# A temperature-dependent molecular switch to adjust behavior to changing environmental conditions

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**Summary**

Circadian clocks increase fitness of organisms by adapting physiological and behavioral rhythms to the daily changes of light and temperature, caused by the Earth' 24-hr rotation around its own axis. They consist of self-sustained molecular oscillators, maintained by negative feedback regulation of several clock genes, including *period* (*per*) and *timeless* (*tim*) in the fruit fly *Drosophila melanogaster*. This molecular clock is synchronized by light:dark and temperature cycles (Zeitgeber), and in turn drives rhythmic biological outputs, like the daily locomotor activity rhythms. While light generally is considered to be the more dominant, daily temperature cycles are sufficient for stable circadian clock synchronization. In *Drosophila* constant light leads to break down of the molecular clock and arrhythmic behavior, but clock function can be restored by simultaneously exposing the flies to temperature cycles, indicating their particular importance in regions experiencing long photoperiods or constant light. Here, we reveal that during temperature cycles, the deleterious effects of constant light on the clock are avoided by repressing the activity of the photoreceptor CRYPTOCHROME (CRY), which normally leads to light-dependent degradation of TIM. We show that CRY levels are repressed by Gq-PLC-ß signaling, operating within central clock neurons, thereby stabilizing TIM and promoting clock function during constant light and temperature cycles. Consistent with these findings, we reveal that a recently evolved and less light sensitive form of TIM, does not require Gq-PLC-ß signaling for maintaining clock function during constant light and temperature cycles. In summary, our results supply the molecular explanation for temperature synchronization in constant light, and how fruit flies can maintain clock function and rhythmic behavior in northern latitudes.

## Introduction

Like most organisms, *Drosophila melanogaster* rely on their endogenous circadian regulate rhythmic physiological and behavioral outputs. This timer is equipped with two core clock proteins CLOCK(CLK) and CYCLE(CYC)to activate the transcription of the clock genes *period* (*per*) and *timeless* (*tim*). The translated PER and TIM proteins then terminateMi their own transcription through negative feedback [1]. This transcription/translation feedback loop, constitutes the molecular oscillator of the biological clock, which runs with a period of approximately 24 hours, even in absence of environment cues. On the other hand, this robust timing system interacts with the environment and resets itself by daily cues like fluctuating light and temperature (so called 'Zeitgeber'). The main circadian photoreceptor CRYPTOCHROME (CRY), is a blue light photoreceptor expressed in the *Drosophila* eye as well as in subsets of the clock neurons, which are composed of about 75 neurons expressing core clock genes in each brain hemisphere [2–4]. This central pacemaker contains 7 anatomically well-defined clusters: 3 groups of dorsal neurons (DN1-3), the lateral posterior neurons (LPN), the dorsal lateral neurons (LNd) and the large and small ventral lateral neurons (l- and s-LNv). Together, they orchestrate timing of the locomotor activity patterns with external light and temperature fluctuations. When flies are exposed to light, CRY is activated and binds to TIM and the F-box protein JETLAG (JET), triggering TIM and CRY degradation in the proteasome to reset the clock network [5–8]. Therefore, exposure of flies to constant light (LL) leads to arrhythmicity, due to the constitutive degradation of TIM in clock neurons, mediated by CRY [9,10]. Another important Zeitgeber to synchronize circadian rhythms is temperature. In mammals, temperature cycles (TC) with an amplitude of 1.5°C induce robust circadian gene expression in cultured tissues [11]. Moreover, the daily fluctuation of body

temperature (36°C -38.5°C) generated by the suprachiasmatic nucleus (SCN) is employed to enhance internal circadian synchronization [12]. In *Drosophila*, unlike cell autonomous light resetting by CRY, clock neurons receive temperature signals from peripheral thermo sensory organs including the aristae and mechanosensory chordotonal organs [13–15]. Interestingly, robust molecular and behavioral entrainment to temperature cycles was observed under LL [16,17], suggesting that cycling temperature can somehow rescue clock neurons from the effects of constant light.

*norpA* encodes Phospholipase C-ß (PLC-ß), a key component of the canonical phototransduction cascade. After rhodopsin-mediated activation of the heterotrimeric G-protein Gq, NORPA catalyzes the hydrolysis of PIP2 to generate IP3 and DAG, a crucial step for depolarizing the photoreceptor neurons by opening of the TRP and TRPL channels (reviewed by [18]. Loss of function *norpA* mutations block this pathway and cause visual blindness [19]. It's been shown that *norpA* also contributes to circadian entrainment by light [3,20–22]. In addition, *norpA* plays an important role in temperature sensation. In different stages of *Drosophila* larval development, *norpA* functions downstream of Rhodopsins [23,24], to regulate TRPA1-dependent temperature preference behavior [25]. *norpA* also contributes to seasonal adaptation by temperature-dependent modulation of *per* splicing [26,27]. Most likely this mechanism is not relevant for temperature entrainment, as daily changes in *per* splicing dynamics are not observed during temperature cycles [16,28].

*norpA* mutants impact molecular and behavioral synchronization to temperature cycles in LL [16]. However, the molecular mechanisms of temperature entrainment in LL mediated by *norpA* remain to be determined. Here we show that *norpA* is specifically required for light-dependent

(LL) temperature synchronization, but not during constant darkness (DD). Further, immunostaining reveals that *norpA* is required for TIM cycling in clock neurons in LL and temperature cycles. We show that other canonical photo-transduction components such as Gq also involved in LL-temperature entrainment. Spatially restricted rescue of *norpA* expression restores temperature synchronization defects of *norpA* mutants, indicating that this PLC-ß functions within clock neurons. Finally, we show that NORPA inhibits CRY in subsets of $CRY^+$ neurons to rescue TIM level during LL and temperature cycles. In summary, our work reveals that a novel 'thermo transduction' pathway, involving Gq and NORPA, and repressing the activity of CRY, is required for temperature synchronization in LL.

## Results

### *norpA* is required for synchronization to temperature cycles in constant light.

We previously reported that Phospholipase C-ß (PLC-ß) encoded by the *norpA* gene mediates temperature synchronization in constant light (12 h 25°C : 12 hr 18°C temperature cycle in LL, [16]). To test whether *norpA* is also involved in temperature synchronization during constant darkness (DD), we exposed two loss-of-function *norpA* alleles ($norpA^{P24}$ and $norpA^{P41}$) as well as wild type flies (*y w*; *s-tim*) to a 12 h 25°C : 12 h 16°C temperature cycle (TC) for 8 eight days after initial synchronization to light:dark cycles (LD 25°C, Figure S1). The temperature cycle was phase-advanced by 6 hours compared to previous the light-dark cycles in order to better visualize synchronization. As expected, control flies synchronized their activity to the LD cycle and showed the typical morning and evening bouts of activity, separated by a period of inactivity during the

day (siesta) and night [29]. During TC, the flies slowly adjusted their main activity peak to occur in the middle of the warm the phase, similar as observed before under these conditions (e.g., [30,31]). As expected, both *norpA*$^{P24}$ and *norpA*$^{P41}$ mutant flies synchronized normally to LD (Figure S1). Interestingly, during TC they are not constantly active during the warm phase as reported for LL and TC [16], but instead adjust their main activity peak to the beginning of the warm phase (Figure S1). Both control and *norpA* mutant flies show a startle response to the sudden temperature increase, but the additional activity peak in *norpA* mutants can clearly be distinguished from the startle response in the histograms. This indicates that the *norpA* mutants do synchronize to TC, and that only the phase of the activity peak occurs several hours earlier compared to the controls. Synchronization of the *norpA* mutants is also indicated by the phase of the activity during the final free-run portion of the experiment (DD and constant 25°C), which is aligned with the activity of the last day during TC (Figure S1). Moreover, both *norpA* mutants showed normal rhythmicity and period-lengths' in DD at constant 25°C after TC (Figure S1B-D). These results show that *norpA* mutants can synchronize to TC in DD and indicate that more sever synchronization defects depend on the presence of LL.

To test this idea, we repeated the experiments in LL and TC. Flies were initially exposed to LL and constant temperature (25°C), which elicits arrhythmic behavior in wild type and *norpA* mutant flies [9,32] (Figure 1 A). After 2 days, flies were exposed to LL and TC for 8 days, followed by another LL TC cycle that was phase advanced by 6 hr compared to the first one (Figure 1). Control flies exhibit the typical phase of their main activity peak towards the end of the warm phase as previously reported (e.g. [16,31]). In contrast, both *norp*A mutants failed to show robust peaks but only exhibited constant high activity in the warm phase (Figure 1A). To confirm the defects

observed in mutants are due to the lack of PLC-ß, we performed genetic rescue experiments using the Gal4/UAS system [33]. Indeed, a wild-type behavioral pattern under LL TC was restored in *norpA*$^{P41}$ mutant flies expressing an *UAS-norpA* cDNA in all clock cells driven by *timeless-Gal4* (Figure 1B).

To quantify the synchronization defects, we calculated an 'entrainment index (EI)' by determining the ratio between the overall activity during the activity peak in *y w* flies and the total activity of the entire warm phase ([31], Experimental Procedures). Consistent with the visual inspection of the behavior described above, the EI's of *norpA* mutant flies were significantly lower compared to the wild type and rescue flies in LL TC (Fig1C). These results demonstrate that *norpA* is required for temperature synchronization in LL.

**_norpA_ is required for synchronized TIM expression in clock neurons during temperature cycles in constant light**

To understand whether the observed temperature synchronization defects in *norpA* mutants are caused by altered clock protein expression, we quantified TIM levels in the different clusters of clock neurons at different time points during LLTC. TIM expression was rhythmic and synchronized within all the groups of clock neurons of control flies with a peak at ZT9 (Figure 2A). This is a somewhat surprising, because peak expression times for TIM and PER usually occur during the cold phase during TC (e.g. [13,34]), but conditions were always considerably different from the ones used in the current study (i.e., DD and/or different temperatures). Nevertheless, and because we restricted our analysis to 4 time points (ZT4, ZT9, ZT16, ZT22), it is possible that

the peak of TIM expression during LL 16°C : 25°C occurs at a later time-point that was not represented in our time course (e.g., ZT14). Unlike wild type, TIM was at very low levels at all time points in all clock neurons of *norpA*$^{P41}$ mutants, except for the l-LNvs, which showed different peak expression at ZT21 instead of ZT9 in control flies (Figure 2A). TIM and PER oscillations in l-LNv were also observed in arrhythmic wild type flies during LL and constant temperature [7], suggesting that these remaining oscillations in the l-LNv of *norpA*$^{P41}$ mutants are not induced by temperature cycles. We also performed antibody staining using the same flies exposed to DDTC. TIM signals were quantified at ZT4 and ZT20, which are known trough and peak times of TIM expression in these conditions [34]. Consistent with our behavioral data, TIM oscillations in clock neurons of the *norpA*$^{P41}$ mutants is normal in DDTC, except for the DN3, which exhibit slightly lower levels at the peak time of expression (Figure 2B). Therefore, in agreement with our behavioral results, *norpA* is required for temperature synchronization of clock neurons in constant light.

**Temperature synchronization defects of *norpA* mutants can be rescued by stabilizing TIM**

Two natural *tim* alleles, *s-tim* and *ls-tim*, exist, encoding TIM isoforms with different light-dependent protein stability. While *s-tim* flies encode the N-terminally truncated SHORT-TIM (S-TIM) protein, *ls-tim* encodes both for S-TIM and LONG-TIM (L-TIM) [35]. Due to a stronger light-dependent interaction with CRY, light-dependent degradation of S-TIM is more efficient compared to that of L-TIM protein [8,36]. As a consequence *s-tim* flies are more sensitive to light compared to *ls-tim* flies [7,36,37]. Because the *norpA*-induced temperature synchronization defects

are light dependent and the original *norpA*$^{P41}$ allele we used was associated with the *s-tim* allele, we asked if introducing *ls-tim* into the *norpA* mutant background would restore temperature synchronization in LL. We therefore analyzed the behavior of control *ls-tim* and *norpA*$^{P41}$ *ls-tim* flies in LL TC using the assay described in Figure 1. As predicted, control *y w ls-tim* flies behaved similar to the *y w s-tim* flies, apart from a more narrow activity peak observed in *ls-tim* flies (compare Figure 3A left, with Figure 1A left). Introducing one copy of *ls-tim* is not sufficient restore temperature synchronization in *norpA* mutants by TC (Figure 3B). In contrast, both *norpA*$^{P41}$ and *norpA*$^{P24}$ flies homozygous for *ls-tim* exhibited a pronounced activity peak in the warm phase with a similar phase as *y w ls-tim* flies (Figure 3A). EI calculation confirmed that introducing the *ls-tim* allele lead to a significant improvement of temperature synchronization in both *norpA* mutant alleles, comparable to wild type controls in *s-tim* background (Figure 3B).

To see if the *ls-tim* allele also influenced molecular oscillations in the clock neurons, we performed anti-TIM stainings in control *ls-tim* and *norpA*$^{P41}$ *ls-tim* flies during LL TC and compared the results to the same genotypes in an *s-tim* background (Figure 3C). All clock neuronal groups analyzed in control *ls-tim* flies showed oscillating TIM levels with a peak around ZT16, 6 hr later compared to the peak in *s-tim* control flies (Figure 3C, Figure S4A-B). This delay is consistent with the behavioral results, as the activity peak of *y w; ls-tim* occurs about 3-4 hr later compared to that of the *y w; s-tim* flies (Figure S4C). The difference is most likely due to reduced light-sensitivity of *ls-tim* (see Discussion). Intriguingly, TIM cycling was largely restored in clock neurons of *norpA*$^{P41}$ *ls-tim* with a peak around ZT9 (DN1-3) or ZT16 (LNd and s-LNvs). These data indicate that TIM is the target of PLC-ß and CRY-mediated degradation during LL and TC.

Consistent with the light-dependent effects of *norpA* mutants on temperature synchronization, we did not observe any alterations on the free-running period of locomotor activity at different constant temperatures (18°C, 25°C, and 29°C) in DD, demonstrating that *norpA* is not required for temperature compensation (Figure S3).

**Factors of the canonical phototransduction cascade are required for temperature synchronization in constant light.**

PLC-ß is a key enzyme in the canonical Rhodopsin phototransduction cascade, generating the second messenger molecules DAG and IP3 through $PIP_2$ hydrolysis, after Gq-activation by Rhodopsin [18]. Moreover, Rhodopsin, Gq, and PLC-ß have recently been implicated to regulate temperature preference behavior in *Drosophila* larvae (see Introduction). We therefore asked if in addition to PLC-ß other components of the phototransduction cascade contribute to temperature synchronization in LL. We first analyzed the potential effects of the severe hypomorphic allele $Gq^1$ [38] on temperature synchronization in LL. Similar to what we observed for *norpA s-tim* mutants, $Gq^1$ *s-tim* flies largely failed to show a distinct activity peak, particularly in the final TC (Figure 4A). As with *norpA* mutants, introducing the *ls-tim* background fully restored temperature synchronization in $Gq^1$ mutants (Figure 4 A, B). To see, if the involvement of phototransduction molecules also extends to the photo receptor Rhodopsin, we also analyzed a loss of function allele ($ninaE^{17}$) of the *ninaE* gene, which encodes the major Rhodopsin1, expressed in the R1-R6 photoreceptor cells. Although the overall activity of $ninaE^{17}$ *s-tim* flies is drastically reduced during LL TC compared to wild type flies and the other mutant alleles, they

do show an organized activity peak in the 2nd half of the warm phase. Changing the genetic background to *ls-tim* lead to increased overall activity levels and a more pronounced activity peak extending from the 2nd half of the warm phase into the beginning of the cold phase (Figure 4 A, B). Although Rhodopsin1 has been shown to be required for temperature discrimination between 18°C and 24°C in *Drosophila* larvae [23], based on the clear activity peak observed in *ninaE*$^{17}$ *s-tim* flies we conclude that Rhodopsin1 is not required for temperature synchronization in constant light.

**_norpA_ expression in Cryptochrome expressing cells contributes to temperature synchronization in constant light.**

*norpA* is predominantly expressed in photoreceptors, including the adult Hofbauer-Buchner eyelet, which also contacts circadian clock neurons [39,40]. In addition, less abundant *norpA* expression has been observed in neurons of the optic lobes and central brain [41] and recently in l-LNv clock neurons [42]. In an attempt to determine which *norpA* expressing cells contribute to temperature synchronization in LL, we performed a set of rescue experiments restoring *norpA* function in different tissues using the Gal4/UAS system. As already shown in Figure1, expression of *UAS-norpA* in all clock cells using the *tim-gal4* driver restored temperature synchronization in *norpA*$^{P41}$ mutant flies. However, in addition to the clock neurons that drive the behavioral rhythms, *tim* is expressed in all photoreceptor cells, and many glia cells throughout the brain. Likewise, *cry* is widely expressed in the fly head, including about 50% of the central clock neurons and all photoreceptors [4]. As expected from this and the *tim-gal4* result, *norpA* rescue with *cry-*

*gal4:39 (Gentile et al., 2013b)*also resulted in wild type behavior during LL TC (Figure 5A, B). Interestingly, expression of *norpA* either in all clock neurons (*Clk856-gal4*) or LNvs (*Pdf-gal4*) could also restore temperature synchronization in LL (Figure 5A). Clear activity peaks could be discerned in the actograms and histograms of the *Clk856-gal4* and *Pdf-gal4* rescue flies, although calculation of the EI indicate a less robust rescue with *Pdf-gal4*, compared to the *tim-gal4* and *cry-gal4* rescue (Figure 5B). In addition, expression of *norpA* in a subset of DN1 (*Clk4.1-gal4*) fails to clearly rescue temperature synchronization in LL, resulting in no significant EI difference compared to *norpA* mutants. This is in agreement with a previous study, showing that lateral ventral clock neurons but not dorsal neurons mediate synchronization to 16°C : 25°C temperature cycles in LL [31].

Our behavioral data above predicts that *norpA* is expressed in other clock neurons apart from the l-LNv. To validate the expression of *norpA* in clock neurons, we performed gene expression analysis using single cell RNAseq data from the *Drosophila* brain [43]. The photoreceptor and DN1 cluster were previously annotated [43], we also define a LNv cluster based on high expression of *Pdf* (Experimental Procedures, Figure 6 bottom). As expected, *rh1-4* are enriched only in the photoreceptor cluster, Figure 6 upper panel) whereas the core clock genes *tim* and *per,* as well as *cry* are expressed in both clock (LNv and DN1) and photoreceptor neuronal clusters as reported (add references) (Figure 6 middle panel). In contrast, the *Pdf receptor* (*Pdfr*) gene, which is expressed in LNv and DN1 clock neurons [44], was also enriched in these clusters according to the singe cell RNAseq data (Figure 6, middle right), demonstrating the validity of this approach. Consistent with our behavioral results, *rh1* is not detectable in LNv and DN1 clusters. However, in addition to photoreceptors, *norpA* and *Gq* are also expressed in both clock neuronal clusters

(Figure 6 bottom panel), supporting our behavioral results. Although we could not identify a prominent role for the DN1 cluster in synchronization to 16° : 25°C TC in LL, *norpA* and *Gq* may be involved in other temperature responses of this cluster (see recent review by [45]).

**Removal of CRY rescues temperature synchronization defects of *norpA* mutants in constant light**

While CRY is not required for temperature synchronization, *cry* mutations enhance synchronization both at the behavioral and molecular level [31]. We therefore reasoned that the extremely low levels of TIM in clock neurons of $norpA^{P41}$ mutants, may result from increased CRY levels or function, which could then explain the light-dependence of the $norpA^{P41}$ temperature synchronization defects. To test this idea, we analyzed the locomotor activity pattern of (*s-tim*) $norpA^{P41}$ $cry^{b}$ double mutants in LL TC. Indeed, removing functional CRY restored the ability of *norpA* mutants to synchronize to temperature cycles (Figure 7 A, B). To confirm that the rescue is not due to genetic background effects, we repeated the same experiment with trans-heterozygous $cry^{b}/cry^{out}$ mutants, and obtained the same result (Figure 7A, B).

Consistent with the behavioral results, removal of CRY in $norpA^{P41}$ mutants significantly restored TIM levels and oscillations in all of the clock neurons compared to $norpA^{P41}$ single mutants (Figure 7 C). Interestingly, except for the CRY-negative DN2 and DN3, TIM levels in the double mutants were significantly increased compared to control *s-tim* flies and remained high, or even increased, after ZT9 (Figure 7 C). The results suggest that the activity or presence of PLC-ß inhibits the function or levels of CRY, thereby regulating TIM oscillations during LL TC. Moreover, the fact that

TIM levels drastically decrease during the 2nd part of the cold phase in the *norpA*$^{P41}$ *cry*$^{b}$ double mutants, suggests the existence of a CRY-independent TIM degradation mechanism in LLTC (cf. [46,47]).

**_norpA_ regulates CRY levels in clock neurons during temperature cycles in constant light**

To investigate the potential role of *norpA* in regulating CRY, we determined the CRY levels in clock neurons of the wild type and *norpA*$^{P41}$ mutant flies. CRY is expressed in a subset of clock neurons, including the DN1, LNd, and all LNv [4]. To evaluate the specificity of the anti-CRY antibody, we first tested CRY levels at two different time points in LD and constant temperature in *s-tim* control, and *cry*$^{02}$ flies. As expected, we detected CRY expression in 3 LNd and all l-LNv and s-LNvs at ZT22, but only very weak signals at ZT1, and no expression in *cry*$^{02}$ brains (Figure 8A, B). We also saw weak expression of CRY in subsets of DN1 at ZT22 (Figure 8 A). Next, we asked whether *norpA* may inhibit CRY expression in clock neurons during LLTC. We exposed wild type as well as *norpA*$^{P41}$ mutant flies to LLTC for 6 days and performed anti-CRY immunostaining at ZT1 on the 7th day. As expected, we did not detect any significant CRY signals in clock neurons of wild type flies (Figure 8 D, F). In contrast, CRY expression was readily detectable in LNd and LNv of *norpA*$^{P41}$ mutant flies (Figure 8 D, F). This *norpA*$^{P41}$-dependent increase in CRY expression was specific to the LLTC conditions, because there was no difference between *norpA*$^{P41}$ mutants and controls at ZT1 during LD (Figure 8 C, E). These data are consistent with a direct or indirect inhibitory effect of *norpA* on CRY expression, explaining how wild type flies can synchronize to temperature cycles in constant light

## Discussion:

### Distinct roles for *norpA* in photo and thermo entrainment

Light and temperature serve as two universal Zeitgebers to time the circadian clock in *Drosophila*. Previous results have shown that *norpA*, in combination with *cry*, belong to two major pathways that reset central clock neurons to light [3,20,22]. Our results reveal a novel role for *norpA* for temperature entrainment in constant light, which is independent its role of light entrainment: 1. $norpA^{41}$ $cry^{b}$ double mutants severely affect light entrainment but show normal activity patterns in LLTC (Figure 7A). 2. Genetic rescue of $norpA^{41}$ mutants in subsets or all clock neurons restored their rhythmic activity pattern under temperature cycles, demonstrating a role for *norpA* in clock neurons.

### The *Gq-norpA* pathway serves as a thermo-switch to maintain clock function and synchronization during constant light

Exposure to constant light breaks down the clock machinery leading to arrhythmic locomotor activity [10,32]. This is likely due to constitutively low TIM levels in clock neurons caused by constant activation of the circadian photoreceptor CRY [5,8,9]. Cycling temperature, on the other hand, serves as another potent Zeitgeber to synchronize circadian clock independent of light, suggesting the circadian thermo input is distinct from the light input at the circuit level. Interestingly, temperature cycles can 'override' the effects of constant light and restore

rhythmicity, both at the molecular and behavioral level [16,17], although the underlying mechanism remained unclear. Core clock proteins such as TIM and PER abolish their oscillation when exposed to constant light, but the rhythmic expression of those proteins is restored by temperature cycles in both peripheral tissues and central pacemakers, suggesting the existence of a functional clock in these conditions. But which molecules protect TIM from constant degradation by light during temperature cycles was so far an unresolved question. Our data show that *norpA, Gq,* and *cry* play crucial roles in this process: First, *norpA* and *Gq* are required for temperature entrainment in LL at the behavioral level. Second, *norpA* maintains TIM levels in most of the clock neurons by repressing CRY levels to protect TIM in clock neurons during LL and TC. Therefore, our results propose that the canonical Gq- PLC-ß components could work as a 'thermo-transduction' pathway to maintain the required TIM levels to restore behavioral rhythmicity under constant light and temperature cycles.

Our data also suggest that different populations in *Drosophila melanogaster* may employ different molecular strategies to respond to light and temperature changes in the environment. Wild type flies carrying *s-tim* allele, require *norpA* and *Gq* to maintain their functional clock during temperature cycles in constant light. In contrast, *ls-tim* flies do not require *Gq* and *norpA* function for normal behavior under LL and temperature cycling conditions. The *ls-tim* allele arose approximately 10000 years ago in southern Europe from where it spread northward by directional selection [37]. *ls-tim* enhances diapause, which presumably served as driving force for this natural selection, by providing advantages in coping with the shorter day-length and earlier winter onset in higher latitudes [37]. So far this was solely attributed to the lower light-sensitivity of *ls-tim* flies compared to *s-tim* flies, due to the weaker light-dependent interaction of L-TIM

with CRY compared to that of S-TIM [7,36]. In addition to an earlier onset of winter, northern latitudes are also characterized by extremely long photoperiods in the summer, and north of the Arctic circle even constant light. Our finding that *ls-tim* also provides an advantage in synchronizing to temperature cycles in constant light (because they are less dependent on additional factors), may therefore indicate that this allele provides an additional advantage during long photoperiods, by strengthening circadian clock synchronization through temperature cycles.

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**Experimental Procedures**

**Fly stains**

Flies were reared on cornmeal-sucrose food at 18 °C or 25°C under 12 hr : 12 hr LD cycles and 60% humidity until used in experiments. *y w* flies in either *s-tim* or *ls-tim* genetic background served as wild type controls. The following stains were used in this study: *norpA*$^{P24}$ [3], *norpA*$^{P41}$ [48], *Gq*$^{1}$ [38], *rh1*$^{17}$ [49], *cry*$^{b}$ [3], *cry*$^{out}$ [4], *tim-gal4:67* [50], *Pdf-gal4* [51], *cry-gal4:39* [52], *Clk856-gal4* [53], *Clk4.1M-gal4* (referred as *DN1-gal4* in this study, [54], *UAS-hid* (BL65403), *UAS-norpA* (gift from Dr.Francois

Rouyer). All mutants and trans-genes were made homozygous for *s-tim* or *ls-tim* to obtain the respective genetic background, followed by PCR confirmation of the *tim* allele [37].

**Behavioral assay**

3-5 days old male flies were used for locomotor activity tests with the *Drosophila* Activity Monitor System (DAM,Trikinetics Inc). Fly activity was recorded in light- and temperature-controlled incubators (Percival,USA) every five minutes for several days depending on the experiment. For temperature cycles in constant light (LL), flies were initially exposed to LL at 25°C to erase previous synchronization to LD. They were subsequently transferred to two temperature cycles of 12 h 25°C : 12 h 16°C under LL (LLTC 25°C:16°C) for 6-7 days, the second of which was 6 hr advanced compared to the first one. For temperature synchronization experiments in constant darkness (DDTC), flies were first entrained to light:dark cycles (LD) at 25°C for 3 days, followed by a 6hr phase-advanced temperature cycle of 12 h 25°C : 12 h 16°C (DDTC 25°C:16°C) for 8 days followed by release to constant darkness at 25°C for 4-5 days. For testing temperature compensation, flies were kept at constant 18°C, 25°C and 29°C for 7 days after initial LD entrainment for 3 days. A fly tool box implemented in MATLAB (Math Works) was employed for plotting actograms and rhythm analysis. Rhythmic strength (RS) and period length values were determined by the autocorrelation function, and only the flies with RS>1.5 were considered as rhythmic. Daily averages and entrainment index (EI) were analyzed using a customized Excel Marco, as described before [31]. EI was defined as the activity ratio between the activity in a specified time window over the activity during the entire warm phase. The time window was determined to cover the main activity peak of wild type *y w; s-tim* flies (ZT6-11). To distinguish

the clock-controlled activity peaks from temperature induced response peaks, a smoothing filter was applied for the four activity bins during the 2 hr directly following each warm/cold transition [31].

**Immunohistochemistry and quantification**

Immunostaining of whole-mount brains was performed as descried in Chen et al., 2018. In brief, flies for LLTC experiments were reared in LL and 25°C for 3 days, followed by 6 days of 25°C: 16°C LLTC. For DDTC experiments, flies were kept in LD 25°C for 3 days, followed 25°C: 16°C DDTC for 6 days. Brains were fixed and dissected on the day 7 of the temperature cycles at the indicated time points. Primary rat anti-TIM (1:1,000; [55]), rabbit anti-CRY(1:1,000, [4], gift from Dr. Charlotte Forster) and secondary rat AlexaFluor-594 (Invitrogen, 1:500), rabbit AlexaFlour-488(Invitrogen, 1:500) were applied for 12 hours at 4°C before the brains were mounted in Vectashield mounting medium. Brains were observed with a Leica TCS SP8 confocal microscope. To quantify the staining signals, pixel intensity of stained neurons and background for each neural groups were measured using ImageJ (NIH), the signal intensities were determined by subtracting average background signals from neuronal signals from pixel values of two surrounding regions. Average intensities for each time point and neuronal group represent at least 8 hemispheres for each genotype. Data were normalized by setting the peak value to 1 and the ratio from each time point was then divided by the peak value.

**Single cell gene expression analysis**

Gene expression single cell RNA-seq data of the *Drosophila* brain were download from the public online database (http://scope.aertslab.org). A set of selected genes were analyzed in the following two previously annotated clusters [43]: DN1 (n=29) and photoreceptors (n=40), and a novel LNv cluster (n=25), which we defined in this study by extracting *Pdf*$^{+}$ cells from clock neurons annotated in [43]. 'n' refers to the number of single cells per cluster. A customized Python package was applied for data plotting (reference https://jupyter.org/).

**Statistics**

All data were plotted and statistically analyzed using Graphpad (Prism). For behavioral data quantification, Kruskal-Wallis with Dunn's post hoc tests were performed to determine the difference between groups. For TIM and CRY staining data, two-way ANOVA with Bonferroni multiple comparisons was applied.

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**Figure 1: Synchronization to temperature cycles in constant light requires *norpA* function in clock cells.** Flies of indicated genotypes were kept at 25°C in LL for 2 days followed by two 6-day temperature cycles (TCs) with 12h : 12h 25°C : 16°C. The 2$^{nd}$ TC is 6h advanced compared to the 1$^{st}$ one. Yellow shading indicates warm, and blue indicates cooler temperature. (A, B) top row shows double plotted actograms of locomotor activity. Middle and bottom rows show daily average of the last 3 days during each TC. Numbers on the y-axis indicates activity in 30mins. (C)

An entrainment index (EI) was calculated and plotted to quantify behavioral activity patterns (see Experimental Procedures and Gentile et al 2013). Number of tested flies is shown on the bottom of each column. **:p<0.01, ****:p<0.0001.

**Figure 2: Rhythmic TIM expression in clock neurons during temperature cycles in constant light requires *norpA*.** (A) Quantification of TIM levels in different clusters of clock neurons during LL TC (25°C :16°C). Numbers below the x-axis indicate different Zeitgeber time points (ZTs), and orange and blue bars 25°C and 16°C, respectively. 8-10 brain. Error bars indicate SEM. (B) Quantification of TIM levels in clock neurons during DDTC (25°C : 16°C) at ZT4 and ZT20 (4 hr after or before the temperature was raised to 25°C, respectively). For (A, B) at least 8 brain hemispheres of each genotype were analyzed per time point. Error bar indicates SEM. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, while n.s. indicates no significance.

**Figure 3: *norpA*-dependend temperature synchronization defects are modulated by different isoforms of TIMELESS**. (A) Double plotted average actogram (top) and daily averages (middle and bottom) of *norpA* mutants and controls homozygous for the *ls-tim* allele exposed to shifted LLTC 16°C : 25°C (same conditions and color coding as described in Figure 1). (B) EI quantification of *y w*, $norpA^{P24}$, and $norpA^{P41}$ flies in homozygous *s-tim* (*s-tim* +, *ls-tim* -), homozygous *ls-tim* (*s-tim -, ls-tim* +), or heterozygous *s-tim/ls-tim* (*s-tim* +, *ls-tim* +) genetic backgrounds. (C) Quantification of TIM levels in different clusters of clock neurons of *y w; s-tim* (dashed black line), $norpA^{P41}$; *s-tim* (dashed red line), *y w; ls-tim* (solid grey line) and $norpA^{P41}$*; ls-tim* (solid green line). Data for

*s-tim* backgrounds are replotted from Figure 2A for comparison to *ls-tim*. Brains were dissected on the 5th day of LLTC (25°C : 16°C) and 8-10 brain hemispheres genotype were analyzed at the ZTs indicated below the x-axis. Orange and blue bars indicate 25°C and 16°C, respectively. Error bars indicate SEM. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001

**Figure 4**: **Components of canonical phototransduction signalling are required for temperature synchronization in constant light.** (A) Top row: double-plotted average actograms during 25°C : 16°C shifted LLTC. Middle and bottom rows: Daily averages of the last three days of each TC (same conditions and color coding as described in Figure 1). B) EI calculation of the indicated genotypes in homozygous *s-tim* or *ls-tim* backgrounds. ** p<0.01.

**Figure 5**: ***norpA* expression in central clock neurons is sufficient for synchronization to temperature cycles in constant light**. (A) Top row: double-plotted average actograms during 25°C : 16°C shifted LLTC. Middle and bottom rows: Daily averages of the last three days of each TC (same conditions and color coding as described in Figure 1). B) EI calculation of the indicated genotypes in homozygous *s-tim* background. * p<0.05, ** p<0.01, *** p<0.001, n.s:not significant.

**Figure 6: Single cell RNAseq reveals expression of phototransduction genes in clock neurons.** Log-normalized gene expression (y-axis) in different cell types (x-axis) for the gens indicated. The digital gene expression matrix for all genes (http://scope.aertslab.org/) from 57,000 scRNAseq profiles was downloaded, and here we show the violin plot representing the distribution of the selected genes for

individual cells in 3 previously annotated clusters: LNv clock neurons (n=25), DN1 (n=29), and photoreceptors (n=40), where the 'n refers to the number of single cells analyzed per cluster. The white circle in the middle represents the median of the gene expression, the black bar behind it represents the interquartile range, and the black lines extending from the bar represent the first and third quartile respectively. *Pdf* expression was threshold to an average of 9 +/-3 SD to represent reliable *Pdf* transcript detection across all clusters. No thresholds were applied to remaining genes. See Experimental Procedures for details.

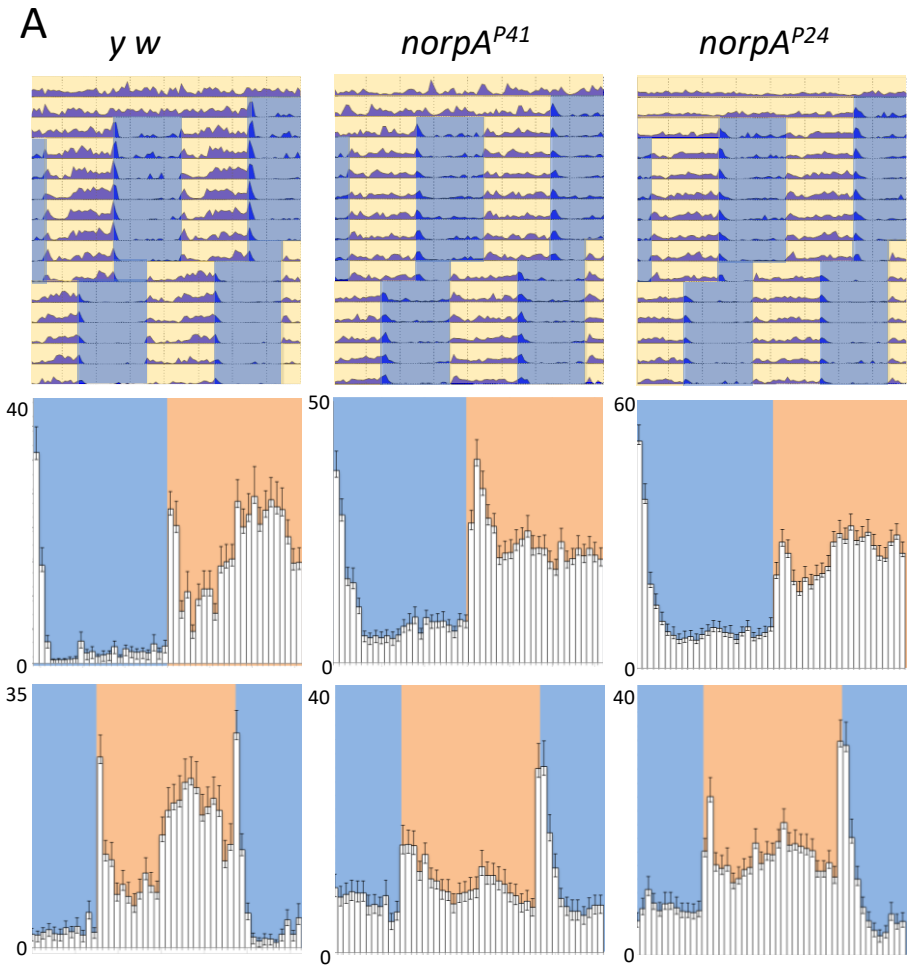
**Figure 7: CRY-depletion rescues behavioral and molecular synchronization of *norpA* mutants to temperature cycles in constant light.** (A) Top row: double-plotted average actograms during 25°C : 16°C shifted LLTC. Middle and bottom rows: Daily averages of the last three days of each TC (same conditions and color coding as described in Figure 1). B) EI calculation of the indicated genotypes in homozygous *s-tim* background. (C) Quantification of TIM levels in different clusters of clock neurons of the indicated genotypes. Brains were dissected on day 5 of TC (25:16°C) in constant light and 8-10 brain hemispheres genotype were analyzed at the ZTs indicated below the x-axis. Orange and blue bars indicate 25°C and 16°C, respectively. Error bars indicate SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

**Figure 8: NORPA represses CRY levels during temperature cycles in constant light.** (A) Representative anti-CRY staining of different clock neuronal clusters in LD 25°C at ZT22 in *y w* flies. $cry^{02}$ served as a negative control for antibody specificity. (B) Quantification of CRY staining of *y w* flies at ZT1 and ZT22 in LD25°C. (C) Representative anti-CRY staining in LD25°C and (D)

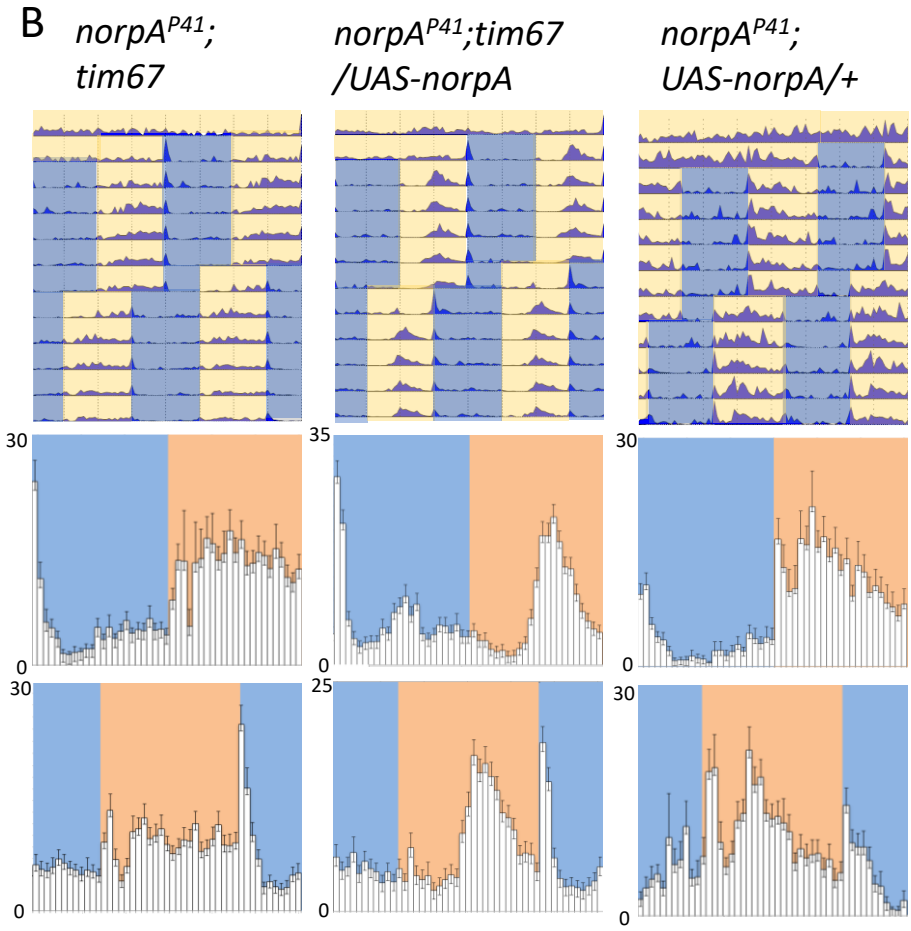
LLTC25°C : 16°C at ZT1. (E-F) Quantification of immunostaining with anti-CRY in different clusters of clock neurons at ZT1 in LD 25°C (E) or LLTC 25° : 16°C (F). (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$). scale bar=10µm.

Figure1

A




B

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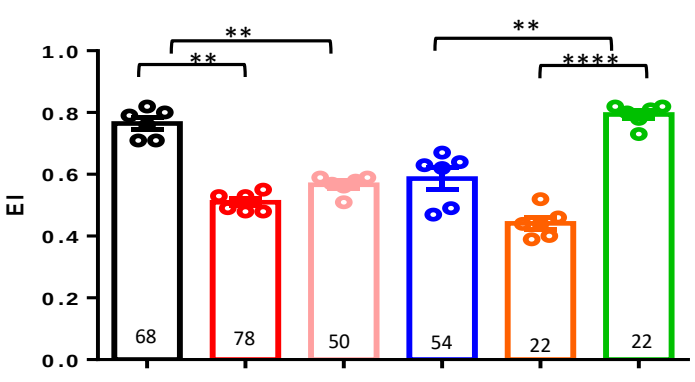
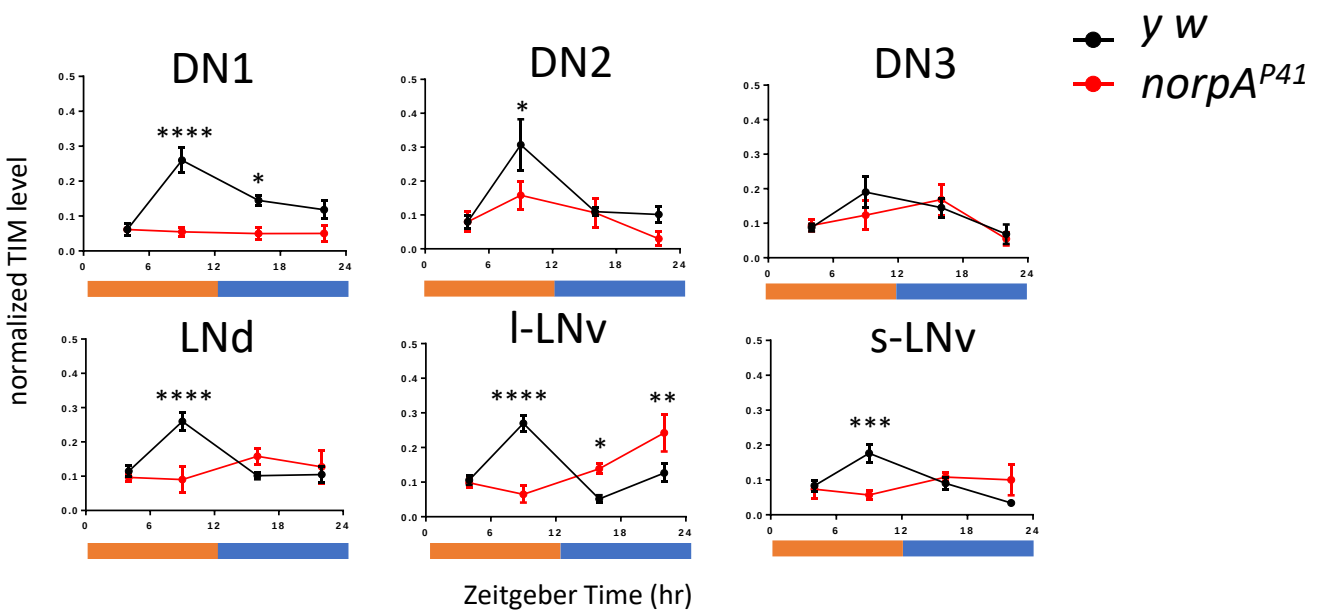



- *y w;s-tim*
- *norpA^P41;s-tim*
- *norpA^P24;s-tim*
- *norpA^P41;s-tim;tim-gal4/+*
- *norpA^P41;s-tim;UAS-norpA6M*
- *norpA^P41;s-tim;tim-gal4/UAS-norpA6M*

Figure2

A


y w
norpA^P41
DN1
DN2
DN3
LNd
l-LNv
s-LNv
normalized TIM level
Zeitgeber Time (hr)

B

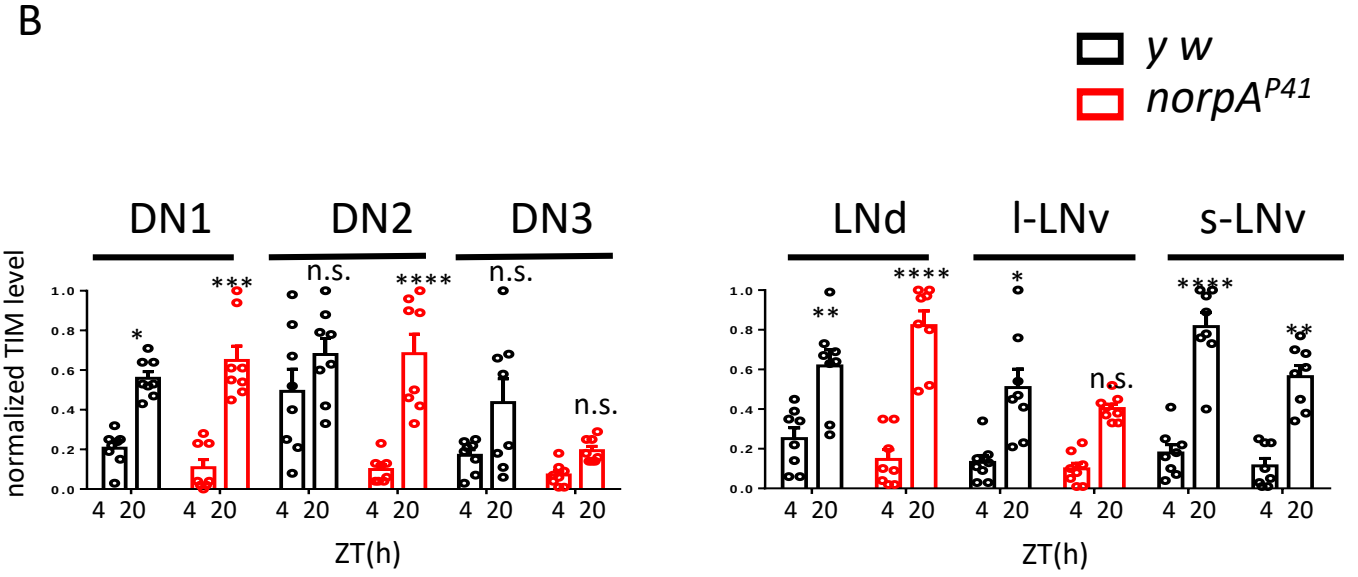
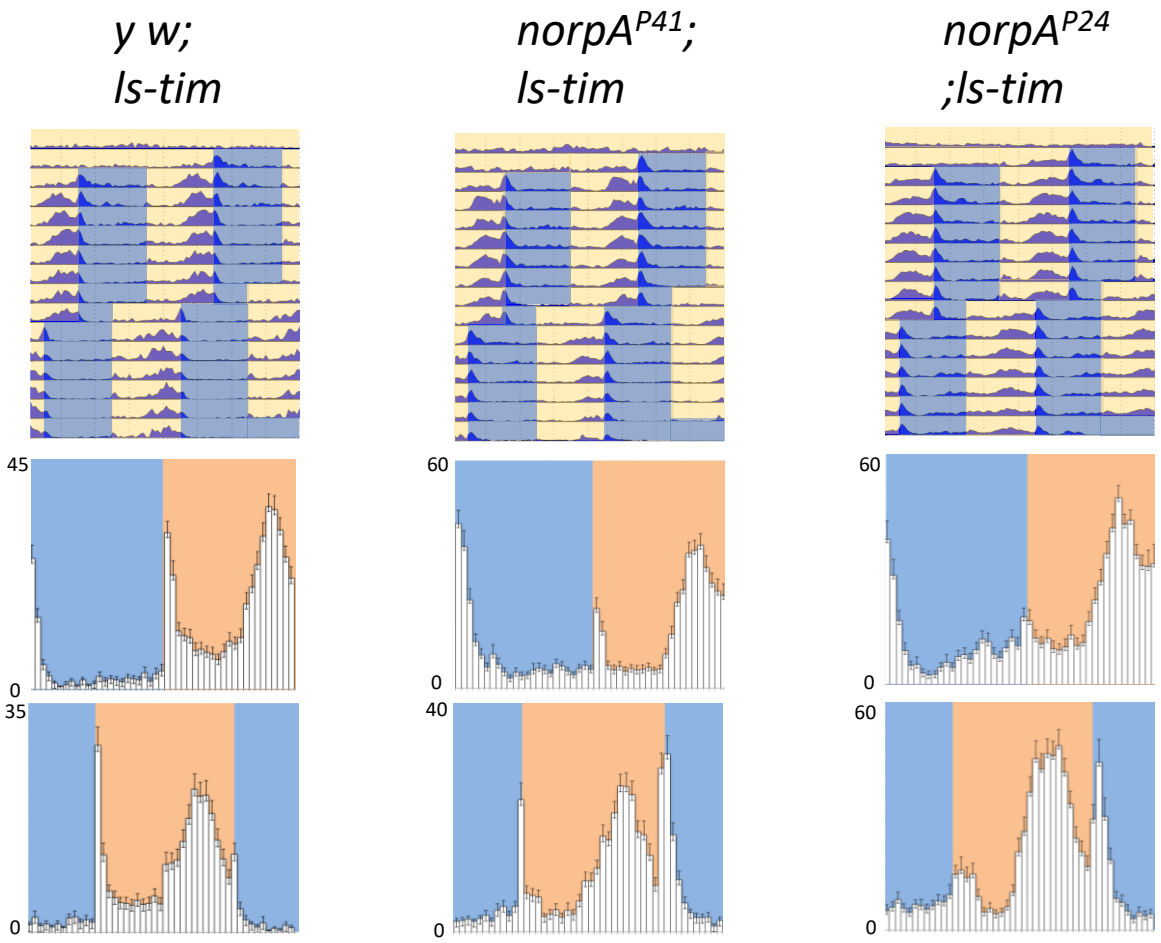

y w
norpA^P41
DN1
DN2
DN3
LNd
l-LNv
s-LNv
normalized TIM level
ZT(h)
n.s.

Figure3

A


y w;
ls-tim
norpA$^{P41}$;
ls-tim
norpA$^{P24}$
;ls-tim
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35
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60
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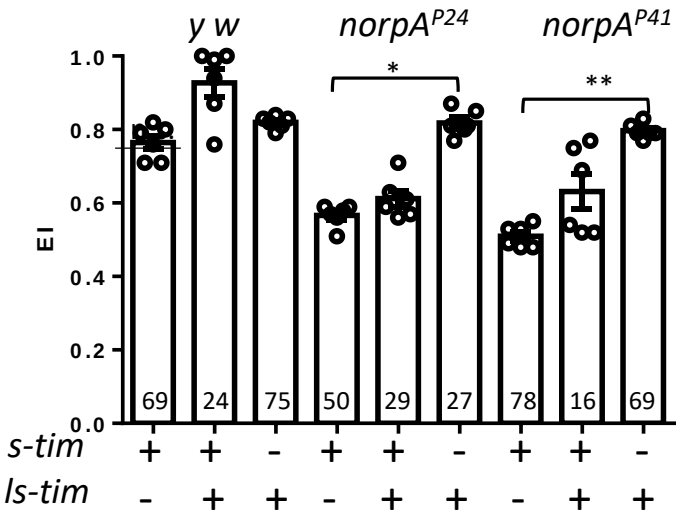
60
0
60
0

B


y w
norpA$^{P24}$
norpA$^{P41}$
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0.6
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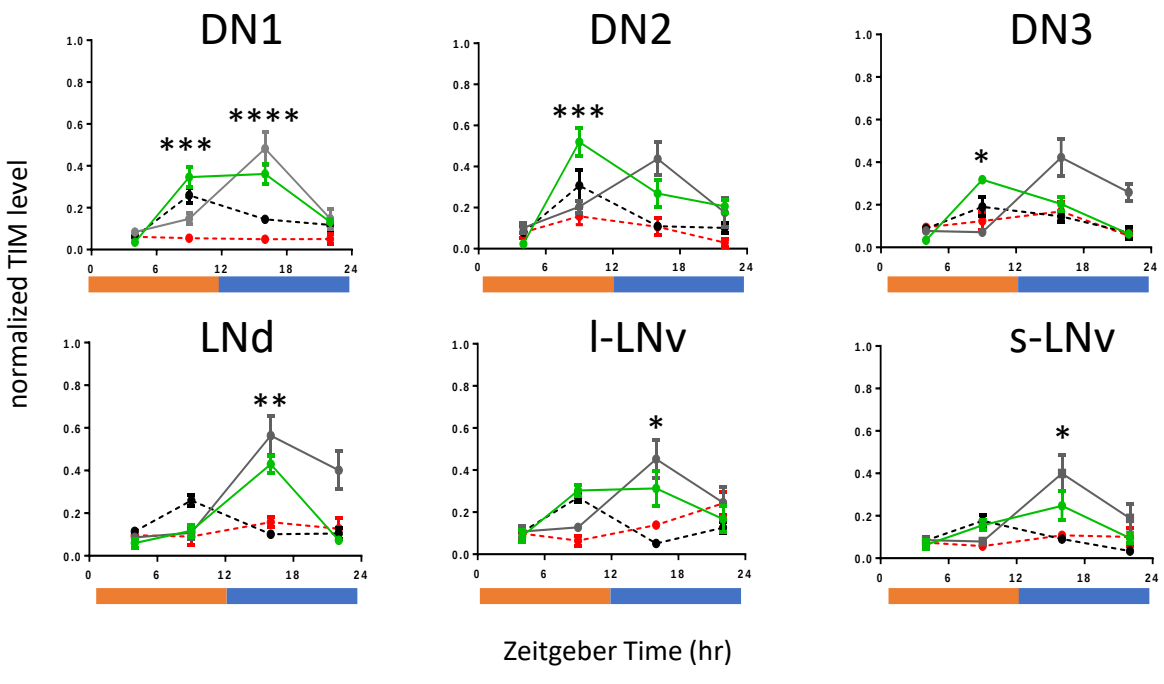
50
29
27
78
16
69
s-tim + + - + + - + + -
ls-tim - + + - + + - + +

C


DN1
DN2
DN3
LNd
l-LNv
s-LNv
normalized TIM level
Zeitgeber Time (hr)
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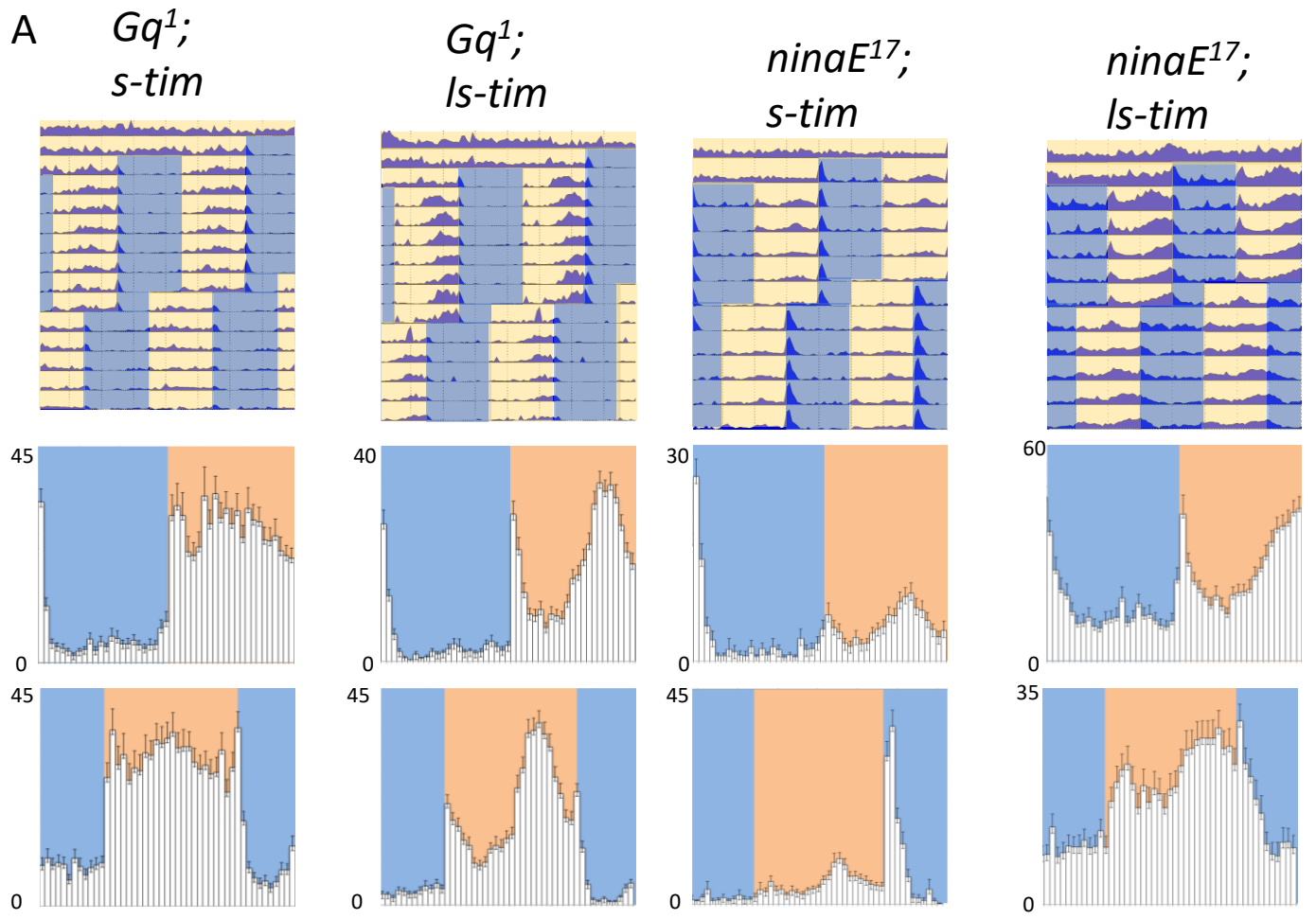
0
6
12
18
24

Figure4

A


Gq1; s-tim
Gq1; ls-tim
ninaE17; s-tim
ninaE17; ls-tim
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40
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30
0
60
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45
0
45
0
45
0
35
0

B

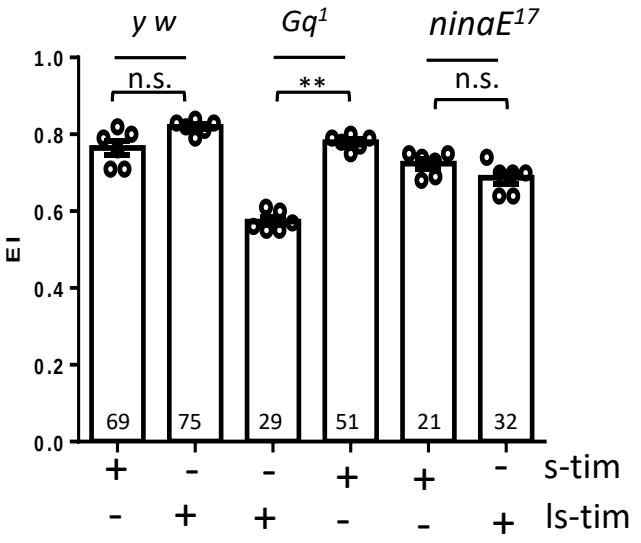

y w
Gq1
ninaE17
n.s.
**
n.s.
EI
1.0
0.8
0.6
0.4
0.2
0.0
69
75
29
51
21
32
+ - - + + - s-tim
- + + - - + ls-tim

Figure5

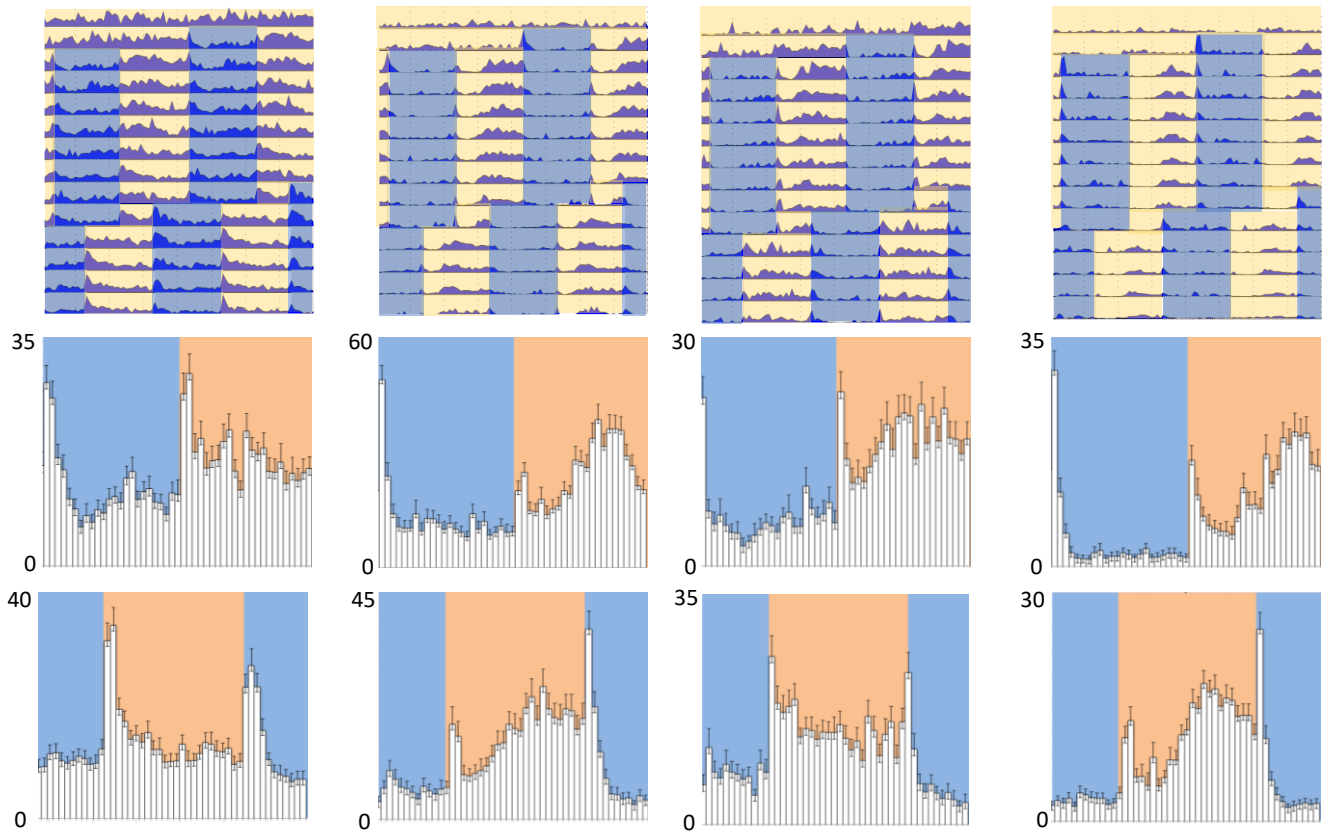
A

*norpA$^{P41}$; Clk-gal4*

*norpA$^{P41}$;Clk-gal4/+ ;+/UAS-norpA*

*norpA$^{P41}$; ; Cry-gal4*

*norpA$^{P41}$;;Cry-gal4 /UAS-norpA*

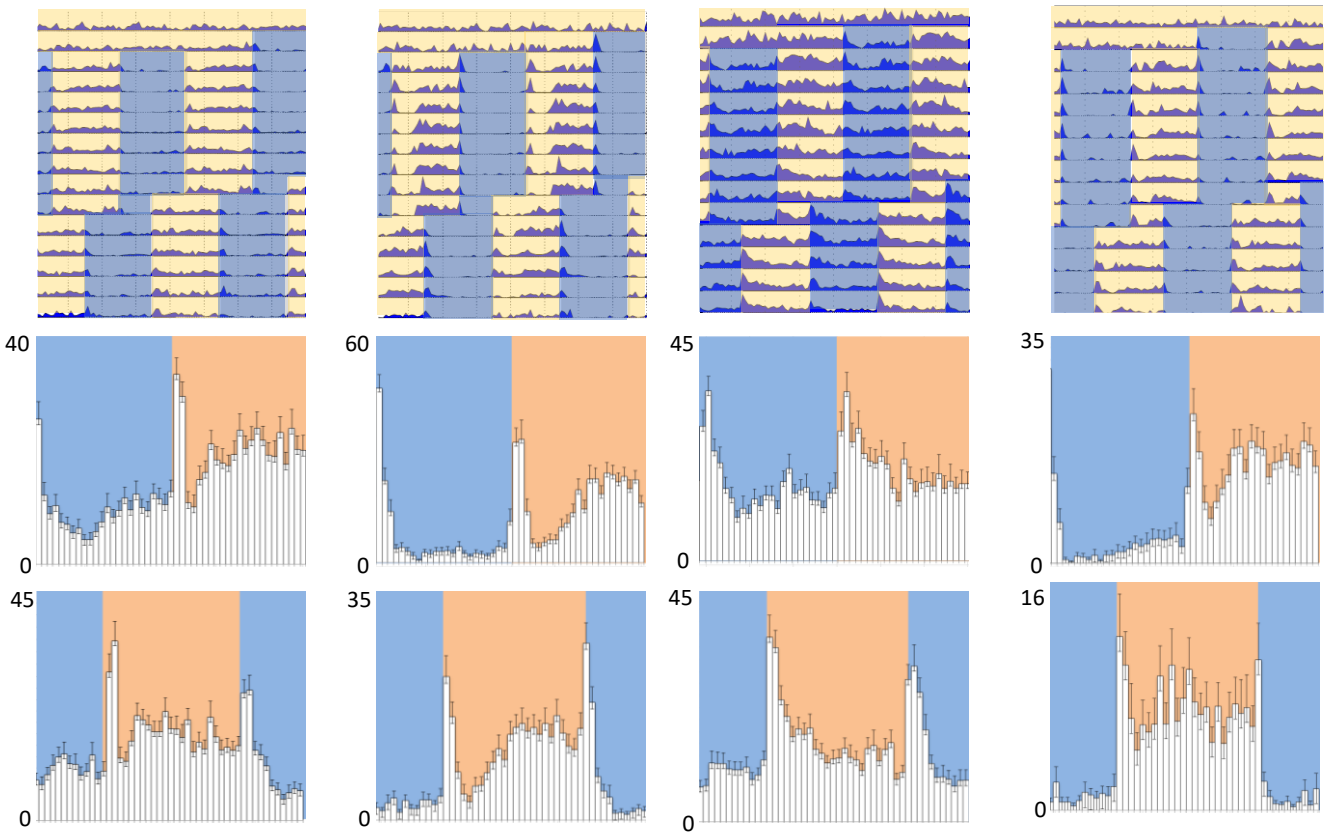



*norpA$^{P41}$; pdf-gal4*

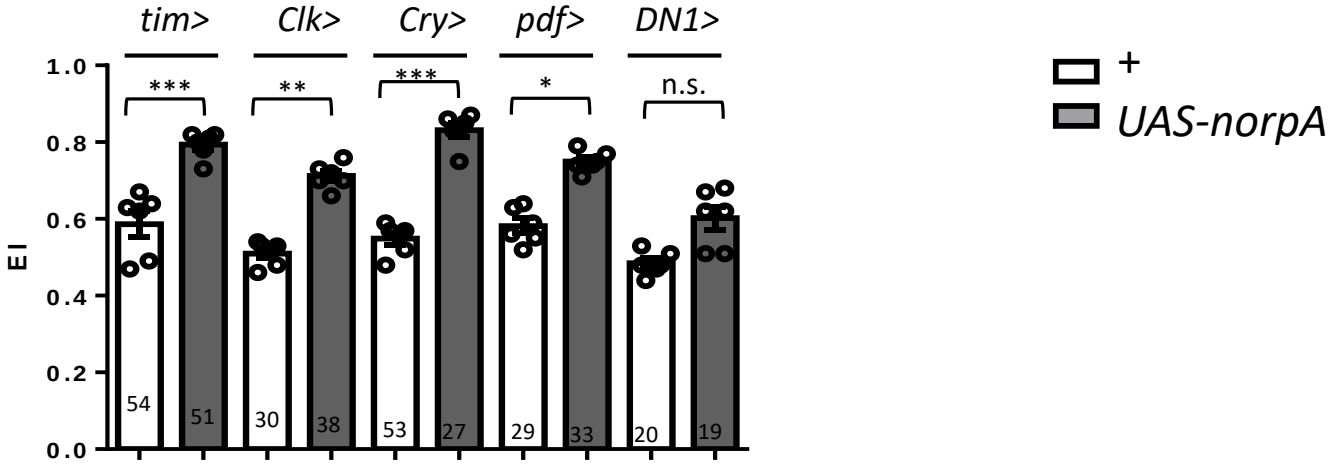
*norpA$^{P41}$;pdf-gal4/UAS-norpA*

*norpA$^{P41}$; DN1-gal4*

*norpA$^{P41}$;DN1-gal4 /UAS-norpA*




B



# Figure6

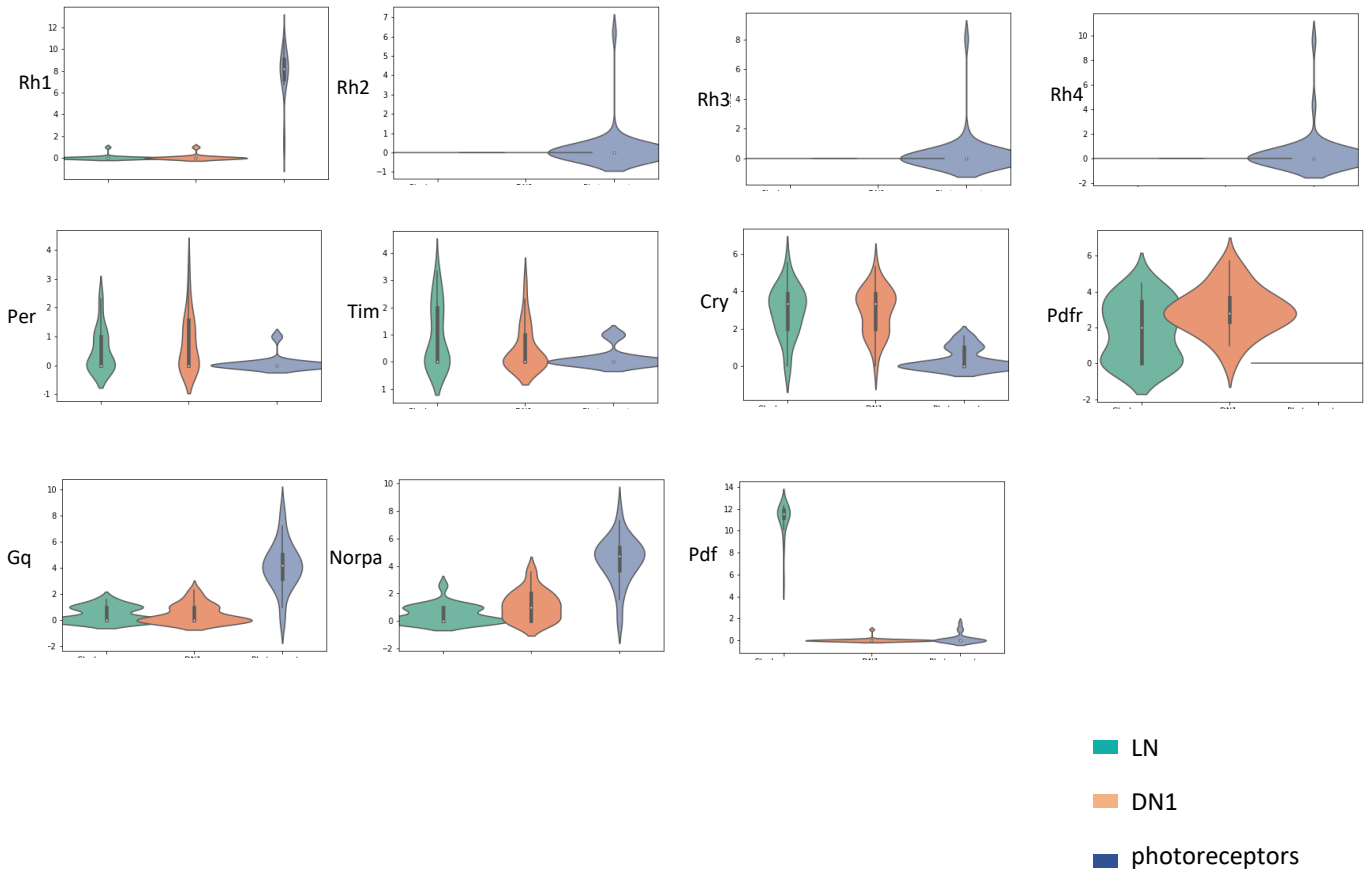
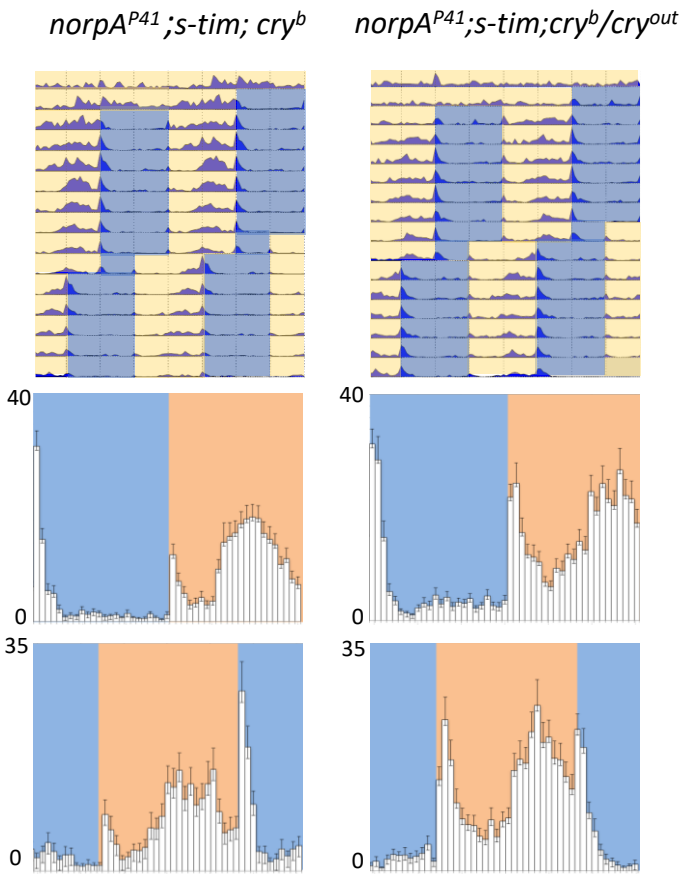
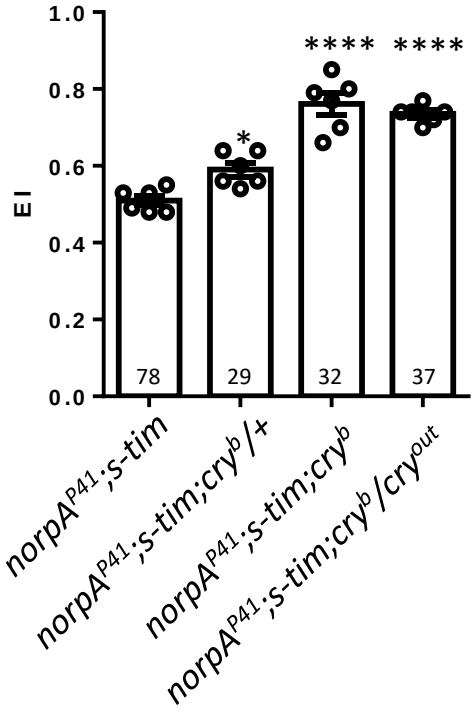

Rh1
Rh2
Rh3
Rh4
Per
Tim
Cry
Pdfr
Gq
Norpa
Pdf
LN
DN1
photoreceptors

Figure7

A


norpA^P41;s-tim; cry^b
norpA^P41;s-tim;cry^b/cry^out
40
0
35
0
40
0
35
0

B


EI
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78
29
32
37
norpA^P41;s-tim
norpA^P41;s-tim;cry^b/+
norpA^P41;s-tim;cry^b
norpA^P41;s-tim;cry^b/cry^out

C

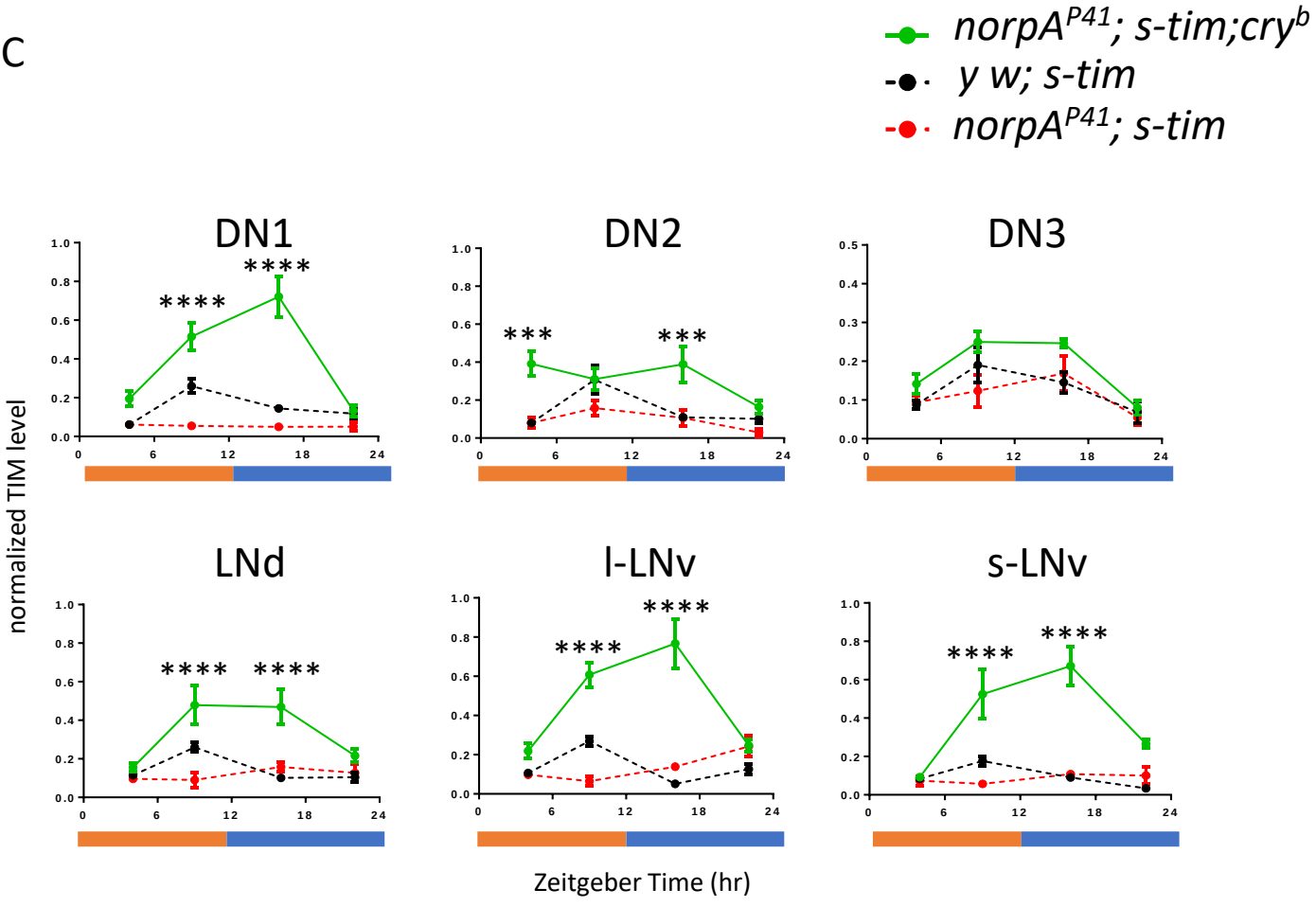
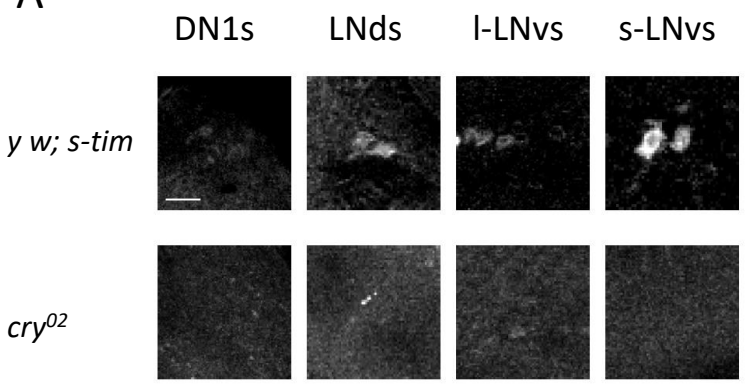
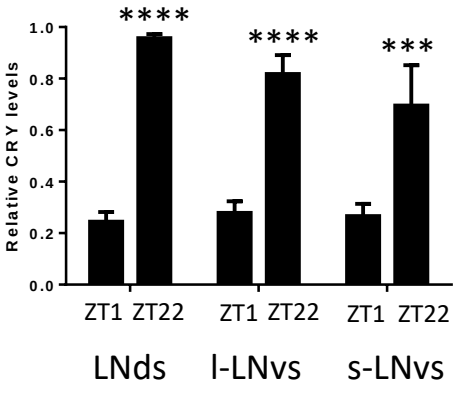

norpA^P41; s-tim;cry^b
y w; s-tim
norpA^P41; s-tim
DN1
DN2
DN3
LNd
l-LNv
s-LNv
normalized TIM level
Zeitgeber Time (hr)
****
***

Figure8

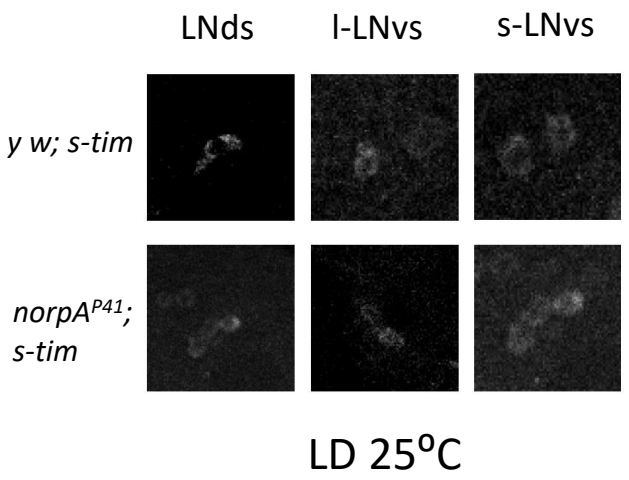
A



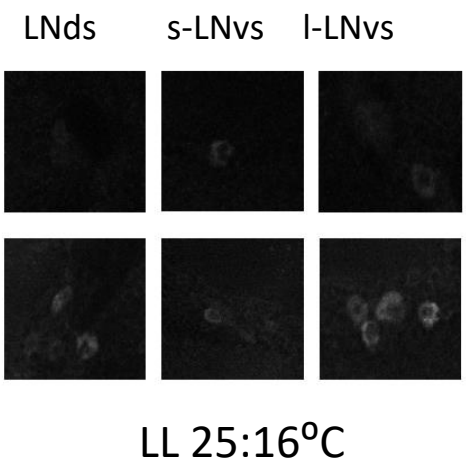

B



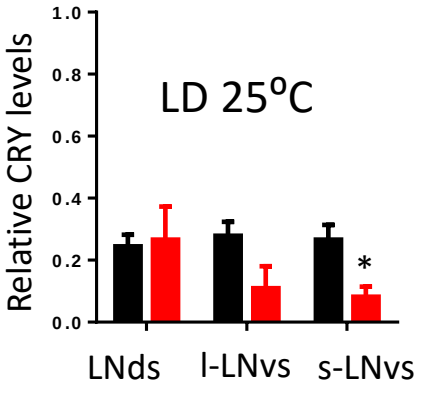

C

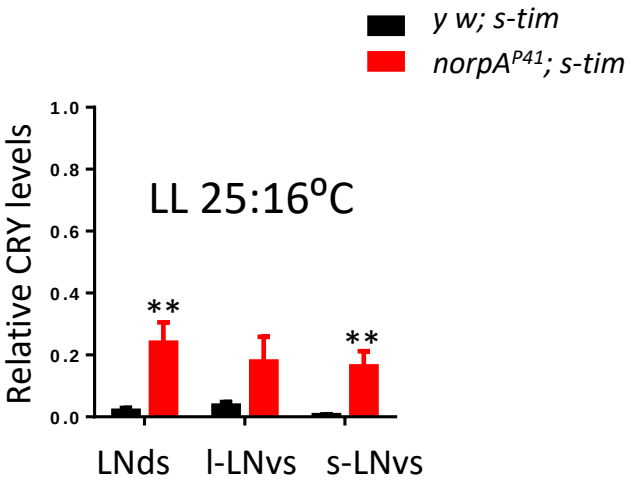



D




E




F