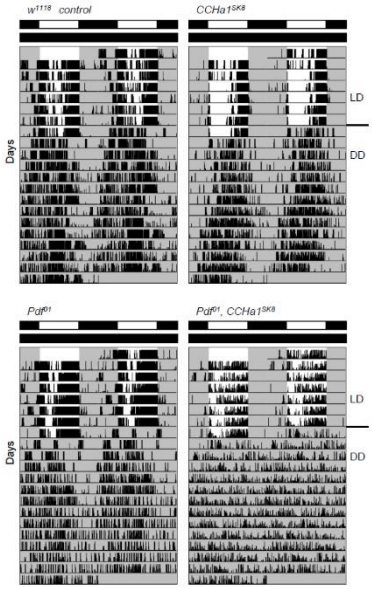

Pigment-dispersing factor and CCHamide1 in the *Drosophila* circadian clock network

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Abstract

Animals possess a circadian central clock in the brain, where circadian behavioural rhythms are generated. In the fruit fly (*Drosophila melanogaster*), the central clock comprises a network of approximately 150 clock neurons, which is important for the maintenance of a coherent and robust rhythm. Several neuropeptides involved in the network have been identified, including Pigment-dispersing factor (PDF) and CCHamide1 (CCHa1) neuropeptides. PDF signals bidirectionally to CCHa1-positive clock neurons; thus, the clock neuron groups expressing PDF and CCHa1 interact reciprocally. However, the role of these interactions in molecular and behavioural rhythms remains elusive. In this study, we generated Pdf^{01} and $CCHa1^{SK8}$ double mutants and examined their locomotor activity-related rhythms. The single mutants of Pdf^{01} or $CCHa1^{SK8}$ displayed free-running rhythms under constant dark conditions, whereas approximately 98% of the double mutants were arrhythmic. In light-dark conditions, the evening activity of the double mutants was phase-advanced compared with that of the single mutants. In contrast, both the single and double mutants had diminished morning activity. These results suggest that the effects of the double mutation varied in behavioural parameters. The double and triple mutants of per^{01}, Pdf^{01}, and $CCHa1^{SK8}$ further revealed that PDF signalling plays a role in the suppression of activity during the daytime under a clock-less background. Our results provide insights into the interactions between PDF and CCHa1 signalling and their roles in activity rhythms.

Running Head: PDF and CCHa1 in the *Drosophila* clock

Keywords: neuropeptide, neural network, clock protein, activity rhythm, masking effect

Introduction

Circadian rhythms in living processes, which are synchronised with environmental cycles, are important for animal survival (DeCoursey et al. 2000; Horn et al. 2019). These rhythms are generated by the molecular mechanism of the circadian clock comprising clock genes and their proteins that are highly conserved across animal species. The molecular clock that controls behavioural and other rhythms resides in specific neurons of the brain, also called as clock neurons.

In the brain of *Drosophila melanogaster*, there are approximately 150 clock neurons that express clock genes, such as *period* (*per*) and *timeless* (*tim*), which generate molecular oscillations at the mRNA and protein levels (Kaneko and Hall 2000; King and Sehgal 2020; Beer and Helfrich-Förster 2020). The *Drosophila* clock neurons are classified into nine groups according to their locations in the brain, cell size, and neurotransmitter content. The lateral neuron groups are located on the lateral side of the midbrain and are divided into five subgroups as follows: small ventral lateral neurons (s-LNv), large ventral lateral neurons (l-LNv), 5^{th} small lateral neuron (5^{th} s-LNv), dorsal lateral neurons (LNd), and lateral posterior neurons (LPN). The dorsal neuron groups are located in the dorsal brain and are divided into four subgroups as follows: anterior dorsal neurons 1 (DN1a), posterior dorsal neurons 1 (DN1p), dorsal neurons 2 (DN2), and dorsal neurons 3 (DN3). These neurons are believed to form a circuit to exchange circadian or zeitgeber information for synchronisation with each other and generation of a coherent rhythm as a unified biological clock. However, the wiring mechanism of the clock neuron circuit remains elusive.

Drosophila shows locomotor activity rhythms, with two peaks in the morning and evening in light-dark cycles (LD) (Hamblen-Coyle et al. 1992). Activity rhythms can persist under constant darkness (DD), with a period of approximately 24 h, termed as free-running circadian rhythms (Konopka and Benzer 1971). Light is the most important environmental factor for resetting the circadian clock. When subjected to a jet-lag experiment of LD, namely a phase-shift in LD, wild-type flies are immediately entrained by the new LD phase. This photic entrainment is mediated by three light-input pathways, namely Cryptochrome (CRY), compound eyes, and Hofbauer–Buchner eyelets (Helfrich-Förster et al. 2001). Light information is transmitted to certain clock neurons and to other clock neurons through a network (e.g., Tang et al. 2010; Seluzicki et al. 2014).

Pigment-dispersing factor (PDF) is the most prominent neurotransmitter that connects s-LNv neurons with other clock neurons (Shafer et al. 2008; Im and Taghert 2010) and is expressed in both s-LNv and l-LNv groups (Helfrich-Förster 1995). The loss of PDF causes an internal desynchronisation among clock neurons and reduces the amplitude of molecular oscillations in the brain, leading to a fragile free-running activity rhythm in DD (Renn et al. 1999; Peng et al. 2003; Lin et al. 2004; Klarsfeld et al. 2004; Yoshii et al. 2009b). In LD, the behavioural phenotypes of *Pdf* mutants include reduced morning activity and phase-advanced evening activity. PDF in s-LNv neurons is involved in DD free-running rhythm and morning activity, whereas in l-LNv neurons, it is involved in the adjustment of the evening activity phase (Grima et al. 2004; Stoleru et al. 2004; Shafer and Taghert 2009; Cusumano et al. 2009; Menegazzi et al. 2017).

As the PDF-receptor (PDF-R) is expressed in several clock neurons, it

93 is evident that PDF signalling is spread throughout the clock neuron network. In
94 particular, it is important for generating normal bimodal activity rhythms to
95 receive PDF in LNd, 5th s-LNv, and DN1p neurons (Lear et al. 2009; Zhang et
96 al. 2010; Schlichting et al. 2016). Additionally, PDF-R is expressed in non-clock
97 neurons, through which PDF signals are transmitted to downstream neurons
98 (e.g., Im and Taghert 2010; Parisky et al. 2008; Pírez et al. 2019).
99 Other neurotransmitters mediating between clock neurons have been
100 identified in the *Drosophila* circadian clock. For example, glutamate mediates
101 signalling from DN1p, LNd, and 5th s-LNv neurons to s-LNv neurons (Collins et
102 al. 2014; Duhart et al. 2020). Moreover, glycine (Frenkel et al. 2017),
103 acetylcholine (Duhart et al. 2020), diuretic hormone 31 (DH31) (Goda et al.
104 2018), and allatostatin C (Díaz et al. 2019) play roles in circadian intercellular
105 couplings. Thus, the entire neuronal wiring of the *Drosophila* clock network
106 comprises neurons that synthesize several different neurotransmitters. The
107 suprachiasmatic nucleus (SCN), the mammalian central clock, is composed of
108 20,000 neurons, with vasoactive intestinal polypeptide, arginine vasopressin,
109 gastrin-releasing peptide, and gamma-aminobutyric acid as intercellular
110 synchronisers (Mieda 2020; Ono et al. 2021). Thus, the *Drosophila* clock
111 network consists of a much smaller number of neurons than the mammalian
112 SCN; however, the number of intercellular synchronisers in the *Drosophila* clock
113 network is abundant.
114 CCHamide1 (CCHa1) was recently identified as a neuropeptide
115 expressed in DN1a neurons (Fujiwara et al. 2018). Mutants of *CCHa1* displayed
116 reduced overall activity, especially in the morning, and a phase-delayed evening
117 activity. Although CCHa1 is also expressed in non-clock cells in the brain,

similar phenotypes to *CCHa1* mutants were observed in flies with *CCHa1* knockdown in DN1a neurons, suggesting that CCHa1 in DN1a neurons plays a role in daily activity pattern. DN1a neurons are synaptically connected to clock neuron groups, namely s-LNv, l-LNv, LNd, 5th s-LNv, and DN3 (Song et al. 2021; Reinhard et al. 2022a), and non-clock neurons, e.g., the dorsal fan-shape body involved in the sleep homeostat (Liu et al. 2016; Donlea et al. 2018; Ni et al. 2019). The CCHa1-receptor (CCHa1-R) is expressed in s-LNv, l-LNv, and many other non-clock neurons, and s-LNv neurons respond to the CCHa1 peptide (Fujiwara et al. 2018). DN1a neurons extend their projections toward s-LNv neurons, and the projections of the s-LNv neurons are adjacent to those of the DN1a (Fujiwara et al. 2018; Song et al. 2021). DN1a neurons express PDF-R and respond to the PDF peptide (Shafer et al. 2008; Im and Taghert 2010). Thus, s-LNv and DN1a neurons are suggested to be reciprocally coupled via PDF and CCHa1. However, the only phenotype shared by *Pdf* and *CCHa1* mutants is decreased morning activity.

This study aimed to investigate the respective and joint effects of PDF and CCH1a peptides on circadian locomotor rhythms and clock protein levels. The double mutants of *Pdf^{01}, CCHa1^{SK8}* had a reduced morning activity and PER levels, which were also observed in the single mutants. In contrast, the percentage of arrhythmic flies in the double mutants was significantly increased compared to that in the single mutants. Furthermore, the phase of evening activity was even more advanced in the double mutants than in the single mutants. Thus, our results suggest that PDF and CCHa1 have two output pathways: shared and independent pathways.

Materials and Methods

Fly strains

The following *D. melanogaster* strains were used: Pdf^{01} (Renn et al. 1999), $CCHa1^{SK8}$ (Ren et al. 2015), and per^{01} (Konopka and Benzer, 1971), with $white^{1118}$ (w^{1118}; BDSC #5905) as a control strain. To minimize the effects of the genetic background, Pdf^{01} and $CCHa1^{SK8}$ mutants were outcrossed at least six times into w^{1118} control flies. The 2nd and 3rd chromosomes of per^{01} mutants were exchanged with those of the w^{1118} control. Flies were reared at 25 °C under 12 h of light and 12 h of dark conditions (LD12:12) on *Drosophila* medium (0.7% agar, 8.0% glucose, 3.3% yeast, 4.0% cornmeal, 2.5% wheat embryo, and 0.25% propionic acid).

Activity recording and data analysis

Male flies aged 3-4 days were used to record locomotor activity rhythms. Flies were confined to recording tubes containing agar/sugar food (2% agar and 4% sucrose) for the *Drosophila* Activity Monitor (Trikinetics Inc. Waltham, MA, USA). The monitors were placed in an incubator (CN-40A, Mitsubishi Electric, Tokyo, Japan) held at a constant temperature of 20 (± 0.25) °C. Standard cool white light emitting diodes (LEDs) or red LEDs (630 nm) were placed above the monitors in the incubator and controlled by an LC4 light controller (Trikinetics Inc.). The light intensity used in all experiments was 100 lux (32 $\mu W \cdot cm^{-2}$ for the white LEDs and 50 $\mu W \cdot cm^{-2}$ for the red LEDs).

We recorded the activity of files in LD12:12 for 7 days, followed by 13 days of DD. The activity of flies was also recorded in 12 h of red light and 12 h

of dark conditions (RD12:12) for 7 days. For long (LD16:08) and short (LD08:16) photoperiod conditions, flies were first entrained to LD12:12 for 4 days, photoperiods were changed, and fly activity was recorded for an additional 7 days.

Raw data were visualized as actograms using ActogramJ (http://actogramj.neurofly.de/)(Schmid et al. 2011). The average daily activity patterns were calculated using the data from days 3–7 (for 5 days). The first two days were excluded from the calculation, as the flies needed to be re-entrained to the new photoperiods. The phase of evening activity peak in LD was determined using the PHASE software (Persons et al. 2022). The period of free-running rhythm in DD, recorded for 10 days, was determined using a chi-square periodogram analysis. If a robust peak above the 95% confidence level appeared in the periodogram, the period was designated as statistically significant (Sokolove and Bushell 1978).

Immunohistochemistry analysis

Whole flies were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) with 0.1% Triton X-100 at room temperature (RT, approximately 25 °C) for 2.5 h. Fixed flies were washed thrice in PBS, and brains were dissected and washed thrice with PBS containing 0.5% Triton X-100 (PBS-T), after which they were blocked in PBS-T containing 5% normal donkey serum for 1 h at RT and subsequently incubated with primary antibodies at 4 °C for 48 h. After washing six times with PBS-T, the brains were incubated with secondary antibodies at RT for 3 h, washed six more times in PBS-T, and mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). The primary

antibodies used were as follows: goat anti-PER (1:1000) (sc-15720, Santa Cruz Biotechnology, TX, USA), rat anti-TIM (1:3000; kindly provided by Jadwiga Giebultowicz), mouse anti-PDF (1:500; Developmental Studies Hybridoma Bank) (Cyran et al. 2005), and rabbit anti-PDF (1:16000) (Abdelsalam et al. 2008). The following fluorescence-conjugated secondary antibodies were used at 1:1000 dilution: Alexa Fluor®-488 (donkey anti-goat, goat anti-mouse), Alexa Fluor®-555 nm (donkey anti-mouse), Alexa Fluor®-647 nm (goat anti-rabbit) (all Life Technologies, Carlsbad, CA, USA), and goat anti-rat Cy3 (Millipore, Billerica, MA, USA).

Staining was visualized using laser scanning confocal microscopes (Olympus FV1200, Olympus, Tokyo, Japan). To quantify PER and TIM staining intensity, the confocal microscope settings were kept consistent throughout the experiments. For each time point, the hemispheres of 20 (for PER) or 16 (for TIM) different brains were analyzed. Measurement of staining intensity was performed using Fiji software (Schindelin et al. 2012) as described previously (Yoshii et al. 2009a).

Statistical analysis

The Kolmogorov–Smirnov test was used to test for normality. One-way analysis of variance (ANOVA) with Tukey's multiple comparison test was used for normally distributed data, whereas the Kruskal–Wallis test and post-hoc Mann–Whitney *U*-test with Holm correction were used for non-normally distributed. All statistical tests were carried out using EZR software (Kanda 2013).

Results

Free-running activity rhythms of CCHa1 *and* Pdf *mutants*

The DD free-running rhythm of *CCHa1*^{SK8} mutants was comparable to that of control flies (Fujiwara et al. 2018), whereas the *Pdf*^{01} mutants displayed weak rhythms, with a free-running period shorter than that of wild-type flies (Renn et al. 1999). The phenotype of *CCHa1*^{SK8} mutants was the same as reported previously (Fig. 1 and Table 1). The power and rhythmicity of the *Pdf*^{01} mutants were also consistent with those reported previously (Renn et al., 1999). However, the free-running periods of the *Pdf*^{01} mutants were not shorter than those of the control flies (Table. 1). This discrepancy might be attributed to differences in genetic background across studies; in this study, all strains were outcrossed with the *w*^{1118} strain. Only 1.6% of the *Pdf*^{01}*, CCHa1*^{SK8} double mutants were rhythmic in DD. Thus, CCHa1 and PDF signalling pathways may be important to maintain DD free-running rhythms.

Photic entrainment of CCHa1 *and* Pdf *mutants*

To examine whether *Pdf*^{01}, *CCHa1*^{SK8} double mutants were impaired in photic entrainment, flies were subjected to an 8 h phase-delay of LD12:12, and their activity rhythms were recorded. All strains were immediately re-entrained by the 8 h phase-delay (Fig. 2A). There was no clear difference between the strains.

The rapid photic entrainment in *Drosophila* is mediated by the blue photoreceptive protein, CRY (Emery et al. 2000; Helfrich-Förster et al. 2001; Yoshii et al. 2015). To exclude CRY-mediated entrainment, the re-entrainment experiments were conducted using red LEDs (630 nm), to which CRY does not respond. In *w*^{1118} control flies, the evening activity peak was gradually re-entrained by an 8 h phase-shift of RD, and four to five days were required for

full entrainment (Fig. 2B). Similar results were obtained in the *CCHa1^{SK8}* mutants. *Pdf^{01}* mutants displayed a large activity peak, starting from lights on under RD12:12, consistent with a previous study (Cusmano et al. 2009). After the 8 h RD shift, the large activity peak of *Pdf^{01}* mutants was immediately phase-shifted on the first day. However, it is difficult to determine whether flies were entrained or simply responded to the lights on. Nevertheless, the double mutants of *Pdf^{01}, CCHa1^{SK8}* had a similar activity pattern to *Pdf^{01}* mutants. Thus, we did not detect any specific phenotype in the double mutants under LD and RD re-entrainment conditions.

Daily activity patterns of* CCHa1 *and* Pdf *mutants

Next, we focused on the activity profiles in LD12:12. Control flies displayed the distinct morning (M) and evening (E) activity peaks (Fig. 3A). The activities of the two main peaks started to increase before light transitions (lights on and lights off), so-called anticipatory activity. The total activity of the *CCHa1^{SK8}* and *Pdf^{01}* single mutants and their double mutants were significantly lower than that of control flies ($p<0.05$) (Fig. 3B). The M peaks of the *CCHa1^{SK8}* and *Pdf^{01}* single mutants were reduced as reported previously, and those of the double mutants were reduced as much as those of the *CCHa1^{SK8}* mutants (Fig. 3C). Thus, the phenotypes in total activity and M peak were common in the three mutants. The phase of E peak of the *CCHa1^{SK8}* mutants did not differ from that of control flies (Fig. 3A, D). The *Pdf^{01}* mutants had an increase in E activity earlier than the control flies and *CCHa1^{SK8}* mutants, reaching a plateau before lights off (Fig. 3A, D). Furthermore, the *CCHa1^{SK8}*, *Pdf^{01}* double mutants reached a plateau even earlier than the *Pdf^{01}* mutants (Fig. 3A, D).

In LD12:12, the M and E peaks usually appeared at lights on and lights off, respectively, making it difficult to determine their actual phases. Under short and long photoperiods, the phases of the M and E peaks were shifted from the light transitions (Rieger et al. 2003). Therefore, we used those conditions to further investigate the M and E phenotypes. Flies were subjected to a short photoperiod condition (LD08:16; Fig. 4A). The increase in anticipatory M activity before lights on was more pronounced in control flies than in flies subjected to LD12:12. However, this was reduced in *CCHa1*^{SK8} and *Pdf*^{01} single mutants, and their double mutants (Fig. 4B), consistent with the results in LD12:12.

Furthermore, flies were subjected to a long photoperiod condition (LD16:08) to examine E activity peaks (Fig. 5A). Control flies showed increased activity from zeitgeber time (ZT) 8, peaking at lights off. The *CCHa1*^{SK8} mutants had a slight delay in activity increase, reached a peak earlier than the control flies, and had a decrease in activity before lights off (Fig. 5A, B). Meanwhile, the *Pdf*^{01} mutants had an increase in activity earlier and reached a peak earlier than the control flies and *CCHa1*^{SK8} mutants (Fig. 5A, B). Thus, the CCHa1 and PDF signalling pathways differently modulate E activity.

In the *CCHa1*^{SK8}, *Pdf*^{01} double mutants, E activity increased earlier than that in *Pdf*^{01} single mutants but reached its peak and started to decrease earlier than *Pdf*^{01} single mutants (Fig. 5A, B). To clarify whether the E activity of the *CCHa1*^{SK8}, *Pdf*^{01} double mutants started to decrease before lights off, we calculated the difference in the total activity between ZT12 to ZT14 and ZT14 to ZT16. The total activity of the control flies between ZT14 to ZT16 was higher than that between ZT12 to ZT14, indicating that the E activity increased from ZT12 to ZT16 (Fig. 5C). In contrast, the differences were smaller and negative

293 in the other three mutants, indicating a decrease in the E activity from ZT12 to
294 ZT16. The *CCHa1*^{SK8}, *Pdf*^{01} double mutants showed the lowest value, which is
295 significantly different from the two single mutants. Thus, these results suggest
296 that the phase determination of E peak, including its cessation, is regulated by
297 both the CCHa1 and PDF pathways.
298
299 ***PER and TIM expression of* CCHa1 *and* Pdf *mutants***
300 The levels of PER and TIM clock proteins showed circadian oscillations, with a
301 peak in late night in LD (Siwicki et al. 1998; Myers et al. 1996). PDF plays a role
302 in determining the phase and amplitude of molecular clock oscillations (Peng et
303 al. 2003; Lin et al. 2004; Klarsfeld et al. 2004; Yoshii et al. 2009b; Seluzicki et al.
304 2014; Sabado et al. 2017). A similar role has been reported for CCHa1
305 (Fujiwara et al. 2018).
306 To investigate whether the *Pdf*^{01}, *CCHa1*^{SK8} double mutants have
307 different levels of PER and TIM compared to the two single mutants, we
308 performed immunostaining analysis for PER and TIM on brain samples
309 collected at peak time points ZT20 and ZT2 (only for PER) and quantified their
310 levels in the following clock neuron groups: s-LNv, l-LNv, and LNd (Fig. 6).
311 The effects on the PER level varied according to the cell group and time
312 point. In s-LNv neurons at ZT20, the PER level of the *CCHa1*^{SK8} mutants was
313 similar to that of the control flies, whereas that of the *Pdf*^{01} mutants was
314 significantly reduced ($p<0.01$) (Fig. 6A). However, at ZT2, all mutants showed
315 reduced levels of PER compared with control flies (Fig. 6B). In LNd neurons,
316 the PER level of the *CCHa1*^{SK8} mutants was significantly reduced at both time
317 points ($p<0.01$), whereas in the *Pdf* mutants, a significant reduction was

detected only at ZT2 ($p<0.01$). The double mutants did not show an additional reduction in the PER level compared with the single mutants. All mutants had reduced PER level compared with control flies.

In contrast, the levels of TIM in the cell groups of the three mutants were comparable to those of the control flies (Fig. 6C), although there were slight differences between the mutants and between cell groups, which may be attributed to phase shifts of molecular oscillations.

Daily activity patterns in CCHa1 *and* Pdf *mutants with* per^{01} *mutant background*

We usually assume that the molecular clock consisting of clock genes is located upstream of PDF and CCHa1 and somehow controls their outputs. However, it is unclear whether the outputs of PDF and CCHa1 stop when the clock is stopped. To investigate this, we generated double and triple mutant strains of per^{01}, Pdf^{01}, and $CCHa1^{SK8}$ and recorded their activity in LD12:12 (Fig. 7). As shown previously, the per^{01} mutants displayed two strong peaks at the light transitions, known as the masking effects of light. The double mutants of $per^{01};;CCHa1^{SK8}$ displayed a very similar activity pattern to that of the per^{01} mutants; however, their overall activity was reduced, probably because of the effect of the $CCHa1^{SK8}$ mutation. Interestingly, the double mutants of $per^{01};;Pdf^{01}$ displayed a large activity peak after lights on, which gradually declined until lights off, resembling the activity pattern of Pdf^{01} single mutants under RD12:12 (Fig. 2B). The activity pattern of the triple mutants of $per^{01};;Pdf^{01}, CCHa1^{SK8}$ was similar to that of the double mutants of $per^{01};;Pdf^{01}$, although the total activity was reduced as seen in the double mutants of

343 *per*^{01}*;;CCHa1*^{SK8}.

344 The unique phenotype of the *per*^{01};;*Pdf*^{01} double mutants persisted
345 under a short photoperiod condition (Fig. 8A) and under RD12:12 (Fig. 8B).
346 Thus, PDF reduces activity after lights on in *per*^{01} mutants.

347

348 ## Discussion

349 SCN cells, the mammalian central clock, retained intrinsic circadian rhythms
350 after they are detached from other tissues or even after individual cells are
351 dispersed (Yamaguchi et al. 2003). However, the rhythms of the dispersed cells
352 were less robust than those of the intact SCN, and there was a large variation in
353 the free-running periods between individual cells, suggesting that the network
354 linking individual SCN cells is important to maintain the robustness and
355 coherence of cellular rhythms (Ono et al. 2021). In *Drosophila*, PDF is the
356 primary neuropeptide involved in signalling within the circadian neuronal
357 network (Peng et al. 2003; Lin et al. 2004; Klarsfeld et al. 2004; Shafer et al.
358 2008; Yoshii et al. 2009b; Im et al. 2010); however, there may be many more
359 unknown factors playing a similar role.

360 Here, we focused on the interactions between CCHa1 and PDF. The
361 *CCHa1*^{SK8} and *Pdf*^{01} single mutants displayed an attenuated M activity and
362 reduced PER levels in LD, and the same phenotypes were observed in the
363 double mutants. In contrast, rhythmicity in DD was regulated by both CCHa1
364 and PDF, and the phase of E activity in LD showed a complex phenotype in the
365 double mutants. Thus, we speculate that CCHa1 and PDF use a shared and
366 independent pathway to control activity rhythms.

367

DD rhythmicity

Pdf^{01} mutants displayed weak activity rhythms in DD (Renn et al. 1999). *Pdf* RNA knockdown in s-LNv neurons alone was sufficient to recapitulate this phenotype (Shafer and Taghert 2009). Here, we showed that the double mutants of *Pdf* and *CCHa1* were arrhythmic in DD. If DN1a neurons were located upstream of s-LNv neurons and this was a one-track pathway, a loss of PDF in s-LNv neurons would be sufficient to shut off CCHa1 signalling. However, as the double mutants showed a more severe DD phenotype than the single mutants, DN1a neuronal output would not only be directed to s-LNv neurons, but also to other neurons. *trans*-Tango experiments exploring post-synaptic neurons revealed that DN1a neurons contact many more clock neuron groups, such as LNd, 5th s-LNv, and DN3, in addition to s-LNv and l-LNv groups (Reinhard et al. 2022a). Moreover, DN1a neurons contact non-clock neurons, for example, the dorsal fan-shape body involved in the sleep homeostat (Liu et al. 2016; Donlea et al. 2018; Ni et al. 2019). Thus, CCHa1 signalling from DN1a neurons links clock and non-clock neurons, which may be important for preserving the weak rhythms of the *Pdf* mutants.

The PDF signalling pathway affects not only synchrony between clock neurons, but also molecular oscillations of clock proteins (Peng et al. 2003; Lin et al. 2004; Klarsfeld et al. 2004; Yoshii et al. 2009b). The effect of PDF on the molecular clock is mediated by transcriptional and post-transcriptional regulation (Li et al. 2014; Seluzicki et al. 2014; Sabado et al. 2017). The CCHa1 signalling pathway also plays roles in clock neuron synchrony and molecular oscillations (Fujiwara et al. 2018). Therefore, the arrhythmicity in *Pdf*^{01}, *CCHa1*^{SK8} double mutants might be attributed to weakened oscillation.

However, the levels of PER and TIM in the double mutants were not significantly reduced compared with those in the single mutants (Fig. 6). Based on these observations, we assume that uni- or bi-directional pathway(s) between DN1a (CCHa1) and s-LNv (PDF) increase PER levels; however, DD arrhythmicity in double mutants is caused by a different output pathway.

A similar genetic interaction was reported in PDF and diuretic hormone 31 (DH31) neuropeptides (Goda et al. 2019). DH31 is a neuropeptide expressed in DN1p and LPN neurons (Kunst et al. 2014; Reinhard et al. 2022b). Whereas the activity of *Dh31* null mutants was rhythmic in DD, similar to that of the *CCHa1*^{SK8} mutants, that of the *Pdf* and *Dh31* double mutants were arrhythmic. Thus, the weak free-running rhythms of the *Pdf* mutants are fragile and easily disrupted by shutting down one output pathway, either that of CCHa1 or DH31.

We speculate that CCHa1 signalling in non-clock neurons might affect DD rhythmicity, since CCHa1 is expressed in many non-clock neurons in the brain (Fujiwara et al., 2018). The same problem might occur in M and E peak phenotypes as discussed below. Notably, PDF signalling does not only target DN1a neurons, but is also transmitted to other clock and non-clock neurons. Thus, establishing strains that enable DN1a neuron-specific knockdown of *CCHa1* or *pdf-r* and s-LNv neuron-specific knockdown of *Pdf* or *CCHa1-r*, in combination with *Pdf* or *CCHa1* mutants, might be useful for the investigation of the pathway between DN1a and s-LNv neurons.

Morning and evening activity

Pdf mutants lacked an anticipatory activity before lights on (Grima et al. 2004;

418 Cusumano et al. 2009), strongly supporting a model wherein PDF-positive s-
419 LNv neurons contain an oscillator important for M activity. *CCHa1*^{SK8} mutants
420 also lacked anticipatory activity in the morning (Fujiwara et al. 2018), and the
421 morning phenotype of the *Pdf*^{01}*, CCHa1*^{SK8} double mutants was comparable to
422 that of the single mutants (Fig. 3A, C; Fig. 4A, B), suggesting that CCHa1 and
423 PDF work in the same pathway to generate M activity.
424 The reception of PDF by DN1p neurons is important for the generation
425 and phasing of the M activity peak (Yao et al. 2016; Chatterjee et al. 2018). PDF
426 level showed circadian changes at the terminals of s-LNv neurons (Park et al.
427 2000); however, the change of PDF level was smaller in the *CCHa1*^{SK8} mutants
428 (Fujiwara et al. 2018). Therefore, DN1a neurons may be located upstream of s-
429 LNv neurons, possibly forming a DN1a > s-LNv > DN1p sequential pathway.
430 DN1a neurons are involved in the integration of light and cold temperature
431 information (Alpert et al. 2020) and a startle response to a light pulse during the
432 night phase (Song et al. 2021). Both are strongly related to activity or sleep in
433 the morning. Thus, DN1a neurons might process light and temperature
434 information in the morning and transmit it to s-LNv neurons, whereby
435 appropriate M activity is determined under various temperature and light
436 conditions.
437 In contrast to the morning phenotype, E activity in the *Pdf*^{01}*, CCHa1*^{SK8}
438 double mutants showed a complex effect, which became more evident under a
439 long-day condition (Fig. 3; Fig. 5). We mainly attribute the phase advance of E
440 activity in the double mutants to the *Pdf* mutation. PDF-R is expressed in the
441 oscillators controlling E activity, such as 5th s-LNv and LNd, DN1p neurons (Im
442 and Taghert 2010), and s-LNv neurons influence the E oscillators (Yao et al.

2016; Chatterjee et al. 2018). The additional *CCHa1* mutation further facilitated the phase advance of E activity (Fig. 3; Fig. 5). We speculate that CCHa1 signalling from DN1a and PDF signalling from s-LNv converge on E oscillators (5^{th} s-LNv and LNd). A relatively clear picture of the DN1a > s-LNv sequential pathway can be assumed in the regulation of M activity; however, regulation of E activity may be more complex.

PDF in* per *null background

We were interested in elucidating the effects of CCHa1 and PDF under a clock-less condition. The $CCHa1^{SK8}$ mutants displayed reduced activity throughout the day compared with control flies (Fig. 3B). The activity level of the $per^{01};;CCHa1^{SK8}$ double mutants was also reduced compared with that of the per^{01} single mutants, indicating a clock-independent phenotype (Fig. 7). More intriguingly, the $per^{01};;Pdf^{01}$ double mutants displayed a large activity peak that started after lights on, gradually declining toward evening. This is a quite similar to the activity pattern of the Pdf^{01} mutants under RD12:12 and that of the *Pdf* (or *pdf-r*) and *cry* double mutants (Fig. 2B; Cusumano et al. 2009; Zhang et al. 2009).

Although this behavioural phenotype was first explained by a large phase advance of E activity into the morning phase, Im et al. (2011) reported that normal PER rhythms (in amplitude, phasing, and subcellular localization) in the ion transport peptide-positive LNd and 5^{th} s-LNv neurons were lost in the *pdf-r* and *cry* double mutants. Hence, we assume that a masking effect of light contributes to the activity patterns of the $per^{01};;Pdf^{01}$ double mutants and *Pdf* and *cry* double mutants. Under the *cry* mutant background, clock neurons rely

on the visual system for light input, whereas the *Pdf* mutation blocks LNv signals to the other clock neurons (Tang et al. 2010). PDF-negative clock neurons also receive light input from the visual system (Li et al. 2018). Thus, light entrainment of PDF-negative clock neurons through the visual system may not be sufficient to shape normal bimodal activity rhythms, and the masking effect outcompetes the activity regulated by the clock.

In addition, the activity pattern of the $per^{01};;Pdf^{01}$ double mutants was distinct from that of the per^{01} single mutants. Thus, PDF is released even in a clock-less condition and plays a role in suppressing activity after lights on. This is in contrast to the effect of PDF when the clock is functional, as the Pdf^{01} mutants lacked M activity. PDF is released most probably in the morning in a clock-dependent manner (Park et al. 2000; Klose et al. 2021). Without the clock, PDF might be released throughout the day to suppress activity in response to light. Moreover, PDF release may be stimulated by light. If so, activity rhythms might be adapted to LD conditions by synchronising the oscillation of the clock with the timing of PDF release in response to light in the morning.

Conclusions

PDF is the first circadian neurotransmitter identified in *Drosophila*, and its mutants show distinct phenotypes in activity rhythms (Helfrich-Förster 1995; Renn et al. 1999). Mutants or knockdown strains of other neurotransmitters show modest phenotypes compared to that of *Pdf*. This means that PDF is a main circadian neurotransmitter, whereas the other neurotransmitters only partly affect the rhythm of locomotor activity. However, these minor effects of non-PDF circadian neurotransmitters may jointly contribute to shaping the normal bimodal

activity rhythm, which might result in overall robust rhythms. Functional analysis of the combination of multiple output factors would provide further insights into the basic principles of the neural network of the clock common to all animals.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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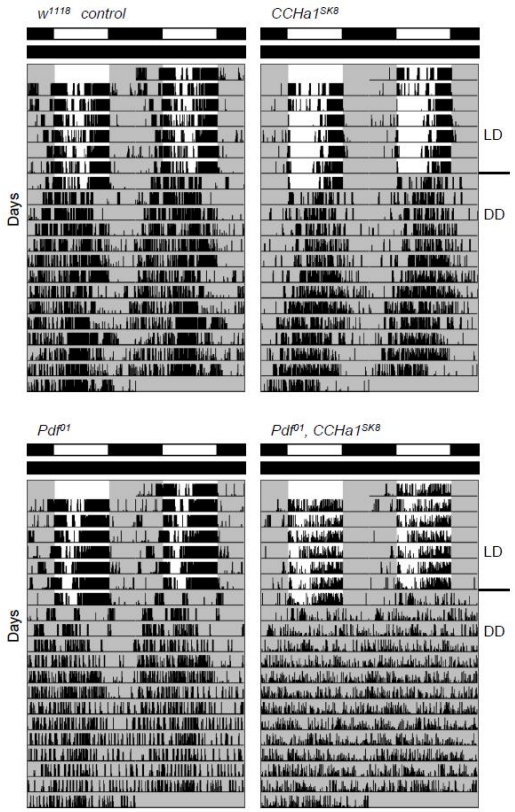
762

763 # Figure legends



764

765 **Figure 1**

766 Representative actograms of a *w*^{1118} control fly, *CCHa1*^{SK8} mutant, *Pdf*^{01} mutant,

767 and *Pdf*^{01}, *CCHa1*^{SK8} double mutant. Activity rhythms were first recorded at

768 LD12:12 for 7 days and at DD for 13 days. The gray areas in the actograms

769 indicate a dark phase.

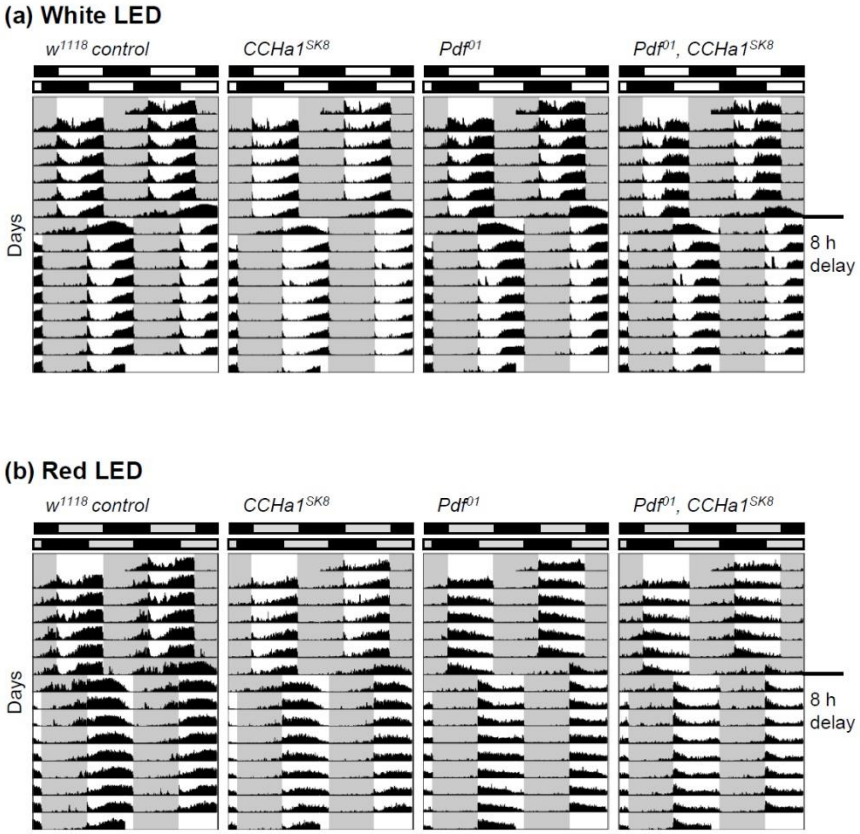
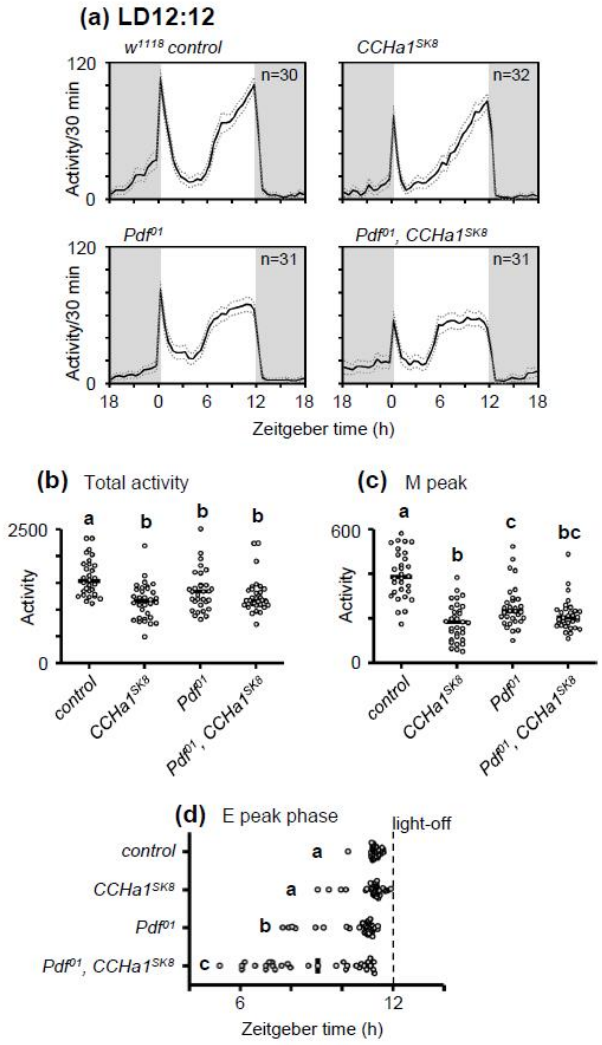


Figure 2

(A) Mean actograms of *w*^{1118} control flies (n=32), *CCHa1*^{SK8} mutants (n=31), *Pdf*^{01} mutants (n=32), and *Pdf*^{01}, *CCHa1*^{SK8} double mutants (n=32) in LD12:12 with white LEDs. Flies were subjected to an 8 h phase-delay of LD12:12 to examine light entrainability. **(B)** Mean actograms of *w*^{1118} control flies (n=32), *CCHa1*^{SK8} mutants (n=31), *Pdf*^{01} mutants (n=31), and *Pdf*^{01}, *CCHa1*^{SK8} double mutants (n=32) in RD12:12 with red LEDs.



778

779 **Figure 3**

780 **(A)** Mean ± standard error of the mean (SEM) activity profiles of *w*^{1118} control
781 flies (n=30), *CCHa1*^{SK8} mutants (n=32), *Pdf*^{01} mutants (n=31), and *Pdf*^{01},
782 *CCHa1*^{SK8} double mutants (n=31) in LD12:12. Lines and gray dotted lines
783 indicate the mean and SEM, respectively. The gray areas in the graphs indicate
784 the dark phase. **(B)** Total activities during the day. **(C)** Activity during the
785 morning phase (from ZT22 to ZT02). **(D)** The phases of E activity peaks of each
786 genotype. Each circle indicates the activity or E phase of individual flies,
787 averaged from 5 days of data. The horizontal bar indicates the mean. Different

letters indicate significant differences ($p < 0.05$, analyzed by ANOVA with Tukey's multiple comparison test or post-hoc Mann–Whitney U-test with Holm correction).

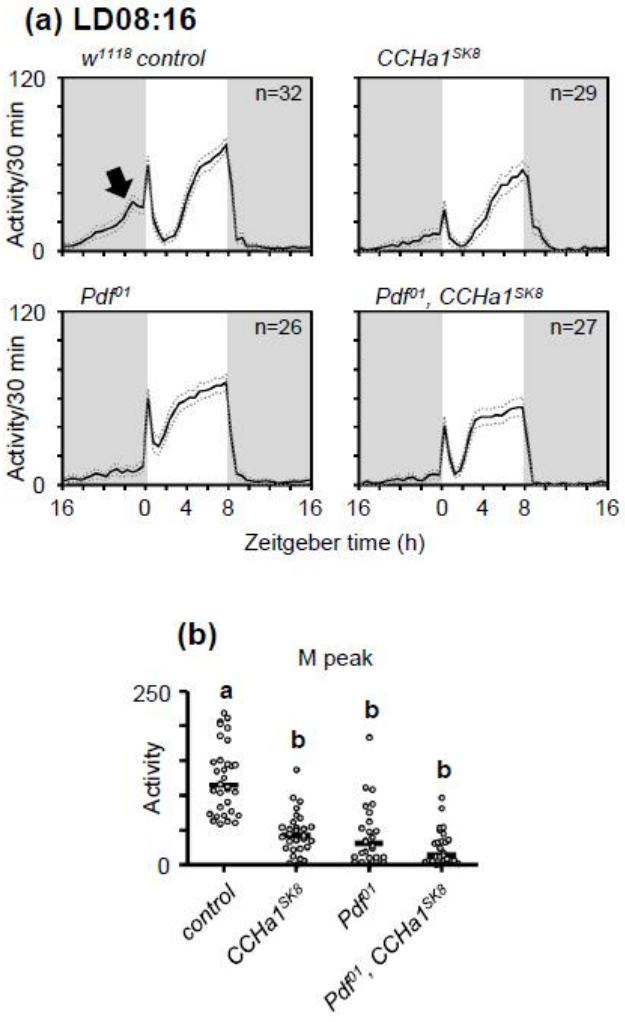


Figure 4

(A) Mean ±SEM activity profiles in LD08:16. The arrow indicates anticipatory activity before lights on. **(B)** Activity during the morning phase (from ZT22 to ZT02) in LD08:16. Each circle represents the data of individual flies; horizontal bars indicate the mean. Different letters indicate statistically significant differences at $p < 0.05$ (ANOVA with Tukey's multiple comparison test).

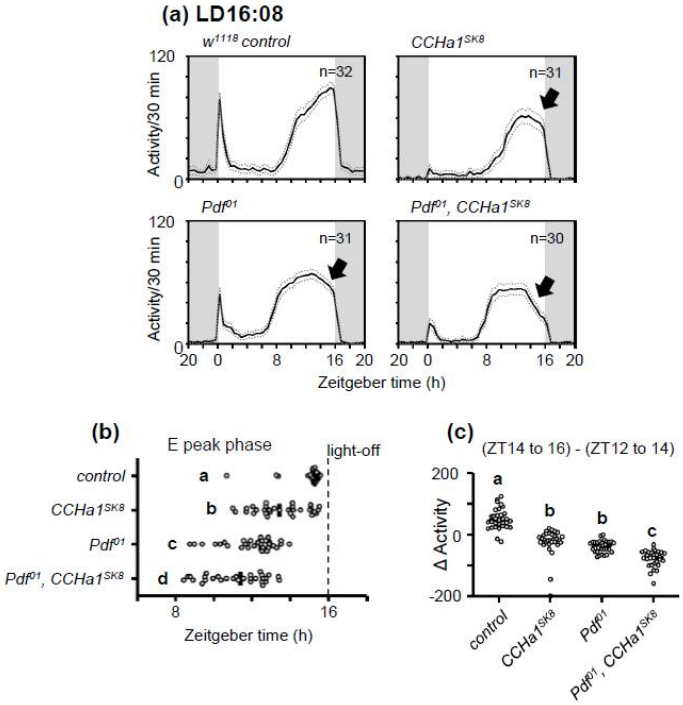


Figure 5

(A) Mean ± SEM activity profiles in LD16:08. The arrows indicate the activity on the downward slope after the E peak. **(B)** The phases of E activity peaks of each genotype. **(C)** To examine whether the activity is on the downward slope before lights off in LD16:08, the activity between ZT14 and ZT16 was subtracted from that between ZT12 and ZT14. Positive values indicate an increasing trend in activity, while negative values indicate a decreasing trend. Each circle represents the data of individual flies; horizontal bars indicate the mean. Different letters indicate significant differences ($p < 0.05$ analyzed by ANOVA with Tukey's multiple comparison test or post-hoc Mann–Whitney U-test with Holm correction).

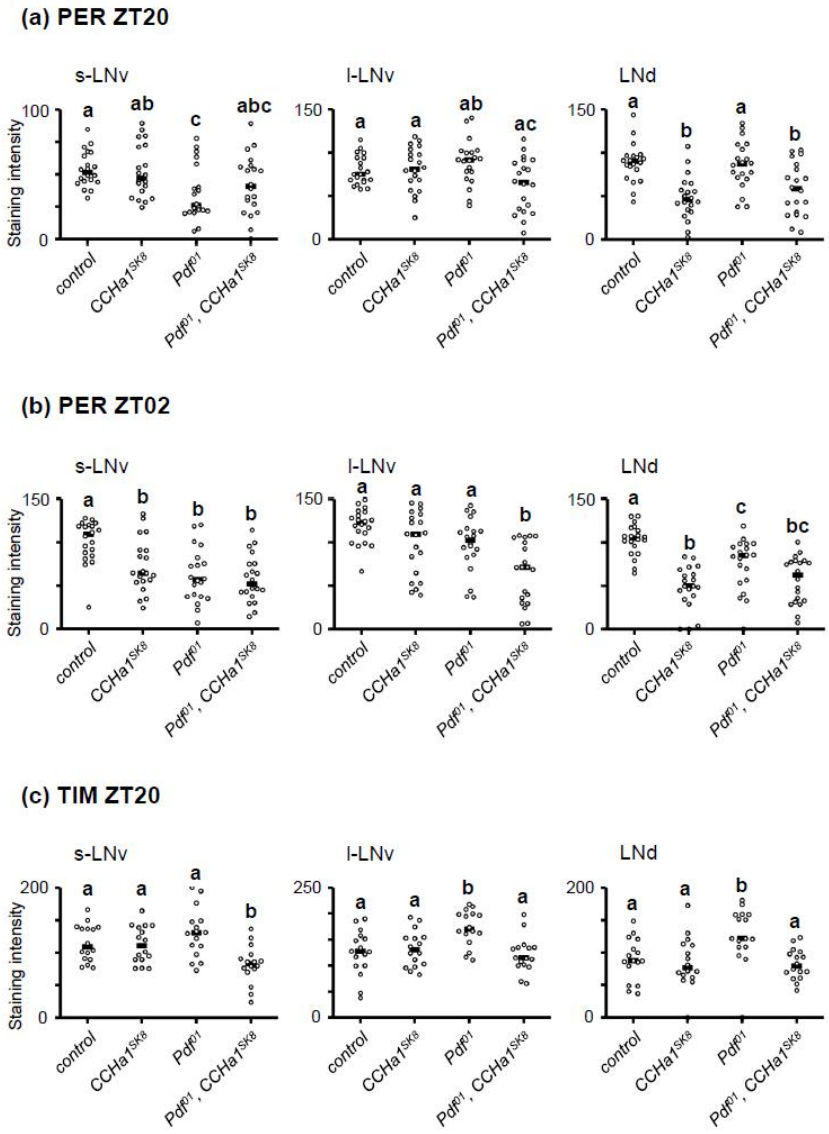
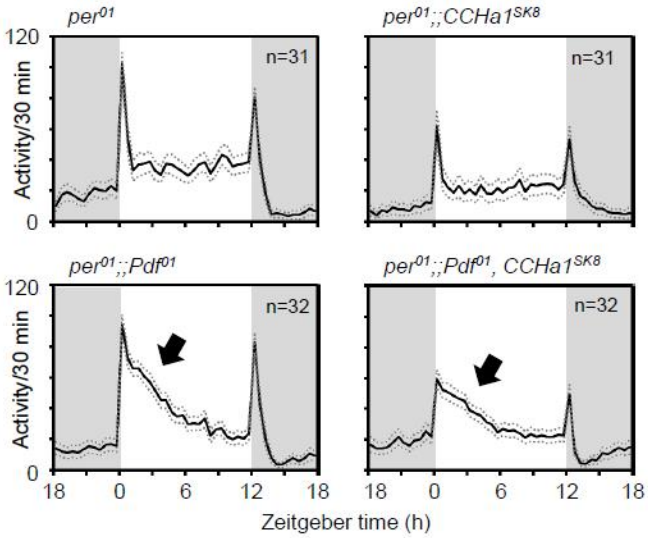


Figure 6

Levels of PER of *w*^{1118} control flies, *CCHa1*^{SK8} mutants, *Pdf*^{01} mutants, and *Pdf*^{01}, *CCHa1*^{SK8} double mutants at ZT20 **(A)** and ZT02 **(B)**, and levels of TIM at ZT20 **(C)**. Each circle represents the data of individual flies; horizontal bars indicate the mean. Different letters indicate significant differences ($p < 0.05$ analyzed by ANOVA with Tukey's multiple comparison test or post-hoc Mann–Whitney U-test with Holm correction).



822

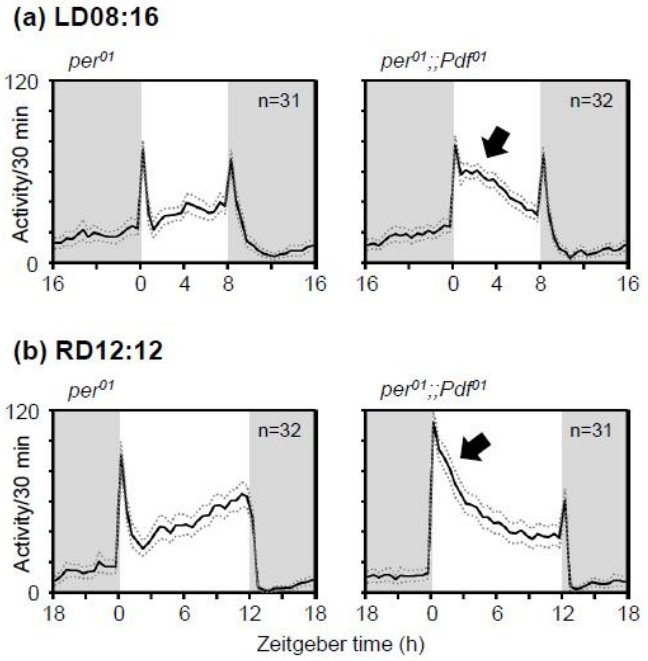
823 **Figure 7**

824 Mean ± SEM activity profiles of *per*^{01} mutants, *per*^{01}*;;CCHa1*^{SK8} double mutants,

825 *per*^{01}*;;Pdf*^{01} double mutants, and *per*^{01}*;;Pdf*^{01}*, CCHa1*^{SK8} triple mutants in

826 LD12:12. The arrows indicate a large activity peak after lights on.

827



828

829 **Figure 8**

830 Mean ± SEM activity profiles of *per*^{01} mutants and *per*^{01}*;;Pdf*^{01} double mutants in

831 LD08:16 with white LEDs (A) and RD12:12 with red LEDs (B). The arrows

832 indicate a large activity peak after lights on.

Table 1. Free-running rhythms in DD

Genotype	Period (h)	Power	% Rhythmicity (n)
w^{1118}	23.94 ± 0.03	626.24 ± 28.58	86.7 (52/60)
$CCHa1^{SK8}$	23.77 ± 0.05	581.55 ± 28.19	88.7 (55/62)
Pdf^{01}	23.84 ± 0.05	456.84 ± 16.77 *	46.0 (29/63)*
Pdf^{01}, $CCHa1^{SK8}$	24	349.3	1.6 (1/63)*

Data for the period and power are presented as the mean ± SEM.

*p<0.05 vs. w^{1118} control (analyzed by one-way ANOVA with Tukey's multiple comparison test for period and power and Chi-square test with Holm correction for rhythmicity).