NIH-PA Author Manuscript

NIH Public Access
Author Manuscript
Curr Biol. Author manuscript; available in PMC 2011 April 13.

Published in final edited form as:
Curr Biol. 2010 April 13; 20(7): 591–599. doi:10.1016/j.cub.2010.02.056.

A Subset of Circadian Neurons Coordinates Light and PDF Signaling to Produce Robust Daily Behavior in *Drosophila*

Luoying Zhang[1], **Brian Y. Chung**[1], **Bridget C. Lear**[1], **Valerie L. Kilman**[1], **Yixiao Liu**[2], **Guruswamy Mahesh**[2], **Rose-Anne Meissner**[1], **Paul E. Hardin**[2], and **Ravi Allada**[1,*]

[1]Department of Neurobiology and Physiology, Northwestern University, Evanston, Illinois, 60208, USA

[2]Department of Biology and Center for Biological Clocks Research, Texas A&M University, College Station, Texas, 77845, USA

Summary

Background—Daily behaviors in animals are determined by the interplay between internal timing signals from circadian clocks and environmental stimuli such as light. How these signals are integrated to produce timely and adaptive behavior is unclear. The fruit fly *Drosophila* exhibits clock-driven activity increases that anticipate dawn and dusk and free-running rhythms under constant conditions. Flies also respond to the onset of light and dark with acute increases in activity.

Results—Mutants of a novel ion channel, *narrow abdomen (na)*, lack a robust increase in activity in response to light and show reduced anticipatory behavior and free-running rhythms, providing a genetic link between photic responses and circadian clock function. We used tissue-specific rescue of *na* to demonstrate a role for ~16-20 circadian pacemaker neurons, a subset of the DN1p, in mediating the acute response to the onset of light as well as morning anticipatory behavior. Circadian pacemaker neurons expressing the neuropeptide PIGMENT DISPERSING FACTOR are especially important for morning anticipation and free-running rhythms and send projections to the DN1p. We also demonstrate that DN1p *Pdfr* expression is sufficient to rescue, at least partially, *Pdfr* morning anticipation defects as well as defects in free-running rhythms including those in DN1 molecular clocks. Additionally, these DN1 clocks in wild-type flies are more strongly reset to timing changes in PDF clocks than other pacemaker neurons, suggesting they are direct targets.

Conclusion—Taken together, we demonstrate that the DN1p lie at the nexus of PDF and photic signaling to produce appropriate daily behavior.

Introduction

The daily activity of many animals, including the fruit fly *Drosophila melanogaster*, is largely determined by two processes: internal circadian timing signals and environmental stimuli, such as light and temperature. Circadian clocks allow animals to anticipate daily changes in the environment, while environmental stimuli drive behavior in response to these changes. The latter is often referred to as masking for its ability to mask underlying circadian rhythms.

*To whom correspondence and request for materials should be addressed. r-allada@northwestern.edu.

Experimental Procedures: Available as Supplementary Online Material.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Together, these pathways determine an animal's temporal niche, i.e., whether an animal is active during the day or night.

In *Drosophila*, circadian behavior is mediated by about 150 circadian clock neurons that drive anticipatory activity peaks before lights-on (morning) and lights-off (evening) transitions in light-dark (LD) cycles. Within these neurons, the CLOCK/CYCLE (CLK/CYC) heterodimeric transcription factor activates *period (per)* and *timeless* (*tim*), which in turn feed back to repress CLK/CYC activity in the principal clock feedback loop which times neural activity and behavior [1]. Morning anticipation is directed by PIGMENT DISPERSING FACTOR (PDF)-expressing large and small ventral lateral neurons (l-LNvs, s-LNvs). Loss of PDF or its receptor (PDFR) results in reduced morning anticipation [2-5], while a functional clock in these PDF neurons (termed morning or M-cells) is sufficient for morning anticipation [5,6]. On the other hand, ablation of the dorsal lateral neurons (LNds), PDF(-) s-LNv, and several dorsal neurons (DN1s), i.e., evening (E) cells, virtually eliminates evening anticipation [5].

M- and E-cells are coupled and cooperate to regulate circadian behavior. Flies with clocks only in E-cells are capable of driving morning behavior, suggesting that E-cells may communicate with M-cells [5,7]. Flies that lack PDF signaling exhibit phase advanced evening anticipation [2-4,8] and alterations in molecular oscillations of in the sLNv, LNd, and at least some of the DN1 in constant darkness (DD) [9-12], although for the LNd see also [11]. Changing the pace of M-cells in DD dictates the pace of non-PDF pacemaker neurons as well as overt behavioral period [13]. In addition, M-cells directly target E-cells via PDF [14] to regulate morning anticipation, the phase of evening anticipation, and behavioral period in DD [15]. However, it remains unclear how the distinct subsets of E-cells mediate different aspects of PDF-controlled behavior.

In contrast to our understanding of circadian networks, the neural circuits important for transmitting light information, especially through the central brain, remain unclear. Immediately following lights-on, flies display a robust burst of activity, referred to as normal masking. However, prolonged light exposure, particularly at high intensities (>1000 lux) results in activity suppression [16]. In addition, flies experience an activity burst immediately after dark onset, referred to as paradoxical masking. Flies employ multiple photoreceptor organs of which compound eyes, ocelli, and Hofbauer-Buchner eyelets [17,18] may sense light to regulate masking behavior.

While circadian and masking behavior can be separated, considerable evidence suggests they are interdependent. Null mutants of the circadian clock component *per* (*per^{01}*) lack circadian behavior, yet show robust masking responses [19], indicating the clock is not required for masking. However, several other mutants with poor or absent rhythms also exhibit masking defects, including mutants for the core clock transcription factors *Clk*, *cyc*, and the ion channel *narrow abdomen* (*na*). These mutants lack an acute, robust increase in activity immediately following lights-on yet retain a robust response to light-off [20-22]. The intensity of masking response is also under circadian regulation [23]. Yet how clock and light signals are appropriately integrated is unclear. Here we identify a subset of circadian pacemaker neurons that can coordinate light and clock signals in daily behavior.

Results

Identification of GAL4 Drivers Expressed Specifically in the Circadian DN1p Neurons

As part of studies to identify enhancers important for the tissue-specific expression of *Clk*, we identified a fragment of the *Clk* promoter that drives GAL4 expression specifically in a subset of dorsal circadian pacemaker neurons. We analyzed two independent inserts of this line (*Clk4.1M*-GAL4 and *Clk4.5F*-GAL4) to drive expression of nuclear GFP (UAS-*nGFP*) and

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

examined expression in pacemaker neurons using PER co-immunolabeling. We observe GFP expression in approximately four DN1s for *Clk4.5F*-GAL4 (Figure 1A) and approximately 8-10 DN1s for *Clk4.1M*-GAL4 (Figure 1B) in each hemisphere, with no obvious GFP expression in other brain regions. The DN1s can be divided into two subgroups based on their anatomical localization: the two anterior DN1s (DN1as) and the rest, which are posterior DN1s (DN1ps) [24]. The DN1as are thought to first appear in larvae and send projections ventrally to the vicinity of the s-LNvs, whereas the projections of DN1ps are restricted to the dorsal brain [24, 25]. To find out whether *Clk4.5F*-GAL4 and *Clk4.1M*-GAL4 are expressed in the DN1as, we used these two lines to drive expression of membrane GFP (UAS-*mGF*P) and examined the projection pattern of GFP(+) cells. We do not observe GFP(+) projections that extend ventrally to the s-LNvs as previously reported for the DN1as [24]. Instead, the projections of the GFP(+) DN1s in both UAS-*mGFP*/+;*Clk4.5F*-GAL4/+ and UAS-*mGFP*/+;*Clk4.1M*-GAL4/+ appear primarily in the dorsal brain (Figure 1C and 1D). Projections that do extend ventrally terminate in the vicinity of the LNds, suggesting that these GFP(+) cells are DN1ps. To further confirm this, we examined whether *Clk4.5F*-GAL4 and *Clk4.1M*-GAL4 drive expression in the two larval DN1s, which are believed to develop into the DN1as in adults [24]. We do not observe GFP expression in larval DN1s (data not shown), consistent with the idea that these two GAL4 drivers are DN1p specific. Therefore, we believe both *Clk4.5F*-GAL4 and *Clk4.1M*-GAL4 drive expression specifically in the DN1ps. Given its broader expression within the DN1p, we focus mainly on the *Clk4.1M*-GAL4 line here.

Rescue of *na* Expression in the DN1ps Is Sufficient to Restore Morning Anticipatory Behavior and Light-Induced Activity

The gene *narrow abdomen* (*na*), which encodes a putative cation leak channel [26], has been established as a circadian output gene that regulates the timing of locomotor behavior [27]. Despite having intact PER oscillations in the LNs and DNs, *na* mutants display severe defects in both morning and evening anticipatory behavior and quickly lose behavioral rhythmicity after release into DD [27]. Additionally, unlike wild-type flies, exposure to light produces both an acute and sustained suppression of activity in *na* mutants [20], suggesting a role for *na* in light-mediated control of diurnal behavior. Rescue studies have implicated *na* function within pacemaker neurons and potentially the DNs in the regulation of morning behavior and lights-on response [27], yet the lack of a driver specific to these cells has prevented further rigorous assessment of this hypothesis.

To further examine the involvement of the DN1ps in mediating circadian output behavior and light-mediated activity, we rescued *na* expression (UAS-*na*; [27]) exclusively in DN1ps using the *Clk4.1M*-GAL4 driver in a na^{har} mutant background. na^{har} mutants are strong hypomorphs with no detectable NA expression [20]. To quantify the direct effect of light on fly locomotor behavior in these flies, we assayed the acute masking index (AMI) which reflects the magnitude and direction of the activity response immediately following lights-on, and sustained masking index (SMI) which reflects the response after prolonged exposure to light. We find that DN1p rescue of *na* expression restores only the acute responsiveness to lights-on (AMI) but not SMI compared to na^{har} mutants (Figure 2, Table 1; One-Way ANOVA, Tukey HSD post-hoc, $p<0.001$). No change in AMI was observed in flies when *na* is overexpressed in a wild-type background (Table 1, Figure S1).

We also asked whether DN1p expression could rescue LD and constant darkness (DD) circadian phenotypes in *na* mutants. Since morning activity can be masked by the lights-on response in LD, we quantified morning anticipation at the transition between the last day of LD and the first day of DD (DD1; Figure 2). We find that morning anticipatory behavior is also restored to near wild-type levels in the *Clk4.1M*-GAL4 rescue genotype (Figure 2, Table 1; One-Way ANOVA, Tukey HSD post-hoc, $p<0.001$). We do not observe a change in

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

anticipatory activity upon *na* overexpression in wild-type flies (Table 1, Figure S1). In contrast to morning rescue, we fail to observe rescue of evening behavior under LD conditions. However, on DD1 we can discern an evening peak (Figure 1), suggesting that evening anticipation may be suppressed by light in LD. Yet, DD rhythms are not sustained (Table 1). Thus, DN1p rescue demonstrates robust rescue of both the acute response to light and morning anticipation.

PDFR Expression in the DN1ps is Sufficient to Rescue Morning Anticipatory Behavior and DD Phenotypes in *Pdfr* Mutants

Given the role of PDF neurons in driving morning behavior and DD rhythms and that the s-LNv send projections to the vicinity of the DN1ps [28], we asked whether the DN1p might be direct targets of PDF. We used the *Clk4.1M*-GAL4 used previously as well as the more narrowly expressing *Clk4.5F*-GAL4 lines (Figure 1) to express PDFR in the DN1ps of *Pdfr* null mutants ($Pdfr^{han5304}$; [3]). In LD, *Pdfr* mutants exhibit phase-advanced evening anticipation (Figures 3A and 3B), consistent with previous studies [3,4]. This evening phase phenotype is not rescued by DN1p expression of PDFR (Figures 3C and 3D,S2A and S2B; Table S1). As previously described [3,4], *Pdfr* mutants also exhibit much reduced morning anticipation (Figures 3E and F; Table 2; Student's t-test, $p<0.05$). In contrast to the evening phase phenotype, morning anticipation is rescued by both *Clk4.5F*-GAL4 (Figures 3G and S2C, Table 2; Student's t-test, $p<0.05$) and *Clk4.1M*-GAL4 driven expression of PDFR (Figures 3H and S2D, Table 2; Student's t-test, $p<0.05$). Thus, similar to *na* rescue in LD, we observe rescue of morning but not evening phenotypes in *Pdfr* mutants.

Behavioral rhythms of *Pdfr* mutants damp in DD and are accompanied by a shorter period (Table 2). We find that the short DD period phenotype of *Pdfr* mutants is rescued by *Clk4.1M*-GAL4 driven expression of PDFR (Table 2, Figure S3; Student's t-test $p<0.001$). We also observe significant improvement of rhythm amplitude with *Clk4.1M*-GAL4 driven expression of PDFR (Table 2, Figure S3; Student's t-test, $p<0.05$), but not with the more restricted *Clk4.5F*-GAL4 line. Taken together, these results indicate that PDFR expression in the DN1ps is sufficient for normal DD period and for improved rhythms.

PDFR Expression in the DN1ps of *Pdfr* Mutants Alters the DN1 Clock

Weakened free-running behavioral rhythms are accompanied by defects in the core molecular clock of circadian pacemaker neurons in *Pdf* or *Pdfr* null mutants [10-12,27]. On day 9 of DD (DD9), PER oscillation is phase-dispersed in the PDF(+) s-LNvs and phase-advanced with reduced amplitude in the LNds [10]. PER oscillations are damped in the DN1ps during DD [11,12]. To test whether DN1p expression of PDFR in *Pdfr* mutants suppresses the molecular phenotypes in circadian pacemaker neurons, we assayed PER oscillation in the s-LNvs, LNds and DN1s of $Pdfr^{han5304}$;;UAS-*Pdfr*/+ and $Pdfr^{han5304}$;;*Clk4.1M*-GAL4/UAS-*Pdfr* flies by performing PER immunolabeling at different time points on DD9. Here we observe a weak PER oscillation ($p<0.01$) in the DN1s of $Pdfr^{han5304}$;;UAS-*Pdfr*/+ flies that peaks at Circadian Time 12 (CT12, i.e. 12 hours after subjective lights-on), whereas in $Pdfr^{han5304}$;;*Clk4.1M*-GAL4/UAS-*Pdfr* flies, PER levels peak between CT18 and CT1 in the DN1s (Figures 4A and 4C). The phase change is consistent with the observation that *Clk4.1M*-GAL4 drives expression in the DN1ps and that PDFR function in the DN1ps sustains and resets PER oscillations [11]. In contrast to the DN1s, we observe only modest changes in PER oscillations in the s-LNvs and LNds (Figures 4B and 4C). In DN1p rescue flies, there is a change in the s-LNv rhythm and a phase delay in LNd rhythms, which may reflect coupling between the DN1ps and LNs (Figures 4B and 4C). Indeed, the DN1ps are in close vicinity to the PDF(+) s-LNv dorsal projections [28] and the LNd (Figure 1). Ultrastructural studies have identified potential sites of synaptic inputs on these dorsal projections, suggesting they could receive DN1p inputs [29]. Behavioral studies have also suggested reciprocal feedback from non-PDF neurons to

NIH-PA Author Manuscript

PDF neurons [5,7]. Overall, these results suggest that PDFR expression in the DN1ps of *Pdfr* mutants alters the molecular clock of DN1 pacemaker neurons, further supporting the role of this receptor in this neuronal subset.

The DN1 Clock is Highly Sensitive to PDF Resetting

Changing the period of PDF neural clocks in DD changes the speed of non-PDF oscillators, including the LNds, DN1s and DN3s [13], highlighting the master pacemaker role for PDF clocks. In this study, the molecular oscillations had been examined on DD4. To better understand the kinetics of this phase-resetting process, we changed the pace of PDF neuron clocks and examined how this affects the clock in other pacemaker neurons on day 2 of DD (DD2) by performing PER immunolabeling. Here we used GAL4 under the control of the *Pdf* promoter (*Pdf*-GAL4; [2]) to express RNAi directed against the beta subunit of the circadian kinase CK2 (UAS-*CK2βRNAi*; [4]). This manipulation lengthens DD behavioral period by ~6 hours. Consistent with the idea that DD period is determined by the PDF(+) s-LNvs [13], we find that PER oscillations in the PDF(+) s-LNvs of *Pdf*-GAL4,UAS-*CK2βRNAi*/+ peak at CT14 on DD2, which is ~12 hours out of phase with UAS-*CK2βRNAi*/+ controls (Figures 5A and 5C). This is consistent with a ~6 hour phase delay per day.

To examine the effects of the phase-delaying PDF(+) s-LNv clock on non-PDF pacemaker neurons, we focused on subsets of E-cells, including the LNds, PDF(-) s-LNv and DN1s. We find that PER oscillations in the LNds and PDF(-) s-LNv of *Pdf*-GAL4,UAS-*CK2βRNAi*/+ is comparable to that of the wild-type control (Figures 5A and 5C), i.e., these cells fail to synchronize to the slower s-LNv clocks. In contrast, the DN1 PER oscillation in *Pdf*-GAL4,UAS-*CK2βRNAi*/+ is damped (Figures 5B and 5C). One possibility is that conflicting signals from the s-LNv and LNd may result in desynchrony between subsets of DN1s. To test this hypothesis, we examined the variation of PER intensities within a cluster, where desynchronization would result in high variance between individual cells. However, we do not observe a larger within-cluster standard deviation among the DN1s of *Pdf*-GAL4,UAS-*CK2βRNAi* compared to UAS-*CK2βRNAi/*+ flies (Table S2; Materials and Methods). Thus, the lack of evident PER oscillations in the DN1s is likely the result of a damped PER oscillations at the single cell level. Consistently, we observe damped behavioral rhythms by DD2, in *Pdf*-GAL4/UAS-*CK2βRNAi* flies (Figures S4A and S4B), consistent with a role for the DN1 clock in sustaining DD rhythmicity. Taken together, the DN1s are more sensitive than the LNd and PDF(-) s-LNv to the period change in the PDF(+) s-LNv.

To further investigate the sensitivity of DN1 clocks to PDF neurons, we expressed a temperature-sensitive dominant-negative dynamin *shibire*^{ts1} (*shi*^{ts1}) in PDF neurons. We previously showed that this manipulation results in a more modest ~2 hour longer DD behavioral period [30]. We hypothesized that *shi* modulates feedback to regulate the pace of the core clock. In order to accumulate a sizable phase shift in the molecular oscillations of pacemaker neurons that would be easily detectable by PER immunolabeling, we examined PER oscillations in *Pdf*-GAL4/+ and *Pdf*-GAL4/+;UAS-*shi*^{ts1}/+ flies on DD3. We find that PER oscillations in the PDF(+) s-LNvs of *Pdf*-GAL4/+;UAS-*shi*^{ts1}/+ are approximately 4-8 hours delayed compared to *Pdf*-GAL4/+ (Figures S5A and S5C), consistent with ~2 hour phase delay per day. The DN1 clock also appears to be phase delayed with peak/trough shifted by 4-8 hours (Figures S5B and S5C), whereas the LNd clock does not exhibit a clear delay (Figures S5A and S5C). Taken together, the *shi*^{ts1} data provide further independent evidence of the sensitivity of the DN1 clocks to PDF neuron phase-resetting signals, in contrast to those of the LNds and PDF(-) s-LNv.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

Discussion

We have demonstrated here that the DN1p pacemaker cells coordinate both circadian signals and light information to regulate diurnal activity in *Drosophila*. Using novel GAL4 drivers that target the DN1ps, we find that *na* expression selectively in the DN1ps is sufficient to rescue the acute masking response, morning anticipation, and partially rescue DD1 evening behavior. These same neurons are likely direct targets of PDF, as *Pdfr* expression in the DN1ps is sufficient to rescue morning anticipatory behavior and DD period phenotypes of *Pdfr* mutants. Consistent with behavioral rescue, we also observe changes in molecular oscillations of the DN1s of *Pdfr* mutants expressing PDFR in the DN1ps relative to *Pdfr* mutant controls. Changing the pace of PDF-neuron clocks in DD affects the DN1 clock but much less so the clocks in the other E-cell subsets [i.e., the LNds and PDF(-) s-LNv], suggesting that the DN1s are more sensitive to PDF signaling than other E-cell subsets.

While considerable efforts have focused on the role of circadian clocks in regulating daily behavior, relatively little is known about the neural substrates of masking and even less about the central brain targets mediating photic responses. Using rescue of *na* mutants, we identify a small cluster of ~16-20 DN1p neurons that play a critical role in mediating acute response to light onset. Notably, these neurons are also identifiable by their expression of the transcription factor GLASS (GL), which is important for the development and/or maintenance of photoreceptor cells and the DN1ps [28,31,32]. *gl* mutants also exhibit masking phenotypes with suppressed activity immediately following lights-on, similar to *na* mutants. While the visual system is likely an important contributor to masking response [18], the masking phenotypes of *gl* mutants are reported to be more severe than loss of the visual system alone [12,32]. Our data suggest that the loss of the DN1p in *gl* mutants could account for this difference. Given that the DN1p are GL expressing, it raises the possibility that the DN1p themselves may be directly photoreceptive, perhaps through the DN1 expressed blue light photoreceptor CRYPTOCHROME (CRY) [33-35] or other opsin based systems [25]. Consistent with the former (CRY), PDF-dependent molecular defects are especially evident in the CRY+ subset of the DN1p and here we demonstrate *Clk4.1M*-GAL4 rescue of PER phase/ rhythms in the DN1 consistent with *Clk4.1M*-GAL4 expression in this CRY+ subset. It is also been observed that *Clk4.1M*-GAL4 drives expression at least in the CRY+ subset of DN1p neurons[36]. Nonetheless, the masking defects present in *na* mutants are more severe than those observed in *cry* mutants suggesting that multiple or other photoreceptors are involved.

In addition to the role of the DN1p in mediating acute light responses, we also demonstrate a role for the DN1p in mediating morning anticipation. Using DN1p-specific rescue of two independent mutants, *na* and *Pdfr*, we find rescue of their morning anticipation phenotypes. Prior reports had demonstrated a role for non-LNv clocks in driving behavior at the morning phase, but this was likely due to antiphase molecular oscillations in these cells[37,38]. In contrast, the DN1 of *Pdfr* mutants do not display an altered clock in LD [15], these studies further support the idea that PDF engages DN1p neural output to regulate morning anticipation. This idea is consistent with prior reports showing acute effects of PDF on neuronal excitability [39]. One intriguing possibility is that the connection between PDF and excitability may be mediated by NA as NA orthologs in mammals may function downstream of peptide receptors [40].

In contrast, we do not observe rescue of evening anticipation in *na* mutants in LD or of evening phase in *Pdfr* mutants. We previously had shown that *Pdfr* expression in E-cells [DN1, LNd, and the PDF(-) s-LNv] could rescue evening phase or anticipation in both *Pdfr* and *na* mutants [15,27]. Thus, our data suggest that either expression in *Clk4.1M*-GAL4 defined DN1p is not sufficient or that expression in the non-*Clk4.1M*-GAL4-expressing E-cells mediates this behavior. Taken together, it is possible that the non-*Clk4.1M*-GAL4 E-cells [i.e., the non-

NIH-PA Author Manuscript

Clk4.1M-GAL4 DN1p, DN1a, the LNds, and the PDF(-) s-LNv] regulate the phase of evening anticipation, while the *Clk4.1M*-GAL4 DN1ps regulate morning anticipation and DD period.

In addition to PDF effects on DN1p output, we also observe PDFR resetting effects on DN1p clocks. We observe a DN1 clock with altered phase and amplitude in mutants relative to the DN1p rescue flies. PDF-dependent changes in the pace and rhythm of non-LNv clocks have been observed[11,41]. Published studies have demonstrated that molecular oscillations in the DN1ps damp in *Pdf* mutants [9,11,12], but these studies were performed earlier in DD (on or before day 5 of DD) and/or with only two-time point resolution. Here on DD9 with four-time point resolution, we observe a weak PER oscillation (~1.5 fold) in *Pdfr* mutants. Notably, crude phase is altered by ~12 hours in the DN1p PDFR rescue flies relative to the mutants, consistent with a ~1 hour shorter period in *Pdfr* mutants that is rescued by PDFR expression in the DN1ps over the 9 days of DD. These results suggest that improvement in period and rhythmicity observed in DN1p rescued *Pdfr* mutants may be based in part on changes in DN1p clocks.

To further examine the strength of the PDF/DN1p connection, we slowed the PDF neuron clock by expressing *CK2βRNAi* or *shi*^{ts1}, which lengthen the period of the clock by ~6 hours and ~2 hours, respectively, and assessed the downstream effects on different pacemaker neural clusters. We find that these manipulations do not substantially affect the clocks in the LNds and PDF(-) s-LNv, whereas it damps (in the case of *CK2βRNAi*) or phase-delays (in the case of *shi*^{ts1}) the clock in the DN1s. Consistent with the notion that the DN1 are PDF-sensitive is the finding that the LNd clocks cycle robustly for several days in DD in *Pdf*^{01} while the DN1p clocks quickly damp in DD [10-12]. It has been noted that changing the pace of PDF neuron clock by expression of the clock kinase SHAGGY (SGG) which shortens period by 3-4 hours results in phase-advanced clocks in both the LNds and DN1s by DD4 [13]. However, a closer examination of their data suggests that the LNd effects may lag those in the DN1, consistent with our observations. In addition, in the case of *shi*^{ts1} and *CK2βRNAi* we looked earlier (DD2 or DD3) and/or we more strongly affected clock speed (i.e., *CK2βRNAi*). Thus, we may have observed different clocks shifting at different rates in response to PDF neuron manipulations.

NIH-PA Author Manuscript

We observe a lack of PER oscillation in the DN1s of *Pdf*-GAL4,UAS-*CK2βRNAi*/+ flies. This is also consistent with studies in mammals showing that the oscillations in some peripheral tissues damp after being subjected to large, abrupt phase shifts [42]. At the same time, we observe reduced amplitude behavioral oscillations in these *Pdf*-GAL4,UAS-*CK2βRNAi*/+ flies, implicating a role for the DN1 clock and/or output in regulating DD behavior. Consistent with the DN1 specific effects observed in *Pdf*-GAL4,UAS-*CK2βRNAi*/+, we find that PER oscillation in the DN1s of *Pdf*-GAL4/+;UAS-*shi*^{ts1}/+ is phase delayed relative to the control. Thus, changing the pace of the PDF neuron clock changes the pace of the DN1 clock but not the LNd clock. Taken together, these results suggest that the molecular clock in the DN1s is more sensitive to PDF neuron/PDF signaling than that of the LNds and PDF(-) s-LNv, further supporting the notion that the DN1s are bona fide targets of the PDF neurons. It will be of interest to determine the molecular basis of the differential sensitivity to PDF resetting signals within the clock network.

Given the important role of the DN1p in light- and PDF-driven behaviors, we hypothesize that these two pathways may be linked. Considerable evidence has linked PDF, and especially the l-LNv to light-driven arousal [43-45]. These light-driven PDF effects may target the DN1p leading to elevated locomotor activity. However, flies lacking PDF still retain responses to lights-on, suggesting a role for PDF-independent pathways in acute light responses.

NIH-PA Author Manuscript

One possibility is that clock-driven PDF effects and/or the DN1 clock might gate behavioral light responses. We find that in DN1p rescued *na* mutants, we observe a robust response to

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

lights on but a light-mediated suppression of evening anticipation, evidenced by comparing evening behavior in LD versus DD1 (Figure 2). We believe the clock, acting directly within the DN1s and/or LNv-derived PDF, may gate photic responses such that light in the morning stimulates activity while light in the evening suppresses activity. Notably, the clock in the larval s-LNv serves a similar gating function in mediating acute responses to light [46]. Consistent with this hypothesis are the robust lights-on masking defects observed in *Clk* and *cyc* mutants. In vertebrates, there is also a close link between circadian behavior and the direct effects of light on behavior. For example, diurnal and nocturnal animals have both anti-phase free-running rhythms and opposing behavioral responses to light [47]. Given the close conservation in the circadian systems between flies and mammals, it will be of interest to determine the anatomical loci of light and clock signaling in higher organisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Patrick Emery for communication of results prior to publication and Michael Rosbash for providing the PER antibody. We thank Adam Seluzicki for help with larval brain dissections. This work was supported by NIH grants R01NS059042 and R01NS052903 to R.A. and R01NS51280 to P.E.H., National Research Service Award F31NS062551 to B.Y.C. from the National Institute of Neurological Disorders and Stroke, and NIH Pathway to Independence Award K99GM080107 to B.C.L. from the National Institute of General Medical Sciences.

References

1. Zheng X, Sehgal A. Probing the relative importance of molecular oscillations in the circadian clock. Genetics 2008;178:1147–1155. [PubMed: 18385110]
2. Renn SC, Park JH, Rosbash M, Hall JC, Taghert PH. A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in Drosophila. Cell 1999;99:791–802. [PubMed: 10619432]
3. Hyun S, Lee Y, Hong ST, Bang S, Paik D, Kang J, Shin J, Lee J, Jeon K, Hwang S, et al. Drosophila GPCR Han is a receptor for the circadian clock neuropeptide PDF. Neuron 2005;48:267–278. [PubMed: 16242407]
4. Lear BC, Merrill CE, Lin JM, Schroeder A, Zhang L, Allada R. A G protein-coupled receptor, groom-of-PDF, is required for PDF neuron action in circadian behavior. Neuron 2005;48:221–227. [PubMed: 16242403]
5. Stoleru D, Peng Y, Agosto J, Rosbash M. Coupled oscillators control morning and evening locomotor behaviour of Drosophila. Nature 2004;431:862–868. [PubMed: 15483615]
6. Grima B, Chelot E, Xia R, Rouyer F. Morning and evening peaks of activity rely on different clock neurons of the Drosophila brain. Nature 2004;431:869–873. [PubMed: 15483616]
7. Rieger D, Wulbeck C, Rouyer F, Helfrich-Forster C. Period gene expression in four neurons is sufficient for rhythmic activity of Drosophila melanogaster under dim light conditions. J Biol Rhythms 2009;24:271–282. [PubMed: 19625729]
8. Mertens I, Vandingenen A, Johnson EC, Shafer OT, Li W, Trigg JS, De Loof A, Schoofs L, Taghert PH. PDF receptor signaling in Drosophila contributes to both circadian and geotactic behaviors. Neuron 2005;48:213–219. [PubMed: 16242402]
9. Peng Y, Stoleru D, Levine JD, Hall JC, Rosbash M. Drosophila free-running rhythms require intercellular communication. PLoS Biol 2003;1:E13. [PubMed: 12975658]
10. Lin Y, Stormo GD, Taghert PH. The neuropeptide pigment-dispersing factor coordinates pacemaker interactions in the Drosophila circadian system. J Neurosci 2004;24:7951–7957. [PubMed: 15356209]
11. Yoshii T, Wulbeck C, Sehadova H, Veleri S, Bichler D, Stanewsky R, Helfrich-Forster C. The neuropeptide pigment-dispersing factor adjusts period and phase of Drosophila's clock. J Neurosci 2009;29:2597–2610. [PubMed: 19244536]

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

12. Klarsfeld A, Malpel S, Michard-Vanhee C, Picot M, Chelot E, Rouyer F. Novel features of cryptochrome-mediated photoreception in the brain circadian clock of Drosophila. J Neurosci 2004;24:1468–1477. [PubMed: 14960620]
13. Stoleru D, Peng Y, Nawathean P, Rosbash M. A resetting signal between Drosophila pacemakers synchronizes morning and evening activity. Nature 2005;438:238–242. [PubMed: 16281038]
14. Shafer OT, Kim DJ, Dunbar-Yaffe R, Nikolaev VO, Lohse MJ, Taghert PH. Widespread receptivity to neuropeptide PDF throughout the neuronal circadian clock network of Drosophila revealed by real-time cyclic AMP imaging. Neuron 2008;58:223–237. [PubMed: 18439407]
15. Lear BC, Zhang L, Allada R. The neuropeptide PDF acts directly on evening pacemaker neurons to regulate multiple features of circadian behavior. PLoS Biol 2009;7:e1000154. [PubMed: 19621061]
16. Rieger D, Fraunholz C, Popp J, Bichler D, Dittmann R, Helfrich-Forster C. The fruit fly Drosophila melanogaster favors dim light and times its activity peaks to early dawn and late dusk. J Biol Rhythms 2007;22:387–399. [PubMed: 17876060]
17. Helfrich-Forster C, Edwards T, Yasuyama K, Wisotzki B, Schneuwly S, Stanewsky R, Meinertzhagen IA, Hofbauer A. The extraretinal eyelet of Drosophila: development, ultrastructure, and putative circadian function. J Neurosci 2002;22:9255–9266. [PubMed: 12417651]
18. Rieger D, Stanewsky R, Helfrich-Forster C. Cryptochrome, compound eyes, Hofbauer-Buchner eyelets, and ocelli play different roles in the entrainment and masking pathway of the locomotor activity rhythm in the fruit fly Drosophila melanogaster. J Biol Rhythms 2003;18:377–391. [PubMed: 14582854]
19. Wheeler DA, Hamblen-Coyle MJ, Dushay MS, Hall JC. Behavior in light-dark cycles of Drosophila mutants that are arrhythmic, blind, or both. J Biol Rhythms 1993;8:67–94. [PubMed: 8490212]
20. Nash HA, Scott RL, Lear BC, Allada R. An unusual cation channel mediates photic control of locomotion in Drosophila. Curr Biol 2002;12:2152–2158. [PubMed: 12498692]
21. Rutila JE, Suri V, Le M, So WV, Rosbash M, Hall JC. CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of *Drosophila period* and *timeless*. Cell 1998;93:805–814. [PubMed: 9630224]
22. Allada R, White NE, So WV, Hall JC, Rosbash M. A mutant *Drosophila* homolog of mammalian *Clock* disrupts circadian rhythms and transcription of period and timeless. Cell 1998;93:791–804. [PubMed: 9630223]
23. Lu B, Liu W, Guo F, Guo A. Circadian modulation of light-induced locomotion responses in Drosophila melanogaster. Genes Brain Behav 2008;7:730–739. [PubMed: 18518924]
24. Shafer OT, Helfrich-Forster C, Renn SC, Taghert PH. Reevaluation of Drosophila melanogaster's neuronal circadian pacemakers reveals new neuronal classes. J Comp Neurol 2006;498:180–193. [PubMed: 16856134]
25. Veleri S, Brandes C, Helfrich-Forster C, Hall JC, Stanewsky R. A self-sustaining, light-entrainable circadian oscillator in the Drosophila brain. Curr Biol 2003;13:1758–1767. [PubMed: 14561400]
26. Lu B, Su Y, Das S, Liu J, Xia J, Ren D. The Neuronal Channel NALCN Contributes Resting Sodium Permeability and Is Required for Normal Respiratory Rhythm. Cell 2007;129:371–383. [PubMed: 17448995]
27. Lear BC, Lin JM, Keath JR, McGill JJ, Raman IM, Allada R. The ion channel narrow abdomen is critical for neural output of the Drosophila circadian pacemaker. Neuron 2005;48:965–976. [PubMed: 16364900]
28. Helfrich-Forster C. The neuroarchitecture of the circadian clock in the brain of Drosophila melanogaster. Microsc Res Tech 2003;62:94–102. [PubMed: 12966496]
29. Yasuyama K, Meinertzhagen IA. Synaptic connections of PDF-immunoreactive lateral neurons projecting to the dorsal protocerebrum of Drosophila melanogaster. J Comp Neurol 518:292–304. [PubMed: 19941354]
30. Kilman VL, Zhang L, Meissner RA, Burg E, Allada R. Perturbing dynamin reveals potent effects on the Drosophila circadian clock. PLoS ONE 2009;4:e5235. [PubMed: 19384421]
31. Moses K, Ellis MC, Rubin GM. The glass gene encodes a zinc-finger protein required by Drosophila photoreceptor cells. Nature 1989;340:531–536. [PubMed: 2770860]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

32. Helfrich-Forster C, Winter C, Hofbauer A, Hall JC, Stanewsky R. The circadian clock of fruit flies is blind after elimination of all known photoreceptors. Neuron 2001;30:249–261. [PubMed: 11343659]
33. Benito J, Houl JH, Roman GW, Hardin PE. The blue-light photoreceptor CRYPTOCHROME is expressed in a subset of circadian oscillator neurons in the Drosophila CNS. J Biol Rhythms 2008;23:296–307. [PubMed: 18663237]
34. Hao Z, Ng F, Yixiao L, Hardin PE. Spatial and circadian regulation of cry in Drosophila. J Biol Rhythms 2008;23:283–295. [PubMed: 18663236]
35. Yoshii T, Todo T, Wulbeck C, Stanewsky R, Helfrich-Forster C. Cryptochrome is present in the compound eyes and a subset of Drosophila's clock neurons. J Comp Neurol 2008;508:952–966. [PubMed: 18399544]
36. Zhang Y. Light and temperature control the contribution of a subset of Dorsal Neurons 1 to Drosophila circadian behavior. Curr Biol. 2010
37. Zhang L, Lear BC, Seluzicki A, Allada R. The CRYPTOCHROME photoreceptor gates PDF neuropeptide signaling to set circadian network hierarchy in Drosophila. Curr Biol 2009;19:2050–2055. [PubMed: 19913424]
38. Cusumano P, Klarsfeld A, Chelot E, Picot M, Richier B, Rouyer F. PDF-modulated visual inputs and cryptochrome define diurnal behavior in Drosophila. Nat Neurosci 2009;12:1431–1437. [PubMed: 19820704]
39. Schneider NL, Stengl M. Pigment-dispersing factor and GABA synchronize cells of the isolated circadian clock of the cockroach Leucophaea maderae. J Neurosci 2005;25:5138–5147. [PubMed: 15917454]
40. Lu B, Su Y, Das S, Wang H, Wang Y, Liu J, Ren D. Peptide neurotransmitters activate a cation channel complex of NALCN and UNC-80. Nature 2009;457:741–744. [PubMed: 19092807]
41. Choi C, Fortin JP, McCarthy E, Oksman L, Kopin AS, Nitabach MN. Cellular dissection of circadian peptide signals with genetically encoded membrane-tethered ligands. Curr Biol 2009;19:1167–1175. [PubMed: 19592252]
42. Yamazaki S, Numano R, Abe M, Hida A, Takahashi R, Ueda M, Block GD, Sakaki Y, Menaker M, Tei H. Resetting central and peripheral circadian oscillators in transgenic rats. Science 2000;288:682–685. [PubMed: 10784453]
43. Shang Y, Griffith LC, Rosbash M. From the Cover: Feature Article: Light-arousal and circadian photoreception circuits intersect at the large PDF cells of the Drosophila brain. Proc Natl Acad Sci U S A 2008;105:19587–19594. [PubMed: 19060186]
44. Chung BY, Kilman VL, Keath JR, Pitman JL, Allada R. The GABA(A) receptor RDL acts in peptidergic PDF neurons to promote sleep in Drosophila. Curr Biol 2009;19:386–390. [PubMed: 19230663]
45. Sheeba V, Fogle KJ, Kaneko M, Rashid S, Chou YT, Sharma VK, Holmes TC. Large Ventral Lateral Neurons Modulate Arousal and Sleep in Drosophila. Curr Biol. 2008
46. Mazzoni EO, Desplan C, Blau J. Circadian pacemaker neurons transmit and modulate visual information to control a rapid behavioral response. Neuron 2005;45:293–300. [PubMed: 15664180]
47. Dardente H, Menet JS, Challet E, Tournier BB, Pevet P, Masson-Pevet M. Daily and circadian expression of neuropeptides in the suprachiasmatic nuclei of nocturnal and diurnal rodents. Brain Res Mol Brain Res 2004;124:143–151. [PubMed: 15135222]

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

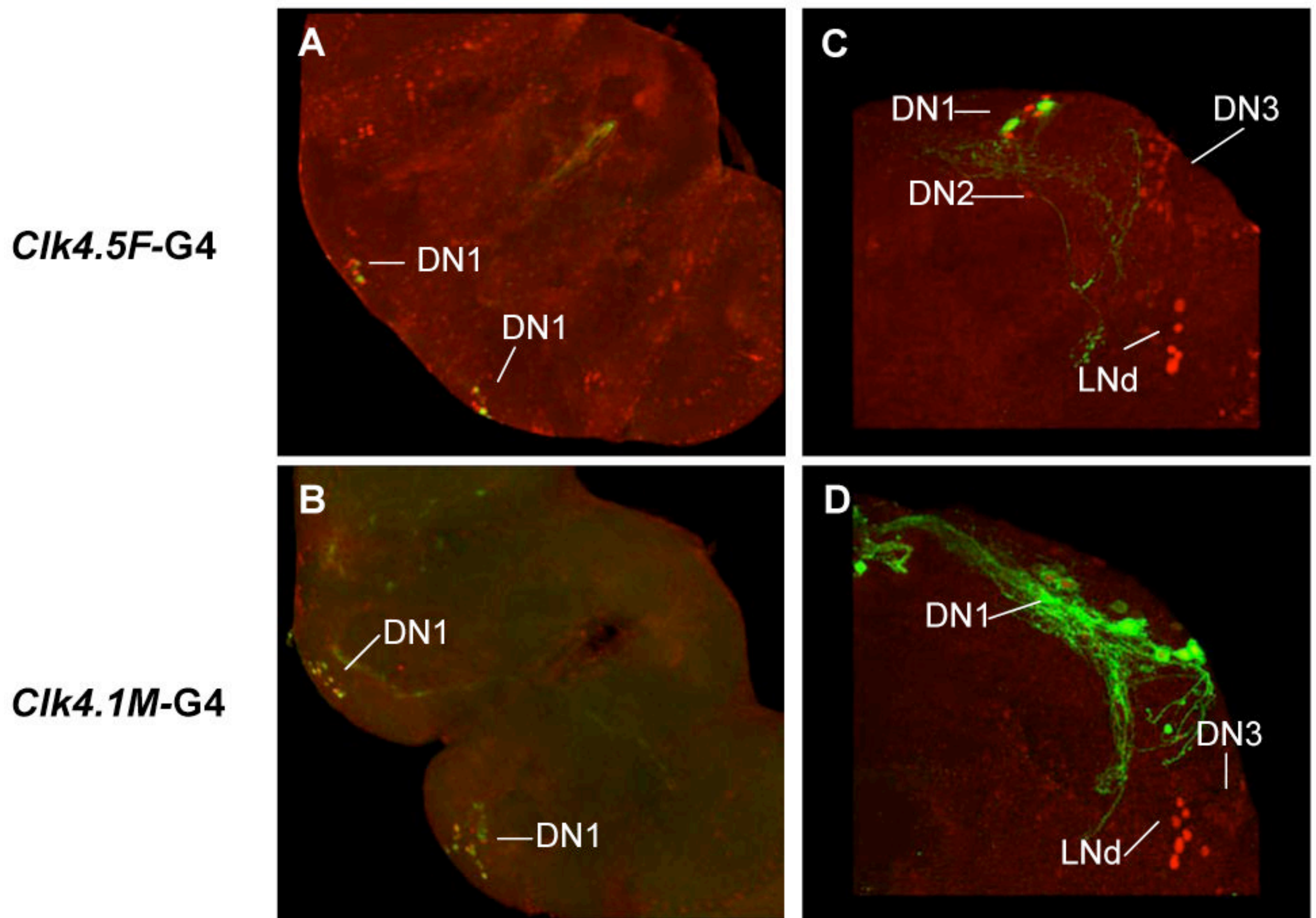

Figure 1. Expression Pattern of two DN1p Specific *Clk*-GAL4 Lines

(A and B) Maximum projections of confocal sections taken in representative adult *Clk4.5F*-GAL4/UAS-*nGFP* (A, green) and *Clk4.1M*-GAL4/UAS-*nGFP* (B, green) brains labeled with PER antibody (red). (C and D) Maximum projections of confocal sections taken in representative adult UAS-*mGFP*/+;*Clk4.5F*-GAL4/+ (C) and UAS-*mGFP*/+;*Clk4.1M*-GAL4/+ (D) brains labeled with GFP (green) and PER antibody (red). The DN1s are indicated by lines.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

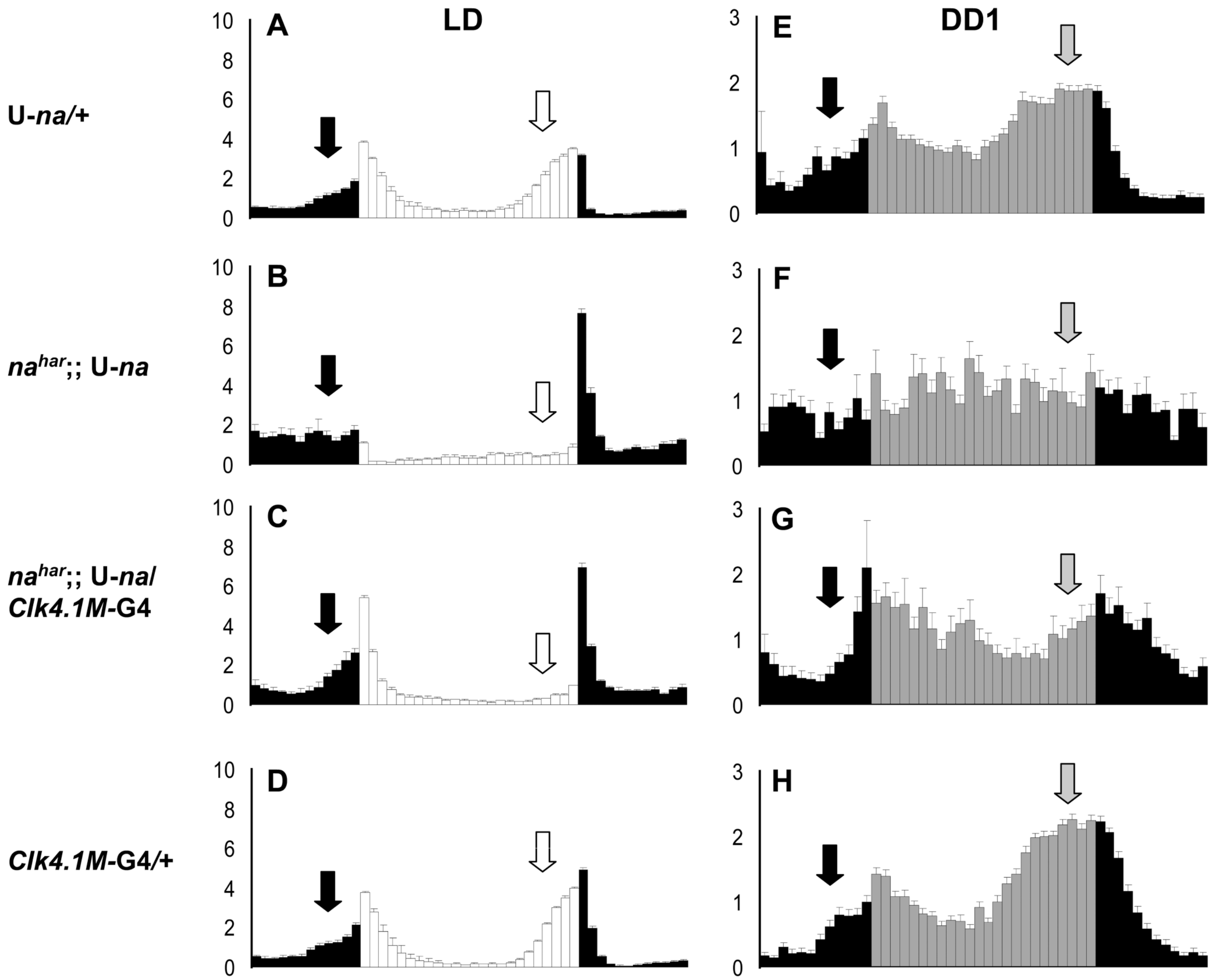

Figure 2. Rescue of *na* Expression in a Subset of DN1 Cells Restores Morning Anticipation, Lights-On Arousal Response, and DD1 Evening Anticipation

Normalized activity plots for adult male populations averaged across either (A-D) 4 days of LD or (E-H) the first day of DD. For (A-D), light and dark phases are indicated by horizontal white and black bars, respectively. For (E-H), subjective light and dark phases are indicated by horizontal gray and black bars, respectively. White arrows indicate LD evening behavior, gray arrows indicate DD1 evening behavior, and black arrows indicate morning behavior. Genotypes shown are (A,E) na^{har};;UAS-*na*/*Clk4.1M*-GAL4, (B,F) na^{har};;UAS-*na*/+, (C,G) UAS-*na*/+, and (D,H) *Clk4.1M*-GAL4/+. n=49-76. Error bars indicate standard error of mean (SEM). See also figure S2 for additional data.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

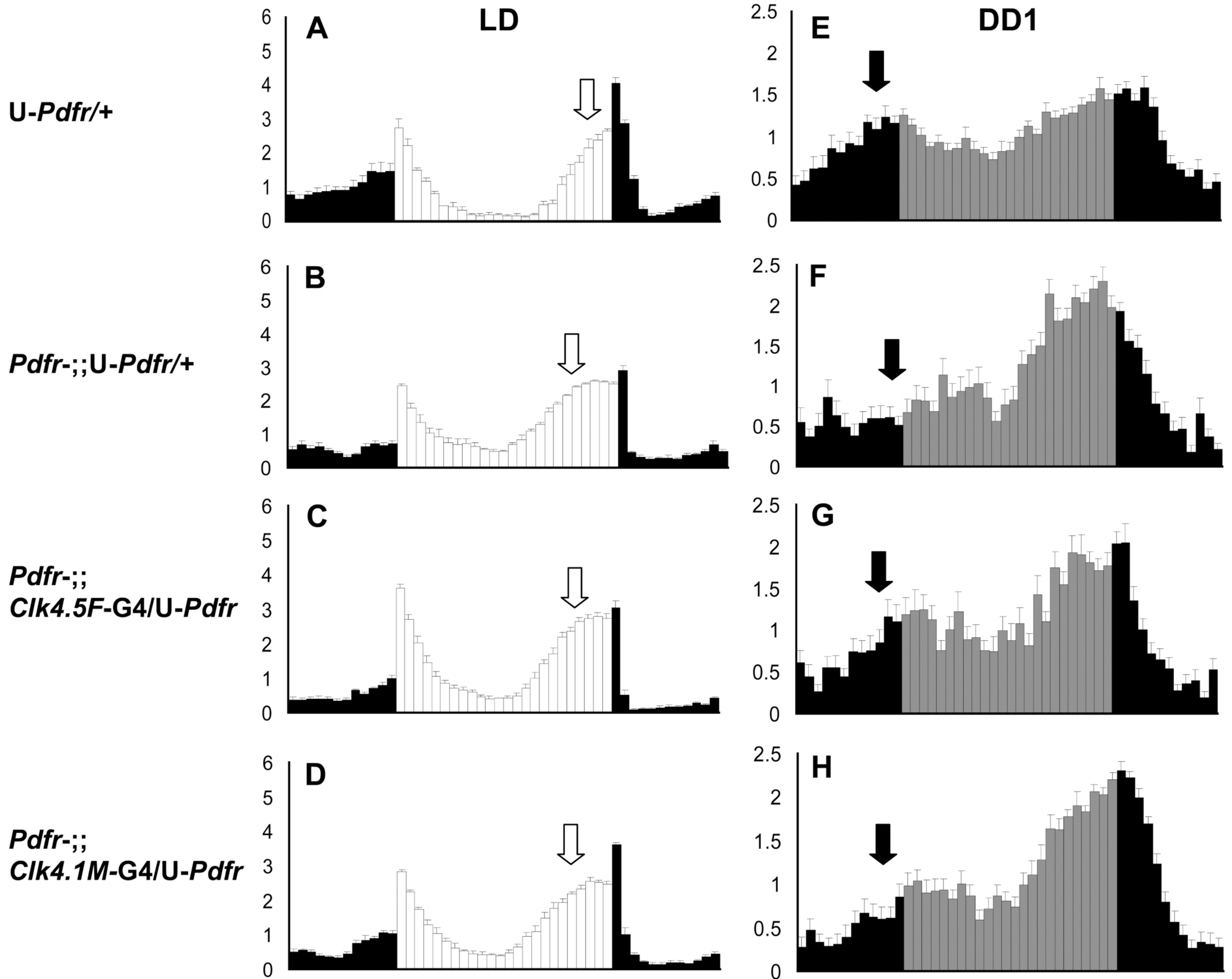

Figure 3. PDFR Expression in the DN1ps is Sufficient for Robust Morning Anticipatory Behavior
(A-D) Normalized activity plots for adult male populations averaged over four days of 12 hour light: 12 hour dark entrainment. The light phase is indicated by white bars, while the dark phase is indicated by black bars. (E-H) Normalized activity plots of adult male populations over the last 6 hours of LD (ZT18-CT0) followed by the first 18 hours of DD (CT0-18). Subjective light phase (CT0-12) is indicated by dark gray bars while subjective dark phase is indicated by black bars. (A and E) UAS-*Pdfr*/+; (B and F) *Pdfr*^{han5304};;UAS-*Pdfr*/+; (C and G) *Pdfr*^{han5304};;*Clk4.5F*-GAL4/UAS-*Pdfr*; (D and H) *Pdfr*^{han5304};;*Clk4.1M*-GAL4/UAS-*Pdfr*. Error bars represent standard error of the mean (n=25-53). White arrows indicate evening behavior and black arrows indicate morning behavior. See also figure S2 and table S1 for additional data.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

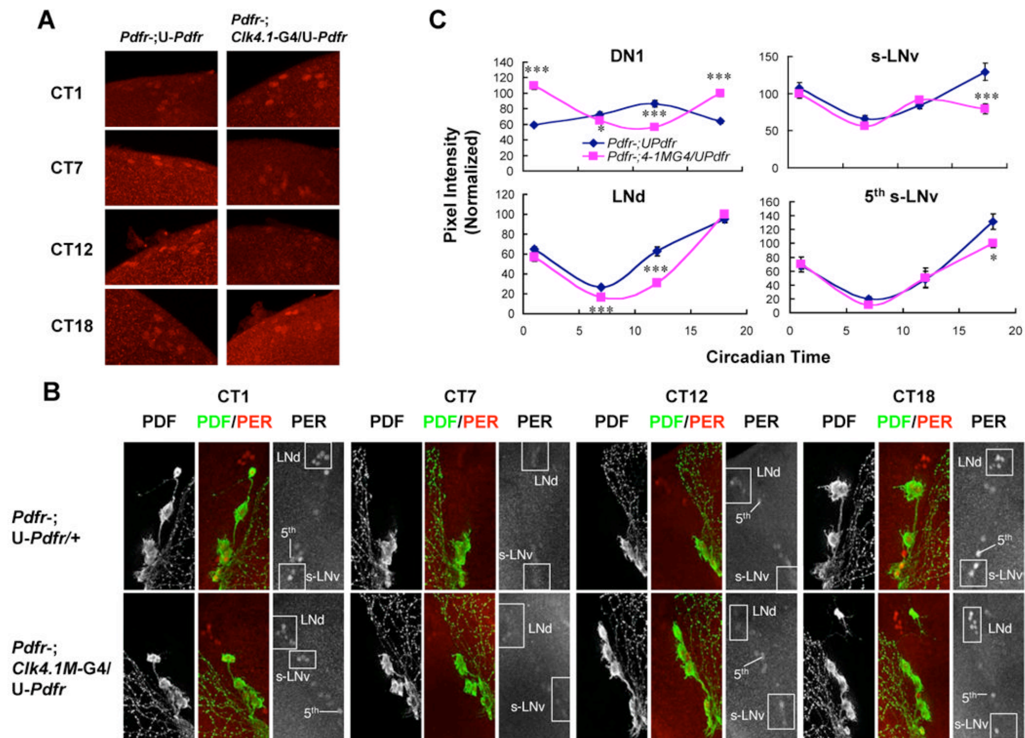

Figure 4. PDFR Expression in the DN1ps Alters the Molecular Oscillation in the DN1s of *Pdfr* Mutants

(A) Maximum projections of confocal sections taken in representative adult *Pdfr*^{han5304};;UAS-*Pdfr*/+ and *Pdfr*^{han5304};;*Clk4.1M*-GAL4/UAS-*Pdfr* brains labeled with PER antibody. Sections contain the DN1s at CT1, 7, 12, and 18. (B) Maximum projections of confocal sections taken in representative adult *Pdfr*^{han5304};;UAS-*Pdfr*/+ and *Pdfr*^{han5304};;*Clk4.1M*-GAL4/UAS-*Pdfr* brains labeled with with PER and PDF antibodies. Sections contain the LNs at CT1, 7, 12, and 18. The PDF(+) s-LNvs and LNds are in boxes, while the 5th s-LNv is indicated by line. (C) Plots of average normalized pixel intensity versus circadian time for each pacemaker cell group. See experimental procedures for details of quantification method. Error bars represent standard error of mean. The results are a combination of two independent experiments: s-LNv, n=32-57; LNd, n=56-91; 5th s-LNv, n=8-17; DN1, n=143-226. Asterisks mark significant differences between genotypes (Student's t-test, *$p<0.05$, **$p<0.01$, ***$p<0.001$).

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

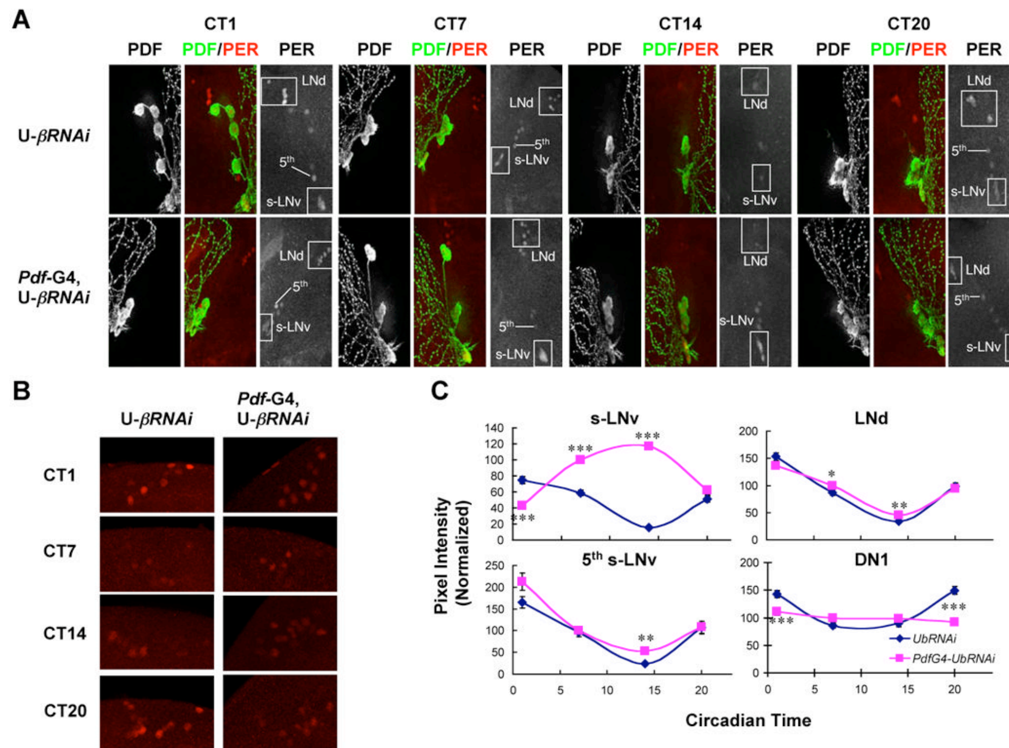

Figure 5. Manipulating the Clock in the PDF(+) s-LNvs Alters the Clock in the DN1s but Not the Other E-cell Subsets

(A and B) Maximum projections of confocal sections taken in representative adult UAS-*CK2βRNAi*/+ and *Pdf*-GAL4,UAS-*CK2βRNAi*/+ brains labeled with with PER and PDF antibodies. Sections contain the LNs at CT1, 7, 14, and 20. The PDF(+) s-LNvs and LNds are in boxes, while the 5th s-LNv is indicated by line. (B) Maximum projections of confocal sections taken in representative adult UAS-*CK2βRNAi*/+ and *Pdf*-GAL4,UAS-*CK2βRNAi*/+ brains labeled with PER antibody. Sections contain the DN1s at CT1, 7, 14, and 20. (C) Plots of average normalized pixel intensity versus circadian time for each pacemaker cell group. See experimental procedures for details of quantification method. Error bars represent standard error of mean. The results are a combination of three independent experiments: s-LNv, n=32-76; LNd, n=54-94; 5th s-LNv, n=10-18; DN1, n=104-189. Asterisks mark significant differences between genotypes (Student's t-test, *$p<0.05$, **$p<0.01$, ***$p<0.001$). See also figures S4 and S5 and table S2 for additional data.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

Table 1

DN1p *na* Rescue LD Masking and DD Circadian Phenotypes Observed

Genotype	AMI	SMI	DD1 Morning Index	Period (hr)	Power	%R
UAS-*na*/+	0.40 ± 0.02	-0.11 ± 0.05	1.04 ± 0.11	24.1 ± 0.0	55 ± 4	93
na^{har}; ; UAS-*na*/+	-0.37 ± 0.04	-0.53 ± 0.04	-0.07 ± 0.13	27.0	2 ± 1	3
na^{har}; ; UAS-*na/Clk4.1M*-GAL4	0.27 ± 0.04	-0.51 ± 0.04	1.38 ± 0.27	25.7 ± 0.0	4 ± 2	11
Clk4.1M-GAL4/+	0.34 ± 0.03	-0.25 ± 0.06	1.08 ± 0.07	23.8 ± 0.0	99 ± 4	100
UAS-*na*/*Clk4.1M*-GAL4	0.38 ± 0.02	-0.36 ± 0.04	0.97 ± 0.07	23.9 ± 0.0	69 ± 3	94

Acute masking index (AMI), sustained masking index (SMI), first day of DD morning anticipation index (DD1 Morning Index), DD free-running period (Period), DD rhythmic power (Power), and percent of flies with detectable DD rhythmicity (%R) values were calculated as described in experimental procedures. All values are shown as “average ± SEM”. n=38-87.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Table 2

DN1p PDFR Rescue DD Circadian Phenotypes

Genotype	DD1 Morning Index	Period (hr)	Power	%R
UAS-*Pdfr*/+	0.74 ± 0.14	23.9 ± 0.0	76 ± 9	92
$Pdfr^{han5304}$; ; UAS-*Pdfr*/+	0.30 ± 0.14	23.2 ± 0.1	25 ± 4	65
$Pdfr^{han5304}$; ; *Clk4.5F*-GAL4/UAS-*Pdfr*	0.76 ± 0.15	23.2 ± 0.1	14 ± 2	41
$Pdfr^{han5304}$; ; Clk*4.1M*-GAL4/UAS-*Pdfr*	0.66 ± 0.09	23.8 ± 0.2	51 ± 9	81
Clk4.5F-GAL4/UAS-*Pdfr*	0.67 ± 0.10	24.0 ± 0.0	70 ± 8	95
Clk4.1 M-GAL4/UAS-*Pdfr*	0.52 ± 0.13	23.9 ± 0.1	72 ± 14	80

First day of DD morning anticipation index (DD1 Morning Index), DD free-running period (Period), DD rhythmic power (Power), and percent of flies with detectable DD rhythmicity (%R) values were calculated as described in experimental procedures. All values are shown as “average ± SEM”. n=15-53. See also figure S3 for additional data.