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PDF-modulated visual inputs and cryptochrome define diurnal behavior in *Drosophila*

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Morning and evening circadian oscillators control the bimodal activity of *Drosophila* in light-dark cycles. The lateral neurons evening oscillator (LN-EO) is important for promoting diurnal activity at dusk. We found that the LN-EO autonomously synchronized to light-dark cycles through either the cryptochrome (CRY) that it expressed or the visual system. In conditions in which CRY was not activated, flies depleted for pigment-dispersing factor (PDF) or its receptor lost the evening activity and displayed reversed PER oscillations in the LN-EO. Rescue experiments indicated that normal PER cycling and the presence of evening activity relied on PDF secretion from the large ventral lateral neurons and PDF receptor function in the LN-EO. The LN-EO thus integrates light inputs and PDF signaling to control *Drosophila* diurnal behavior, revealing a new clock-independent function for PDF.

Day-night cycles entrain endogenous circadian clocks to synchronize living organisms with the solar day. In most animals, a brain clock controls rest-activity rhythms[1]. In laboratory light-dark conditions, *Drosophila melanogaster* adults display morning and evening activity peaks anticipating lights on and lights off, whereas they mostly rest at night and take a mid-day nap that is more pronounced in males. The evening anticipating activity is usually more prominent than the morning one, thus defining *Drosophila* as a diurnal animal. The position of this evening activity depends on temperature and day length, with high temperature and long days inducing a temporal delay that allows the insects to escape from the heat of summer days[2–5].

The *Drosophila* brain clock that controls daily activity rhythms relies on a network of about 150 neurons that are organized into seven groups[6,7]. The lateral neurons are the best characterized clock neurons. In each brain hemisphere, the PDF-expressing (PDF^+) lateral neurons consist of four to five large ventral lateral neurons (l-LN_vs) and four small ventral lateral neurons (s-LN_vs), whereas the PDF-negative (PDF^-) lateral neurons consist of a single s-LN_v (the fifth s-LN_v) and six dorsal lateral neurons (LN_ds). All LN_vs, but only a subset of three LN_ds, express the blue light–sensitive protein CRY[8–12].

Morning and evening bouts of activity in light-dark are controlled by different neuronal groups[13]. Briefly, PDF^+ neurons control morning activity, whereas PDF^- neurons control evening activity in light-dark conditions[14,15]. Among the lateral neurons, the PDF^+ LN_vs drive free-running rhythms in constant darkness[14–16], whereas the CRY^+ PDF^- lateral neurons drive free-running rhythms in constant light, provided that CRY-dependent photoreception is diminished[10]. We previously defined a morning oscillator in the PDF^+ lateral neurons (LN-MO) and an evening oscillator in the CRY^+ PDF^- lateral neurons (LN-EO)[10].

Light-dark cycles entrain the activity rhythms through two light-input pathways. Flies with reduced or no CRY show altered clock responses to light[17–19]. Such responses are restored by CRY expression in the clock cells, suggesting that CRY signaling is cell autonomous[8], although non-autonomous synchronization also occurs[11,12,20]. The visual system, which includes the compound eye and the extra-retinal Hofbauer-Büchner eyelet, provides rhodopsin-dependent light inputs[4,21–23]. The behavioral clock becomes blind only when both CRY and the visual system are absent[9,24].

Flies with no PDF or no PDF receptor (PDFR) lose morning anticipation and display a 2–3 h advance of the evening activity[14,25–29]. In the absence of light, PDF^+ neurons are involved in the internal synchronization of the circadian network[9,29–34], but how PDF signaling influences the light entrainment of the evening activity is unknown.

We analyzed the contribution of CRY and the visual system pathways to the entrainment of the lateral neurons' morning and evening oscillators and their interaction with PDF signaling. We found that each of the two lateral neuron 5′ oscillators was able to use either CRY or the visual system to synchronize to light-dark cycles. The control of evening activity by the LN-EO involved two different light-input pathways: CRY in the LN-EO neurons and PDF-modulated visual system inputs. Our results indicate that the phase control of evening activity by PDF is a clock-independent function of the l-LN_vs.

RESULTS

Four PDF^- lateral neurons can drive evening activity

PER cycling restricted to the PDF^+ lateral neurons produces only morning activity, whereas PER cycling in all lateral neurons produces both morning and evening activity in light-dark cycles[14]. In addition, PER expression in only the l-LN_vs did not restore a morning peak[14]. To assay the s-LN_vs' contribution, we used the *R6-Gal4* line, in which Gal4 is expressed in the s-LN_vs but not the l-LN_vs[7]. *per^0; R6-Gal4/+; UAS-per16/+* flies showed morning anticipation that was abolished by inhibition of PER expression with *pdf-Gal80*, indicating that the s-LN_vs constitute an autonomous morning oscillator in light-dark cycles (**Fig. 1a** and **Supplementary Fig. 1**). We thus define the LN-MO as the s-LN_vs hereafter.

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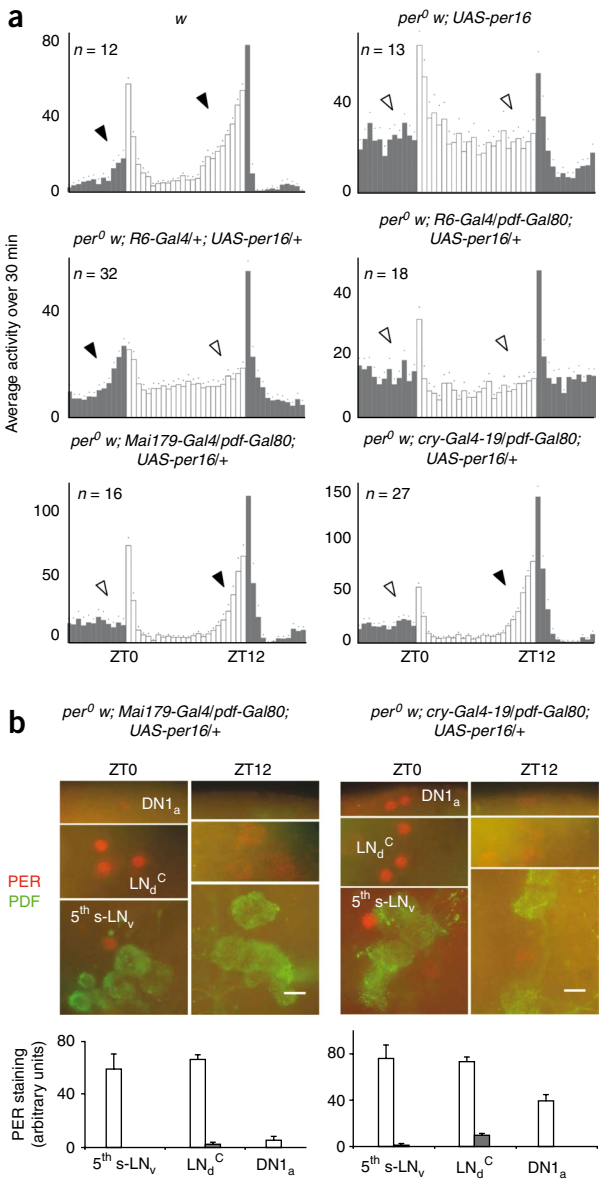

Figure 1 Characterization of the LN-MO and LN-EO. (**a**) Averaged light-dark locomotor activity profiles of LN-MO and LN-EO of PER-rescued flies. Top, wild-type and *per*^{0} controls. Middle, *R6-Gal4*–driven PER expression in the s-LN$_v$s (LN-MO only) with (right) or without (left) inhibition by *pdf-Gal80* in the PDF$^+$ LN$_v$s. Bottom, *Mai179-Gal4*–driven PER expression in PDF$^+$ LN$_v$s and PDF$^-$ lateral neurons inhibited by *pdf-Gal80* in the PDF$^+$ LN$_v$s (LN-EO only) (left) and *cry-Gal4-19*–driven PER expression in PDF$^+$ LN$_v$s, PDF$^-$ lateral neurons and DN1$_a$s inhibited by *pdf-Gal80* in the PDF$^+$ LN$_v$s (LN-EO + DN1$_a$s) (right). Full and empty arrowheads indicate the activity peaks that were present or absent, respectively, as judged from comparison with relevant controls (see also anticipation indexes in **Supplementary Fig. 1**). *n* represents the number of flies. (**b**) PER and PDF immunoreactivity of LN-EO PER-rescued flies in light-dark. Top, PER and PDF staining at ZT0 and ZT12. Scale bar represents 10 µm. Very weak PER labeling was detected in two DN1$_a$s (see ref. 6) of the *Mai179-Gal4* flies at ZT0, as described in the absence of *pdf-Gal80* (see Online Methods). CRY$^+$ LN$_d$s, LN$_d^C$. Bottom, PER quantification at ZT0 (white) and ZT12 (gray) for each neuronal subset. Error bars represent the s.e.m. ZT12 values are zero for the fifth s-LN$_v$ and DN1$_a$s in *Mai179-Gal4* flies (left) and for DN1$_a$s in *cry-Gal4-19* flies (right).

The autonomous ability of the PDF$^-$ lateral neurons (LN-EO) to generate evening activity was tested in flies expressing PER in the three CRY$^+$ LN$_d$s and the fifth PDF$^-$ s-LN$_v$ (see ref. 10). *per*^{0}*; Mai179-Gal4/pdf-Gal80; UAS-per16/+* flies showed a robust evening peak of locomotor activity in light-dark cycles (**Fig. 1a**), with strong PER labeling at Zeitgeber time 0 (ZT0) and faint PER at ZT12 in the four LN-EO cells, consistent with PER cycling (**Fig. 1b**). *per*^{0}*; cry-Gal4-19/pdf-Gal80; UAS-per16/+* flies had additional PER cycling in the two dorsal neuron 1$_a$ (DN1$_a$) neurons, but displayed a similar light-dark activity (**Fig. 1b**). PER cycling in only the LN-EO was thus sufficient to drive evening activity in light-dark.

Autonomous entrainment of morning and evening oscillators

Flies require either CRY or the visual system to synchronize to light-dark cycles[9,24], but how these two pathways specifically entrain the LN-MO and LN-EO is unknown. We tested flies expressing PER in the PDF$^+$ lateral neurons (LN-MO + l-LN$_v$s) or in the CRY$^+$ PDF$^-$ LN-EO, which were either depleted for CRY or in which the visual system was genetically ablated (**Fig. 2**, **Supplementary Fig. 1** and **Table 1**). In the absence of CRY (**Fig. 2a**) or the visual system (**Fig. 2b**), flies with PER in only the PDF$^+$ cells anticipated lights on, whereas flies with PER in only the CRY$^+$ PDF$^-$ cells anticipated lights off. This suggested that both the LN-MO and LN-EO oscillators could be entrained by either CRY or the visual system. However, the presence or absence of a peak in light-dark could reflect masking effects produced by light. The flies were thus released in constant conditions to determine whether the light-dark anticipations persisted as entrained circadian components. Flies with PER in the PDF$^+$ neurons are rhythmic in constant darkness[14] and were thus analyzed in such conditions. In the absence of either CRY (**Fig. 2a** and **Supplementary Table 2**) or the visual system (**Fig. 2b** and **Supplementary Table 2**), the light-dark morning peak persisted as a broader component in constant darkness. *cry*^{b} flies with PER in only the LN-EO are arrhythmic in constant darkness but are rhythmic in constant light[10]. These flies showed a broad constant light activity component that was clearly derived from the light-dark evening peak (**Fig. 2a** and **Supplementary Table 2**). The behavioral arrhythmicity of *cry*$^+$ flies in constant light prevented us from assaying the persistence of their evening component in constant conditions (**Fig. 2b** and **Supplementary Table 2**).

Finally, the transgenic flies were submitted to an 8-h shift of the light-dark schedule. Flies carrying either a functional LN-MO or a functional LN-EO were able to synchronize to the new light-dark schedule in the absence of CRY or of the visual system (**Supplementary Fig. 2**). We therefore conclude that both the LN-MO and the LN-EO can be entrained through either CRY or the visual system in the absence of any other functional clock neurons.

Visual system–mediated entrainment is modulated by PDF

Because PDF is known to slightly affect the phase of the evening activity in light-dark[25], we asked whether it could specifically control the phase of the evening oscillator when entrained through either CRY or the visual system. In light-dark cycles, *pdf*^{0} flies with or without a visual system showed a similar 2–3 h phase advance of the evening activity (**Fig. 3a** and **Supplementary Table 1**). In contrast, *pdf*^{0}*cry*^{b} double mutants displayed a very different activity pattern, with a complete disappearance of the evening peak. A similar phenotype was observed in *cry*^{b} flies in which the PDF$^+$ LN$_v$s were genetically ablated (**Fig. 3a**). However, we found entrained free-running rhythms and PER cycling in the LN-EO of *pdf*^{0}*cry*^{b} flies when they were transferred from light-dark to constant light[10]. To investigate this paradox, we carefully analyzed the phase of the *pdf*^{0}*cry*^{b} constant light free-running

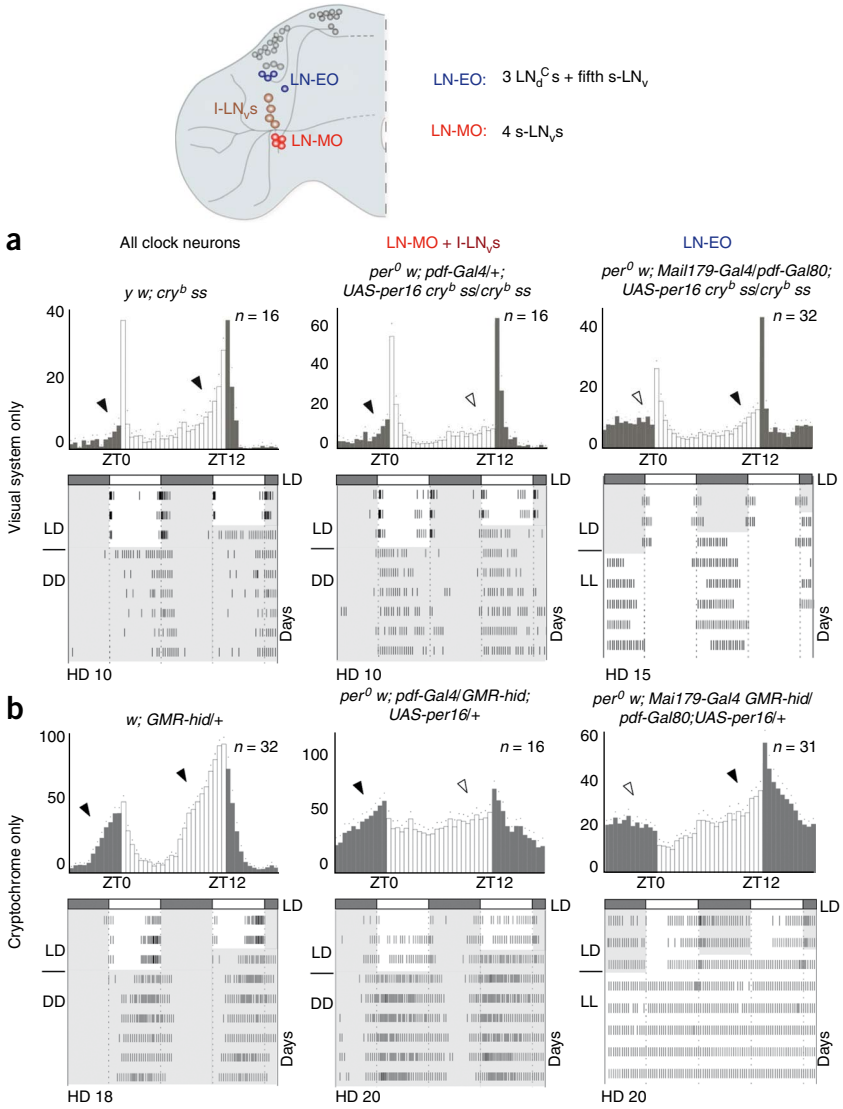

Figure 2 Light entrainment of flies lacking either CRY or the visual system. (**a**,**b**) CRY-deficient flies (**a**) and visual system–deficient flies (**b**). Top, averaged light-dark locomotor activity profiles of PER$^+$ (left), LN-MO (middle) or LN-EO (right) PER-rescued flies. Bottom, averaged actograms over 3 d of light-dark (LD) and 6 d of constant darkness (DD, shaded) or constant light (LL, white). White and gray bars above each panel indicate light and dark periods of the light-dark cycle, respectively. Hash density (HD) varied according to the genotype (see Online Methods) and true activity levels are reported in **Supplementary Table 2**. Full and empty arrowheads indicate the activity peaks that were present or absent, respectively (see also **Supplementary Fig. 1**). *n* represents the number of flies.

rhythms after light-dark entrainment (**Fig. 3b** and **Supplementary Tables 1** and **3**). Although *cry^b* mutants showed constant light activity originating from the light-dark evening peak, *pdf^{0}cry^b* double mutants showed an opposite phase, with the constant light activity now originating from the light-dark morning peak. *pdf^{0}cry^{01}* flies behaved exactly like *pdf^{0}cry^b* flies (data not shown). A similar morning phase was observed in constant light with *cry^b* flies ablated for the PDF$^+$ LN$_v$s, indicating that this rhythmic behavior was not generated by the LN-MO (**Fig. 3b**). The period shortening (about 22.5 h) observed in the absence of PDF or of PDF$^+$ cells was previously reported[10].

To confirm these results, we tested *pdf^0* mutants in red light, which does not activate CRY and fails to entrain flies depleted for long wavelength–sensitive rhodopsins[35]. Unlike wild-type flies, *pdf^0* flies did not show an evening peak in red light-dark cycles (**Supplementary Fig. 3**), which is consistent with the *pdf^{0}cry^b* phenotype in light-dark. We then compared *pdf^0* and wild-type flies that were first entrained under light-dark or red light-dark cycles and then released into constant red light, where both genotypes showed robust free-running rhythms. Wild-type flies displayed an evening phase in constant red light after both light-dark and red light-dark entrainment (**Supplementary Fig. 3** and **Table 3**). In contrast, *pdf^0* mutants showed an evening phase after light-dark entrainment and a morning phase after red light-dark entrainment (**Supplementary Fig. 3** and **Table 3**), indicating that the absence of both PDF and short-wavelength light (<600 nm) induced a phase reversal of the evening activity. Thus, PDF signaling is required to maintain evening activity in the absence of CRY, whereas it is not required for doing so in the absence of the visual system. In wild-type flies, the interaction between PDF and the visual system may be critical when environmental conditions do not sufficiently activate CRY. We asked whether very low white-light conditions could similarly affect the evening activity even in the presence of CRY. Low-light light-dark 12 h:12 h cycles at 20 °C did not strongly affect the evening activity of *pdf^0* mutants (**Supplementary Fig. 4**, compare with **Fig. 3a**). However, in winter-type conditions (light-dark 8:16 h at 15 °C), low light, but not bright light, induced the disappearance of evening activity in *pdf^0* mutants (**Supplementary Fig. 4**). Altogether, these results indicate that PDF controls the presence of evening activity through the visual system–mediated light entrainment when CRY signaling is absent or reduced. Notably, this PDF function is clock independent, as evening anticipation was observed in *cry^b* mutants with no PER in the LN$_v$s (**Fig. 2a**).

Antiphasic PER cycling in the LN-EO of *pdf^{0}cry^b* flies

To investigate the molecular basis of the absence of evening activity in *pdf^{0}cry^b* flies, we analyzed PER oscillations in light-dark in their clock neurons. In *pdf^0* and *cry^b* single mutants, LN-MO and LN-EO neurons had a wild-type PER oscillation peaking at ZT0–3, as did the DN1 neurons (**Fig. 4a**,**b**, **Supplementary Fig. 5** and **Supplementary Table 1**). As previously reported[24], the dorsal neuron 2 (DN2) neurons did not cycle in *cry^b* mutants. In *pdf^{0}cry^b* double mutants, LN-EO neurons showed robust, but completely phase reversed, PER oscillations, peaking at ZT15. The phase reversal was specific for the LN-EO, with LN-MO neurons still showing PER cycling with a peak at ZT0–3 and DN1–2 neurons showing no PER cycling (**Fig. 4c**, **Supplementary Fig. 5** and **Table 1**). Similar antiphasic PER oscillations in the LN-EO were observed in *cry^b* flies ablated for PDF$^+$ LN$_v$s (data not shown). In the absence of CRY and PDF, the LN-EO cycled with a phase that was opposite to that of wild-type flies, strongly suggesting that the

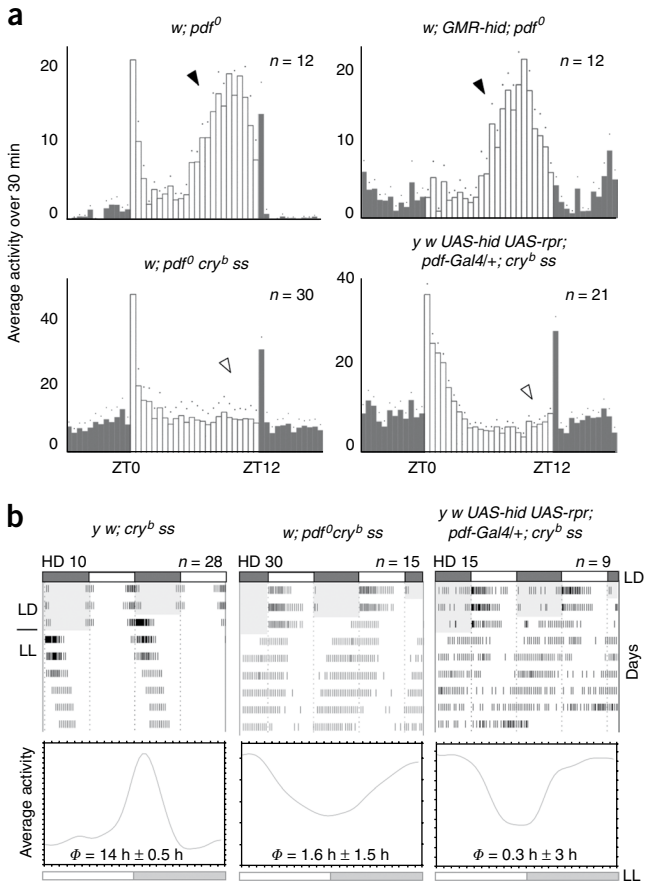

Figure 3 Light-dark activity and light entrainment of PDF-depleted flies. (**a**) Averaged locomotor activity profiles in light-dark 12:12 h, 20 °C and standard lighting conditions (see Online Methods). Full and empty arrowheads indicate the presence or absence, respectively, of the evening activity peak (see also **Supplementary Fig. 1**). (**b**) Top, averaged actograms over 3 light-dark days and 6 constant light days. Hash density varied according to the genotype and true activity levels are reported in **Supplementary Table 3**. Bottom, mean waveforms represent the averaged distribution of activity through a circadian cycle in constant light. The *x* axis corresponds to one whole period for the considered genotype, scaled to 24 h (that is, in circadian time; see **Supplementary Table 3**). Φ, mean phase of activity peak ± 95% confidence limit, in circadian time (see Online Methods). *n* represents the number of flies.

absence of evening activity in light-dark and reversed phase of free-running rhythms in constant light are a consequence of antiphasic PER cycling in the LN-EO. Thus, PDF controls the phasing of the LN-EO molecular clock when entrained by the visual system, in the absence of CRY signaling.

We then asked whether the phasing of PER oscillations by PDF requires *per* transcriptional control in the LN-EO. pdf^0 flies with *Gal4*-driven *per* expression in the LN-EO did not display evening activity in red light-dark cycles, as expected in the absence of both PDF and CRY signaling (**Supplementary Fig. 6**). In light-dark cycles, CRY signaling restored evening activity in these flies (**Supplementary Fig. 6**). Notably, PDF signaling was able to induce evening activity without *per* transcriptional control in the LN-EO in red light-dark cycles (**Supplementary Fig. 6**) or in the absence of CRY (**Fig. 2a**). This indicates that PDF does not set the phase of the visual system–entrained LN-EO through *per* transcription.

CRY and PDF signaling function in the LN-EO

To determine where CRY and PDF signaling were required to generate evening activity, we carried out CRY and PDFR rescue experiments. Because *Mai179-Gal4* expression was strongly downregulated in pdf^0 and han^{5304} (PDFR) mutants (P.C., E.C. and F.R., unpublished observations), we used *cry–Gal4-19* in the rescue experiments. Expression of a *cry* transgene was targeted to either the LN-MO or the LN-EO of pdf^0cry^b double mutants. CRY expression in the LN-EO restored evening activity (although slightly advanced, as expected in the absence of PDF), whereas CRY expression in the LN-MO did not (**Fig. 5a**, **Supplementary Fig. 1** and **Table 1**). CRY photoreception can therefore act autonomously in the LN-EO neurons to generate evening activity in the absence of PDF.

We then asked whether PDF signaling acts directly through PDFR in the LN-EO to control the evening activity in the absence of CRY (**Fig. 5b**, **Supplementary Fig. 1** and **Table 1**). As expected, han^{5304} mutants showed no evening anticipation in red light-dark cycles, which do not allow CRY activation. No restoration of the evening peak was observed when PDFR was expressed only in the LN-MO neurons, whereas a robust evening activity was induced when PDFR was expressed in both the LN-MO and LN-EO neurons. Although a contribution of the $DN1_a$s cannot be excluded, this strongly suggests that PDF signaling is required in the LN-EO to generate evening activity in the absence of CRY.

l-LN$_v$s control visual system–entrained evening activity

Both the s-LN$_v$s and l-LN$_v$s express PDF[36], but PER expression in the s-LN$_v$s is sufficient to induce morning activity (see above), whereas PER restricted to the l-LN$_v$s is not[14]. We asked whether the l-LN$_v$s could be involved in the phasing of evening activity by targeting PDF expression in pdf^0 mutants. In the brain, *pdf-Gal4* is expressed in the

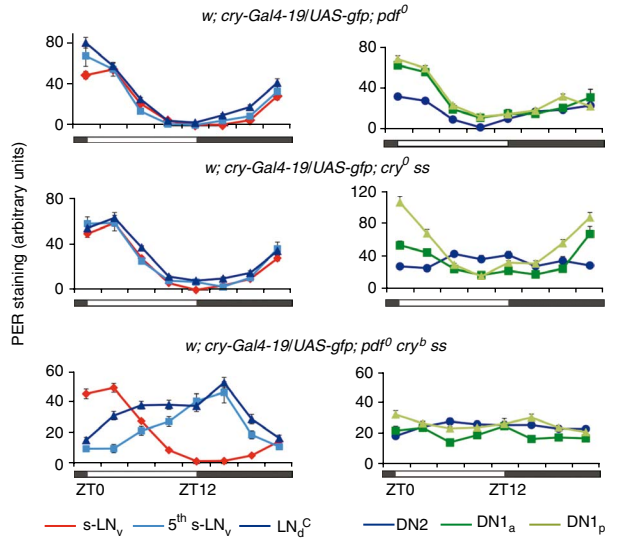

Figure 4 Oscillations of PER levels in the clock neurons in the absence of PDF and/or CRY. Flies were entrained in light-dark for 5 d and then dissected every 3 h. PER immunoreactivity was quantified in the LN-MO (red) and LN-EO (blue) (left), but also in the DN1 (green) and DN2 (blue) neurons (right). The different neuronal groups were identified by PDF and GFP expression, as well as by position in the brain. White and gray bars indicate light and dark periods of the light-dark cycle. pdf^0 mutants are shown in **a**, cry^b mutants in **b** and pdf^0cry^b mutants in **c**.

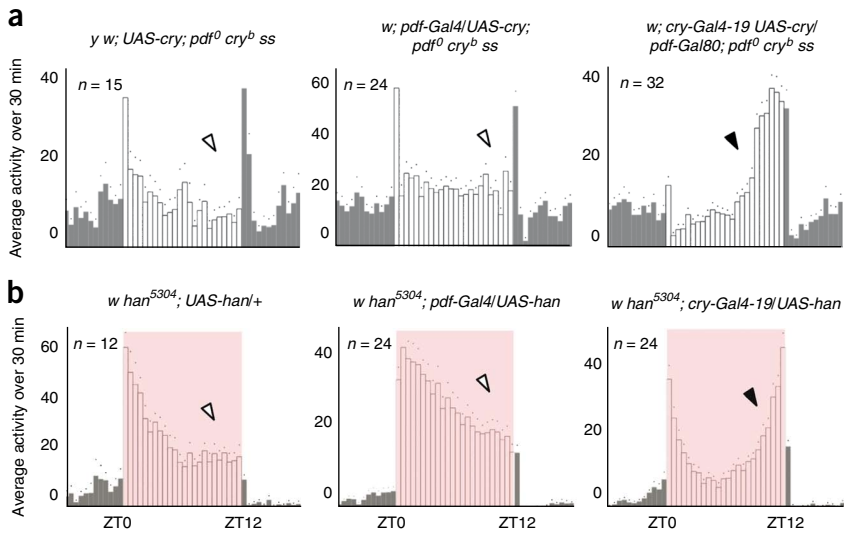

Figure 5 Averaged locomotor activity profiles of rescued pdf^0cry^b and *pdfr* mutants. Full and empty arrowheads indicate the presence or absence, respectively, of the evening activity peak (see also **Supplementary Fig. 1**). (**a**) Light-dark conditions. Control flies (left) and pdf^0cry^b mutants with CRY expression in the PDF$^+$ (middle) or PDF$^-$ (right) lateral neurons. (**b**) Red light-dark conditions. Controls (left) and han^{5304} mutants with PDFR expression in only the PDF$^+$ lateral neurons (middle) or in the PDF$^+$ lateral neurons and the LN-EO. Red shading indicates the red light phase in red light-dark. *n* represents the number of flies.

s-LN$_v$s and l-LN$_v$s[37], whereas *C929-Gal4* is expressed in the l-LN$_v$s and in nonclock cells, but not in the s-LN$_v$s[38].

As expected, *pdf-Gal4*–driven PDF expression restored evening activity in pdf^0cry^b flies (**Fig. 6a**, **Supplementary Fig. 1** and **Table 1**). The rescue was almost as strong with *C929-Gal4*–driven PDF expression and was further improved with two copies of both the *Gal4* and *UAS-pdf* transgenes (**Fig. 6a**, **Supplementary Fig. 1** and **Table 1**). No evening activity was observed when *pdf-Gal80* was used to inhibit PDF rescue in the l-LN$_v$s. These results indicated that PDF expression in the l-LN$_v$s is sufficient to restore evening activity in the pdf^0cry^b flies. They support PDF secretion by the l-LN$_v$s as being important for the control of evening activity (**Fig. 6b**).

DISCUSSION

Autonomous light entrainment of the lateral neuron oscillators

Recent work has shown that the brain clock that controls rest-activity rhythms in *Drosophila* relies on several groups of clock neurons with different behavioral functions[13]. In light-dark conditions, PER expression in the PDF$^+$ neurons (LN-MO) restores a morning peak of activity to per^0 flies, whereas additional PER expression in the four CRY$^+$ PDF$^-$ lateral neurons (LN-EO, see ref. 10) restores both morning and evening activity peaks[14]. We restricted this LN-MO to the four PDF$^+$ s-LN$_v$s and found that the LN-EO autonomously drives evening activity in light-dark conditions.

The brain clock can use either CRY or the visual system to synchronize to light-dark cycles[9,24]. Our light-dark experiments indicate that flies with PER in only the PDF$^+$ lateral neurons or in only the LN-EO neurons can use CRY and the visual system to reset their single morning or evening peak, respectively. Both oscillators thus have autonomous entrainment pathways. This is consistent with CRY being expressed in both groups of neurons[9–12]. Moreover, the accessory medulla receives projections from the visual system and from CRY-expressing PDF$^+$ and PDF$^-$ lateral neurons[7,12]. This provides a likely place for connecting the visual system with the LN-MO and LN-EO neurons. The presence of light-dark–entrained constant light rhythms in cry^b flies ablated for the PDF$^+$ neurons indicates that these cells are not required to relay entraining visual inputs to the LN-EO.

PDF and CRY set the molecular phase of the LN-EO

We found that pdf^0cry^b flies in light-dark and pdf^0 flies in red light-dark lost evening anticipation. Because the double mutants are rhythmic and synchronized to light-dark cycles in constant light, a light-driven oscillator is present and entrained through the visual system[10]. Consistent with the complete phase reversal of their constant light activity, PER cycling was reversed in the LN-EO with

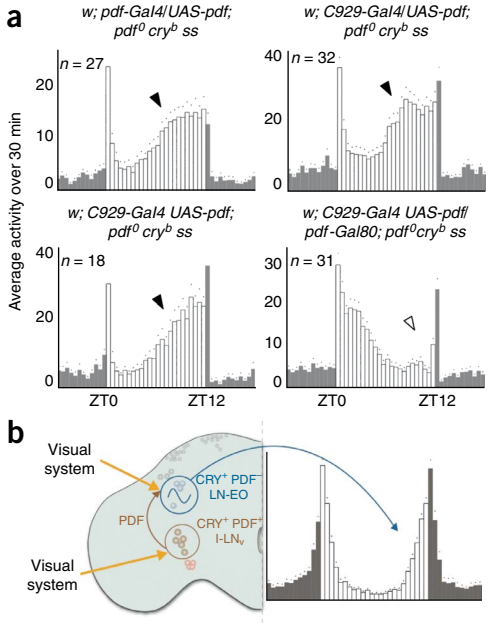

Figure 6 Light-dark activity of rescued pdf^0cry^b mutants and model for the new PDF function. (**a**) Averaged light-dark locomotor activity profiles of pdf^0cry^b mutants expressing PDF under the control of *pdf-Gal4* (top left), *C929-Gal4* (top right and bottom left) or *C929-Gal4* with *pdf-Gal80*–driven inhibition (bottom right). (**b**) A model for the control of *Drosophila* diurnal activity by PDF. The four PDF$^-$ CRY$^+$ neurons of the LN-EO (blue) are three LN$_d$s and the fifth s-LN$_v$. The LN-EO autonomously controls evening activity in light-dark and can be entrained by both CRY and the visual system (directly or indirectly). PDF interacts with the visual system input to define the phase of the evening activity, upstream of the LN-EO molecular oscillator, when CRY signaling is absent or strongly reduced (brown arrow). The LN-EO phasing function of PDF is carried by four to five PDF$^+$ CRY$^+$ l-LN$_v$s (brown) in a clock-independent manner and relies on PDFR expression in the LN-EO. Light activates the l-LN$_v$s through both CRY and the visual system (directly or indirectly)[44]. Other clock neurons are the four PDF$^+$ CRY$^+$ s-LN$_v$s (LN-MO, red) that autonomously control morning activity as well as the three PDF$^-$ CRY$^+$ LN$_d$s (large gray circles) and a large set of dorsal neurons (small gray circles), whose function in light-dark is not characterized.

peak levels around ZT12–15, whereas it was phased normally with a peak at ZT0–3 in the LN-MO. The DN1 neurons stopped cycling in the pdf^0cry^b flies, indicating that they use PDF signaling from the LN_vs to receive entraining visual inputs in the absence of CRY function. In the larval brain, the CRY^- DN2s show similar antiphasic oscillations in light-dark cycles[39] that can be phase reversed by ectopic CRY expression[9]. However, they stop cycling in pdf^0 mutants[40]. PDF is therefore involved at two different levels in the synchronization of the clock network by the visual system. Some cells (adult DN1s, larval DN2s) require PDF to be entrained by the visual system, whereas other cells (CRY^+ LN_ds + adult fifth s-LN_v) require PDF for proper phasing when entrained by the visual system.

The phase reversal of PER cycling in the LN-EO could be expected to generate a new morning peak in the double mutants. However, the low activity levels of pdf^0 mutants, especially at the end of the night[41], as well as the inhibition of the LN-EO output by darkness[10], may prevent morning anticipation in the double mutants. The occurrence of a phase reversal in the absence of CRY, but not in the absence of the visual system, indicates that PDF signaling phases the LN-EO by interacting with its visual system–entrainment pathway. Our results suggest that the default phase of the visual system–entrained LN-EO is a morning one and that inputs from CRY and PDF shift it toward the evening. The absence of evening activity in pdf^0 flies subjected to short days, low temperatures and low light suggests that PDF can affect the phase of the LN-EO even in the presence of CRY. Moreover, the strength of the different inputs is likely to undergo daily variations. First, the spectral composition of daylight changes, with more blue light being present in the middle of the day. Second, light-induced CRY degradation decreases CRY levels at the end of the day[12,42]. Light inputs to the LN-EO are therefore probably dominated by CRY during the day and by the visual system at dusk. Finally, PDF release shows daily variations[37,43], adding another level of regulation to the phasing of diurnal activity.

The rescue of the evening peak by PDFR expression in the LN-EO neurons of *pdfr* mutants indicates that PDF signaling acts in these cells, supporting an interaction between PDF signaling and visual system inputs in the LN-EO neurons. As the cAMP signaling pathway is probably involved in PDFR signal transduction[26,28,32], it could control the visual input signals that entrain the clock to set the phase. Our data indicate that, in the absence of CRY signaling, normal phasing of evening activity by PDF occurs in flies without *per* transcriptional control in the LN-EO, suggesting that PDFR signaling affects molecular oscillations at a post-transcriptional level (or through *tim* transcription). Deciphering this mechanism will require determining how visual inputs reset the molecular oscillations, which remains unknown in *Drosophila*.

A new clock-independent function for PDF

The PDF-dependent phasing of the LN-EO in cry^b mutants can be observed in flies with no PER cycling in the LN-MO, indicating that this PDF function is clock independent. We found that it is carried by the l-LN_vs, in which PER expression is not sufficient to drive morning or evening activity, nor any rhythms in constant darkness[14]. The l-LN_vs have been recently described as light-activated neurons that promote arousal during daytime[20,41,44]. Their PDF output is probably regulated by light, providing additional control for the synchronization of the LN-EO to day-night cycles.

Our results indicate that the LN-EO neurons integrate visual system inputs and PDF signaling from the l-LN_vs to shape *Drosophila* diurnal activity, when CRY signaling is absent or strongly reduced. Switches from nocturnal to diurnal activity were observed in mutant mice with very reduced visual system inputs or in wild-type animals maintained in light-dark cycles with very low light[45]. Such a behavioral switch was recently associated with a phase reversal of the suprachismatic nucleus (SCN) molecular oscillations[46]. In the SCN, vasoactive intestinal peptide neuropeptide signaling is involved in synchronizing and/or phasing, as is PDF in *Drosophila*[47]. It will be interesting to see whether vasoactive intestinal peptide interacts with the retinal inputs to the SCN. Whatever the underlying mechanisms, one could imagine that animal species without CRY photoreceptive function may be more prone to diurnal-nocturnal switches.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

P.C., A.K. and F.R. conceived and designed the experiments. P.C., A.K. and E.C. performed the experiments and analyzed the data. M.P. contributed to behavioral analyses. B.R. contributed to microscopy analysis. P.C., A.K. and F.R. wrote the paper.

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ONLINE METHODS

***Drosophila* strains.** The $cry^{b}ss$ (ref. 17), cry^{01} (ref. 19), pdf^{0} (ref. 25), *UAS-per16* (ref. 48), *UAS-cry* (ref. 8), *UAS-pdf* (ref. 25), han^{5304} and *UAS-han* (ref. 26) stocks have been previously described. Among clock cells, *pdf-Gal4* is expressed in the PDF-positive LN_vs[25], *Mai179-Gal4* is expressed in the s-LN_vs, three CRY^+ LN_ds and occasionally one of the two $DN1_a$[10,14], and *cry-Gal4-19* is expressed in the LN_vs, three CRY^+ LN_ds and both $DN1_a$s[10]. Both *Mai179-Gal4* and *cry-Gal4-19* cover the LN-EO and gave identical results when we were able to compare them. However, in the $per^{0}w$; *Mai179-Gal4/+; UAS-per16/+* flies, PER labeling in the few labeled DN1a was very weak in light-dark and cycling was completely abolished in constant light[10]. This strongly supports a very small contribution, if any, of the DN1a to the LN-EO. *R6-Gal4* is expressed in the PDF-positive s-LN_vs[7]. Because its expression requires PDF (P. Taghert, personal communication), it could not be used to drive transgenes in pdf^{0} mutants. We used the *pdf-Gal80* line 96A, which contains two insertions and completely abolishes *pdf-Gal4*–driven expression in the PDF^+ LN_vs[15]. Expression of *C929-Gal4* has been described in about 100 peptidergic neurons of the adult brain, including the l-LN_vs, but not the s-LN_vs[38]. For cell-ablation experiments the *pdf-Gal4* line was crossed with the *UAS-hid; UAS-rpr* line. The *GMR-hid* strain[49] expressed the apoptotic gene *hid* under the control of a glass multimer–response element. The different combinations of *Gal4/UAS* lines and mutants were obtained through standard genetic crosses. *Drosophila* cultures were usually maintained on a 12 h:12 h light-dark cycle on standard corn meal–yeast agar medium at 25 °C.

Behavioral analysis. Locomotor activity experiments were performed at 20 °C with 1–4-d-old male flies, using commercial activity monitors (TriKinetics). Light was provided by standard, white, fluorescent low-energy bulbs. Light intensity at the fly level was in the range of 300–1,000 μW cm^{-2}. For low-light conditions, gray filters were used that diminished light intensity to 0.03–0.1 μW cm^{-2}. For red-light experiments, red plastic filters were used to cut out wavelengths below 600 nm (data not shown); intensity was reduced approximately fourfold. Identical results were obtained when white bulbs were replaced with red light-emitting diodes (Lunartec, Pearl Diffusion), with emission between 620 and 660 nm (data not shown).

Data analysis was performed with the FaasX software, which is derived from the Brandeis Rhythm Package[50] and is available on request (Mac OSX only). Actograms are double-plotted graphs representing the absolute activity levels for each 0.5-h interval, averaged over groups of flies of a given genotype. The hash density (number of activity events per hash) varied from 10 to 30 according to the activity levels of the genotype. This allows the comparison of activity profiles between genotypes that have very different activity levels. For light-dark analysis, flies were entrained in light-dark 12 h:12 h for 3 d and then analyzed for 4–6 d. Histograms represent the distribution of the activity through 24 h, in 30-min bins, averaged for *n* flies over 4–6 light-dark cycles. White and gray bars indicate day and night phases, respectively. Dots indicate the s.e.m. of the activity for each 30-min bin. The morning and the evening anticipation index were calculated from the last five bins before lights on and the last eight bins before lights off (six bins before advanced peak of pdf^{0} genotypes), respectively. Excel linear regression function was used to derive a slope (increase or decrease in activity events per bin) with associated s.e.m. and statistics to obtain a significance value.

For constant light and constant darkness experiments, flies were entrained in light-dark for 3 d, transferred to constant conditions and analyzed for 7 d starting from the second day of constant light or constant darkness. Rhythmic flies were defined by χ^2 periodogram analysis with the following criteria (filter on): power ≥ 20, width ≥ 2 h, with selection of 24 ± 6 h on period value. Power and width are the height and width of the periodogram peak, respectively, and give the significance of the calculated period. Mean daily activity (number of events per 0.5 h) was calculated over the whole period in constant conditions. Waveforms of individual rhythmic flies are activity profiles averaged over several circadian cycles (which are defined for each fly according to its own period, as computed by FaasX). They allow determination of the phase of activity peak for that fly, also expressed in circadian time. A mean waveform and a mean phase (Φ) can then be computed for each genotype. Circular statistics are used to determine significance and 95% confidence limits of mean phases. All behavioral experiments were reproduced two or three times with very similar results.

Immunolabeling. All experiments were done on whole-mounted adult brains. Flies were entrained for 4–5 light-dark days at 20 °C and then dissected at the indicated ZT times. Staining was carried out on 15–20 brain hemispheres for genotype and each time point. GFP-reporter expression, PER and PDF labeling were done as previously described[9,22]. Fluorescence signals were analyzed with a Zeiss Axioplan2 epifluorescence microscope or a Zeiss Z1 with an apotome structured illumination module. Fluorescence intensity was quantified from digital images with the NIH ImageJ software. For each neuronal group, we applied the formula $I = \frac{100n(S-B)}{B(yN)}$, which gives the fluorescence percentage above background (where *n* is the total number of labeled cells from that group in *y* brain hemispheres, *S* is the average fluorescence intensity, *B* is the average intensity of the region adjacent to the positive cells and *N* is the number of cells expected in the group; four for the PDF^+ s-LN_vs, one for the fifth PDF^- s-LN_v, three for the CRY^+ PDF^- LN_ds and two for the $DN1_a$s).

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