Circadian rhythm of temperature preference and its neural control in *Drosophila*

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Abstract

A daily body temperature rhythm (BTR) is critical for the maintenance of homeostasis in mammals. While mammals use internal energy to regulate body temperature, ectotherms typically regulate body temperature behaviorally [1]. Some ectotherms maintain homeostasis via a daily temperature preference rhythm (TPR) [2], but the underlying mechanisms are largely unknown. Here, we show that *Drosophila* exhibit a daily circadian clock dependent TPR that resembles mammalian BTR. Pacemaker neurons critical for locomotor activity are not necessary for TPR; instead, the dorsal neuron 2s (DN2s), whose function was previously unknown, is sufficient. This indicates that TPR, like BTR, is controlled independently from locomotor activity. Therefore, the mechanisms controlling temperature fluctuations in fly TPR and mammalian BTR may share parallel features. Taken together, our results reveal the existence of a novel DN2- based circadian neural circuit that specifically regulates TPR; thus, understanding the mechanisms of TPR will shed new light on the function and neural control of circadian rhythms.

Keywords

circadian rhythm; body temperature rhythm; temperature preference; period; timeless; *Drosophila*

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Contributions

F.N.H and H.K designed research. F.N.H, H.K, L.M.H and X.T performed TPR behavioral experiments. J.L and P.E performed locomotor activity behavioral experiments and *Clk9M-Gal4* staining. Y.L and P.E.H made *Clk9M-Gal4* fly lines. F.N.H, H.K and L.M.H wrote the manuscript.

RESULTS

Mammalian body temperature varies rhythmically throughout the day. This BTR contributes to their homeostasis by regulating sleep and metabolic energy usage [2–4]. While mammals control body temperature by generating heat, ectotherms use behavioral strategies to regulate body temperature [1]. Since ectotherms adapt their body temperature to their surrounding temperature, TPR is believed to be a strategy for ectotherms to achieve homeostasis [2]. For example, slugs, pill bugs, crayfish and goldfish, have been shown to exhibit TPR. Some lizards also exhibit TPR, which causes the temperature of their skin to exhibit a daily rhythm [5]. Although BTR and TPR are well-known phenomena in animal physiology, the mechanisms by which the circadian clock controls rhythms in body temperature are largely unknown in both mammals and ectotherms. Fly-based research has contributed to the discovery of many genes crucial for circadian rhythms, which has subsequently resulted in fundamental discoveries relevant to the workings of molecular clocks in several animals including mammals [6, 7]. Therefore, we sought to understand the molecular and cellular mechanisms that control TPR in *Drosophila*. As a first step, we determined whether flies exhibit TPR.

Drosophila exhibit a circadian clock dependent temperature preference rhythm

We previously showed that *Drosophila* exhibit a robust temperature preference behavior [8]. To determine if this preference is rhythmic, behavioral assays were performed at different times of day. Control fly strains w^{1118}, *Canton-S* (*cs*) and (*yw*) were raised under 12h light / 12h dark cycles (LD) at 25°C, mimicking natural day and night cycles. During each ZT (Zeitgeber Time) zone (ZT1–3, ZT4–6, ZT7–9, ZT10–12, ZT13–15, ZT16–18, ZT19–21 and ZT22–24), temperature preference behavioral assays were performed for 30 min each using a temperature gradient ranging from ~17–33 °C (Figure 1A). We found that the distribution of preferred temperature shifted from warmer to colder temperatures, and vice versa, depending on the time of day (Figure S1A–D). By plotting their average preferred temperature, we found that their preferred temperature oscillated over the course of 24 hours (ANOVA, P<0.0001) (Figure 1B). The preferred temperature gradually increased from morning (ZT 1–3) to evening (ZT10–12), and reached its peak in the evening at ZT10–12. The preferred temperature was lowest at ZT13–15 and had a second small peak at ZT19–21 (Figure 1B). Thus, we conclude that the fly displays TPR.

TPR is under clock control

To assess if TPR is clock-regulated or driven by light-dark cycles, we tested flies in "free-running" conditions in DD (constant dark) and LL (constant light) (Figure 2I). We found that w^{1118} control flies still exhibited TPR during DD day 2 (ANOVA, P=0.0004) (Figure 2A, Table S1) and DD day 4 (ANOVA, P<0.0001) (Figure 2B, Table S1). The phase of these TPR oscillations in DD was the same as under LD condition (Figures 2A and 2B). Thus, TPR is controlled by an endogenous clock. Previous studies using locomotor activity have shown that oscillator functions are abolished by day 4 in LL conditions [9, 10]. Nonetheless, we found that flies kept in LL for 4 days and 8 days still exhibited TPR (Figures 2C and D; Table S1), although the oscillations amplitude was lower on day 8 (Figure 2D and Table S1). Next, we investigated whether the essential circadian clock genes *period* (*per*) and *timeless* (*tim*) are required for TPR. We found that null mutant, tim^{01}, showed arrhythmic TPR in DD day 2 and in LL day 4 (Figures 2G; Table S1). In the null mutant per^{01}, TPR was also profoundly disrupted under DD and LL. A weak rhythm appeared to be present in LL day 4, but the preferred temperatures in each time zone were not statistically different from ZT1–3 (Figures 2E; Table S1). We therefore conclude that in LL4 also, per^{01} is arrhythmic as expected. In DD2, per^{01} showed constant low temperature preference, except at ZT22–24 (Figures 2E; Table S1). The reason for this sudden increase,

completely out of phase with the normal peak of TPR in wild-type flies, is unclear. In summary, we conclude that under constant conditions, TPR is profoundly disrupted in both *per* and *tim* null mutants, and that TPR is therefore driven by the circadian clock.

Interestingly, we also observed a "masking" effect of the LD cycle, in which per^{01} and tim^{01} flies preferred a higher temperature during the day (ZT1–12) and a lower temperature during the night (ZT13–24) (Figures 2F and H; Table S1) [11]. This shows that when the flies are exposed to light they choose a higher temperature. It is consistent with the fact that w^{1118} flies kept in LD prefer higher temperatures during the daytime than those kept in DD2 (Figure 2A) and that per^{01} and tim^{01} flies kept in LL4 prefer a higher temperature than those kept in DD2 (Figures 2E and G). Therefore, light affects the fly's temperature preference independent of the circadian clock. Importantly however, in per^{01} and tim^{01} flies, time-dependent changes in temperature preferences are completely lost during the course the day and during the course of the night, even in LD (Figures 2F and H).

The ~1–1.5 °C increase in temperature preference observed during the course of the day – which we will refer to as "daytime TPR" - is the most salient, robust and reproducible feature of clock-controlled TPR (Figures 1B and 2J). Particularly important for the studies described below, daytime TPR is highly consistent in various genetic backgrounds in LD (Figures S1E and F). In order to understand the neural mechanisms controlling circadian TPR, we therefore focused on daytime TPR.

The Morning and Evening oscillators are neither necessary nor sufficient for daytime TPR

There are ~150 central pacemaker cells in the fly brain, which are functional homologs of mammalian SCN neurons [12]. The pacemaker cells are divided into groups of lateral neurons (s-LN$_v$, l-LN$_v$, 5th-sLN$_v$, LN$_d$ and LPN) and dorsal neurons (DN1, DN2 and DN3) based on their brain location (Figure S2B) [13]. *Drosophila*'s locomotor activity has two peaks in the morning and evening. While the morning activity is controlled by M oscillators (s-LNv), the evening activity is controlled by E oscillators (5th-sLN$_v$, LN$_d$). The function of the DNs and LPNs is not yet well understood, but they appear to play a particularly important role in the detection of environmental inputs such as temperature and light [13–18]. In TPR, we found that the preferred temperature started to rise in the morning (dawn) and reached its peak in the evening (dusk) when locomotor activity is high (Figure 1), indicating that TPR is synchronized with locomotor activity and might thus be controlled by the same pacemaker neurons. Therefore, we first determined whether the Morning and Evening oscillators are necessary and/or sufficient for daytime TPR by targeting subsets of pacemaker cells with well-characterized *Gal4* lines with specific spatial expressions (Figure S2A).

During the daytime, per^{01} and tim^{01} flies constantly preferred ~26 and 26.5 °C respectively and did not exhibit daytime TPR in LD (Figures 3A and B). For locomotor activity, tissue-specific expression of PER by either *elav-Gal4* (a pan-neuronal driver) or *tim-Gal4* (a driver for all circadian cells) is known to rescue the per^{01} arrhythmic phenotype in LD and DD [19, 20]. For TPR, we found that both the *elav-* and *tim*-GAL4 drivers were able to rescue daytime TPR in per^{01} under LD conditions (Figures 3C, D and G). This result indicates that PER expression in circadian neurons is sufficient for daytime TPR.

We then tried to rescue per^{01} flies only in the M cells with *pdf-Gal4* and then in both the M and E cells with *cry13-Gal4* [19, 21–23]. *pdf-Gal4* is well known to rescue morning activity, whereas *cry13-Gal4* restores both morning and evening activities [21]. We reexamined the locomotor activity in LD and reached the same conclusion (Figure S3A, B, E and F). Surprisingly, however, neither driver was able to rescue daytime TPR under LD conditions, although *pdf-Gal4* was able to drive a weaker-amplitude rhythm with an abnormal phase

(Figures 3E, F and G). Thus, the M and E cells are not sufficient for daytime TPR. Furthermore, to determine whether Morning and Evening oscillators are necessary, we ablated them by expressing the proapoptic gene *hid* with *pdf-Gal4* and *cry13-Gal4*. We confirmed that *pdf*-neuron ablated flies exhibited loss of the morning peak of locomotor activity and advanced evening activity, whereas *cry*-neuron ablated flies exhibited loss of both the morning and evening peak of locomotor activity (Figure S3G-K), as previously reported [19, 22]. However, interestingly, both Morning oscillators ablated (*pdf-Gal4/UAS-hid*) and Morning/ Evening oscillators ablated (*cry-Gal4/UAS-hid*) flies were still able to increase their preferred temperature to similar levels as in the control flies during LD (Figure 3H). This data indicates that Morning and Evening oscillators are neither necessary nor sufficient for daytime TPR in LD.

The neuropeptide PDF is a key molecule expressed in s-LN$_v$ and l-LN$_v$ cells. Both PDF and its receptor PDF-Receptor (PDFR) are required for normal circadian locomotor behavior in LD and DD [22, 24, 25]. However, the null mutants of these genes, *pdf^{01}* and *pdfr^{Han5304}*, exhibited daytime TPR similar to those of wild type flies during LD (Figures 3H and S2C-D), which suggests that PDF and PDF-R are also dispensable for daytime TPR in LD. This result is consistent with the data in Figures 3E-H that suggested s-LN$_v$s and l-LN$_v$s cells are not required for daytime TPR.

DN2 neurons are sufficient for daytime TPR

We then determined whether a subset of dorsal pacemaker neurons might control daytime TPR. DN2 neurons are suspected to be important for temperature entrainment of circadian neurons in larvae [15]. However, the functions of the DN2s in the adult are largely unknown, although they still seem particularly sensitive to temperature cycles [16]. To analyze the functions of DN2 cells in daytime TPR, we created new *Gal4* lines by using a DNA fragment containing the first three *Clk* introns and exons 2 and 3. We found that *Clk9M-Gal4* is selectively expressed in both s-LN$_v$s and DN2s (Figures 4A and S2A). Since *pdf-Gal80* can eliminate *Gal4*-dependent expression in the s-LN$_v$s, expression in *Clk9M-Gal4::pdf-Gal80* flies is limited to the DN2 neurons (Figures 4B and S2A). By using these flies (*per^{01}; Clk9M-Gal4/+; UAS-per/+* and *per^{01}; Clk9MGal4/+;pdf-Gal80/UAS-per*), we found that PER expression in DN2 cells rescues ~46% and ~66% of the *per^{01}* mutant phenotypes for daytime TPR, respectively (Figures 4C–F). This result is consistent with the data in Figures 3E–H that *cry13-Gal4* expressing neurons are not required for daytime TPR, because *cry13-Gal4* is not expressed in the DN2 neurons (Figure S2A) [19, 23]. Thus, this result indicates that PER expression in DN2 neurons is sufficient for daytime TPR.

DN2s regulate TPR independently from circadian locomotor behavior

To determine whether the DN2s also influence circadian locomotor behavior, we tested locomotor activity using *per^{01}; Clk9M-Gal4/+; pdf-Gal80/UAS-per* flies. We found that these flies are not able to rescue the *per^{01}* locomotor activity phenotypes under either light-dark (LD) or temperature (TC) cycles (Figures S3A–D and S3L–O). These flies were also arrhythmic in DD (data not shown). Therefore, our data indicates that adult DN2 only regulates TPR, not locomotor activity. Taken together, our data indicate that locomotor activity and TPR are controlled by distinct pacemaker cells in adult flies.

DISCUSSION

Here, we show that *Drosophila* exhibit a daily TPR– low in the morning, high in the evening - that follows a similar pattern as in humans [26]. This study is not only the first demonstration of fly TPR, but also the first systematic analysis of the molecular and neural mechanisms underlying TPR. We found that TPR is controlled by the DN2s, which might

explain why temperature preference remains rhythmic in LL in Figure 2C and D. The DN2s do not express CRYPTOCHROME (CRY), a blue-light sensor crucial for circadian photoreception [27–29]. Arrhythmicity in LL is caused by constant activation of CRY and thus constant degradation of TIM [23, 30]. Therefore, CRY negative DN2 neurons may maintain residual rhythms in LL for a longer period of time than CRY positive circadian neurons. To explore this possibility, we performed immunostaining of brains with TIM-antibody and analyzed the staining levels of DN2 cells in LL 4 days. Although we found TIM signal to be weakly rhythmic in DN2 neurons, these oscillations were not statistically significant. Further studies will thus be needed to verify that DN2 neurons maintain residual rhythm in LL. Since locomotor activity is controlled by CRY positive circadian neurons and rapidly becomes arrhythmic in LL, the maintenance of TPR rhythms in LL also supports the conclusion that locomotor activity and TPR are controlled by independent circadian neural pathways.

Our data reveals striking parallel features between fly TPR and mammalian BTR, although the modes of heat production are not the same. We demonstrate here that flies exhibit robust temperature increases during the daytime, which is the same phenomenon as diurnal mammalian BTR. Furthermore, ablation studies in rats show that BTR is controlled by SCN neurons that target a different subset of subparaventricular zone neurons than those that control locomotor activity [31]. Thus, both fly TPR and mammalian BTR exhibit circadian clock dependent temperature fluctuations, independently regulated from locomotor activity. Taken together, our data raises the possibility that mammalian BTR and fly TPR are evolutionarily conserved, which may be because temperature fluctuation in an animal's body is fundamental for maintenance of its homeostasis.

Why do flies exhibit TPR? Flies probably exhibit TPR primarily to maintain homeostasis, like mammals [2]. Mammalian BTR has been shown to have a clear interaction with sleep [4], and it has been reported that mechanisms controlling fly sleep are analogous to those controlling mammalian sleep [6, 32–34]. Therefore, fly TPR may also have a relationship with sleep. Intriguingly, we did observe that PER expression limited to *pdf* neurons can generate a weak TPR with an abnormal phase in per^{01} mutants (Figure 3F), suggesting that *pdf* neurons may have a role in the TPR circuits. *pdf* positive (sLNv and lLNv) neurons regulate sleep [34] and sLNvs are known to project near the DN2s [35]. Therefore, *pdf* neurons may be able to modulate DN2 activity even when these neurons are arrhythmic, and may represent the neural basis for an interaction between TPR and sleep.

Additionally TPR may provide feedback to circadian pacemakers. Ambient temperature fluctuations can entrain not only peripheral clocks in mammals [36, 37] and flies [38] but also circadian pacemaker neurons in the fly brain, which contribute to Morning and Evening locomotor activity [14, 16, 39, 40]. Since TPR can generate temperature fluctuations in the fly body, the output of TPR may thus reinforce or refine circadian rhythm entrainment. For circadian locomotor behavior, the DN2s could actually participate in the reinforcement, since in the larval brain the DN2s help the sLNvs entraining to temperature cycles [15]. Therefore, by further exploring this newly discovered circadian rhythm, *Drosophila* TPR might not only help understanding the mechanisms underlying body temperature control in animals, but also contribute to a greater understanding of circadian rhythm's plasticity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HIGHLIGHTS

We identify a novel circadian behavior in flies, temperature preference rhythm (TPR).

Drosophila TPR follows a similar pattern as body temperature rhythm in humans.

Drosophila TPR is regulated independently from circadian locomotor activity.

TPR is controlled by a newly identified DN2- based pacemaker circuit in the brain
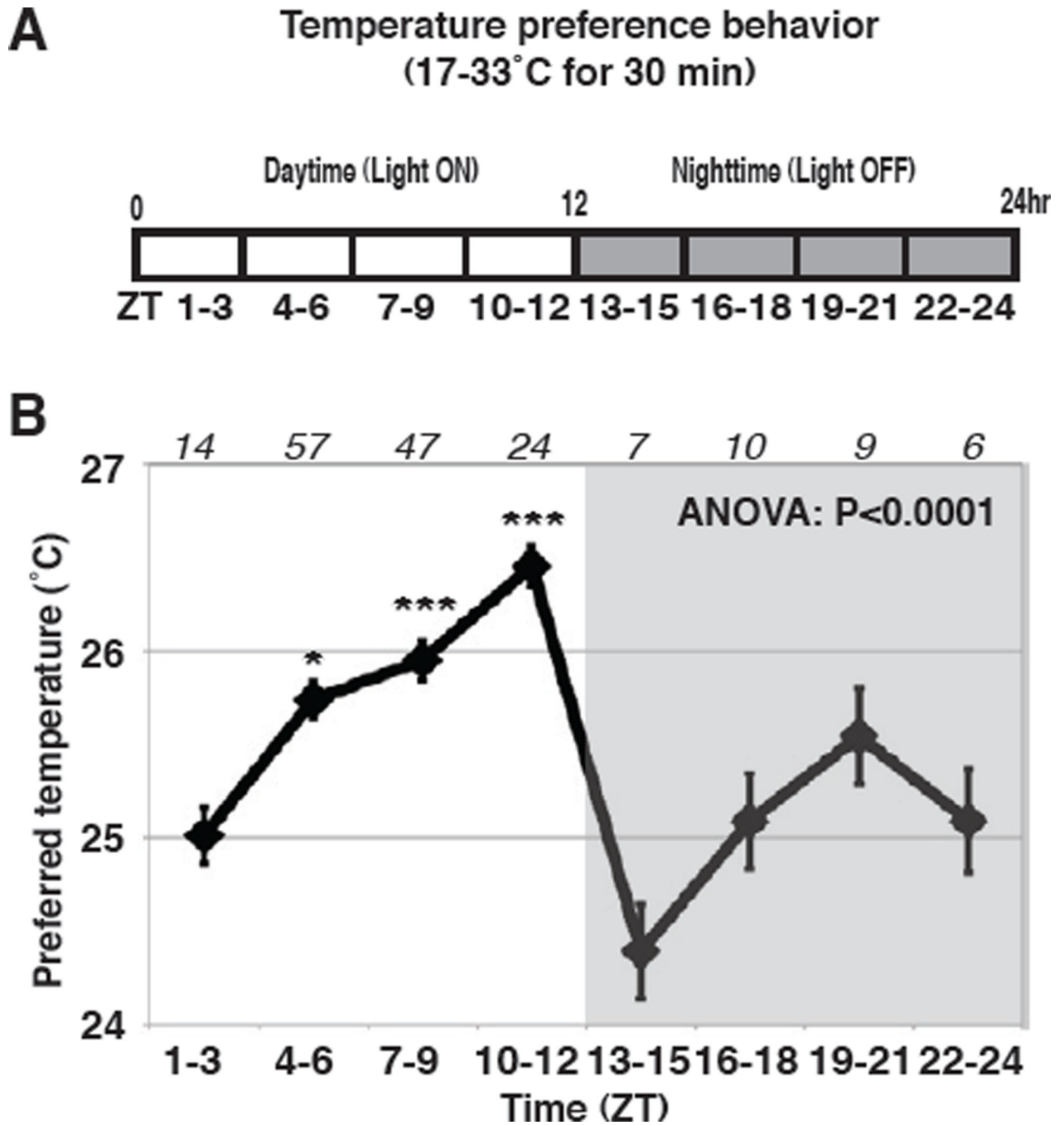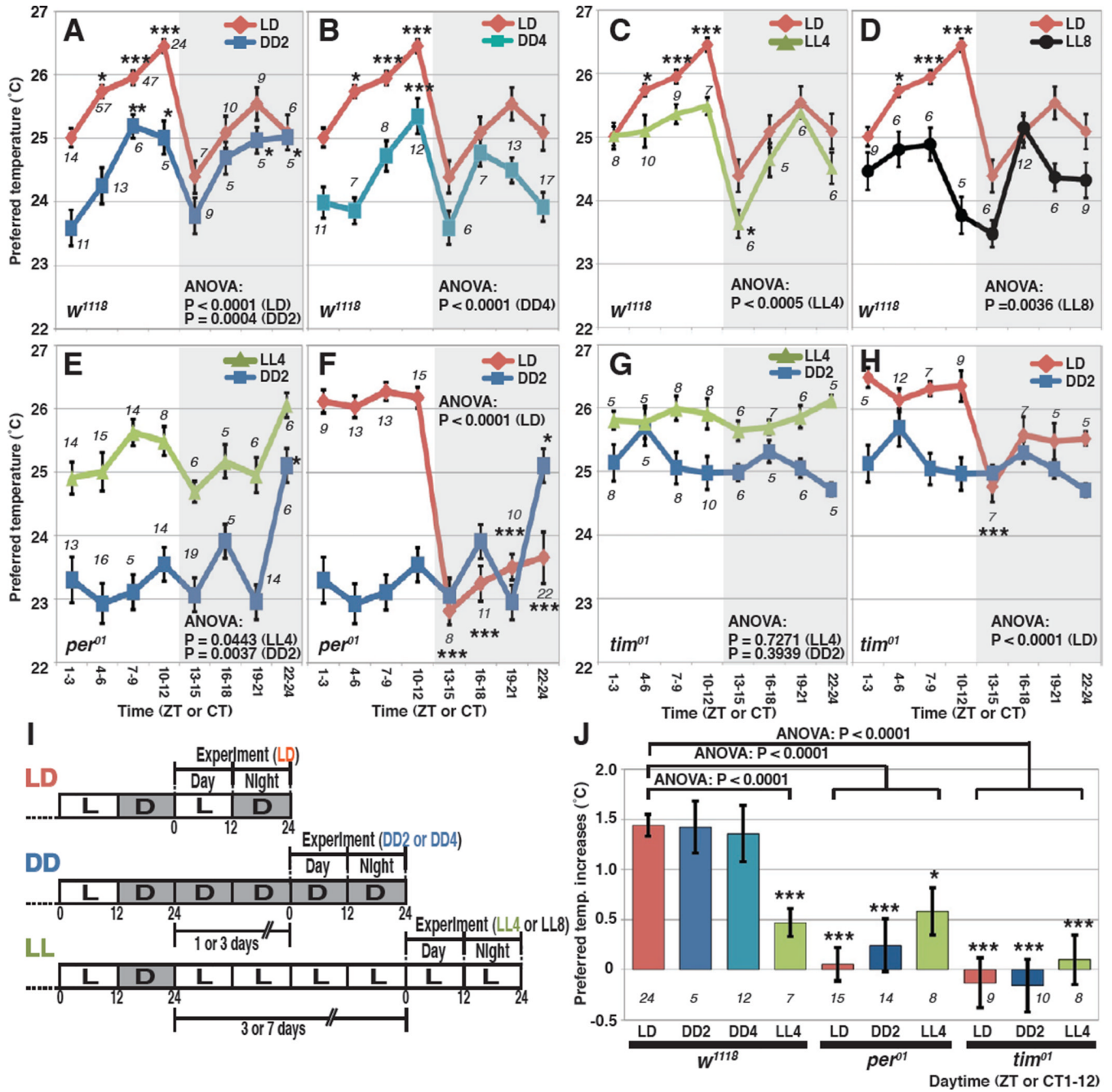
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Temperature preference behavior
(17-33°C for 30 min)

Figure 1. Fly's temperature preference is rhythmic over the course of a day
(A) Schematic of experimental condition. Temperature preference behavior assays were performed for 30 min in each of the eight different time zones (ZT 1–3, 4–6, 7–9, 10–12, 13–15, 16–18, 19–21 and 22–24). Zeitgeber Time (ZT) (12h light/dark cycle; ZT0 is lightson, ZT12 is lights-off). (B) TPR of w^{1118} flies over 24 hrs. Preferred temperatures were calculated using the distribution of flies in temperature preference behavior (Figure S1). Data are shown as the mean preferred temperature in each time zone. Numbers represent the number of assays. ANOVA, P<0.0001. Tukey-Kramer test compared to ZT1–3, ***P<0.001, **P<0.01 or *P<0.05. By Tukey-Kramer test, compared to ZT13–15, the

preferred temperature at ZT4–6, 7–9, 10–12 (P<0.001) and ZT19–21 (P<0.05) were statistically significant (see Table S1).

Figure 2. The fly's TPR is circadian clock-dependent

(A–H) Comparison of TPR over the course of 24 hours in w^{1118}, per^{01} and tim^{01} flies kept in LD (red lines), DD2 (blue lines), DD4 (light blue lines), LL4 (green lines) and LL8 (black lines). w^{1118} flies kept in LD and DD2 (A), LD and DD4 (B), LD and LL4 (C) and LD and LL8 (D). The same LD data from Figure 1B were used in A-D. per^{01} flies kept in LL4 and DD2 (E), and LD and DD2 (F). The same DD2 data for per^{01} were used for the comparison in E–F. tim^{01} flies kept in LL4 and DD2 (G), and LD and DD2 (H). The same DD2 data for tim^{01} were used for the comparison in G–H. Numbers represent the number of assays. CT is Circadian Time in DD or LL. Tukey-Kramer test comparison to ZT1–3, ***P<0.001, **P<0.01 or *P<0.05. More detailed statistics are shown in Table S1.

(**I**) Schematic of experimental conditions. w^{1118} flies were raised under LD conditions and were transferred to DD or LL conditions. Then, TPR assays were performed over the course of 24 hours in LD, DD2, DD4, LL4 or LL8.

(**J**) The bar graph shows daytime temperature increases (ZT or CT 1–12) for w^{1118}, per^{01} and tim^{01} in the daytime TPR in LD (red), DD2 (blue), DD4 (light blue) and LL4 (green) conditions. The value of temperature increases during the daytime was calculated by subtracting the preferred temperature at ZT or CT1-3 from the preferred temperature at ZT or CT10–12. ANOVA was performed on the following groups; w^{1118} (LD, DD2, DD4 and LL4); per^{01} (LD, DD2 and LL4) *and* w^{1118} (LD); and tim^{01} (LD, DD2 and LL4) *and* w^{1118} (LD). Tukey-Kramer test comparison to w^{1118} in LD.
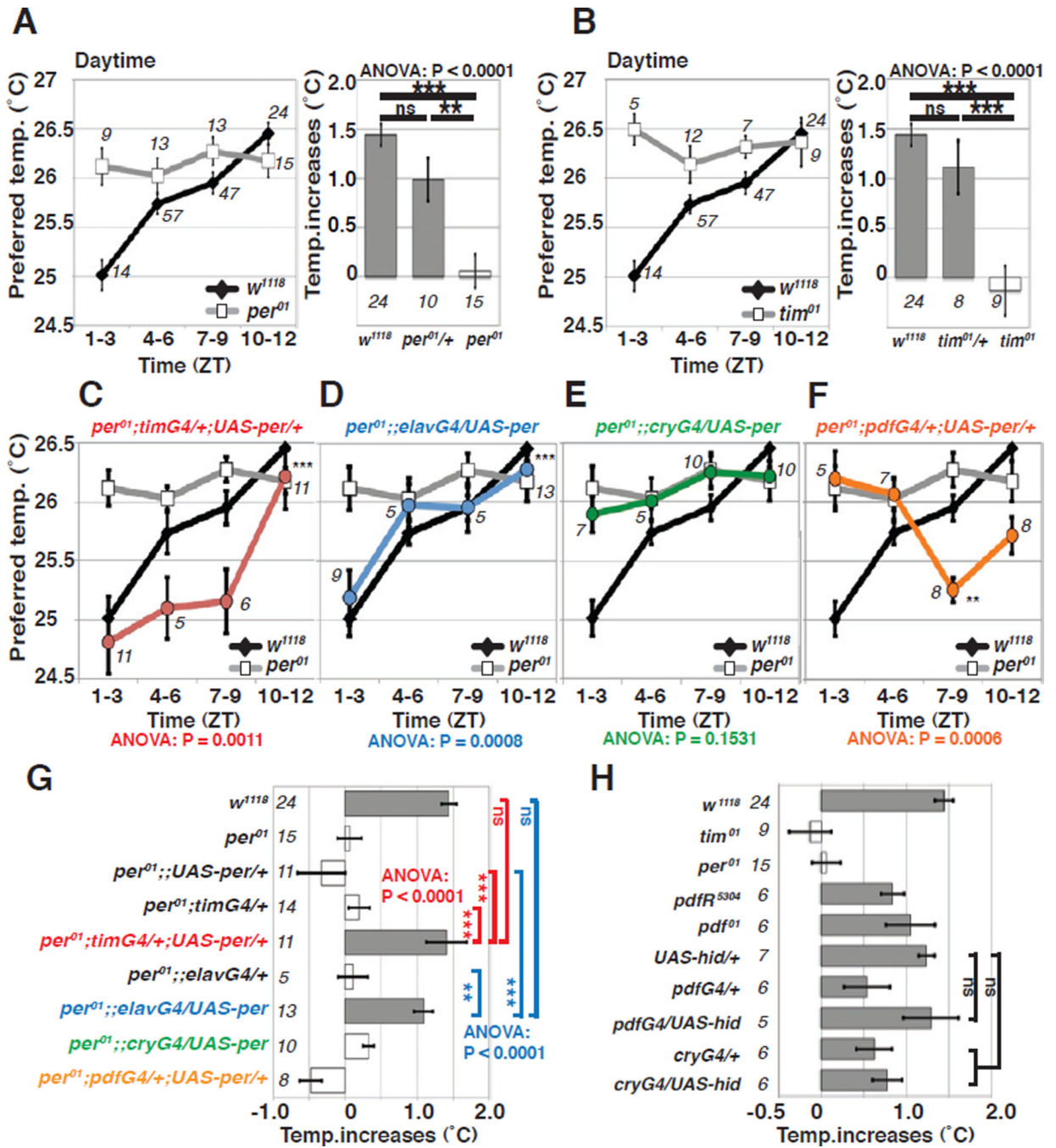
Figure 3. The Morning and Evening oscillators are not necessary for daytime TPR in LD
(A–B) The line graphs show preferred temperatures of w^{1118} and null mutants per^{01} (A), and w^{1118} and null mutants tim^{01} (B) during the daytime (ANOVA, P=0.77 (per^{01}), P=0.67 (tim^{01})). The bar graphs show daytime TPR (ZT1–12) in LD condition for each genotype. The same LD daytime data from Figures 1B, 2F and 2H were used for w^{1118}, per^{01} and tim^{0}, respectively. Tukey-Kramer test comparison to w^{1118}, ***P<0.001, **P<0.01 or *P<0.05. (C–F) The line graphs show preferred temperatures of w^{1118} and rescue fly lines in the null mutant per^{01} background during the daytime in LD. PER expression using tim-Gal4 (C, red) and $elav$-Gal4 (D, blue) are sufficient to rescue the per^{01} mutant phenotype for daytime TPR

(ZT1–12). PER expression using *cry13-Gal4* (E, green) and *pdf-Gal4* (F, orange) are not sufficient to rescue the *per^{01}* mutant phenotype for daytime TPR in LD.

(G) The bar graph shows the daytime TPR (ZT1–12) in LD for each genotype shown in Figure 3A, C–F. The Morning and Evening oscillators are not sufficient. ANOVA was performed on the following groups; *w^{1118}*, *per^{01};;UAS-per/+*, *per^{01};timG4/+*, and *per^{01};timG4/+;UAS-per/+* (red); as well as *w^{1118}*, *per^{01};;UAS-per/+*, *per^{01};;elavG4/+* and *per^{01};;elavG4/UAS-per* (blue). Tukey-Kramer test comparison to each control fly line.

(H) The Morning and Evening oscillators are not necessary. The bar graph shows the daytime TPR (ZT1–12) in LD of flies with ablated Morning and/or Evening oscillator cells. The flies were still able to increase their preferred temperature to similar levels as the control flies during LD. The patterns of the TPR of *pdf^{01}* and *pdfR^{han5304}* are shown in Figures S2C–D.
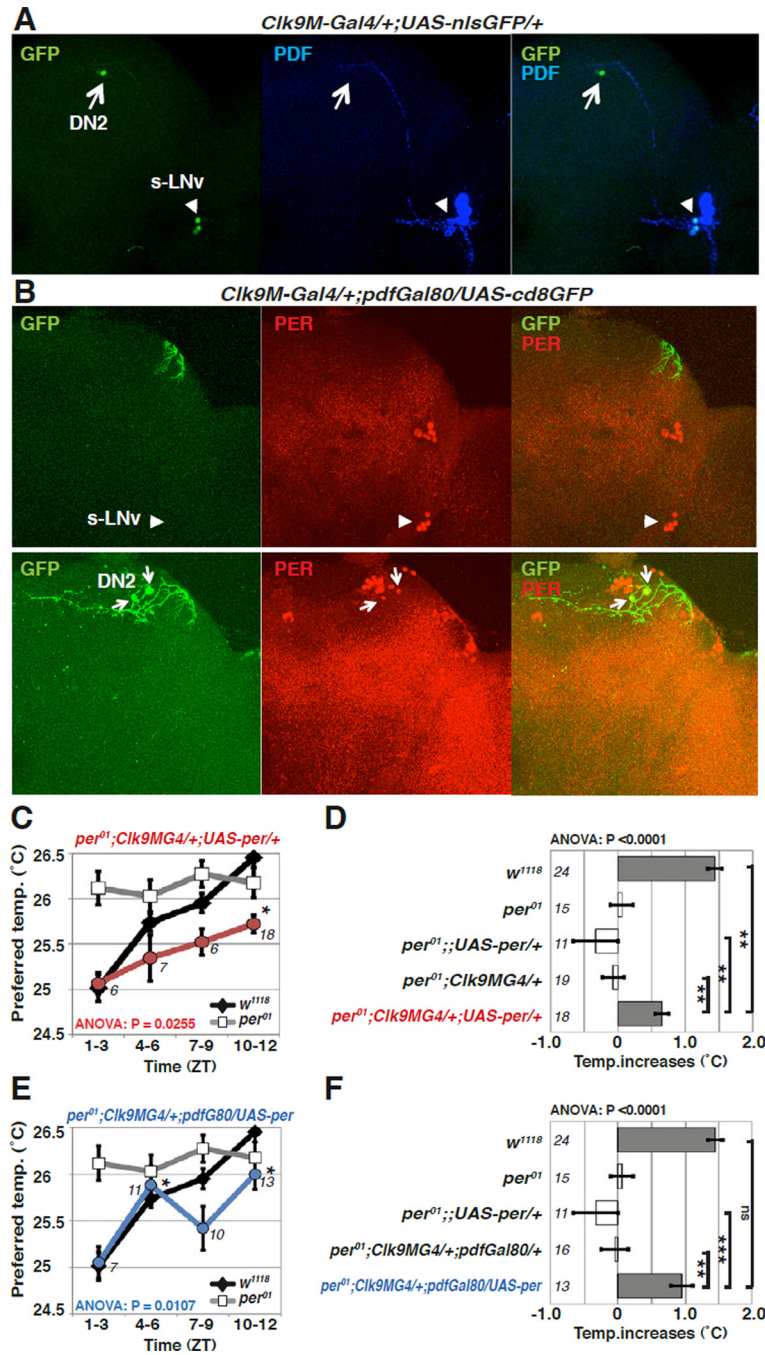
Figure 4. DN2 neurons are sufficient to generate daytime TPR in LD

(**A-B**) *Clk9M-Gal4; pdf-Gal80* is selectively expressed in DN2s. (**A**)*Clk9M-Gal4::UAS-nlsGFP* (nuclear GFP) flies were stained with anti-GFP (green) and anti-PDF (blue). The PDF antibody labels the s-LNv (arrowhead) and l-LNvs neurons. *Clk9M-Gal4* labels both DN2 (arrow) and s-LNv neurons (arrowhead). 2 DN2 cells are observed in *Clk9MGal4::UAS-nGFP* flies, but GFP staining was frequently weaker in one of them. (**B**) *Clk9M-Gal4, pdf-Gal80/ UAS-cd8GFP* flies are stained with anti-GFP and anti-PER. The PER antibody labels circadian pacemaker cells in the brain. *pdf-Gal80* eliminates the expression in s-LNv neurons. *Clk9M-Gal4, pdf-Gal80/ UAS-cd8GFP* is selectively

expressed in the DN2 neurons. Upper and lower pictures show different Z-stacks of the same brain.

(**C–F**) PER expression in DN2 neurons is sufficient to rescue daytime TPR of *per^{01}* in LD. The line graphs (**C**, **E**) show preferred temperatures of *w^{1118}* and rescue fly lines in the null mutant *per^{01}* background during the daytime. The same LD daytime data from Figures 1B, and 2F were used for *w^{1118}* and *per^{01},* respectively. PER expression in *Clk9M-Gal4, pdf-Gal80* (**E**, blue) is sufficient to rescue the *per^{01}* mutant phenotype for daytime TPR (ZT1–12), while PER expression in *Clk9M-Gal4* (**C**, red) partially rescues it. The bar graphs (**D**, **F**) show the daytime TPR (ZT1–12) for each genotype. ANOVA was performed on the following groups; *w^{1118}*, *per^{01};;UAS-per/+*, *per^{01}; Clk9M-Gal4/+*, and *per^{01}; Clk9M-Gal4/+;UAS-per/+* (**D**), as well as *w^{1118}*, *per^{01};;UAS-per/+*, *per^{01}; Clk9MGal4/+; pdf-Gal80/+*, and *per^{01}; Clk9M-Gal4/+; pdf-Gal80/UAS-per* (**F**), Tukey-Kramer test comparison to ZT1–3 (**C, E**) and each control fly line (**D, F**). ***P<0.001, **P<0.01 or *P<0.05.