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Report

Circadian Pacemaker Neurons Change Synaptic Contacts across the Day

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Summary

Daily cycles of rest and activity are a common example of circadian control of physiology. In *Drosophila*, rhythmic locomotor cycles rely on the activity of 150–200 neurons grouped in seven clusters [1, 2]. Work from many laboratories points to the small ventral lateral neurons (sLNvs) as essential for circadian control of locomotor rhythmicity [3–7]. sLNv neurons undergo circadian remodeling of their axonal projections, opening the possibility for a circadian control of connectivity of these relevant circadian pacemakers [8]. Here we show that circadian plasticity of the sLNv axonal projections has further implications than mere structural changes. First, we found that the degree of daily structural plasticity exceeds that originally described [8], underscoring that changes in the degree of fasciculation as well as extension or pruning of axonal terminals could be involved. Interestingly, the quantity of active zones changes along the day, lending support to the attractive hypothesis that new synapses are formed while others are dismantled between late night and the following morning. More remarkably, taking full advantage of the GFP reconstitution across synaptic partners (GRASP) technique [9], we showed that, in addition to new synapses being added or removed, sLNv neurons contact different synaptic partners at different times along the day. These results lead us to propose that the circadian network, and in particular the sLNv neurons, orchestrates some of the physiological and behavioral differences between day and night by changing the path through which information travels.

Results and Discussion

Temporal Dynamics of the Structural Plasticity

Circadian remodeling of the small ventral lateral neuron (sLNv) dorsal terminals was first described at the peak and trough levels of pigment-dispersing factor (PDF) immunoreactivity, that is at zeitgeber time 2 (ZT2) and ZT14 (2 hr after lights ON and lights OFF, respectively), as well as their counterparts under constant darkness (DD) (circadian time 2 [CT2] and CT14) [8]. For a more precise examination of the extent of structural remodeling, a time course was carried out. An inducible GAL4 version termed GeneSwitch [10, 11] restricted to PDF neurons (*pdf*-GS) [12] combined with a membrane-tethered version of GFP (mCD8GFP) was used as control. As expected from our original observations, a significant reduction in complexity of the axonal arbor—measured as total axonal crosses—could be seen between CT2 and CT14 and between CT18 and CT22 (Figures 1A and 1B), which remained unchanged at nighttime. However, toward the end of the subjective night (CT22), the primary processes appeared to be shorter. To more precisely describe this additional form of plasticity, we measured the length of the maximum projection from the lateral horn toward the midbrain. This analysis revealed that toward the end of the subjective night (CT22), PDF projections are significantly shorter than at the beginning of the day (CT2; Figure 1C). These observations imply that mechanisms other than the proposed changes in the degree of fasciculation are recruited during circadian plasticity [8, 13]. To get a deeper insight into the nature of the phenomena, we monitored the changes in brain explants kept in culture for 48 hr after dissection. Transgenic *pdf*-GAL4*;* UAS-*mCD8RFP* flies (from now on referred to as *pdf >RFP*) were dissected under safe red light, and brains were maintained under DD. Imaging of individual brains at two different time points highlighted three types of changes experienced by axonal terminals: (1) changes in the degree of fasciculation/defasciculation, more common in primary branches, (2) the addition/retraction of new processes, mostly affecting those of secondary or tertiary order, and (3) positional changes of minor terminals (Figures 1D and 1E), thus confirming and extending our previous observations. Altogether, these results indicate that a rather complex remodeling process takes place on daily basis in the axonal terminals of PDF neurons.

Morphological Plasticity Correlates with Changes in Synaptic Markers

The level of structural remodeling occurring at the dorsal terminals suggested that synapses themselves could undergo changes in a time-dependent fashion. We first examined the presynaptic protein SYNAPTOTAGMIN (SYT) at different times across the day as an indicator of vesicle accumulation. A GFP-tagged version of SYT was expressed in PDF neurons (*pdf >syt^{GFP}*), and both the number and area span by SYT^{+} puncta (most likely describing the accumulation of several dense core vesicles [14]) were analyzed separately at the sLNv dorsal terminals (Figures S1A–S1C available online). No statistical differences were observed in the number of SYT^{+} puncta (although there is a tendency for higher numbers in the early morning), perhaps as a result of the nature of the signal, which is too diffuse for precise identification of individual spots (Figure S1B). On the other hand, SYT^{+} puncta were larger and, as a result, the area covered by SYT^{+} immunoreactivity was significantly different at CT2 compared to CT14, but not between CT22 and CT2, perhaps reflecting that vesicles started to accumulate at the end of the day in preparation for the most dramatic membrane change taking place between CT22 and the beginning of the following morning (Figure S1C).

The observation that a more complex structure correlated with a larger area covered by presynaptic vesicles reinforced the notion that indeed the number of synapses could be

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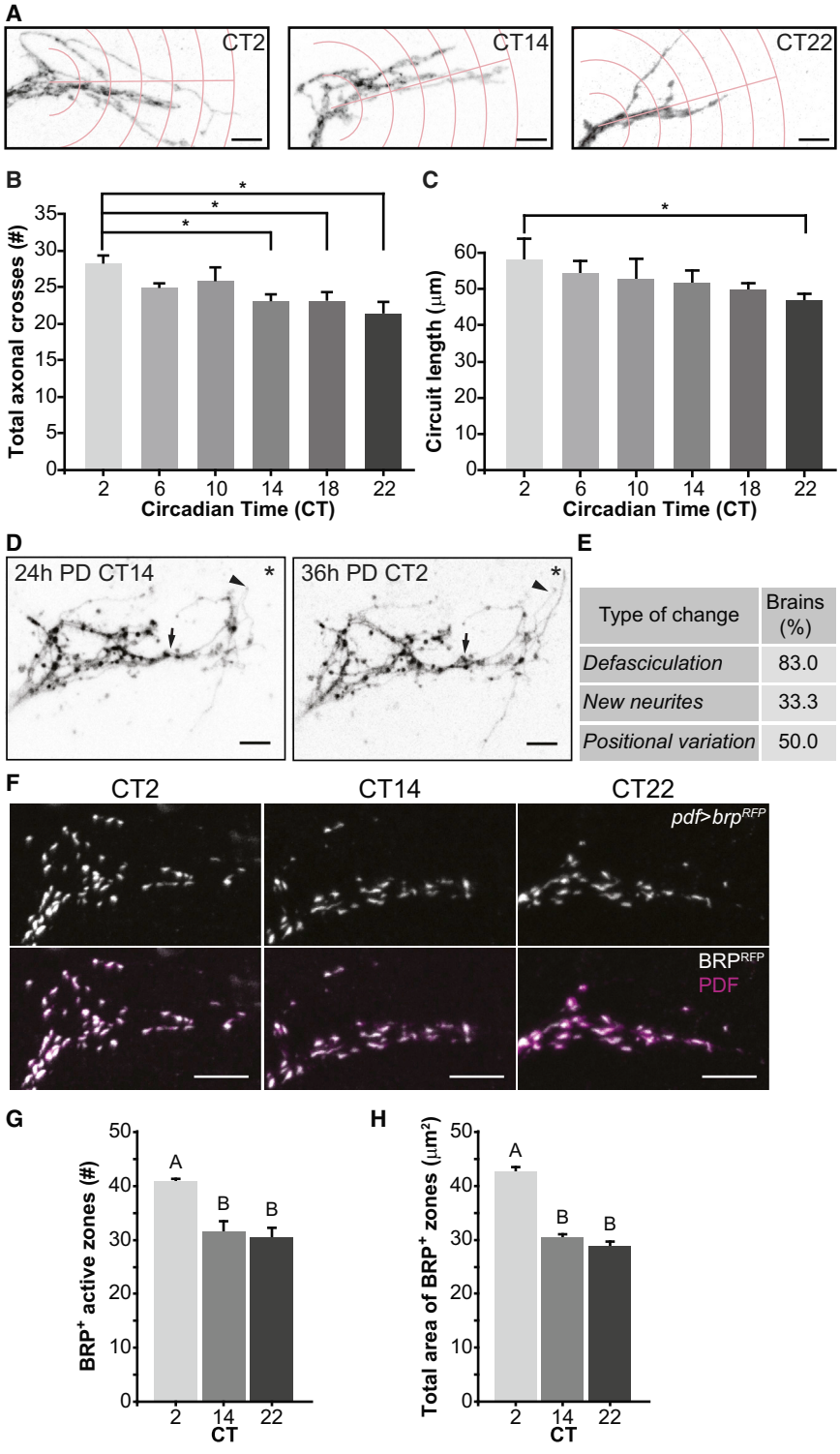


Type of change	Brains (%)
Defasciculation	83.0
New neurites	33.3
Positional variation	50.0

Figure 1. Severe Morphological and Synaptic Changes Occur during the Dark to Light Transition

(A) Representative confocal images taken at CT2, CT14, and CT22. During early subjective day (CT2), axonal projections are more complex and extended, reaching further toward the medial region, whereas at CT22, PDF projections are less complex (as in CT14) and appear to be shorter.

(B and C) Quantitation of total axonal crosses (B) and the longest axonal branch (C) at CT2, CT6, CT14, CT18, and CT22 for control brains (*pdf*-GS > *mCD8GFP*). Dissections were performed on the fourth day of DD. Dark gray represents subjective night, and light gray represents subjective day. * indicates significant differences with $p < 0.05$. Statistical analysis included blocked ANOVA (total axonal crosses, $p = 0.0002$; circuit length, $p = 0.0417$) with a Tukey post hoc test ($p < 0.05$; total axonal crosses least significant difference = 3.40; circuit length least significant difference = 10.98 μm).

(D) Representative confocal images of dorsal sLNv projections from cultured brains. Brains were cultured 72 hr and imaged 24 hr postdissection (PD; left), which equals CT14, and 36 hr PD, which equals CT2 (right). A fasciculation/defasciculation process could be appreciated in the principal branches (arrows), whereas in secondary neurites, different phenomena were observed: addition/retraction (asterisk) and positional changes (arrowhead).

(E) Quantitation of changes seen in different cultured brains (n = 6).

(F) Representative confocal images of fly brains stained for BRP^{RFP} (white) and PDF (magenta) dissected at CT2, CT14, and CT22 on the fourth day of DD.

(G and H) Quantitation of BRP^+ active zones (G) and the total area covered by them (H). Control *pdf*-GS>brp^{RFP} flies display circadian changes in BRP^+ active zones and the area covered by BRP^+ immunoreactivity. Significant differences were found in both variables between subjective day and night but not between time points taken at nighttime. The same letters indicate no significant differences. Statistical analysis included one way ANOVA (BRP^+ active zones, $p = 0.0069$; BRP^+ area, $p < 0.0001$) with a Tukey post hoc test ($p < 0.05$; BRP^+ active zones least significant difference = 6.99; BRP^+ area least significant difference = 3.35 μm^2).

Scale bars represent 10 μm. Bars and error bars indicate mean ± SEM. See also Figure S1.

changing throughout the day and prompted us to analyze BRUCHPILOT (BRP), a well-established indicator of active zones [15–18]. Expressing a tagged version of BRP in PDF neurons, we quantitated the number of BRP^+ puncta as a proxy for active zones [19] at times when the most dramatic changes in structure had been detected (i.e., CT2, CT14, and CT22; Figures 1F–1H). Interestingly, the number of active zones was significantly larger at CT2 than at CT14 or CT22; in fact, no statistical differences were observed between the last two time points, underscoring that axonal remodeling can occur (i.e., pruning of major projections taking place toward the end of the night) without significantly affecting overall connectivity. Thus, circadian structural plasticity is accompanied by changes in the number of synapses. Not only are more vesicles recruited toward CT2, but also a higher number of active zones are being established.

Circadian changes in the abundance of the presynaptic active zone BRP have also been shown in the first optic neuropil of the fly brain, although BRP abundance in the lamina increases in the early night under DD conditions [20], in contrast

to the oscillations in BRP levels observed at the dorsal protocerebrum that peak in the early subjective day just described. In addition, rhythmic changes in the number of synapses have also been described in the terminals of adult motor neurons in *Drosophila* [21] examined through transmission electron microscopy, as well as BRP^{+} light confocal microscopy, underscoring the validity of the approach employed herein. Interestingly, in different brain areas, the level of presynaptic markers (such as BRP^{RFP} or SYT^{GFP}) also changes in response to the sleep/wake "state," being high when the animals are awake and lower during sleep [19, 22, 23]; this observation led to the proposal that sleep could be involved in maintaining synaptic homeostasis altered during the awaking state. This trend coincides with our observation of higher levels during the subjective morning and lower levels at the beginning of the subjective night; however, we could not detect changes through the night, suggesting that, at least in clock neurons, there is a circadian rather than a homeostatic control of synaptic activity. Given that clock outputs are predominantly regulated at the transcriptional level [24] and that there is circadian regulation of MEF2, a transcription factor that turns on a program involved in structural remodeling [13], this correlation opens the provocative possibility that the circadian clock is controlling the ability of assembling novel synapses in particularly plastic neurons, which might become recruited and/or stabilized, or otherwise pruned (disassembled), toward the end of the day.

Activity Dependent and Independent Mechanisms Underlie Structural Plasticity

Adult-specific electrical silencing of PDF neurons reduces the complexity of dorsal arborizations, although a certain degree of circadian remodeling of the axonal terminals still takes place [12]. To examine whether electrical alterations could affect circadian changes in the number of active zones, we expressed either Kir2.1 or NaChBac (to hyperpolarize or depolarize PDF neurons, respectively). To avoid any undesired developmental defects, we used *pdf*-GS to drive expression of the channels only during adulthood. Interestingly, Kir2.1 expression abrogated circadian changes in the number of active zones. In fact, PDF neurons displayed a reduced number of active zones compared to controls at CT2 and remained at similar levels throughout the day, indistinguishable from nighttime controls (Figure S1D). On the other hand, when neurons were depolarized through NaChBac expression, the number of active zones did not change along the day and was maintained at daytime levels even at CT14 and CT22 (Figure S1E).

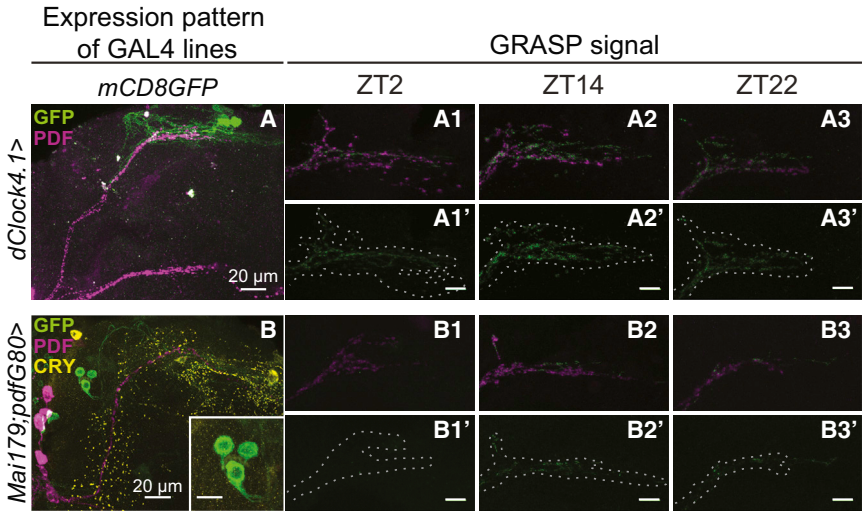
It has recently been shown that MEF2, a transcription factor involved in activity-dependent neuronal plasticity and morphology in mammals [25], is circadianly regulated and mediates some of the remodeling of PDF dorsal terminals through the regulation of Fasciclin2 [13]. On the other hand, adult-specific silencing (and depolarization) of PDF neurons abolishes cycling in the number of BRP^{+} active zones (Figures S1D and S1E), despite the fact that it does not completely obliterate the remodeling of the axonal terminals [12], suggesting that some of the mechanisms underlying structural plasticity are clearly activity independent and are most likely the result of additional clock-controlled output pathways still to be identified.

Circadian Changes in the sLNv Connectome

Since structural remodeling of PDF neurons results in the formation and disappearance of new synapses on daily basis, we anticipated that not only the number but also the postsynaptic partners of these contacts could concomitantly be changing. To shed light on this possibility, we employed GFP reconstitution across synaptic partners (GRASP), which labels contacts between adjacent membranes [9, 26]. In brief, two complementary fragments of GFP tethered to the membrane are expressed in different cells. If those cells are in contact, GFP is reconstituted and becomes fluorescent. GRASP has previously been employed to monitor synapses in adult flies [26–29]. Given the complex arborization at the dorsal protocerebrum, we inquired whether specific subsets of circadian neurons projecting toward that area [1] could be contacting across the day. Perhaps not surprisingly, an extensive reconstituted GFP signal could be observed between the sLNv dorsal projections and those of the posterior dorsal neuron 1 cells (DN1ps, lighted up by the *dClk4*.1-GAL4 line [30, 31]), suggesting contacts along the entire area (Figures 2A and 2C), which are detectable across all time points analyzed (ZT2, ZT14, and ZT22). Consistent with our observations, extensive physical contact between the sLNv projections and those of the DN1p neurons has just been reported at the dorsal protocerebrum with no clear indication of the time of day examined [32, 33]. We next examined whether a subset of dorsal LNs (LNds), projecting toward both the accessory medulla and the dorsal protocerebrum (through the combined expression of *Mai179*-GAL4; *pdf*-GAL80), could also contact the profuse dorsal arborization of sLNv neurons; this genetic combination enables expression of split-GFP in a restricted number of circadian cells (which are part of the evening oscillator [4], i.e., up to four LNds, including at least a CRYPTOCHROME-positive one, and the fifth sLNv), as well as others located within the pars intercerebralis (PI), a neurosecretory structure recently identified as part of the output pathway relevant in the control of locomotor behavior [32]. In contrast to the extensive connections between DN1p and sLNv clusters, only very discreet reconstituted puncta were detected. Quite strikingly, the degree of connectivity appeared to change across the day, reaching a maximum (when almost every brain exhibited reconstituted signal) at ZT22 (Figures 2B and 2C). However, due to the nature of the signal, no quantitation of its intensity was attempted. Although a more detailed analysis is required to define the identity (i.e., whether it is one or several LNds, the fifth sLNv, or both groups that directly contact the sLNvs), this finding highlights a potentially direct contact between the neuronal substrates of the morning and evening oscillators. In sum, through GRASP analysis, we have begun to map the connectivity within the circadian network; commensurate with a hierarchical role, the sLNvs appear to differentially contact specific subsets in a distinctive fashion.

Transmitting Time of Day Information to Noncircadian Targets

To address the possibility that PDF neurons could be contacting noncircadian targets at different times across the day, we carried out an enhancer trap screen employing a subset of GAL4 enhancers selected on the basis of their expression pattern in the adult brain, i.e., known to drive expression in the dorsal protocerebrum, and an additional requirement imposed was that none of the selected GAL4 lines could direct expression to the sLNv neurons to avoid internal GFP reconstitution. Reconstitution of the GFP signal at the sLNv dorsal terminals by recognition through specific antibodies was assessed at three different time points for each independent GAL4 line (ZT2, ZT14, and ZT22). Some of the GAL4 lines



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GAL4 line	Relative frequency of brains with GRASP$^+$ signal		
	ZT2	ZT14	ZT22
dClock4.1	0.98	0.98	0.97
Mai179;pdfG80	0.47	0.60	0.91

Figure 2. GRASP Analysis on Putative Clock Partners Reveals Constant and Plastic Changes in sLNv Connectivity

(A and B) Images represent examples of putative synaptic partners of PDF neurons. Expression profiles of (A) *dClock4.1*-GAL4 to light up DN1p neurons and (B) *Mai179*-GAL4*; pdf*-GAL80 expression on a restricted subset of circadian-relevant neurons, including the fifth sLNv, up to four LNds and PI cells are shown. PDF and GFP signals are shown in magenta and green, respectively.

(A1–A3) Representative confocal images of a *pdf*-lexA>lexAop-*CD4::GFP*^{11}/*dClock4.1*-GAL4> UAS-*CD4GFP*$^{1-10}$ brain dissected during early day (ZT2; A1), early night (ZT14; A2), and late night (ZT22; A3).

(A1′–A3′) Reconstituted GFP$^+$ signal is shown; the structure of PDF projections is outlined by a dashed line (encircling the PDF signal) to improve visualization of the reconstituted GFP. GFP$^+$ signal was observed at all time points analyzed.

(B1–B3 and B1′–B3′) Intersection between PDF and *Mai179*-GAL4*; pdf*-GAL80 neurons (the so-called evening oscillator [8]). The reconstituted signal changes across the day, becoming more pervasive at nighttime.

(C) Quantitative analysis confirms constant contacts between sLNvs and DN1p clusters, but plastic ones between sLNvs and the evening oscillator, with a statistically significant increase at ZT22 (Kruskal-Wallis test, $p < 0.01$). Scale bars represent 10 μm unless otherwise noted.

showed reconstituted GFP signal at every time point analyzed (see, for example, the *11-8* line shown in Figure 3A or the *4-93* line in Figure S2D), suggesting that those neuronal projections are indeed in close contact across the day and might represent stable synaptic contacts. No GFP signal was detected in the negative parental controls (Figures S2A and S2B). Despite the fact that several GAL4 drivers directed expression to the proximity of the PDF dorsal terminals, some of the selected lines did not result in reconstituted GFP signal (about 20% of the samples analyzed; Figures S2C and S2D).

Quite remarkably, a proportion of the GAL4 lines showed GFP$^+$ signal only at a specific time point. One such example is line *3-86*, where reconstitution was detected in most of the brains analyzed at ZT2, but not at nighttime (Figure 3B). Being able to identify putative postsynaptic contacts to the sLNvs in the early morning is consistent with the observation of a higher number of BRP$^+$ active zones in the early day (Figures 1F–1H). This enhancer trap spans different neuropils, such as the mushroom body (MB) lobes and lateral horn, and directs expression to particularly high levels in the PI (Figure 3B and Figure S2E), a structure that has recently been implicated in the rhythmic control of locomotor activity [32]. In fact, some yet unidentified somas in the PI appear to arborize profusely near the PDF dorsal terminals, underscoring a potential link between the two neuronal groups. These direct contacts are unlikely to be the ones reported by *Mai179*-GAL4; *pdf*-GAL80 since those connect to the sLNv neurons preferentially at night (Figure 2). Interestingly, a subset of neurons in the PI is relevant in mediating the arousal promoting signal from octopamine [34]; in addition, sleep promoting signals are also derived from a different subset of neurons in the PI [35], opening the attractive possibility that both centers could be under circadian modulation.

GRASP analysis also uncovered a different neuronal cluster (*4-59*) that contacts PDF neurons preferentially during the early night (ZT14), which is in itself striking, since this time point corresponds to that with fewer arborizations and an overall decrease in the number of synapses (Figure 3C). This enhancer trap is expressed in the MBs, subesophagic ganglion, antennal lobes, and accessory medulla (Figure S2G). Among those structures, the MBs are important for higher-order sensory integration and learning in insects [36]. Interestingly, circadian modulation of short-term memory [37] and memory retrieval after sleep deprivation [38] was reported; short-term memory was found to peak around ZT15–ZT17, coinciding with the window of GFP reconstitution, thus providing a functional connection to the synaptic plasticity observed. To corroborate whether there is a direct contact between the two neuronal clusters, we employed the extensively used GAL4 driver OK^{107}, which is expressed in the α′/β′and the γ lobes of the MBs and to a lower extent in the PI [39] (Figure S2H), for GRASP analysis. Surprisingly, reconstituted GFP signal could be observed at every time point analyzed, suggesting that MB lobes contact PDF neurons throughout the day but that specific clusters (for example those highlighted by the *4-59* line) establish plastic, time-of-day-dependent physical contact with PDF neurons (Figure 3D).

We next inquired whether these prospective postsynaptic targets of PDF neurons could play a role in the output pathway controlling rhythmic locomotor activity. To address this possibility, we examined the impact of adult-specific alteration of excitability of distinct neuronal groups through expression of TRPA1. Interestingly, adult-specific depolarization of specific neuronal populations triggered a clear deconsolidation of the rhythmic pattern of activity, which resulted in less-rhythmic flies accompanied by a significant decrease in the strength of the underlying rhythm (Table 1). These results lend support to the notion that the underlying neuronal clusters are relevant in the control of rest/activity cycles.

Over the years, it has become increasingly clear that the circadian clock modulates structural properties of different

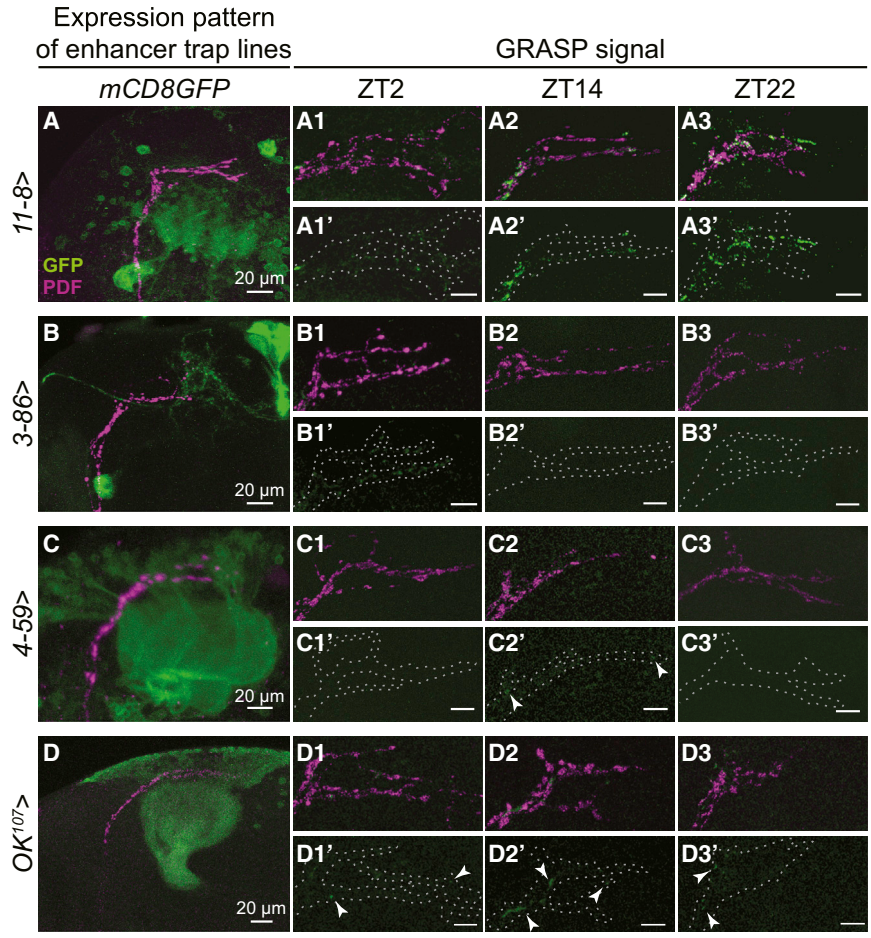

Figure 3. A GRASP Screen Uncovers Changes in Connectivity to Noncircadian Targets

Images represent examples of putative synaptic partners of PDF neurons contacting them in different time windows: throughout the day (A), during ZT2 (B), or during ZT14 (C). Expression profiles of *11-8* (A), *3-86* (B), *4-59* (C), and OK^{107} (D) neuronal clusters are shown. PDF and GFP signals are shown in magenta and green, respectively. *3-86* is expressed in the PI and sends neurites proximal to sLNv dorsal projections. *4-59* and *11-8* are both expressed in the calyx of the MBs, although different subgroups of Kenyon cells appear to be included in each line. OK^{107} is a widely used MB driver.

(A1–A3) Representative confocal images of *pdf*-lexA>lexAop-*CD4::GFP*^{11}/*11-8*-GAL4>UAS-*CD4GFP*$^{1-10}$ brains dissected during early day (ZT2; A1), early night (ZT14; A2), and late night (ZT22; A3).

(A1′–A3′) Reconstituted GFP$^+$ signal is shown; the overall structure is outlined by a dashed line (encircling PDF signal) to improve visualization of the reconstituted GFP. GFP$^+$ signal was observed at the three analyzed time points.

(B1–B3 and B1′–B3′) Intersection between PDF and *3-86* neurons. Reconstitution signal was observed only at ZT2.

(C1–C3 and C1′–C3′) A similar analysis was carried out with the *4-59* enhancer-trap line. Reconstitution was observed at ZT14.

(D1–D3 and D1′–D3′) Synaptic contacts between PDF neurons and the mushroom bodies evidenced by GRASP at ZT2, ZT14 and ZT22.

Arrows indicate synaptic reconstitution. Scale bars represent 10 μm unless otherwise indicated. See also Figure S2.

cells (reviewed in [40]). In fact, a number of years ago, it was reported that the projections of a subset of core pacemaker fly PDF$^+$ [8] and mammalian VIP$^+$ [41] neurons undergo structural remodeling on daily basis. The work presented herein lends support to our original hypothesis that circadian plasticity represents a means of encoding time-of-day information. By changing their connectivity, PDF neurons could drive time-specific physiological processes. As new synapses assemble while others are dismantled, the information flux changes, allowing PDF neurons to promote or inhibit different processes at the same time. This type of plasticity adds a new level to the complex information encoded in neural circuits, where PDF neurons could not only modulate the strength in the connectivity between different partners, but also define which neuronal groups could be part of the circadian network along the day. Although further analysis of the underlying process is ensured, evidence so far supports the claim that structural plasticity is an important circadian output.

Experimental Procedures

Strains and Fly Rearing

Flies were reared and maintained at 22 (locomotor activity assays) or 25°C in vials containing standard cornmeal medium under 12:12 hr light:dark (LD) cycles, with the exception of those including RU486 (mifepristone, Sigma) that were treated as previously described [12]. A list of the stocks employed throughout this work is included in the Supplemental Experimental Procedures.

Brain Cultures

For brain cultures, we used the protocol previously described [42] with minor changes. In brief, flies reared in LD were cold anesthetized and washed with 70% ethanol. Brains were quickly dissected in ice-cold Schneider medium (Invitrogen) and placed on a Millicell Low Height Culture Plate Insert (Millipore), previously coated with laminin (BD Biosciences) and polylysine (Sigma), on a Petri dish with culture medium, which was kept at 25°C under DD conditions. The first observation was made 24 hr postdissection (PD). The culture medium was supplemented with penicillin, streptomycin, fetal bovine serum (Natocor), and insulin and was replaced on daily basis.

Locomotor Behavior Analysis

Flies were crossed and maintained at 22°C while being entrained to a 12 hr LD cycle. Newly eclosed adult males were placed in glass tubes containing standard food and monitored for locomotor activity using the DAM system (TriKinetics). Isolated males were kept in LD conditions for 3 days, followed by 6 days at 22°C on DD. On day 7, the temperature was raised to 28°C, and flies were transferred to fresh tubes under red light and kept in the incubator for additional 7 days. Period, FFT, and rhythmicity were estimated using ClockLab software (Actimetrics) as previously described [12, 43].

Dissection and Immunofluorescence

Dissection and immunostaining of adult fly brains was performed as previously described [12]. The primary antibodies employed were rabbit anti-GFP 1:500 (Invitrogen), rabbit anti-RFP 1:500 (Rockland), chicken anti-GFP 1:500 (Upstate), rabbit anti-PDF 1:1500 (custom made by NeoMPS, France), and homemade rat anti-*Drosophila*-PDF 1:500 [12]. Secondary antibodies used were Cy2- and Cy3-conjugated anti-rabbit, Cy2-conjugated anti-chicken, and Cy5- and Cy3-conjugated anti-rat (Jackson ImmunoResearch). Images were taken on a Zeiss Pascal laser scanning microscope (LSM), a Zeiss LSM 510 meta confocal microscope, or a Zeiss LSM 710 two-photon microscope. After acquisition, images were processed with Fiji, an ImageJ-based image-processing environment [44].

Table 1. Deconsolidation of Rhythmic Activity upon Adult-Specific Activation of Specific Neuronal Clusters

Genotype	τ (hr)	Rhythmicity (%)	Power FFT	n
UAS-*TrpA1*/+	23.34	91.54	0.06^{B}	124
OK^{107}-GAL4/+	23.36	100.00	0.10^{C}	42
4-59-GAL4/+	23.67	100.00	0.07^{B}	37
3-86-GAL4/+	23.70	98.08	0.06^{B}	54
OK^{107}>*TrpA1*	22.55	47.62	0.04^{A}	58
4-59>TrpA1	23.52	68.95	0.03^{A}	71
3-86>TrpA1	23.43	73.22	0.04^{A}	79

Circadian rhythmicity is affected when noncircadian contacting neurons are depolarized. Average period, percentage of rhythmicity, and fast Fourier transform (FFT) in control and TrpA1-expressing groups at 28°C (activated condition) are presented; the period under free-running conditions is shown. Statistically significant differences could be observed in FFT for control groups and treatments. "n" refers to the number of individuals analyzed per experimental group. Two to four locomotor activity experiments were carried out. Statistical analysis included a Kruskal-Wallis test with pairwise comparisons. Same letters indicate no significant differences, and different letters indicate statistically significant differences ($p < 0.01$).

Quantitation of the Axonal Branching and Axonal Length

Structural plasticity was analyzed as reported [8]. The Zeiss LSM Image Browser software was used to measure the length of the sLNv dorsal arborization. The starting point was set at the lateral horn, and the maximal length was measured toward the mid brain, following the path of the largest neurite.

BRP and SYT Quantification

Images were processed with Fiji. First, a z projection of the stacks was made. Then, a region of interest around the dorsal arborization of the sLNvs was selected. The threshold image was adjusted in order to mark most of the BRP^{RFP} or SYT^{GFP} puncta. Finally, the "analyze particles" tool was employed to measure the total area and number of fluorescent puncta.

GRASP

A GRASP screen was carried out with a subset of the Heberlein's enhancer trap collection [45], and the analysis was performed at three time points (ZT2, ZT14, and ZT22). The mouse monoclonal anti-GFP antibody from Sigma recognized the reconstituted GFP molecule, but not the GFP^{1-10} or GFP^{11} fragments alone, and was employed for GRASP analysis. A minimum of 15 brains were analyzed per genotype and time point. A positive GFP signal at a given time point was considered only if more than half of the brains presented reconstituted GFP signal. Only in those GAL4 lines that supported GFP reconstitution at some of the time points studied did we confirm that parental strains (*pdf*-lexA>lexAop-CD4GFP^{11} and *X*-GAL4>UAS-CD4GFP$^{1-10}$) did not present a GFP^{+} signal.

Statistical Analysis

Statistical analyses were performed with InfoStat (Grupo InfoStat, FCA, Universidad Nacional de Córdoba). Whenever possible, ANOVA was performed. Normality was tested using Shapiro-Wilks test, and the homogeneity of variance was assessed with Levene's test. $p < 0.05$ was considered statistically significant. When in a two-way ANOVA an interaction between factors was significant, interaction contrast was performed and p values were informed.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures and two figures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.07.063.

Author Contributions

E.A.G., A.D.-C., and M.F.C. designed the experiments. E.A.G., A.D.-C., L.F., and N.P. performed and analyzed experiments. E.A.G. and M.F.C. wrote the manuscript. A.D.-C., L.F., and N.P. revised and improved the manuscript.

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