PDF Cycling in the Dorsal Protocerebrum of the *Drosophila* Brain Is Not Necessary for Circadian Clock Function

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Abstract In *Drosophila*, the neuropeptide pigment-dispersing factor (PDF) is a likely circadian molecule, secreted by central pacemaker neurons (LNvs). PDF is expressed in both small and large LNvs (sLNvs and lLNvs), and there are striking circadian oscillations of PDF staining intensity in the small cell termini, which require a functional molecular clock. This cycling may be relevant to the proposed role of PDF as a synchronizer of the clock system or as an output signal connecting pacemaker cells to locomotor activity centers. In this study, the authors use a generic neuropeptide fusion protein (atrial natriuretic factor–green fluorescent protein [ANF-GFP]) and show that it can be expressed in the same neurons as PDF itself. Yet, ANF-GFP as well as PDF itself does not manifest any cyclical accumulation in sLNv termini in adult transgenic flies. Surprisingly, the absence of detectable PDF cycling is not accompanied by any detectable behavioral phenotype, since these transgenic flies have normal morning and evening anticipation in a light-dark cycle (LD) and are fully rhythmic in constant darkness (DD). The molecular clock is also not compromised. The results suggest that robust PDF cycling in sLNv termini plays no more than a minor role in the *Drosophila* circadian system and is apparently not even necessary for clock output function.

Key words circadian, clock, *Drosophila*, pigment-dispersing factor, lateral neurons, locomotor activity

Circadian rhythms are widespread in nature, ranging from cyanobacteria to humans. In all systems, a central circadian pacemaker that generates endogenous rhythms of about 24 h is connected to environmental signals through input pathways that measure light or temperature changes. Recent progress in mammalian systems has highlighted the role of specialized melanopsin photoreceptors in the ganglion cell layer of the retina (Berson, 2003; Brown and Robinson, 2004; Hastings and Herzog, 2004; Kavakli and Sancar, 2002; Panda et al., 2002b) and the cryptochrome protein being a key circadian photoreceptor in *Drosophila* (Busza et al., 2004; Emery et al., 1998, 2000a, 2000b). Pacemakers are also connected to many effector systems and organs through output pathways. Although some of these are electrical, there has been increasing interest in humoral connections via hormones and neuropeptides, for example between

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the central pacemaker of the suprachiasmatic nucleus and peripheral clocks and tissues in mammals (Antle and Silver, 2005; Hastings and Herzog, 2004; Panda et al., 2002a).

In insects, the neuropeptide pigment-dispersing factor (PDF) is a likely circadian clock molecule secreted by central pacemaker neurons. PDF belongs to the family of the crustacean pigment-dispersing hormones (PDH), which are responsible both for dispersion of black pigment in the chromatophores of the body integument and for the migration of screening pigment in the distal pigment cells of the compound eyes (Rao and Riehm, 1993). PDF only serves the 2nd, circadian function in insects, and "factor" implies that it is not released into the general circulation (Rao et al., 1987). In *Drosophila* larvae, PDF is first expressed in a small group of larval neurons, the small lateral neurons (sLNvs) within the brain hemispheres as well as in the abdominal PDF-containing neurons (PDFAb) within the ventral ganglion. In adults, PDF is also expressed in the large lateral neurons (lLNvs), which do not exist in larvae. The adult sLNvs send projections to the dorsal protocerebrum, whereas the lLNvs contact the second optic neuropile (the medulla). Projections from lLNvs also connect the central lateral brain hemisphere via the posterior optic tract (POT) (Homberg et al., 1991; Helfrich-Forster and Homberg, 1993).

The large and small LNvs coexpress numerous clock genes (e.g., *period*, *timeless*, and *cry*) along with PDF, and these cells are thought to be the main pacemaker neurons in insects (Blau and Young, 1999; Emery et al., 2000a; Helfrich-Forster, 1995; Kaneko and Hall, 2000). Despite these similarities, the 2 cell groups are likely to serve nonidentical functions (Helfrich-Forster and Homberg, 1993; Kaneko and Hall, 2000). For example, in a strain mutant for the circadian photoreceptor gene *cry*, the small cells maintain robust cycling of PER and TIM proteins in LD conditions, whereas the large neurons do not (Stanewsky et al., 1998). The small cells also show a uniform timing of PER nuclear accumulation, which is different by 3 to 4 h from that of the large cells (Shafer et al., 2002).

Because ablation of the LNvs affects only limited aspects of behavioral rhythms under LD conditions, there might be other important cells that contribute to circadian behavior (Renn et al., 1999). Indeed, cell-specific ablation with other circadian drivers showed that neurons governing the timing of morning and evening activity peaks derive from 2 distinct groups of circadian cells: morning activity from sLNvs and evening activity from another group of cells including the dorsal lateral neurons (Stoleru et al., 2004; Grima et al., 2004). Although these 2 oscillators can function autonomously, they are functionally coupled (Stoleru et al., 2004, 2005).

There are striking circadian oscillations of PDF staining intensity in the small cell termini, with a peak at ZT2-3 and a trough at ZT13-15. There is no comparable cycling in lLNv termini (Park et al., 2000). The PDF cycling in sLNv termini requires a functional molecular clock; that is, there is no terminal cycling in the arrhythmic per^{01} genotype (Park et al., 2000). As *pdf* mRNA does not oscillate and protein staining does not oscillate in sLNv cell bodies (i.e., cycling is restricted to cell termini), posttranscriptional mechanisms must govern the circadian accumulation of PDF in or secretion from small cell termini. Based on the prominent role of the small cells in DD rhythmicity and in LD morning activity, it has been proposed that the cycling PDF in small cell termini connects these pacemaker cells to other circadian-relevant neurons (Park et al., 2000).

These observations beg the questions, what role does the PDF small cell cycling play, and what is the function(s) of PDF more generally in *Drosophila*? Although pdf^{01} flies show quite normal locomotor activity under LD conditions, they are not able to anticipate lights-on, and their evening activity peak is advanced by about 1 h. More importantly, this *pdf*-null mutation disrupts true circadian behavior because most pdf^{01} flies show little or no rhythmicity in DD. The residual rhythmic population manifests a somewhat short period, consistent with the modest activity peak advance observed under LD conditions. Thus, it has been proposed that LNvs, and specifically PDF, are important in coordinating a circadian network within the fly brain (Peng et al., 2003; Lin et al., 2004). In this scenario, a complete lack of PDF desynchronizes clock cells under DD conditions. PDF is probably an important fraction of the lateral neuron output because the phenotypes of LNv-ablated flies are very similar to those of the pdf^{01} (*pdf*-null mutant) strain.

A related function comes from experiments in cockroaches, which described the response to PDF injections in the vicinity of the accessory medulla of cockroaches during the late subjective day; this protocol caused phase delays of circadian wheel-running activity rhythm, indicating that PDF may act as a synchronizer between pacemaker centers

(Petri and Stengl, 1997). The observation fits with the POT staining, which connects the 2 bilaterally symmetric rhythms centers in cockroaches as well as in *Drosophila*. It also fits with recent period-resetting experiments in *Drosophila* (Stoleru et al., 2005)

To address the function of PDF cycling in sLNvs, we made transgenic *Drosophila* expressing a mammalian neuropeptide-GFP fusion gene (pre-pro-atrial natriuretic factor–green fluorescent protein [ANF-GFP]). ANF-GFP has been previously used to visualize the fate of a neuropeptide during regulated secretion in mammalian tissue culture systems; release is measured as a decrease in fluorescence intensity (Lang et al., 1997; Burke et al., 1997; Han et al., 1999; de Bree et al., 2000). It has also been used to study the developmental expression, processing, transport, and release of neuropeptides in *Drosophila* neurons (Rao et al., 2001), and in vivo imaging of neuropeptide vesicles is possible with transgenic animals expressing GFP-tagged peptides (Levitan, 2004). When expressed with the panneural promoter *elav*, ANF-GFP was first seen to accumulate in synaptic regions of the embryonic central nervous system. At the larval neuromuscular junction, it was concentrated in nerve terminals. Moreover, the depolarization-induced release from synaptic boutons was observed as a decrease in fluorescence intensity (Rao et al., 2001). This construct has also been used to study ecdysis and identify neurosecretory cells involved in control of this behavior (Husain and Ewer, 2004) and to study changes in vesicle release induced by mating in female flies (Heifetz and Wolfner, 2004).

Our strategy was similar, that is, we expressed this generic neuropeptide-GFP fusion in the LNvs of live flies. Would GFP then cycle in sLNv termini indistinguishably from PDF? We also considered the possibility that high level expression of the ANF-GFP fusion protein in the LNv cell population might act in a dominant negative fashion and inhibit PDF function. Although the results were complicated and did not fulfill any of these simple predictions, they did suggest that visible PDF cycling in the small cell termini plays no more than a minor role in circadian locomotor activity of adult *Drosophila*.

MATERIALS AND METHODS

Drosophila melanogaster strains were reared on medium containing agar, yeast, corn meal, dextrose, and a mold inhibitor (Lexgard), in 12:12 h LD cycles at 25 °C.

Experimental Procedures

Larval entrainment. Flies were laying eggs for 5 d under LD. Subsequently, adult flies were removed; there were no climbing larvae at this time. Larvae were then entrained for subsequent 3 d in DD, and 3rd instar larvae were selected. They were therefore exposed to 3 d of DD after exposing earlier larval stages and embryos to 2 to 3 d of LD.

Adult entrainment. In case of LD (12:12) experiments, flies were entrained for 3 d and dissected at indicated time points. In case of DD experiments, flies were entrained in LD, released into DD, and dissected on the 3rd day of DD at the indicated time points.

The circadian driver lines containing pdf-gal4 have been previously described (Renn et al., 1999). The UAS-ANF-GFP fly strain was also previously described (Burke et al., 1997).

GFP Expression Analysis

To visualize the axon projections from the sLNvs, adult or 3rd instar larval brains of pdf-gal4; UAS-ANF-GFP flies were dissected in phosphate buffer saline (PBS) and fixed in 3.7% paraformaldehyde in PBS for 5 min. After 3 rinses in PBS, brains were mounted in Vectashield mounting medium (Vector Laboratories). Fluorescence intensity was scored blindly in all samples; genotypes either had obvious PDF cycling (e.g., differences between ZT15 and ZT3 were always present and easy to discern), or it was absent by eye. Moreover, the whole-mount brains were examined with a Zeiss Axiophot microscope equipped with a Photometrics SenSys CCD camera (Photometrics Ltd, Tucson, AR), and images (using a 20× lens magnification) were used for quantification of fluorescence signals. For a given time point and genotype, at least 3 to 5 termini from different brains were photographed in the fluorescence field. The mean pixel intensity was measured directly from the square area defined inside the terminals. Average pixel intensity was calculated per time point, and a corresponding mean pixel intensity of the background within the brain and adjacent to the termini was measured and subtracted. To get relative staining intensity (note: the stronger the fluorescence from the measured area, the lower mean pixel intensity

with OpenLab software), it was necessary to convert average intensity values to an arbitrary scale of 1-200, in which 1 denotes darkness and 200 brightness.

PDF Staining

Brains were dissected in PBS at different time points of LD or DD and fixed in 3.7% paraformaldehyde for 30 min. After washing in 0.1 M PBT (PBS + 0.5% bovine serum albumin and 0.5% Triton) 5 × 5 min, the brains were incubated overnight at 4 °C with rabbit anti-PDF polyclonal serum (1:6000 dilution; a gift from Dr. Kenji Tomioka). Anti-PDF serum was visualized with Texas Red–conjugated secondary antibody (Texas Red anti-rabbit, 1:400 dilution; Jackson Labs) for at least 2 h at room temperature and washed 5 × 5 min in PBT. Finally, brains were mounted in Vectashield (Vector Laboratories).

In situ mRNA hybridization on adult brain whole mounts. In situ hybridization of *tim* mRNA was performed as described previously (Zhao et al., 2003).

Behavioral analysis. Flies were entrained for 3 to 5 days in LD before release into DD. Locomotor activities of individual flies were monitored using Trikinetics *Drosophila* Activity Monitors (Waltham, Mass). The analysis was done using a signal processing toolbox (Levine et al., 2002). Autocorrelation and spectral analysis were used to assess rhythmicity and to estimate period. Rhythm strength (RS) was assessed with Rhythm Index (Levine et al., 2002).

RESULTS

We used the GAL4/UAS system to express the ANF-GFP fusion under control of a *pdf* promoter (genotype: pdf-gal4; UAS-ANF-GFP, homozygous inserts). We expected that ANF-GFP fusion would act as a reporter for PDF expression. Indeed, GFP expression was detected in the 4 larval sLNvs in each brain hemisphere as well as 4 cell pairs in the ventral ganglion (data not shown). In adult flies, GFP expression was observed in the 4 sLNvs, the 4 to 5 lLNvs (4-5 large cells), and their projections into the dorsal protocerebrum as well as projections connecting the 2 brain hemispheres (POT; Fig. 1). We could also see the GFP signal in the medulla. All of this is indistinguishable from PDF staining in wild-type flies. Moreover, the sLNv and lLNv cell bodies in adult fly brains showed a strong GFP signal, further indicating that the ANF-GFP transgene was well transcribed in PDF cells.

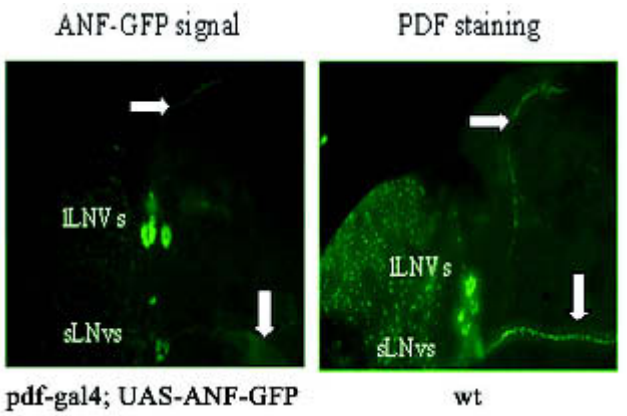

Figure 1. Atrial natriuretic factor–green fluorescent protein (ANF-GFP) exhibits the same expression pattern as pigment-dispersing factor (PDF). Adult brains are shown. ANF-GFP expression (left) and PDF staining (right). lLNvs and sLNvs cell bodies are shown with their projections (right arrow indicates sLNv terminals, down arrow indicates lLNv projections).

ANF-GFP Acts as a Reporter for Neuropeptide Release in Larvae

We then assayed ANF-GFP cycling in transgenic pdf-gal4; UAS-ANF-GFP 3rd instar larvae under LD conditions (Fig. 2A, 2C). GFP showed obvious cyclical accumulation in sLNv termini (4 independent experiments). Fluorescence intensity was stronger at the beginning of the day (ZT3) and weaker at the beginning of the night (ZT15), exactly like PDF in wt flies. Moreover, the rhythm of cyclical ANF-GFP accumulation in larval sLNv terminals was also observed under DD conditions (3rd day of DD; Fig. 2B, 2C). As in LD, the termini had a much stronger GFP signal at the beginning of the subjective day than at the beginning of the subjective night. Similar results were obtained in at least 4 independent experiments. Quantification of ANF-GFP fluorescence under both LD and DD conditions in larval brains is shown in a histogram (Fig. 2C; left graph represents LD, right graph DD).

The ANF-GFP cycling in the termini of sLNvs in DD suggests that this phenomenon is controlled by the circadian clock. Furthermore, this implies that any neuropeptide expressed in sLNvs will be handled in a similar manner, that is, that this phenomenon constitutes a general property of sLNvs.

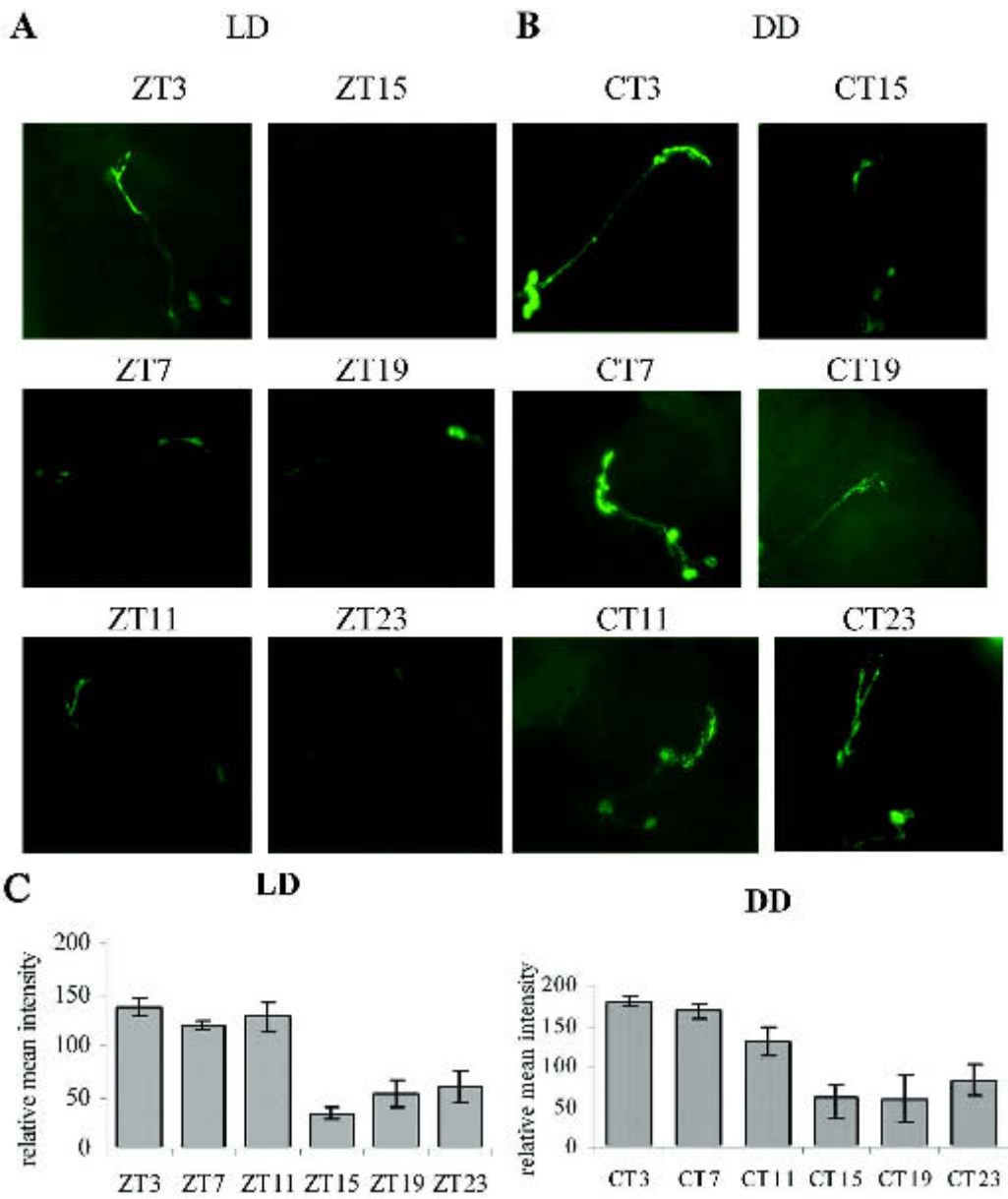

Figure 2. Atrial natriuretic factor–green fluorescent protein (ANF-GFP) phenocopies pigment-dispersing factor (PDF) cycling in larvae. In larvae of transgenic pdf-gal4; UAS-ANF-GFP flies, ANF-GFP shows cyclical accumulation in the termini of sLNvs both under LD and DD conditions. (A) Images showing sLNv terminals in larval brains under LD conditions. (B) Images of larval brain sLNv terminals under DD conditions. (C) Quantification of ANF-GFP fluorescence under LD and DD conditions in larval brains. The relative fluorescence intensity was quantified and plotted as mean ± SEM for each time point (*N* = 4-5 brains) (see Materials and Methods).

No ANF-GFP Cycling in Adults

Encouraged by the results from larvae, we assayed ANF-GFP cycling in adult brains (Fig. 3 and data not shown). We first compared fluorescence intensity at ZT3 and ZT15 in the sLNv terminals under LD conditions. However, we could not see any significant difference between these 2 time points. To check for a possible phase change, we subsequently assayed ANF-GFP intensity every 4 h during an LD cycle. Also in this case, there was no reproducible difference between the 6 time points (Fig. 3A). Moreover, we did not observe any circadian oscillations of ANF-GFP signal in sLNv terminals under DD conditions (Fig. 3B). As in LD, the termini showed comparable weak fluorescence across all 6 time points in at least

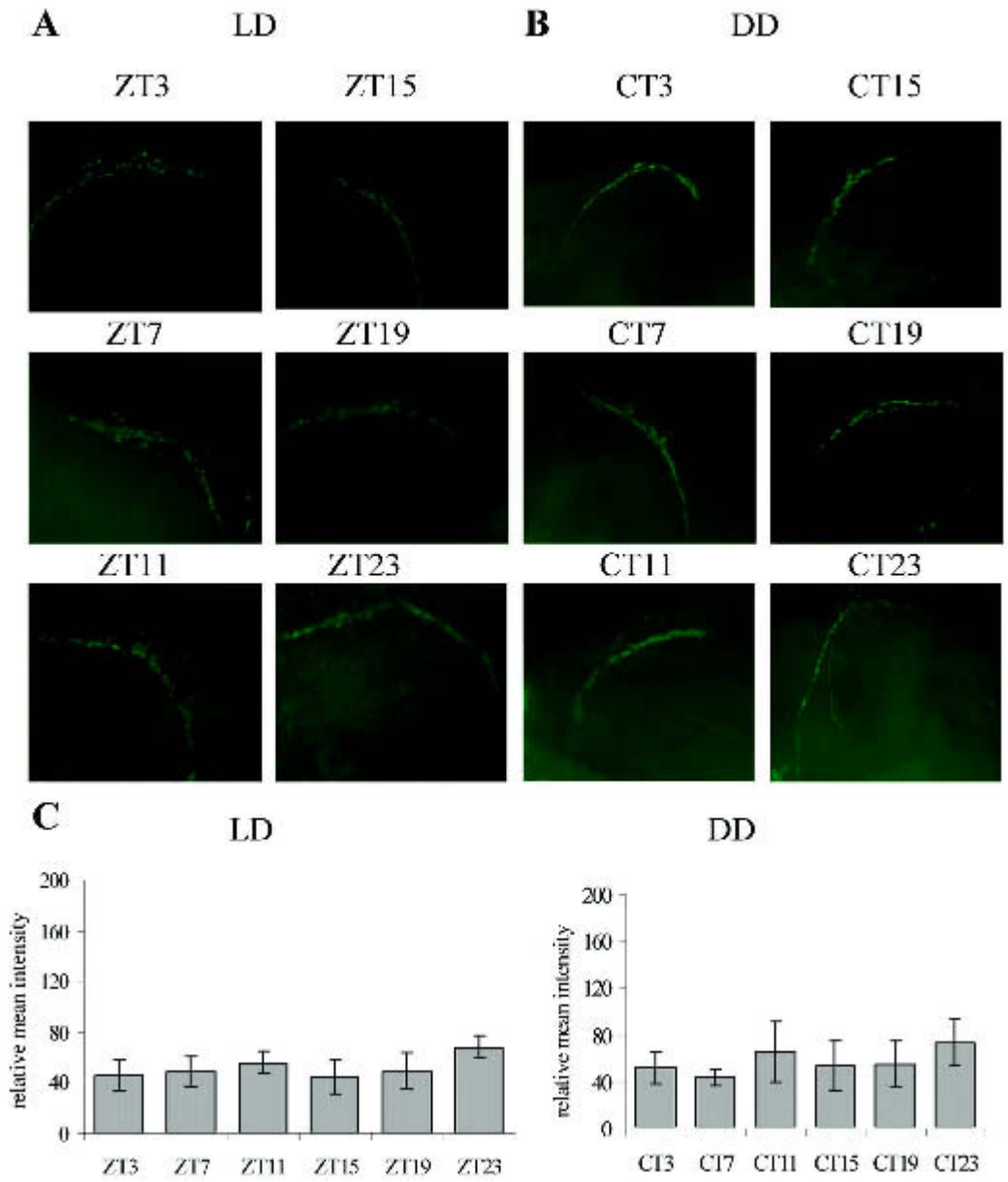

Figure 3. No atrial natriuretic factor–green fluorescent protein (ANF-GFP) oscillations in adult flies. In adults of transgenic pdf-gal4; UAS-ANF-GFP flies, ANF-GFP shows no cyclical accumulation in the termini of sLNvs both under LD and DD conditions. (A) Images of adult sLNv terminals under LD conditions. (B) Images of adult sLNvs terminals under DD conditions. (C) Quantification of ANF-GFP fluorescence under LD and DD conditions. The relative fluorescence intensity was quantified as in Figure 2.

5 independent experiments. Similar results were obtained in heterozygous pdf-gal4; UAS-ANF-GFP flies, as these sLNv termini also had uniform, weak fluorescence intensity compared to sLNv cell bodies (data not shown). Quantification of ANF-GFP fluorescence in adult brains under both LD and DD conditions is shown in a histogram (Fig. 3C).

The failure to observe ANF-GFP oscillations in the adult sLNv termini suggests that fusion gene expression might act in a dominant negative fashion and inhibit PDF as well as its own cycling within these cells. This predicts that PDF also would no longer be cyclically accumulated in adult terminals. To test this possibility, we used anti-PDF serum to stain adult brains of the transgenic strains. We first repeated the results of Park et al. (2000) by staining brains of wild-type flies (CS and yw) with anti-PDF serum (a gift from K. Tomioka). Consistent with the published findings,

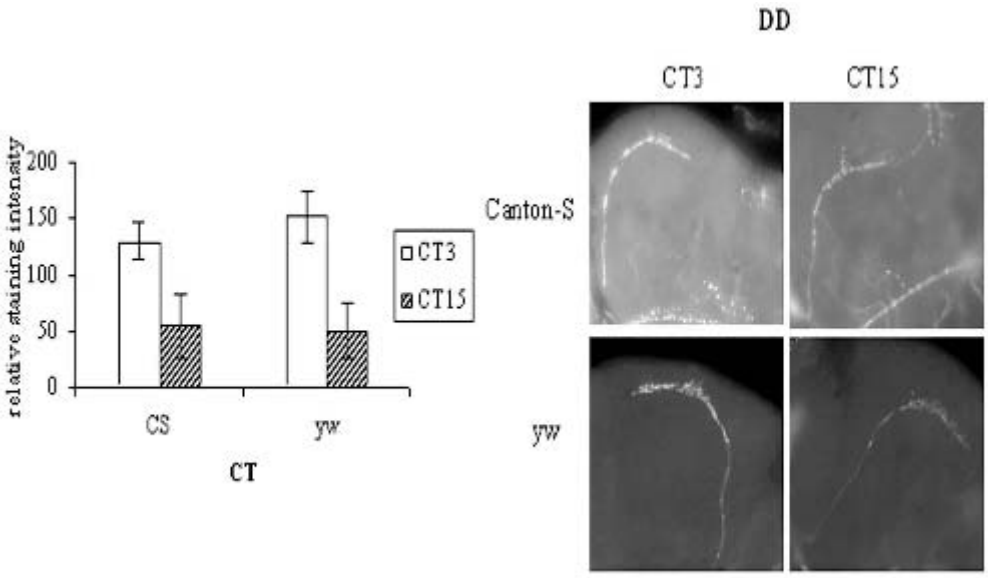

Figure 4. Pigment-dispersing factor (PDF) cycling in adult CS and yw flies under DD conditions. PDF expression shows daily oscillations in terminals of sLNvs under DD conditions with a peak at CT3 and a trough at CT15. Histograms show relative staining intensity after immunostaining with anti-PDF serum in DD. The relative staining intensity was quantified as in Figure 2.

we observed cyclical PDF staining intensity in the dorsal protocerebrum, with a peak at the beginning of the day and a trough at the beginning of the night (data not shown). Moreover, this rhythm persists in constant darkness (DD; CT3 vs. CT15, where CT0 is the beginning of the subjective day and CT12 is the beginning of the subjective night; Fig. 4 and data not shown). No PDF cycling was observed in lLNv termini, exactly as reported by Park et al. (2000).

PDF Cycling Is Not Observed in Adult pdf-gal4; UAS-ANF-GFP Flies

Consistent with the dominant negative hypothesis, pdf-gal4; UAS-ANF-GFP flies did not show any PDF oscillations in sLNv termini under both LD and DD conditions at the 2 key time points when PDF shows maximal and minimal expression in wild-type flies (beginning of the day, ZT3, and beginning of the night, ZT15, respectively). The same negative result was obtained in at least 5 independent experiments (Fig. 5). The lack of cyclic terminal staining presumably reflects the absence of cyclic neuropeptide release. According to this view, strong terminal staining mirrors a storage pool of neuropeptide at a given time point, whereas a weak fluorescence signal indicates recent neuropeptide release. Although only 2 time points are shown, failure to detect oscillations was also observed at additional time points taken every 4 h during a 24-h period (e.g., ZT3, 7, 11, 15, 19, and 23; data not shown). Moreover, PDF terminal staining always showed strong and uniform fluorescence intensity at all 6 time points under DD as well as LD conditions. This was contrary to the signal intensity of ANF-GFP in the PDF termini, which was weak and uniform at all time points.

To explore further this putative dominant negative hypothesis, we performed PDF staining in the 2 parental strains (i.e., yw; pdf-gal4 and UAS-ANF-GFP), first at the 2 key LD time points (e.g., ZT3 and ZT15). In UAS-ANF-GFP flies, we observed canonical PDF cycling in the sLNv termini, that is, stronger fluorescence intensity at the beginning of the day and weaker fluorescence intensity at the beginning of the night (Fig. 5, at least 4 independent experiments). Cycling PDF was also observed in a 6-time-point experiment (data not shown). However, we could not detect any PDF oscillations in the other parental strain, namely, yw; pdf-gal4 flies. In this strain, PDF showed indistinguishable staining intensity between ZT3 and ZT15 (Fig. 5) as well as at other time points spanning the whole 24-h period in at least 5 independent experiments (data not shown). A graphical representation of the relative staining intensity in different strains is shown in a histogram (Fig. 5). This suggests that this dominant negative feature may be due to the presence of an additional copy of the

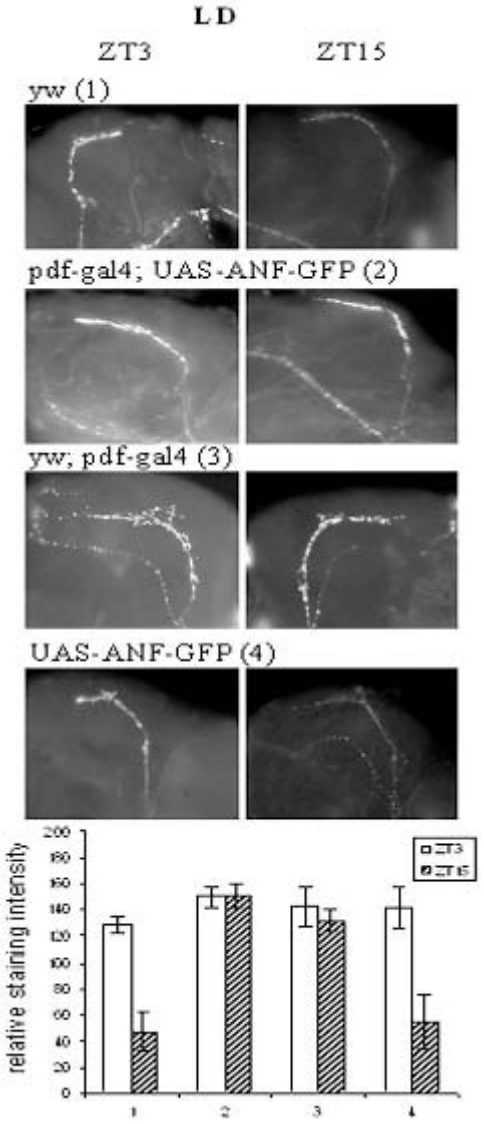

Figure 5. Transgenic-strain pigment-dispersing factor (PDF) staining in LD. PDF shows cyclical accumulation in the sLNv terminals in wild-type flies (panel 1) and UAS-ANF-GFP flies (panel 4) under LD conditions. There is no cyclical accumulation in the terminals of pdf-gal4; UAS-ANF-GFP flies (panel 2) or yw; pdf-gal4 flies (panel 3). The histogram shows the quantification. The relative staining intensity was quantified as in Figure 2.

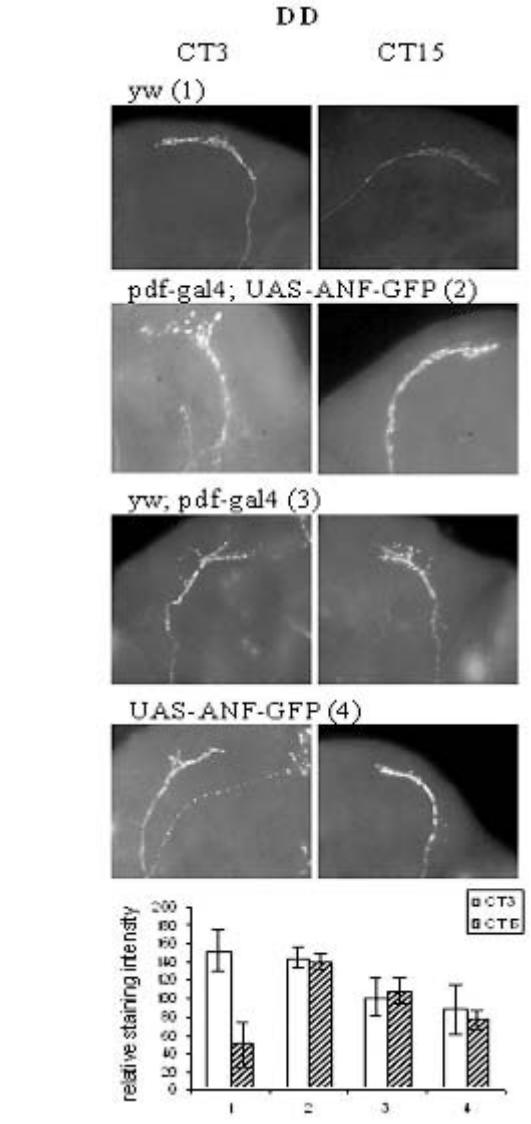

Figure 6. Transgenic-strain pigment-dispersing factor (PDF) staining in DD. PDF shows cyclical accumulation in the sLNv terminals only in control, yw flies in DD (panel 1). There are no PDF oscillations in pdf-gal4; UAS-ANF-GFP flies (panel 2), yw; pdf-gal4 flies (panel 3), or UAS-ANF-GFP flies (panel 4) in DD. The histogram shows PDF-staining quantification.

pdf promoter rather than ANF-GFP expression. However, this explanation appears unlikely, since there is no impact of an additional copy of the *pdf* promoter on ANF-GFP cycling in larvae (Fig. 2).

Moreover, there was no PDF cycling in all 3 transgenic lines in DD, for example, pdf-gal4; UAS-ANF-GFP and the 2 parental strains: yw; pdf-gal4, and yw; UAS-ANF-GFP (Fig. 6 and data not shown). PDF always showed strong fluorescence intensity in the sLNv termini across all 6 time points examined (data not shown); that is, it did not differ significantly between subjective day and subjective night. A graphical representation of relative staining intensity under DD conditions in the different strains is shown in a histogram (Fig. 6). This indicates that PDF terminal cycling is significantly weaker in the transgenic strains than in standard wild-type strains such as Canton-S or yw.

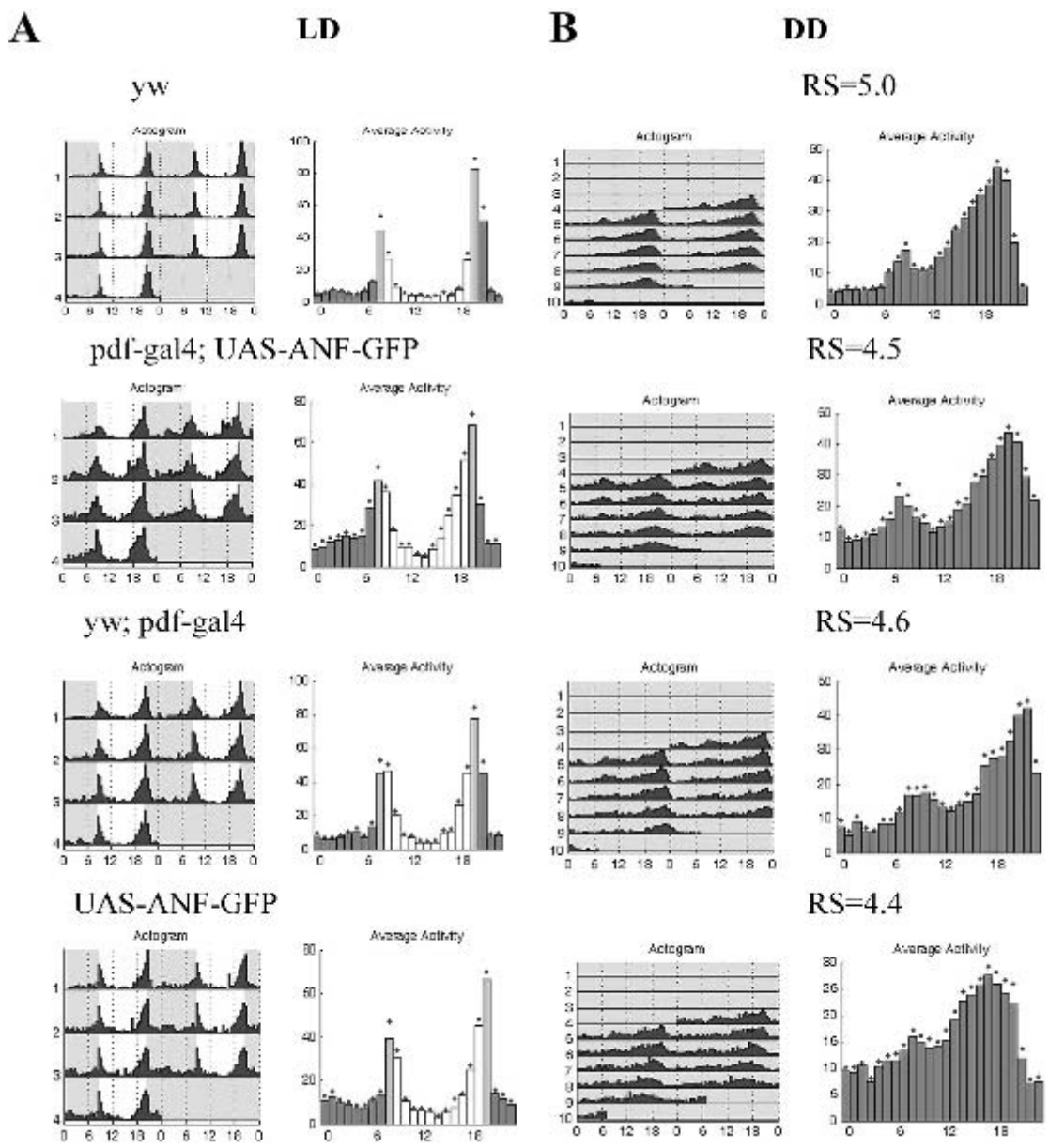

Figure 7. Normal, wild-type–like behavioral activity without cyclical PDF accumulation. Locomotor activity of yw, pdf-gal4; UAS-ANF-GFP, UAS-ANF-GFP, and yw; pdf-gal4 flies under LD (A) and DD (B) conditions is shown. All transgenic flies have locomotor activity patterns indistinguishable from wild-type flies under both conditions. For each genotype, actograms and average activity are shown (for details, see Materials and Methods). Moreover, in case of DD experiments, the rhythm strength (RS) index is shown for each genotype (Materials and Methods).

Locomotor Activity Rhythms Are Intact without Cycling PDF Accumulation

We also examined carefully the locomotor activity rhythms of pdf-gal4; UAS-ANF-GFP flies. These flies showed normal LD morning and evening activity peaks, indistinguishable from the activity pattern of the control yw strain (Fig. 7). A lack of detrimental effects of the ANF-GFP transgene in LD is also consistent with previous observations (Rao et al., 2001; Heifetz and Wolfner, 2004; Husain and Ewer, 2004). Surprisingly, they also had normal, wild-type locomotor activity profiles in DD, and they did not lose the ability to predict morning and evening activity peaks. Moreover, the percentage of rhythmic flies in DD was identical to that observed in wild-type flies

and very different from that observed in the *pdf* ^{01} loss of function strain (data not shown; Fig. 7).

We also analyzed locomotor activity levels in pdf-gal4; UAS-ANF-GFP flies. The assay was based on the observation by Helfrich-Forster et al. (2000) that excess PDF release from the sLNvs has an inhibitory effect on locomotor activity, whereas excess PDF in other locations near sLNv termini increases locomotor activity and severely inhibits rhythmicity. However, we did not observe alterations in gross locomotor activity levels in any of the transgenic strains missing detectable PDF cycling in sLNv termini, for example, pdf-gal4; UAS-ANF-GFP flies (data not shown). Moreover, we could not detect any impact on locomotor activity rhythms that correlated with the constant PDF staining intensity: as the rhythm strength of pdfgal4; UAS-ANF-GFP flies under DD conditions was not significantly different from that of control flies (Fig. 7). This suggests that cycling PDF accumulation in or release from sLNv terminals plays no more than a minor role in the circadian system.

PDF Cycling in the sLNvs Terminals Is Not Required for Synchronization of the Circadian Network

Finally, we examined whether the function of sLNv terminal cycling might be to drive the rhythmicity of dorsal neurons (DNs), which lie near these terminals. To this end, we performed *tim* in situ hybridization on fly brains at CT14 and CT2. In wild-type flies, *tim* mRNA cycles with the peak at ZT14 (CT14 under DD conditions) and a trough at ZT2 (CT2). The *tim* mRNA cycling as well as the general *tim* mRNA expression pattern was unaffected in all clock neurons of pdf-gal4; UAS-ANF-GFP flies. More importantly, *tim* was still cycling in DNs and in other *tim*-positive neurons of the transgenic strain under DD conditions (Fig. 8). This indicates that the circadian molecular program is functional in this region of the fly brain, even without apparent PDF terminal cycling.

DISCUSSION

Here, we used a transgenic *Drosophila* line that expresses an ANF-GFP fusion protein under control of the *pdf* promoter. As a result, GFP is expressed and presumably secreted from adult-brain lateral neurons. In transgenic larvae, this fusion protein shows circadian staining intensity both under LD and DD conditions. However, in adult pdf-gal4; UAS-ANF-GFP flies, ANF-GFP is not cycling in sLNv terminals. Surprisingly, the lack of PDF cycling is not accompanied by any unusual or mutant behavioral activity. This is because these transgenic flies are rhythmic under DD conditions and have wild-type–like morning and evening activity peaks in LD. The molecular clock is also not compromised in this strain. Our results suggest that PDF cycling in sLNv termini plays no essential role in the adult circadian system.

It has previously been shown that the ANF-GFP fusion is recognized by the *Drosophila* machinery, proteolitically processed, sorted into secretory granules, transported down axons and concentrated in nerve terminals at neuromuscular junctions; that is, ANF-GFP appears to be indistinguishable from a simple neuropeptide (Rao et al., 2001; Han et al., 2002; Levitan, 2004). This behavior presumably reflects the generic features of ANF, for example, the nucleotide sequence of pre-pro-ANF is typical for a eukaryotic neuropeptide mRNA sequence. This includes a hydrophobic stretch of N-terminal amino acids typical of secreted proteins, which makes it a good candidate for a naïve secreted peptide in *Drosophila* (Seidman et al., 1984). In mammals, atrial natriuretic factor affects water and salt homeostasis and vasodilation (Strand, 1999). The fact that expression in *Drosophila* produced no evident deleterious effects suggests that ANF has no biological activity in flies, consistent with the fact that the *Drosophila* genome contains no similar peptide sequence (Rao et al., 2001; Heifetz and Wolfner, 2004; Husain and Ewer, 2004).

If transgenic ANF-GFP were processed indistinguishably from PDF in wild-type fly brains, we expected to see cyclical changes in fluorescence intensity, namely, stronger fluorescence at the beginning of the day/subjective day and weaker fluorescence at the beginning of the night/subjective night in the sLNv termini of the dorsal protocerebrum. This would mirror the cyclical staining intensity of PDF (Park et al., 2000). It is thought that this phenomenon reflects circadian PDF secretion: low at the beginning of the day when staining intensity is high, and high at the beginning of the night when staining intensity is low. Moreover, such rhythmic oscillations of fluorescence intensity may be activity dependent, as it has been recently shown that substantial neuropeptide release is observed within minutes of in vivo electrical stimulation of type Ib boutons (Shakiryanova et al., 2005).

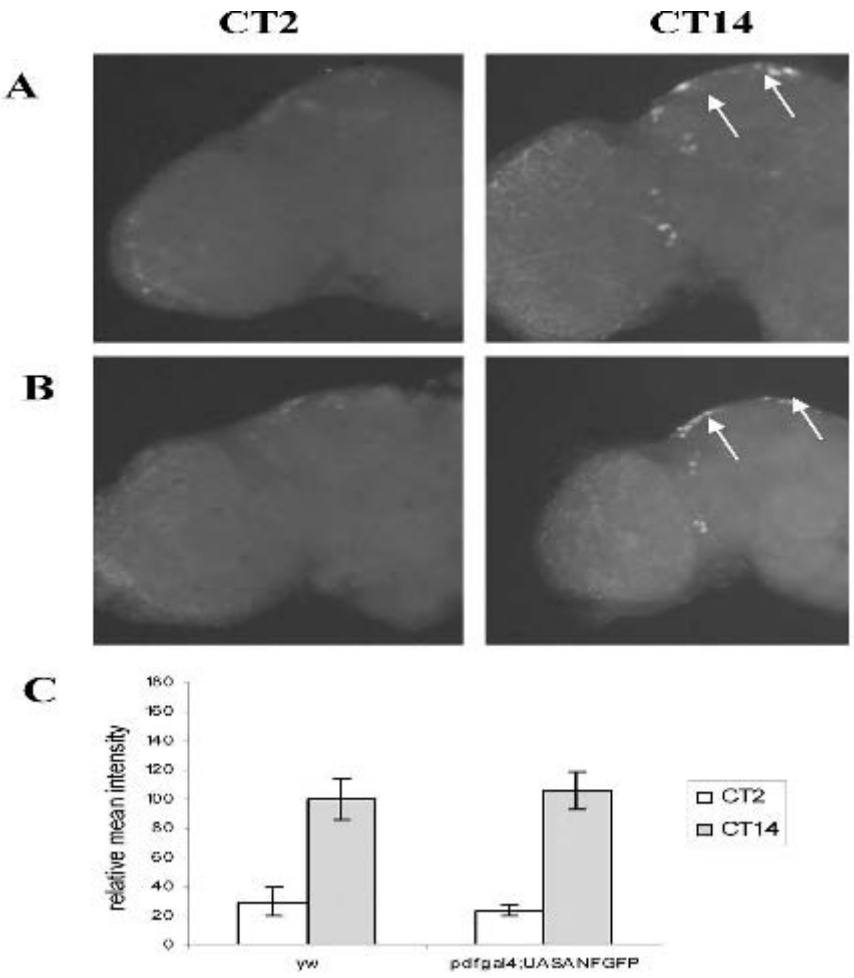

Figure 8. Normal *tim* mRNA oscillations in dorsal neurons without cyclical pigment-dispersing factor (PDF) accumulation. (A) Images of brain, whole amount in situ hybridization of yw flies and (B) pdf-gal4; UAS-ANF-GFP flies. Arrows indicate dorsal neurons. (C) Quantification of *tim* expression in dorsal neurons in DD. The relative staining intensity was quantified and plotted as in Figure 2.

However, we observed no cyclical accumulation of ANF-GFP in adult sLNv terminals, as terminal GFP fluorescence was uniformly weak. This was accompanied by strong cell body staining, suggesting that the absence of cycling may reflect inefficient processing or transport of ANF-GFP between cell bodies and termini. Alternatively, constitutive, efficient ANF-GFP release might compromise its ability to phenocopy circadian features of PDF regulation. Because pdf-gal4; UAS-ANF-GFP flies also failed to manifest PDF staining oscillations (under LD as well as DD conditions), we imagined that some aspect of fusion gene processing, transport, or secretion inhibits PDF cycling. However, transgenic lines expressing only the driver (yw; pdf-gal4) or only the UAS transgene (UAS-ANF-GFP) also show no reproducible cycling PDF staining. PDF and GFP noncycling, or cycling in control genotypes, was always evident by visual inspection as well as by quantification (see Materials and Methods). The only exception was the line containing the UAS transgene, which showed PDF cycling in sLNv termini only under LD conditions (Fig. 5). Although this may indicate that an extra copy of the *pdf* promoter as well as ANF-GFP expression inhibits PDF cycling, we prefer the interpretation that the cycling is not present in all strains and is generally sensitive to subtle changes in genotype, with DD cycling even more sensitive than LD cycling.

Cyclical PDF release has been proposed to be a crucial step in synchronizing other clock neurons as well as serving as a potent output signal from sLNvs (Park et al., 2000). It was therefore unexpected that flies without sLNv PDF cycling have normal, wild-type

locomotor activity under both LD and DD conditions (Fig. 7). The percentage of rhythmic flies in DD was indistinguishable from the positive control Canton-S or yw flies and very different from the negative control pdf^{01} strain. Consistent with the wild-type behavior, *tim* mRNA cycling in DNs was unaffected in pdf-gal4; UAS-ANF-GFP flies, under both LD and DD conditions. The lack of a molecular as well as behavioral phenotype is surprising, especially in light of articles indicating an important role of PDF in locomotor activity rhythms (e.g., Grima et al., 2004; Stoleru et al., 2004). One possibility is that these strains maintain a reduced amplitude of circadian PDF accumulation/secretion, sufficient for biological activity but below the level of detection by staining. Another is that PDF cycling interfaces with other regulatory circuit(s); for instance, one can imagine circadian regulation of PDF receptor activity (Hyun et al., 2005; Lear et al., 2005; Mertens et al., 2005); circadian rhythmicity would then require only a single functional circuit. A 3rd possibility, related to the 2nd, is that only temporally constant PDF secretion is necessary for wild-type locomotor activity rhythms; cycling PDF serves a different function, which we have not assayed.

Despite a lack of evidence for circadian control of geotaxis, we note in this context the surprising effects of *pdf* genotype on *Drosophila* geotaxis behavior, discovered through a microarray screen of different geotaxis strains. Homozygous pdf^{01} males show an extreme phenotype, which is ameliorated by adding 1 copy of the *pdf* transgene. Geotaxis is apparently very sensitive to small changes in PDF levels, due to gene dosage and sex effects on *pdf* expression (Toma et al., 2002). There are also indications that PDF may have additional functions in other insects. It functions as a central neuromodulator and circulating hormone in *Locusta migratoria*, and PDF application to the terminal abdominal ganglion triggers the firing of action potentials in motor neurons, which have axons in the genital nerves of males and the 8th ventral nerve of females (Persson et al., 2001). PDF levels do not differ between day and night in locust hemolymph.

Yet another possibility is that PDF cycling contributes predominantly to larval rhythms, with a reduced importance in adults. This hypothesis is based on the prominent GFP as well as PDF cycling in ANF-GFP larvae, under DD as well as LD conditions. The markedly reduced cycling and signal amplitude in adult sLNv termini presumably reflects differences between larval and adult sLNv properties, for example larger adult cells with increased gene expression but a reduced capacity to undergo circadian processing or secretion. Although a more prominent contribution to larval rhythms could be quantitative (a similar but quantitatively less important function in adults), an extreme view is that sLNv terminal PDF cycling only contributes to larval rhythms and that adult PDF cycling reflects a passive inheritance of the cycling machinery and/or mechanism from larvae. Perhaps PDF cycling is only important for an aspect of larval circadian behavior or for establishing neuronal identity during larval development. Indeed, it is conceivable that all features of PDF function, static as well as cycling, are only important for larvae. This more extreme view suggests that the robust larval GFP and PDF cycling as well as the adult phenotypes of pdf^{01} flies reflect a PDF contribution to the establishment of circadian and output circuitry during larval and pupal development, including circuitry important for adult geotaxis. To our knowledge, no adult-only or larval-only rescue of the arrhythmic pdf^{01} genotype has been reported. The results of both strategies should be interesting and will shed light on the true contribution of PDF terminal cycling to adult behavioral phenotypes.

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