Experimental Assessment of the Network Properties of the *Drosophila* Circadian Clock

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ABSTRACT

Circadian rhythms are conserved across kingdoms and coordinate physiology and behavior for appropriate timekeeping. The neuronal populations that govern circadian rhythms are described in many animal models, and the current challenge is to understand how they interact to control overt rhythms, remaining plastic enough to respond and adapt to a changing environment. In *Drosophila melanogaster*, the circadian network comprises about 150 neurons, and the main synchronizer is the neuropeptide pigment-dispersing factor (PDF), released by the well-characterized central pacemaker neurons, the small ventral lateral neurons (sLNvs). However, the rules and properties governing the communication and coupling between this central pacemaker and downstream clusters are not fully elucidated. Here we genetically manipulate the speed of the molecular clock specifically in the central pacemaker neurons of *Drosophila* and provide experimental evidence of their restricted ability to synchronize downstream clusters. We also demonstrate that the sLNv-controlled clusters have an asymmetric entrainment range and were able to experimentally assess it. Our data imply that different clusters are subjected to different coupling strengths, and display independent endogenous periods. Finally, the manipulation employed here establishes a suitable paradigm to test other network properties as well as the cell-autonomous mechanisms running in different circadian-relevant clusters. J. Comp. Neurol. 523:982–996, 2015.

INDEXING TERMS: *Drosophila*; circadian; complex rhythms; entrainment; *shaggy*; BMP; *schnurri*

Adaptations that handle the adjustment to the 24-hour environmental fluctuations are the result of the action of the circadian clock, which is present in all living organisms and roughly all cell types. Interestingly, independent biological clocks within a neuronal network displaying distinct intrinsic angular velocities and/or phase relationships can give rise to different emergent outputs such as those required for seasonal adaptation. This could be achieved by altering the coupling and/or dominance between these independent clocks (Yamaguchi et al., 2003; Stoleru et al., 2007; Yoshii et al., 2009; Schwartz et al., 2011; Bywalez et al., 2012). Defining how ensembles of neurons are set in place to integrate environmental cues and coordinate overt rhythms remains a challenge (Muraro et al., 2013). In this regard, *Drosophila* offers an ideal model organism because the circadian network is well characterized and anatomically spread, the molecular clock is comprehensively defined, and the fly permits the genetic manipulation necessary to address complex behavior.

Identification of the molecular components underlining the *Drosophila* circadian clock (for a detailed review of the molecular circadian clock see Ozkaya and Rosato, 2012) has facilitated the localization of its neuronal substrates. About 150 neurons encompass the circadian network, among them the dorsal lateral neurons (LNd), four groups of dorsal neurons (DN1a, DN1p, DN2, and DN3) and the small and large ventral lateral neurons (sLNvs and lLNvs, respectively). Determination of their molecular identity as well as the neuropeptides and neurotransmitters released/received by them has helped to indicate further subdivisions of the network

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(Muraro et al., 2013). Importantly, three of the six LNds, the DN1as, some DN1ps, and the LNvs express cryptochrome (CRY), a key player in cell-autonomous light entrainment (Ceriani et al., 1999; Busza et al., 2004). In contrast, the remaining LNds and DN1ps, the DN2s, and DN3s are CRY-negative. Moreover, the lLNvs and four of the sLNvs express pigment-dispersing factor (PDF), which is absent from the fifth sLNv. The PDF-positive sLNvs (unlike the lLNvs) are considered the central pacemaker, and PDF the key synchronizer of the fly clock (Renn et al., 1999; Peng et al., 2003; Lin et al., 2004; Sheeba, 2008; Shafer and Taghert, 2009). *pdf* mutants are largely arrhythmic, although some of them exhibit a short period phenotype of ~22.5 hours and a weak rhythm strength (Lin et al., 2004). Further characterization has demonstrated that the CRY-positive LNds and DN1ps, along with the fifth sLNv, cycle with a short endogenous period and control behavior when the output of the sLNvs is impaired (Wu et al., 2008; Yoshii et al., 2009). In contrast, CRY-negative neurons display a ~24-hour endogenous period (Yoshii et al., 2009; Zhang et al., 2009) and to some extent follow the command of the sLNvs (Stoleru et al., 2005). Moreover, recent reports challenge the idea of a unique master pacemaker and propose that overt behavioral rhythms emerge from multiple independent oscillators (Dissel et al., 2014; Yao and Shafer, 2014). Thus, several lines of evidence indicate that the *Drosophila* circadian network includes a heterogeneous population, underscoring the existence of a hierarchical organization and coupling systems, as is the case for pacemaker structures in other invertebrate and vertebrate species (Vansteensel et al., 2008). However, despite this heterogeneity, locomotor rhythms are controlled by the network with a precision in the range of minutes.

Clocks become synchronized by a variety of cues that entrain circadian oscillators to species-specific combinations of photic and nonphotic cycles collectively known as *zeitgebers*. A *zeitgeber* achieves entrainment of an oscillator when the period and phase-relationship between them is locked. Arguably, if both the endogenous and zeitgeber periods run at a similar pace, entrainment is easier to achieve, and this also depends on *zeitgeber* strength. For a given zeitgeber strength, the maximum and minimum *zeitgeber* period at which entrainment is achieved define the entrainment range of the oscillator (Granada et al., 2011). Under free-running conditions the main pacemaker cells of the *Drosophila* circadian clock, the sLNvs, control the pace of overt rhythms, and thus they operate as a master oscillator for the downstream clusters that ultimately shape circadian locomotor activity.

Defining the rules that govern the communication between different clusters will illuminate how neuronal networks translate genetic and environmental information into coherent behavior. Here, we inquire about the properties of the circadian network, focusing on how the sLNVs can drive the rest of the network. Through genetic manipulations of the molecular clock in main pacemaker neurons we show that the master oscillator has a restricted ability to coordinate the function of downstream clusters, probably shaped by the connectivity among them. We found that at least two groups of neurons differentially coupled to the sLNvs act in concert to control locomotor behavior. We propose that a heterogeneous array of molecular clocks and their specific connectivity to the central pacemaker underlie the organization of coherent rhythms and the ability to synchronize to a changing environment.

MATERIALS AND METHODS

Fly rearing and strains

Flies were reared on cornmeal medium complemented with yeast and maintained in constant temperature incubators at 25°C under 12 hours of light and 12 hours of darkness (LD12:12). Acceleration of the endogenous clock through overexpression of the kinase *shaggy* (*sgg*) was achieved with the strains uas-sgg^{WT} (RRID:BDSC_11008) and uas-sgg^{CA} (Martinek et al., 2001, RRID:BDSC_5362); as an alternative strategy to speed up the clock, the *doubletime* (*dbt*) kinase was also employed, specifically uas-dbt^{S} (Muskus et al., 2007). To slow down the pace of the clock, uas-sgg^{RNAi} (VDRC, transformant ID 101538), and P[UAS]^{756} (Beckwith et al., 2013), which allows *shn* overexpression, were used. To enhance RNAi efficiency, uas-*dicer2* (VDRC, transformant ID 25090) was employed. To control gene expression in the circadian-relevant clusters, the *pdf*GAL4 (Renn et al., 1999, RRID:BDSC_6900), *tim*-GAL4 (Emery et al., 1998, RRID:BDSC_7126), and *clk*^{856}GAL4 (Gummadova et al., 2009) strains were used, referred to here as as *pdf*>, *tim*>, and *clk*^{856}>, respectively. *pdf*> and *tim*> were obtained from the Bloomington Stock Center (Bloomington, IN). Strains were crossed to w^{1118} (RRID:BDSC_5905) to generate heterozygote controls.

Recording the locomotor activity of flies

The locomotor activity of male flies was quantified by using *Drosophila* Activity Monitors (TriKinetics, Waltham, MA) following standard proceedures (Rosato and Kyriacou, 2006). In brief, flies were entrained to LD12:12 cycles during development, and 0–3-day-old adult males were placed in glass tubes containing standard food.

Data were acquired every 30 minutes. Activity was monitored in LD conditions for 3–4 days, followed by 9 or 15 days of constant darkness depending on the experiment.

Data analysis

Collected data were analyzed by chi-square periodogram analysis employing the ClockLab software (Actimetrics, Wilmette, IL). Flies with a single peak over the significance line ($\alpha = 0.05$) in a chi-square analysis were scored as rhythmic, which was confirmed by visual inspection of the actograms. Activity patterns were categorized as complex rhythms when two or three clear peaks over significance were present. Flies exhibiting multiple and similarly low peaks were classified as weakly rhythmic, and when no peak reached significance flies were considered arrhythmic. Rhythmic power was calculated with the ClockLab software as previously reported (Yao and Shafer, 2014).

A minimum of three independent experiments, including at least 15 flies that survived throughout the experiment, were analyzed per genotype. The comparisons of period and rhythmicity between genotypes in Figures 1 and 2 were conducted by one-way analysis of variance (ANOVA) followed by Tukey comparisons using $\alpha = 0.05$. In all cases normality was assessed by the Shapiro–Wilk test, and variance homogeneity assumption was evaluated by Levene's test. Statistical analysis was performed with the InfoStat package (Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina).

RESULTS

Entrainment of the *Drosophila* circadian network is asymmetric

We attempted to evaluate the network properties of the *Drosophila* circadian system when driven by the sLNvs. Thus, we took advantage of previously described strategies to manipulate the pace of the molecular clock within the sLNs in a cell-autonomous fashion, leaving the rest of the network to run with its wild-type (wt) endogenous period. First, we overexpressed *shaggy* (*sgg*) specifically in pacemaker neurons by means of the *pdf*GAL4 line. SGG (*Drosophila* GSK3), together with the kinases DOUBLETIME (DBT) and CASEIN KINASE II (CKII) and the protein phosphatases PP1 and PP2A, regulates the subcellular localization and stability of the core clock proteins PERIOD (PER) and TIMELESS (TIM) in the first transcriptional–translational negative feedback loop (Ozkaya and Rosato, 2012). Overexpression of a wt version of SGG (*pdf*>*sgg*^{WT} flies) clearly resulted in a period shortening of ~2 hours (Fig. 1A,B and Stoleru et al., 2005), characterized by fragmentation of the activity patterns, a significantly reduced degree of rhythmicity reflected in less pronounced peaks in the periodogram analysis, and a significant reduction in rhythmic power (Fig. 1A,C, Table 1). Conversely, *sgg* knock-down through the expression of a specific RNAi had no effect on the coordination of locomotor activity and resulted in a reciprocally long period phenotype of half an hour, which did not reach statistical significance (Fig. 1). The concomitant overexpression of *dicer2* (to enhance the efficiency of the RNAi machinery; Dietzl et al., 2007) further slowed the underlying clock, giving rise to a highly rhythmic population displaying an ~2-hour longer endogenous period compared with controls (Fig. 1, Table 1).

To independently regulate the pace of the clock in these cells, we activated the bone morphogenetic protein (BMP) pathway in PDF-positive neurons. We recently showed that this manipulation inhibits *dClk* transcription, and therefore modulates the pace of the molecular clock (Beckwith et al., 2013). Thus overexpression of the nuclear BMP pathway component *schnurri* (*shn*) slowed down the molecular oscillator and triggered a long period phenotype of ~1.5 hours, during which flies behaved in a perfectly rhythmic fashion (Fig. 1A–C, Table 1). So far we have shown that accelerating or slowing down the pace of the clock within PDF-expressing neurons accomplishes different results. Acceleration of the molecular clock by ~2 hours (compare *pdf*>*sgg*^{WT} with parental controls) disrupts proper behavior, leading to poor rhythmicity, probably as a result of a deficit in the communication between clusters. In contrast, slowing down the clock appears to achieve proper synchronization of the downstream clusters even when the resulting period is 2 hours longer than the wt one, suggesting an asymmetric restriction in the propagation of information delivered by the sLNvs. Next, we inquired what happens to behavior when genetic manipulation intended to speed up the clock is driven into the entire circadian network. So we altered the pace of the clock with the same tools but operating on the entire circadian network by means of the *tim* promoter. Interestingly, BMP pathway activation in all circadian neurons triggered a similar behavioral phenotype as the one observed when the manipulations were restricted to the LNvs (Figs. 1, 2; for the effect of *sgg* knock-down on the *tim* domain, see Fig. 5 and Table 2). However, when *sgg*^{WT} overexpression was directed to TIM-positive neurons, a substantial improvement of rhythmicity was observed compared with its effect on PDF-positive neurons (compare Figs. 1 and 2). This experiment strongly suggests that coherent molecular oscillators among circadian clusters likely result in coordinated locomotor activity.

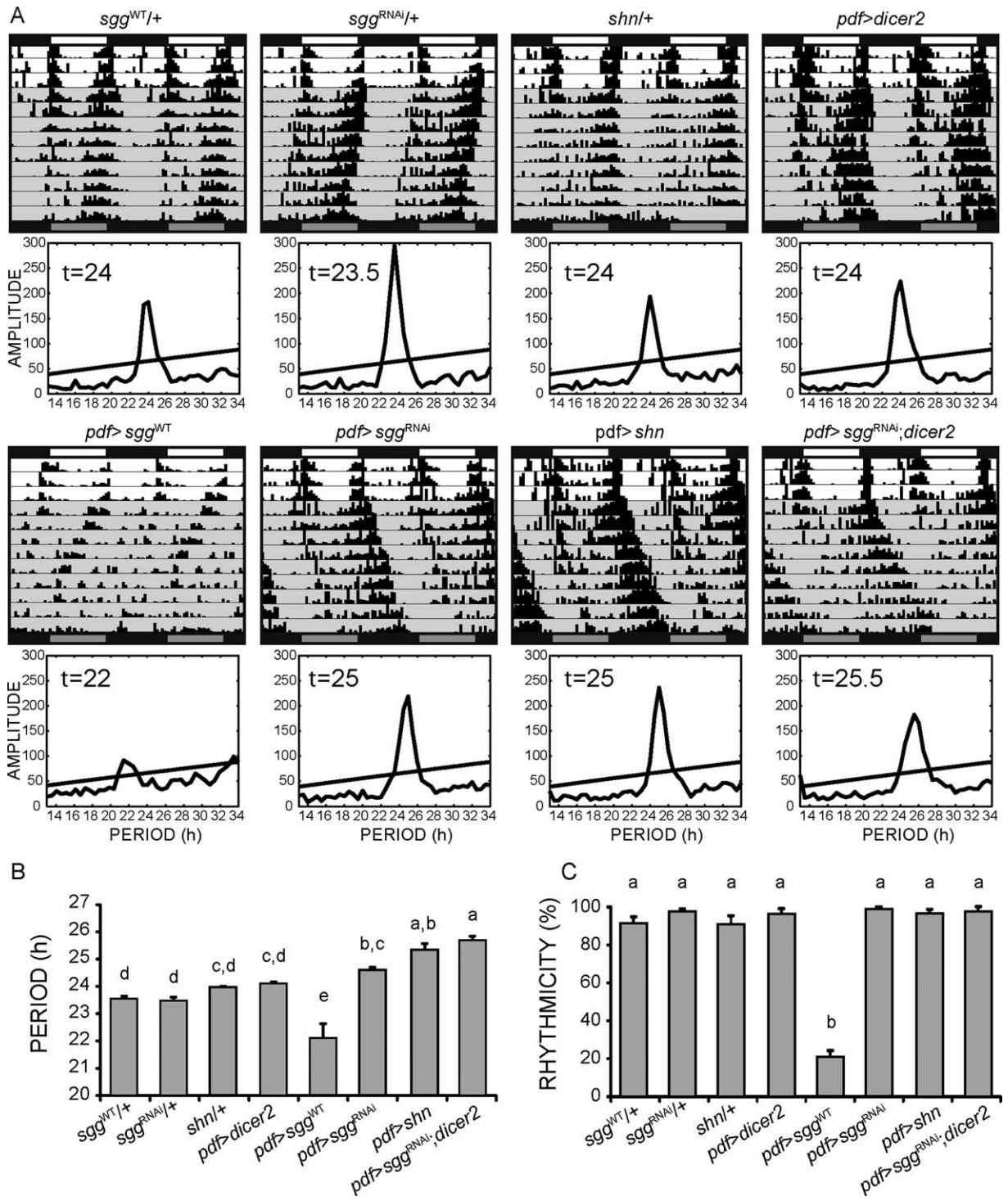

Figure 1. Asymmetric entrainment of non-pacemaker neurons. **A:** Representative double-plotted actograms of individual flies of the indicated genotypes. During the experiments, flies were kept at 12:12-hour light:dark (LD) for 3 days, then switched to constant darkness (DD; shaded gray area), and monitored for 9 additional days. **B,C:** Bar graph shows the quantitation of period and rhythmicity for the indicated genotypes, respectively. Analysis included one-way ANOVA (period: $F_{(7,21)} = 31.27$, $P < 0.0001$, Tukey's test $\alpha = 0.05$; means with a common letter are not significantly different; least-significant difference 0.97 hours; rhythmicity: $F_{(7,21)} = 125.66$, $P < 0.0001$, Tukey's tes $\alpha = 0.05$, means with a common letter are not significantly different; least-significant difference 11.8%; rhythmic power $F_{(7,21)} = 8.37$, $P = 0.0001$, Tukey's test $\alpha = 0.05$; least-significant difference 54.91; see data in Table 1). Letters indicate statistically different treatments. Experiments were independently repeated four times, with 20–32 flies analyzed per genotype/experiment.

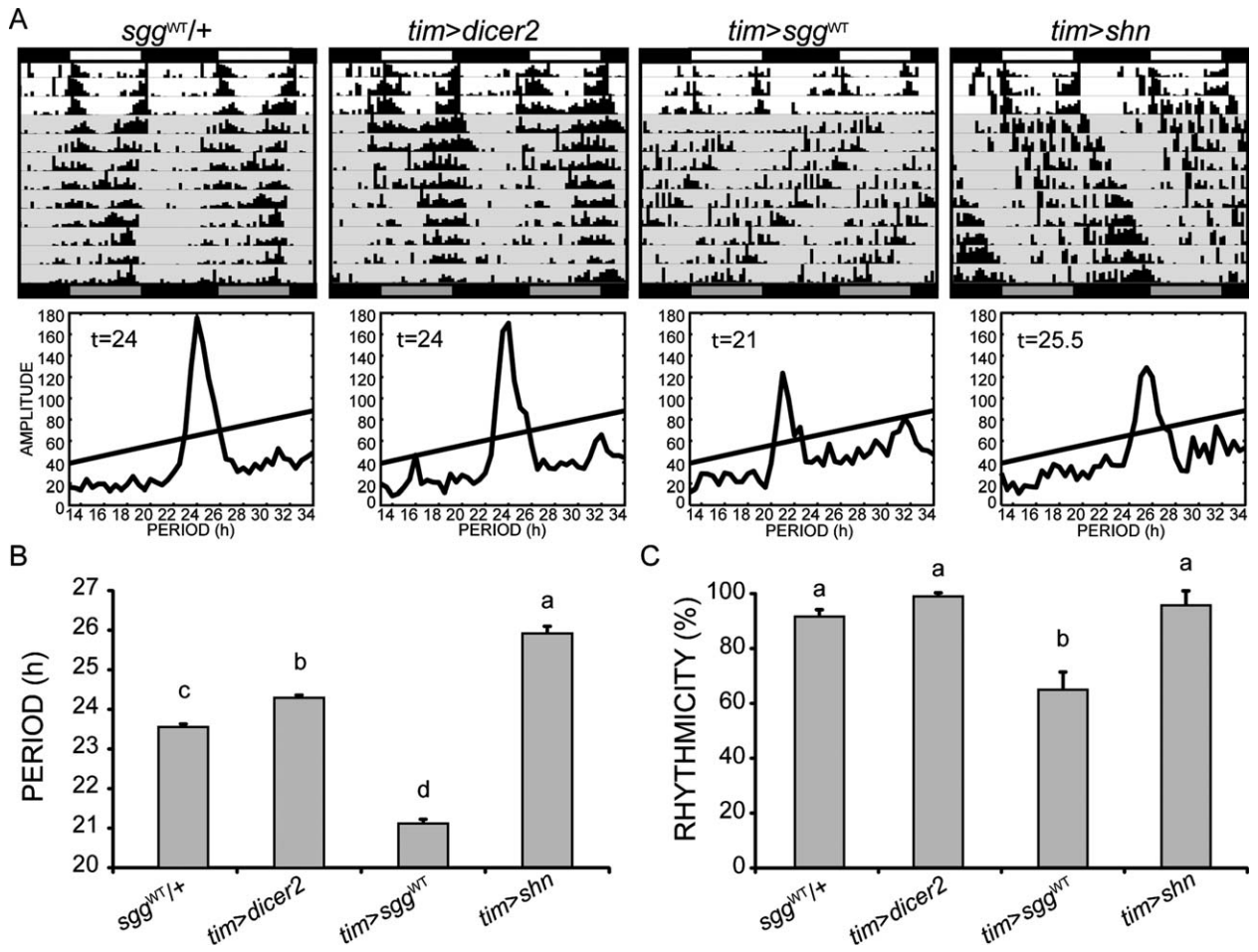

Figure 2. Network coherence ensures rhythmicity. **A:** Representative double-plotted actograms of individual flies of the indicated genotypes. During the experiments, flies were kept in LD for 3 days, then switched to DD (shaded gray area), and monitored for 9 additional days. **B,C:** Bar graph shows the quantitation of period and rhythmicity for the indicated genotypes, respectively. Analysis included one-way ANOVA (period: $F_{(3,8)} = 447.79$, $P < 0.0001$, Tukey's test $\alpha = 0.05$; means with a common letter are not significantly different; least-significant difference 0.43 hours; rhythmicity: $F_{(3,8)} = 18.7$, $P = 0.0006$, Tukey's test $\alpha = 0.05$; means with a common letter are not significantly different; least-significant difference 16.3%; rhythmic power $F_{(3,21)} = 28.28$, $P < 0.0001$, Tukey's test $\alpha = 0.05$; least-significant difference 24.0; see data in Table 1). Experiments were independently repeated three times, with 20–32 flies analyzed per genotype/experiment.

In sum, even though the circadian network as a whole supports different periodicities and the main pacemaker neurons can entrain downstream clusters, the entrainment properties of the network are not symmetric.

Alternative strategies to accelerate the pacemaker endogenous period

To independently manipulate the clock gears in the sLNvs and assess its impact on the rest of the network, we employed two alternative approaches: overexpression of a constitutively active version of *sgg* (*sgg*^{CA}), and overexpression of yet another kinase, *dbt*, in a mutated version that leads to short periods (*dbt*^{S}). Although the three alternative strategies to speed up the clock resulted in the expected period shortening (Fig. 3, Table 2) (Martinek et al., 2001; Yao and Shafer, 2014), the most prominent phenotype observed with the three genetic manipulations was the lack of clear rhythms; in some cases the emergence of complex rhythms became evident, characterized by the superimposition of two rhythmic components driving behavior.

In particular, the extreme phenotype achieved by overexpression of *dbt*^{S} led to the lack of rhythmic locomotor components in 85% of the analyzed flies, with the remaining animals displaying a period phenotype ~6 hours shorter than wt (Fig. 3, upper panels, Table 2).

TABLE 1.

The circadian neuronal network has Asymmetric Entrainment Properties[1]

Genotype	*N*	*n*	Period	% of Rhythmicity	Rhythmic power
Controls					
pdf>dicer2	109	4	24.1 ± 0.1	96.5 ± 2.8	137.3 ± 13.9
tim>dicer2	77	3	24.3 ± 0.1	98.9 ± 1.3	103.4 ± 6.3
sgg^{WT}/+	87	3	23.6 ± 0.1	91.6 ± 3.3	125.9 ± 7.9
sgg^{RNAi}/+	93	3	23.5 ± 0.1	97.8 ± 1.3	125.7 ± 11.6
shn/+	92	4	24.0 ± 0.0	91.0 ± 4.5	78.1 ± 19.9
Manipulation of the central pacemakers					
$pdf>sgg^{WT}$	119	4	22.1 ± 0.5	21.0 ± 3.2	40.1 ± 5.5
$pdf>sgg^{RNAi}$	113	4	24.6 ± 0.1	99.2 ± 0.9	119.8 ± 13.6
$pdf>sgg^{RNAi}$; *dicer2*	95	3	25.7 ± 0.1	97.9 ± 2.5	117.7 ± 21.2
pdf>shn	99	4	25.3 ± 0.2	96.7 ± 2.2	113.3 ± 5.4
Manipulation of the entire circadian network					
$tim>sgg^{WT}$	85	3	21.1 ± 0.1	64.9 ± 6.4	65.1 ± 3.6
tim>shn	77	3	25.9 ± 0.2	95.7 ± 5.3	117.4 ± 5.3

[1]Values are expressed as mean ± SEM. *N*, total number of analyzed flies; *n*, number of independent experiments. All the analyses shown in the table were performed with 9 days of free running.

Moreover, overexpression of sgg^{CA} resulted in an intermediate period phenotype, with 50% of the population exhibiting complex rhythms characterized by a short (as expected for this manipulation) and an ~24-hour component (see the multiple peaks in the periodogram analysis in Fig. 3, middle panel, and Table 3). This result suggests that in addition to the fast sLNvs, other clusters are in command of behavior in this experimental condition. Interestingly, an extended analysis of the sgg^{WT}-overexpressing flies confirmed the short period phenotype and the lack of coordinated locomotor activity patterns in the vast majority of the flies (compare Figs. 1 and 3 and

TABLE 2.

Strong Alterations in the Endogenous Period of Pacemaker Neurons Lead to Complex Rhythms[1]

Genotype	*N*	*n*	Period	% of Rhythmicity	% of Complex rhythms	Rhythmic power
Controls						
pdf>+	61	3	24.4 ± 0.1	93.3 ± 8.2	0.0	175.2 ± 16.6
pdf>dicer2	74	3	24.1 ± 0.1	95.3 ± 3.4	0.0	127.5 ± 20.4
tim>+	*68*	*3*	24.4 0.0	97.1 1.8	0.0	115.2 ± 5.4
tim>dicer2	*63*	*2*	24.0 ± 0.0	96.8 ± 0.1	1.6 ± 2.2	167.7 ± 30.7
clk^{856}>+	*59*	*3*	23.8 ± 0.1	100.0 ± 0.0	0.0	202.2 ± 23.8
sgg^{WT}/+	*54*	*3*	23.8 ± 0.1	100.0 ± 0.0	0.0	218.5 ± 22.1
sgg^{CA}/+	*66*	*3*	23.9 ± 0.1	100.0 ± 0.0	0.0	237.7 ± 23.0
dbt^{S}/+	*68*	*3*	24.1 ± 0.0	98.6 ± 1.7	0.0	143.5 ± 14.4
Manipulation of the central pacemakers						
$pdf>sgg^{WT}$	*53*	*3*	–[2]	24.2 ± 3.7	18.2 ± 17.0	–[2]
$pdf>sgg^{CA}$	*83*	*3*	–[2]	9.7 ± 4.1	49.8 ± 10.8	–[2]
$pdf>dbt^{S}$	*81*	*3*	18.2 ± 0.1	14.8 ± 4.6	1.2 ± 1.5	21.6 ± 7.9
$pdf>sgg^{RNAi}$	82	3	24.8 ± 0.3	97.9 ± 2.6	0.0	165.9 ± 18.2
$pdf>sgg^{RNAi}$;*dicer2*	109	4	26.0 ± 0.1	87.3 ± 10.6	0.0	139.1 ± 31.7
pdf>shn;dicer2	79	4	25.3 ± 0.2	88.6 ± 3.2	0.0	137.3 ± 40.5
$pdf>shn;sgg^{RNAi}$	97	3	–[2]	28.1 ± 20.9	47.3 ± 16.9	–[2]
$pdf>shn;sgg^{RNAi}$;*dicer2*	128	4	–[2]	9.4 ± 4.6	81.8 ± 5.4	–[2]
Manipulation of the entire circadian network						
$tim>sgg^{WT}$	65	3	21.2 ± 0.1	84.8 ± 8.3	0.0	65.4 ± 14.7
$clk^{856}>sgg^{CA}$	79	3	19.8 ± 0.2	61.1 ± 24.2	15.8 ± 17.0	72.3 ± 29.9
$tim>dbt^{S}$	78	3	18.1 ± 0.1	85.0 ± 6.9	0.0	70.0 ± 11.1
$tim>sgg^{RNAi}$	59	2	24.9 ± 0.0	89.3 ± 15.2	0.0	138.7 ± 32.1
$tim>sgg^{RNAi}$; *dicer2*	61	2	26.4 ± 0.0	90.1 ± 0.7	1.6 ± 2.2	110.7 ± 25.2
tim>*shn;dicer2*	56	2	25.5 ± 0.3	80.0 ± 28.3	6.7 ± 9.4	132.4 ± 17.3
tim>shn; sgg^{RNAi}	39	2	26.2 ±0.1	89.4 ± 2.7	0.0	147.8 ± 51.8
tim>shn; sgg^{RNAi};*dicer2*	52	2	28.2 ± 0.2	63.7 ± 31.1	30.4 ± 27.8	102.5 ± 33.8

[1]Values are expressed as mean ± SEM. *N*, total number of analyzed flies; *n*, number of independent experiments. All the analyses shown in the table were performed with 15 days of free running.

[2]Analysis of endogenous periods and the rhythmic power of the rhythms of these genotypes are described in Table 3.

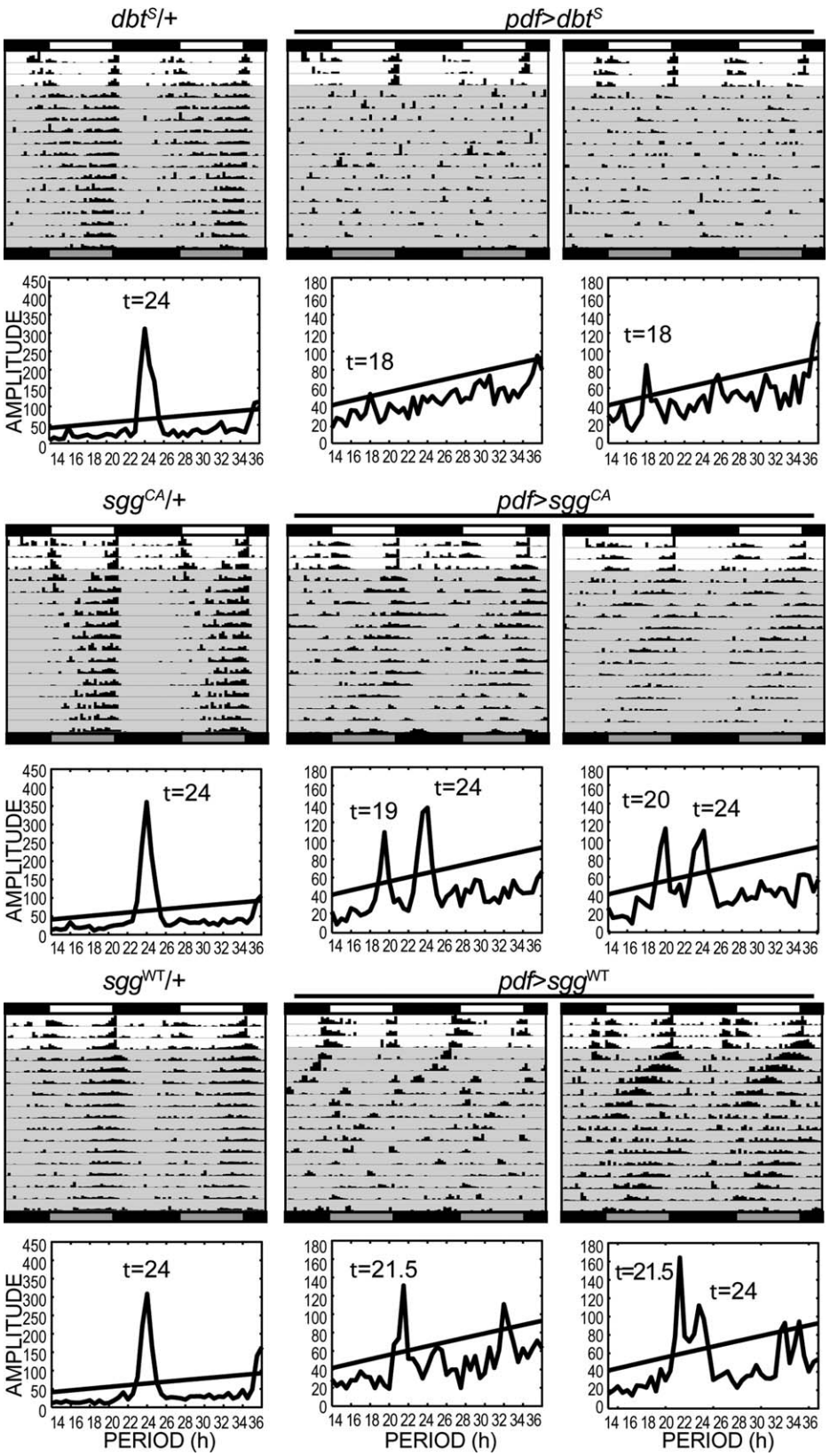

Figure 3. Acceleration of pacemaker neurons results in lack of rhythmicity and/or the emergence of multiple period components. Representative actograms of indicated genotypes and their respective periodograms. During the experiments, flies were kept in LD for 4 days, then switched to DD (shaded gray area), and monitored for 15 additional days. To better describe the complexity of the behavior resulting from the acceleration of pacemaker neurons, two representative individuals are depicted per genotype.

TABLE 3.
Animals With Complex Behaviors Express Two or Three Characteristic Components[1]

	Short component		Middle component		Long component	
Genotype	Period	Rhythmic power	Period	Rhythmic power	Period	Rhythmic power
pdf>sgg^{WT}	21.7 ± 0.1	13.2 ± 10.3	23.6 ± 0.1	39.9 ± 15.2	–	–
pdf>sgg^{CA}	19.3 ± 0.2	20.8 ± 2.3	23.9 ± 0.1	33.5 ± 4.8	–	–
pdf>shn; *sgg*^{RNAi}	21.4 ± 0.2	27.0 ± 11.0	24.8 ± 0.2	33.0 ± 9.4	28.1 ± 0.2	50.1 ± 21.7
pdf>shn; *sgg*^{RNAi};*dicer2*	22.2 ± 0.1	32.1 ± 4.2	24.8 ± 0.2	60.8 ± 6.0	27.9 ± 0.4	27.4 ± 9.7

[1]Values are expressed as mean ± SEM. Short component: periods shorter than 23.5 hours; middle component: periods between 23.5 and 26.5 hours; and long component: periods longer than 26.5 hours. All the analyses shown in the table were performed with 15 days of free running.

Tables 1 and 2). Extended analysis of the free running behavior revealed that a fraction of the population also displayed complex rhythms (Fig. 3, bottom panel, Table 3). As already shown for *dbt*^{S}, both genetic manipulations revealed a short and a ~24-hour component.

Overall, these experiments confirm that the sLNvs can drive period shortening but that their ability to impose this period on the remaining clusters is restricted. Under these conditions, the contribution of the non-sLNvs clusters on overt behavior is revealed.

Slowing down the pace of the sLNvs also leads to complex rhythms

To further address to what extent the master clock is able to influence the period of the remaining circadian relevant clusters ultimately controlling behavior, we combined the previously described genetic interventions in an effort to further slow down the pace of the clock exclusively in the sLNvs. Strikingly, concomitant *sgg* knock-down and upregulation of the BMP pathway (*pdf>sgg*^{RNAi},*shn*) led to either very long locomotor activity rhythms (of around 28 hours) or a complex pattern in the activity profile, characterized by the appearance of more than one peak in the periodogram analysis (Fig. 4, middle panels). The coexistence of flies with rhythmic behavior (those displaying a single peak) and complex rhythms within the *pdf>sgg*^{RNAi},*shn* population suggests that in a proportion of the flies the LNvs are able to transmit a 28-hour period to clusters running with a faster (~24-hour) clock; in contrast, the existence of complex rhythms is a clear indication that there is a limit to that ability, as previously reported for the overexpression of wt and constitute active versions of *sgg* (Fig. 3). These results support the idea of an asymmetric entrainment curve, suggesting that a 28-hour period (4 hours longer than the 24-hour endogenous period) could be the upper limit of the entrainment range, whereas an ~22-hour period (only 2 hours apart from the wt free running one) seems to be its lower limit (see Discussion and Fig. 7).

To increase the difference in endogenous period between the LNvs and the rest of the network, we employed *dicer2* overexpression (which should enhance the silencing efficacy of the targeted RNAi; Dietzl et al., 2007), delivering at least 1 extra hour of period difference between clusters (compare genotypes *pdf>sgg*^{RNAi} and *pdf>sgg*^{RNAi};*dicer2* in Fig. 1). Interestingly, this condition led to a more penetrant phenotype of complex locomotor behavior when flies were kept under free running conditions (Fig. 4, bottom panel, Table 3).

To begin to characterize the array of phenotypes, behavior was classified into four categories (rhythmic, weakly rhythmic, complex rhythms, and arrhythmic; described in Materials and Methods; see also Nitabach et al., 2006). Interestingly, the most extreme period disagreement between clusters (*pdf>sgg*^{RNAi},*shn*;*dicer2*) gave rise to a higher proportion of flies displaying complex rhythms, in contrast to what was observed in a somewhat moderate genotype (*pdf>sgg*^{RNAi},*shn*; Table 2). Additionally, the frequency of rhythmic flies inversely correlated with those displaying complex rhythms (Table 2).

In sum, sLNvs with either a very slow or a very fast molecular clock are unable to efficiently coordinate behavior. It is worth noting that when a very slow clock operates in the sLNvs, rhythmic behavior is not prevented because the vast majority of flies exhibit complex rhythms (Table 2), highlighting the fact that circadian control of behavior is probably executed by other neuronal clusters.

A coherent network entails coherent behavior

Intrigued by the emergence of complex rhythms when accelerating and slowing down the main pacemaker neurons, we manipulated clock speed in a pan-circadian fashion. Regardless of which maneuver was employed, altering clock period in the entire circadian network resulted in clear activity patterns underlying the strong improvement in rhythmicity (Fig. 5, Table 2).

In conclusion, the circadian network has an inherent ability to sustain very short or very long endogenous periods, but a coherent network is a requisite for a consolidated activity pattern. Along these lines, previous work employing pleiotropic mutations in the core clock

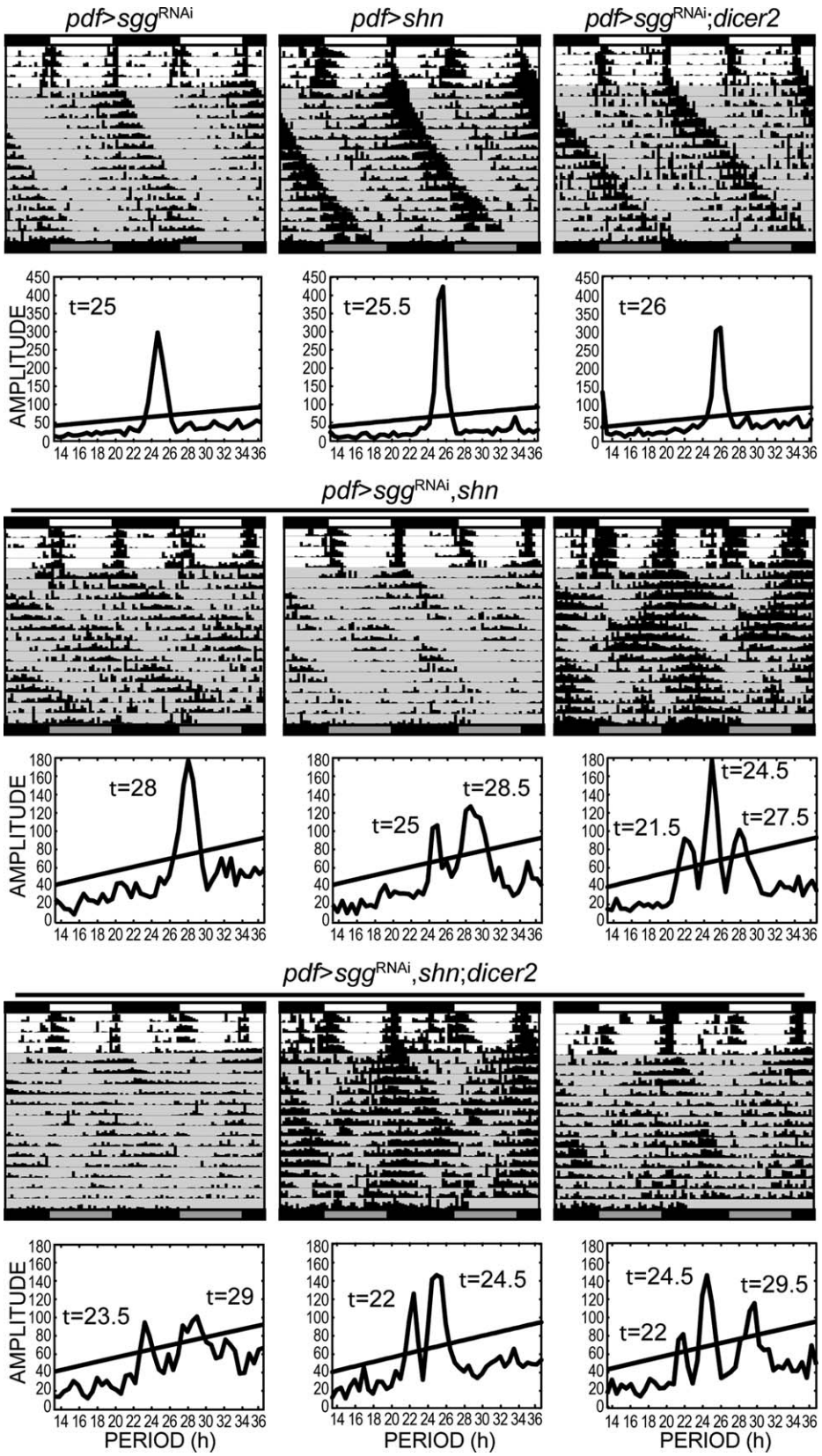

Figure 4. SGG downregulation coupled to SHN overexpression leads to complex rhythms. Representative actograms of indicated genotypes and their respective periodograms. During the experiments, flies were kept in LD for 4 days, then switched to DD (shaded gray area), and monitored for 15 additional days. Genotypes *pdf>sgg*^{RNAi},*shn* and *pdf>sgg*^{RNAi},*shn*;*dicer2* display a variety of different behaviors, so the figure includes three different individuals spanning representative ones.

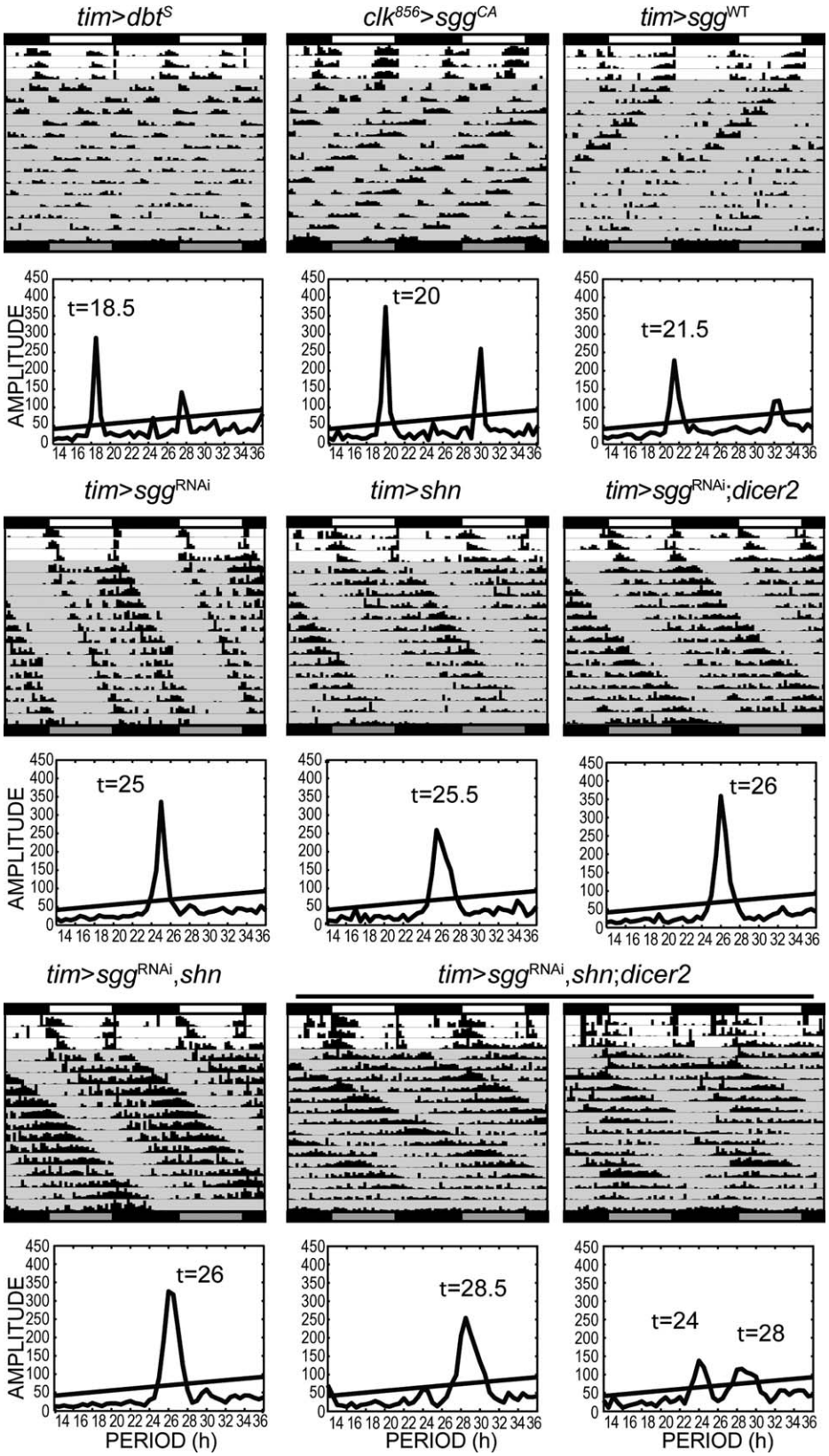

Figure 5. Pancircadian manipulation of clock speed triggers coherent rhythms. Representative actograms of indicated genotypes and their respective periodograms. During the experiments, flies were kept in LD for 4 days, then switched to DD (shaded gray area), and monitored for 15 additional days. Note that for pancircadian expression of the constitutively active shaggy (*sgg*^{CA}) we employed the strain *clk*^{856}Gal4 (Gummadova et al., 2009) because the use of the *tim*Gal4 strain results in lethality. Periodograms in the upper panels display two peaks that are not indicative of complex rhythms, rather they stem from the resonant frequency of a single endogenous period. *tim>sgg*^{RNAi}*,shn;dicer2* flies show extremely long period phenotypes, but complex rhythms persist in a minority of the individuals (22.8%), represented in the bottom-right actogram.

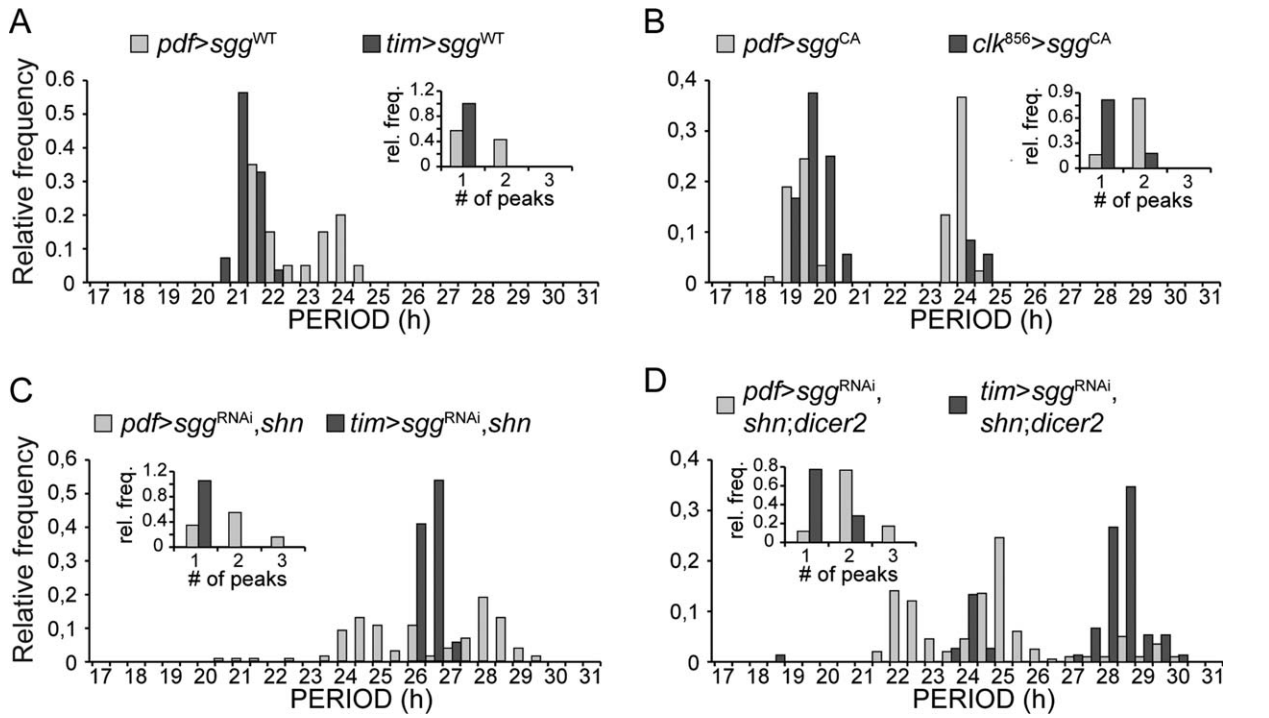

Figure 6. Ultrafast or ultraslow sLNvs become disconnected from the network controlling locomotor behavior. Detailed analysis of the experiments shown in Figures 3–5. Histograms show the relative frequency of statistically significant periods identified through the periodogram analysis for flies of the indicated genotypes as a function of period (in hours). The insets show histograms of the relative number of flies as a function of the number of statistically significant periodogram peaks.

genes *per* and *tim* showed that the circadian network is able to sustain ultrashort or ultralong free running periods without impaired rhythmicity (Rothenfluh et al., 2000). Taken together, these data lend further support to the idea of a complex network with multiple relevant components.

Very fast or slow LNvs are disconnected from the network

A detailed analysis of periodograms revealed that control flies with no alteration of the endogenous period in the LNvs, along with genotypes with moderate differences between clusters (such as *pdf>sgg*^{RNAi};*dicer2* or *pdf>shn*) exhibited a single peak. In contrast, only a minor proportion of the flies displaying strong alterations of the endogenous period in the main pacemaker neurons showed a single peak (*pdf>sgg*^{WT}, *pdf>sgg*^{CA}, *pdf>sgg*^{RNAi},*shn*, and *pdf>sgg*^{RNAi},*shn*;*dicer2*). Among these, we found flies displaying two stable locomotor activity patterns, and a smaller fraction even presenting three peaks in the periodogram analysis (Fig. 4, bottom and middle panels, Table 3).

A careful analysis of the distribution of endogenous periods exhibited by flies with complex rhythms highlighted an interesting phenomenon. Flies overexpressing *sgg*^{CA} or *sgg*^{WT} in the PDF domain showed a bimodal distribution of rhythmic components, including a short rhythm reflecting the sLNv period and a second one of around 24 hours (Fig. 6A,B); however, there is a difference between these two genotypes. The more spread out the two components were (as is the case for *sgg*^{CA}), the higher the proportion of flies observed displaying a 24-hour rhythm, perhaps reflecting a more pronounced disconnection among the clusters. Similar observations were made when we analyzed flies with slowed-down main pacemaker neurons. In *pdf>sgg*^{RNAi},*shn* flies (the intermediate phenotype), the most represented periods were those around 24.5 hours and a longer—and more robust—component centered on 28 hours (Fig. 6C, Table 3). In addition, these flies exhibited a third (underrepresented and poorly defined) component ranging from 20 to 22.5 hours. In contrast, in the case of the most extreme phenotype (*pdf>sgg*^{RNAi},*shn*;*dicer2*), the longest component almost disappeared and the shortest one was substantially more frequent (Fig. 6D), whereas the ~24-hour component became preponderant (Table 3). The absence of the long component in most of these flies strongly suggests that the sLNvs were disconnected from

downstream clusters. Moreover, the appearance of novel rhythmic component(s) reinforces the possibility that when the sLNvs are not able to impose their own endogenous period on the rest of the network, then other clusters in the circadian network are able to take control over behavior; the genetic strategy put forward here allowed assessment of the endogenous periods of those clusters (Fig. 6).

DISCUSSION

We took advantage of the well-characterized molecular clock of *Drosophila* together with the large genetic tool box available to address network properties of the circadian neuronal clock. By forcing apart the endogenous period of the main pacemaker from the rest of the network, we were able to address the ability of the sLNs to entrain other oscillators under free running conditions, a manipulation that ultimately led to split locomotor activity patterns. Previous work demonstrated that the sLNvs are able to shorten (Stoleru et al., 2005; Yu et al., 2011) or lengthen (Blanchard et al., 2010; Lim et al., 2011) the period of locomotor activity, establishing the idea of a master pacemaker ruling slave oscillators. In agreement with the data presented here, the previously accepted model has recently been challenged (Dissel et al., 2014; Yao and Shafer, 2014). Yao and Shafer demonstrated that independent neuronal oscillators within the network can drive bouts of rhythmic behavior when the sLNvs are disconnected (i.e., as in the absence of PDF signaling) or when there is a large discrepancy regarding clock speed between clusters, a situation that entails a conflict between them. Thus, the sLNvs, through PDF signaling, can impose their own free running period to the network within a restricted range. We established that this entrainment range is between 22 and 28 hours, namely, the sLNvs have an asymmetric capability to impose coherent activity on the rest of the network; beyond that range other circadian oscillators run independently of sLNv control and contribute to shape behavior (Fig. 7).

The observed asymmetry described here contrasts with the estimations reported by Yao and Shafer (2014); even though both data sets suggest a restriction in period shortening when oscillators are running about 2 hours apart, our results suggest that a synchronous period lengthening can be achieved when individual oscillators are maintained almost 4 hours apart (*pdf>shn,sgg*^{RNAi} flies described in Fig. 4, middle panel). Along these lines, overexpression of constitutively active forms of the BMP pathway receptors *thickveins* and *saxophone* in PDF-positive neurons led to a 27-hour

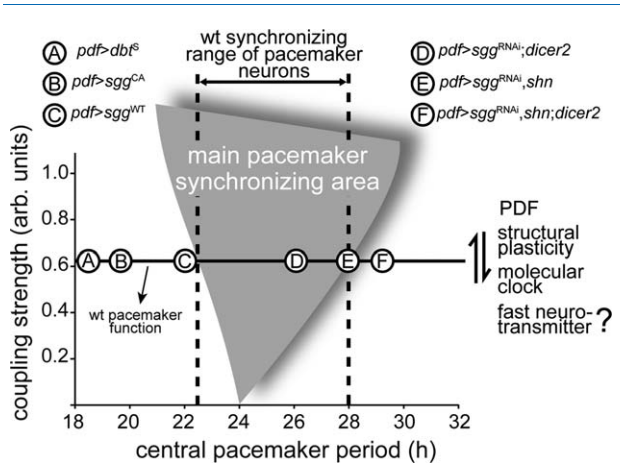

Figure 7. A model for the restricted entrainment. The sLNvs operate as pacemakers for the rest of the circadian system; the ability of these neurons to shorten the period of the rest of the network reaches its limit at around 22 hours. Instead, their ability to lengthen the period of downstream targets goes beyond that restricted 2-hour window, because the sLNvs can impose a 28-hour period on the network governing locomotor activity. Such restrictions may arise from the nature and strength of the temporal cues that could be modulated from and to the sLNvs by PDF signaling, the structural plasticity of the sLNvs dorsal projections, the intrinsic properties of the different oscillators, and the release of an unknown fast neurotransmitter from the sLNvs. A–F represent six of the genotypes evaluated.

endogenous period with no impact on behavioral rhythmicity (Beckwith et al., 2013). Finally, these results constitute a previously unexplored strategy that allows the assessment of the endogenous period of non-LNv clusters, which resulted in observations that confirm previous estimations (Yoshii et al., 2009).

Complex rhythms as an instrument to assess chronobiological properties

In addition to a variety of manipulations leading to complex rhythms (Page, 1983; Tomioka et al., 1991; Abe et al., 1997; Ushirogawa et al., 1997; Helfrich-Forster et al., 2000; Lavie, 2001; de la Iglesia et al., 2004; Nitabach et al., 2006; Rieger et al., 2006; Cambras et al., 2007; Schwartz et al., 2009; Umezaki et al., 2011; Casiraghi et al., 2012; Wotus et al., 2013), such behavior can be brought about through forcing a very fast or slow molecular clock exclusively in the sLNvs. Together, *sgg* up- or downregulation along with *shn* overexpression allowed us to generate conflicts between the main pacemaker neurons and other oscillators in the network, as suggested by the emergence of different free running components in locomotor behavior. Under these conflicting conditions, different periods arose: an extremely long (or short) component, driven by the genetically manipulated LNvs; an ~24-

hour pattern, probably reflecting the endogenous period of loosely coupled oscillators, present in every genetic condition examined here; and, only in the most extreme pattern, a short ~22-hour component, most likely driven by a strongly coupled oscillator now released into *free running* conditions. Previous work has described such oscillators, and very thorough analyses have attempted their identification. The two CRY-positive LNds also expressing the short neuropeptide F (sNPF) neuropeptide, along with the fifth sLNv, and the CRY-positive DN1a and DN1p neurons probably constitute a fast oscillator strongly coupled to the sLNvs, whose period is lengthened through PDF signaling (Johard et al., 2009; Yoshii et al., 2009; Yao and Shafer, 2014). Likewise, the DN3 neurons also seem to be strongly coupled to the sLNvs, although their intrinsic period has not been estimated (Stoleru et al., 2005). Finally, the CRY-negative and PDFR-negative LNds, the CRY-negative DN1p, and the DN2 neurons are loosely coupled to the PDF oscillator, and run with a slightly longer period (Stoleru et al., 2005; Yoshii et al., 2009; Yao and Shafer, 2014). Hence, a clearer description of the circadian network controlling locomotor behavior is emerging.

Synchronization of the network

In terms of the coupling mechanisms, the exact molecules that couple the different oscillators within the *Drosophila* circadian network have not yet been determined. Undoubtedly, PDF exerts a major synchronizing role, and it is also clear that PDF action is not the same in each cluster (Helfrich-Forster et al., 2000; Yoshii et al., 2009). Interestingly, these different effects could be partially explained by slightly different signaling pathways recruited in target cells. Along these lines, it has been shown that the adenylate cyclase present in the LNvs is different from that expressed in the LNds (Duvall and Taghert, 2012, 2013), but a complete analysis of the signaling pathway that is switched on after PDF receptor activation in each cluster is ongoing. Beyond PDF, other neuropeptides like sNPF and ion transport peptide (ITP) are relevant as network synchronizers and output signals (Hermann et al., 2012; Hermann-Luibl et al., 2014; Yao and Shafer, 2014), and could be the substrate of coupling differences.

In addition, the recruitment of different components to the molecular machinery, i.e., the presence or absence of CRY, could also contribute to fine-tuning of the intrinsic properties of the oscillators and their coupling abilities (Shafer et al., 2006; Yoshii et al., 2008; Fogle et al., 2011). Moreover, the existence of a fast neurotransmitter within the sLNvs opens many possibilities worth exploring (Yasuyama and Meinertzhagen, 2010). Neuropeptide release together with a fast neurotransmitter could impose gating properties or modulate neuropeptide function in several ways depending, for example, on the receptors expressed in the downstream targets. Moreover, cyclical changes in axonal terminals (that could impact on both the neuropeptide and/or the fast neurotransmitter release or activity) could influence network synchronization, resulting in a differential effect of the sLNv neurons on each contacting cluster throughout the day (Gorostiza et al., 2014).

In sum, signals driving synchronization, together with the intrinsic properties of individual oscillators and their ability to respond to synchronizing cues, define communication within the circadian network and sustain behavioral rhythmicity.

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CONFLICT OF INTEREST STATEMENT

The authors declare they have no competing financial interests.

ROLE OF AUTHORS

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: EJB and MFC. Acquisition of data: EJB. Analysis and interpretation of data: EJB and MFC. Drafting of the manuscript: EJB. Critical revision of the manuscript for important intellectual content: MFC. Obtained funding: MFC.

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