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A *pdf* Neuropeptide Gene Mutation and Ablation of PDF Neurons Each Cause Severe Abnormalities of Behavioral Circadian Rhythms in *Drosophila*

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Summary

The mechanisms by which circadian pacemaker systems transmit timing information to control behavior are largely unknown. Here, we define two critical features of that mechanism in *Drosophila*. We first describe animals mutant for the *pdf* neuropeptide gene, which is expressed by most of the candidate pacemakers (LN_v neurons). Next, we describe animals in which *pdf* neurons were selectively ablated. Both sets of animals produced similar behavioral phenotypes. Both sets entrained to light, but both were largely arrhythmic under constant conditions. A minority of each *pdf* variant exhibited weak to moderate free-running rhythmicity. These results confirm the assignment of LN_v neurons as the principal circadian pacemakers controlling daily locomotion in *Drosophila*. They also implicate PDF as the principal circadian transmitter.

Introduction

Circadian clocks function within multicellular organisms to provide a temporal framework and allow biological events to anticipate changing environmental conditions. A coherent picture of the clock mechanism has recently emerged through molecular genetics (reviewed by Dunlap, 1999). However, relatively little is known about the chemical messengers, cellular pathways, or downstream signaling events that link the daily oscillations of pacemaker cells to overt behavioral rhythms. Many questions are raised by such issues of clock output, and most resolve to consideration of two initial points: what are the critical circadian pacemaker neurons, and which specific substances transfer information from these neurons to other brain centers.

In mammals, the pacemaker for daily activity rhythms resides in the suprachiasmatic nucleus (SCN) of the hypothalamus (reviewed by Weaver, 1998). In insects, the pacemaker for daily activity rhythms resides in neurons associated with brain visual centers (reviewed by Helfrich-Fôrster et al., 1998). Both pacemaking centers are distinguished by expression of clock genes such as *period* (reviewed by Dunlap, 1999), although other cell types also display clock gene expression. For insects, various lines of evidence suggest that clock gene expression in certain lateral neurons (LNs) is required for their pacemaker activities (reviewed by Kaneko, 1998). LN neurons are divisible into dorsal (LN_d) and ventral clusters (LN_v); the ventral cluster is further divided into small and large groups (*s*-LN_v and *l*-LN_v). LN_v neurons may be especially important for production of circadian behavioral rhythms (Helfrich-Fôrster et al., 1998).

One strategy to identify clock outputs has involved searching for factors whose abundance fluctuates in a circadian fashion. While such screens have identified rhythmically expressed factors in many organisms (e.g., Anderson and Kay, 1997; Lopez-Molina et al., 1997), only some of these have been shown to be under direct clock regulation (e.g., Van Gelder and Krasnow, 1996; Loros, 1998; Jin et al., 1999). In particular, certain neuropeptides or neuropeptide RNAs cycle within the SCN (Inouye, 1996). A candidate transmitter for *Drosophila* clock neurons was suggested by the demonstration that LN_v neurons express neuropeptides related to crustacean pigment-dispersing hormone (Helfrich-Fôrster, 1995). This substance (β-PDH) belongs to the large pigment-dispersing factor (PDF) family of neuropeptides; in crustacea, α-PDH and β-PDH cause diurnal movements of pigment granules in retinal cells and their dispersion in epithelial chromatophores (Rao and Riehm, 1993).

In several insects, β-PDH-like immunoreactivity is found in a very limited number of neurons (Nässel et al., 1991; Homberg et al., 1991), and β-PDH has pharmacological properties consistent with a circadian role (Pyza and Meinertzhagen, 1996; Petri and Stengl, 1997). Helfrich-Fôrster et al. (1998) have hypothesized that this neuropeptide is a clock output signal. These studies raise several questions as to whether PDF is released, what behavioral roles it might have, and whether LN_v neurons release other transmitters in performing their pacemaker functions. Here, we have used genetics to address the functional roles of the PDF neuropeptide system and LN_v neurons. The results we present support the contention that the LN_v neurons are critical pacemaker neurons underlying daily locomotor rhythms. Furthermore, they refine the hypothesis that proPDF-derived neuropeptides are obligate circadian transmitters that help to organize behavior.

Results

Identification of a *Drosophila* Mutant for the *pdf* Neuropeptide Gene

LN_v neurons were first shown to express PDF-like immunoreactivity with an antibody to crustacean β-PDH. Figures 1A–1C demonstrate colocalization of β-PDH-like antigens with those detected by a new antibody to distinct sequences of the *Drosophila* proPDF precursor (anti-PAP; see the Experimental Procedures). These experiments ($n = 20$) confirmed that *pdf* gene products are found in three cell types (Figure 2B; J. H. P. and

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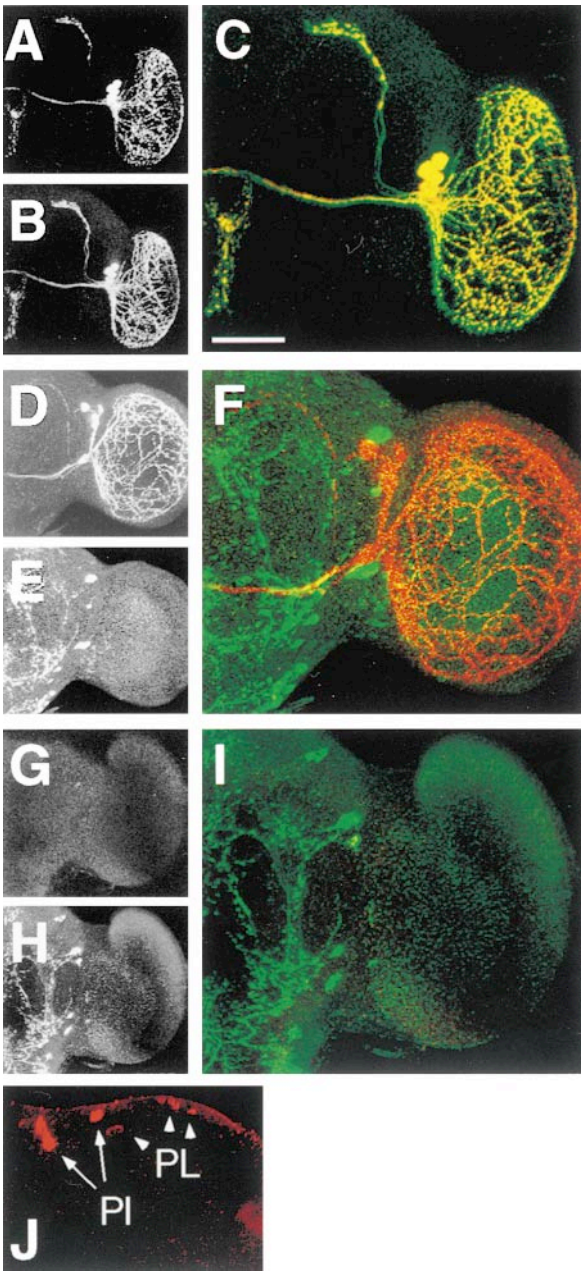

Figure 1. *Drosophila* Strains Lacking *pdf* Neuropeptide Expression

(A) through (I) depict ~150 μm confocal stacks. LN_v neurons coexpress anti-PAP ([A], also green in [C]) and anti-β-PDH ([B], also red in [C], generating yellow) immunoreactivities (n = 25). The cell bodies are marked by an arrowhead. W33 animals display wild-type PAP immunoreactivity in LN_v neurons ([D], also red in [F]) in relation to wild-type pro-dFMRFamide immunoreactivity ([E], also green in [F]) (n = 30). W15 animals do not display any PAP immunoreactivity ([G], also red in [I]) while maintaining wild-type pro-dFMRFamide immunoreactivity ([H], also green in [I]) (n = 30). (J) A 15 μm stack enhanced to show weak β-PDH immunosignals that persist in protocerebral cells of W15 animals (PI, arrow; PL, arrowhead). Scale bar, 50 μm for (C), (F), (I), and (J).

J. C. H., unpublished data). These include the LN_vs (Figures 1A–1C), two to four tritocerebral cells (PDF-Tri), and four to six abdominal cells (PDF-Ab).

We discovered *pdf*-null animals among laboratory stocks as a fortuitous consequence of studying ectopic P element reporter gene expression. The pWF6–84 stock (Schneider et al., 1993) contains a *dFMRF–lacZ* fusion gene that produces ectopic reporter expression in a pattern that includes the LN_v neurons. We mobilized pWF6–84 and found several lines that lacked all anti-PAP immunostaining and lacked β-PDH immunostaining in most β-PDH-positive cells. Figure 1 depicts results from two derivative lines: a PAP-positive line (W33; Figures 1D–1F) and a PAP-negative one (W15; Figures 1G–1I). The lack of PAP immunostaining was heritable and specific for the PDF transmitter system. W33 and W15 lines were both positive when stained with other anti-neuropeptide antibodies (e.g., proFMRF) (Figures 1E and 1H).

In W15 animals, β-PDH signals were absent in LN_v ($n > 100$), in PDF-Tri ($n = 10$, 1 day adults), and in PDF-Ab ($n = 20$) neurons. However, signals were retained in neurons of the pars intercerebralis (PI) and protocerebrum (PL) (Figure 1J). We found 0.4 ± 0.8 PL neurons per brain hemisphere in W33 flies ($n = 212$) and 0.9 ± 1.3 in W15 flies ($n = 194$). PI neurons were rarely seen but were present in both W33 ($n = 5$) and W15 ($n = 2$) flies. Neurons lacking β-PDH signals in W15 correspond to those that express *pdf* mRNA and PDF in wild-type (J. H. P. and J. C. H., unpublished data); this supports the hypothesis that PI and PL neurons do not express the *pdf* gene product but instead express cross-reacting PDF related–antigen(s).

The pWF6–84 transgene is inserted at 86B on chromosome 3. Several of the derivative lines contained small deletions at that insertion site, but there was no correlation between such molecular damage and the mutant phenotype (data not shown). We therefore considered the *pdf* locus itself within third chromosome region 97B (Park and Hall, 1998). Two deletions of that region each failed to complement the transmitter phenotype associated with W15 (Figure 3). We sequenced the *pdf* gene from W15 animals and found a nonsense mutation at prepro-PDF residue 21, converting a Tyr to a stop codon (Figure 2A). We refer to this mutation as pdf^{01}. The conceptual pdf^{01} precursor is consistent with the immunostaining phenotype because it is truncated before epitope positions assayed by anti-β-PDH, PDF, or PAP antibodies. With a PCR-based assay, we found the pdf^{01} mutation in each of the 22 derivative lines that displayed the phenotype (data not shown). The same assay revealed that pdf^{01} is present in various of our laboratory stocks at a range of frequencies: some are homozygous for the allele. In a survey of 38 $y\ w^{67c23}$ adults, the stock into which the pWF6–84 DNA was injected (Schneider et al., 1993), we found 9 flies homozygous and 20 heterozygous for pdf^{01}.

These genetic and molecular data suggest that disruption of the *pdf* gene is directly responsible for the immunostaining phenotype. To confirm this hypothesis, we transformed mutant animals with a P element containing wild-type *pdf* genomic sequences. We analyzed nine independent lines and confirmed by PCR that they contained both the pdf^{01} and pdf^{+} sequences (data not shown). Immunocytochemistry revealed a restoration of β-PDH immunoreactivity in LN_vs, as well as in PDF-Tri and PDF-Ab cells (Figure 2B). Seven lines mimicked the

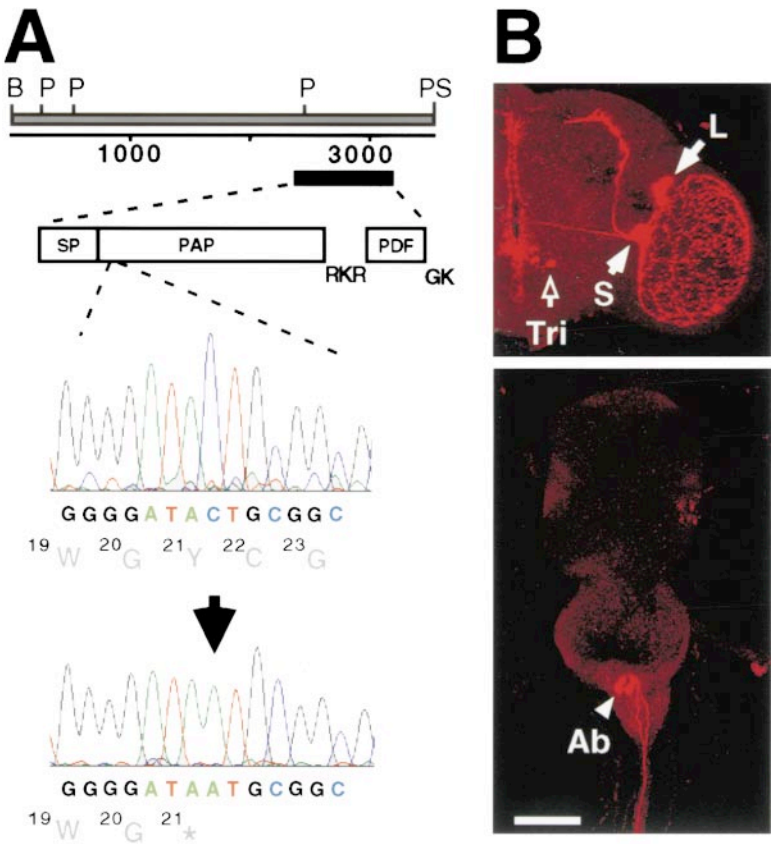

Figure 2. *pdf*^{01} DNA Sequence and Rescue of Its Immunostaining Phenotype

(A) The genomic map of the *pdf* locus shows the approximately 3.5 kb BamHI (B) to SalI (S) fragment used for genomic rescue and includes PstI (P) restriction sites. The black bar indicates the entire open reading frame, expanded below to show presumed processing sites (subscripts below ORF) for the signal peptide (SP), PDF-associated peptide (PAP), and the amidated PDF peptide (PDF). Electropherogram of the PAP/β-PDH-immunopositive stock W33, compared to that of the PAP/β-PDH immunonegative stock W15 (these flies are homozygous for *pdf*^{01}). The single base pair difference (C versus A) is indicated by an arrow, and the predicted mutation of tyrosine 21 to a stop codon is represented in the amino acid sequence.

(B) Confocal reconstruction of a day 1 adult brain from W15 mutant animal (β-PDH immunostaining). This animal is transgenic for an approximately 3.5 kb insertion that contains the entire wild-type *pdf* gene. Specific immunostaining is restored in the three cell types otherwise absent in W15 mutants: the small (s) and large (l) LN_vs (solid arrows), the PDF-Tri's (hollow arrow), and the PDF-Ab's (arrowhead). Scale bar, 100 mm.

normal PDF spatial pattern (n ≥ 6 specimens per line), and most displayed expression comparable to wild type. Two lines (Res F and Res X) displayed anomalous expression in protocerebral cells (n ≥ 6 cells; data not shown) in addition to the normal pattern. A second rescue design employed the GAL4 × UAS system. Assayed by PDF immunostaining, *pdf*-GAL4 (J. H. P. and J. C. H., unpublished data) drove UAS-*pdf* in LN_v neurons in *pdf*^{01} animals (n = 11). However, that staining was weaker than wild type (data not shown). These two rescue tests demonstrate that lack of PAP/β-PDH in the derivative lines is likely due to the *pdf*^{01} mutation.

The lack of peptide expression could result from either the absence of PDF neurons or merely a lack of peptide transmitter expression in otherwise normal neurons. To determine the state of *pdf* neurons in animals containing the transmitter mutation, we used *pdf*-GAL4 to produce β-galactosidase (β-gal) in flies that contained a single *pdf*$^{+}$ allele (Figures 3A–3C; n = 6) or that were hemizygous for *pdf*^{01}. In all *pdf*^{01}/deletion specimens (n = 5), the number and morphology of LN_v, PDF-Tri, and PDF-Ab neurons were normal (Figures 3D–3F).

The *pdf*-Null Mutation Disrupts Circadian Behavior

Over the course of 24 hr in light:dark (LD) cycles, wild-type flies are active at dawn, quieter at midday, then active again toward evening (e.g., Hamblen-Coyle et al., 1992). One feature of flies' "entrainment" to LD cycling is the anticipation of transitions between lights-on and -off (Figures 4A–4C). Rhythmic behavior persists when wild-type flies proceed from LD into constant darkness (Figures 4A–4C, 5A, 6A, and 6B).

We examined the locomotor activity rhythms of the *pdf*^{01} mutant. Homozygous and hemizygous *pdf*^{01} flies were well entrained during LD cycles (Figures 4D, 4G, 4J, 5B–5E, and 6C). However, *pdf*^{01} behavior in LD was not entirely normal. The evening activity peak was advanced by approximately 1 hr (compare Figure 4A to Figure 4D; see Table 1 for analysis of phase). Also, there was a lack of lights-on anticipation (compare Figure 4A to Figures 4D, 4G, and 4J).

Free-running behavior of *pdf*^{01} in constant darkness (DD) was severely abnormal and included several features that distinguish this mutation from others that disrupt circadian behavior. By periodogram analysis, flies homozygous and hemizygous for *pdf*^{01} (Figures 5B–5D) were much less rhythmic in DD as were *pdf*$^{+}$ controls (Figure 5A; Table 1). Of these mutants, 50%–98% exhibited no detectable rhythmicity for the duration of 9 DD days (Table 1). Actograms of *pdf*^{01} individuals suggested that most were rhythmic for 2 or 3 days in DD but later lost rhythmicity (Figures 5B–5E). Separate average activity histograms for DD days 1–2 and DD days 3–9 reveal the severity of the *pdf*^{01} phenotype (Figures 4E, 4H, and 4K versus Figuress 4F, 4I, and 4L). A higher proportion of mutant individuals was arrhythmic during DD days 3–9 than during DD days 1–9 (Table 1). The minority of *pdf*^{01} animals that maintained DD rhythmicity (Figures 5C and 5E) had free-running periods approximately 1 hr shorter (t ≈ 23 hr) than wild-type or *pdf*^{01}/+ heterozygous flies (Table 1).

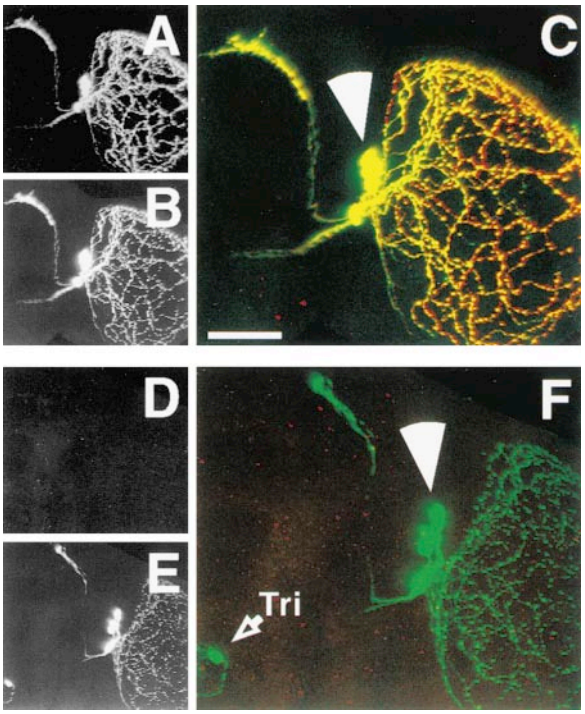

Figure 3. Morphology of *pdf* Neurons in the *pdf*^{01} Mutant

Flies hemizygous for *pdf*$^{+}$ or *pdf*^{01} were stained with antibodies to β-PDH and to *lacZ*. β-gal was produced by the combination of *pdf-GAL4 and UAS-lacZ* transgenes. In a *pdf*$^{+}$*/Df(3R)TI-X* hemizygous animal, β-PDH ([A] and red [C]) colocalizes with β-gal ([B] and green [C]) in LN_v neurons (arrowhead marks the cell bodies). In a *pdf*^{01}*/Df(3R)TI-X* hemizygote, LN_v neurons lack β-PDH staining ([D] and red [F]) but retain *lacZ* expression ([E] and green [F]) and have a normal appearance. Scale bar, 50 mm.

We used signal-to-noise analysis (SNR; Dowse and Ringo, 1987) to obtain measures that are linearly related to rhythm strength. (Table 1). SNR stems from Maximum Entropy Spectral Analysis (MESA) of the locomotor records: it provides single values for each fly's behavior and permits quantitation of weak rhythmicity, such as displayed by *pdf*^{01} flies (see Figure 5). The average of SNRs for *pdf*$^{+}$ flies (Table 1) depended on the background genotype and ranged from 1.7 for wild type (cf., Dowse and Ringo, 1987; Dushay et al., 1990) to 1.0 for *y w*. Both stocks displayed normal proportions of rhythmic individuals and periods (Table 1). The range of SNR values associated with the behavior of wild-type flies is very wide (0.4–4; Figure 7). In contrast, the distributions for the behaviorally arrhythmic *per*^{01} and *disco* mutants are skewed to the left (Figure 7B), as expected (Dowse and Ringo, 1987). The distributions of *per*^{01} SNRs for DD days 1–9 and those for the DD days 3–9 were congruent (Table 1). This reflects the fact that this *period* mutant is arrhythmic throughout the DD period (e.g., Wheeler at al., 1993). SNRs for *pdf*^{01} mutant animals were similar to those of *per*^{01}, even when computed for the entire DD 1–9 (Table 1). Hence, the residual rhythms displayed by *pdf*^{01} animals in DD were very weak.

Two strategies were used to rescue the *pdf*^{01} behavioral phenotype. First, we analyzed the activity of seven independent *pdf*^{01} lines that were transgenic for wild-type *pdf* genomic sequences (Figure 6; Table 2). Most showed a partial reversal of the *pdf*^{01} phase-advance LD phenotype (Figures 6E and 6G). The arrhythmicity *pdf*^{01} homozygotes and hemizygotes in DD days 3–9 was partially reversed by expression of the *pdf*$^{+}$ transgene in the majority (6/7) of lines examined (Figures 6F and 6H). The transgenic animals gave SNR values more similar to those of matched *pdf*$^{+}$ controls (W33), compared to values of *pdf*^{01} homozygotes tested in parallel (Table 2).

The second rescue design used the GAL4 × UAS system and tested the efficiency of this binary transgene approach in the context of a quantifiable behavioral phenotype. The two parental genotypes (*pdf*-GAL4*;pdf*^{01} and UAS-*pdf;pdf*^{01}) displayed aberrant rhythms (similar to *pdf*^{01}; Table 2). However, the majority of offspring from their cross was rhythmic by periodogram analysis ($t \approx 24$ hr). The rescue was only partial (Table 2): cross progeny exhibited stronger rhythms (SNR = 0.8) than did *pdf*^{01} (SNR = 0.3) but were less rhythmic than the parental types (*pdf*-GAL4 − SNR = 1.1; UAS-*pdf* − SNR = 1.5).

Transgenic Ablation of PDF Neurons

The *pdf*^{01} mutant phenotype retains several rhythmic features and so may be revealing the actions of other LN_v transmitters, or the actions of other (non-PDF) pacemaker neurons, or both. To address this issue, we effected cell ablation using *pdf*-GAL4 transgenic animals to express the cell death genes UAS-*rpr* or UAS-*hid*. Previously, McNabb et al. (1997) used this approach to study neurons expressing the neuropeptide eclosion hormone.

Larval and adult progeny from a control cross (*y w* × UAS-*rpr*) showed wild-type PDF immunostaining patterns (Figure 8A; Table 3). In contrast, no PDF-positive cell bodies were detected in the CNS of third instar larvae bearing *pdf*-GAL4 and UAS-*rpr* (Table 3). In some of these larval CNSs, however, a few residual processes in the dorsal brain were very faintly stained (data not shown). In adults bearing both transgenes, neither *s*-LN_v cell bodies nor dorsal processes were stained, whereas some *l*-LN_vs were stained (Table 3; Figure 8D). *p35* encodes a caspase inhibitor that can rescue *rpr*- or *hid*-mediated cell death (Clem et al., 1991; Zhou et al., 1997). When *p35* was coexpressed with *rpr*, most of the larval LNs (80%), adult *s*-LN_vs (70%), and *l*-LN_vs (80%) survived (Table 3). The PDF-Tri neurons (which normally cease to express *pdf* after adult days 1–2) were persistently PDF immunoreactive in *rpr*-rescued brains, up to adult day 10 (Table 3). This suggests that PDF-Tri cells normally die in young adults.

Ectopic expression of *hid* produced stronger effects than did *rpr* (Table 3). None of the normal PDF-positive cells was detected in larvae or adults that coexpressed *pdf-GAL4 and UAS-rpr* (Table 3). Moreover, coexpression of *p35* did not maintain PDF neurons as effectively as it did in experiments with *rpr*: we saw only one or two *s*-LN_vs (and rarely one *l*-LN_v) in about 60% of brains (Table 3). Also, *p35* did not lead to the anomalous persistence of PDF-Tri cells in *hid*-expressing animals (Table 3).

We evaluated the activity rhythms of flies with PDF cell ablations. Progenies from the control cross (*y w* × UAS-*rpr* or UAS-*hid*) exhibited normal behavior in both LD and DD (Figure 8B and 8C; Table 4). All cell-ablated flies entrained to LD cycles (Figure 8E; Table 4), but their

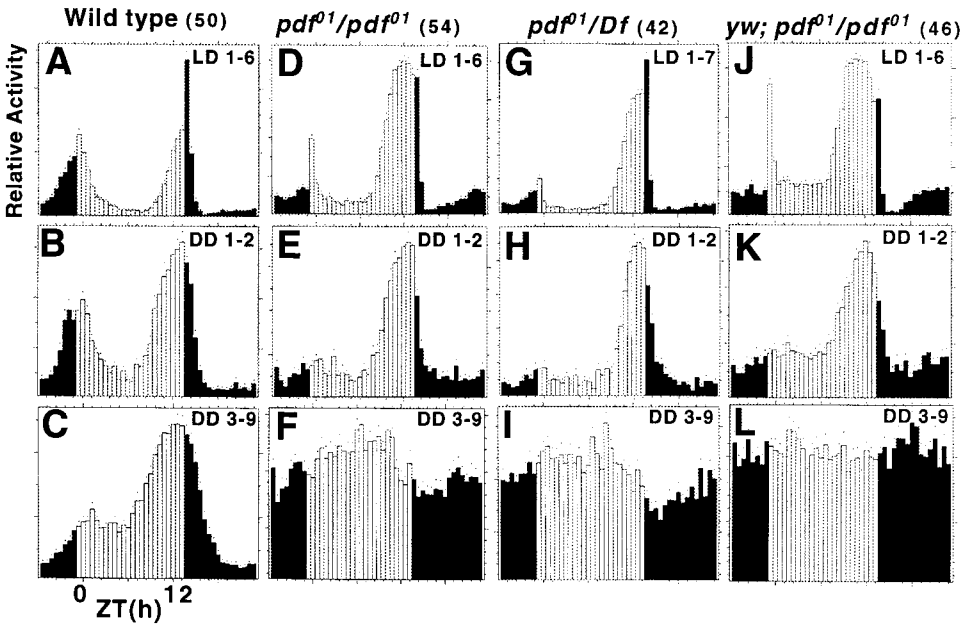

Figure 4. Locomotor Activity of Normal Flies and the *pdf*^{01} Mutant

Average activity histograms indicating relative levels of locomotion. Open and black bars designate the day and night phases in LD, respectively (Hamblen-Coyle et al., 1989, 1992). n, number of flies tested. For the constant dark (DD) plots (second two rows), open bars designate the subjective day. Dots indicate SEM values for that time bin, with reference to the average level of activity per fly. (A, B, and C), Canton-S wild type; (D, E, and F), *pdf*^{01} mutant in an otherwise normal genetic background; (G, H, and I), flies heterozygous for *pdf*^{01} and a deletion of the locus [a mixture of *pdf*^{01}/*Df(3R)Tl-X* and *pdf*^{01}/*Df(3R)Tl-84*^{cRPA} hemizygotes]; (J, K, and L), *pdf*^{01} mutant in a *y w* background (see Table 1 for behavior of the *y w* control). (A, D, G, and J), 6 days worth of LD behavior; (B, E, H, and K), behavior during DD 1–2; (C, F, I, and L), behavior during 3–9.

evening locomotor phases were advanced by at least 0.5 hr (Table 4). Analysis of DD behavior showed that 63% of *rpr*-ablated flies were rhythmic for the entire period, whereas only 17% of *hid*-ablated ones sustained such rhythmicity (Table 4). As in the case of *pdf*^{01}, separate periodogram analysis of DD days 3–9 revealed decreased proportions of rhythmic individuals in cell-ablated flies (Table 4). The subnormal SNR values

Table 1. Locomotor Behavior Affected by the *pdf*^{01} Mutation Compared to Controls

		DD 1-9				DD 3-9			LD Phases (ZT)	
Genotype	n	n Rhythmic (%)	τ (h)	SNR	Events/ Bin	n Rhythmic (%)	τ (h)	Events/Bin	Morning	Evening
Wild type	50	50 (100)	24.2 ± 0.1	1.73 ± 0.16	19 ± 1	48 (96)	24.3 ± 0.1	2.11 ± 0.21	0 ± 0.1	11.5 ± 0.1
y w	45	41 (91)	23.8 ± 0.1	0.97 ± 0.09	18 ± 1	42 (93)	23.7 ± 0.1	1.11 ± 0.1	0.1 ± 0.2	10.9 ± 0.1
w^{+}; *pdf*^{01}	54	13 (24)	22.9 ± 0.2	0.30 ± 0.02	10 ± 0	6 (11)	23.3 ± 0.3	0.32 ± 0.02	0.1 ± 0.2	10.4 ± 0.1
y w; pdf^{01}	46	1 (2)	21.0	0.24 ± 0.01	16 ± 1	0 (0)	NA	0.28 ± 0.02	0.8 ± 0.2	9.9 ± 0.1
w^{+}; *pdf*^{01}/+	20	18 (90)	24 ± 0.1	1.38 ± 0.19	16 ± 1	18 (90)	23.9 ± 0.1	1.48 ± 0.22	23.8 ± 0.2	11.8 ± 0.1
pdf^{01}/*Df(3R)Tl-X*	22	11 (50)	23.2 ± 0.2	0.44 ± 0.05	12 ± 1	5 (23)	22.8 ± 0.5	0.4 ± 0.04	23.6 ± 0.2	11.2 ± 0.1
pdf^{01}/*Df(3R)Tl-84*^{cRPA}	19	9 (47)	22.9 ± 0.2	0.4 ± 0.06	9 ± 1	4 (21)	22.9 ± 0.3	0.37 ± 0.05	23.5 ± 0.1	10.8 ± 0.1
w disco^{1}*f*	15	2 (13)	22.5 / 22.9	0.53 ± 0.06	29 ± 1	0 (0)	NA	0.5 ± 0.06	1.1 ± 0.3	11 ± 0.3
y per^{01} *w*	15	0/15 (0)	NA	0.37 ± 0.06	31 ± 2	0 (0)	NA	0.44 ± 0.06	ND	ND

The genotypes of mutant, normal, and transgenic flies tested are listed at left; see text for details of relevant mutations and deletions (*Df*); *pdf*$^{+}$-bearing third chromosomes were from a Canton-S wild-type or a *y w* strain. n, number of flies. The analysis of free-running behavior covers both the entire 9 days (DD 1–9) and just the last 7 (DD 3–9). Free-running period (τ): means (±SEM) resulting from χ-square periodogram analyses (see also Figure 5). NA, not applicable due to complete arrhythmicity. By ANOVA, the period differences among wild type, *y w*, and *pdf*^{01} were highly significant ($p < 0.0001$). Subsequent pairwise comparisons showed wild type and *y w* each to have longer periods compared to *pdf*^{01} ($p < 0.001$). Wild-type and *y w* periods were marginally different from each other ($p < 0.05$). SNR, signal-to-noise ratios (mean ± SEM). Numbers of locomotor events are means of means (± SEM): average events/30 min/fly, averaged for all individuals of a given genotype. These values indicate *pdf* mutant animals are no less active than are wild-type controls. LD phases, times of peak activity during LD; mean peak times (± SEM) are given as ZT values (ZT0 = time of D-to-L transition; ZT12 = time of L-to-D transition). ND, not determined; these flies do not anticipate L/D transitions and are DD arrhythmic (Wheeler et al., 1993). Evening peak values from *pdf*$^{+}$, from *y w*, and from *y w; pdf*^{01} were significantly different by ANOVA ($p = 0.0000$). The significance for pairwise comparisons is as follows: $p < 0.001$ for wild type and *pdf*^{01}, $p < 0.001$ for *y w* and *y w; pdf*^{01}, and $p < 0.05$ for wild-type and *y w*.

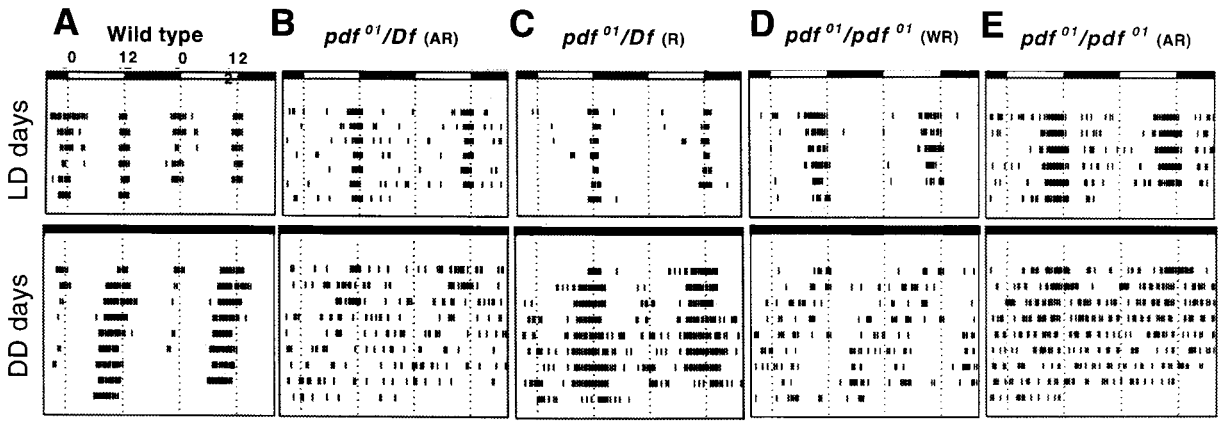

Figure 5. Representative Actograms of Flies Bearing Various *pdf* Genotypes

The *Df* indicated is a deletion of the *pdf* locus. Horizontal bars: open, day; closed, night (in an LD cycle, or the subjective night in DD); gray = subjective day in DD. The records are double plotted: top line, days 1–2; next line, days 2–3; etc. Each tick represents an activity greater than 25 events per 30 min bin. AR, arrhythmic; R, rhythmic by periodogram analysis. For DD days 1–9, the rhythmic wild type (A) displayed power of 195 and width of 5 (actograms with strong rhythmic behavior have power of >100 and width of 5–7); the SNR value for this wild-type record was 3 (DD days 1–9). Power and width values for (B) were 1 and 1 (SNR, 0.2); for (C), 100 and 5 (SNR, 0.9); for (D), 17 and 3 (SNR, 0.3); and for (E), 1 and 1 (SNR, 0.2).

computed for both the *rpr*- and *hid*-ablated flies (Table 4; Figure 7) are consistent with their abnormal free-running behavior. Finally, the *rpr* and *hid* ablation individuals that were persistently rhythmic in DD tended to manifest short periods (t ≈ 22–23 hr).

Despite having a slightly lower number of PDF cells (Table 3), animals that coexpressed *rpr* and *p35* displayed essentially normal behavior (Figures 8H and 8I; Table 4). The free-running period of the rescued group was approximately 0.5 hr longer than control values (Table 4). In contrast to animals that coexpressed *p35* and *rpr*, only about 70% animals that coexpressed *p35* and *hid* were rhythmic in DD, and the SNRs for this group are intermediate between those for controls and for *hid* ablation (Table 4). This incomplete behavioral rescue paralleled the histological findings (Table 3).

Discussion

Behavioral Phenotypes of *Drosophila* Lacking LN_v Pacemakers or *pdf*

The most severe phenotype displayed by pdf^{01} and by PDF cell-ablated animals is that the majority is arrhythmic in constant darkness. Both sets of animals were rhythmic over the first 1–2 days of constant conditions. Their locomotor patterns became arrhythmic gradually over a 9 day period. We conclude that this circadian behavior is largely independent of *pdf* and LN_v neurons

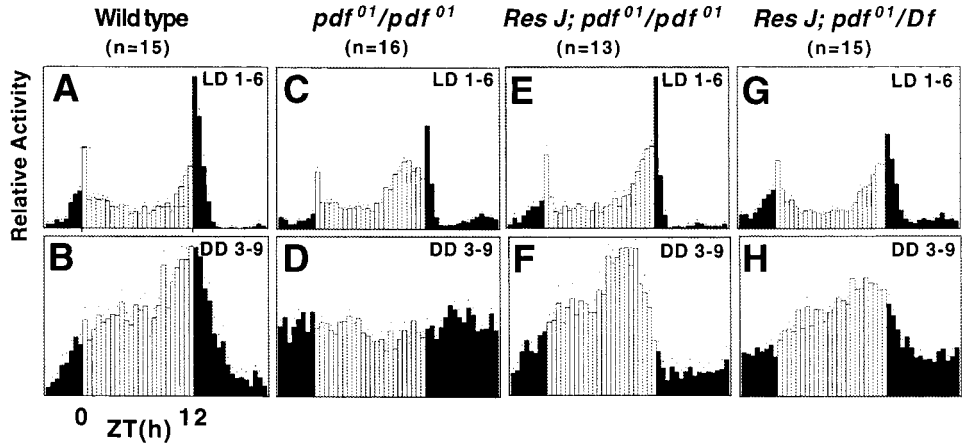

Figure 6. Locomotor Activity of *pdf* Mutant Flies Transgenic for pdf^{+} Sequences

Average activity histograms for groups of flies, plotted as described in Figure 4. Res J is one of six different *pdf* (genomic) transgenic lines that were analyzed in the homozygous pdf^{01} background (E and F) and in the hemizygous $pdf^{01}/Df(3R)TI\text{-}X$ background (G and H). Results from Res J animals are representative of the behavior observed with the different Res lines that carry the 3.5 kb of pdf^{+} DNA at other genomic locations (see Table 2).

Table 2. Rescue of *pdf*^{01}-Affected Locomotor Behavior by *pdf*$^{+}$-Containing Transgenes

		DD 1-9				DD 3-9			LD Phases (ZT)	
Genotype	n	n Rhythmic (%)	τ (h)	SNR	Events/ Bin	n Rhythmic (%)	τ (h)	SNR	Morning	Evening
Genomic Rescue										
Res B; *pdf*^{01}	16	12 (75)	23.6 ± 0.1	0.57 ± 0.09	13 ± 1	12 (75)	24.8 ± 0.9	0.58 ± 0.09	0.2 ± 0.2	10.7 ± 0.3
Res B; *pdf*^{01}/*Df(3R)Tl-X*	15	13 (87)	23.9 ± 0.2	0.64 ± 0.14	20 ± 1	11 (73)	23.9 ± 0.2	0.59 ± 0.06	23.6 ± 0.2	11.5 ± 0.2
Res J; *pdf*^{01}	13	13 (100)	23.8 ± 0.1	0.65 ± 0.09	12 ± 1	10 (77)	23.7 ± 0.1	0.52 ± 0.07	0.4 ± 0.1	10.8 ± 0.3
Res J; *pdf*^{01}/*Df(3R)Tl-X*	15	13 (87)	24.4 ± 0.4	0.59 ± 0.1	18 ± 2	10 (66)	23.9 ± 0.2	0.52 ± 0.10	0.2 ± 0.2	11.4 ± 0.2
y w;; pdf^{+}	16	15 (94)	24.2 ± 0.1	0.57 ± 0.09	16 ± 1	14 (88)	24 ± 0.1	0.48 ± 0.06	0.6 ± 0.2	11.9 ± 0.2
y w;; pdf^{01}	16	10 (63)	22.6 ± 0.7	0.30 ± 0.03	13 ± 1	0 (0)	NA	0.34 ± 0.04	0.9 ± 0.2	10.5 ± 0.1
GAL4 × UAS Rescue										
UAS-*pdf*	16	15 (94)	24.1 ± 0.1	1.1 ± 0.09	20 ± 2	15 (94)	24.1 ± 0.1	1.15 ± 0.11	0.8 ± 0.1	11.6 ± 0.2
UAS-*pdf; pdf*^{01}	21	5 (24)	21.5 ± 0.4	0.28 ± 0.02	14 ± 1	1 (5)	22.5	0.32 ± 0.02	0.1 ± 0.2	10.6 ± 0.1
pdf-GAL4	16	16 (100)	24.6 ± 0.1	1.5 ± 0.15	16 ± 1	16 (100)	24.8 ± 0.1	1.52 ± 0.2	0.5 ± 0.1	12 ± 0.1
pdf-GAL4; *pdf*^{01}	17	0 (0)	NA	0.26 ± 0.01	18 ± 2	0 (0)	NA	0.33 ± 0.03	0.6 ± 0.2	10.5 ± 0.2
UAS-*pdf*/*pdf*-GAL4; *pdf*^{01}	32	31 (97)	23.8 ± 0.1	0.77 ± 0.11	19 ± 1	26 (81)	23.7 ± 0.1	0.64 ± 0.09	23.7 ± 0.2	10.9 ± 0.1

The format for this table is similar to that for Table 1. Genomic rescue: analysis of *pdf*^{01} animals transformed with a genomic fragment bearing the entire *pdf* locus. Res B and Res J: independent lines both homozygous for the transgene. Figure 6 shows examples of behavior in line Res J. In four additional lines tested, the percent of rhythmic individuals (in homozygous *pdf*^{01} or *pdf*^{01}/*Df* genetic backgrounds) ranged from 46%–100% during DD days 1–9 and from 31%–93% for DD 3–9. For DD1–9, average SNRs among the seven lines ranged from 0.4 to 1.1. GAL4 × UAS rescue: analysis of activity in animals bearing *pdf*-**GAL4** and *UAS-pdf* transgenes, along with single-transgene controls, all in a *pdf*^{01} background.

during LD and short term DD but reveals such requirements during sustained constant conditions.

What features of LN_v neurons and *pdf* signaling could explain this phenotype? In cockroaches, injection of β-PDH into the brain caused phase delays in daily locomotor activity, consistent with a role for the peptide in a nonphotic clock input (Petri and Stengl, 1997). The morphology of *l*-LN_v neurons suggests a basis for how secreted *pdf* gene products could access the pacemaker neurons. As previously described, a subset of *l*-LN_v cells projects axons across the midline to the area containing the contralateral LN_v cell bodies. Therefore, rhythmic *l*-LN_v release of PDF could produce a phase delay in pacemakers of the opposite side and, thus, contribute to bilateral synchrony.

This scenario predicts that in *Drosophila* that are mutant for *pdf*, the circadian clock will operate with advanced phase in LD and run more quickly in DD. Those were our behavioral observations for *pdf*-null animals. The deterioration of free-running rhythmicity over DD days 1–3 may therefore reflect a gradual loss of synchronization between bilateral pacemaker centers. The *disco* mutant displays a progressive damping of rhythmicity and also lacks LN_v neurons (Wheeler et al., 1993; Helfrich-Fôrster, 1998). Likewise, the ablation of the pineal gland produces an analogous behavior in sparrows: when transferred from LD cycling to DD conditions, operated animals display a progressive loss of behavioral rhythmicity (Menaker and Zimmerman, 1976). This effect was shown to derive from lack of melatonin, which normally helps to maintain a mutual synchronization between the pineal and other pacemaker structures (Cassone and Menaker, 1984).

The dorsally projecting *s*-LN_v cells may have a greater role in regulating circadian locomotor rhythms than the *l*-LN_vs. Our cell ablation studies are consistent with the proposition that a single LN_v is competent to organize behavioral rhythmicity. This same conclusion was reached in an analysis of *disco* mutants (Helfrich-Fôrster, 1998). Likewise, the circadian regulators *Clock* and *cycle* regulate *pdf* expression in *s*-LN_vs, but not in *l*-LN_vs (J. H. P., C. Helfrich-Fôrster, et al., unpublished data). That result argues that neuropeptide expression by *s*-LNs neurons is especially important for the circadian behavioral

Table 3. PDF Immunocytochemistry Following PDF Cell Ablation

	Larva	Adult		
	LN (n)	*s*-LN_v (n)	*l*-LN_v (n)	PDF-Tri (n*)
y w × UAS-*rpr*	4 ± 0 (18)	3.5 ± 0.1 (13)	3.9 ± 0.1 (13)	0 (7)
pdf-**GAL4** × UAS-*rpr*	0 (82)	0 (34)	0.5 ± 0.1 (34)	0 (17)
pdf-**GAL4**, UAS-*p35* × UAS-*rpr*	3.2 ± 0.1 (50)	2.8 ± 0.2 (25)	3.2 ± 0.2 (25)	1.5 ± 0.2 (13)
y w × UAS-*hid*	NA	3.1 ± 0.2 (16)	3.6 ± 0.1 (16)	0 (8)
pdf-**GAL4** × UAS-*hid*	NA	0 (31)	0 (31)	0 (16)
pdf-**GAL4**, UAS-*p35* × UAS-*hid*	NA	0.4 ± 0.1 (36)	0.1 ± 0.0 (36)	0 (14)

Averages of PDF-immunoreactive cell counts (± SEM). n, numbers of brain hemispheres; n*, numbers of brains. NA, not available due to homozygous lethality of the transgene.

Table 4. Effects of Ablating PDF Neurons on Locomotor Behavior

		DD 1-9				DD 3-9			LD Phases (ZT)	
Genotype	n	n Rhythmic (%)	τ (h)	SNR	Events/ Bin	n Rhythmic (%)	τ (h)	SNR	Morning	Evening
y w × UAS-*rpr*	31	30 (97)	24.0 ± 0.1	1.22 ± 0.12	17 ± 1	30 (97)	23.9 ± 0.1	1.51 ± 0.17	0.2 ± 0.1	11.9 ± 0.1
pdf-GAL4 × UAS-*rpr*	32	20 (63)	23.3 ± 0.1	0.49 ± 0.05	19 ± 1	15 (47)	22.9 ± 0.1	0.47 ± 0.05	0.5 ± 0.1	11.4 ± 0.1
pdf-GAL4, UAS-*p35* × UAS-*rpr*	32	32 (100)	24.5 ± 0.1	1.16 ± 0.1	21 ± 1	32 (100)	24.5 ± 0.1	1.35 ± 0.12	0.4 ± 0.1	12 ± 0.1
y w × UAS-*hid*	11	11 (100)	25.1 ± 1.1	1.39 ± 0.25	20 ± 2	10 (91)	24.0 ± 0.1	1.73 ± 0.32	0.8 ± 0.1	12.1 ± 0.1
pdf-GAL4 × UAS-*hid*	24	4 (17)	21.9 ± 0.5	0.37 ± 0.04	13 ± 1	3 (13)	22.3 ± 0.2	0.38 ± 0.04	0.8 ± 0.1	11.5 ± 0.1
pdf-GAL4, UAS-*p35* × UAS-*hid*	24	17 (71)	24.0 ± 0.1	0.73 ± 0.1	14 ± 1	16 (67)	23.9 ± 0.2	0.80 ± 0.14	0.9 ± 0.1	12 ± 0.1

The format of this table is comparable to that of Table 1. Comparisons of free-running period values among the singly (control), doubly (ablated), and triply (rescued) transgenic types showed the three *rpr*-related cases to be different by ANOVA ($p < 0.0001$). Subsequent pairwise comparisons showed UAS-*rpr* and rescued each to have longer period compared to *pdf*-GAL4 × UAS-*rpr* (both p's < 0.001). UAS-*rpr* and rescued were also different ($p < 0.01$). For the periods of *hid*-related transgenics, the three types were different by ANOVA ($p = 0.0015$). Pairwise comparisons showed UAS-*hid* and rescued each to be longer than *pdf*-GAL4 × UAS-*hid* (both p's < 0.01). UAS-*hid* and rescued were not significantly different ($p > 0.05$). Comparisons of the evening phases (in LD) showed significant differences. For the three *rpr*-related types, they were different by ANOVA ($p < 0.001$). Pairwise comparisons showed UAS-*rpr* and rescued each to have later phases compared to *pdf*-GAL4 × UAS-*rpr* (both p's < 0.01). In contrast, the UAS-*rpr* control was not different from rescued ($p > 0.05$). Values for the three *hid* types were different by ANOVA ($p < 0.0009$). Pairwise comparisons indicated that UAS-*hid* and rescued were later than *pdf*-GAL4 × UAS-*hid* (both p's < 0.01). In contrast, UAS-*hid* and rescued were not significantly different ($p > 0.05$).

regulation we have inferred from analysis of pdf^{01} animals.

While the current results strongly support the hypothesis that LN_vs are critical circadian pacemaker neurons, analyses of both pdf^{01} and PDF-ablated flies revealed minorities of animals that maintain weak rhythmicity in DD. These low proportions of rhythmic individuals suggest the involvement of secondary pacemaker neurons involved in the circadian regulation of behavior. We do not know their cellular identities, but on the assumption that they will express the clock genes *period* and *timeless*, we propose three specific candidates. The first is a fifth *per*-positive, *pdf*-negative LN_v neuron; Kaneko et al. (1997) found a cell with such properties in larvae and hypothesized it may also exist in adults; if so, we presume its activities were not affected in the mutants we studied. The second candidate cell type is represented by the dorsolateral LN_d cluster of neurons (Kaneko, 1998). The third plausible candidate cell type is represented by the Dorsal Neurons (DNs) of posterior–medial brain regions (Kaneko, 1998). The second and third candidate cell types are both *pdf* negative (Helfrich-Fôrster, 1995). Free-running rhythms are more severely disrupted in *disco* flies than in pdf^{01} flies (Dushay et al., 1989); we propose this is because *disco* flies lack almost all *per*-positive LN neurons, not just the ventral LN group.

The pdf^{01} and neuron-ablated animals entrain to a 24 hr light:dark cycle and show considerable rhythmicity. This feature is noticeably different from other clock mutants, which are solely driven by photoperiod (Wheeler et al., 1993). This difference suggests the clock is still running in pdf^{01} and cell-ablated flies and that PDF is therefore not a central component of the clock mechanism. However, both pdf^{01} and cell-ablated flies display phase-advanced evening activity peaks in LD, and if rhythmic in DD, they display a short free-running period. We propose that the same physiological mechanism underlies both of these phenotypes. Both features are also displayed by per^{Clk} (Dushay et al., 1990) and *norpA* mutants (Hamblen-Coyle et al., 1992). As discussed by Hamblen-Coyle et al. (1992), light resets these fast-paced clocks by about 1 hr per day to 24 hr. Hence, pdf^{01}, like per^{Clk} and *norpA* mutants, produce fast-paced clock movements in LD and DD. However, pdf^{01} variants have additional phenotypes: they fail to anticipate a lights-on transition in LD and are largely arrhythmic in DD. This suggests that a fast clock is not the only or appropriate explanation for all phenotypes associated with the pdf^{01}-mutated and cell-ablated flies.

The PDF Neuropeptide as a Circadian Signal in *Drosophila*

The predicted *pdf* gene product is a neuropeptide precursor, pro-PDF, which we presume is processed to two or more final peptide products that include the PAP and amidated 18–amino acid PDF molecules (Nässel et al., 1993). The pharmacological activities of injected β-PDH peptide in other insects (Pyza and Meinertzhagen, 1996; Petri and Stengl, 1997) are consistent with the hypothesis that it represents a secreted agent. While the results of this study suggest that PDF is the principal circadian messenger in *Drosophila*, certain details remain ambiguous and will require further study. Two sets of results warrant comment. First, we demonstrated a role for PDF neurons but did not implicate LN_v neurons exclusively. The lack of transmitter in PDF-Tri and PDF-Ab neurons, or their genetic ablation, may have contributed to the phenotypic defects. We consider this unlikely, as neither cell type expresses clock genes (Kaneko, 1998). Furthermore, PDF-Tri cells normally undergo apoptosis before the stage when we measured locomotion. Second, our experiments do not tell us when the lesions we studied have their effects: lack of transmitter or lack of *pdf* neurons at an early, preadult stage may covertly affect behavioral periodicity, as well as having later physiological effects. The normal morphology of LN_v neurons in mutant animals argues against this possibility, and PDF

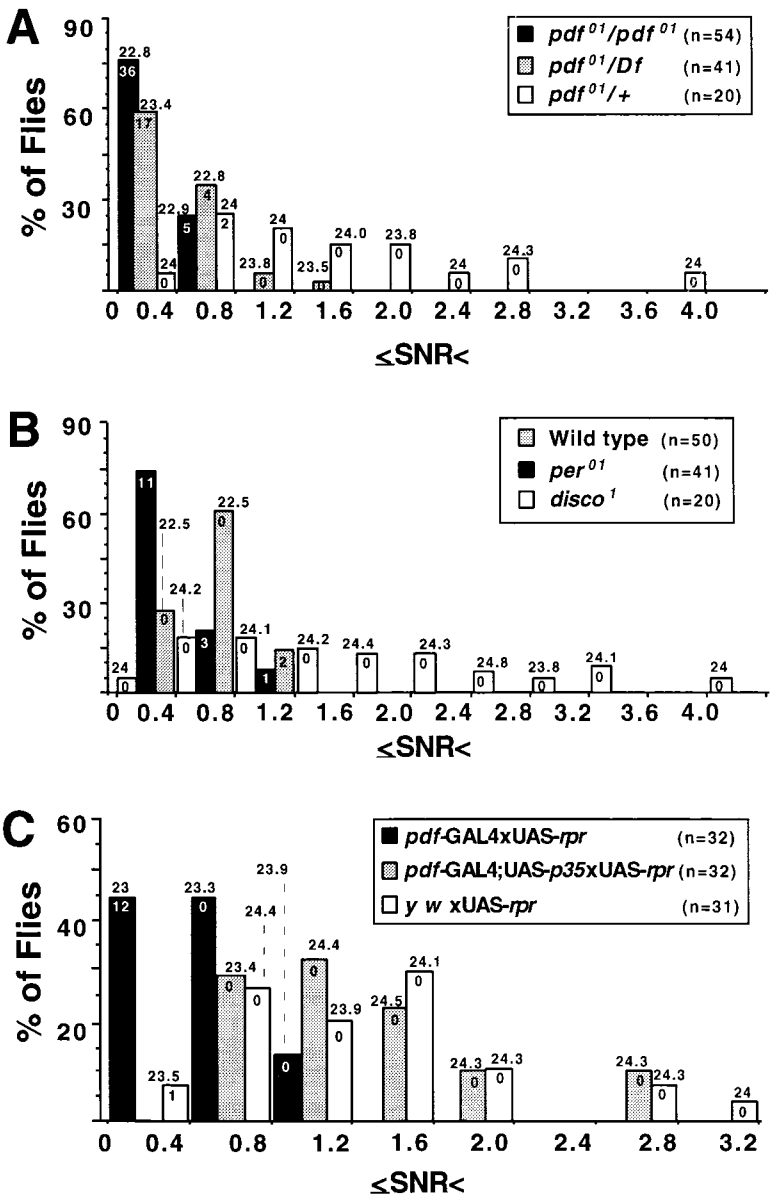

Figure 7. Numerical Indices of Varying Behavioral Rhythm Strengths

Signal-to-noise ratios (SNRs) for the entirety of the free-running records (DD days 1–9; see also Table 1). SNRs values were divided into increments of 0.4; the ordinate values are the percent of total flies whose SNR falls within that interval. The number of flies scored arrhythmic by periodogram analysis are within histogram bars. For the rhythmic individuals, the free-running periods indicated (means; SEMs all $\leq$ 0.3 hr) stemmed from Maximum Entropy Spectral Analyses and are independent of those in Table 1. The three panels contain SNR distributions for (A) *pdf*^{01} homozygous, *pdf*^{01}/+ heterozygous, and *pdf*^{01}/*Df* hemizygous flies [a mix of *Df(3R)TI-X* and *Df(3R)TI-84*^{cRPA} deletions]; (B) wild-type, *per*^{01} (*period*-null mutant), and *disco*^{1} flies, which are positive and negative controls for locomotor activity rhythmicity; and (C) *rpr*-ablated, control, and ablated but *p35*-rescued transgenic types.

neuropeptides have not previously been implicated in developmental functions. However, future experiments employing conditional manipulations will be necessary to evaluate this possibility.

The role of *pdf* in the *Drosophila* circadian system is notable—a neuropeptide essential for its output. In rodents, the *vasopressin* neuropeptide gene is rhythmically expressed in SCN under the influence of the *Clock* gene (Jin et al., 1999). However, a functional role for vasopressin in SCN regulation of locomotor behavior has not been defined (Boer et al., 1999). Several neuropeptides can reset the phase of daily rhythms (e.g., Harrington et al., 1999), but their effects appear to mimic natural inputs to circadian cycling, not its output. NE is released from sympathetic nerve terminals in a circadian manner (Cassone and Menaker, 1983), but these neurons represent a distant, polysynaptic target of the neuronal output emanating from SCN (Li et al., 1998). To date, PDF-related peptides have been found only in arthropods (Rao and Riehm, 1993) and mollusks (Elekes and Nässel, 1999). Whether related PDF-like peptides have analogous circadian functions in vertebrates, or whether a nonrelated transmitter has such functions, remains to be determined.

Experimental Procedures

Immunostaining

The anti-PAP antibody was made by injection of a BSA-conjugated synthetic peptide (H-YPLILENSLGPSVPI-OH; Multiple Peptide Systems, San Diego, CA; corresponding to amino acids 65–79 of preproPDF [Park and Hall, 1998]) into each of two guinea pigs (Covance Co.). Whole-mount immunocytochemistry was performed as previously described (Benveniste and Taghert, 1999), with a modified fixative (4% paraformaldehyde/7% picric acid in 0.1 M phosphate buffer [pH 7.4]). Rabbit anti-β-PDH (1:5000; Dirckson et al., 1987), mouse anti-β-galactosidase (Promega; 1:2000), and anti-PAP

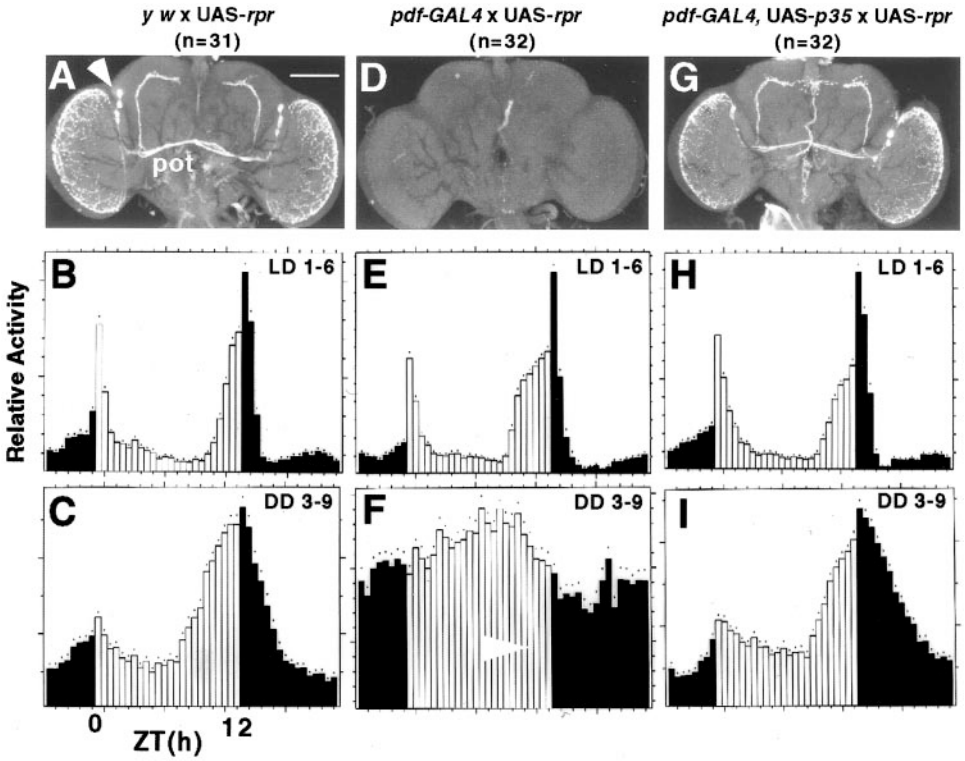

Figure 8. PDF Immunoreactivity and Behavior of Cell Ablation Animals

(A, D, and G) Adult CNSs from the progeny of crosses indicated at the tops of the panels were immunostained for PDF. LN_v cell bodies are indicated by an arrowhead (see also Table 3). pot, posterior optic tract. (A) Brains in control flies. (D) Staining of doubly transgenic progeny: notice the absence of PDF-immunoreactive cells. (G) Maintenance of PDF-immunoreactive cell bodies and their axonal processes *p35*. Scale bar, 100 μm.

(B, E, and H). Locomotor behavior in LD, plotted as in Figure 4; n, number of animals in behavioral analyses.

(C, F, and I) Behavior of the same ablated and control flies in DD. Flies ablated of PDF LN_v neurons were well entrained to LD cycles ([E], compare to [B]), as were those coexpressing the *p35* transgene (H). Cell-ablated animals displayed poor free running rhythmicity (F). Animals containing only the *UAS–rpr* fusion (C), or that coexpressed *rpr* and *p35* (I), produced more normal behavior. See also Tables 3 and 4.

(1:2000) were visualized with Cy3-conjugated (1:500) or FITC-conjugated (1:200) secondary antibodies (Jackson Labs). Double-antibody experiments were performed with sequential antibody application to minimize cross-reactivities. Tissues were examined using a Zeiss microscope or using an Olympus confocal microscope with Fluoview software. Constant PMT voltage sensitivity was maintained between scans for preparations requiring direct comparison. Use of interference filters and manipulation of the relative powers of the lasers were employed to minimize bleed-through. Images were assembled in Photoshop. For whole-mount stains using anti-β-PDH and anti-fly-PDF, third instar larval or 10 day adult tissues were processed essentially as described by Helfrich-Fôrster (1997) and will be described in full by J. H. P. et al. (unpublished data).

PCR Screen for *pdf*^{01}

Sequence analysis of the *pdf* gene used four primers (bp −177 to −160, ACACCGATACTGACGCTC; bp +19 to +36, TTGTCGCC CTTGTGCTTC; bp +459 to +442, CTCTATTAGATGACTACA; bp +691 to +674, TGGCTTTCATTGGTTCGC). A PCR screen was designed to identify the *pdf*^{01} C-to-A transition. Using a primer that includes a single-base mismatch (bp +84 to +63, CCGGCATGGC CATGGCGCTGCA) and another primer (bp −121 to −110, CAGTGG GTTATCCAGTCCAGGT), the PCR product from wild-type DNA incorporated a PstI site into a 205 bp product, while *pdf*^{01} genomic DNA did not. These products were amplified by 30 cycles of 96°C, 15 s; 65°C, 15 s; 72°C, 1 min, using a final [$MgCl_2$] of 2.0 mM, PstI restricted, and gel analyzed.

Drosophila Strains and Genetic Crosses

Flies were maintained on a cornmeal, agar, molasses, yeast, Tegosept medium at 25°C, in 12 hr:12 hr LD cycles. Details concerning *pdf*^{01}-bearing lines are described in the first section of the Results. Other basic strains applied were Canton-S wild type, *yellow white* and *y w*^{67c23} mutant markers, a *period* gene null mutant (*per*^{01}, linked to the *y* and *w* markers) or a *disconnected* mutant (*disco*^{1}, linked to the *w* and *forked* markers). Three deletions [*Df(3R)TI-X* (97B;97D1–2), *Df(3R)TI-P* (97A;98A1–2), and *Df(3R)TI-84*^{RPA} (97A9–10;97D2)] were balanced by *In(3LR)TM3, Sb* or *In(3LR)TM3, Ser*, and used to generate *pdf* hemizygous mutants by crossing to *pdf*^{01} homozygotes.

Constructs for Rescue and Ablation

The genomic rescue construct contained a 3530 bp fragment of the *pdf* locus (see Figure 2) cloned into the BamHI and XhoI sites of pW8 (Klemenz et al., 1987). Relative to the translation–initiation site, the first bp of the fragment is −2503 and the last bp is +927. The DNA at 0.5 mg/ml was injected into embryos of the stock pWF6–84 W15, along with helper plasmid Pπ25.7^{wc} (at 0.2 mg/ml). Transformants were backcrossed to pWF6–84 W15. Stocks were analyzed for insert copy number by Southern Blot and for presence of the *pdf*^{01} allele by PCR as described above. The *pdf*–GAL4 construct fused 2.4 kb of 5′-flanking *pdf* DNA to yeast GAL4 (J. H. P. and J. C. H., unpublished data); the 5′ end of this construct and the approximately 3.5 kb genomic fragment (above) are the same. The UAS-*pdf* construct was generated by PCR amplifying the *pdf* open reading frame; forward (5′-GCGAATTCATGGCTCGCTACACGTAC-3′) and reverse (5′-CGCGGATCCTTCTTCAGCATTTTCCGGGC-3′) primers were applied, using the entire *pdf* cDNA as a template (see Park and Hall,

1998, for details of the PCR conditions). The PCR fragment was digested with EcoRI–BamHI and subcloned into pBluescript, from which the EcoRI–XbaI insert was transferred to pUAST (Brand and Perrimon, 1993) and used for transformation as will be described by J. H. P., C. Helfrich-Fôrster, G. Lee, L. Liu, M. R., and J. C. H. Of three independent UAS-*pdf* lines, a second chromosome insert was used in the present work.

For cell ablation experiments, a *pdf*-GAL4 line was crossed to UAS-*reaper* (*rpr*) (third chromosome insertion) and to UAS-*head-involution-defective* (*hid*)/*In(2LR)0,Cy*(*CyO*) line (*Cy0* is a second chromosome balancer). We used UAS-*rpr* and UAS-*hid* lines generated by McNabb et al. (1997), although not the identical strains reported by them. We produced a double-transgenic line homozygous for both UAS-*p35* (Zhou et al., 1997) and *pdf*-GAL4 (each on the second chromosome). The *pdf*-GAL4, UAS-*p35* doubly homozygous transgenics were crossed to UAS-*rpr* or UAS-*hid/CyO*.

Behavioral Analysis

Adult males (1–5 days old) were monitored for locomotor events at 25°C as described in Hamblen et al. (1986, 1998). We first monitored 7–8 days in 12 hr:12 hr LD conditions; free-running locomotion was monitored in constant darkness (DD) for 9–10 further days. Numbers of activity events were recorded for every half-hour bin, and average numbers of activity events per bin per fly were calculated (Hamblen-Coyle et al., 1989).

The times of morning and evening activity peaks (phase values) associated the flies' behavior in LD were computed (Hamblen-Coyle et al., 1992). Evening peak phases (computed from each fly's behavior) were statistically analyzed by the Kruskal-Wallis Nonparametric ANOVA Test, followed by Dunn's Multiple Comparisons Test (using Instat programs, GraphPad Software, San Diego, CA). χ-square periodogram analyses (Sokolove and Bushell, 1978) were performed to generate power and width values; flies showing periods with a power of greater than or equal to 10 and width of greater than or equal to 2 were considered rhythmic. For more sensitive determinations of free-running periodicities, the activities were subjected to a low-pass digital filter (Dowse and Ringo, 1987), then analyzed by MESA (Dowse and Ringo, 1987; Hamblen-Coyle et al., 1989). Subsequently, a MESA-based SNR value for each fly was computed (Dowse and Ringo, 1987).

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