LETTERS

A resetting signal between *Drosophila* pacemakers synchronizes morning and evening activity

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The biochemical machinery that underlies circadian rhythms is conserved among animal species and drives self-sustained molecular oscillations and functions, even within individual asynchronous tissue-culture cells[1–3]. Yet the rhythm-generating neural centres of higher eukaryotes are usually composed of interconnected cellular networks, which contribute to robustness and synchrony as well as other complex features of rhythmic behaviour[4–7]. In mammals, little is known about how individual brain oscillators are organized to orchestrate a complex behavioural pattern. *Drosophila* is arguably more advanced from this point of view: we and others have recently shown that a group of adult brain clock neurons expresses the neuropeptide PDF[8] and controls morning activity (small LN_v cells; M-cells), whereas another group of clock neurons controls evening activity (CRY^+, PDF^- cells; E-cells)[6,9]. We have generated transgenic mosaic animals with different circadian periods in morning and evening cells. Here we show, by behavioural and molecular assays, that the six canonical groups of clock neurons[10] are organized into two separate neuronal circuits. One has no apparent effect on locomotor rhythmicity in darkness, but within the second circuit the molecular and behavioural timing of the evening cells is determined by morning-cell properties. This is due to a daily resetting signal from the morning to the evening cells, which run at their genetically programmed pace between consecutive signals. This neural circuit and oscillator-coupling mechanism ensures a proper relationship between the timing of morning and evening locomotor activity.

Overexpression of the TIM kinase SHAGGY (SGG; *Drosophila* GSK3) shortens the period by 3–4 h (ref. 11). We first drove SGG expression in all clock cells by crossing *tim-GAL4* (ref. 12) with flies carrying an EP element inserted at the *SGG* locus (*EP1576*, referred to as *UAS-SGG*)[11,13]. The locomotor activity rhythm of *tim-GAL4*/*UAS-SGG* (*timSGG*) flies in constant darkness (DD) confirmed previous results[11] (Fig. 1a and Supplementary Fig. S1b), in that the period was about 3 h shorter than that of control flies ($\tau_{timSGG} = 20.6 \pm 0.3$ h; $\tau_{UAS\text{-}SGG} = 23.7 \pm 0.2$ h; τ is the average locomotor period; $\pm$s.e.m.).

We next expressed SGG only in LN_v cells by constructing a *Pdf-GAL4*/*UAS-SGG* genotype. The *Pdf-GAL4* driver is well characterized[8] and drives gene expression only in two clock-cell groups: the PDF^+ small LN_v (s-LN_v) cells (that is, M-cells)[9] and the PDF^+ large LN_v (l-LN_v) cells. The driver is inactive in the CRY^+PDF^- evening cells[6]. *Pdf-GAL4*/*UAS-SGG* (*PdfSGG*) flies also manifested a short period ($\tau_{PdfSGG} = 21.7 \pm 0.2$ h; Fig. 1c). The period shortening was less than that of *timSGG* flies, probably because of weaker expression from *Pdf-GAL4* driver in LN_v cells[6]. (SGG expression from an even weaker driver, cry_{13}-*GAL4*, did not affect behavioural period (Supplementary Fig. S1a).)

A closer inspection of the behavioural actograms (Supplementary Fig. S1b) revealed that the period of evening activity is significantly shorter in *PdfSGG* flies (with a daily advance of about 2 h; Supplementary Fig. S1b). This indicates that the pace of E-cells was accelerated, although the period manipulation was restricted to M-cells. An advanced evening peak, without an increase in E-cell SGG expression, indicates that the faster M oscillator might be setting the E-cell pace. We therefore proposed that the PDF^+ cells influence molecular circadian events within E-cells.

To investigate this possibility, we estimated the molecular period (cycle duration) of each clock-cell group in these different genotypes: *UAS-SGG* (control), *timSGG* and *PdfSGG*. Fly brains were analysed by *in situ* hybridization for *tim* RNA expression pattern after 4 days in DD, so that a barely detectable daily advance by 2–3 h would result in an aggregate advance of 8–12 h on DD4 (fourth day of DD). Indeed, SGG overexpression in all clock neurons (*timSGG*) markedly shifted the interval of high *tim* mRNA expression on DD4 by about 12 h, from between CT10 and CT18 to before CT6 (compare Fig. 1b with Fig. 1a, right panels). (CT is the circadian time within a constant-darkness experiment; CT0 is the hour of the last lights-on event.) All neurons expressing clock genes showed a similar temporal pattern, consistent with the expected SGG-induced period shortening in all clock cells, and with a deterministic relationship between the molecular period and the locomotor activity period.

However, the *PdfSGG tim* RNA profiles were strikingly different and unexpected (Fig. 1c and Supplementary Figs S6 and S7). Whereas the s-LN_v cells showed a roughly 8 h advance in DD4, expected from a period shortening of 2 h per day, the l-LN_v cells showed no appreciable change from those in control flies; that is, their molecular programme was apparently unaffected by SGG overexpression within these cells (Fig. 1c and Supplementary Figs S6 and S7). Also surprising were the DN1 and DN3 profiles, which showed a roughly 8 h advance, as were the LN_d cells, which were advanced by about 6 h relative to those in control flies (Fig. 1a, c; Supplementary Figs S2 and S6). As *PdfSGG* flies do not overexpress SGG in these three cell groups, their molecular programmes behave in a non-cell-autonomous manner. Because the E-cells are included within these groups[6] and because the s-LN_v cells (the M-cells)[9] are the only cells with a cell-autonomous programme that matches the behavioural period of the flies, the M-cells apparently determine the clock pace of these other neuronal groups, including the E-cells.

The l-LN_v cells and DN2 cells emerged as the only clock-gene-expressing neurons that evaded control of the M-cells and maintained a wild-type-like phase of *tim* RNA cycling in *PdfSGG* flies. Because DN2 cells are genotypically wild type in these flies, we infer that they oscillate with cell-autonomous properties and are the best candidates for determining the non-cell-autonomous wild-type-like characteristics of the l-LN_v cells. As a consequence there are at least two parallel clock-cell circuits in the *Drosophila* brain in constant

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darkness: the M–E circuit controls locomotor activity rhythms and is driven by the M-cells (s-LN_v cells), whereas the DN2–l-LN_v circuit has as yet unknown functions and is driven by the DN2 cells.

To verify and extend these concepts, we generated a genotype in which the E-cells should run faster than M-cells. By adding the previously described *Pdf-GAL80* repressor construct to the *tim-GAL4;UAS-SGG* background[6], SGG was expected to be overexpressed in all clock neurons with the exception of PDF-expressing cells. As these include the M-cells (s-LN_v cells), they should run more slowly (24 h) than the E-cells (about 21 h). A 'faster takes all' rule predicts that the short-period E-cells will dominate over the normal 24 h M-cells in this genotype and generate a behavioural rhythm of about 21 h. Alternatively, dominant M-cells will give rise to a behavioural period of 24 h despite the faster endogenous oscillator in the E-cells.

Consistent with a dominant M-cell model was the observation that *timSGG/PdfGAL80* flies had an almost wild-type period in DD, $\tau = 23.8 \pm 0.2$ h (Fig. 2a, left). The molecular analysis was also consistent, as the s-LN_v cells manifested a wild-type-like programme: *tim* mRNA peaked between CT12 and CT20 on DD4 (Fig. 2a, right). Despite SGG overexpression, the LN_d cells, DN1 cells and DN3 cells had a similar and wild-type-like pattern of *tim* expression (Fig. 2a, and Supplementary Figs S8 and S9). As described above, this indicates that all three cell groups behave non-autonomously and are probably driven by the s-LN_v cells. This result is supported by the anatomical pattern of s-LN_v neuronal processes, which project towards the brain regions populated by LN_d, DN1 and DN3 cells[12,14]. DN2 cells were again the only SGG-overexpressing cells in which the phase of *tim* RNA oscillation corresponded to the predicted accelerated pace (Fig. 2b). The l-LN_v cells, despite lacking SGG overexpression (because of the *PdfGAL80* transgene), also showed a comparable advance of *tim* expression (Fig. 2b). These *timSGG/PdfGAL80* results confirm that the s-LN_v cells determine the phase of LN_d, DN1 and DN3 cells and that an independent cellular network includes the DN2 and l-LN_v cells. Because the behavioural period was wild-type-like and paralleled the molecular clock within the s-LN_v cells, the results confirm that these M-cells assign the circadian period in the absence of light cues.

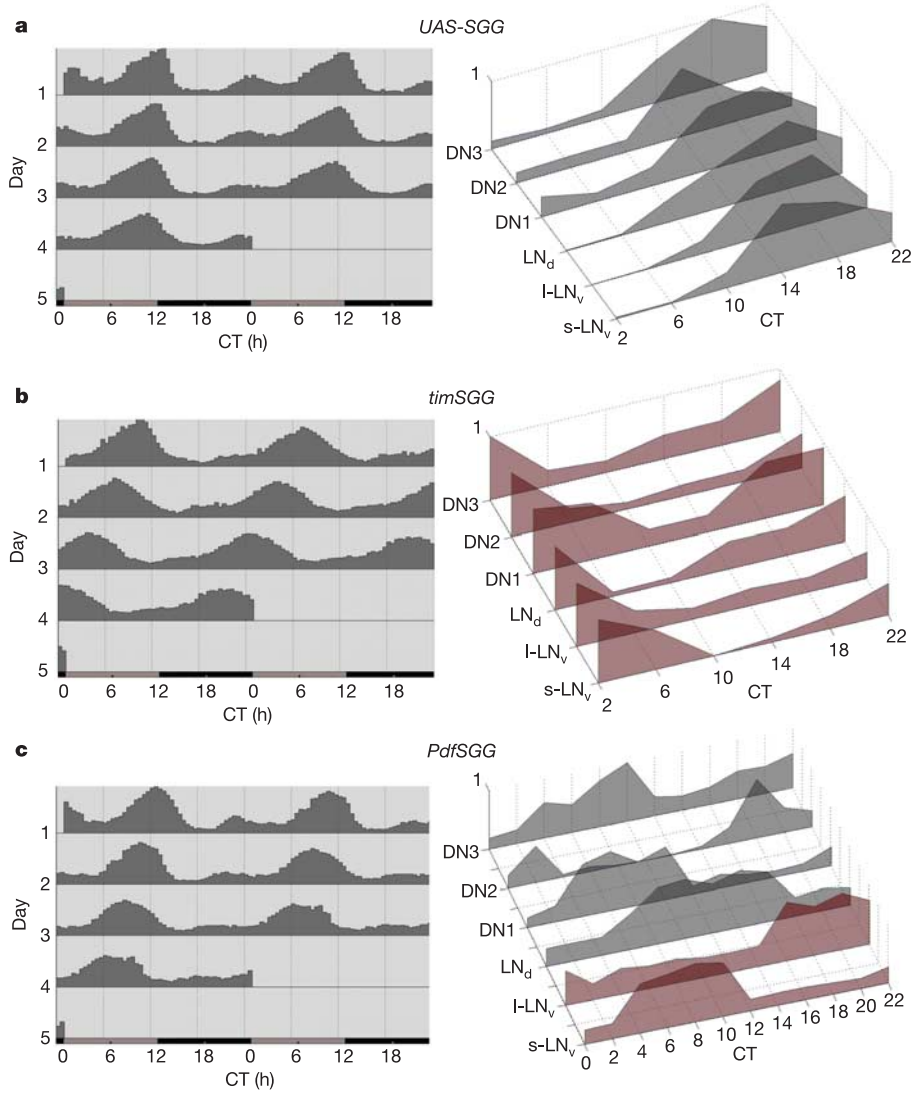

Figure 1 | The M-cell clock influences the timing of events related to E-cells. Characterization of behavioural and molecular phenomena in flies in which different neuronal groups run molecular circadian programmes with different periods. Left panels: double-plotted actograms of locomotor behaviour, during the first four days in constant darkness (DD1–4). Right panels: plots representing the temporal changes in *tim* RNA levels during DD4. The cell groups in which SGG was overexpressed are plotted in red. **a**, *UAS-SGG* flies ($n = 110$; $\tau = 23.6$ h); **b**, *timSGG* flies ($n = 100$; $\tau = 20.8$ h); **c**, *PdfSGG* flies ($n = 125$; $\tau = 21.7$ h). For a more detailed cell-group-specific analysis of the *tim* signal oscillations, see the main text and Supplementary Figs S2–S11.

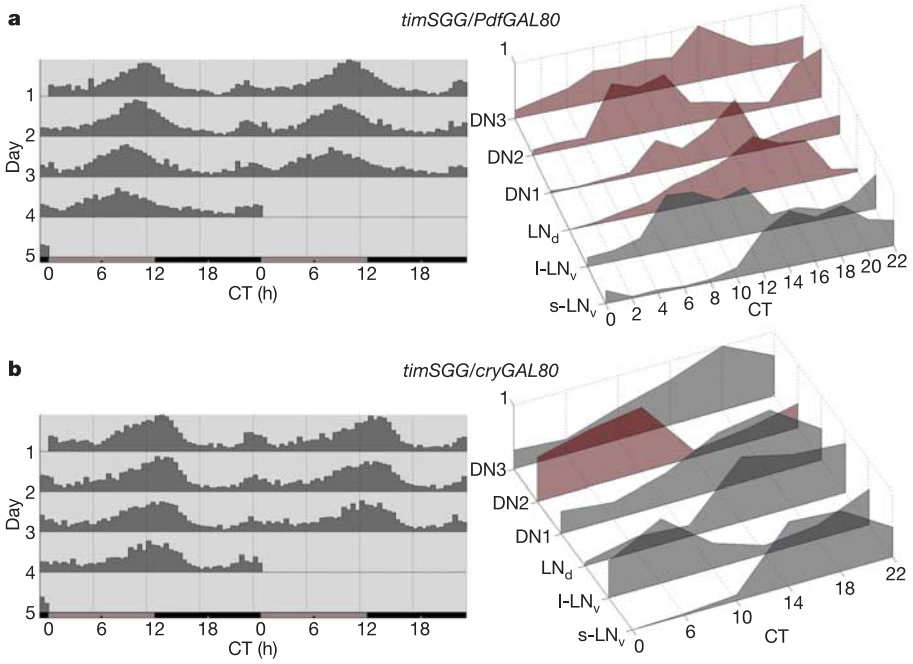

Figure 2 | The M-cell clock dictates the period and phase of E-cell molecular oscillation, and DN2 cells control the phase of l-LN$_v$ cells. **a**, *timSGG/PdfGAL80* flies ($n = 32$; $\tau = 23.7$ h); **b**, *timSGG/cryGAL80* flies ($n = 32$; $\tau = 23.8$ h). The panels are structured as in Fig. 1.

To confirm the lack of a contribution of DN2/l-LN$_v$ to the E–M network function and to locomotor rhythms, we also examined the *timSGG/cryGAL80* genotype. It is similar to the *timSGG/PdfGAL80* genotype described above, except that SGG overexpression is repressed in a wider group of cells. These include most if not all of the E-cells and l-LN$_v$ cells as well as the M-cells[6]. As DN2 cells are the only clock cells in which *cry* promoter-driven expression was not detected[6], we expected that the faster clock in *timSGG/cryGAL80* would be limited to CRY$^-$ cells, including the apparently cell-autonomous DN2 cells.

Indeed, *tim* hybridization *in situ* confirmed that the period of DN2 rhythm was shortened by about 2–3 h per day (Fig. 2b and

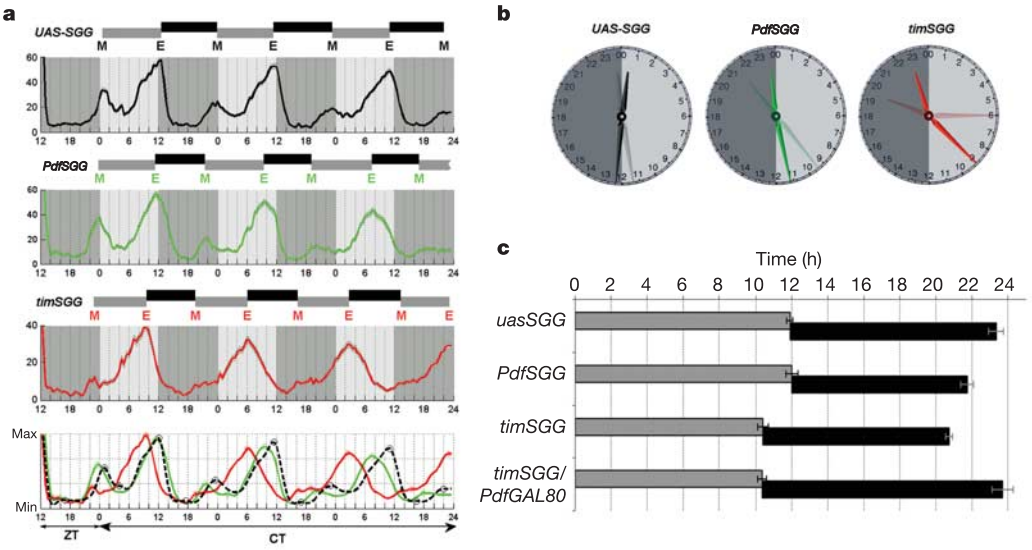

Figure 3 | The duration of subjective day is correlated with the genotype of E-cells, whereas the period is correlated with the genotype of M-cells. Phase comparison of locomotor activity for three genotypes: *UAS-SGG* (black), *PdfSGG* (green) and *timSGG* (red). **a**, The top three panels show a time-based analysis of DD locomotor activity peaks for each genotype: the starting time point coincides with the last light-off event, that is, the last night of LD (ZT12–24) and the following three days in DD are shown. The timing is indicated by different background shades of grey: light grey for CT0–12 (corresponding to light interval in LD) and dark grey for CT12–24 (corresponding to dark interval in LD). The M and E peaks are indicated above each panel. The bottom panel shows a three-way genotype comparison of the peak phases. The automatically identified peaks are marked with open circles (control), asterisks (*timSGG*) and diamonds (*PdfSGG*). The *x* axis shows ZT and CT time in hours, and the *y* axis shows mean activity. **b**, The actual timing of morning and evening peaks during the first 48 h after lights-off. The numbers on the 24 h dials are as shown in **a**. The short arm indicates the time of the morning peak, the long one the time of evening activity. The full-coloured indicators represent the timing of events during the first cycle of activity, and the shaded ones refer to the second cycle after lights-off. **c**, Comparison of average duration of subjective day (M–E, in grey), during DD1–4. Black bars show the length of subjective night (E–M). Error bars represent s.d.

Supplementary Figs S10, S11). The l-LN$_v$ neurons were shifted to about the same extent, which is consistent with the notion that they behave non-cell-autonomously and follow the pace of the DN2 clock programme. All other clock cells maintained a pattern similar to that of control flies (Fig. 2b). Because *timSGG/cryGAL80* flies had a normal behavioural period ($\tau = 23.9 \pm 0.1$ h; Fig. 2a), these results confirm that l-LN$_v$ and DN2 cells do not contribute detectably to locomotor activity rhythms. This conclusion is in agreement with previous results showing that wild-type flies have persistent DD behavioural rhythms, despite protein oscillation idiosyncrasies of the l-LN$_v$ and DN2 cells[15,16].

How does the M-cell (s-LN$_v$) clock determine the period of E-cells (LN$_d$ cells/DN cells)? Although our previous work indicated possible oscillator coupling[6] and a direct effect of LN$_v$ on the transcriptional oscillations of other clock cells[5], it was difficult to envision how the M-cells could override the intrinsic molecular timing of the E-cells. We therefore considered a second possibility, namely that the E-cells maintain an unaltered intrinsic clock programme but receive a daily resetting signal from the M-cells. This model predicts that the timing of the evening activity within every cycle (between two consecutive mornings) reflects the status of the endogenous clock of E-cells, whereas the overall period exhibited by the evening peaks reflects the pace of the M-cell resetting signal.

To examine this possibility, we assayed the different transgenic strains for their average evening activity phase within a cycle, by using the leading morning peak as a reference and then measuring the average time until the subsequent evening peak (M–E interval = subjective day; Fig. 3). The overall DD period correlated with the genotype of M-cells as expected, but the length of the subjective day (M–E interval) correlated only with the genotype of the E-cells. In control flies (*UAS-SGG*) with a period of about 24 h, the subjective day was roughly 12 h, similar to the duration of subjective day of *PdfSGG* (Fig. 3b, c). The latter strain features a wild-type-like E-oscillator but a fast, SGG-expressing M-oscillator and a period of about 22 h (Fig. 1a). In contrast, *timSGG* flies express SGG in both E-cells and M-cells, and both the average length of subjective day and the period (M–M) are reduced (M–E = 10.45 h; period more than 3 h shorter; Figs 1b and 3b, c). Taken together with the molecular data (Fig. 1, right panels; Supplementary Figs S2–S7), the results indicate that the E-cells run an autonomous clock programme whose starting (or ending) points are determined by daily resetting signals from the M-cells.

A DD unidirectional M → E resetting mechanism also predicts that a slower (24 h) M-cell clock and a faster E-cell clock will have a normal morning peak phase but an advanced evening peak phase. To test this prediction we compared the behavioural outputs of *timSGG/PdfGAL80* and *timSGG/cryGAL80* flies, which differ only in the genotypes of their E-cells (Fig. 4). Both strains have periods of about 24 h, but the former should give rise to a fast E-cell molecular programme, whereas the latter should have an E-clock of 24 h as a result of suppression of SGG expression.

Indeed, the evening phase of *timSGG/cryGAL80* is similar to that of control flies (Fig. 4b, middle), and it always occurs about 2.5 h later than that of *timSGG/PdfGAL80* (Fig. 4). The evening phase of *timSGG/PdfGAL80* is more similar to that of *timSGG* (Fig. 4b, right), although the latter genotype ($\tau = 20.6$ h) has a much shorter period than the former ($\tau = 23.8$ h). The length of subjective day of *timSGG/PdfGAL80* flies (M–E = 10.35 h; that is, similar to that of *timSGG*; Fig. 3c) further confirms that the evening phase within each cycle is a reflection of the endogenous E-cell rhythm, whereas the period of the cycle (M–M) correlates with the intrinsic M-cell clock.

These comparisons indicate that the circadian network is modulated by intercellular communication signals, which achieve and maintain circadian coherence—the proper relationship between morning and evening activity. The dominant M-clock determines the period of the entire system by providing a daily reset signal to the E-clock in darkness and is therefore a true cellular Zeitgeber. Because the M-cells can delay as well as advance E-cells, the resetting signal may be required for E-cell oscillations. The usual candidate for this signal is the M-cell-specific neuropeptide PDF. It contributes to the normal synchrony and/or rhythmicity in constant darkness[5,7], with a striking similarity to the mammalian neuropeptide VIP[4,17]. Moreover, injecting PDF into the cockroach brain causes circadian phase delays[18]. Other principles and/or molecules may also be relevant to the M–E subnetwork, because E-cells can drive clockless M-cells to manifest cyclical behavioural outputs under 12 h light/12 h dark (LD) conditions[6].

The l-LN$_v$ and DN2 cells are the two neuronal groups that escape the M-cell reset signal in DD. They constitute a second circadian subnetwork with no apparent effect on locomotor activity rhythms and no known function. The DN2 cells are among the few clock-gene-expressing brain cells in larvae[12] and are also the only clock cells that do not change their morphology after eclosion[19]. Larval DN2 cells are apparently devoid of CRY and manifest anti-phase oscillations of TIM and PER[10,19]. It is therefore likely that both the DN2 cells and the l-LN$_v$ cells impart circadian regulation to unknown physiological functions relevant to both larvae and adults. More generally, we expect that the organizational principles of the two subnetworks described here will also be relevant to mammalian neuronal networks with important behavioural functions, for example the relationship between different oscillators in the SCN[20,21].

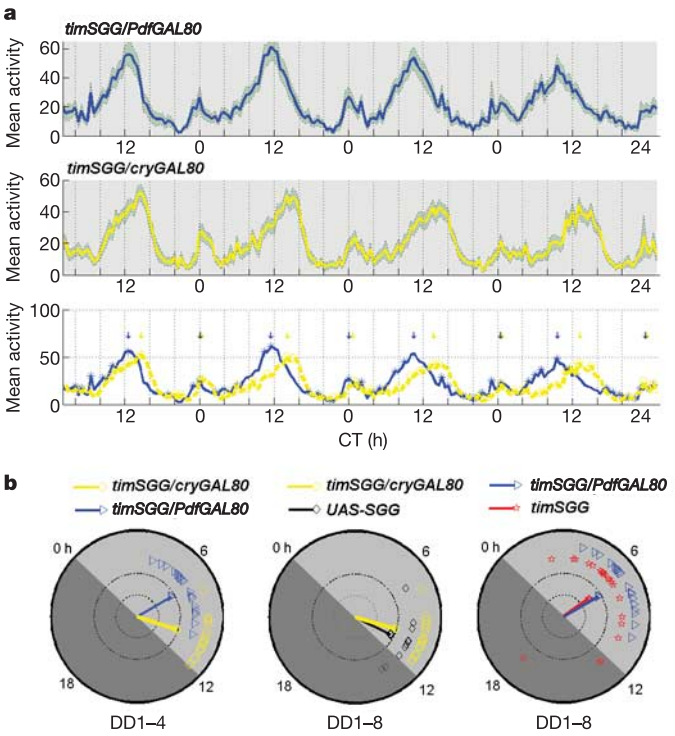

Figure 4 | The evening oscillator times its output according to its own intrinsic clock programme. a, Phase comparison of the DD locomotor activity of *timSGG/PdfGAL80* flies (blue) and *timSGG/cry-GAL80* flies (yellow). In the bottom panel, the M and E peaks of each genotype are indicated by small arrows of corresponding colour. The graph shows counts of activity over time. **b**, Circular phase analysis of DD1–4 and DD1–8 locomotor activity[23]. The vectors indicate the average phase of the evening peak for the whole group[23]. The length of the vector is proportional to the statistical strength and coherence within the group[23]. The evening locomotor activity of *timSGG/cryGAL80* flies has a similar phase to that of control flies (bivariate test; confidence = 99.9%), and the evening activity of *timSGG/PdfGAL80* is similar to that of *timSGG* (bivariate test; confidence = 100%).

METHODS

Fly strains. *Pdf-GAL4* (ref. 8), *tim-GAL4* (refs 6, 12), *cry*$_{13}$*-GAL4*, *Pdf-GAL80*$_{96A}$ and *cry-GAL80*$_{2e3m}$ (ref. 6) transgenic lines have all been characterized previously. The *EP1576* line was obtained from the Bloomington Stock Center (http://fly.bio.indiana.edu/). All molecular and behavioural experiments were performed at 25 °C.

Behavioural analysis. Flies were entrained for 5 days in LD conditions before being released into conditions of constant darkness (DD). Locomotor activities of individual male flies were monitored with Trikinetics *Drosophila* activity monitors. Analysis was performed with a signal-processing toolbox[22] implemented in MATLAB (MathWorks). Autocorrelation and spectral analysis were used to assess rhythmicity and calculate period. Phase estimation was achieved by using circular statistics[23]. For the calculation of M–E intervals in DD (Fig. 3b, c), we took the following steps: we applied a Butterworth filter to the activity time courses to smooth out random peaks (those oscillating with periods of less than 20 h)[22]. We then manually chose the first peak between two consecutive evening events (which were detected unambiguously by the software[22]) and identified its corresponding phase as morning CT time. The time interval between every morning peak and the following evening peak was computed for the first four days in darkness, averaged and represented in Fig. 3c. The interval between two subsequent morning peaks was similarly calculated and represented. To reduce noise, more than 100 flies from each genotype were used. Alternative methods of estimating the phase of morning/evening activities (including the activity onset, centre of activity mass, increasing the number of individuals in a group to about 300 or varying the values of the smoothing filter) were also used and gave similar results (data not shown).

Imaging techniques. Fly brains were dissected and mounted as described previously[5]. *In situ* hybridization of *Pdf* and *tim* was also conducted as described[24]. At least ten brains were examined for each time and genotype. The maximum projection images taken with a Leica laser scanning confocal microscope were used for quantifying *tim* RNA signals with ImageJ software (http://rsb.info.nih.gov/ij). Values for individual cells were computed as the ratio between the pixel intensity of the cell and the average intensity of three background areas in neighbouring brain regions. Six to ten single-cell *tim* signals from at least four different hemispheres were averaged to obtain the mean values for each of the six individual cell groups (for example the DN1 group) for every time point, and standard deviations were calculated. Mean values within every given experiment were then normalized to the maximum value of the six (Figs 1a, b and 2c, right panels) or 12 (Figs 1c and 2a) time points. The detailed quantification results are shown in Supplementary Information, together with samples of brain pictures (Supplementary Figs S2–S11). The identification of PDF$^-$ LN$_d$ and DN cells was based on their specific location and anatomy (size and number). The particularly problematic distinction between DN1 cells and the two DN2 cells was based mainly on the position of the latter group in a slightly more posterior coronal section (layer).

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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