

Differential regulation of circadian pacemaker output by separate clock genes in *Drosophila*

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Regulation of the *Drosophila* pigment-dispersing factor (*pdf*) gene products was analyzed in wild-type and clock mutants. Mutations in the transcription factors CLOCK and CYCLE severely diminish *pdf* RNA and neuropeptide (PDF) levels in a single cluster of clock-gene-expressing brain cells, called small ventrolateral neurons (*s-LN_vs*). This clock-gene regulation of specific cells does not operate through an E-box found within *pdf* regulatory sequences. PDF immunoreactivity exhibits daily cycling, but only within terminals of axons projecting from the *s-LN_vs*. This posttranslational rhythm is eliminated by *period* or *timeless* null mutations, which do not affect PDF staining in cell bodies or *pdf* mRNA levels. Therefore, within these chronobiologically important neurons, separate elements of the central pacemaking machinery regulate *pdf* or its product in novel and different ways. Coupled with contemporary results showing a *pdf*-null mutant to be severely defective in its behavioral rhythmicity, the present results reveal PDF as an important circadian mediator whose expression and function are downstream of the clockworks.

Daily rhythms of physiology and behavior are generated by endogenous circadian oscillators. In *Drosophila*, this time-keeping is controlled by several clock genes (reviewed in ref. 1). The current model posits that the *Clock*- and *cycle*-encoded products—basic helix–loop–helix proteins that dimerize by means of their PAS domains—activate *period* (*per*) and *timeless* (*tim*) transcription by interactions with E-boxes located in the 5'-flanking region of these clock genes and a circadian enhancer upstream of (at least) *per*'s transcription unit (2, 3). After PER and TIM proteins accumulate in the cytoplasm and form heterodimers, they translocate to the nucleus and negatively regulate their own genes by interfering with CLK:CYC function (3, 4).

A similar negative-feedback loop operates in the circadian clocks of other animals. Mammalian homologs of *Drosophila* clock genes have been identified or originally discovered in mice and humans (1). Circadian oscillations of *mPer* mRNAs occur in the suprachiasmatic nucleus of mouse, a circadian-pacemaker structure in the brain. The amplitude of these mRNA rhythms is reduced in the murine *Clock* mutant, consistent with the fly paradigm (5). In addition to the central time-keeping functions of clock transcription factors, they may also regulate downstream rhythm-relevant genes, which are thought to control physiological and behavioral rhythms. In this respect, CLK:BMAL1 heterodimers can activate transcription of a clock-regulated vasopressin gene in mice (5). In *Drosophila*, cyclically expressed genes whose mRNA oscillations are affected by clock mutations have been described (6, 7). Additional factors putatively functioning as clock outputs were originally identified by mutations (8, 9). However, the manner by which clock genes act—presumably upon these and other output factors—to effect the pacemaker's control of an overt rhythm is unknown.

It has been suggested that a neuropeptide, pigment-dispersing factor (PDF), is involved in clock output in insects (reviewed in ref. 10). PDF is also known as pigment-dispersing hormone, which was named after its function in crustaceans; it does not appear to play such a role in insects (11). PDH-immunoreactive

neurons in *Drosophila melanogaster* co-express the *per* gene (12). These brain cells—called Lateral Neurons—are believed to play an important role in the control of circadian behavioral rhythms (reviewed in ref. 13).

To permit examination of a possible role for PDF as a circadian effector, we previously isolated the *pdf* gene in *Drosophila* (14). Subsequently we presented evidence that PDF is necessary for normal locomotor-activity rhythms (15). Yet, this behavioral genetic study did not address the question of whether *pdf* or its encoded product are influenced by the circadian pacemaker. In this report, we show that this gene and PDF are regulated by clock genes in remarkably diverse and unexpected ways. That a *pdf* mutant is rhythm-defective (15) could mean that the neuropeptide is a generic maintenance factor for behavioral rhythmicity, but the current study reveals that PDF exhibits daily cycling in a subcellular compartment of certain brain neurons.

Materials and Methods

Fly Strains. Most of the *per*, *tim*, *Clk*, and *cyc* mutant strains used were as described previously (16, 17). A new *cyc*⁰ mutant was found in a screen (of ca. 6,000 lines descended from chemically mutagenized flies) independent of that which resulted in *cyc*⁰¹. *cyc*⁰² is a recessive locomotor-arrhythmic, as is *cyc*⁰¹; the mutations are noncomplementing both for behavior and for a severe reduction in levels of PER protein (data not shown; cf. ref. 17). By sequence analysis like that performed on *cyc*⁰¹—showing it to be a nonsense mutation in the PAS-encoding region (17)—*cyc*⁰² was found to be similarly mutated (Q113/CAA → UAA) within a part of the gene that specifies amino acids between the relatively N-terminal basic helix–loop–helix domain and the PAS domain. To obtain *Clk*^{JK} hemizygotes, flies carrying a 3rd-chromosome deletion missing the *Clock* locus [*Df(3L)pbl-X/In(3LR)TM6B, Tb Hu* (16)] were crossed to mutant homozygotes.

RNA Assessments. *In situ* hybridizations were performed as in ref. 18 on dissected central nervous systems (CNSs). The reagents and procedures were held constant among specimens to permit quantitative analysis of the signals. Numbers of brain cells stained after application of the *pdf* probe (full-length cDNA, digoxigenin-labeled) were counted blindly with respect to genotype. For temporal analysis and mutant vs. normal comparisons of *in situ*-hybridized brains, staining levels were scored blindly (on a scale of 0 to 4, in increments of 1) with respect to time of sacrifice and genotype. In the temporally controlled experiment, wild-type male flies were put through five 12-h:12-h light–dark (12:12 LD) cycles, then killed at four time-points for

Abbreviations: PDF, pigment-dispersing factor; *s-LN_vs* and *l-LN_vs*, small and large ventrolateral neurons; LNs, Lateral Neurons; CNS, central nervous system; LD, light–dark cycle; ZT, Zeitgeber time in LD cycle.

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in situ hybridization. Staining scores for the *per*⁰¹ and *tim*⁰¹ mutants were statistically analyzed by the Kruskal–Wallis non-parametric ANOVA test, followed by Dunn’s multiple comparisons test. Northern blotting was performed and quantified as in ref. 14.

Immunohistochemistry. The *Drosophila* PDF 18-mer was synthesized on the basis of its inferred amino acid sequence (14). The product was amidated at its C terminus, as is the case for naturally occurring PDH (11), then conjugated separately to three different carrier proteins (ovalbumin, BSA, keyhole limpet hemocyanin). These materials were injected sequentially into two rats; subsequent bleedings led to antisera, which from each rat produced identical immunohistochemical results. Whole-mounted CNSs were dissected 2–5 h after lights-on (ZT2–5) and subjected to staining procedures as in ref. 19, except for varying incubation times: overnight for the primary antibody (dilution 1:500 for anti-fly-PDF, 1:2000 for anti-crab-PDH); and 2- to 5-h incubations for the fluorescein isothiocyanate (FITC)-, tetramethylrhodamine (TRITC)-, or peroxidase-conjugated secondary antibody (dilution 1:200). Both peroxidase- and FITC-mediated signals led to the cell counts in the legend to Fig. 2, which were obtained blindly with respect to genotype.

Temporally based immunostaining of sectioned material was performed by sacrificing flies at 3-h intervals in 12:12 LD cycles or constant darkness; heads were fixed, dehydrated, embedded in paraffin, sectioned at 7 μ m, and stained with anti-PDH as in ref. 19. Signal intensities were scored as in ref. 20, blindly with respect to time-points and *per* genotypes. Period values were determined by applying curve-fitting software. To compare the signal intensities at two times within an LD cycle, dissected whole-mounted adult brains were obtained from 10-day-old flies (that had been entrained to 12:12 LD) at ZT1 and ZT13; anti-PDH immunohistochemistry (involving TRITC) was performed as in ref. 21. Pictures of the stained specimens were taken with a digital camera mounted on a fluorescence microscope; the images were converted to “gray scale” by a COREL PHOTO PAINT program (Corel, Dublin, Ireland). The gray values of the stained structures and of the background were scored by the DOKU program (Olympus) with a scale from 0 (black) to 255 (white). Gray values of the background were subtracted from corresponding ones of the stained nerve terminals to obtain the intensity indices on the ordinate of Fig. 4D. These values were statistically analyzed as indicated for the quantified *in situ* hybridizations.

pdf Transgenics. A genomic fragment from the locus (14), inserted into pBluescript, was digested with *Eco*NI, ligated with *Eco*RI linker, cut with *Bam*HI/*Eco*RI, then subcloned into pBluescript (P2.4-pBS). This DNA fragment, called P2.4 (promoter-region, 2.4 kb), was sequenced; its 3’ end extends 16 bp downstream of the transcription-start site and terminates 58 bp upstream of the translation-start (see ref. 14). An *Xba*I/*Eco*RI fragment from P2.4-pBS was subcloned into the pPTGAL vector (which includes the mini-*w*⁺ marker gene), then injected into *y w* embryos with pUChps Δ 2–3 helper plasmid. For the “promoter-bashed” constructs, P2.4-pBS was digested with *Bsa*AI/*Eco*RI (for P1.4), *Acc*I/*Eco*RI (for P1.0), or *Nsi*I/*Eco*RI (for P0.8); then each of these fragments was subcloned into pBluescript, from which *Xba*I/*Eco*RI fragments were isolated and subcloned to pPTGAL vector. To produce the most severely bashed P0.5 construct, P2.4-pBS was *Nhe*I/*Spe*I-digested; then the vector was isolated and religated intramolecularly, from which *Xba*I/*Eco*RI fragments were isolated and subcloned into pPTGAL. After generating transgenic strains from the five construct types, such flies were crossed to ones from a UAS-*lacZ* reporter strain, which produces cytoplasmic β -galactosidase (β -GAL) in the presence of GAL4 (22). 5-Bromo-4-chloro-3-indolyl β -D-

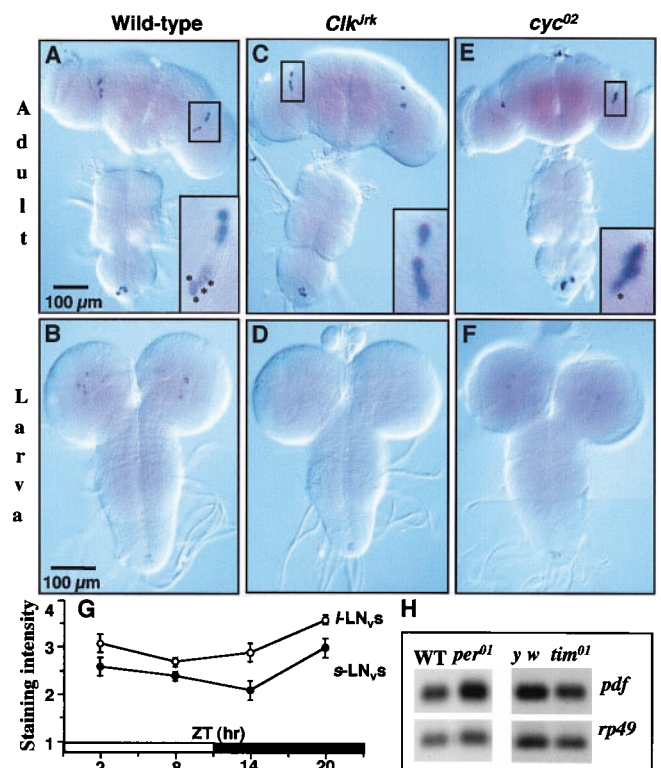


Fig. 1. Spatial and temporal expression of *pdf* RNA. Whole-mounted nervous systems were processed for *pdf* *in situ* hybridization. (A) Wild-type (Canton-S) adults ($n = 5$ dissected CNSs). Almost invariably, there were four signal-containing s-LN_vs and four l-LN_vs in each of the 10 brain hemispheres. The boxed area is a 4 \times magnification of the lateral-brain region; asterisked cells are the s-LN_vs. (B) Wild-type larvae ($n = 6$); four signal-containing brain cells were invariably stained in each of the 12 brain hemispheres. (C) *Clk*^{rk} mutant adults ($n = 7$); there were no discernible s-LN_vs in any of the 14 hemispheres. (D) *Clk*^{rk} larvae ($n = 6$); no *pdf*-RNA-containing cells were detected in the 12 hemispheres. (E) *cyc*⁰ mutant adults. The results depicted here for *cyc*⁰² ($n = 7$) were very similar those obtained from *cyc*⁰¹ ($n = 8$); in both allelic mutant types, there were subnormal numbers of s-LN_vs labeled by the *pdf* probe; this is exemplified by the one asterisked cell in the 4 \times -magnified box for the lateral-brain region of this *cyc*⁰² adult (compare with the box in A); the reductions in s-LN_v cell numbers in *cyc*⁰ adults were best appreciated after immunohistochemistry (see Fig. 2). (F) *cyc*⁰ larvae; for *cyc*⁰² (as exemplified in this image), 2.6 \pm 0.3 lateral neurons (LN) were labeled per hemisphere ($n = 14$), and these RNA signals were weak compared with wild-type; corresponding values for *cyc*⁰¹: 3.4 \pm 0.2 ($n = 8$). Near the bottom of A–F is the abdominal ganglion, within which there is a small cluster of *pdf* cells. (G) Timecourse of *pdf* mRNA in separate clusters of peptide-containing neurons; *in situ*-hybridized specimens were blind-scored for staining levels, using 7–16 adult brains per time-point (the scoring scale ranged from 0 to 4, but only 3% of the individual values ranged as low as 1); ZTs are Zeitgeber times in which light comes on at noon during a 12-h light (open bar)–12-h dark (black bar) cycle. (H) *pdf* RNA levels in *period* and *timeless* mutants; the Northern-blot results were obtained from head extracts after sacrificing adults at ZT2–4; normalizing the *per*⁺ and *tim*⁺ levels (set = 1.00) to the *rp49* loading control, the abundance in *per*⁰¹ was 1.07 and that for *tim*⁰¹ was 0.95. *In situ* hybridizations on *y per*⁰¹ *w*, *y w*; *tim*⁰¹, and *y w* control brains (dissected from adults at ZT2–5) led to the following staining scores (as on the ordinate of G): s-LN_vs, in the genotypic order just given: 2.1 \pm 0.2 ($n = 16$ brain hemispheres), 1.8 \pm 0.3 ($n = 10$), 2.4 \pm 0.2 ($n = 8$); l-LN_vs: 3.0 \pm 0.2, 2.7 \pm 0.3, 3.6 \pm 0.2; by ANOVA, the differences among s-LN_v scores were not significant ($P = 0.1$); those among l-LN_v scores were significant ($P = 0.03$), but only one pairwise comparison was marginally so (*tim*⁰¹ vs. control, $P \approx 0.05$).

galactoside (X-Gal) histochemistry was performed on dissected CNSs of the progeny as in ref. 20. Five or more independent *pdf*-GAL4 lines for each construct were obtained; three or more lines for each construct were crossed to UAS-*lacZ*, and at least

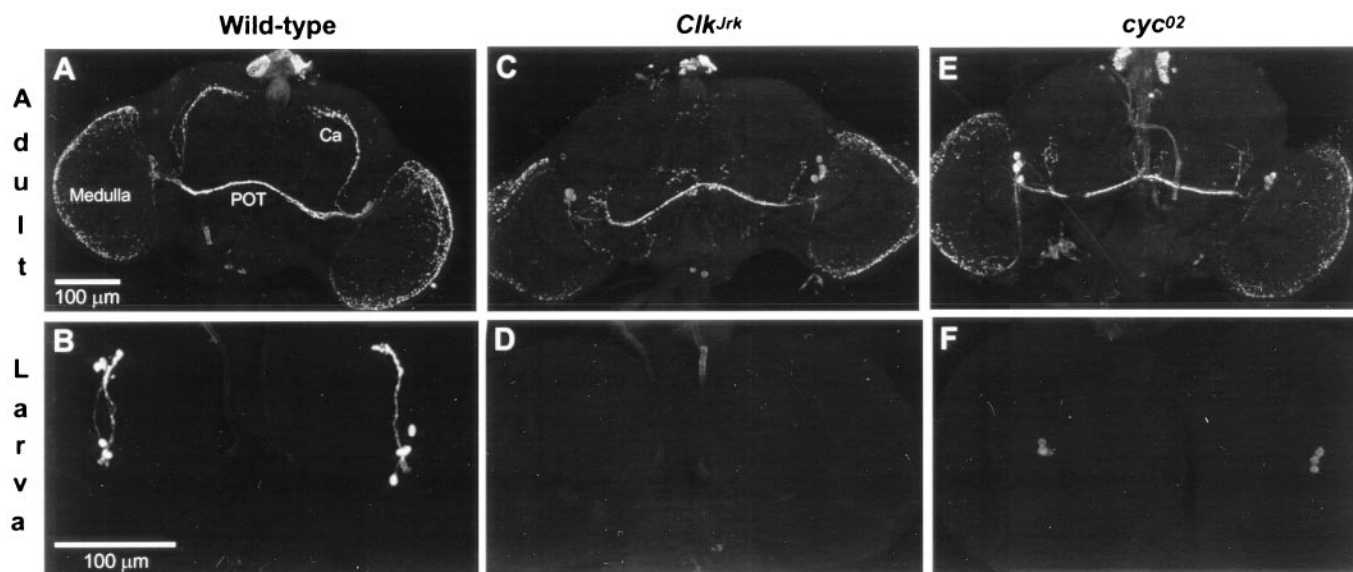


Fig. 2. Effects of *Clock* and *cycle* mutations on PDF immunostaining in brain neurons. (A) Wild-type adults ($n = 14$); numbers of signal-containing cells per brain hemisphere: $s\text{-LN}_v$ s, 3.0 ± 0.2 ; $l\text{-LN}_v$ s, 3.6 ± 0.1 ($n = 27$ scorable hemispheres). Ca, calyx of the dorsal-brain mushroom body, in the vicinity of which are termini of neurites projecting from $s\text{-LN}_v$ cells; POT, posterior optic tract, one type of projection from $l\text{-LN}_v$ cells. (B) Wild-type larvae ($n = 6$); four PDF-containing LNs were invariably detected in the 12 brain hemispheres examined. (C) *Clk^{Jrk}* adults ($n = 10$); no $s\text{-LN}_v$ s were detected in the 20 brain hemispheres examined for mutant homozygotes, nor were there any such signals in 7 brains of flies heterozygous for *Clk^{Jrk}* and a deletion (*Df*) of the locus; counts of stained $l\text{-LN}_v$ s in *Clk^{Jrk}/Clk^{Jrk}*: 3.8 ± 0.2 ($n = 17$ scorable hemispheres). Arrow, abnormal projections. (D) *Clk^{Jrk}* larvae ($n = 14$); no LNs were detected in any of the 28 hemispheres examined, nor were any observed in the brains of 8 larvae heterozygous for *Clk^{Jrk}* and the *Df*. (E) *cyc⁰²* adults; this *cyc⁰²* specimen exemplifies the subnormalities and abnormalities of $s\text{-LN}_v$ cells exhibited by the 10 adults examined that were homozygous this allele—e.g., no PDF neurites extending toward the top of the brain. Arrowhead, abnormal projections. For the companion *cyc⁰¹* mutant ($n = 9$ adult CNSs dissected and stained) cell counts: $s\text{-LN}_v$ s, 1.0 ± 0.2 ; $l\text{-LN}_v$ s, 3.4 ± 0.2 ($n = 15$ scorable hemispheres). (F) *cyc⁰¹* larvae. Consistent with the *in situ* hybridizations (Fig. 1), the staining intensities in PDF cells were much weaker than in wild-type and variable within a given specimen; regardless of the number of LNs detected in either *cyc⁰¹* or *cyc⁰²* larvae ($n = 16$ and 10, respectively), no stained axonal processes were detectable. Cell counts: *cyc⁰¹*, 3.1 ± 0.3 ($n = 32$ hemispheres); *cyc⁰²*, 2.9 ± 0.3 ($n = 20$).

four larval and four adult CNSs from among the progeny of each cross were examined for β -GAL expression.

Results

Expression of a Neuropeptide-Encoding Gene in the CNS. To assess the effects of clock mutations on *pdf* expression, we first determined the normal cellular distribution of the *Drosophila* gene's native products. By *in situ* hybridization, we showed that the expression pattern of *pdf* RNA is very similar to that determined previously with anti-crab-PDH (19, 23). There are four positive cells in each larval brain hemisphere (Fig. 1B); these persist into adulthood (Fig. 1A, C, and E) and become the small ventrolateral neurons ($s\text{-LN}_v$ s), whose neurites project into a dorsal region of the adult brain. Four large ventrolateral neurons ($l\text{-LN}_v$ s) also express *pdf* (Fig. 1A); these emerge during metamorphosis and send projections into the optic lobe and across the brain midline. Larvae and adults also contain *pdf* mRNA in the posterior extremity of the CNS (Fig. 1).

Northern blots revealed no daily rhythm of *pdf* mRNA abundance (14), but they could have failed to detect *pdf* mRNA cycling in a subset of the cells. Thus we performed temporal *in situ* hybridizations; neither category of *pdf*-expressing neurons exhibited systematic fluctuations in signal intensities (Fig. 1G). Therefore, there is no *pdf* mRNA rhythm for clock mutations to affect.

Our newly produced anti-*Drosophila* PDF antibodies led to cell labeling identical to that obtained by *in situ* hybridization (Fig. 2A and B). Neither method led to marking of cells in the dorsal brain of adults that are stained by anti-crab-PDH (23). This and other evidence (Fig. 3, below; and ref. 15) indicates that the dorsally located antigen is cross-reacting material and does

not have to be considered in terms of effects of clock mutations on *pdf* expression.

Cell-Specific Regulation of *pdf* mRNA and PDF by *Clock* and *cycle*.

Expression of *pdf* in the arrhythmic *Clk^{Jrk}* mutant was found to be strikingly abnormal. In *Clk^{Jrk}* brains, neither *pdf* mRNA nor PDF was detectable in larval LN cells and in the $s\text{-LN}_v$ s of adults (Figs. 1 and 2). The same defects were observed in mutant animals heterozygous for *Clk^{Jrk}* and a deletion of the locus (legend to Fig. 2). These results suggest that CLK is required for *pdf* transcription, although only in certain cells: the larval LNs and the $s\text{-LN}_v$ s into which they develop. Dorsally projecting axonal processes arising from the $s\text{-LN}_v$ cells terminate near the calyx of the dorsal-brain mushroom body. In accord with the absence of perikaryal $s\text{-LN}_v$ immunoreactivity, these projections are absent from *Clk^{Jrk}* brains (Fig. 2C). In contrast, expression in the $l\text{-LN}_v$ s and abdominal-ganglionic cells of adults was apparently unaffected by *Clk^{Jrk}* (Figs. 1C and D and 2C and D); this included normal staining of centrifugal and interhemispheric projections within the fly's head (Fig. 2A). However, certain features of projections from $l\text{-LN}_v$ cells are aberrant in *Clk^{Jrk}*. Approximately 50% of the mutant brains showed abnormal projections (as exemplified in Fig. 2C); in others, one or two axons from this region projected farther and irregularly toward a dorsal or median region of the brain. None of these projections was similar to the more dorsal-reaching projections in the brains of wild-type adults (Fig. 2A).

Because the CYC protein cooperates with CLK in their transcriptional-activation roles (1), we also analyzed *pdf* expression in *cycle* mutants (Figs. 1 and 2). The effects were similar to but less severe than those caused by *Clk^{Jrk}*. Most of the larval LNs homozygous for either of two *cyc⁰* mutations showed much

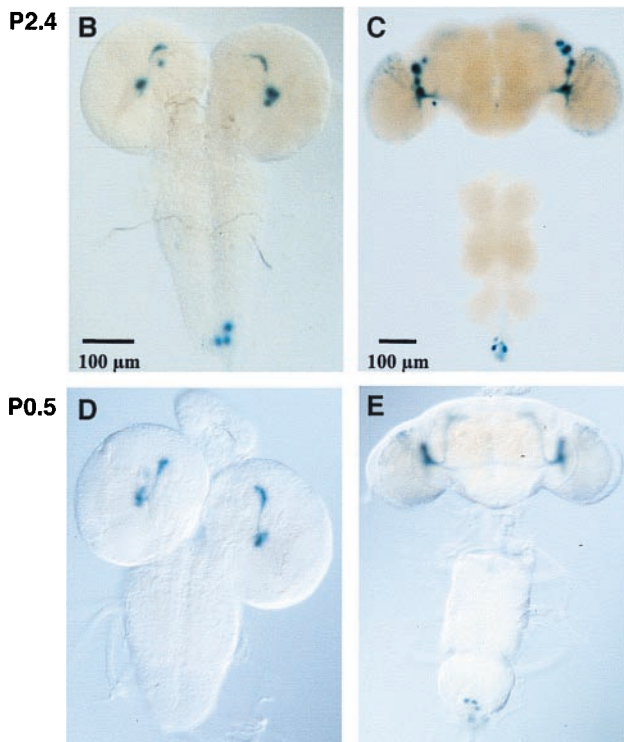
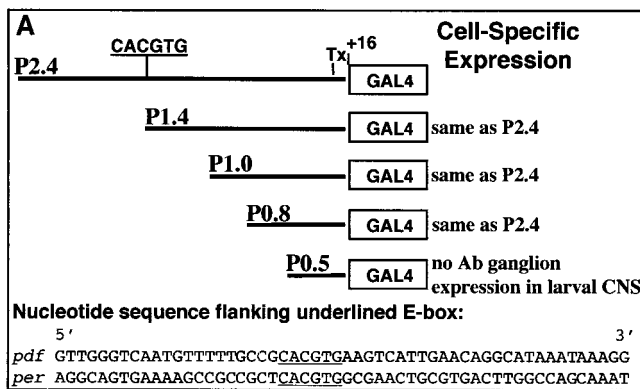


Fig. 3. Analysis of the *pdf* promoter region. (A) E-box sequence ca. 1.4 kb upstream of the transcription start site (Tx) in the P2.4 construct. Half of the E-box (GTG) is present in P1.4 but eliminated in the other three constructs; note that there is no homology in the nucleotide sequences flanking *per*'s and *pdf*'s E-boxes (*per* upstream sequence: ref. 2). (B) 5-Bromo-4-chloro-3-indolyl β -D-galactoside (X-Gal) histochemistry for a P2.4 + UAS-*lacZ* larval CNS. (C) Adult CNS of the same transgenic type as in B. Note that in the imaginal specimen there are only LN signals, no dorsal signals (cf. Figs. 1 and 2). (D) X-Gal-mediated staining in a P0.5 + UAS-*lacZ* larval CNS. There is no reporter expression in the abdominal ganglion. (E) Adult CNS of the same transgenic type as in D.

weaker expression of both mRNA and peptide compared with wild type, but the mutant expression levels were variable even within a single brain hemisphere: some cells contained signal, whereas others were extremely difficult to detect. While this report was in preparation, we learned that similar *Clk^{Jrk}* and *cyc⁰¹* effects on *pdf* expression in larvae were obtained by others (9). The numbers of antibody-stained *s*-LN_vs in *cyc⁰* adults were well above zero (details in the legend to Fig. 2), compared with the elimination of such signals in *Clk^{Jrk}* flies. Numbers of *l*-LN_v cells in the brains of *cyc*-mutated adults were normal (Fig. 2), similar to the results obtained in the *Clk^{Jrk}* background. About 25% of the adult *cyc⁰* brains exhibited an

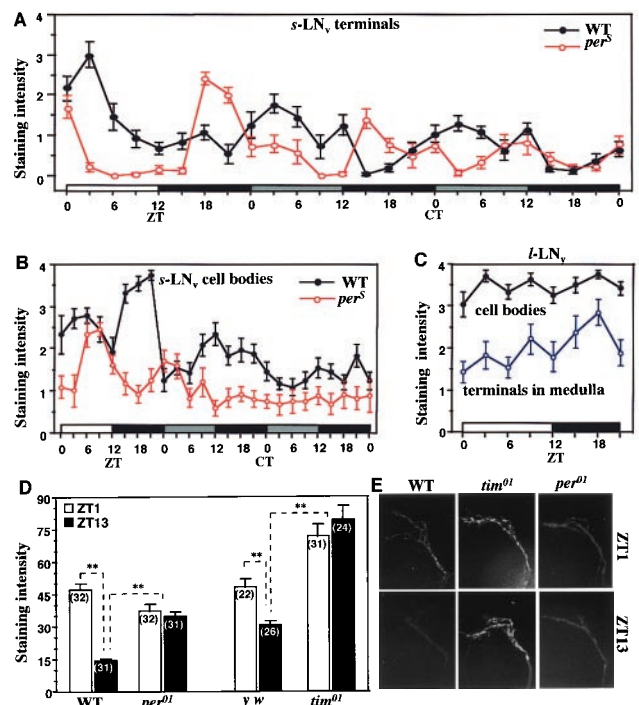


Fig. 4. Temporal analysis of PDF-like immunoreactivity in brain cells and their projections. (A) Immunohistochemical time-course of wild-type head sections and those of the *per^S* and *per⁰¹* mutants, in both LD-cycling and constant-dark (DD) conditions ($n = 10$ –13 per time-point for each genotype). ZTs, as in Fig. 1; CT, circadian times during DD (gray bar, subjective day, corresponding to actual daytime in the preceding LD condition; black bar, subjective night). The apparent oscillations were formally analyzed for the DD portions of the records, resulting in 24.2-h and 19.9-h free-running period estimates for wild-type and *per^S*, respectively. In *per⁰¹* specimens, examined at eight equally spaced times in LD, the nerve-terminal signals were chronically low: at five time-points, the staining levels were 0; at the other three, the average scores ranged from 0.1 to 0.2. (B) *s*-LN_v cell-body staining scores for the same specimens as in A. (C) *l*-LN_v cell staining for cell bodies and centrifugally projecting fibers; the specimens scored are a subset of those that led to the scoring for A and B (here, only the flies that were in the LD cycle). (D) Immunohistochemical comparison of wild type (WT) to two clockless mutants. The nerve-terminal signals were examined in wild type, *per⁰¹*, and *tim⁰¹* at the peak and trough times (cf. A) in an LD cycle; these brains were whole-mounted, and the relevant dorsally located anti-PDH-mediated signals were quantified as described in the text; *y w* is a *tim⁺* control for the arrhythmic loss-of-function *timeless* mutant (which was in a *y w* genetic background); based on the numerical read-outs from analysis of the nerve-terminal staining intensities, certain of the average signals were determined to be significantly different ($P < 0.05$), as indicated by **; the numbers of brain hemispheres examined are shown in parentheses; error bars, SEM. (E) Representative dorsal-brain images of *s*-LN_v nerve terminals (cf. Fig. 2A), stained after sacrificing flies of three genotypes in the early morning (ZT1) or the early night (ZT13).

abnormal dorsal projection similar to that shown in Fig. 2E. In approximately 30% of these mutant specimens, the projections were asymmetric within a single individual: one hemisphere would contain a bundle of dorsally projected axons; but in the contralateral hemisphere, only one or two axons projected into the dorsal brain. In other *cyc⁰* adults, axons stemming from the region indicated by an arrow in Fig. 2E projected irregularly into a median brain region.

The major conclusions from examining *pdf* expression in the *Clock* and *cycle* mutants are that (i) both genes appear to be positive regulators of *pdf* RNA levels but only in the *s*-LN_vs and their larval precursors; (ii) the effects of *Clk^{Jrk}* are stronger than those of the *cyc⁰* mutations; and (iii) there are developmental

defects, as PDF-containing processes in the adult CNS are aberrant in both types of mutants.

A *pdf* E-Box Is Not Necessary for Normal Spatial Expression. Do CLK and CYC activate *pdf* transcription directly? If that were the case, there could be an E-box in this gene's regulatory region (cf. refs. 2 and 3). Indeed, within a 2.4-kb segment 5' to the *pdf* ORF we found a CACGTG sequence \approx 1.4 kb upstream of the transcription-start site (Fig. 3A). The 2.4-kb DNA fragment was fused to the (yeast) *GAL4* gene; transgenic strains were generated and crossed to flies carrying UAS-*lacZ*. The doubly transgenic progeny showed faithful β -galactosidase-reported expression of *pdf* (Fig. 3). To determine whether the E-box is important for the *pdf*'s transcriptional activation, further transgenics were generated (Fig. 3A). Deletions missing the half or all of the E-box were sufficient to drive brain expression indistinguishable from that observed in wild type (Fig. 3). Interestingly, the smallest 5'-flanking region examined mediated the normal brain pattern but did not lead to abdominal-ganglionic expression in the larval CNS (Fig. 3D). That the influences on *pdf* expression of *Clock* and *cycle* do not operate through a circadian E-box, and thus seem to be indirect, is consistent with the lack of *pdf* mRNA cycling and *Clock/cycle*-independent expression in the *l*-LN_v cells.

Posttranscriptional Regulation of PDF by *period* and *timeless*. No effect of a *period*-null mutation on *pdf* mRNA levels was detectable in previous Northern blottings (14). We confirmed and extended this result by showing that neither *per*⁰¹ nor a *timeless*-null mutation affects the RNA's abundance, by Northern blottings and by *in situ* hybridizations (Fig. 1H and accompanying legend).

To search further for regulation by *per* or *tim*, we stained adult brains with anti-PDH at different times of day and night. Strikingly, nerve terminals in a dorsal region of the central brain exhibited rhythms of anti-PDH-mediated staining. The neurites that terminate in this region project from the *s*-LN_v cells (13). In an LD cycle, the peak and trough times for the nerve-terminal cycling were 1 h after lights-on and lights-off, respectively (Fig. 4A). Staining levels in the perikarya of *s*-LN_vs exhibited some fluctuations but no regular pattern (Fig. 4B). The adult-specific, larger PDF neurons also exhibited no appreciable cycling of anti-PDH-mediated staining, either in *l*-LN_v cell bodies or in the termini of their neurites that ramify over the surface of the medulla optic lobe (Fig. 4C).

The dorsal-brain, nerve-terminal cycling persisted in constant darkness with an \approx 24-h period in wild type. In that condition the cycle duration was shortened to \approx 20 h by the *per*^S mutation (Fig. 4A), which causes behavioral periodicities to be about 5 h shorter than normal (e.g., ref. 24). In the dorsal brains of the *per*⁰¹ null mutant, nerve-terminal cycling was abolished, and the signal strengths were very low (legend to Fig. 4A). However, the immunohistochemical procedure performed on these brain sections is not very sensitive. Therefore, we used a quantitative fluorescence method, better to judge PDF staining intensities in whole-mounted brains (Fig. 4D). At the peak and trough time-points, nerve-terminal signals in wild type were again higher in the early morning compared with the early night. This temporal difference was not observed in the dorsal brains of the arrhythmic *per*⁰¹ and *tim*⁰¹ mutants. In *per*⁰¹, the staining intensities at both times were nearly identical and at levels intermediate between the *per*⁺ peaks and troughs (Fig. 4D and E). In *tim*⁰¹, the PDF terminal signals were also the same at the two time-points but significantly higher than in *tim*⁺ (Fig. 4D and E). The mutational effects of these clock genes on daily fluctuations of PDF abundance at certain nerve terminals indicate that an aspect of this peptide's regulation is on the one hand clock controlled, and on the other posttranslationally regulated.

Discussion

This paper focuses on the remarkable cell-type specificity manifest by *Drosophila* clock mutations. They affect a particular subset of adult neuropeptide-containing cells, small neurons in a ventrolateral region of the brain (Figs. 1 and 2). The larval precursors of such cells are similarly affected (Fig. 1), an observation also made by others (9) for two of the mutants, *Clk*^{Jrk} and *cyc*⁰¹. Although the larval assay makes a connection between these clock mutants and *pdf* expression, it does not detect the exquisite cell-type selectivity of the mutations. This is because the affected precursor cells are the only PDF-expressing ones in larvae. In adults, a second class of PDF cells has arisen. These, the *l*-LN_vs, are largely insensitive to effects of *Clk* and *cyc*⁰ (Figs. 1 and 2). Analogous cell-specific effects were uncovered for *per* and *tim* mutations (Fig. 4), whose effects on the PDF oscillation are manifest only in the small cells despite the presence of cycling PER and TIM in both the *l*-LN_vs and the *s*-LN_vs (25).

These results provide further evidence pointing to the *s*-LN_v cells as the most important circadian-pacemaker neurons in the fly's circadian system, at least insofar as behavioral rhythmicity is concerned (cf. ref. 15). Supporting this inference are the facts that (i) *s*-LN_v cells are the only clock-gene-expressing LNs projecting to a central brain region—as opposed to out into the visual system, which is dispensable for behavioral rhythmicity (13); (ii) a brain-damaged mutant, in which LNs are mostly eliminated, exhibits behavioral rhythms very rarely, and this is almost always correlated with the presence of centripetal (PDF-stained) projections from the *s*-LN_vs (21); (iii) a *cryptochrome* (*cry*^b) mutation that eliminates *per* and *tim* gene-product cyclings throughout most of the fly permits behavioral rhythmicity, which is correlated with PER and TIM cycling in the *s*-LN_vs (25); (iv) the precursors of *s*-LN_v cells are present throughout postembryonic development (19, 20), and adult locomotor-activity rhythms can be entrained as early as the first-instar larval stage (24, 26).

The cell-type specificity of these mutants is unprecedented, as the central pacemaker mechanism—the CLK:CYC/PER:TIM feedback loop—is believed to operate similarly in a large number of clock cells all over the fly. So why is PDF expression uniquely sensitive to *Clk*^{Jrk} and *cyc*⁰ in the *s*-LN_vs? There must be additional clock-relevant elements or developmental events that distinguish between the small and large cells. An extreme view of the second possibility is that CLK:CYC or some related circadian clock transcription complex is necessary for *s*-LN_v specification; thus, these cells would be absent from the *Clk*^{Jrk} and *cyc*⁰ mutants. A developmental role for these genes is consistent with the aberrant projections of certain PDF-containing neurites in the mutant animals (Fig. 2). However, the key PDF cells are not eliminated by these mutations, in that the *s*-LN_vs are retained in the *Clk*^{Jrk} and *cyc*⁰ mutants. This retention was revealed by weak *per* and *tim* expression within these neurons (27), even though the levels of the latter two gene products are grossly subnormal in *Clk*^{Jrk} and *cyc*⁰ flies (16, 17). Moreover, the *cry* gene, which is co-expressed with *pdf* in larval LNs (28), marks those cells as present in the *Clk*^{Jrk} and *cyc*⁰¹ mutants (P. Emery and M.R., unpublished observations).

Why does *Clk*^{Jrk} have more severe effects on *pdf* expression than do the *cyc*⁰ mutations? This question is especially intriguing, because the former is not a null mutant; the semidominance of this mutation is a reflection of the residual activity of CLK^{Jrk} (16). In contrast, both *cyc*⁰ mutants are likely to be complete loss-of-function mutants (*Materials and Methods*; ref. 17). The milder effects of *cyc*⁰s on *pdf* (Figs. 1 and 2) suggest the existence of another PAS-containing transcription factor, which in *cyc*⁰ mutants would partner with CLK and provide a chronobiologically relevant function. The stronger effects of *Clk*^{Jrk} on *pdf* expression could be due to this mutant protein's ability to

sequester in inactive complexes not only CYC but also the other putative PAS factor.

In the *Clock* mutant mouse, the aberrant product is similar to that encoded by *Clk^{Jrk}* in *Drosophila*; both proteins are missing substantial portions of their activation domains (1). By analogy to the cell specificity of *Clk^{Jrk}* effects on *pdf*, *Clock* eliminates a vasopressin mRNA rhythm in the mouse suprachiasmatic nucleus (SCN), whereas the (normally) constitutive level of that transcript in another hypothalamic nucleus is unaffected (5). Regulation of the vasopressin RNA rhythm in the SCN probably results from a canonical interaction of the mCLK:BMAL1 heterodimer with an E-box present in the 5'-flanking region of the vasopressin gene (5). As implied by Fig. 3, clock regulation of *pdf* expression within *Drosophila* lateral neurons is unlikely to be based on similar principles.

Against a background of the transcriptional feedback-loop paradigm that dominates the current circadian landscape (1), it is remarkable that the robust temporal oscillation of PDF involves the peptide itself and is restricted to the axon terminals projecting from certain cells. Moreover, this cycling and its alteration by *per* and *tim* mutations (Fig. 4) do not stem from changes in *pdf* mRNA abundance (Fig. 1). Although the implied posttranslational regulation could take place at any level (such as peptide processing or axonal transport), we prefer the idea that the clock regulates release of PDF from the centripetally projecting s-LN_v terminals. Under this hypothesis, there are abundant PDF-containing axonal termini early in the morning; clock-mediated release would occur during the day, such that

there are relatively few PDF-positive termini in the early night (Fig. 4). Inappropriately high nighttime levels of PDF in *per⁰¹* and *tim⁰¹* (especially the latter: Fig. 4D) suggest that release is inhibited in these mutants. Consistent with this supposition is that PDF axonal signals normally rise in the morning (Fig. 4) as PER and TIM levels are falling (1). This is not to say that "high PER and TIM" directly potentiate nighttime release of PDF. These clock proteins could contribute to temporal gating of release by hindering the production of an inhibitor of that process.

In summary, our results indicate remarkable cell-type specificity of several circadian clock genes as well as an important role for posttranslational regulation of the PDF neuropeptide. Taken together with the effects of a *pdf* mutation (15), these findings present insights into how a behaviorally meaningful humoral output factor is regulated by central pacemaker cells and clock genes functioning within them. Given the similarities between circadian-clock mechanisms in *Drosophila* and mouse, it will be interesting to see whether these features of the insect system also apply to mammals.

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