 0.5	33	76	23.6 ± 0.1
	per-gal4-2	33	48	24.1 ± 0.1	8	0	
	per-gal4-3	27	33	24.2 ± 0.1	20	25	24.0 ± 0.2
	tim-gal4-26	32	9	25.0 ± 0.6	14	14	33.8 ± 3.8
	tim-gal4-27	57	63	24.0 ± 0.1	24	0	
	tim-gal4-82	30	20	23.8 ± 0.4	5	0	
	none	30	100	24.0 ± 0.0	41	100	23.7 ± 0.1
None	*per-gal4*-1b	35	100	24.0 ± 0.0	34	100	23.6 ± 0.0
	per-gal4-2	35	100	24.0 ± 0.0	33	100	23.5 ± 0.1
	per-gal4-3	35	100	24.0 ± 0.0	34	100	23.4 ± 0.1
	tim-gal4-26	34	100	24.0 ± 0.0	31	100	24.2 ± 0.1
	tim-gal4-27	35	100	24.0 ± 0.0	31	100	24.3 ± 0.1
	tim-gal4-82	35	100	24.0 ± 0.0	33	100	24.2 ± 0.1
	none	40	100	24.0 ± 0.0	40	100	23.6 ± 0.1

For the light-dark cycling (LD) and constant-dark (DD) conditions, *n* is the numbers of flies that gave valid data (activity records that lasted for 6 days in LD, and those with more than 300 0.5-h bins in DD). In the "Entrained" and "Rhythmic" columns are given percentages of flies that were diagnosed as rhythmic (with respect to periodogram-based metrics; see Materials and Methods) among those that resulted in valid records.

* Values are means ± S.E.M.

the GAL4-driver transgenes did not cause a decrease in the amount of average activity per 0.5-h bin in DD, with one exception: flies carrying TNT-G and *per-gal4*-2 showed substantially lower activity compared to controls carrying only TNT-G or *per-gal4*-2 (see Fig. 1). However, flies carrying *per-gal4*-2 and TNT-E (even those flies diagnosed as arrhythmic by periodogram) did not show such sluggishness, implying that TeTxLC expression under the control of this particular line of *per-gal4* can cause arrhythmicity in DD without affecting the overall amount of activity. Therefore, the arrhythmicity observed in flies carrying TNT-G and *per-gal4*-2 does not appear to be a direct consequence of low levels of locomotion. For the rhythmic individuals in these DD experiments, circadian periods of most flies were not dramatically affected by expression of active or inactive toxins under the control of *per-gal4* or *tim-gal4* (Table 1). Note, however, that flies carrying *tim-gal4* (irrespective of the presence of UAS-*tetxlc*) gave slightly longer periods than did those carrying *per-gal4* (Table 1).

TIM Immunoreactivity in Toxin-Containing Flies

As TeTxLC specifically cleaves neuronal synaptobrevin (see Introduction), one would not expect this toxin

Table 2 Average Activity of Flies Expressing UAS-Fusion Genes under the Control of *per-gal4* or *tim-gal4*.

UAS-*tetxlc*	*gal4*	*n*	Activity/Bin Day	Activity/Bin Night
A. Tetanus toxin				
IMPTNT	*per-gal4*-1b	15	31.5 ± 3.2	13.4 ± 3.0
	per-gal4-2	25	26.8 ± 1.6	7.7 ± 0.8
	per-gal4-3	19	29.1 ± 2.7	7.2 ± 1.1
	tim-gal4-26	29	27.8 ± 1.7	17.8 ± 2.3
	tim-gal4-27	14	20.3 ± 1.6	10.8 ± 1.4
	tim-gal4-82	17	18.4 ± 1.4	9.5 ± 1.2
	none	14	27.1 ± 2.8	8.1 ± 1.4
TNT-E	*per-gal4*-1b	16	25.6 ± 1.8	35.2 ± 6.9
	per-gal4-2	15	24.1 ± 2.8	30.7 ± 3.8
	per-gal4-3	12	21.7 ± 2.9	36.4 ± 5.9
	tim-gal4-26	34	29.6 ± 2.1	47.5 ± 2.9
	tim-gal4-27	15	22.4 ± 3.2	52.2 ± 5.8
	tim-gal4-82	12	31.7 ± 7.5	35.4 ± 4.7
	none	15	45.4 ± 6.7	14.2 ± 2.8
TNT-G	*per-gal4*-1b	13	47.9 ± 9.3	49.9 ± 7.1
	per-gal4-2	31	22.6 ± 2.4	38.1 ± 3.5
	per-gal4-3	13	30.3 ± 4.7	35.0 ± 6.0
	tim-gal4-26	24	23.2 ± 4.1	21.3 ± 2.1
	tim-gal4-27	14	23.0 ± 3.5	29.3 ± 5.9
	tim-gal4-82	14	26.5 ± 8.1	23.9 ± 3.8
	none	10	52.1 ± 10.8	17.1 ± 5.5
None	*per-gal4*-1b	35	40.5 ± 2.6	17.1 ± 1.7
	per-gal4-2	35	32.8 ± 2.0	11.9 ± 0.9
	per-gal4-3	35	41.3 ± 3.3	14.2 ± 2.0
	tim-gal4-26	34	36.9 ± 2.6	16.5 ± 2.1
	tim-gal4-27	35	35.3 ± 2.7	13.1 ± 1.4
	tim-gal4-82	35	35.0 ± 2.6	13.5 ± 1.3
	none	40	34.8 ± 3.4	13.0 ± 1.4

Background	*gal4*	UAS-*per*	*n*	Activity/Bin Day	Activity/Bin Night
B. UAS-*per*					
per$^+$	*per-gal4*-1b	+	12	27.7 ± 3.8	35.4 ± 3.8
		−	10	23.9 ± 1.5	20.8 ± 2.9
	per-gal4-2	+	11	21.3 ± 2.5	38.9 ± 4.5
		−	10	18.9 ± 1.8	23.4 ± 1.4
	per-gal4-3	+	8	18.9 ± 3.4	25.6 ± 3.2
		−	8	16.6 ± 2.4	13.1 ± 1.7
	tim-gal4-62	+	7	30.7 ± 4.2	53.0 ± 4.4
		−	20	29.4 ± 2.5	38.7 ± 2.9
	tim-gal4-86	+	7	26.2 ± 6.3	44.5 ± 9.6
		−	16	22.2 ± 2.6	25.1 ± 2.4
	tim-gal4-82	+	9	21.4 ± 1.9	47.5 ± 5.0
		−	19	15.6 ± 1.2	14.7 ± 1.1
	tim-gal4-27	+	19	22.9 ± 1.8	36.5 ± 2.6
		−	18	17.6 ± 1.0	20.6 ± 1.4
	None	+	33	19.7 ± 1.0	36.0 ± 2.8
per^0	*per-gal4*-1b	+	9	23.5 ± 1.8	23.1 ± 2.0
		−	32	26.6 ± 2.3	25.4 ± 2.7
	per-gal4-2	+	11	21.1 ± 2.6	33.8 ± 3.9
		−	30	21.1 ± 1.3	26.8 ± 1.8
	per-gal4-3	+	12	23.9 ± 3.1	35.6 ± 5.1
		−	22	34.3 ± 3.5	37.6 ± 3.8
	tim-gal4-62	+	10	30.6 ± 6.0	50.7 ± 9.4
		−	34	28.3 ± 1.7	35.1 ± 2.2
	tim-gal4-86	+	9	35.9 ± 10.4	56.2 ± 9.4
		−	33	28.2 ± 2.4	34.5 ± 3.4
	tim-gal4-82	+	11	15.0 ± 1.6	31.3 ± 2.6
		−	34	22.7 ± 3.3	28.2 ± 4.1
	tim-gal4-27	+	10	25.9 ± 3.1	44.6 ± 7.7
		−	37	27.1 ± 2.4	29.1 ± 2.6
	none	+	33	19.4 ± 1.2	28.3 ± 1.9

A: Numbers of locomotor events (infrared light-beam crossings) were obtained as the output from the LD activity monitoring experiments for each of the 0.5-h time bins (after which such events are written to disc). These events per 0.5-h bin were averaged for each fly and each genotype (average activity per bin per fly) and are shown for each combination of GAL4-driver transgene and UAS-*tetxlc,* one value for each of day and night phases in LD cycling conditions. The Wilcoxon/Kruskal–Wallis nonparametric tests (Sokal and Rohlf, 1995) were performed to assess these differences in activity level (either daytime or nighttime) among different genotypes. Significance levels were adjusted to .005, owing to the experiment-wise error computed for these data (Sokal and Rohlf, 1995). In addition to the systematic effects of TeTxLC (active) on nighttime activity, that in the daytime may be reduced by strong TeTxLC expression—in which GAL4 drives TNT-G—with one exception: *per-gal4*-1b/TNT-G flies did not show decrease in daytime activity compared to the control carrying only *per-gal4*-1b. The apparent activity reduction caused by GAL4/TNT-E was not statistically significant.

B: Average activities per 0.5-h bin during light and dark phases of LD cycles are given for each *per* genetic background (1st column) and GAL4-driver type (2nd column), with (+) or without (−) the UAS-*per* transgenes (3rd column). For flies carrying only a GAL4-driver in the *per*^{01} genetic background, 5 days' worth of data (instead of the usual 6) were analyzed. In the Wilcoxin/Kruskal–Wallis tests (Sokal and Rohlf, 1995), performed to check differences among genotypes, significance levels were adjusted to .007, for flies carrying the *per*^{01} mutation, and .004 for flies carrying endogenous *per*$^+$ gene, owing to experiment-wise error (Sokal and Rohlf, 1995). No systematic effect of *per* expression under the control of *per-gal4* or *tim-gal4* on the overall daytime activity or nighttime activity was detected.

to affect the intracellular molecular feedback loop responsible for circadian oscillations. Nevertheless, we looked into this matter by staining clock-gene–expressing cells with anti-TIM. The experimental flies examined histologically—at the normal TIM trough and peak times (cf. Hunter-Ensor et al., 1996; Myers et al., 1996; Zeng et al., 1996)—carried the active form of TeTxLC (encoded by TNT-G or -E) and *tim-gal4*. Controls carried the inactive form of TeTxLC and *tim-gal4*. Flies were entrained in 12-h : 12-h LD cycles for 4 days at 25°C before they were sacrificed and processed for immunohistochemistry. Staining intensities for eight categories of clock-gene–expressing cells were scored (subjectively but blindly): six neuronal clusters (cf. Kaneko, 1998), brain glia, and photoreceptor cells in the compound eye. At Zeitgeber Time 9 (the trough, with respect to ZT 0 being the time of lights-on) and ZT 21 (TIM

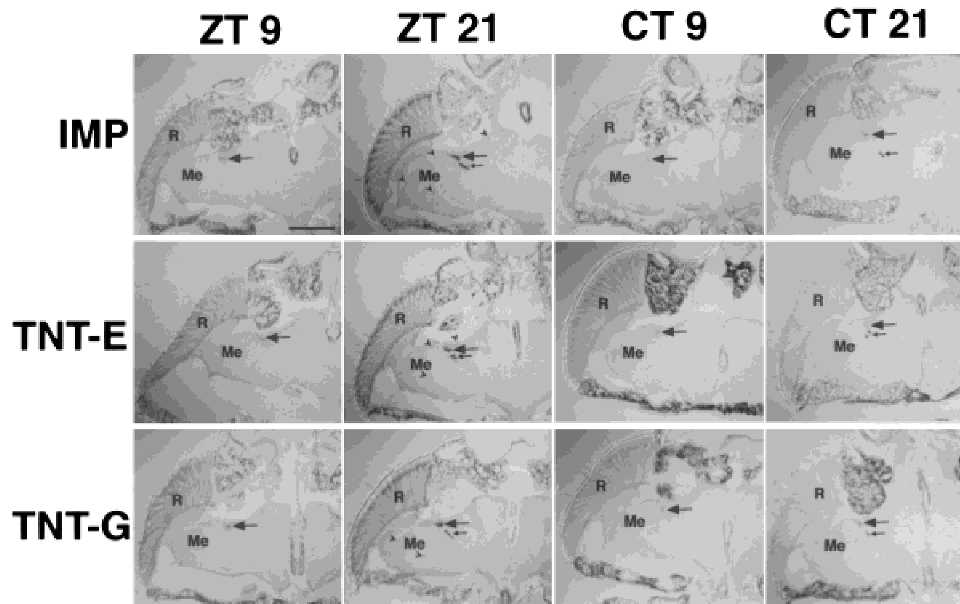

Figure 2 TIM staining in flies expressing TeTxLC under the control of *tim-gal4*. TIM immunoreactivity in horizontal sections through adult heads of flies carrying *tim-gal4* (line 27) and UAS-active *tetxlc* (TNT-E and TNT-G) or inactive *tetxlc* (IMP) is shown. The animals whose representative sections are in the two left-hand columns were sacrificed at ZT 9 and ZT 21 in LD-cycling conditions (9 h after lights-on and 9 h after lights-off). The two right-hand columns present examples from flies in constant darkness, sacrificed at CT 9 and CT 21 (9 h after subjective dawn and dusk, respectively) on the second DD day. Large arrows point to the relatively large, ventrally located, clock-gene–expressing lateral neurons (LN_vs); small arrows to small LN_vs, and arrowheads to glial cells; R, retina; Me, medulla optic lobe. Bar, 100 μm. No genotype-dependent differences in staining intensities were found among the three doubly transgenic types, for any of the cell types shown; nevertheless, temporally dependent anti-TIM staining differences were observed (very weak or no signals at ZT 9 or CT 9), and these were the same among genotypes.

peak, 9 h after lights-off), the immunoreactivity levels were clearly different: higher at the latter (nighttime) phase compared with ZT 9, in all the cell types within flies of experimental and control genotypes (Figs. 2, 3). No differences in staining intensities were detected for any of the cell types among different genotypes. Therefore, expression of TeTxLC in *tim*-expressing neurons does not affect TIM oscillations in these cells in the head.

In addition to examining clock-gene expression in the anterior CNS and PNS, glia in the thoracic and abdominal nervous system, along with cells in the gut and Malpighian tubules, were checked for TIM immunoreactivity. Although there was a low level of background staining in these tissues, strong nuclear signals were observed at ZT 21 but not at ZT 9 in flies of all genotypes (data not shown), indicating that TeTxLC has no effect on TIM oscillations in these posterior tissues. This is consistent with the fact that TIM cycles in the Malpighian tubules in an apparently autonomous manner (Giebultowicz et al., 2000).

To address the issue of toxin-affected pacemaker functioning in free-running (DD) conditions, in which chemical transmission from clock-gene–expressing cells may be necessary for the maintenance of molecular cycling in certain cells, a histochemical experiment was performed on flies expressing TeTxLC: the flies were sacrificed at Circadian Time (CT) 9 and CT 21 on the second day of DD (CT 0 and CT 12 correspond to the beginning and the end of the subjective day, during which it is of course dark; but CT 0–12 extrapolates back to the daylight hours within the pre-DD environmental cycles). In contrast to the result in LD conditions, robust cycling of TIM was observed only in three neuronal clusters (Figs. 2, 3), two of them located in lateral-brain regions (small $LN_{\text{-ventral}}$ and LN_{dorsal} cells), one dorsally (DN1). Almost no cycling of TIM was detected in the eyes, although within the large LN_v cells TIM immunoreactivity was slightly higher at CT 9 than at CT 21 (Fig. 3). A key feature of these DD results is that no differences in staining intensity were observed among flies expressing different amounts or forms of the

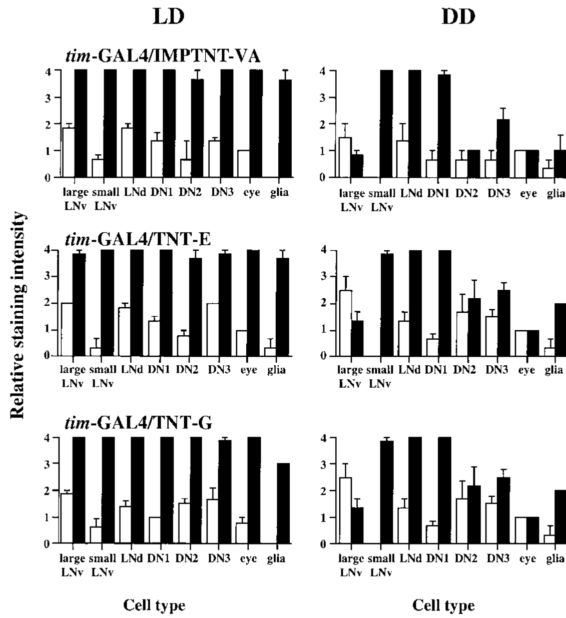

Figure 3 Quantification of TIM staining intensities in flies expressing TeTxLC under the control of *tim-gal4* (line 27). The immunohistochemical signals were quantified using sections of the type exemplified in Figure 2; subjective staining levels were scored blindly as in Kaneko et al. (1997). For each timepoint, cell type, genotype and experimental condition, three flies were scored, with two exceptions: four flies were scored for doubly transgenic *tim-gal4*/TNT-G at ZT 9 (in LD) and for those carrying IMPTNT-V and *tim-gal4* at CT 9 (in DD). Black bars indicate average intensity scores for ZT 21 (LD) or CT 21 (DD), and white bars for ZT 9 (LD) or CT 9 (DD). Error bars, S.E.M.

toxin in any of the TIM-expressing cells at either timepoint (Figs. 2, 3). Therefore, expression of active or inactive TeTxLC in clock neurons does not affect molecular cycling in various clock cells in DD as well as in LD conditions. Reduction of TIM cycling in DD in the retina and in the large LN_vs was also observed in a control strain that carries only the *white* eye-color mutation (without any transgenes; data not shown).

These data suggest that TeTxLC expression in clock cells does not affect intracellular oscillatory mechanisms; nor does it affect entrainment of molecular cycling in many clock cells, including the pacemaker neurons essential for behavioral rhythmicity. Therefore, anomalous locomotor activity rhythms in these flies are not due to defects in entrainment or molecular pacemaking mechanisms, but most likely to the inability of the pacemaker neurons to convey rhythmic signals to their downstream targets. This result also suggests that there is no feedback from the clock output pathways to the molecular pacemakers mediated by synaptic transmission that can be blocked by TeTxLC.

Locomotor Rhythms of Flies Expressing TeTxLC under the Control of a Neuropeptide Gene

TeTxLC expression under the control of *per-gal4* or *tim-gal4* resulted in abnormal rhythmic behavior during LD cycles as well as in DD. As *per-gal4* and *tim-gal4* drive marker-gene expression in most classes of clock neurons in the brain, it is not clear which such cells are responsible for the TeTxLC effect on behavioral rhythms. In order to narrow down the neuronal substrates responsible for this phenotype, we expressed TeTxLC under the control of another GAL4-driver transgene, *pdf-gal4,* in which 5′-flanking sequences from a gene encoding the neuropeptide pigment-dispersing factor (PDF; Park and Hall, 1998) were fused to the GAL4-encoding gene (Park et al., 2000). *pdf-gal4* drives expression of the *lacZ* marker gene only in eight *per/tim*-expressing LN_vs per brain hemisphere, including four small LN_vs and four large ones, in addition to four neurons in the abdominal ganglion that also express *pdf* (Park et al., 2000). Therefore, TeTxLC is expected to be expressed in most of the *per/tim*–expressing LN_vs under the control of *pdf-gal4* (exception: one PDF-negative, but PER-positive small LN_v cell; Kaneko et al., 1997).

Flies expressing TeTxLC under the control of *pdf-gal4* did not show appreciable defects in rhythmicity in LD cycles as well as in DD (Table 3; Fig. 4). The proportion of flies synchronized to LD cycles was the same for all the genotypes tested (those expressing inactive toxin or active toxin under the control of *pdf-gal4,* and controls carrying only UAS-inactive *tetxlc* or UAS-active *tetxlc*) (Table 3). TeTxLC expression controlled by *pdf-gal4* had no effect on the pattern of activity during LD cycles: although activity during the night seemed to be higher in flies expressing active toxin (via the TNT-G transgene), compared to those expressing inactive one (by IMPTNT-V), a similar effect of the TNT-G insert was observed in the absence of *pdf-gal4* (Fig. 4). In DD, only a small decrease in the proportion of rhythmic flies was observed in flies carrying TNT-G and *pdf-gal4,* compared to that of controls expressing inactive toxin or to the behavior of flies carrying only UAS-*tetxlc* (Table 3). However, even the three *pdf-gal4*/TNT-G animals diagnosed as arrhythmic by periodogram exhibited weak rhythmicity, judging from eye examination of actograms (see the DD actogram for one of the formally arrhythmic flies carrying *pdf-gal4* and TNT-G in Fig. 4). The behavioral cycle durations for rhythmic flies were slightly longer in flies carrying *pdf-gal4* compared with those without the GAL4-driver transgene ($p < .0001$, $\alpha = .01$ in each of the following comparisons: *pdf-gal4*/IMPTNT vs. IMPTNT/+, and

Table 3 Rhythmicity and Periods of Locomotor Activity for *Drosophila* Expressing the *tetxlc* Gene, Controlled by *pdf-gal4*

		LD			DD	
UAS-*tetxlc*	*pdf-gal4*	*n*	Entrained (%)	Behavioral cycle* (h)	Rhythmic (%)	Behavioral cycle* (h)
IMPTNT	+	14	86	24.0 ± 0.0	100	24.3 ± 0.1
	−	15	87	24.0 ± 0.0	100	23.6 ± 0.1
TNT-G	+	16	88	24.1 ± 0.0	81	24.5 ± 0.1
	−	16	88	24.0 ± 0.1	100	23.6 ± 0.0

The first column gives the UAS-*tetxlc* transgene carried by the flies tested. The presence or absence of the *pdf-gal4* transgene in a given group of flies is indicated by "+" and "−", respectively, in the 2nd column. *n* is the number of flies that gave valid data (see Materials and Methods).

* Values are means ± S.E.M.

pdf-gal4/TNT-G vs. TNT-G/+) (Table 3). However, circadian periods of flies expressing inactive toxin under the control of *pdf-gal4* were not significantly different from those of *pdf-gal4*/TNT-G double transgenics ($p = .15$, $\alpha = .01$) (Table 3). Therefore, TeTxLC expression under the control of *pdf-gal4* did not cause changes in locomotor periodicities.

Thus, TeTxLC expression under the control of *pdf-gal4* has a substantially milder effect on locomotor activity rhythms compared to cases in which the same toxin is driven by *per-gal4* or *tim-gal4* in a wider array of brain cells.

Locomotor Rhythms Disrupted by Anomalous *per* Expression

To study the effects of *period*-gene overexpression, flies carrying two copies of UAS-*per* (on chromosome *3* in homozygous condition, hence four copies per fly) were crossed to those carrying *per-gal4* or *tim-gal4.* Progeny from these crosses—carrying two copies of UAS-*per* and one copy of *per-gal4* or *tim-gal4,* in a per^+ genetic background—were monitored for locomotor activity rhythms at 29°C. This rather high temperature was used, because GAL4-mediated gene expression increases in such conditions (e.g., Morimura et al., 1996).

The majority of the flies expressing *per* under the control of *per-gal4* or *tim-gal4* synchronized to LD cycles, although the proportions of entrained flies were slightly lower than in the case of controls carrying only UAS-*per, per-gal4,* or *tim-gal4;* the percentage of (experimental) entrainees was most subnormal when UAS-*per* was combined with *tim-gal4* (Table 4).

Patterns of locomotor activity (in LD) were qualitatively anomalous when the extra (transgene-carried) per^+ allele was driven by GAL4, especially when UAS-*per* and *tim-gal4* were combined (Fig. 5). In these flies, the activity increase that normally occurs before lights-off was not observed; instead there were high levels of locomotion just after lights-off. Therefore, the *tim-gal4;*UAS-*per* flies do not anticipate lights-off, but respond to this environmental change by what has been termed a "startle response"—a behavior exhibited by the clockless per^{01} mutant (exemplified here in the average-activity plot for per^{01}*;* UAS-*per* control flies in Fig. 5; cf. Hamblen-Coyle et al., 1989; Wheeler et al., 1993). Flies whose genotypes permit clock functioning exhibit a startle response as well, but this is superimposed on more gradual rises and falls of locomotor activity around the L-to-D transition time (e.g., Wheeler et al., 1993). In this regard, after an initial response to lights-off, activity levels decreased in control flies carrying only a GAL4 driver or two copies of UAS-*per* (Fig. 5). In contrast, flies overexpressing PER driven by *tim-gal4* stayed relatively active all through the night. Moreover, unlike control flies, these double-transgenics did not exhibit activity increases a few hours before lights-on. After lights-on, the activity of *tim-gal4;* UAS-*per* flies decreased gradually during the day, reaching a minimum just before lights-off. Flies carrying *per-gal4* and UAS-*per* behaved in a relatively normal manner (compared with *tim-gal4;* UAS-*per* flies) in terms of the qualitatively appreciated patterns of activity in LD. However, the usual pre-lights off increase in locomotion was not as prominent as in controls carrying only GAL4 driver or UAS-*per* (Fig. 5).

Because the average-activity plots showed that flies overexpressing PER stay relatively active all through the night, activity counts were compared among genotypes separately for the light and for the dark phases. No significant effect of GAL4-driven PER expression on overall daytime activity was found, especially when double-trangenic flies were compared to a control carrying only UAS-*per* [Table

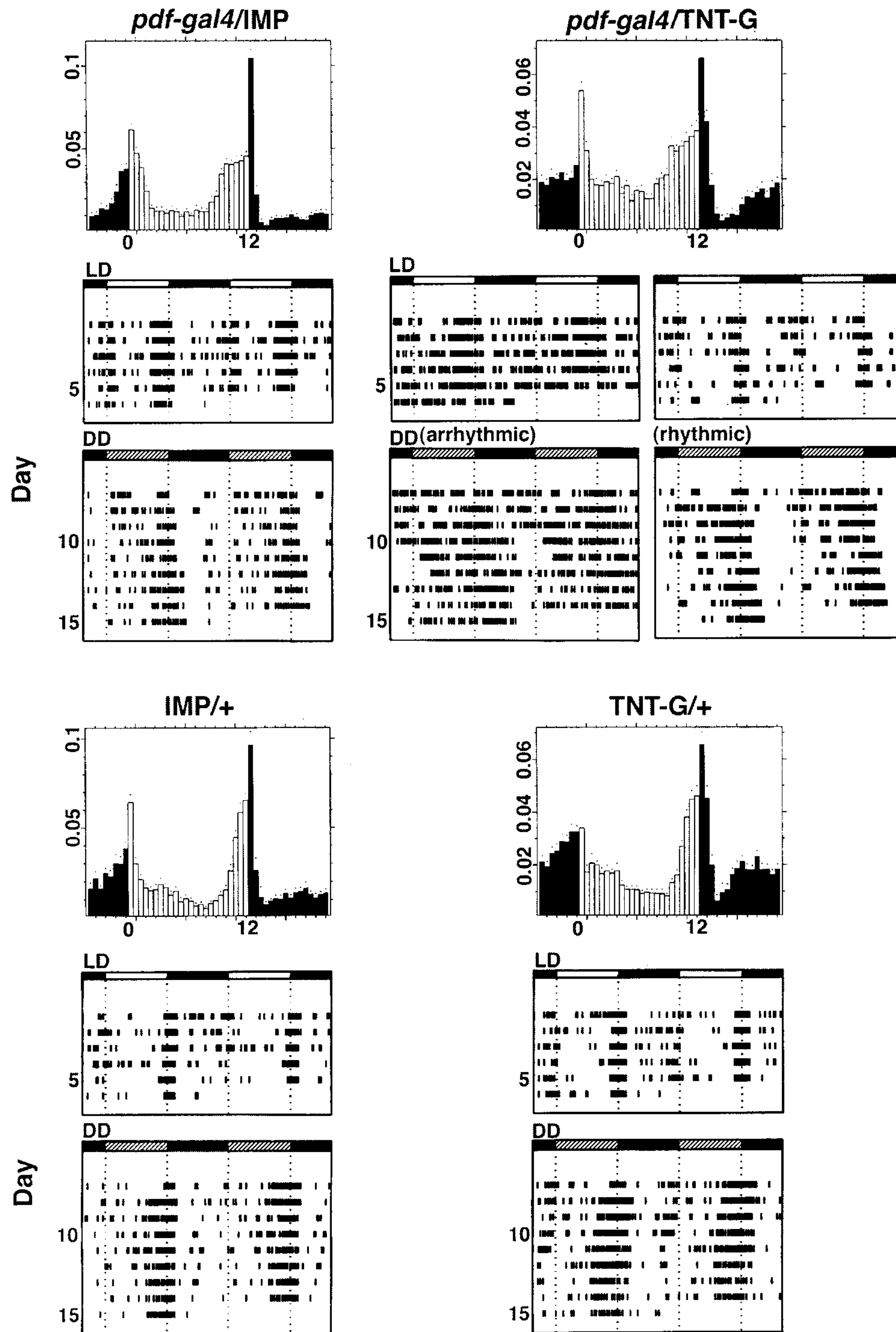

Figure 4 Average-activity plots and actograms for flies expressing UAS-*tetxlc* under the control of *pdf-gal4*. Sixteen flies were combined for each activity-average plot (generated as described in Fig. 1). Actograms for LD (top) and DD (bottom) conditions derived from a single fly is given separately for each control of *pdf-gal4*/IMPTNT-V, IMPTNT-V/+, and TNT-G/+. For the experimental (*pdf-gal4*/TNT-G), Two such pairs (LD and DD) of actograms are given: actograms on the left are from a fly diagnosed as arrhythmic in DD and on the right from a fly with robust rhythmicity in DD as well as in LD.

Table 4 Rhythmicity and Periods of Locomotor Activity of *Drosophila* Expressing *per* under the Control of *per-gal4* or *tim-gal4* in per^{+} or per^{01} Genetic Backgrounds

			LD			DD		
Background	UAS-*per*	*gal4*	*n*	Entrained (%)	Behavioral cycle* (h)	*n*	Rhythmic (%)	Behavioral cycle* (h)
per^{+}	+	*per-gal4*-1b	39	100	24.0 ± 0.0	32	97	24.1 ± 0.1
	+	*per-gal4*-2	45	98	24.0 ± 0.0	35	63	24.1 ± 0.2
	+	*per-gal4*-3	39	90	24.0 ± 0.0	35	89	23.9 ± 0.1
	+	*tim-gal4*-27	35	89	24.0 ± 0.1	32	19	23.8 ± 0.6
	+	*tim-gal4*-62	38	92	23.7 ± 0.3	34	12	27.0 ± 0.6
	+	*tim-gal4*-82	35	91	23.6 ± 0.4	37	27	24.9 ± 0.5
	+	*tim-gal4*-86	38	89	23.6 ± 0.6	33	39	24.4 ± 0.7
	+	None	33	97	24.0 ± 0.0	33	91	23.8 ± 0.1
per^{01}	+	*per-gal4*-1b	24	100	24.0 ± 0.0	17	18	28.3 ± 0.3
	+	*per-gal4*-2	43	91	24.0 ± 0.0	34	15	27.8 ± 0.4
	+	*per-gal4*-3	27	96	24.0 ± 0.0	17	41	26.9 ± 0.5
	+	*tim-gal4*-27	34	88	24.0 ± 0.0	33	12	27.4 ± 1.9
	+	*tim-gal4*-62	36	83	24.0 ± 0.0	32	16	24.6 ± 0.9
	+	*tim-gal4*-82	37	86	24.0 ± 0.1	35	37	25.0 ± 0.3
	+	*tim-gal4*-86	38	92	23.0 ± 0.6	32	31	24.9 ± 0.6
	+	None	33	97	24.0 ± 0.0	33	3	28.5
per^{+}	−	*per-gal4*-1b	35	100	24.0 ± 0.0	31	100	24.0 ± 0.1
	−	*per-gal4*-2	35	100	24.0 ± 0.0	35	97	23.9 ± 0.1
	−	*per-gal4*-3	35	97	24.0 ± 0.0	34	97	24.0 ± 0.1
	−	*tim-gal4*-27	35	97	24.0 ± 0.0	35	91	24.4 ± 0.1
	−	*tim-gal4*-62	36	100	24.0 ± 0.0	31	74	24.1 ± 0.1
	−	*tim-gal4*-82	35	100	24.0 ± 0.0	35	94	24.5 ± 0.1
	−	*tim-gal4*-86	34	94	24.0 ± 0.0	32	88	24.8 ± 0.3

The 1st column gives the genetic background (with regard to the endogenous *per* allele) of the flies tested. "+" in the 2nd column indicates that a given group of flies carried two copies of UAS-*per,* and "−" means that the animals did not carry that transgene. GAL4-driver transgenes carried by a given group of flies are in the "*gal4*" column. For both LD and DD conditions, *n* is the number of flies that yielded valid data (see Materials and Methods). Data for flies carrying only a GAL4-driver transgene in the per^{01} genetic background were similar to those for flies carrying UAS-*per* in this mutant genetic background (data not shown).

* Values are means ± S.E.M.

2(B)]. Similarly, no systematic changes of nighttime activity due to PER expression controlled by *per-gal4* or *tim-gal4* were detected [Table 2(B)].

In constant darkness and temperature (here, 29°C), a large proportion of *tim-gal4;* UAS-*per* flies became arrhythmic, whereas the controls carrying only *tim-gal4* or UAS-*per* were rhythmic (Table 4; Fig. 5). For the rhythmic *tim-gal4;*UAS-*per* individuals (in this per^{+} genetic background), variations of circadian periods were larger (22–29 h) than in the controls (23–25 h) (Table 4). Statistical significance tests could not be performed on these period values due to small sample sizes for some of the relevant genotypes (not many double-transgenic individuals were rhythmic) (Table 4).

Scrutiny of certain control results revealed slight decrements in DD rhythmicity for some of the *tim-gal4* flies that did not carry UAS-*per* (Table 4); this effect of the fusion gene was not observed in DD at 25°C (Table 1). The generalized increase in activity of the GAL4 protein at higher temperature (Morimura et al., 1996) may affect the flies' behavior.

When *per-gal4* drivers were combined with UAS-*per,* similar but less marked reductions of free-running rhythmicity were observed, again in comparison to controls that carried only *per-gal4* or UAS-*per.* The poorest rhythmicity in DD caused by *per-gal4* occurred when driver lines 2 and 3 were combined with UAS-*per* (Table 4). Circadian periodicities of flies carrying *per-gal4* and UAS-*per* were indistinguishable from those of controls (Table 4). One feature of the control results bears mentioning: the periodicities of flies carrying *per-gal4* were shorter than for those carrying *tim-gal4,* similar to what was observed at 25°C (Table 1).

We also tested whether *per* driven by GAL4 rescues the arrhythmicity that is caused by a null mutation of the *period* gene. UAS-*per* transgenes (two

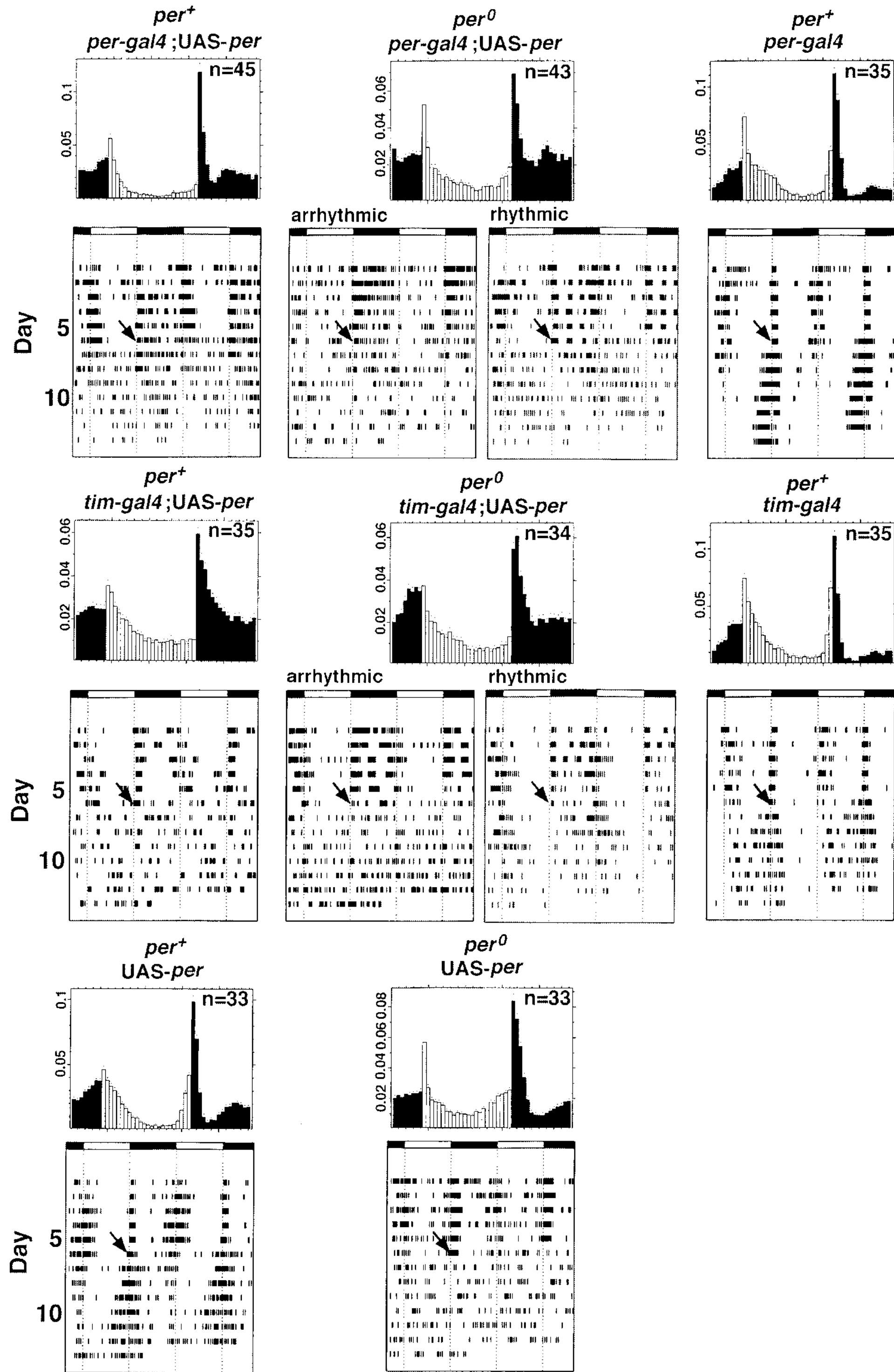

Figure 5 Average-activity plots and actograms for flies expressing UAS-*per* under the control of *per-gal4* or *tim-gal4*. Numbers of flies that were combined for each average-activity plot (generated as described in Fig. 1) are shown on the top right corner of each plot. Arrows on the actograms point to the time of the final lights-off (cf. Fig. 1). Plots for doubly transgenic progeny carrying *per-gal4* from line 2 and *tim-gal4* from line 27 are shown. The effects of *per* expression under the control of *per-gal4*-(2) on behavioral rhythms—reducing the robustness of such rhythmicity—were stronger than in double transgenics carrying the GAL4 driver from the two other *per-gal4* lines. The activity patterns of flies expressing UAS-*per* under the control of *tim-gal4*-(27) were similar to the behavior of double transgenics in which *tim-gal4* was derived from any of the other three lines. For each of per^{01}*;per-gal4;*UAS-*per* and per^{01}*;tim-gal4;*UAS-*per,* two actograms are shown, one derived from a DD-arrhythmic fly and the other a rhythmic one.

copies) were combined with *per-gal4* or *tim-gal4* in flies whose genetic background was per^{01}. In LD cycles, the activity patterns of these double-transgenic flies were similar to those of per^{+} ones expressing *per* under the control of *per-gal4* or *tim-gal4* (Fig. 5). When *per-gal4* lines 1b and 3 were used to drive *per,* increases in activity before lights-on and lights-off were slightly more prominent than for per^{01} controls carrying only UAS-*per* or *per-gal4;* this indicates a weak rescue of the per^{01} effect in these conditions (by itself, this mutation causes a lack of anticipation of the environmental transitions).

In DD, per^{01} flies carrying the UAS-*per* and either *per-gal4* or *tim-gal4* were appreciably rhythmic, more so than in controls for which this *per*-null mutation should not be rescued (i.e., when only a GAL4 driver or UAS-*per* was present). In the doubly transgenic cases, 10–40% of the individuals behaved rhythmically in DD, depending on the line that was the source of the GAL4 driver (Table 4). For the flies whose arrhythmicity was rescued by the simultaneous presence of *per-gal4,* along with UAS-*per,* the circadian periods were longer than normal (Fig. 5; Table 4). Circadian periods of per^{01} flies rescued by *tim-gal4* and UAS-*per* varied much more than is typically observed for wild-type flies: 23–30 h compared to ca. 23.5–24.5 h (e.g., Hamblen et al., 1986).

PER and TIM Levels Influenced by *per* Overexpression

To determine whether expression of PER and TIM in putative circadian-pacemaker cells in flies fits with the behavioral effects of *per-gal4* or *tim-gal4,* driving an extra per^{+} allele, we assayed these doubly transgenic flies histochemically. The animals were sacrificed at time points within an LD cycle (at 29°C) that correspond to the usual peaks and troughs for the levels of these clock proteins: ZT 23 and ZT 11 (Zerr et al., 1990) for PER; ZT 21 and ZT 9 for TIM (Myers et al., 1996; Hunter-Ensor et al., 1996; Zeng et al., 1996).

In the control strain carrying only UAS-*per,* differences in PER staining intensities comparing ZT 11 to ZT 23 were observed in *per*-expressing neuronal clusters, brain glia, and in retinal photoreceptors (Figs. 6, 7). Compared to this control, PER immunoreactivities at ZT 23 in the neuronal clusters and in the eyes were elevated in flies expressing PER under the control of *per-gal4.* The *per-gal4* driver (derived from line 2) did not lead to higher than normal immunoreactivity in glia at ZT 23 (Figs. 6, 7); this is consistent with the fact that this fusion construct (in this particular transgenic line, 2) does not mediate glial expression of UAS-marker proteins (Kaneko and Hall, 2000). At ZT 11 as well, relatively high levels of PER

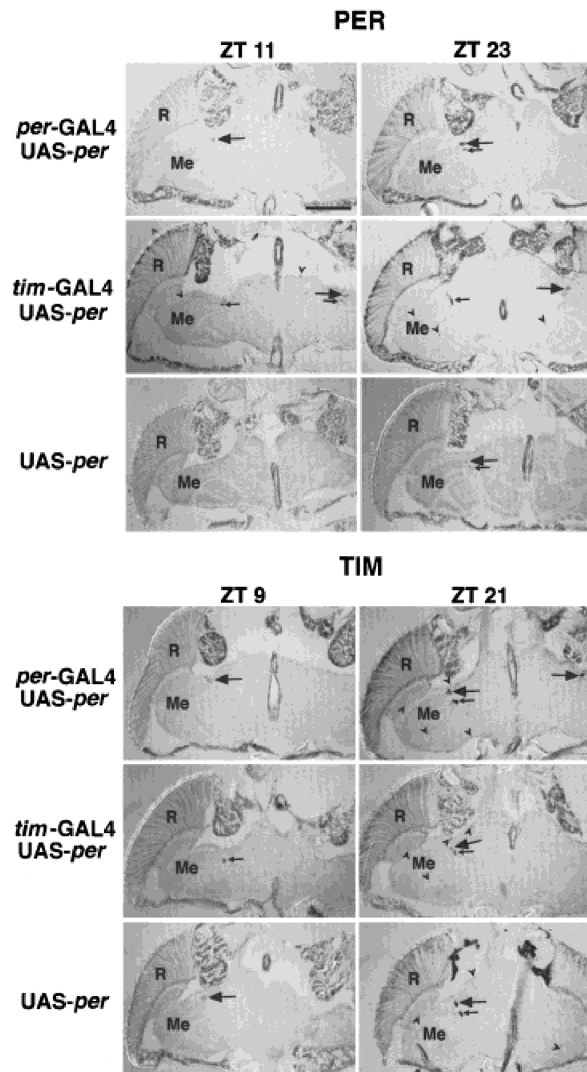

Figure 6 PER and TIM stainings of adult-head sections from flies expressing UAS-*per* under the control of *per-gal4* or *tim-gal4.* The genetic background was per^{+}, and the condition was LD cycles. PER immunoreactivities in head sections at ZT 11 and ZT 23 (approximate trough and peak timepoints for histochemically scored levels of such signal, cf. Zerr et al., 1990) in flies carrying *per-gal4* (line 2), and those carrying *tim-gal4* (line 27)—each in addition to UAS-*per*—are shown on upper panels. TIM immunoreactivities for flies of the same genotypes at ZT 9 and ZT 21 are in the lower panels. As controls, PER and TIM immunoreactivities on flies carrying only UAS-*per* are also shown in the bottom pair of images within each group. The transgene types carried by the flies are shown to the left of each row. Large arrows point to the large LN_vs, small arrows to the small LN_vs, and arrowheads to glial cells R, retina; Me, medulla. Bar (upper left), 100 μm (same for all 12 panels). Note that PER immunostaining in this particular experiment was weak; thus, for the "UAS-*per* only," the sole staining that is evident in this particular image is within LN_vs (but not in the retina). However, weak staining was indeed detected in photoreceptor cells of the retina (see Fig. 7).

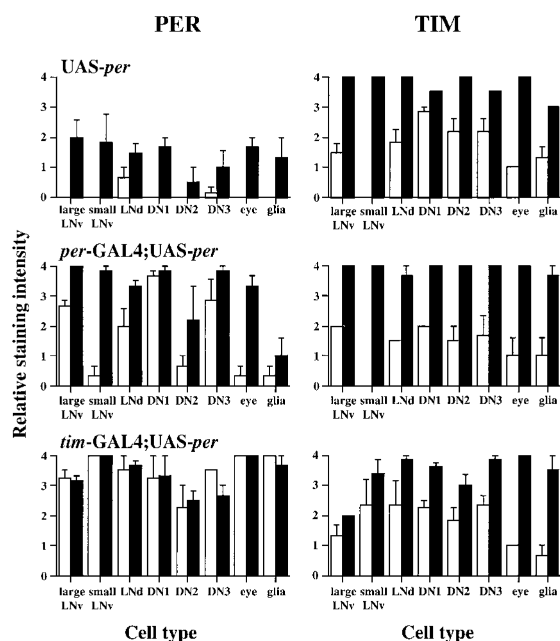

Figure 7 Quantification of staining intensities for PER and TIM immunoreactivities in flies expressing *per* under the control of *per-gal4* (line 2) or *tim-gal4* (line 27). Anti-TIM and -PER stainings on head sections (like those shown in Fig. 6) were scored subjectively and blindly as usual (cf. Fig. 3). For each time point, cell type, genotype and antigen, three flies were scored, with the following exceptions: two for PER immunostaining in flies carrying *tim-gal4* in addition to UAS-*per* at ZT 9; 1 for TIM staining in control flies, carrying only UAS-*per,* ZT 21; four for TIM staining in flies carrying *tim-gal4* in addition to UAS-*per,* ZT 21. Black bars represent average intensity scores at ZT 21, and white bars at ZT 9. Error bars, S.E.M.

staining were found in several clusters of clock-gene–expressing neurons: large LN_vs, LN_ds, DN1s, and DN3s (Figs. 6, 7). As a result, clear cycling of PER was observed only in the small LN_vs and the eyes of *per-gal4*/UAS-*per* flies.

Within the heads of the flies carrying *tim-gal4* and UAS-*per* both at ZT 11 and ZT 23, all clock-gene–expressing neuronal clusters as well as in glia and the retina exhibited high levels of PER (at a gross level). However, closer observation of the retinal staining revealed that the strong signals in the apical region of this tissue seemed to correspond to nonphotoreceptive structures (n = 2 at ZT 11, and n = 3 at ZT 23; specimens from the same experiment that led to Figs. 6, 7). These (PER-containing) structures seemed to be pigment cells, judging by their position just underneath the lens and their round shapes, as opposed to the more oval appearance of photoreceptor nuclei (cf. Cagan and Ready, 1989). A small number of photoreceptor nuclei were strongly stained in these flies at both ZT 11 and ZT 23 (data not shown). Given such anomalous immunohistochemical results for the retinal signals, meaningful high and noncycling levels of PER can be said to occur only in neurons and glia under the influence of *tim-gal4.* Normal *per* expression in such CNS locations is likely to be behaviorally significant (e.g., Ewer et al., 1992; Frisch et al., 1994; Helfrich-Förster 1998), whereas clock-gene expression in the retina has no known chronobiological meaning.

The histochemical results just described measure PER expression originating from two different sources of per^+ gene, an endogenous one and UAS-*per* (whose expression is driven by *per-gal4* or *tim-gal4*). To assess the pure effect of UAS-*per* and *per-gal4* or *tim-gal4* on PER levels, two further immunohistochemical studies (using anti-PER antibodies) were performed. One used flies carrying *per-gal4* (line 2) and UAS-*per,* in a per^{01} genetic background using polyclonal rat anti-PER; flies were sacrificed at ZT 11 (n = 3) and at ZT 23 (n = 4) in LD at 25°C. The other experiment involved flies carrying *tim-gal4* (line 27) or *per-gal4* (line 2) and UAS-*per* in a per^{01} genetic background stained by polyclonal rabbit anti-PER; flies were stained at ZT 9 and at ZT 21 in LD at 29°C (n = 3 to 4 for each timepoint and genotype). In both experiments, PER cycling was not detectable in many cell types of doubly transgenic flies that normally express PER cyclically in wild type (data not shown). Flies carrying *tim-gal4* and UAS-*per* in a per^{01} genetic background showed constitutive levels of PER in all the brain neurons (except LN_d cells, which showed a slight difference between two time points) and glia (data not shown); this is similar to the results obtained from doubly transgenic flies in a per^+ genetic background (Figs 6, 7). The results for per^{01}*; per-gal4;*UAS-*per* flies varied between two experiments: the first of these, performed at 25°C, detected PER cycling only in the photoreceptor cells but not in brain neurons; the latter, at 29°, found low-amplitude PER cycling in a few clusters of brain neurons (DN3s and small LN_vs) but not in the photoreceptor cells (data not shown). These differences could be due to variabilities associated with experimental temperature, antibodies, or time points. In the second experiment, per^+; UAS-*per* (no GAL4 driver) controls (n = 3 for each time point) were compared to the test flies, which carried GAL4 drivers and UAS-*per* in a per^{01} genetic background. In many cell types, these particular experimental flies showed higher PER immunoreactivities than did the controls (data not shown). The general conclusions drawn from these experiments are that PER, solely driven by GAL4 driver and UAS-*per* (no endogenous per^+), is higher than PER at peak time points in wild type in several

cell types, and such high levels of PER oscillate with low amplitudes.

Although normal PER protein is produced by the combination of *per-gal4* and UAS-*per,* the histochemical control just described suggests that these two transgenes alone (without endogenous per^{+}) do not sustain the normal molecular feedback loop responsible for PER cycling, in that there is a temporally constant level of this protein in brain neurons. Immunohistochemical data from per^{+} double-transgenic flies suggest that PER expression stemming from UAS-*per* and a GAL4-driver transgene also disrupt the feedback loop mediated by endogenous per^{+} in many clock cells. This deleterious effect of the double transgenes on the feedback loop was stronger when UAS-*per* was combined with *tim-gal4* compared to the *per-gal4*/UAS-*per* transgenics (Figs. 6, 7). This correlates well with the stronger activity of the *tim* promoter compared to that of *per* (for example, according to quantitative bioluminescence signals mediated by *tim-luc* and *per-luc;* Stanewsky et al., 1998).

TIM immunoreactivity was also checked in flies expressing PER under the control of *per-gal4* or *tim-gal4.* TIM immunoreactivity was not appreciably affected by the (simultaneous) presence of *per-gal4* and UAS-*per* (compared to controls carrying only UAS-*per*), in any of the cell types studied (Figs. 6, 7). However, in flies carrying *tim-gal4* and UAS-*per,* strong stainings were observed in the small LN_vs both at ZT 9 as well as at ZT 21 (Fig. 7). The (usual) temporally dependent difference in TIM staining intensity for this neuronal cluster was reduced compared to wild type (Fig. 7). In the large LN_vs, *tim-gal4*/UAS-*per*–influenced TIM immunoreactivity was relatively low, both at ZT 9 and ZT 21. In contrast, robust cycling of TIM was observed in the eye and glia in the case of *tim-gal4* combined with UAS-*per.* This result is consistent with the high and temporally constitutive level of PER in these flies, which may in turn lead to anomalous TIM oscillations at least in some central-brain neurons.

Real-time Reporting of Clock-Gene Cycling Influenced by Overexpression of Such Genes

As PER negatively regulates transcription of *per* as well as *tim,* the double transgenic situation (implied by the title of this section) can be expected to cause a reduction of the overall amounts, as well as the cyclings, of *per* RNA and *tim* RNA. We applied a *per-luciferase* reporter to ask whether this is so. Bioluminescence cycling mediated by a *per-luc* transgene (BG-*luc*) containing *per* 5′-flanking sequences (the *per* promoter and 5′-untranslated material) and those encoding the N-terminal two-thirds of PER fused to firefly *luc* reflects *per* mRNA oscillations (Stanewsky et al., 1997b). An *X*-chromosomal BG-*luc* transgene was combined with two other transgene types: GAL4-drivers (*per-gal4* or *tim-gal4* on chromosome *2*) and UAS-*per* (double inserts on chromosome *3*). As controls, flies carrying only a GAL4 driver or UAS-*per* (double inserts) or no transgenes in addition to BG-*luc* were monitored automatically for luminescence fluctuations. The conditions were LD cycles and 25°C (cf. Stanewsky et al., 1997b).

When UAS-*per* was placed under *per-gal4* control, only modest reductions of cycling and luminescence levels were observed compared to the control time-courses [Fig. 8(A)]—especially those involving UAS-*per* only (no GAL4 driver). Although peak levels were decreased, *luc*-reported indices of rhythmicity in flies carrying *per-gal4* and UAS-*per* were as robust as control values (i.e., "Rel-Amp errors," relatively low values for which indicate strong cycling; cf. Stanewsky et al., 1997b) (Table 5). In contrast, when *tim-gal4* and UAS-*per* were present along with the reporter construct, a marked decrease in cycling amplitude and overall levels of luminescence occurred compared to the controls [Fig. 8(B)]. Although the amplitude of reported *per* cycling was low in flies carrying *tim-gal4* and UAS-*per,* ca. 80% of the individual animal records were weakly rhythmic (Table 5).

Periodicities of these bioluminescence rhythms were similar in all the genotypes tested (Table 5), as they should be in LD (Stanewsky et al., 1997a). Phase values for the luminescence peaks did not vary significantly among the six genotypes ($p = .14$, $df = 5$). Thus, PER expression under the control of *tim-gal4* or *per-gal4* did not affect the phase of *per*'s (reported) mRNA rhythm.

Luciferase reporting of *tim* mRNA cycling was also tested in the face of "extra" *period* gene expression. For this, we combined a *timeless* promoter-*luc* fusion (*tim-luc:* Stanewsky et al., 1998) with GAL4-drivers (*per-gal4* or *tim-gal4*) and UAS-*per.* Similar to the case of BG-*luc, tim-luc* luminescence was greatly reduced in flies carrying *tim-gal4* and UAS-*per* (data not shown); whereas only modest reductions of reporter signals occurred in flies carrying *per-gal4* and UAS-*per* compared to controls (GAL4 driver only, or UAS-*per* only, or no transgene present other than *tim-luc*).

As *tim-gal4* induces stronger expression of PER in many clock cells compared to the effect of a *per-gal4* driver (Figs. 6, 7), the results of monitoring reported mRNA cycling of these two clock genes are consis-

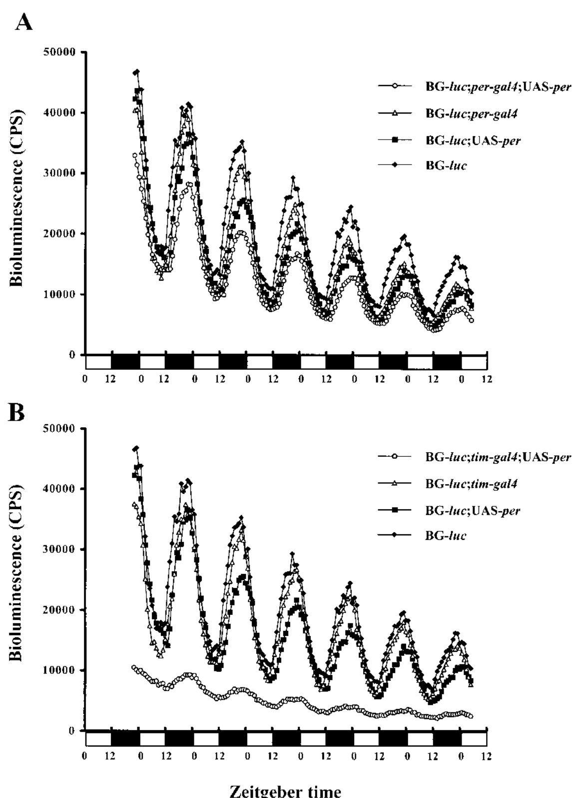

Figure 8 Luminescence oscillations mediated by a *per-luc* reporter gene in flies expressing UAS-*per* under the control of *per-gal4* or *tim-gal4.* (A) *per-gal4* (from line 2); (B) *tim-gal4* (line 27). (A) *per-luc* reporter gene (BG-*luc*) in which 5′-flanking *per* sequences (including the promoter), the 5′ untranslated region, and sequences encoding the N-terminal two-thirds of PER were fused to *luc* and used to monitor *per* mRNA cycling in live flies fed with food containing 10 m*M* luciferin (cf. Stanewsky et al., 1997b). The experiment was performed in LD cycles (12-h light: 12-h dark, as used throughout this study) for 6.25 days. Levels of luminescence (counts per second, CPS) were monitored automatically (cf. Stanewsky et al., 1997b) and plotted against Zeitgeber Time (ZT 0 and 12-h lights-on lights-off, respectively, as usual); white and black horizontal boxes at the bottom of each plot represent light and dark phases, respectively. In (A), per-fly average luminescence counts for flies expressing UAS-*per* under the control of *per-gal4* ($n = 53$) are plotted along with those of three controls: flies carrying only *per-gal4* ($n = 61$), only UAS-*per* ($n = 68$), or no transgene ($n = 71$) other than BG-*luc.* In (B), averaged luminescence fluctuations for flies expressing UAS-*per* under the control of *tim-gal4* ($n = 48$) are plotted with those for controls carrying only *tim-gal4* ($n = 78$), UAS-*per* or no transgene other than BG-*luc;* the latter two control results are re-plotted from (A). BG-*luc*-mediated luminescence in flies expressing UAS-*per,* under the control of GAL4 drivers from different *per-gal4* or *tim-gal4* insertion lines, was also tested in a separate experiment; it gave results similar to those shown, in terms of the modest vs. marked reductions in cycling amplitude and luminescence levels in flies (respectively) carrying *per-gal4* (1b, 2, and 3) or *tim-gal4* (62, 82, and 86). The numbers of flies tested for the back-up experiment ranged from one to eight (depending on the GAL4-driver used) for experimental flies carrying the three transgene types; the numbers of flies for controls (those carrying GAL4 driver, UAS-*per,* or no transgene in addition to BG-*luc*) were 2–10. A further, separate set of experiments involved luminescence cycling mediated by *tim-luc* (cf. Stanewsky et al., 1998); the test flies overexpressed PER under the control of *per-gal4* (1b, 2, and 3) or *tim-gal4* (27, 62, 82, and 86); similar to the BG-*luc* results shown here, dramatic reductions in *tim-luc* cycling amplitude and luminescence levels resulted from *tim-gal4*/UAS-*per* combinations, compared to controls carrying only *tim-gal4,* or UAS-*per,* or no transgene other than *tim-luc.* The numbers of flies monitored in this experiment ranged from 1 to 13 for the experimental tests and 3 to 30 for controls.

tent with the behavioral results presented earlier. Thus, flies carrying *tim-gal4* and UAS-*per* were markedly diminished in terms of *per* and *tim* cyclings, correlated with this double-transgenic combination leading to the strongest decrements in behavioral rhythmicity.

DISCUSSION

Neurons Responsible for Tetanus-Toxin Effects on Behavioral Rhythmicity

TeTxLC expression in clock cells, controlled by the *per-gal4* or *tim-gal4* fusion genes, caused abnormalities of behavioral rhythms. These flies showed substantially decreased rhythmicity in constant darkness and either reduced synchronization to LD cycles or complete loss of such entrainment. In LD conditions, the level of locomotion during the night was anomalously high, and the usual morning peak of activity was reduced under the influence of TeTxLC. These effects of the toxin were qualitatively similar when two types of GAL4-driver transgenes—containing *per* or *tim* regulatory sequences—were compared. However, there were varying TeTxLC effects on behavior, which depended upon the chromosomal locations of a given GAL4-driver; this kind of position effect is commonly encountered for *gal4*-containing transgenes in *Drosophila* and is the principle underlying enhancer-trap screens that involve mobilization of such engineered transposons (e.g., Wilson et al., 1990; Kaiser 1993).

The similar behavioral effects of TeTxLC expression in flies carrying *tim-gal4* or *per-gal4* (in addition to UAS-*tetxlc*) are presumed to be the result of putting this toxin into the same chronobiologically relevant

Table 5 Quantitative Analysis of BG-*luc* Bioluminescent Oscillations in Flies Expressing PER under the Control of *per-gal4* or *tim-gal4*

gal4	UAS-*per*	*n*	Rhythmic (%)	Rel-Amp error*	Period (h)*	Phase (ZT)*
per-gal4	+	53	100	0.20 ± 0.01	24.4 ± 0.0	21.1 ± 0.2
per-gal4	−	60	100	0.21 ± 0.01	24.4 ± 0.0	20.4 ± 0.1
tim-gal4	+	47	81	0.47 ± 0.03	24.2 ± 0.1	22.7 ± 0.4
tim-gal4	−	78	100	0.22 ± 0.01	24.3 ± 0.0	20.5 ± 0.1
None	+	68	100	0.31 ± 0.01	24.4 ± 0.1	21.5 ± 0.2
None	−	71	99	0.23 ± 0.01	24.4 ± 0.1	19.9 ± 0.2

Numerical summary of the *per*-controlled luminescence oscillations shown in Figure 8. The GAL4-driver transgene carried by a given group of flies is indicated in the "*gal4*" column. "+" in the 2nd column indicates that the given group of flies carried two copies of UAS-*per*. *n* is the number of flies tested. Percentages of flies with rhythmic bioluminescence cycling are given under the "Rhythmic (%)" column; a fly that was significantly rhythmic in terms of luciferase-reported oscillations had Rel-Amp errors < .7, as described in Stanewsky et al. (1997b). Additional components of the analytical procedures described in that report led to Period determinations for each fly's luminescence record and Phase values (peak times). To calculate a mean of these phase values that take a circular rather than a linear distribution, each of these phase values was then transformed into a vector of length 1 and a certain angle (e.g., ZT 0 = 0°, and ZT 12 = 180°; cf. Batchelet, 1965): then the angle of the mean vector (angular mean) was calculated, transformed back to ZT, and given as the average ZT hour when the luminescence peaks occurred (all such values are ca. 8 h after the L-to-D transition, i.e., ZT 20). This procedure was applied, because it is the most accurate method of calculating mean phases that take a circular rather than a linear distribution. However, approximation by arithmetic means gave errors of less than 0.3 h for the current data, implying that this approximation should be valid for phases of BG-*luc*–mediated bioluminescence cycling in LD cycles.

* Values are means ± S.E.M.

neurons in the double-transgenic situation. Indeed, *per-gal4* and *tim-gal4* lead to overlapping GAL4 expression in many brain neurons, those known from other evidence to contain PER and TIM (Kaneko and Hall, 2000). Note, however, that the GAL4-driven, UAS-marker-reported expression of these two clock genes is not identical. Nonetheless, we surmise that the extent of *per/tim* coexpression, at least insofar as laterally and dorsally located brain neurons is concerned (cf. Kaneko, 1998), leads to the toxin-induced behavioral-rhythm abnormalities.

It is possible that TeTxLC expressions in parts of brain other than the location of clock-gene–expressing lateral and dorsal neurons—such as cell bodies near the tritocerebrum, subesophageal ganglion, and the anterior cortex near the antennal lobes—are in part responsible for the behavioral defects. These regions express marker genes under the control of *per-gal4* and/or *tim-gal4* (Kaneko and Hall, 2000) but are not immunoreactive for PER or TIM. In this regard, neurons in the anterior cortex, where strong marker expression is driven by *per-gal4,* project to the central complex (Kaneko and Hall, 2000). That brain structure is indicated in the control of walking behavior (Strauss and Heisenberg, 1993), albeit not necessarily the rhythmic features thereof. Nevertheless, toxin-disrupted synaptic function at nerves innervating in the central complex could affect the fly's locomotion—here, revealed by monitoring walking over the course of many hours. The idea that disruption of central-complex function contributes to toxin-induced behavioral abnormalities is undermined by the fact that marker-gene expression in the central complex is less prominent in *tim-gal4*-containing flies compared to *per-gal4* ones (Kaneko and Hall, 2000). This does not correlate with the effects on rhythmic behavior observed in *tim-gal4*/UAS-*tetxlc,* which in general were stronger than those caused by the *per-gal4*/UAS-*tetxlc* combination (Fig. 1; Table 1).

In contrast to the TeTxLC expression that was driven by the two clock-gene promoters, expression of the same toxin under the control of *pdf-gal4* had a remarkably mild effect on adult locomotor-activity rhythms. These modest subnormalities observed could be due to lower toxin expressed within the LN cells, via *pdf-gal4,* compared to toxin expression within the same set of cells induced by *per-gal4* or *tim-gal4.* However, the *pdf* promoter seems to be a strong one: *pdf* transcripts can be easily detected by Northern-blot hybridization on total RNA in spite of the small number of cells that express this gene (Park and Hall, 1998) (*per* mRNA, for example, is difficult to detect on Northern blots; Hardin et al., 1990). Furthermore, TeTxLC expression induced by *pdf-gal4* within the LN_vs is expected to be higher than or at least comparable to that via *per-gal4:* secondary marker-gene expression under the control of *per-gal4* was not reliably detected in small LN_v cells (Kaneko and Hall, 2000), indicating very weak *per*-mediated expression of TeTxLC in this neuronal cluster. In contrast marker-gene expression controlled by *pdf-gal4* is readily detectable in the same LN cluster (Park et al., 2000), which suggests that reasonably high levels of TeTxLC in these neurons are elicited by the *pdf* promoter. Therefore, the mild effect of TeTxLC in flies that carried *pdf-gal4,* compared to the rather

severe rhythmic defects observed in flies expressing the same toxin under the control of *per-gal4* or *tim-gal4,* may be because more neurons contain TeTxLC in the transgenic types involving clock-gene promoters. Neurons that express *per* and *tim,* but are not PDH-immunoreactive, include three dorsal neuronal clusters—DN1, DN2, and DN3—and the more dorsally located of the lateral neuronal clusters (LN_d); also there is a single LN_v that is PDH-negative (Helfrich-Förster, 1995; Kaneko et al., 1997; Kaneko, 1998). Therefore, some of these neurons may be responsible for the abnormal behavioral rhythms observed in flies carrying UAS-*tetxlc* and *per-gal4* or *tim-gal4.*

A neuroanatomical mutant *disconnected* (*disco*) is largely arrhythmic in DD and lacks the brain's lateral neurons, including PDF-expressing LN_vs and PDF-negative LN_ds (Dushay et al., 1989; Zerr et al., 1990; Helfrich-Förster, 1998). Although the defect in *disco* is qualitatively different from that in TeTxLC-expressing flies, it is useful to compare the behavioral effects of these two kinds of genotypes. Flies expressing TeTxLC under the control of *per-gal4* or *tim-gal4* (except in the case of *per-gal4*-1b) are also largely arrhythmic in constant conditions, that is, similar to the behavior of *disco* mutants. However, *disco* flies and the toxin-expressing transgenics differ in their LD behaviors: 85–90% of *disco* individuals entrain to LD cycles (Hardin et al., 1992; Wheeler et al., 1993; Helfrich-Förster, 1998). Although a similar (high) percentage of *per-gal4* (1b) flies expressing TeTxLC entrained to LD cycles, the other lines of *per-gal4* and all those carrying *tim-gal4* transgenes (along with TNT-G) exhibited mediocre synchronization to LD cycles: only 10–60% of the individuals entrained (Table 1). The different effects of *disco* mutations compared to *per-gal4*– or *tim-gal4*–mediated TeTxLC expression suggest that the toxin's presence in cells other than LNs are involved in, and possibly responsible for, the abnormal LD behavior of the doubly transgenic flies. Intriguingly, however, the average-activity plots that resulted from monitoring *tim-gal4*/UAS-*tetxlc* or *per-gal4*/UAS-*tetxlc* flies in LD revealed that the toxin causes a reduction of both the anticipation of dawn and of the ensuing morning peak amplitude. This is similar to *disco*'s behavior in these conditions. Moreover, both genotypes cause subnormal increases in locomotion during the second half of the daytime (Hardin et al., 1992; Wheeler et al., 1993; Helfrich-Förster, 1998, cf. to Fig. 1 in the current report). Therefore, it can be argued that the combined effects of TeTxLC on the functioning of LNs (including non–PDF-expressing LN_ds, which are eliminated by *disco*), together with effects on clock-gene–expressing cells that are unaffected by *disco* lead to the overall pattern of behavioral-rhythm defects exhibited by the double-transgenic types.

In spite of the rather severe rhythm abnormalities exhibited by the *per*- or *tim-gal4*/UAS-*tetxlc* flies, we acknowledge that certain clock cells that normally contribute to behavioral rhythms may not be affected by toxin expression. That is, the cells may be functioning as pacemakers in spite of TeTxLC expression. The neurons in question could contribute to behavioral rhythms, but it may be necessary to eliminate them to disrupt the relevant functions, instead of putting a toxin into them that is not necessarily relevant to the outputs from those cell types. For instance, TeTxLC expression in *pdf*-expressing LN_vs, whose importance for generation of robust behavioral rhythmicity have been demonstrated (Frisch et al., 1994; Helfrich-Förster, 1998; Stanewsky et al., 1998; Renn et al., 1999), barely affected behavioral rhythmicity.

One goal of the current study was to reveal as many neural substrates of *Drosophila*'s locomotor rhythms as may be relevant—against a background of the many cells within the brain of this insect that express clock genes. Thus, the dorsal LNs, cells within DN clusters, or combinations of such neurons could be imagined to contribute to the regulation of behavioral rhythmicity. Many of the *per/tim*-expressing neurons—small LN_vs, LN_ds, DN1s, DN2s, and DN3s—send their neuronal processes into the superior protocerebrum (Kaneko and Hall, 2000), and fibers from some of these neurons are closely situated in this dorsal area of the brain. Therefore, all these clock neurons may participate in the generation of the rhythmic behavior by communicating with each other or with a common target in the superior protocerebrum.

The increase in nighttime activity caused by *per-gal4*- or *tim-gal4*-driven TeTxLC suggests that normal function of some of the *per/tim*-expressing neurons involves an inhibition of locomotion during the night. TeTxLC may release this inhibition by blocking chronologically relevant chemical signals that are normally transmitted from clock neurons. In this regard, both anatomical and functional perturbations of the mushroom bodies, whose calyces are also situated in the superior protocerebrum, lead to increased locomotion (Martin et al., 1998). Therefore, some of the clock neurons may send positive signals to the mushroom bodies to suppress walking.

Might Tetanus Toxin Affect Release of Neuropeptides from Circadian-Pacemaker Neurons?

Although TeTxLC blocks "fast-chemical" transmission mediated by classical neurotransmitters, it should

not eliminate electrical communication between neurons, because the latter is not dependent on the function of the target of this toxin, neuronal synaptobrevin (Sweeny et al., 1995). TeTxLC might not affect the release of neuropeptides—from the dense-cored vesicles, whose release mechanism is relatively understudied (Martin, 1994)—although an effect of this toxin on the release of at least certain such substances in vertebrates as well as in invertebrates has been demonstrated (Dayanithi et al., 1994; Whim et al., 1997).

In this regard, *pdf-gal4*/UAS-tetanus toxin-containing flies showed remarkably normal rhythmic behavior both in LD and DD (with only a slight decrement of DD rhythmicity). TeTxLC expression as controlled by *pdf-gal4* should occur in most of the *per/tim*-expressing LN_vs, including the LN_vs that have relatively small somata (Helfrich-Förster, 1995). Those particular neurons seem to be essential for robust rhythmicity in DD (Helfrich-Förster, 1998; Stanewsky et al., 1998). Therefore, if TeTxLC expression in these neurons disrupts all the means of communication from them to downstream motor centers—along with the possibility that input to these LN cells could also be affected by the toxin (cf. Baines et al., 1999)—this substance should lead to severely abnormal rhythmic behavior in the doubly transgenic flies. It really should, because transgene-mediated elimination of the ventrolateral neurons in question (applying the same *pdf-gal4* driver) leads to severe subnormalities of locomotor rhythmicity, albeit not their complete elimination (Renn et al., 1999). The most likely explanation for relatively normal rhythmicity of *pdf-gal4*/UAS-*tetxlc* flies is insufficient disruption of cellular communication machinery in the LN_vs of these flies. One possibility is that chemical synaptic transmission—hypothetically involving classical neurotransmitters as well as neuropeptides—is not completely blocked in the LN_vs because of insufficient levels of the toxin. This scenario is unlikely, because the *pdf* promoter seems to be a strong one (see above). Another possibility is that electrical transmission, which is not affected by TeTxLC, is involved in pacemaker communication with downstream motor centers. The last and most plausible possibility is that TeTxLC does not block the release of neuropeptides in *Drosophila* in a scenario for which such humoral factors (released from the LN_vs) are involved in output from the pacemaker cells to rhythmic behavior. This kind of circulating chronobiological factor was implied from the results of brain-transplant experiments (Handler and Konopka, 1979). PDF, which is expressed in most of the ventrally located lateral neurons (including small and large LN_vs), is a candidate for such a specific releasable substance that influences behavioral rhythmicity (reviewed by Helfrich-Förster et al., 1998).

Effects of GAL4-Mediated PER Expression

In attempts to overexpress PER protein, *per-gal4* or *tim-gal4* was combined with UAS-*per.* Because PER negatively regulates transcription of *per* itself as well as *tim* (Zeng et al., 1994; Darlington et al., 1998), the design of our experiments was expected to reduce *per*- or *tim*-promoter–mediated GAL4 expression in flies carrying UAS-*per.* Therefore, this approach may not result in ultimate overexpression of PER, because that protein may stay at a level equilibrated by the GAL4-mediated expression of *per* and PER-mediated suppression of GAL4 expression. In fact, in flies producing PER under the control of *tim-gal4,* GAL4-mediated PER expression dampened the cycling as well as the level of luciferase-reported clock-gene expression emanating from additional transgenes (*per-luc* or *tim-luc*) that were added to these flies. This implies that the levels of *per* mRNA and *tim* mRNA transcribed from endogenous per^+ and tim^+ genes were also low and cycled with abnormally low amplitude within clock neurons (cf. Stanewsky et al., 1997b, 1998). However, PER immunoreactivity in these double-transgenic flies increased in many clock cells compared to the control flies carrying only UAS-*per.* Therefore, in spite of the low levels of endogenous *per* RNA and *tim* RNA and a hypothetical decrement of RNA transcribed from the *tim-gal4* transgene, GAL4-mediated expression of UAS-*per* seems to result in more than normal PER in many cells. This high level of PER seems to be responsible for the low level of *per* RNA and *tim* RNA that were inferred from luciferase-reported *per* and *tim* expression in the *tim-gal4*/UAS-*per* flies: the luminescence levels were abnormally low and the cycles were associated with low amplitudes.

Recently, positive effects of PER and TIM on the expression of the positive transcription factor of *per* and *tim,* CLOCK (CLK) have been suggested, because mRNA and protein encoded by the *Clk* gene are at low levels in per^{01} and tim^{01} genetic backgrounds (Bae et al., 1998; Lee et al., 1998). However, this positive effect of PER on *Clk* should not increase *per* RNA and protein levels or *gal4* expression controlled by the *per* or *tim* promoter, because this action of PER is best interpreted as a disinhibition of *Clk* (Glossop et al., 1999). Thus, the activity of CLK, notwithstanding its derepressed level, should be inactivated by excessive amounts of PER in our doubly transgenic animals.

An exception to PER overexpression mediated by

GAL4-controlled UAS-*per* expression involved photoreceptors in flies carrying *tim-gal4* and UAS-*per;* most such cells were barely stained by anti-PER (data not shown). This lack of PER immunoreactivity in so many photoreceptors (in each doubly transgenic individual) is not due to the absence of GAL4 expression in these cells, because UAS-marker expression, driven by *tim-gal4,* was observed within all retinal photoreceptors (Kaneko and Hall, 2000). Therefore, the equilibrium alluded to above seems shifted in the direction of a collapse of feedback-loop function in the compound eyes of *tim-gal4*/UAS-*per* transgenics (such a "collapse" would involve low production of PER from the latter transgene and from the endogenous per^{+} gene as well). It should be noted that this particular histological experiment (Figs. 6, 7) involving PER immunohistochemistry performed with the doubly transgenic type just indicated, as well as with singly transgenic controls carrying only UAS-*per,* gave relatively low PER staining intensities; this was true for the control, even at the peak time point of PER. Therefore, the lack of PER staining in photoreceptor cells of flies carrying *tim-gal4* and UAS-*per* does not necessarily mean that there is little PER expression in that tissue. An intermediate level of PER (lower than the peak level but higher than the trough level of wild type) may occur constitutively in photoreceptor cells of *tim-gal4*/UAS-*per* flies.

The full-length *rhodopsin-1* promoter fused to *per* can drive extremely high level of PER in photoreceptor cells, which eventually abolishes expression of the endogenous per^{+} gene (Zeng et al., 1994). However, effects of this tissue-specific overexpression of PER on chronobiological phenotypes could not be tested, because none is known in *Drosophila* photoreceptors, and neither the compound eyes nor ganglia within the visual system are necessary for free-running behavioral rhythmicity or entrainment (Helfrich-Förster, 1996).

Our current studies delved deeper into the meaning of the negative feedback loop that functions within the circadian pacemakers in *Drosophila.* We show that forced PER expression and its disruption of clock-gene-product oscillations affects an actual rhythmic output, owing to the *tim-gal4*/UAS-*per* combination's success in causing such anomalous expression within the pacemaker cells that are known to regulate behavioral rhythmicity. Furthermore, correlating the strength of molecular oscillations with rhythmic behavior (or the lack thereof) revealed that PER and TIM oscillations in the small LN_vs and locomotor rhythmicity are affected in parallel. Thus, with the *per-gal4*/UAS-*per* combination, robust PER and TIM oscillations were observed in the small LN_vs (Figs. 6, 7), and relatively high proportions of the behaviorally monitored adults were rhythmic (Table 5). In these flies, GAL4-mediated PER expression may not be strong enough to significantly impinge on PER and TIM oscillations in the small LN_v cells. In contrast, flies carrying *tim-gal4* and UAS-*per* had almost no PER oscillations and reduced TIM oscillations in that neuronal cluster, and they were largely arrhythmic in constant conditions. A high level of TIM immunoreactivity at ZT 9, which we observed within some of the small LN_v cells, was not expected, because the levels of this protein have been shown to decrease rapidly in response to light (Hunter-Ensor et al., 1996; Myers et al., 1996; Zeng et al., 1996). It seems as if the anomalously high level of PER (in the current transgenics) partly overcomes this light-dependent disappearance of TIM. A similar effect of PER overexpression on TIM abundance (although not necessarily during the day) has been reported for flies carrying an extra copy of *per* and the tim^{rit} mutation (Matsumoto et al., 1999). In any case, the *tim-gal4*/UAS-*per* transgenics in such LD conditions were behaviorally impaired (Fig. 5) as well as anomalous on histochemical criteria (Figs. 6, 7).

Increasing the levels of *period* gene products by extra copies of the per^{+} allele generally shortens free-running periods of wild-type flies (Smith and Konopka, 1982). This appears to contradict the present results, in which periods of rhythmic double-transgenic flies that express PER under the control of *per-gal4* or *tim-gal4* were not shorter than control flies carrying only a GAL4 driver or UAS-*per.* In the case of flies carrying high dosage of the normal *per* gene, the products of it are solely supplied by wild-type alleles; thus, the feedback loop functions relatively normally, and the molecular oscillations as well as behavioral rhythms are only accelerated. In contrast, constitutive levels of PER induced by *tim-gal4*–mediated UAS-*per* expression causes disruption of the loop, leading to a reduction of rhythmicity rather than a shortening of free-running period.

In another genetic situation—where the per^{01} background genotype allows for no behavioral rhythms—adding per^{+} function solely via the GAL4/UAS system led to weak rescue of arrhythmicity (Table 4). It is likely that low-amplitude oscillations of *per* and *tim* expression occurred in these flies (at least in terms of protein cycling; cf. Cheng and Hardin, 1998; Matsumoto et al., 1999), although such cycling was not detectable by histochemistry performed on the per^{01} flies carrying the *per-gal4* and UAS-*per* transgenes. It is also conceivable that cycling could occur at the level of phosphorylation or nuclear translocation, which

could not be detected by the histological methods used in this study.

Applicability of the GAL4 System for Dissecting Neural Substrates of Circadian Rhythms

The present study made extensive use of *per-gal4* and *tim-gal4* fusion genes to study the chronobiological functions of cells within *Drosophila* adults that express these clock genes. Tetanus toxin, whose production came under the control of the *per* or *tim* fusion genes, was brought to bear on our behavioral experiments in part because of its limited effect—on synaptic transmission among excitable cells (although this effect may not be limited to synaptic function in the narrowest sense; cf. Baines et al., 1999; Hiesinger et al., 1999). Application of this neural disrupter in *Drosophila* has previously been limited to behavioral studies of simple stimulus–responses or motor functions (Sweeny et al., 1995; Reddy et al., 1997; Tissot et al., 1998; Martin et al., 1998; Heimbeck et al., 1999). We felt it would be valuable to provide entry-level disruptions of at least one additional kind of behavior (whether or not the fly's rhythmic behavior is "simple" in terms of the pertinent neural functions and whole-animal actions). The quite specific effect of tetanus toxin notwithstanding, expression of this substance mediated by *per-gal4* or *tim-gal4* within many CNS neurons made it difficult to pinpoint the cells responsible for a particular features of the fly's rhythmic behavior. Nevertheless, our results suggest that a substantial proportion of *per/tim* neurons—beyond the small handful of these cells on which attention has been focused—are candidates for participating in this organism's rhythm system. Perhaps the ensemble of these neurons (all 70 pairs of them) must act and interact in order that fully normal behavioral rhythmicity can occur. Future experiments may be able to dissect further the broad features of this chrononeurobiological substrate. GAL4-driver lines with more restricted expression patterns could be designed or may be encountered (enhancer traps) and then combined with the UAS-*tetxlc* transgene that we applied. Brain-behavioral experiments resulting from application of these factors might lead to identification of all the individual cell locations and types responsible for the several features of locomotor activity we have analyzed in the current studies: behaviors occurring at various times within a natural cycle of day and night and endogenous, purely clock-driven locomotor rhythmicity that occurs in constant environmental conditions.

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