Drosophila DH31 Neuropeptide and PDF Receptor Regulate Night-Onset Temperature Preference

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Body temperature exhibits rhythmic fluctuations over a 24 h period (Refinetti and Menaker, 1992) and decreases during the night, which is associated with sleep initiation (Gilbert et al., 2004; Kräuchi, 2007a,b). However, the underlying mechanism of this temperature decrease is largely unknown. We have previously shown that *Drosophila* exhibit a daily temperature preference rhythm (TPR), in which their preferred temperatures increase during the daytime and then decrease at the transition from day to night (night-onset) (Kaneko et al., 2012). Because *Drosophila* are small ectotherms, their body temperature is very close to that of the ambient temperature (Stevenson, 1985), suggesting that their TPR generates their body temperature rhythm. Here, we demonstrate that the neuropeptide diuretic hormone 31 (DH31) and pigment-dispersing factor receptor (PDFR) contribute to regulate the preferred temperature decrease at night-onset. We show that PDFR and tethered-DH31 expression in dorsal neurons 2 (DN2s) restore the preferred temperature decrease at night-onset, suggesting that DH31 acts on PDFR in DN2s. Notably, we previously showed that the molecular clock in DN2s is important for TPR. Although PDF (another ligand of PDFR) is a critical factor for locomotor activity rhythms, *Pdf* mutants exhibit normal preferred temperature decreases at night-onset. This suggests that DH31–PDFR signaling specifically regulates a preferred temperature decrease at night-onset. Thus, we propose that night-onset TPR and locomotor activity rhythms are differentially controlled not only by clock neurons but also by neuropeptide signaling in the brain.

Key words: circadian rhythm; DH31; *Drosophila*; PDFR; temperature preference behavior; thermoregulation

Significance Statement

Body temperature rhythm (BTR) is fundamental for the maintenance of functions essential for homeostasis, such as generating metabolic energy and sleep. One major unsolved question is how body temperature decreases dramatically during the night. Previously, we demonstrated that a BTR-like mechanism, referred to as temperature preference rhythm (TPR), exists in *Drosophila*. Here, we demonstrate that the diuretic hormone 31 (DH31) neuropeptide and pigment-dispersing factor receptor (PDFR) regulate preferred temperature decreases at night-onset via dorsal neurons 2. This is the first *in vivo* evidence that DH31 could function as a ligand of PDFR. Although both DH31 and PDF are ligands of PDFR, we show that DH31 regulates night-onset TPR, but PDF does not, suggesting that night-onset TPR and locomotor activity rhythms are controlled by different neuropeptides via different clock cells.

Introduction

Body temperature rhythm (BTR) is fundamental for maintaining homeostasis, such as in generating metabolic energy and sleep

(Aschoff, 1983; Refinetti and Menaker, 1992; Kräuchi, 2002, 2007a,b; Gilbert et al., 2004; Weinert, 2010; Gerhart-Hines et al., 2013). BTR is one of the most robust circadian outputs and can affect the peripheral clocks of mammals (Brown et al., 2002; Buhr et al., 2010). The rhythmic patterns of BTR and locomotor activity rhythms are analogous. For instance, in diurnal mammals, both body temperature and locomotor activity increase during the daytime and decrease at night (Satlin et al., 1995; Weinert and

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Waterhouse, 1998; Refinetti, 1999). Nonetheless, BTR and locomotor activity rhythms are regulated by different subsets of subparaventricular zone (SPZ) neurons, suggesting that these rhythms are controlled independently (Saper et al., 2005a,b).

Mammals are not the only examples of this phenomenon. We have previously shown that *Drosophila* exhibit a daily temperature preference rhythm (TPR), in which their preferred temperatures increase during the daytime and then decrease at the transition from day to night (night-onset) (Kaneko et al., 2012). Because *Drosophila* are small ectotherms, their body temperature is very close to that of the ambient temperature (Stevenson, 1985), suggesting that their TPR generates their BTR. In *Drosophila*, we showed that TPR and locomotor activity rhythms are regulated by different subsets of clock neurons (Kaneko et al., 2012). There are ~150 central pacemaker cells in the fly brain, which are functionally homologous to mammalian suprachiasmatic nucleus (SCN) neurons (Welsh et al., 2010). These pacemaker cells are approximately divided into lateral neurons [LNs; small ventral LNs (s-LNvs), large ventral LNs (l-LNvs), and dorsal lateral neurons (LNDs)] and dorsal neurons (DNs; DN1, DN2, and DN3) based on their location and size (Dubruille and Emery, 2008). We previously found that the clocks in DN2s are critical for regulating TPR during the daytime (Kaneko et al., 2012), but not for regulating locomotor activity rhythms (Kaneko et al., 2012).

Neuropeptides and their receptors have important roles in synchronizing circadian clocks (Taghert and Nitabach, 2012). A class II G-protein-coupled receptor, pigment-dispersing factor receptor (PDFR; Hyun et al., 2005; Lear et al., 2005; Mertens et al., 2005), and its ligand (PDF) play important roles in synchronizing circadian clocks and are required for robust circadian locomotor activity in *Drosophila* (Choi et al., 2009, 2012; Duvall and Taghert, 2012; Taghert and Nitabach, 2012). Notably, PDF and PDFR function in a similar manner to vasoactive intestinal peptide (VIP) and its receptor VPAC2 in mammals, both of which play important roles in the ability of clock neurons to regulate the rhythmicity and synchrony of both locomotor activity rhythms and BTRs (Harmar et al., 2002; Aton et al., 2005; Hannibal et al., 2011; Schroeder et al., 2011).

Recent reports have suggested that, in addition to PDF, diuretic hormone 31 (DH31) also activates PDFR based on *in vitro* experiments (Mertens et al., 2005) and a study that used brain imaging with bath-applied DH31 (Shafer et al., 2008). Moreover, it has been shown that DH31 is expressed in the posterior dorsal neurons 1 (DN1ps) and that it modulates sleep as a wake-promoting signal before dawn but does not affect locomotor activity rhythms in *Drosophila* (Kunst et al., 2014). DH31 is a functional homolog of mammalian calcitonin gene-related peptide (CGRP), which mediates thermosensation and thermoregulation (Coast et al., 2001; Johnson et al., 2005; Seybold, 2009; McCoy et al., 2013). However, it is unknown whether CGRP is involved in the regulation of BTR in mammals.

Here, we demonstrate that DH31 and PDFR play important roles for TPR at night-onset. DN2s are the main clock cells for TPR (Kaneko et al., 2012), and our data suggest that DH31 binding to PDFR in DN2s regulates temperature preference decreases at night-onset, which is the first *in vivo* evidence that DH31 could function as a ligand of PDFR. Therefore, we propose that circadian locomotor activity and night-onset TPR are regulated by different neuropeptides that use the same receptor expressed in different clock cells.

Materials and Methods

Fly lines

All the flies were raised in 12 h light/dark cycles at 25°C; zeitgeber time (ZT) 0 is lights-on, ZT12 is lights-off. *w¹¹¹⁸* flies were used for wild-type (WT) flies. Transgenic flies bearing membrane-tethered DH31 (*t-DH31*; *UAS-t-DH31-ML:B4*), membrane-tethered PDF (*t-PDF*; *UAS-t-PDF-ML:M2a*), or membrane-tethered SCR (*UAS-t-PDF-SCR:2A*) were from Dr. Michael Nitabach (Choi et al., 2009). *Pdf⁵³⁰⁴*, *UAS-Pdf* were from Dr. Paul Taghert. *γw*; *Pdf⁰¹*, *γw*; *Dh31^{#51}* flies (Head et al., 2015) and *Pdf⁵³⁰⁴* were backcrossed with *w¹¹¹⁸* and written as *w*; *Pdf⁰¹*, *w*; *Dh31^{#51}* and *Pdf^{5304-bc}*, respectively. *tim-Gal4* (expressed in all clock neurons), *Clk4.1M-Gal4* (expressed in ~8–10 DN1ps) and *Clk4.5F-Gal4* (expressed in ~4 DN1ps; L. Zhang et al., 2010; Y. Zhang et al.), *Clk9M-Gal4* (expressed in s-LNvs and DN2s) and *Clk9M-Gal4, Pdf-Gal80* (expressed in DN2s; Kaneko et al., 2012) were used. *Pdf-Gal80* was used to suppress the *Clk9M-Gal4* expression in s-LNvs (Stoleru et al., 2005).

Immunohistochemistry

Immunostaining was performed as described previously (Hamada et al., 2008; Tang et al., 2013), with the following modifications: 5% normal goat serum in PBST (PBS plus 0.3 or 0.5% Triton X-100) for blocking and antibody incubations. DH31 staining was performed with the guinea pig or rabbit anti-DH31 antibody (1:200; Kunst et al., 2014) and donkey anti-guinea pig or anti-rabbit Cy5 (1:200; Jackson ImmunoResearch). PDF staining was used with the mouse anti-PDF antibody or the guinea pig anti-VRI (VRILLE) antibody (Glossop et al., 2003; 1:200) and anti-mouse or guinea pig Alexa Fluor 647 (1:200; Jackson ImmunoResearch). Mounted brains were scanned using a Zeiss LSM5 Pascal confocal microscope. Images were digitally projected by Z-stacks.

Temperature preference behavioral assay and data analysis

Temperature preference behavioral (TPB) assay and data analysis were performed as described previously (Kaneko et al., 2012; Goda et al., 2014). The flies were raised at 25°C in 12 h light/dark (LD) conditions. The TPB assays were performed for 30 min in an environmental room maintained at 25°C/65–70% relative humidity. Twenty to 30 flies were used for one trial and were never reused. Control and experimental trials were performed in parallel or independently. After the 30 min behavioral assay, the number of flies completely on the apparatus were counted. Flies partially or completely on the walls of the apparatus cover were not included in the data analysis. The percentage of flies within each 1°C temperature interval on the apparatus was calculated by dividing the number of flies within each 1°C interval by the total number of flies on the apparatus. The location of each 1°C interval was determined by measuring the temperature at six different points on the bottom of the apparatus. Data points were plotted as a percentage of flies within a 1°C temperature interval. The weighted mean of the preferred temperature was calculated by summing the products of the percentage of flies within a 1°C temperature interval and the corresponding temperature (e.g., fractional number of flies × 18.5°C + fractional number of flies × 19.5°C + ... fractional number of flies × 32.5°C).

TPR assay

The TPR assays were performed for 30 min in light during the day and dark during the night and were tested ≥5 times in each time zone (ZT1–ZT3, ZT4–ZT6, ZT7–ZT9, ZT10–ZT12, ZT13–ZT15, ZT16–ZT18, ZT19–ZT21, and ZT22–ZT24). If the SEM of averaged preferred temperatures was not <0.3 after the five trials, we performed more trials until SEM reached <0.3. To have a full curve of 24 h, ≥40 experiments were necessary. In each time zone, the weighted mean preferred temperatures from each trial were averaged together and the SEM was calculated.

Statistical analysis

One-way ANOVA. To determine whether the datasets were of normal distribution, either Shapiro–Wilk normality test or Kolmogorov–Smirnov test was used. If it passed, the one-way ANOVA test and the Tukey–Kramer test were used. If it failed, Kruskal–Wallis test one-way ANOVA on ranks was used (see Tables 2, 3).

Figure 1. *Pdfr* is required for night-onset TPR. **A**, TPR phenotype of *w¹¹¹⁸* (black line) and *Pdfr⁵³⁰⁴* (red line) flies in LD cycles. The preferred temperatures at each time point were normalized by subtracting the preferred temperature at ZT1–ZT3 because *Pdfr⁵³⁰⁴* exhibited a mild warm-temperature preference. The original data are shown in Figure 2F. Error bars indicate the SEM. Italicized numbers represent the number of assays (ZT0 is lights-on, ZT12 is lights-off). Kruskal–Wallis test one-way ANOVA on ranks or one-way ANOVA: $p < 0.0001$ (*w¹¹¹⁸*) and $p = 0.0183$ (*Pdfr⁵³⁰⁴*). *Post hoc* test compared with ZT1–ZT3: **** $p < 0.0001$, *** $p < 0.001$, * $p < 0.05$. The detailed statistical analysis is shown in Table 3. **B**, Comparison of preferred temperature increases during the daytime (ZT1–ZT12) for *w¹¹¹⁸* and *Pdfr⁵³⁰⁴* flies. The values for temperature increases during the daytime were calculated by subtracting the preferred temperature at ZT1–ZT3 from the preferred temperature at ZT10–ZT12 (*w¹¹¹⁸*) or ZT7–ZT9 (*Pdfr⁵³⁰⁴*). *t* test: $p = 0.6981$. **C**, Comparison of preferred temperature decreases at night-onset (ZT10–ZT12 to ZT13–ZT15) for *w¹¹¹⁸* and *Pdfr⁵³⁰⁴* flies. The values for temperature decreases at night-onset were calculated by subtracting the preferred temperature at ZT13–ZT15 from the preferred temperature at ZT10–ZT12. *t* test: $p = 0.0006$. **D**, Comparison of preferred temperature decreases at night-onset (ZT10–ZT12 to ZT13–ZT15) for *Pdfr⁵³⁰⁴* rescue and their control flies. *UAS-Pdfr* was expressed in a subset of clock cells using the following *Gal4* and *Gal80* drivers: *Pdf* (*Pdf-Gal4*), *DN1* (*Clk4.5F-Gal4*) and *DN2* (*Clk9M-Gal4*; *Pdf-Gal80*). One-way ANOVA and Tukey–Kramer test compared with controls: ** $p < 0.01$ (Table 2). Two-way ANOVA between controls and *Pdfr* rescue in DN2s flies showed a significant interaction between genotype and time ($F_{(2,28)} = 3.627$, $p = 0.0397$; Table 4).

Two-way ANOVA. To compare the differences between ZT10–ZT12 and ZT13–ZT15 in control and experimental flies, we used two-way ANOVA particularly focusing on the interaction. Sidak’s multiple-comparison test was used for *Post hoc* analysis (see Table 4).

Behavioral analysis of locomotor activity and sleep

Locomotor activity assay and data analysis were performed as described previously (Head et al., 2015; Umezaki et al., 2012). Flies were reared in 12 h LD cycles at 25°C. One–5-d-old male flies were used in locomotor activity experiments. *Drosophila* Activity Monitoring (DAM) systems (TriKinetics, <http://www.trikinetix.com/>) were placed in an incubator (MIR-154, Sanyo Scientific). Lights in the incubator (a 15 W cool-white fluorescent lamp; FL15D, Toshiba) were connected to an electric timer. Light intensity was ~800 lux. The locomotor activity was monitored in 12 h LD cycles for 5 d and constant dark conditions for >10 d at 25°C. The free-running period was calculated by a χ^2 periodogram (Sokolove and Bushnell, 1978) with Actogram J software (Schmid et al., 2011). The power value was calculated according to the previous report (Umezaki et al., 2012). In sleep data analysis, sleep profiles were obtained using Microsoft Excel-based software as described by Tomita et al. (2011) and averaged across 4 d.

Results

PDFR is required for preferred temperature decreases at night-onset

When WT flies are released onto a temperature gradient (18–32°C), they prefer ~25°C (Hamada et al., 2008). This preferred temperature increases during the daytime (ZT1–ZT12), decreases dramatically at night-onset (ZT10–ZT12 to ZT13–ZT15; Kaneko et al., 2012), and returns to ~25°C before dawn (ZT22–ZT24).

To understand the molecular mechanisms of TPR, we first focused on the role of PDFR in TPR, because the PDFR is a neuropeptide receptor that functions to synchronize the circadian clock. We found that the preferred temperature of *Pdfr⁵³⁰⁴* mutants increased during the daytime (Fig. 1A,B), but decreased only ~0.8°C at night-onset (ZT10–ZT12 to ZT13–ZT15) while WT flies exhibited robust preferred temperature decreases of ~1.5–2°C (Fig. 1A,C). The data suggest that PDFR is involved in regulating TPR at night-onset (ZT10–ZT15). Since WT flies dramatically decrease preferred temperature at night-onset, when

many physiological changes presumably occur to maintain animal homeostasis, we sought to dissect the molecular mechanisms of night-onset TPR (ZT10–ZT12 to ZT13–ZT15) by focusing on the roles of PDFR.

PDFR in DN2s is critical for night-onset TPR

PDFR is expressed in a large subset of clock cells within the brain (Im and Taghert, 2010). To determine which clock neurons are important for night-onset TPR, we performed cDNA rescue experiments by expressing PDFR in a subset of the clock neurons. When PDFR was expressed in LNvs or DN1ps with *Pdf-Gal4* or *Clk4.5F-Gal4*, respectively, PDFR expression did not sufficiently rescue the *Pdfr⁵³⁰⁴* mutant phenotype (Fig. 1D, green and blue).

Next, we focused on DN2s because we have previously shown that DN2 function is important for preferred temperature increases during the daytime (Kaneko et al., 2012). Importantly, the previous data regarding PDFR-MYC expression and brain imaging with bath-applied PDF suggest that PDFR is expressed in DN2s (Shafer et al., 2008; Im and Taghert, 2010). As previously reported, *Clk9M-Gal4*; *Pdf-Gal80* was only expressed in DN2s and not expressed in s-LNvs at 25°C (Kaneko et al., 2012; Fig. 2A,B). Therefore, we used *Clk9M-Gal4*; *Pdf-Gal80* to express *Pdfr* in DN2s in the *Pdfr⁵³⁰⁴* background.

During the daytime, even though the total increases of preferred temperature of the *Gal4* and *UAS* controls in the *Pdfr⁵³⁰⁴* background were lower than that of the rescue flies (Fig. 2D; see Table 2), their preferred temperature still showed a statistically significant increase (Fig. 2C; see Table 3). At night-onset, the preferred temperature decreases of the rescue flies were restored (Figs. 1D, red, 2C,E) and statistically different from that of the *Gal4* and *UAS* controls (see Tables 2, 3). These data indicate that PDFR expression in DN2s is sufficient for night-onset TPR.

It is worth noting that *Pdfr⁵³⁰⁴* mutants preferred a slightly warmer temperature compared with WT and exhibited a phase-advanced phenotype with a peak at ZT7–ZT9 (Fig. 2F).

Figure 2. TPR phenotype of *Pdfr* mutant rescue flies and *Pdfr* mutant backcrossed flies. **A, B**, Immunostaining with anti-PDF (**A**) or anti-VRI (**B**) on the fly brain of *Clk9M-Gal4>UAS-mCD8::GFP* and *Clk9M-Gal4, Pdf-Gal80>UAS-mCD8::GFP* flies at 25°C. These images are the confocal stacks. GFP signals are shown in green. PDF (**A**) or VRI (**B**) signals are shown in red. *Clk9M-Gal4, Pdf-Gal80* was not expressed in s-LNv (**A**). GFP was expressed strongly in only one of the two DN2s in ~80% of *Clk9M-Gal4; Pdf-Gal80* and *Clk9M-Gal4* brains (**B**). **C**, A 24 h profile of the TPR phenotype of *Pdfr⁵³⁰⁴* rescue flies in LD cycles. *UAS-Pdfr* was expressed in DN2 cells using *Clk9M-Gal4, Pdf-Gal80*. Error bars indicate SEM. Italicized numbers represent the number of assays. Kruskal–Wallis test one-way ANOVA on ranks or one-way ANOVA and *post hoc* test compared TPB using ZT1–ZT3 as the standard: ** $p < 0.01$ or * $p < 0.05$ (Table 3). **D**, Comparison of preferred temperature increases during the daytime (ZT1–ZT12) for *Pdfr* rescue in DN2s. Error bars indicate the SEM. One-way ANOVA and Tukey–Kramer test were used to compare with each control: * $p < 0.05$ (Table 2). **E**, Comparison of preferred temperature decreases at night-onset (ZT10–ZT12 to ZT13–ZT15) for *Pdfr* rescue in DN2s. Error bars indicate the SEM. One-way ANOVA and Tukey–Kramer test were used to compare against each control: **** $p < 0.0001$ or *** $p < 0.001$ (Table 2). **F**, TPR phenotype of *w¹¹¹⁸* (black line) and *Pdfr⁵³⁰⁴* (red line) flies in LD cycles. This was the original data of Figure 1A before normalizing. *Pdfr⁵³⁰⁴* exhibited a slightly warmer temperature preference than that of WT and an advanced TPR, where the peak was at ZT7–ZT9. **G**, TPR phenotype of *w¹¹¹⁸* (blue line) and *Pdfr^{5304-BC}* (orange line) flies in LD cycles. Because different apparatuses were used for **F** and **G**, the preferred temperatures of WT (**F**, black; **G**, blue) were slightly different. Importantly, WT flies using either apparatus increased ~1°C preferred temperature during the daytime, showing normal TPR (Table 3). *Pdfr^{5304-BC}* exhibited a similar TPB as WT. **H**, Comparison of preferred temperature increases during the daytime (ZT1–ZT12) for *w¹¹¹⁸* and *Pdfr^{5304-BC}* flies. The values for temperature increases during the daytime were calculated by subtracting the preferred temperature at ZT1–ZT3 from the preferred temperature at ZT10–ZT12. *t* test: $p = 0.1436$. **I**, Comparison of preferred temperature decreases at night-onset (ZT10–ZT12 to ZT13–ZT15) for *w¹¹¹⁸* and *Pdfr^{5304-BC}* flies. The values for temperature decreases at night-onset were calculated by subtracting the preferred temperature at ZT10–ZT12 from the preferred temperature at ZT13–ZT15. *t* test: $p = 0.0005$.

Figure 3. DH31 but not PDF is required for night-onset TPR. **A**, TPR phenotype of w^{1118} (black line) and Pdf^{01} (blue line) flies in LD cycles. Error bars indicate SEM. Italicized numbers represent the number of assays. One-way ANOVA: $p < 0.0001$ (w^{1118}) and $p = 0.0362$ (Pdf^{01} ; $F_{(3,32)} = 3.205$). Tukey–Kramer test compared with ZT1–ZT3: **** $p < 0.0001$ or * $p < 0.05$ in Table 3. **B**, Comparison of preferred temperature increases during the daytime (ZT1–ZT12) for w^{1118} and Pdf^{01} flies. The values for temperature increases during the daytime were calculated by subtracting the preferred temperature at ZT1–ZT3 from the preferred temperature at the peak (w^{1118} : ZT10–ZT12; Pdf^{01} : ZT7–ZT9). t test: $p = 0.3354$. Two-way ANOVA was performed, and there was no significant interaction between w^{1118} and Pdf^{01} flies during the daytime ($F_{(1,88)} = 2.669$, $p = 0.1059$) in Table 4. **C**, Comparison of preferred temperature decreases at night-onset (ZT10–ZT12 to ZT13–ZT15) for w^{1118} and Pdf^{01} flies. The values for temperature decreases at night-onset were calculated by subtracting the preferred temperature at ZT13–ZT15 from the preferred temperature at ZT10–ZT12. t test: $p = 0.1461$. Two-way ANOVA was performed and there was no significant interaction between w^{1118} and Pdf^{01} flies at night-onset ($F_{(1,62)} = 0.8354$, $p = 0.3643$) in Table 4. **D**, TPR phenotype of w^{1118} (black line) and $Dh31^{#51}$ (red line) flies in LD cycles. Error bars indicate the SEM. Italicized numbers represent the number of assays. One-way ANOVA: $p = 0.0103$ ($F_{(3,34)} = 4.386$). Tukey–Kramer test compared with ZT1–ZT3: **** $p < 0.0001$ or ** $p < 0.01$ in Table 3. **E**, Comparison of preferred temperature increases during the daytime (ZT1–ZT12) for w^{1118} and $Dh31^{#51}$ flies. The values for temperature increases during the daytime were calculated by subtracting the preferred temperature at ZT1–ZT3 from the preferred temperature at ZT10–ZT12. t test: $p = 0.1080$. Two-way ANOVA was performed and there was no significant interaction between w^{1118} and $Dh31^{#51}$ flies during the daytime ($F_{(1,89)} = 1.256$, $p = 0.2653$) in Table 4. **F**, Comparison of preferred temperature decreases at night-onset (ZT10–ZT12 to ZT13–ZT15) for w^{1118} and $Dh31^{#51}$ flies. The values for temperature decreases at night-onset were calculated by subtracting the preferred temperature at ZT13–ZT15 from the preferred temperature at ZT10–ZT12. t test: $p < 0.0001$. Two-way ANOVA was performed and there was a significant interaction between w^{1118} and $Dh31^{#51}$ flies at night-onset ($F_{(1,62)} = 6.922$, $p = 0.0107$) in Table 4.

However, when Pdf^{5304} mutants were backcrossed with w^{1118} ($Pdf^{5304-BC}$), they preferred a similar temperature to WT and no longer showed the advanced peak in daytime TPR (Fig. 2G). This suggests that the mild warmer temperature preference and the phase-advanced phenotype of Pdf^{5304} mutants were likely caused by the genetic background. Most importantly, $Pdf^{5304-BC}$ showed a TPR during the daytime similar to that of WT (Fig. 2H) and sustained the abnormal night-onset TPR (Fig. 2I).

PDF is not required for night-onset TPR

PDF is a ligand of PDFR that has important roles in circadian rhythms (Renn et al., 1999; Nitabach and Taghert, 2008; Taghert and Nitabach, 2012). We therefore examined the TPR phenotype of Pdf^{01} . We found that Pdf^{01} mutants exhibited a phase-advanced phenotype with a peak at ZT7–ZT9 (Fig. 3A; see Table 3). However, the preferred temperature of Pdf^{01} mutants still increased during the daytime (Fig. 3B) and decreased normally at night-onset (Fig. 3C). This suggests that PDF is not required for night-onset TPR.

Figure 4. DH31 immunostaining, locomotor activity rhythms, and sleep phenotypes of *Dh31^{#51}* flies. **A**, Immunostaining with anti-DH31 antibody on *w¹¹¹⁸* and *Dh31^{#51}* brains. The images are confocal stacks. Because the epitope regions of the DH31 antibody were deleted in the *Dh31^{#51}* mutant (Kunst et al., 2014), the signals (arrows) in *Dh31^{#51}* brain should be nonspecific signals. **B, C**, DH31 expression in DN1s (arrowhead) was labeled using *Clk4.5F-Gal4* or *Clk4.1M-Gal4* (**B**) or *tim-Gal4* (**C**) with *UAS-mCD8::GFP* (green) and anti-DH31 antibody (red). **D, E**, No DH31 expression in LNvs or DN2s (arrowhead) was observed using *tim-Gal4* with *UAS-mCD8::GFP* (green) and anti-PDF or anti-DH31 antibodies (red). **F**, Averaged locomotor activity rhythms of *w¹¹¹⁸* and *Dh31^{#51}* flies are shown in a double-plotted actograms. The activity was monitored in LD 12:12 for 5 d and subsequently in constant darkness for 10 d. **G**, Sleep profiles for days 2–5 in LD were calculated for *w¹¹¹⁸* and *Dh31^{#51}* flies.

Additionally, we observed that *Pdf⁰¹* mutants preferred a lower temperature during ZT16–ZT24 (Fig. 3A). We noted that PDF-expressing neurons contribute to this lower temperature preference behavior especially before dawn (ZT22–ZT24; X. Tang, F.N. Hamada, unpublished observations). Therefore, PDF is likely involved in this lower temperature preference during ZT16–ZT24. On the other hand, since *Pdf⁰¹* mutants exhibit normal night-onset TPR, PDFR appears to use other ligands to control night-onset TPR.

DH31 is required for night-onset TPR

Because PDFR is also activated by DH31 (Mertens et al., 2005), we used a *Dh31* mutant, *Dh31^{#51}* in which the DH31 active peptide region was ablated (Head et al., 2015). Using an anti-

Table 1. Comparison of the free-running period and power value between *w¹¹¹⁸* and *Dh31^{#51}* flies

Genotype	<i>n</i>	<i>n</i> Rhythmic (%)	Tau (hour)		Power	
			Average	SEM	Average	SEM
<i>w¹¹¹⁸</i>	118	114 (97)	24.0	0.02	1371.7	75.1
<i>Dh31^{#51}</i>	86	80 (93)	24.4	0.06	678.2	46.0

body against the active peptide region of DH31 (Kunst et al., 2014), we found that there was no positive signal in the *Dh31^{#51}* mutant brain (Fig. 4A). In the WT brain, DH31 was expressed in many cells, including a subset of DN1s labeled by *Clk4.5F-Gal4*, *Clk4.1M-Gal4* (Fig. 4B) and *tim-Gal4* (Fig. 4C), but not in other clock cells, such as LNvs or DN2s (Fig. 4D,E).

Figure 5. *t*-DH31 expression in DN2s restores night-onset TPR. **A–C**, Comparison of preferred temperature decreases at night-onset (ZT10–ZT12 to ZT13–ZT15) in *Dh31*^{#51} flies with several rescue constructs. *UAS-t-Dh31/+* (**A**), *UAS-t-Pdf/+* (**B**), or *UAS-t-SCR/+* (Choi et al., 2009) was expressed in a subset of the clock cells using the following Gal4 drivers: *Pdf* (*Pdf-Gal4*) in s-LNvs and l-LNvs, *DN1* (*4.1M*) (*Clk4.1M-Gal4*) or *DN1* (*4.5F*) (*Clk4.5F-Gal4*) in DN1s, and *DN2* (*Clk9M-Gal4*; *Pdf-Gal80*) in DN2s. Kruskal–Wallis test one-way ANOVA on ranks or one-way ANOVA and *post hoc* test were used to compare rescue against each control: **** $p < 0.0001$, *** $p < 0.01$, or * $p < 0.05$. The detailed statistical analysis is shown in Table 2.

Figure 6. A 24 h profile of temperature preference behaviors of *Dh31* mutant rescue flies. **A**, TPR phenotype of *Dh31*^{#51} rescue flies in DN2s (red: *Dh31*^{#51}; *DN2* > *t-Dh31*) and their controls (blue: *Dh31*^{#51}; *DN2* > + or green: *Dh31*^{#51}; *UAS-t-Dh31/+*) in LD cycles. *UAS-t-Dh31* was expressed in DN2 cells using *Clk9M-Gal4*, *Pdf-Gal80*. Error bars indicate the SEM. Italicized numbers represent the number of assays. One-way ANOVA and Tukey–Kramer test were used to compare temperature change based on ZT1–ZT3: **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$. The detailed statistical analysis is shown in Table 3. **B**, Comparison of preferred temperature increases during the daytime (ZT1–ZT12) for *Dh31*^{#51} rescue flies. Error bars indicate the SEM. One-way ANOVA and Tukey–Kramer test were used to compare the rescue with each control: ** $p < 0.01$ and * $p < 0.05$ (Table 2). **C**, Comparison of preferred temperature decreases at night-onset (ZT10–ZT12 to ZT13–ZT15) for *Dh31*^{#51} rescue flies. Error bars indicate the SEM. One-way ANOVA and Tukey–Kramer test were used to compare the rescue with each control: * $p < 0.05$ (Table 2).

A similar pattern of DH31 expression was also shown in a recent study (Kunst et al., 2014). We also examined the sleep and locomotor activity rhythms of *Dh31*^{#51} mutants. Although *Dh31*^{#51} mutants had a relatively higher level of late-night sleep (ZT21–ZT24), they maintained robust locomotor activity rhythms in both the LD and constant dark conditions with a similar period length to WT flies (Fig. 4F, G; Table 1), suggesting that DH31 is involved in sleep regulation but not in circadian rhythmicity. These data were consistent with the results using another *Dh31* mutant shown in a previous study (Kunst et al., 2014).

To determine whether DH31 is responsible for TPR, we tested the TPR behavior of *Dh31*^{#51} mutants and found that they exhibited a normal preferred temperature increase during the daytime similar to that of WT flies (Fig. 3D, E; see Table 3). However, *Dh31*^{#51} mutants interestingly showed only a ~0.6–0.8°C decrease in preferred temperature at night-onset (Fig. 3F), indicating that DH31 is required for night-onset TPR.

t-DH31 expression in DN2s is sufficient for night-onset TPR

Previous studies using imaging analyses have shown that a bath application of DH31 can activate LNvs (Shafer et al., 2008). To examine

Table 2. One-way ANOVA analysis of preferred temperature increases during the daytime (ZT1–ZT12) or preferred temperature decreases at night-onset (ZT10–ZT12 and ZT13–ZT15)

Figure	p value	Tests
1D		
<i>Pdfr</i> ⁵³⁰⁴ ; <i>Pdf</i> > <i>Pdfr</i>		
Compared with <i>Pdfr</i> ⁵³⁰⁴ ; <i>Pdf</i> > +	NS	Passed Shapiro–Wilk normality test; one-way ANOVA: $p = 0.1182$; $F_{(DFn,DFd)}: F_{(2,15)} = 2.471$
<i>Pdfr</i> ⁵³⁰⁴ ; <i>Pdf</i> > <i>Pdfr</i>		
Compared with <i>Pdfr</i> ⁵³⁰⁴ ; <i>UAS-Pdfr</i> /+	NS	
<i>Pdfr</i> ⁵³⁰⁴ ; <i>UAS-Pdfr</i> /+		
Compared with <i>Pdfr</i> ⁵³⁰⁴ ; <i>Pdf</i> > +	NS	
1D		
<i>Pdfr</i> ⁵³⁰⁴ ; <i>DN1(4.5F)</i> > <i>Pdfr</i>		
Compared with <i>Pdfr</i> ⁵³⁰⁴ ; <i>DN1(4.5F)</i> > +	NS	Passed Shapiro–Wilk normality test; one-way ANOVA: $p = 0.1799$; $F_{(DFn,DFd)}: F_{(2,13)} = 1.963$
<i>Pdfr</i> ⁵³⁰⁴ ; <i>DN1(4.5F)</i> > <i>Pdfr</i>		
Compared with <i>Pdfr</i> ⁵³⁰⁴ ; <i>UAS-Pdfr</i> /+	NS	
<i>Pdfr</i> ⁵³⁰⁴ ; <i>UAS-Pdfr</i> /+		
Compared with <i>Pdfr</i> ⁵³⁰⁴ ; <i>DN1(4.5F)</i> > +	NS	
1D		
<i>Pdfr</i> ⁵³⁰⁴ ; <i>DN2</i> > <i>Pdfr</i>		
Compared with <i>Pdfr</i> ⁵³⁰⁴ ; <i>DN2</i> > +	**	Passed Shapiro–Wilk normality test; one-way ANOVA: $p = 0.0030$; $F_{(DFn,DFd)}: F_{(2,14)} = 9.066$
<i>Pdfr</i> ⁵³⁰⁴ ; <i>DN2</i> > <i>Pdfr</i>		
Compared with <i>Pdfr</i> ⁵³⁰⁴ ; <i>UAS-Pdfr</i> /+	**	
<i>Pdfr</i> ⁵³⁰⁴ ; <i>UAS-Pdfr</i> /+		
Compared with <i>Pdfr</i> ⁵³⁰⁴ ; <i>DN2</i> > +	NS	
1D		
<i>Pdfr</i> ⁵³⁰⁴ ; <i>UAS-Pdfr</i> /+		
Compared with <i>Pdfr</i> ⁵³⁰⁴ ; <i>Pdf</i> > +	NS	Passed Shapiro–Wilk normality test; one-way ANOVA: $p = 0.2464$; $F_{(DFn,DFd)}: F_{(3,18)} = 1.508$
<i>Pdfr</i> ⁵³⁰⁴ ; <i>UAS-Pdfr</i> /+		
Compared with <i>Pdfr</i> ⁵³⁰⁴ ; <i>DN1(4.5F)</i> > +	NS	
<i>Pdfr</i> ⁵³⁰⁴ ; <i>UAS-Pdfr</i> /+		
Compared with <i>Pdfr</i> ⁵³⁰⁴ ; <i>DN2</i> > +	NS	
<i>Pdfr</i> ⁵³⁰⁴ ; <i>Pdf</i> > +		
Compared with <i>Pdfr</i> ⁵³⁰⁴ ; <i>DN2</i> > +	NS	
<i>Pdfr</i> ⁵³⁰⁴ ; <i>DN1(4.5F)</i> > +		
Compared with <i>Pdfr</i> ⁵³⁰⁴ ; <i>DN2</i> > +	NS	
2D		
<i>Pdfr</i> ⁵³⁰⁴ ; <i>DN2</i> > <i>Pdfr</i>		
Compared with <i>Pdfr</i> ⁵³⁰⁴ ; <i>DN2</i> > +	*	Passed Shapiro–Wilk normality test; one-way ANOVA: $p = 0.0169$; $F_{(DFn,DFd)}: F_{(2,35)} = 4.593$
<i>Pdfr</i> ⁵³⁰⁴ ; <i>DN2</i> > <i>Pdfr</i>		
Compared with <i>Pdfr</i> ⁵³⁰⁴ ; <i>UAS-Pdfr</i> /+	*	
<i>Pdfr</i> ⁵³⁰⁴ ; <i>UAS-Pdfr</i> /+		
Compared with <i>Pdfr</i> ⁵³⁰⁴ ; <i>DN2</i> > +	NS	

(Table Continues)

Table 2. Continued

Figure	p value	Tests
2E		
<i>Pdfr</i> ⁵³⁰⁴ ; <i>DN2</i> > <i>Pdfr</i>		
Compared with <i>Pdfr</i> ⁵³⁰⁴ ; <i>DN2</i> > +	***	Passed Shapiro–Wilk normality test; one-way ANOVA: $p < 0.0001$; $F_{(DFn,DFd)}: F_{(2,35)} = 15.32$
<i>Pdfr</i> ⁵³⁰⁴ ; <i>DN2</i> > <i>Pdfr</i>		
Compared with <i>Pdfr</i> ⁵³⁰⁴ ; <i>UAS-Pdfr</i> /+	****	
<i>Pdfr</i> ⁵³⁰⁴ ; <i>UAS-Pdfr</i> /+		
Compared with <i>Pdfr</i> ⁵³⁰⁴ ; <i>DN2</i> > +	NS	
5A		
<i>Dh31</i> ^{#51} ; <i>Pdf</i> > <i>t-Dh31</i>		
Compared with <i>Dh31</i> ^{#51} ; <i>Pdf</i> > +	NS	Kruskal–Wallis test: $p = 0.1101$
<i>Dh31</i> ^{#51} ; <i>Pdf</i> > <i>t-Dh31</i>		
Compared with <i>Dh31</i> ^{#51} ; <i>UAS-t-Dh31</i> /+	NS	
<i>Dh31</i> ^{#51} ; <i>UAS-t-Dh31</i> /+		
Compared with <i>Dh31</i> ^{#51} ; <i>Pdf</i> > +	NS	
5A		
<i>Dh31</i> ^{#51} ; <i>DN1(4.1M)</i> > <i>t-Dh31</i>		
Compared with <i>Dh31</i> ^{#51} ; <i>DN1(4.1M)</i> > +	NS	Passed Shapiro–Wilk normality test; one-way ANOVA: $p = 0.7916$; $F_{(DFn,DFd)}: F_{(2,14)} = 0.2377$
<i>Dh31</i> ^{#51} ; <i>DN1(4.1M)</i> > <i>t-Dh31</i>		
Compared with <i>Dh31</i> ^{#51} ; <i>UAS-t-Dh31</i> /+	NS	
<i>Dh31</i> ^{#51} ; <i>UAS-t-Dh31</i> /+		
Compared with <i>Dh31</i> ^{#51} ; <i>DN1(4.1M)</i> > +	NS	
5A		
<i>Dh31</i> ^{#51} ; <i>DN1(4.5F)</i> > <i>t-Dh31</i>		
Compared with <i>Dh31</i> ^{#51} ; <i>DN1(4.5F)</i> > +	NS	Passed Shapiro–Wilk normality test; one-way ANOVA: $p = 0.1446$; $F_{(DFn,DFd)}: F_{(2,14)} = 2.228$
<i>Dh31</i> ^{#51} ; <i>DN1(4.5F)</i> > <i>t-Dh31</i>		
Compared with <i>Dh31</i> ^{#51} ; <i>UAS-t-Dh31</i> /+	NS	
<i>Dh31</i> ^{#51} ; <i>UAS-t-Dh31</i> /+		
Compared with <i>Dh31</i> ^{#51} ; <i>DN1(4.5F)</i> > +	NS	
5A		
<i>Dh31</i> ^{#51} ; <i>DN2</i> > <i>t-Dh31</i>		
Compared with <i>Dh31</i> ^{#51} ; <i>DN2</i> > +	NS	Passed Shapiro–Wilk normality test; one-way ANOVA: $p < 0.0001$; $F_{(DFn,DFd)}: F_{(2,31)} = 14.16$
<i>Dh31</i> ^{#51} ; <i>DN2</i> > <i>t-Dh31</i>		
Compared with <i>Dh31</i> ^{#51} ; <i>UAS-t-Dh31</i> /+	NS	
<i>Dh31</i> ^{#51} ; <i>UAS-t-Dh31</i> /+		
Compared with <i>Dh31</i> ^{#51} ; <i>DN2</i> > +	NS	
5A		
<i>Dh31</i> ^{#51} ; <i>UAS-t-Dh31</i> /+		
Compared with <i>Dh31</i> ^{#51} ; <i>Pdf</i> > +	NS	Passed Shapiro–Wilk normality test; one-way ANOVA: $p = 0.4887$; $F_{(DFn,DFd)}: F_{(4,35)} = 0.8751$
<i>Dh31</i> ^{#51} ; <i>UAS-t-Dh31</i> /+		
Compared with <i>Dh31</i> ^{#51} ; <i>DN1(4.1M)</i> > +	NS	
<i>Dh31</i> ^{#51} ; <i>UAS-t-Dh31</i> /+		
Compared with <i>Dh31</i> ^{#51} ; <i>DN1(4.5F)</i> > +	NS	

(Table Continues)

Table 2. Continued

Figure	p value	Tests
<i>Dh31^{#51}; UAS-t-Dh31/+</i> Compared with <i>Dh31^{#51}; DN2> +</i>	NS	
<i>Dh31^{#51}; Pdf> +</i> Compared with <i>Dh31^{#51}; DN1(4.1M)> +</i>	NS	
<i>Dh31^{#51}; Pdf> +</i> Compared with <i>Dh31^{#51}; DN1(4.5F)> +</i>	NS	
<i>Dh31^{#51}; Pdf> +</i> Compared with <i>Dh31^{#51}; DN2> +</i>	NS	
<i>Dh31^{#51}; DN1(4.1M)> +</i> Compared with <i>Dh31^{#51}; DN1(4.5F)> +</i>	NS	
<i>Dh31^{#51}; DN1(4.1M)> +</i> Compared with <i>Dh31^{#51}; DN2> +</i>	NS	
<i>Dh31^{#51}; DN1(4.5F)> +</i> Compared with <i>Dh31^{#51}; DN2> +</i>	NS	
5B <i>Dh31^{#51}; Pdf> t-Pdf</i> Compared with <i>Dh31^{#51}; Pdf> +</i>	NS	Passed Shapiro–Wilk normality test; one-way ANOVA: $p = 0.1975$; $F_{(DFn,DFd)}: F_{(2,19)} = 1.769$
<i>Dh31^{#51}; Pdf> t-Pdf</i> Compared with <i>Dh31^{#51}; UAS-t-Pdf/+</i>	NS	
<i>Dh31^{#51}; UAS-t-Pdf/+</i> Compared with <i>Dh31^{#51}; Pdf> +</i>	NS	
5B <i>Dh31^{#51}; DN1(4.1M)> t-Pdf</i> Compared with <i>Dh31^{#51}; DN1(4.1M)> +</i>	NS	Passed Shapiro–Wilk normality test; one-way ANOVA: $p = 0.7238$; $F_{(DFn,DFd)}: F_{(2,15)} = 0.3303$
<i>Dh31^{#51}; DN1(4.1M)> t-Pdf</i> Compared with <i>Dh31^{#51}; UAS-t-Pdf/+</i>	NS	
<i>Dh31^{#51}; UAS-t-Pdf/+</i> Compared with <i>Dh31^{#51}; DN1(4.1M)> +</i>	NS	
5B <i>Dh31^{#51}; DN1(4.5F)> t-Pdf</i> Compared with <i>Dh31^{#51}; DN1(4.5F)> +</i>	NS	Passed Kolmogorov–Smirnov test with Dallal–Wilkinson–Lillie for p value; one-way ANOVA: $p = 0.0924$; $F_{(DFn,DFd)}: F_{(2,15)} = 2.803$
<i>Dh31^{#51}; UAS-t-Pdf/+</i> Compared with <i>Dh31^{#51}; DN1(4.5F)> +</i>	NS	
5B <i>Dh31^{#51}; DN2> t-Pdf</i> Compared with <i>Dh31^{#51}; DN2> +</i>	NS	Passed Shapiro–Wilk normality test; one-way ANOVA: $p = 0.026$; $F_{(DFn,DFd)}: F_{(2,24)} = 4.454$
<i>Dh31^{#51}; DN2> t-Pdf</i> Compared with <i>Dh31^{#51}; UAS-t-Pdf/+</i>	*	
<i>Dh31^{#51}; UAS-t-Pdf/+</i> Compared with <i>Dh31^{#51}; DN2> +</i>	NS	

(Table Continues)

Table 2. Continued

Figure	p value	Tests
5B <i>Dh31^{#51}; UAS-t-Pdf/+</i> Compared with <i>Dh31^{#51}; Pdf> +</i>	NS	Passed Shapiro–Wilk normality test; one-way ANOVA: $p = 0.2378$; $F_{(DFn,DFd)}: F_{(4,36)} = 1.45$
<i>Dh31^{#51}; UAS-t-Pdf/+</i> Compared with <i>Dh31^{#51}; DN1(4.1M)> +</i>	NS	
<i>Dh31^{#51}; UAS-t-Pdf/+</i> Compared with <i>Dh31^{#51}; DN1(4.5F)> +</i>	NS	
<i>Dh31^{#51}; UAS-t-Pdf/+</i> Compared with <i>Dh31^{#51}; DN2> +</i>	NS	
<i>Dh31^{#51}; Pdf> +</i> Compared with <i>Dh31^{#51}; DN1(4.1M)> +</i>	NS	
<i>Dh31^{#51}; Pdf> +</i> Compared with <i>Dh31^{#51}; DN1(4.5F)> +</i>	NS	
<i>Dh31^{#51}; Pdf> +</i> Compared with <i>Dh31^{#51}; DN2> +</i>	NS	
<i>Dh31^{#51}; DN1(4.1M)> +</i> Compared with <i>Dh31^{#51}; DN1(4.5F)> +</i>	NS	
<i>Dh31^{#51}; DN1(4.1M)> +</i> Compared with <i>Dh31^{#51}; DN2> +</i>	NS	
5C <i>Dh31^{#51}; Pdf> t-SCR</i> Compared with <i>Dh31^{#51}; Pdf> +</i>	NS	Passed Shapiro–Wilk normality test; one-way ANOVA: $p = 0.0166$; $F_{(DFn,DFd)}: F_{(2,23)} = 4.924$
<i>Dh31^{#51}; Pdf> t-SCR</i> Compared with <i>Dh31^{#51}; UAS-t-SCR/+</i>	*	
<i>Dh31^{#51}; UAS-t-SCR/+</i> Compared with <i>Dh31^{#51}; Pdf> +</i>	NS	
5C <i>Dh31^{#51}; DN1(4.1M)> t-SCR</i> Compared with <i>Dh31^{#51}; DN1(4.1M)> +</i>	NS	Passed Shapiro–Wilk normality test; one-way ANOVA: $p = 0.0092$; $F_{(DFn,DFd)}: F_{(2,22)} = 5.849$
<i>Dh31^{#51}; DN1(4.1M)> t-SCR</i> Compared with <i>Dh31^{#51}; UAS-t-SCR/+</i>	NS	
<i>Dh31^{#51}; UAS-t-SCR/+</i> Compared with <i>Dh31^{#51}; DN1(4.1M)> +</i>	**	
5C <i>Dh31^{#51}; DN1(4.5F)> t-SCR</i> Compared with <i>Dh31^{#51}; DN1(4.5F)> +</i>	NS	Passed Shapiro–Wilk normality test; one-way ANOVA: $p = 0.0030$; $F_{(DFn,DFd)}: F_{(2,19)} = 7.997$
<i>Dh31^{#51}; DN1(4.5F)> t-SCR</i> Compared with <i>Dh31^{#51}; UAS-t-SCR/+</i>	*	

(Table Continues)

Table 2. Continued

Figure	p value	Tests
<i>Dh31^{#51}; UAS-t-SCR/+</i> Compared with <i>Dh31^{#51}; DN1(4.5F)>+</i>	NS	
5C <i>Dh31^{#51}; DN2>t-SCR</i> Compared with <i>Dh31^{#51}; DN2>+</i>	NS	Passed Shapiro–Wilk normality test; one-way ANOVA: $p < 0.0001$; $F_{(DFn,DFd)}: F_{(2,34)} = 21.7$
<i>Dh31^{#51}; DN2>t-SCR</i> Compared with <i>Dh31^{#51}; UAS-t-SCR/+</i>	****	
<i>Dh31^{#51}; UAS-t-SCR/+</i> Compared with <i>Dh31^{#51}; DN2>+</i>	****	
5C <i>Dh31^{#51}; UAS-t-SCR/+</i> Compared with <i>Dh31^{#51}; Pdf>+</i>	**	Passed Shapiro–Wilk normality test; one-way ANOVA: $p < 0.0001$; $F_{(DFn,DFd)}: F_{(4,40)} = 10.76$
<i>Dh31^{#51}; UAS-t-SCR/+</i> Compared with <i>Dh31^{#51}; DN1(4.1M)>+</i>	***	
<i>Dh31^{#51}; UAS-t-SCR/+</i> Compared with <i>Dh31^{#51}; DN1(4.5F)>+</i>	**	
<i>Dh31^{#51}; UAS-t-SCR/+</i> Compared with <i>Dh31^{#51}; DN2>+</i>	****	
<i>Dh31^{#51}; Pdf>+</i> Compared with <i>Dh31^{#51}; DN1(4.1M)>+</i>	NS	
<i>Dh31^{#51}; Pdf>+</i> Compared with <i>Dh31^{#51}; DN1(4.5F)>+</i>	NS	
<i>Dh31^{#51}; Pdf>+</i> Compared with <i>Dh31^{#51}; DN2>+</i>	NS	
<i>Dh31^{#51}; DN1(4.1M)>+</i> Compared with <i>Dh31^{#51}; DN1(4.5F)>+</i>	NS	
<i>Dh31^{#51}; DN1(4.1M)>+</i> Compared with <i>Dh31^{#51}; DN2>+</i>	NS	
<i>Dh31^{#51}; DN1(4.5F)>+</i> Compared with <i>Dh31^{#51}; DN2>+</i>	NS	
6B <i>Dh31^{#51}; DN2>t-Dh31</i> Compared with <i>Dh31^{#51}; DN2>+</i>	*	Passed Shapiro–Wilk normality test; one-way ANOVA: $p = 0.0017$; $F_{(DFn,DFd)}: F_{(2,17)} = 9.562$
<i>Dh31^{#51}; DN2>t-Dh31</i> Compared with <i>Dh31^{#51}; UAS-t-Dh31/+</i>	NS	
<i>Dh31^{#51}; UAS-t-Dh31/+</i> Compared with <i>Dh31^{#51}; DN2>+</i>	**	
6C <i>Dh31^{#51}; DN2>t-Dh31</i> Compared with <i>Dh31^{#51}; DN2>+</i>	*	Passed Shapiro–Wilk normality test; one-way ANOVA: $p = 0.0069$; $F_{(DFn,DFd)}: F_{(2,32)} = 5.835$
<i>Dh31^{#51}; DN2>t-Dh31</i> Compared with <i>Dh31^{#51}; UAS-t-Dh31/+</i>	NS	
<i>Dh31^{#51}; UAS-t-Dh31/+</i> Compared with <i>Dh31^{#51}; DN2>+</i>	*	

whether DH31 acts on LNvs to regulate night-onset TPR, we used t-DH31 (Choi et al., 2009, 2012). This membrane-tethered peptide has both linker and anchor peptides that couple with the cell membrane, resulting in cell-autonomous binding and activation of its receptors on specific cells (Choi et al., 2009, 2012).

First, we expressed t-DH31 in LNvs using *Pdf-Gal4* in the *Dh31^{#51}* background and tested night-onset TPR (Fig. 5A). Although the decrease in the preferred temperature of rescue flies was slightly greater than decreases for *Gal4* and *UAS* controls, the decrease was not statistically significant (Fig. 5A, green; Table 2). This suggests that DH31 acting on LNvs is not sufficient for night-onset TPR. Furthermore, a recent study has shown that DN1s secrete DH31 (Kunst et al., 2014). Because neuropeptides can function in autocrine signaling (Im and Taghert, 2010), we expressed t-DH31 in a subset of DN1s using *Clk4.1M-Gal4* or *Clk4.5F-Gal4* in the *Dh31^{#51}* background. However, the decreases of preferred temperature in these flies were not statistically different from those of their control flies (Fig. 5A, orange and blue; Table 2). Therefore, the data suggest that DH31 acting on DN1s is also not sufficient for night-onset TPR.

Since we showed that PDFR expression in DN2s is important for night-onset TPR (Fig. 1D), we hypothesized that DH31 might function as a ligand of PDFR for night-onset TPR. If so, DH31 may act on DN2s through PDFR to control preferred temperature decreases. To examine this, t-DH31 was expressed in DN2s using *Clk9M-Gal4*; *Pdf-Gal80* (Figs. 5A, 6) in the *Dh31^{#51}* background. The flies displayed restored night-onset TPR, in which the preferred temperature decreases of these rescue flies were statistically different from those of *Gal4* and *UAS* controls (Figs. 5A, red, 6A, C; Tables 2, 3). This indicates that t-DH31 expression in DN2s is sufficient for night-onset TPR.

To examine the specificity of t-DH31 expression in DN2s, t-PDF or scrambled PDF (t-SCR) was expressed in DN2s in the *Dh31^{#51}* background as controls (Fig. 5B, C). Although t-PDF expression in DN2s slightly rescued night-onset TPR compared with *UAS* controls (*UAS-t-Pdf/+* in *Dh31^{#51}*; Fig. 5B, red), the preferred temperature decreases were not statistically different from those of the *Gal4* control flies (*Clk9M-Gal4*; *Pdf-Gal80* in *Dh31^{#51}*; Table 2). Additionally, we observed that the control flies using t-SCR did not restore their night-onset TPR (Fig. 5C). Thus, the data indicate that DH31 acting on DN2s is critical for night-onset TPR.

It should be noted that flies with t-DH31 (both DN2s rescue and *UAS* control flies) showed a smaller preferred temperature increases than the flies without t-DH31 (*Gal4* control flies) during the daytime (Fig. 6A, B). It is possible that the *UAS-t-Dh31* transgene itself or t-DH31 leaky expression might prevent a normal preferred temperature increase during the daytime. Nonetheless, with regard to night-onset (ZT10–ZT12 to ZT13–ZT15; Table 4), only t-DH31 expression in DN2s could rescue the *Dh31* mutant phenotype (Fig. 5A). Also, t-PDF and t-SCR in DN2s did not restore night-onset TPR (Fig. 5B, C). Therefore, we concluded that t-DH31 expression in DN2s is sufficient for regulating night-onset TPR.

Discussion

We showed that both *Dh31* and *Pdfr* mutants exhibited abnormal night-onset TPR and that t-DH31 and PDFR expression in DN2s are sufficient to control night-onset TPR, suggesting that DH31 could be a ligand of PDFR in DN2s (Fig. 7B). Unexpectedly, PDF, an important neuropeptide for locomotor activity, is not required for night-onset TPR. It suggests that PDF and DH31 appear to act on different subsets of PDFR-expressing clock cells to regulate locomotor activity

Table 3. One-way ANOVA analysis to compare TPR at different times of the day

Figure; flies; ZT	Comparison	<i>p</i> value	Tests
1A; <i>w¹¹¹⁸</i> ; ZT1–ZT3	ZT4–ZT6	***	Kruskal–Wallis test: $p < 0.0001$
	ZT7–ZT9	***	
	ZT10–ZT12	****	
1A; <i>w¹¹¹⁸</i> ; ZT13–ZT15	ZT1–ZT3	Not significant	Kruskal–Wallis test: $p < 0.0001$
	ZT4–ZT6	***	
	ZT7–ZT9	****	
	ZT10–ZT12	****	
	ZT16–ZT18	Not significant	
	ZT19–ZT21	Not significant	
	ZT22–ZT24	Not significant	
1A; <i>Pdfr⁵³⁰⁴</i> ; ZT1–ZT3	ZT4–ZT6	Not significant	Passed Shapiro–Wilk normality test; one-way ANOVA: $p = 0.0292$; $F_{(DFn,DFd)}: F_{(3,24)} = 3.559$
	ZT7–ZT9	*	
	ZT10–ZT12	Not significant	
1A; <i>Pdfr⁵³⁰⁴</i> ; ZT13–ZT15	ZT1–ZT3	Not significant	Kruskal–Wallis test: $p < 0.0001$; one-way ANOVA: $p = 0.0183$; $F_{(DFn,DFd)}: F_{(7,45)} = 2.747$
	ZT4–ZT6	Not significant	
	ZT7–ZT9	*	
	ZT10–ZT12	Not significant	
	ZT16–ZT18	Not significant	
	ZT19–ZT21	Not significant	
	ZT22–ZT24	Not significant	
	ZT4–ZT6	Not significant	
2C; <i>Pdfr⁵³⁰⁴</i> ; <i>DN2</i> > +; ZT1–ZT3	ZT7–ZT9	Not significant	Passed Shapiro–Wilk normality test; one-way ANOVA: $p = 0.0408$; $F_{(DFn,DFd)}: F_{(3,38)} = 3.034$
	ZT10–ZT12	*	
	ZT1–ZT3	Not significant	
	ZT4–ZT6	Not significant	
2C; <i>Pdfr⁵³⁰⁴</i> ; <i>DN2</i> > +; ZT13–ZT15	ZT7–ZT9	Not significant	Kruskal–Wallis test: $p = 0.0003$
	ZT10–ZT12	*	
	ZT1–ZT3	Not significant	
	ZT4–ZT6	Not significant	
	ZT7–ZT9	Not significant	
	ZT10–ZT12	Not significant	
	ZT16–ZT18	Not significant	
	ZT19–ZT21	Not significant	
2C; <i>Pdfr⁵³⁰⁴</i> ; <i>UAS-Pdfr</i> / +; ZT1–ZT3	ZT22–ZT24	Not significant	Kruskal–Wallis test: $p = 0.0250$
	ZT4–ZT6	Not significant	
	ZT7–ZT9	Not significant	
	ZT10–ZT12	*	
2C; <i>Pdfr⁵³⁰⁴</i> ; <i>UAS-Pdfr</i> / +; ZT13–ZT15	ZT1–ZT3	Not significant	Kruskal–Wallis test: $p < 0.0001$
	ZT4–ZT6	Not significant	
	ZT7–ZT9	Not significant	
	ZT10–ZT12	Not significant	
	ZT16–ZT18	Not significant	
	ZT19–ZT21	Not significant	
	ZT22–ZT24	Not significant	
	ZT4–ZT6	Not significant	
2C; <i>Pdfr⁵³⁰⁴</i> ; <i>DN2</i> > <i>Pdfr</i> ; ZT1–ZT3	ZT7–ZT9	Not significant	Kruskal–Wallis test: $p = 0.0030$
	ZT10–ZT12	**	
	ZT1–ZT3	Not significant	
	ZT4–ZT6	Not significant	
2C; <i>Pdfr⁵³⁰⁴</i> ; <i>DN2</i> > <i>Pdfr</i> ; ZT13–ZT15	ZT7–ZT9	Not significant	Kruskal–Wallis test: $p < 0.0001$
	ZT10–ZT12	***	
	ZT16–ZT18	Not significant	
	ZT19–ZT21	Not significant	
	ZT22–ZT24	Not significant	
	ZT4–ZT6	Not significant	
	ZT7–ZT9	Not significant	
	ZT10–ZT12	***	
2F; <i>Pdfr⁵³⁰⁴</i> ; ZT1–ZT3	ZT16–ZT18	Not significant	Passed Shapiro–Wilk normality test; one-way ANOVA: $p = 0.0292$; $F_{(DFn,DFd)}: F_{(3,24)} = 3.559$
	ZT19–ZT21	Not significant	
	ZT22–ZT24	Not significant	
2G; <i>w¹¹¹⁸</i> ; ZT1–ZT3	ZT4–ZT6	Not significant	Passed Shapiro–Wilk normality test; one-way ANOVA: $p = 0.0009$; $F_{(DFn,DFd)}: F_{(3,27)} = 7.47$
	ZT7–ZT9	*	
	ZT10–ZT12	**	
2G; <i>Pdfr^{5304-BC}</i> ; ZT1–ZT3	ZT4–ZT6	Not significant	Passed Shapiro–Wilk normality test; one-way ANOVA: $p = 0.0006$; $F_{(DFn,DFd)}: F_{(3,27)} = 7.917$
	ZT7–ZT9	*	
	ZT10–ZT12	**	
3A; <i>Pdfr⁰¹</i> ; ZT1–ZT3	ZT4–ZT6	Not significant	Passed Shapiro–Wilk normality test; one-way ANOVA: $p = 0.0362$; $F_{(DFn,DFd)}: F_{(3,32)} = 3.205$
	ZT7–ZT9	*	
	ZT10–ZT12	Not significant	

(Table Continues)

Table 3. Continued

Figure; flies; ZT	Comparison	p value	Tests
3A; <i>Pdf</i> ⁰¹ ; ZT13–ZT15	ZT1–ZT3	Not significant	Kruskal–Wallis test: $p < 0.0001$
	ZT4–ZT6	Not significant	
	ZT7–ZT9	*	
	ZT10–ZT12	Not significant	
	ZT16–ZT18	Not significant	
	ZT19–ZT21	Not significant	
	ZT22–ZT24	Not significant	
3D; <i>Dh31</i> ^{#51} ; ZT1–ZT3	ZT4–ZT6	Not significant	Passed Shapiro–Wilk normality test; one-way ANOVA: $p = 0.0103$; $F_{(DFn,DFd)}; F_{(3,34)} = 4.386$
	ZT7–ZT9	Not significant	
	ZT10–ZT12	**	
3D; <i>Dh31</i> ^{#51} ; ZT13–ZT15	ZT1–ZT3	Not significant	Kruskal–Wallis test: $p = 0.1072$.
	ZT4–ZT6	Not significant	
	ZT7–ZT9	Not significant	
	ZT10–ZT12	Not significant	
	ZT16–ZT18	Not significant	
	ZT19–ZT21	Not significant	
	ZT22–ZT24	Not significant	
6A; <i>Dh31</i> ^{#51} ; DN2 > <i>t-Dh31</i> ; ZT1–ZT3	ZT4–ZT6	Not significant	Passed Shapiro–Wilk normality test; one-way ANOVA: $p = 0.0011$; $F_{(DFn,DFd)}; F_{(3,28)} = 7.087$
	ZT7–ZT9	**	
	ZT10–ZT12	***	
6A; <i>Dh31</i> ^{#51} ; DN2 > <i>t-Dh31</i> ; ZT13–ZT15	ZT1–ZT3	Not significant	Passed Shapiro–Wilk normality test; one-way ANOVA: $p = 0.0001$; $F_{(DFn,DFd)}; F_{(7,54)} = 5.392$
	ZT4–ZT6	Not significant	
	ZT7–ZT9	**	
	ZT10–ZT12	****	
	ZT16–ZT18	Not significant	
	ZT19–ZT21	Not significant	
	ZT22–ZT24	Not significant	
6A; <i>Dh31</i> ^{#51} ; DN2 > +; ZT1–ZT3	ZT4–ZT6	*	Passed Shapiro–Wilk normality test; one-way ANOVA: $p < 0.0001$; $F_{(DFn,DFd)}; F_{(3,26)} = 12.9$
	ZT7–ZT9	***	
	ZT10–ZT12	****	
6A; <i>Dh31</i> ^{#51} ; DN2 > +; ZT13–ZT15	ZT1–ZT3	*	Passed Shapiro–Wilk normality test; one-way ANOVA: $p < 0.0001$; $F_{(DFn,DFd)}; F_{(7,48)} = 7.449$
	ZT4–ZT6	Not significant	
	ZT7–ZT9	Not significant	
	ZT10–ZT12	Not significant	
	ZT16–ZT18	Not significant	
	ZT19–ZT21	Not significant	
	ZT22–ZT24	Not significant	
6A; <i>Dh31</i> ^{#51} ; <i>UAS-t-Dh31</i> /+; ZT1–ZT3	ZT4–ZT6	Not significant	Kruskal–Wallis test: $p = 0.1896$
	ZT7–ZT9	Not significant	
	ZT10–ZT12	Not significant	
6A; <i>Dh31</i> ^{#51} ; <i>UAS-t-Dh31</i> /+; ZT13–ZT15	ZT1–ZT3	Not significant	Kruskal–Wallis test: $p = 0.0108$
	ZT4–ZT6	Not significant	
	ZT7–ZT9	Not significant	
	ZT10–ZT12	Not significant	
	ZT16–ZT18	Not significant	
	ZT19–ZT21	Not significant	
	ZT22–ZT24	Not significant	

rhythms and night-onset TPR, respectively (Fig. 7A,B). Together, our data suggest that locomotor activity and night-onset TPR are regulated through PDFR by different subsets of clock neurons using different neuropeptides (Fig. 7A,B). Nonetheless, because all *Dh31*, *Pdf*, and *Pdfr* mutants still maintain normal daytime TPR (Figs. 1, 2, 3), the underlying molecular mechanisms of daytime TPR are still obscure.

In humans, body temperature dramatically decreases at night, which is associated with sleep initiation (Gilbert et al., 2004; Kräuchi, 2007a,b). Although it suggests a relationship between BTR and sleep–wake cycles, the underlying mechanisms are largely unclear. A recent study suggested that DH31 mediates sleep–wake cycles and that DH31 secretion functions as a wake-promoting signal before dawn (Kunst et al., 2014) (Fig. 7C). Given that we found that DH31 is required for night-onset TPR,

DH31 could regulate both sleep–wake cycles and TPR with different timing (i.e., at night-onset for TPR and before dawn for sleep; Fig. 7B,C).

While *t-DH31* expression in DN2s rescues the *Dh31*^{#51} phenotype (Fig. 5A), *t-PDF* expression in DN2s slightly rescues in comparison with *UAS* controls (*UAS-t-Pdf*/+; Fig. 5B). This shows that *t-DH31* in DN2s rescues more efficiently than *t-PDF* in DN2s. However, DH31 stimulates PDFR less efficiently than PDF *in vitro* (Mertens et al., 2005). This suggests that either DH31 may more efficiently bind PDFR in DN2s than PDF or that DN2s may express another receptor of DH31 that also regulates night-onset TPR. Notably, although the DH31 receptor (DH31R) is a known receptor for DH31 *in vitro*, we have observed that DH31R is not expressed in DN2s and that the *Dh31r* mutant exhibited normal night-onset TPR (T. Goda, F.N. Hamada, unpublished observations). Therefore, it

Table 4. Two-way ANOVA analysis to compare the preferred temperature at ZT1–ZT3 and ZT10–ZT12 as well as ZT10–ZT12 and ZT13–ZT15

Source of variation	$F_{(DFn,DFd)}$	p value	N		Mean differences	Summary
			ZT10–ZT12	ZT13–ZT15		
Fig. 1D						
Interaction	$F_{(2,27)} = 1.3$	$p = 0.2891$				Not significant
ZT10–ZT12 versus ZT13–ZT15	$F_{(1,27)} = 8.329$	$p = 0.0076$				**
Genotype	$F_{(2,27)} = 0.7226$	$p = 0.4946$				
Sidak's multiple-comparison test						
$Pdfr^{5304}, UAS-Pdfr/+$			5	5	−0.6893	Adjusted p value not significant
$Pdfr^{5304}, Pdf> +$			6	5	−0.7892	Adjusted p value not significant
$Pdfr^{5304}, Pdf> UAS-Pdfr$			7	5	−0.1156	Adjusted p value not significant
Fig. 1D						
Interaction	$F_{(2,25)} = 0.9138$	$p = 0.4140$				Not Significant
ZT10–ZT12 versus ZT13–ZT15	$F_{(1,25)} = 35.73$	$p < 0.0001$				****
Genotype	$F_{(2,25)} = 16.27$	$p < 0.0001$				****
Sidak's multiple-comparison test						
$Pdfr^{5304}, UAS-Pdfr/+$			5	5	−0.6893	Adjusted p value*
$Pdfr^{5304}, DN1(4.5F)> +$			6	5	−1.176	Adjusted p value***
$Pdfr^{5304}, DN1(4.5F)> Pdfr$			5	5	−0.8373	Adjusted p value*
Fig. 1D						
Interaction	$F_{(2,28)} = 3.627$	$p = 0.0397$				*
ZT10–ZT12 versus ZT13–ZT15	$F_{(1,28)} = 35.62$	$p < 0.0001$				****
Genotype	$F_{(2,28)} = 5.463$	$p = 0.0099$				**
Sidak's multiple-comparison test						
$Pdfr^{5304}, UAS-Pdfr/+$			5	5	−0.6893	Adjusted p value not significant
$Pdfr^{5304}, DN2> +$			5	5	−0.7271	Adjusted p value not significant
$Pdfr^{5304}, DN2> Pdfr$			7	7	−1.703	Adjusted p value****
Fig. 3B						
Interaction	$F_{(1,88)} = 2.669$	$p = 0.1059$				Not significant
ZT1–ZT3 versus ZT10–12	$F_{(1,88)} = 15.82$	$p = 0.0001$				***
Genotype	$F_{(1,88)} = 7.163$	$p = 0.0089$				**
Sidak's multiple-comparison test						
w^{1118}			35	39	−1.149	Adjusted p value****
$Pdfr^{01}$			9	9	−0.4798	Adjusted p value not significant
Fig. 3C						
Interaction	$F_{(1,62)} = 0.8354$	$p = 0.3643$				Not significant
ZT10–ZT12 versus ZT13–ZT15	$F_{(1,62)} = 41.08$	$p < 0.0001$				****
Genotype	$F_{(1,62)} = 7.686$	$p = 0.0073$				**
Sidak's multiple comparison test						
w^{1118}			39	12	1.753	Adjusted p value****
$Pdfr^{01}$			9	6	1.315	Adjusted p value**
Fig. 3E						
Interaction	$F_{(1,89)} = 1.256$	$p = 0.2653$				Not significant
ZT1–ZT3 versus ZT10–ZT12	$F_{(1,89)} = 24.34$	$p < 0.0001$				****
Genotype	$F_{(1,89)} = 0.2533$	$p = 0.6160$				Not significant
Sidak's multiple-comparison test						
w^{1118}			35	39	−1.149	Adjusted p value****
$Dh31^{#51}$			9	10	−0.7234	Adjusted p value not significant
Fig. 3F						
Interaction	$F_{(1,62)} = 6.922$	$p = 0.0107$				*
ZT10–ZT12 versus ZT13–ZT15	$F_{(1,62)} = 26.05$	$p < 0.0001$				****
Genotype	$F_{(1,62)} = 4.468$	$p = 0.0386$				*
Sidak's multiple-comparison test						
w^{1118}			39	12	1.753	Adjusted p value****
$Dh31^{#51}$			10	5	0.5604	Adjusted p value not significant
Fig. 5A						
Interaction	$F_{(2,29)} = 1.124$	$p = 0.3389$				Not significant
ZT10–ZT12 versus ZT13–ZT15	$F_{(1,29)} = 25.14$	$p < 0.0001$				****
Genotype	$F_{(2,29)} = 0.3582$	$p = 0.7020$				Not significant
Sidak's multiple-comparison test						
$DH31^{#51}; UAS-t-Dh31/+$			5	5	−0.8157	Adjusted p value*
$DH31^{#51}; Pdf> +$			7	8	−0.5618	Adjusted p value not significant
$DH31^{#51}; Pdf> t-Dh31$			5	5	−1.183	Adjusted p value**
Fig. 5A						
Interaction	$F_{(2,42)} = 0.03909$	$p = 0.9617$				Not significant
ZT10–ZT12 versus ZT13–ZT15	$F_{(1,42)} = 9.247$	$p = 0.0041$				**
Genotype	$F_{(2,42)} = 0.6209$	$p = 0.5423$				Not significant

(Table Continues)

(Table Continues)

Table 4. Continued

Source of variation	$F_{(DFn,DFd)}$	p value	N		Mean differences	Summary
			ZT10–ZT12	ZT13–ZT15		
Sidak's multiple-comparison test						
$DH31^{#51}; UAS-t-Dh31/+$			5	5	−0.8157	Adjusted p value not significant
$DH31^{#51}; DN1(4.1M) > +$			7	21	−0.7021	Adjusted p value not significant
$DH31^{#51}; DN1(4.1M) > t-Dh31$			5	5	−0.6573	Adjusted p value not significant
Fig. 5A						
Interaction	$F_{(2,28)} = 0.803$	$p = 0.4580$				Not significant
ZT10–ZT12 versus ZT13–ZT15	$F_{(1,28)} = 24.61$	$p < 0.0001$				****
Genotype	$F_{(2,28)} = 1.457$	$p = 0.2501$				Not significant
Sidak's multiple-comparison test						
$DH31^{#51}; UAS-t-Dh31/+$			5	5	−0.8157	Adjusted p value not significant
$DH31^{#51}; DN1(4.5F) > +$			7	5	−0.6604	Adjusted p value not significant
$DH31^{#51}; DN1(4.5F) > t-Dh31$			5	7	−1.203	Adjusted p value**
Fig. 5A						
Interaction	$F_{(2,48)} = 4.433$	$p = 0.0171$				*
ZT10–ZT12 versus ZT13–ZT15	$F_{(1,48)} = 64.06$	$p < 0.0001$				****
Genotype	$F_{(2,48)} = 5.161$	$p = 0.0093$				**
Sidak's multiple-comparison test						
$DH31^{#51}; UAS-t-Dh31/+$			5	5	−0.8157	Adjusted p value*
$DH31^{#51}; DN2 > +$			16	7	−0.8666	Adjusted p value***
$DH31^{#51}; DN2 > t-Dh31$			13	8	−1.741	Adjusted p value****

would be interesting to further explore whether additional receptors for DH31 in DN2s are involved in night-onset TPR.

We previously showed that flies in the light prefer a 1°C higher temperature than in the dark (light-dependent temperature preference [LDTP]) (Head et al., 2015) (Fig. 7D), thus demonstrating that light influences temperature preference behavior. Importantly, while LDTP is only affected by light, night-onset TPR is affected by both light and time (ZT10–ZT12 and ZT13–ZT15). Therefore, they are not controlled by the same pathway (Fig. 7C,D). In fact, we showed that PDFR expression using *Clk4.5F-Gal4* rescues the abnormal LDTP phenotype of *Pdfr⁵³⁰⁴* mutants (Head et al., 2015), but it did not rescue night-onset TPR (Fig. 1D, blue).

DH31 is a functional homolog of mammalian CGRP (Johnson et al., 2005). Importantly, CGRP is related to many physiological functions, such as temperature sensation, migraines, and chronic pain in mammals (Arulmani et al., 2004; Tsujikawa et al., 2007; Benemei et al., 2009; Seybold, 2009). CGRP has recently attracted attention for its role in the treatment of migraine attacks, which are associated with body-temperature fluctuations (Durham and Vause, 2010; Ordás et al., 2013) and correlate with the time of day (Fox and Davis, 1998). Additionally, CGRP is expressed in the SCN, but its function in circadian rhythms is not entirely clear (Park et al., 1993). Thus, our findings raise the possibility that CGRP may be involved in BTR regulation and may mediate the connection between the circadian clock and other physiological functions. Moreover, this research will not only expand our

Figure 7. A–E, Schematic summaries of locomotor activity (A), night-onset TPR (B), sleep (C), light dependent temperature preference (LDTP) (D), and clock cells in the brain (E). A, PDF binds to PDFR in M and E cells to regulate locomotor activity. B, DH31 binds to PDFR in DN2s to regulate night-onset TPR. C, DH31 expression in DN1ps mediates sleep–wake cycles. D, PDFR in DN1ps are necessary and sufficient for LDTP.

current understanding of neuropeptidergic regulation, but may also ultimately facilitate a discovery of the fundamental mechanisms of BTR.

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