Circadian photoreceptor CRYPTOCHROME promotes wakefulness under short winter-like days via a GABAergic circuitry

Lixia Chen[1], Danya Tian[1], Chang Su[1] and Luoying Zhang[1,2]*

[1]Key Laboratory of Molecular Biophysics of Ministry of Education, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, Hubei 430074, China

[2] Hubei Province Key Laboratory of Oral and Maxillofacial Development and Regeneration, Wuhan, Hubei 430022, China

*Correspondence: Luoying Zhang, E-mail: zhangluoying@hust.edu.cn

Abstract

A cardinal symptom of seasonal affective disorder (SAD, also known as winter depression) is hypersomnolence, while the cause of this "winter sleepiness" is not known. Here we found that lack of the circadian photoreceptor *cryptochrome* (*cry*) leads to increased sleep under short winter-like days in fruit flies, reminiscent of the hypersomnolence in SAD. CRY functions in neurons that synthesize the major inhibitory neurotransmitter GABA, including the small ventral lateral neurons which are known to be circadian pacemakers, and down-regulates the GABAergic tone. This in turn leads to increased neural activity of the wake-promoting large ventral lateral neurons, a subset of circadian neurons that are inhibited by GABA-A receptor. CRY protein is known to be degraded by light, thus rendering CRY to be functional within this GABAergic circuitry to enhance wakefulness only under short day length. Taken together, we demonstrate a mechanism that specifically regulates wakefulness under short winter-like days, which may provide insights regarding the winter sleepiness in SAD.

Keywords: wakefulness, circadian pacemaker, CRYPTOCHROME, GABA, photoperiod, photoreceptor

Introduction

Seasonal affective disorder (SAD), also known as winter depression, is characterized by the onset of depression in fall/winter months and spontaneous remission in the spring/summer (Rosenthal et al., 1984). It is generally agreed that SAD is caused by lack of day light in the fall/winter months due to the short day length (or photoperiod), as bright light therapy is effective and most commonly used for treating the depression associated with SAD (Golden et al., 2005). The prevalence of SAD is ~1-10% world-wide, with symptoms lasting for approximately 40% of the year (Kurlansik and Ibay, 2012). About 64-80% of SAD patients report a winter increase in sleep, ranging from 30 min to 2 h longer in duration compared to controls, which is considered to be a distinguishing symptom in the characterization and diagnosis of SAD (Wescott et al., 2020). Currently almost nothing is known regarding the underlying mechanism of this winter hypersomnolence.

This phenomenon of winter hypersomnolence in SAD patients implicates distinct mechanisms that regulate sleep under short winter-like photoperiods vs. longer non-winter-like photoperiods, as the sleep of these individuals appear to be selectively perturbed under short photoperiods. However, the mechanisms by which sleep duration is determined under different photoperiods have not been characterized. Since the circadian clock is believed to be important for adaptations to seasonal changes in the environment and in particular, seasonal changes of photoperiod, we hypothesize that the circadian clock may also participate in regulating sleep duration under different photoperiods (Wood and Loudon, 2014).

To address our hypothesis, we tested fruit flies mutant for different circadian clock genes under a range of photoperiods. We found that flies lacking the circadian photoreceptor CRYPTOCRHOME (CRY) display increased sleep duration specifically under short photoperiods, similar to the winter hypersomnolence in SAD. Genetic and pharmacological analysis identified that CRY is functioning in GABAergic neurons and acts upon GABA-A receptor to promote wakefulness. We further narrowed down the neural circuitry mediating the influences of CRY on sleep by demonstrating that *cry* deficiency increases the GABAergic tone and reduces calcium concentration in the wake-promoting large ventral lateral neurons (l-LNvs) which are known to be GABA-A+ (Hamasaka et al., 2005; Parisky et al., 2008). Consistently, inhibiting these neurons increases sleep while activating them blocks the effects of *cry* deficiency on sleep. CRY likely functions in part in the GABAergic small ventral lateral neurons (s-LNvs), and lack of *cry* increases GABA level and the activity of these cells while impairing their GABA transmission suppresses the sleep phenotype of *cry* mutants (Allada and Chung, 2010). In summary, here we identify a potential role for CRY in down-regulating the GABAergic signaling from the s-LNvs to the l-LNvs specifically under short photoperiod. This in turn enhances the neural activity of the wake-promoting l-LNvs, resulting in increased wakefulness during short winter-like days. These findings reveal a mechanism underlying how sleep duration is determined under winter-like photoperiod, while disruptions of this regulatory system may be related to the winter hypersomnolence associated with SAD.

Results

cry mutation increases sleep duration specifically under short photoperiods

We assessed the sleep of flies mutant for circadian clock gene *period* (*per*^{0}), *timeless* (*tim*^{0}), *clock* (*clk*^{jrk}), *cycle* (*cyc*^{0}) and *cry* (*cry*^{b}) under a range of photoperiods (Figure 1A and B)(Allada, 1998; Konopka and Benzer, 1971; Sehgal et al., 1994; Stanewsky, 1998). We found that *cry*^{b} mutation, which is known to be a loss of function or severe hypomorphic allele, leads to increased sleep duration under 4 h light: 20 h dark condition (4L20D) and 8L16D but not under longer photoperiods (Stanewsky, 1998). Since this phenotype recapitulates the winter hypersomnolence of SAD patients, we further characterized the effects of *cry* deficiency on sleep. We focused on sleep under 4L20D, as the extent of sleep increase is slightly larger than that of 8L16D. We also demonstrated that *cry* mutation lengthens sleep duration in both male and female flies, while waking activity is not significantly reduced in the mutants (Figure 1C and D). This means the increased sleep in the mutants is not caused by defects of locomotor ability. We next examined the sleep architecture of these flies and found that *cry* mutation enhances sleep by extending the duration of average sleep bout rather than increasing sleep bout number, indicating that *cry* deficiency promotes sleep consolidation under short photoperiod (Figure 1E and F). Because CRY exerts influences on sleep/wakefulness in a gender-independent manner, we used male flies for the remainder of the study. In addition, we tested the effects of a *cry* knock-out allele (*cry*^{0}) on sleep under 4L20D and also observed significantly prolonged sleep

duration, similar to *cry*^{b} mutation (Figure 1—figure supplement 1) (Dolezelova et al., 2007).

CRY functions in GABAergic neurons and promotes wakefulness via GABA/GABA-A

Upon light activation, CRY binds to the clock protein TIM and results in its degradation, which is followed subsequently by degradation of CRY itself (Busza et al., 2004; Ceriani et al., 1999; Lin et al., 2001). Therefore, we first tested whether the influences of CRY on sleep/wakefulness requires TIM. We monitored sleep in flies mutant for both *cry* and *tim*, and found that the sleep duration of these double mutants are comparable to that of *cry* mutants (Figure 2—figure supplement 1). This indicates that CRY promotes wakefulness in a TIM-independent manner.

To identify anatomical substrates that mediate the effects of CRY on wakefulness, we employed the UAS/GAL4 system to knock down *cry* in different brain structures and cell types and verified that *cry* is indeed knocked down by assessing its mRNA level (Figure 2—figure supplement 2). We found that knocking down *cry* in GABAergic neurons using *vesicular GABA transporter* (*VGAT*)GAL4 or *glutamic acid decarboxylase 1* (*Gad1*)GAL4 results in increased sleep duration under 4L20D but not 12L12D, similar to the *cry* mutant phenotype (Figure 2A-C; Figure 2—figure supplement 3A and B)(Deng et al., 2019). Given that *Gad1*GAL4 generated a more prominent sleep phenotype, we used this driver for the remainder of this study.

Since CRY appears to act in GABAergic neurons to promote wakefulness, we tested whether GABA is involved in this regulatory process. We first took a

pharmacological approach and fed flies with drugs to inhibit GABA transaminase (ethanolamine-O-sulphate, EOS) or GABA transporter (nipecotic acid, NipA). Both of these treatments are known to increase GABA level, and indeed lengthens the sleep duration in WT flies (Ki and Lim, 2019; Leal and Neckameyer, 2002). *cry* mutation fails to enhance sleep when treated with NipA, while the extent of sleep increase caused by *cry* mutation is smaller when treated with EOS compared to the control (Figure 2D; Figure 2—figure supplement 3C and D). Similarly, knocking down *cry* in GABAergic neurons no longer lengthens sleep duration after NipA treatment (Figure 2E; Figure 2—figure supplement 3E). For further validation, we adopted a genetic approach. We found that knocking down *Gad1*, the synthetic enzyme for GABA, suppresses the long sleep phenotype of *cry* RNAi flies (Figure 2F; Figure 2—figure supplement 3F) (Jackson et al., 1990). In addition, we knocked down *VGAT* which encodes an essential transporter responsible for packing GABA into synaptic vesicles (Enell et al., 2007). *cry* deficiency fails to lengthen sleep duration when *VGAT* is knocked down (Figure 2G and H; Figure 2—figure supplement 3G and H). We verified that *Gad1* and *VGAT* are indeed knocked down by measuring their mRNA levels (Figure 2—figure supplement 3I and J). As a control for the genetic interaction experiments, we co-expressed a GFP RNAi and found this does not significantly alter the sleep duration of *cry* RNAi flies (Figure 2—figure supplement 3K). These series of results indicate that CRY acts via GABA to promote wakefulness.

We next sought to identify GABA receptor that mediates the effects of CRY on sleep/wakefulness. We fed flies with agonists of GABA-A (THIP) and GABA-B

141 receptor (SKF-97541) (Ki and Lim, 2019). Both drugs enhance sleep in WT, while
142 *cry* mutation can increase sleep in flies fed with SKF-97541 but not THIP, implicating
143 that CRY acts through GABA-A to promote wake (Figure 3A-C). Consistently,
144 GABA-A receptor antagonist carbamezapine (CBZ) reduces sleep in WT flies while
145 *cry* mutation fails to lengthen sleep duration after CBZ treatment (Figure 3D and E)
146 (Agosto et al., 2008). We also treated *cry* RNAi flies with THIP or CBZ and found
147 this treatment abolished the long sleep phenotype as well (Figure 3F-I). To validate
148 that CRY modulates sleep/wakefulness by acting upon GABA-A receptor, we tested
149 for genetic interaction between *cry* and *Resistant to dieldrin* (*Rdl*), the gene that
150 encodes GABA-A receptor in flies (ffrench-Constant et al., 1993). We found that *Rdl*
151 mutation (Rdl^{MDRR}) blocks the sleep-enhancing effect of *cry* RNAi (Figure
152 3J-L)(Agosto et al., 2008). These findings demonstrate that GABA-A receptor
153 mediates the wake-promoting function of CRY.

154 In short, pharmacological and genetic approaches reveal that CRY acts in
155 GABAergic neurons and impinges on GABA/GABA-A signaling to promote
156 wakefulness.

157 **CRY acts upon l-LNvs to promote wakefulness**

158 Previous studies reported that GABA enhances sleep at least in part by activating
159 RDL on the l-LNvs, thus inhibiting the activities of these cells and reducing the
160 release of the arousal-promoting neuropeptide pigment dispersing factor (PDF)
161 (Chung et al., 2009; Parisky et al., 2008). Therefore, we tested whether the l-LNvs
162 mediate the effects of CRY on sleep/wakefulness. We first verified that the l-LNvs

indeed receive projections from GABAergic neurons. We expressed a GFP-tagged synaptotagmin (*syt-GFP*) which labels axon terminals using *Gad1*GAL4, and GFP signal was observed at the l-LNv soma (Figure 4—figure supplement 1A) (Zhang et al., 2002). We employed the GFP Across Synaptic Partners (GRASP) method with enhanced specificity (t-GRASP) to check for synaptic connections between GABAergic neurons and l-LNvs (Shearin et al., 2018). Pre-mGRASP was expressed in the axon terminal of GABAergic neurons (using *Gad1*GAL4), whereas its t-GRASP partner post-mGRASP was expressed in the l-LNvs (using the driver *Pdf*LexA) (Shang et al., 2008). GFP signal can be detected at the l-LNv soma, which means GABAergic neurons send synaptic projections to the l-LNvs (Figure 4 —figure supplement 1B). We further validated this connection using the *trans*-Tango technique which labels down-stream synaptic targets of GABAergic neurons with the HA tag, and we were able to observe HA expression in the l-LNvs (Figure 4—figure supplement 1C) (Talay et al., 2017). All in all, these results demonstrate that GABAergic neurons project to the l-LNvs and form synaptic connections.

We next examined the effects of *cry* mutation on GABA level at the l-LNv soma by immunostaining. As expected, GABA signal is significantly increased in *cry* mutants under 4L20D but not 12L12D (Figure 4A-D). Consistent with elevated GABA, GCaMP6m signal, an indicator of intracellular calcium concentration, is significantly reduced in the l-LNvs under 4L20D (Figure 4E and F) (Chen et al., 2013). This implies that the neural activity of these cells is down-regulated, possibly due to increased GABA signaling.

To test whether the long sleep phenotype in *cry* mutants is due to reduced activity of the l-LNvs, we electrically silenced these neurons by expressing an inward rectifying potassium channel Kir2.1 (Baines et al., 2001). This results in lengthened sleep duration under 4L20D, mimicking the sleep phenotype of *cry* mutants (Figure 4G; Figure 4—figure supplement 2A). On the other hand, activating the l-LNvs by expressing the temperature-gated depolarizing cation channel TrpA1 or the bacterial depolarization-activated sodium channel NachBac blocks the sleep enhancing effect of *cry* mutation (Figure 4H and I; Figure 4—figure supplement 2B and C) (Hamada et al., 2008; Nitabach et al., 2006). These findings support the notion that *cry* mutation lengthens sleep duration by down-regulating the neural activity of the l-LNvs. Since it has been shown that the l-LNvs promote arousal by releasing PDF, we assessed whether PDF or its receptor PDFR is required by CRY to exert influence on sleep/wakefulness (Chung et al., 2009; Parisky et al., 2008). *cry* mutation fails to lengthen sleep duration on *Pdf* or *Pdfr* mutant background, indicating the necessity of PDF signaling in mediating the arousal-promoting function of CRY (Figure 4J and K; Figure 4—figure supplement 2D and E) (Hyun et al., 2005; Lear et al., 2005; Mertens et al., 2005; Renn et al., 1999).

To summarize, these results strongly suggest that CRY promotes wakefulness by reducing GABA signaling and thus increasing the activity of the l-LNvs, which may in turn lead to increased release of PDF.

CRY acts in the s-LNvs to promote wakefulness

To identify the subset of GABAergic neurons in which CRY functions to regulate

207 wakefulness, we first examined the expression pattern of *Gad1*GAL4 by labeling the
208 GAL4+ cells with a nuclear GFP (nls-GFP) (Shiga et al., 1996). While the l-LNvs are
209 not GFP+, we noticed that the s-LNvs which are close to the l-LNvs and also express
210 PDF appear to be GFP+ (Figure 5A and B). Because the s-LNvs are known to express
211 CRY, we suspected that CRY may be acting in these s-LNvs to regulate the activity of
212 the l-LNvs via GABA signaling (Benito et al., 2008; Yoshii et al., 2008). To test this
213 idea, we knocked down *cry* in the s-LNvs using R6GAL4 while over-expressing
214 *dicer2* (*dcr2*) to enhance RNAi efficiency and observed a modest but significant
215 lengthening of sleep duration (Figure 5C; Figure 5—figure supplement 1A)
216 (Helfrich-Forster et al., 2007). However, when we adopted a *Pdf*GAL80 to block the
217 actions of GAL4 in the PDF neurons in *Gad1*GAL4/UAS*cry*RNAi, this does not alter
218 the long-sleep phenotype (Figure 5—figure supplement 1B) (Stoleru et al., 2004).
219 These series of results suggest that *cry* expression in the s-LNvs are necessary but not
220 sufficient to maintain normal sleep/wakefulness. We were indeed able to detect
221 GABA signal at the s-LNvs, while GABA intensity is enhanced in *cry* mutants under
222 4L20D but not 12L12D (Figure 5D-G). To further validate that the s-LNvs are
223 GABAergic, we knocked down *VGAT* in these cells and observed a decrease of
224 GABA intensity (Figure 5—figure supplement 2A and B).

225 Next, we examined the effects of *cry* deficiency on the activity level of the
226 s-LNvs. We found that *cry* mutation increases calcium concentration in these cells
227 under 4L20D, in stark contrast to that of the l-LNvs (Figure 5H and I). This strongly
228 suggests that *cry* deficiency leads to elevated neural activity in the s-LNvs.

229 Consistently, we observed increased sleep when we activated these cells using TrpA1,
230 similar to the long-sleep phenotype of *cry* mutants (Figure 5J; Figure 5—figure
231 supplement 3A). Because R6GAL4 is also expressed in several other cells in the brain,
232 we combined *Pdf*GAL80 to verify that the sleep-enhancing effect is caused by
233 over-activation of the s-LNvs (Helfrich-Forster et al., 2007). Indeed, we no longer
234 observed the long-sleep phenotype when GAL4 expression is inhibited in the PDF
235 neurons by GAL80 (Figure 5J; Figure 5—figure supplement 3A). Moreover, we found
236 that *cry* mutation can no longer exert effects on sleep when the s-LNvs are activated,
237 further validating that the s-LNvs mediate the influences of CRY on sleep (Figure 5K;
238 Figure 5—figure supplement 3B). Because CRY has been shown to regulate
239 membrane depolarization and input resistance together with the redox sensor of the
240 voltage-gated potassium channel β-subunit HYPERKINETIC (HK), we tested
241 whether CRY and HK also function together in the s-LNvs to regulate arousal
242 (Agrawal et al., 2017; Fogle et al., 2015). We found that while over-expressing *hk* in
243 the s-LNvs of WT flies does not alter sleep duration under 4L20D, over-expressing it
244 in *cry* mutants significantly increases sleep (Figure 5L; Figure 5—figure supplement
245 3C). This genetic interaction indicates that CRY and HK cooperate to regulate
246 wakefulness, possibly by modulating the electric activity of the s-LNvs.

247 Next, we expressed syt-GFP in the s-LNvs and observed GFP signal at the soma
248 of the l-LNvs, indicating that the s-LNvs send axonal terminals to the l-LNvs (Figure
249 6A). *trans*-Tango technique further demonstrated that the s-LNvs project to form
250 synaptic connections with the l-LNvs (Figure 6B). Moreover, when we disrupted

GABA transmission in the s-LNvs by knocking down *VGAT* or *Gad1*, this reduced GABA intensity in the l-LNvs, implicating that the s-LNvs release GABA onto the l-LNvs (Figure 6C-E). At the behavioral level, these manipulations supress the long-sleep phenotype of *cry* mutation (Figure 6F-I).

Taken together, these results suggest that *cry* deficiency in the s-LNvs results in increased neuronal activity and thus enhanced GABA release at the l-LNvs via direct synaptic projections, ultimately leading to decreased wakefulness and increased sleep.

Short photoperiod reduces GABA level at the l-LNvs

Our findings thus far point to an inhibitory role of CRY on the GABAergic tone, and this in turn removes the inhibition on the neural activities of the l-LNvs. Since CRY is degraded by light, we hypothesized that CRY should exert a stronger effect on GABA under short photoperiod (Emery et al., 1998)**.** Consistently, GABA intensity does appear to be down-regulated at the l-LNvs under 4L20D vs. 12L12D (Figure 7A and B). On the other hand, GABA intensity does not exhibit photoperiod-dependent alteration in the s-LNvs (Figure 7C and D). Presumably, this reduced GABA level at the l-LNvs under 4L20D can result in elevated activation of these cells and increased wakefulness. In line with this, WT flies display shortened sleep duration under 4L20D compared to 12L12D (Figure 7E and F). This sleep reduction is a result of decreased sleep bout length but not bout number, while wake activity is not altered by photoperiod (Figure 7G-I). These observations indicate that short photoperiod hampers sleep maintenance, similar to the effects of CRY.

Discussion

Previous studies have shown that CRY mediates light-induced electrical activity of the l-LNvs and acute arousal, which indicates that CRY can immediately act to promote wake and terminate sleep in response to light pulse (Fogle et al., 2015; Sheeba et al., 2008). At the molecular level, this is believed to be accomplished by a direct coupling of light-activated CRY with HK in the l-LNvs (Fogle et al., 2015). Here we found that CRY promotes extended wakefulness under short photoperiod by functioning as an inhibitor of GABAergic tone. In contrast to the previously characterized roles of CRY which are activated by light, we believe this novel function we identify here reflects a role for CRY in the dark. Under short photoperiods, CRY acts in the dark to inhibit GABA signaling and thus promote wakefulness, leading to reduced sleep during the dark phase compared to longer photoperiods. Indeed, CRY is an ideal signal for conveying photoperiodic information, for it is degraded by light and only accumulates during darkness (Emery et al., 1998). It can measure the length of the day/night and thus instruct downstream signaling components to modulate photoperiod-dependent processes. Consistent with this idea, the largest increases of sleep in *cry* mutants occur immediately after lights-off and prior to lights-on (Fig 1B), indicating that CRY functions to promote wakefulness during these time windows which are exactly the time windows that would be affected by photoperiod changes (i.e. there will be light during these time windows under longer photoperiods). In other words, CRY appears to act at the time of the day that is most sensitive to photoperiodic changes, which fits perfectly with the idea that it serves as an instructive signal of day/night length.

While EOS and NipA treatment lengthen sleep duration as previously reported, we are somewhat surprised by the observation that knocking down *Gad1* or *VGAT* in GABAergic neurons also extends sleep duration (Ki and Lim, 2019; Leal and Neckameyer, 2002). We reason this may be due to some sort of over-compensation induced by chronic GABA deficiency to maintain excitation/inhibition balance, as previous studies have reported that the amplitude of glutamatergic current is substantially down-regulated in *Gad1* mutant flies and increased in *Gad1* over-expression flies (Featherstone et al., 2000), (Featherstone et al., 2002). Consistent with this idea, we noticed when *cry* is knocked down in *Gad1/VGAT* RNAi flies, sleep duration is shortened and comparable to that of the controls. This is probably because *cry* depletion enhances the GABAergic tone, thus normalizing GABA signaling in these flies which results in normal sleep duration.

We acknowledge that THIP treatment leads to prominent lengthening of sleep duration, and thus it is possible in this case *cry* deficiency no longer increases sleep duration due to a ceiling effect rather than epistatic interaction. Nonetheless, considering that CBZ and *rdl* mutation also block the effect of *cry* deficiency on sleep duration, it is highly likely that GABA-A receptor mediates the influences of CRY on sleep. One caveat is that the $rdl^{MD\text{-}RR}$ mutation has been shown to diminish the desensitization of GABA-A receptor and is thus believed to be a gain-of-function allele, but here we found that it eliminates the effect of *cry* RNAi on sleep (Zhang et al., 1994). Since treatment with GABA-A agonist THIP leads to substantially increased sleep while under the same condition $rdl^{MD\text{-}RR}$ mutation does not appear to

317 significantly alter sleep duration, we suspect that similar to *Gad1/VGAT* RNAi,
318 chronic enhancement of GABA-A function associated with *rdl*$^{MD\text{-}RR}$ mutation may
319 also trigger some kind of compensatory mechanism that counteracts this increased
320 GABA-A activity. Consequently, the influences of *cry* RNAi on sleep are blocked. Of
321 course, a lot more in depth characterizations are required to elucidate these issues.

322 The s-LNvs have been reported to receive GABAergic inputs possibly via
323 GABA-B receptors, but have not been shown to be able to synthesize GABA (Dahdal
324 et al., 2010; Hamasaka et al., 2005). Here we observed that *Gad1*GAL4 is expressed
325 in these cells, and their GABA intensity is reduced when we use R6GAL4 to knock
326 down *VGAT* in these cells. R6GAL4 drives prominent expression in the s-LNvs with
327 very little if any expression in the l-LNvs, and weaker (and not very consistent)
328 expression in several other neurons in the protocerebrum, pars intercerebralis and
329 subesophageal area which are all believed to lie outside of the circadian neuron
330 network (Helfrich-Forster et al., 2007). Therefore, we reason that the alteration of
331 GABA signal associated with knocking down *VGAT* should arise from VGAT
332 deficiency within the s-LNvs. Taken together, these results suggest that the s-LNvs are
333 GABAergic. Although knocking down *VGAT* or *Gad1* in these cells can suppress the
334 long sleep phenotype of *cry* mutants, knocking down *cry* in these cells only lead to a
335 modest lengthening of sleep duration. Moreover, inhibiting *cry* RNAi expression in
336 PDF neurons does not eliminate the long-sleep phenotype of
337 *Gad1*GAL4/UAS*cry*RNAi flies. Therefore, we suspect that *cry* deficiency in other
338 GABAergic neurons are also required for the long-sleep phenotype. Given that the

339 s-LNvs are known to express CRY and appear to be GABAergic based on our
340 findings here, we believe that CRY acts at least in part in the s-LNvs to promote
341 wakefulness under short photoperiod.

342 The molecular mechanism by which CRY down-regulates the GABAergic tone
343 remains unclear. Besides conducting light-driven depolarization via HK, CRY has
344 also been shown to act in synergy with HK to prevent membrane input resistance
345 from falling to a low level in larval salivary glands (Agrawal et al., 2017; Fogle et al.,
346 2015). In contrast to previous studies, here we found that lack of CRY increases the
347 activity of the s-LNvs. Instead of functioning in synergy with HK, CRY appears to act
348 in the opposite direction of HK as over-expressing *hk* enhances the long-sleep
349 phenotype caused by *cry* mutation. We reason that the coupling between CRY and HK
350 as well as their influences on the electric activity in the s-LNvs may be different from
351 that of the l-LNvs and larval salivary glands. Nonetheless, our results also support an
352 interaction between CRY and HK to promote arousal. We suspect that CRY acts via
353 HK to inhibit the activity of the s-LNvs, which results in decreased GABA release and
354 dis-inhibition of the l-LNvs. Extensive further investigations will be needed to
355 elucidate the mechanism by which CRY regulates the activity of the s-LNvs.

356 In conclusion, here we describe a CRY-controlled GABAergic circuitry involving
357 the l-LNvs and the s-LNvs that adjusts sleep duration in adaptation to changes in day
358 length and propose a mechanistic explanation regarding how this circuitry functions
359 (Fig 7J). Under short photoperiods, more CRY accumulates and inhibits the activity of
360 the GABAergic s-LNvs, leading to a dis-inhibition of the l-LNvs which can release

more PDF and promote arousal. Under longer photoperiods, on the other hand, less CRY can accumulate and thus the s-LNvs will exert more inhibitory influences on the l-LNvs, leading to decreased release of PDF and wakefulness. Notably, almost all neurons in the mammalian pacemaker, the suprachiasmatic nucleus, are GABAergic, and GABA/GABA-A signaling have been shown to mediate neuronal coupling in response to photoperiod changes (Ono et al., 2021). We believe a similar GABAergic circuitry may exist in the mammalian system that adjusts sleep/wakefulness to photoperiodic changes.

Materials and Methods

Fly strains

All strains were obtained from Bloomington *Drosophila* Stock Center, Vienna Drosophila Resource Center and TsingHua Fly Center or as gifts from colleagues. Except for *Gad1*GAL4, all neurotransmitter related GAL4 lines were generated in Dr. Yi Rao's laboratory (Deng et al., 2019). The following fly strains were used in this study: cry^{b} (BDSC:80921), per^{0} (BDSC:80917), tim^{0} (BDSC:80922), clk^{jrk} (BDSC:80927), cyc^{0} (BDSC:80929), Pdf^{01} (BDSC:26654), $Pdfr^{han5304}$ (BDSC:33068), UAS-*cry*RNAi-1 (THU4761), UAS-*cry*RNAi-2 (THU5148), cry^{03} (Dolezelova et al., 2007), UAS-*gad1*RNAi-1 (THU2920), $Rdl^{MD\text{-}RR}$ (BDSC:1675), UAS-*VGAT*RNAi-1 (THU4303), UAS-*VGAT*RNAi-2 (V45916), UAS-*hk* (BDSC:86270), isogenic w^{1118} (BDSC:5905), UAS-*NachBac* (BDSC:9469), *Pdf*GAL4 (BDSC:6899), *Gad1*GAL4 (BDSC:51630), *VGAT*GAL4 (BDSC:58980), UAS-*TrpA1* (BDSC:26263),

383 UAS-*Kir2.1* (BDSC:6596), UAS-*dcr2* (V6009), UAS-syt*GFP* (BDSC:6925),
384 UAS-nls*GFP* (BDSC:4775), UAS-*myr*GFP,QUAS-mtdtomato(3XHA);*Trans*-tango
385 (BDSC:77480)，13XLexAop2-*post*-*t*-GRASP,20XUAS-*pre*-mGRASP (BDSC:79040),
386 *Dop2R*GAL4, *TH*GAL4, *Trh*GAL4, *AdoRGAL4*, *DAT*GAL4, *GABAR1*GAL4,
387 *GABAR3*GAL4, *SerT*GAL4, *GluRIA*GAL4, R6GAL4(Helfrich-Forster et al., 2007),
388 c929GAL4(Park et al., 2008), *Pdf*GAL80(Stoleru et al., 2004),
389 UAS-GCaMP6m-Tdtomato (Chen et al., 2013) and *Pdf*-LexA(Shang et al., 2008). All
390 flies used for sleep monitoring were backcrossed with the isogenic w^{1118} strain for at
391 least 5 times except for cry^{03} which was backcrossed three times. All experiments
392 were conducted in male flies unless otherwise specified.

393 **Fly sleep monitoring and analysis**

394 Flies were raised on standard cornmeal-yeast-sucrose medium and kept in 12L12D at
395 25°C until behavior monitoring. ~3 to 4-day-old flies were entrained under different
396 photoperiods at 25℃ for 4 days, and then their activities in the next 3 days were
397 analyzed. Sleep is defined as 5 min consecutive inactivity. Sleep was analyzed with
398 Counting Macro written in Excel (Microsoft) following previously published protocol
399 (Pfeiffenberger et al., 2010). Flies were fed with agar-sucrose food (2% agar, 5%
400 sucrose) during entire sleep monitoring. TrpA1 flies were raised at 21°C and baseline
401 sleep was monitored at 21°C. Temperature was then raised at lights on to 29°C for
402 further sleep monitoring.

403 **Drug treatment**

404 For pharmacological experiments, drugs were mixed in the fly food at the following

concentrations. For nipecotic acid (10 mg/ml, Sigma) and EOS (10 mM, Sigma), drugs were fed during the entire sleep monitoring. For THIP (10 ug/ml, Sigma), SKF-97541 (10 ug/ml, Tocris) and CBZ (0.15 mg/ml, Sinopharm Chemical Reagent), drugs were fed for 1 day after baseline sleep monitoring. The same amount of solvent was added into the fly food as vehicle control.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Approximately 50 5-day-old flies were collected and frozen immediately on dry ice. Fly heads were isolated and homogenized in Trizol reagent (Life Technologies). Total RNA was extracted and qRT-PCR conducted following our previously published procedures (Bu et al., 2019). The following primers were used to quantify mRNA level, *cry* F: TGCAGGTACCAAGAATGTGG, R: GTCCACGTCCATCAGTTGC. *Gad1* F: TGCCACCACATTGAAGTACC, R: GGTGAACATGTTGGTGTTCG. *VGAT* F: ACGGCTTTAGGCAAGGTAGC, R: TTGGAATTCGTCGATTTTGC.

Immunostaining

Male flies were entrained for 3 days under indicated photoperiod and collected on Day 4. flies were anesthetized with CO_2 and dissected and fixed with 4% paraformaldehyde diluted in PBS at the indicated time points. The brains were then fixed with 4% paraformaldehyde for 30 minutes. Samples were washed with PBT (PBS with 0.3% TritonX-100) for 3 × 10min. Then samples were incubated in PBS with 1% TritonX-100 for 20 min at room temperature. Brain samples were then blocked in PBT with 5% fetal bovine serum (Hyclone) for 30 minutes and subsequently incubated with mouse anti-PDF (1:100, DSHB), rabbit anti-GABA

427 (1:200, Sigma), and anti-HA (1:100, DSHB) for 2-4 days at 4°C (4 days for
428 anti-GABA and 2 days for the other antibodies). After PBT rinses for 3 times, the
429 brains were incubated with donkey anti-mouse Alexa Fluor-594 (1:1000, Life
430 Technologies), donkey anti-rabbit Alexa Fluor-488 (1:1000, Abcam) and donkey
431 anti-rabbit Alexa Fluor-647 (1:1000, Abcam) overnight at 4°C. Then the brains were
432 rinsed 3 times in PBS and mounted and imaged using Olympus FV3000 confocal
433 microscope with a 60× objective lens. The intensity of GABA signal was quantified
434 by ImageJ software. For each cell, the image slice with the strongest signal in the Z
435 stack was selected and average intensity was quantified. A region on the same image
436 slice was then selected as background and its intensity value was subtracted from the
437 average intensity value of the cell. This subtracted value was subsequently normalized
438 to the average intensity of the control group as indicated in figure legends. Sample
439 sizes in the legends indicate number of cells examined per genotype. For *trans*-Tango
440 experiment, offsprings were raised at 18°C for 3-4 weeks before HA and PDF
441 immunostaining.

442 **Calcium imaging**

443 For live imaging experiments (GCaMP and tdTomato), flies 2-3 days old were
444 collected into tubes with standard food and entrained under LD for 3 days. Flies were
445 anesthetized with CO_2 and brains were dissected at the indicated time points in
446 *Drosophila* adult hemolymph-like saline solution. The dissected brain samples were
447 put on glass slide and sealed with cover slide. The duration for dissection and
448 microscopy should be completed within 0.5 hours for each time point. Images were

449 captured with Olympus FV3000 confocal microscopy with a 20× objective lens. The
450 intensity of GCaMP and tdTomato signals were quantified by ImageJ software.
451 Sample sizes in the legends indicate number of cells examined per genotype.

452 **Statistical Analysis**

453 For data that fit normal distribution, two-tailed Student's *t*-test (Microsoft Excel) was
454 used to compare the difference between two genotypes. For data that do not fit normal
455 distribution, Mann-Whitney test (Prism Graphpad) was used to compare the
456 difference between two genotypes. For multiple comparisons, one-way ANOVA with
457 Bonferroni multiple comparison test (Prism Graphpad) was used. Sample size,
458 statistical test, and significance values are indicated in figure legends. Sample size is
459 determined based on previous studies with similar experimental assays.

460

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469

470 ## Author contributions

L.C. and L.Z. designed the experiments. L.C. and D.T. conducted the experiments. L.C., C.S. and L.Z. wrote the paper.

Disclosure and competing interests statement

The authors declare no competing interests.

Data Availability

This study includes no data deposited in external repositories.

References

Agosto, J., Choi, J.C., Parisky, K.M., Stilwell, G., Rosbash, M., and Griffith, L.C. (2008). Modulation of GABA(A) receptor desensitization uncouples sleep onset and maintenance in Drosophila. Nat Neurosci *11*, 354-359.

Agrawal, P., Houl, J.H., Gunawardhana, K.L., Liu, T., Zhou, J., Zoran, M.J., and Hardin, P.E. (2017). Drosophila CRY Entrains Clocks in Body Tissues to Light and Maintains Passive Membrane Properties in a Non-clock Body Tissue Independent of Light. Curr Biol *27*, 2431-2441 e2433.

Allada, R., and Chung, B.Y. (2010). Circadian organization of behavior and physiology in Drosophila. Annu Rev Physiol *72*, 605-624.

Allada, R., White, N.E., So, W.V., Hall, J.C., and Rosbash, M. (1998). A mutant *Drosophila* homolog of mammalian *Clock* disrupts circadian rhythms and transcription of period and timeless. Cell *93*, 791-804.

Baines, R.A., Uhler, J.P., Thompson, A., Sweeney, S.T., and Bate, M. (2001). Altered electrical properties in Drosophila neurons developing without synaptic transmission. J Neurosci *21*, 1523-1531.

Benito, J., Houl, J.H., Roman, G.W., and Hardin, P.E. (2008). The blue-light photoreceptor CRYPTOCHROME is expressed in a subset of circadian oscillator neurons in the Drosophila CNS. J Biol Rhythms *23*, 296-307.

Bu, B., He, W., Song, L., and Zhang, L. (2019). Nuclear Envelope Protein MAN1 Regulates the Drosophila Circadian Clock via Period. Neurosci Bull *35*, 969-978.

Busza, A., Emery-Le, M., Rosbash, M., and Emery, P. (2004). Roles of the two Drosophila CRYPTOCHROME structural domains in circadian photoreception. Science *304*, 1503-1506.

Ceriani, M.F., Darlington, T.K., Staknis, D., Mas, P., Petti, A.A., Weitz, C.J., and Kay, S.A. (1999). Light-dependent sequestration of TIMELESS by CRYPTOCHROME. Science *285*, 553-556.

Chen, T.W., Wardill, T.J., Sun, Y., Pulver, S.R., Renninger, S.L., Baohan, A., Schreiter, E.R., Kerr, R.A., Orger, M.B., Jayaraman, V.*, et al.* (2013). Ultrasensitive fluorescent proteins for imaging neuronal

activity. Nature *499*, 295-300.
Chung, B.Y., Kilman, V.L., Keath, J.R., Pitman, J.L., and Allada, R. (2009). The GABA(A) receptor RDL acts in peptidergic PDF neurons to promote sleep in Drosophila. Curr Biol *19*, 386-390.
Dahdal, D., Reeves, D.C., Ruben, M., Akabas, M.H., and Blau, J. (2010). Drosophila pacemaker neurons require g protein signaling and GABAergic inputs to generate twenty-four hour behavioral rhythms. Neuron *68*, 964-977.
Deng, B., Li, Q., Liu, X., Cao, Y., Li, B., Qian, Y., Xu, R., Mao, R., Zhou, E., Zhang, W., *et al*. (2019). Chemoconnectomics: Mapping Chemical Transmission in Drosophila. Neuron *101*, 876-893 e874.
Dolezelova, E., Dolezel, D., and Hall, J.C. (2007). Rhythm defects caused by newly engineered null mutations in Drosophila's cryptochrome gene. Genetics *177*, 329-345.
Emery, P., So, W.V., Kaneko, M., Hall, J.C., and Rosbash, M. (1998). CRY, a Drosophila clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. Cell *95*, 669-679.
Enell, L., Hamasaka, Y., Kolodziejczyk, A., and Nassel, D.R. (2007). gamma-Aminobutyric acid (GABA) signaling components in Drosophila: immunocytochemical localization of GABA(B) receptors in relation to the GABA(A) receptor subunit RDL and a vesicular GABA transporter. J Comp Neurol *505*, 18-31.
Featherstone, D.E., Rushton, E., and Broadie, K. (2002). Developmental regulation of glutamate receptor field size by nonvesicular glutamate release. Nat Neurosci *5*, 141-146.
Featherstone, D.E., Rushton, E.M., Hilderbrand-Chae, M., Phillips, A.M., Jackson, F.R., and Broadie, K. (2000). Presynaptic glutamic acid decarboxylase is required for induction of the postsynaptic receptor field at a glutamatergic synapse. Neuron *27*, 71-84.
ffrench-Constant, R.H., Steichen, J.C., Rocheleau, T.A., Aronstein, K., and Roush, R.T. (1993). A single-amino acid substitution in a gamma-aminobutyric acid subtype A receptor locus is associated with cyclodiene insecticide resistance in Drosophila populations. Proc Natl Acad Sci U S A *90*, 1957-1961.
Fogle, K.J., Baik, L.S., Houl, J.H., Tran, T.T., Roberts, L., Dahm, N.A., Cao, Y., Zhou, M., and Holmes, T.C. (2015). CRYPTOCHROME-mediated phototransduction by modulation of the potassium ion channel beta-subunit redox sensor. Proc Natl Acad Sci U S A *112*, 2245-2250.
Golden, R.N., Gaynes, B.N., Ekstrom, R.D., Hamer, R.M., Jacobsen, F.M., Suppes, T., Wisner, K.L., and Nemeroff, C.B. (2005). The efficacy of light therapy in the treatment of mood disorders: a review and meta-analysis of the evidence. Am J Psychiatry *162*, 656-662.
Hamada, F.N., Rosenzweig, M., Kang, K., Pulver, S.R., Ghezzi, A., Jegla, T.J., and Garrity, P.A. (2008). An internal thermal sensor controlling temperature preference in Drosophila. Nature *454*, 217-220.
Hamasaka, Y., Wegener, C., and Nassel, D.R. (2005). GABA modulates Drosophila circadian clock neurons via GABAB receptors and decreases in calcium. J Neurobiol *65*, 225-240.
Helfrich-Forster, C., Shafer, O.T., Wulbeck, C., Grieshaber, E., Rieger, D., and Taghert, P. (2007). Development and morphology of the clock-gene-expressing lateral neurons of Drosophila melanogaster. J Comp Neurol *500*, 47-70.
Hyun, S., Lee, Y., Hong, S.T., Bang, S., Paik, D., Kang, J., Shin, J., Lee, J., Jeon, K., Hwang, S., *et al.* (2005). Drosophila GPCR Han is a receptor for the circadian clock neuropeptide PDF. Neuron *48*, 267-278.
Jackson, F.R., Newby, L.M., and Kulkarni, S.J. (1990). Drosophila GABAergic systems: sequence and expression of glutamic acid decarboxylase. J Neurochem *54*, 1068-1078.

Ki, Y., and Lim, C. (2019). Sleep-promoting effects of threonine link amino acid metabolism in Drosophila neuron to GABAergic control of sleep drive. eLife *8*.

Konopka, R.J., and Benzer, S. (1971). Clock mutants of Drosophila melanogaster. Proc Natl Acad Sci U S A *68*, 2112-2116.

Kurlansik, S.L., and Ibay, A.D. (2012). Seasonal affective disorder. Am Fam Physician *86*, 1037-1041.

Leal, S.M., and Neckameyer, W.S. (2002). Pharmacological evidence for GABAergic regulation of specific behaviors in Drosophila melanogaster. J Neurobiol *50*, 245-261.

Lear, B.C., Merrill, C.E., Lin, J.M., Schroeder, A., Zhang, L., and Allada, R. (2005). A G protein-coupled receptor, groom-of-PDF, is required for PDF neuron action in circadian behavior. Neuron *48*, 221-227.

Lin, F.J., Song, W., Meyer-Bernstein, E., Naidoo, N., and Sehgal, A. (2001). Photic signaling by cryptochrome in the Drosophila circadian system. Mol Cell Biol *21*, 7287-7294.

Mertens, I., Vandingenen, A., Johnson, E.C., Shafer, O.T., Li, W., Trigg, J.S., De Loof, A., Schoofs, L., and Taghert, P.H. (2005). PDF receptor signaling in Drosophila contributes to both circadian and geotactic behaviors. Neuron *48*, 213-219.

Nitabach, M.N., Wu, Y., Sheeba, V., Lemon, W.C., Strumbos, J., Zelensky, P.K., White, B.H., and Holmes, T.C. (2006). Electrical hyperexcitation of lateral ventral pacemaker neurons desynchronizes downstream circadian oscillators in the fly circadian circuit and induces multiple behavioral periods. J Neurosci *26*, 479-489.

Ono, D., Honma, K.I., and Honma, S. (2021). GABAergic mechanisms in the suprachiasmatic nucleus that influence circadian rhythm. J Neurochem *157*, 31-41.

Parisky, K.M., Agosto, J., Pulver, S.R., Shang, Y., Kuklin, E., Hodge, J.J., Kang, K., Liu, X., Garrity, P.A., Rosbash, M.*, et al.* (2008). PDF cells are a GABA-responsive wake-promoting component of the Drosophila sleep circuit. Neuron *60*, 672-682.

Park, D., Veenstra, J.A., Park, J.H., and Taghert, P.H. (2008). Mapping peptidergic cells in Drosophila: where DIMM fits in. PLoS One *3*, e1896.

Pfeiffenberger, C., Lear, B.C., Keegan, K.P., and Allada, R. (2010). Processing sleep data created with the Drosophila Activity Monitoring (DAM) System. Cold Spring Harb Protoc *2010*, pdb prot5520.

Renn, S.C., Park, J.H., Rosbash, M., Hall, J.C., and Taghert, P.H. (1999). A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in Drosophila. Cell *99*, 791-802.

Rosenthal, N.E., Sack, D.A., Gillin, J.C., Lewy, A.J., Goodwin, F.K., Davenport, Y., Mueller, P.S., Newsome, D.A., and Wehr, T.A. (1984). Seasonal affective disorder. A description of the syndrome and preliminary findings with light therapy. Arch Gen Psychiatry *41*, 72-80.

Sehgal, A., Price, J.L., Man, B., and Young, M.W. (1994). Loss of circadian behavioral rhythms and per RNA oscillations in the Drosophila mutant timeless [see comments]. Science *263*, 1603-1606.

Shang, Y., Griffith, L.C., and Rosbash, M. (2008). From the Cover: Feature Article: Light-arousal and circadian photoreception circuits intersect at the large PDF cells of the Drosophila brain. Proc Natl Acad Sci U S A *105*, 19587-19594.

Shearin, H.K., Quinn, C.D., Mackin, R.D., Macdonald, I.S., and Stowers, R.S. (2018). t-GRASP, a targeted GRASP for assessing neuronal connectivity. J Neurosci Methods *306*, 94-102.

Sheeba, V., Gu, H., Sharma, V.K., O'Dowd, D.K., and Holmes, T.C. (2008). Circadian- and light-dependent regulation of resting membrane potential and spontaneous action potential firing of Drosophila circadian pacemaker neurons. J Neurophysiol *99*, 976-988.

Shiga, Y., Tanaka-Matakatsu, M., and Hayashi, S. (1996). A nuclear GFP/ beta-galactosidase fusion protein as a marker for morphogenesis in living Drosophila. Dev Growth Differ *38*, 99-106.

Stanewsky, R., Kaneko, M., Emery, P., Beretta, B., Wagner-Smith, K., Kay, S.A., Rosbash, M., and Hall, J.C. (1998). The cry^b mutation identifies cryptochrome as a circadian photoreceptor in *Drosophila*. Cell *95*, 681-692.

Stoleru, D., Peng, Y., Agosto, J., and Rosbash, M. (2004). Coupled oscillators control morning and evening locomotor behaviour of Drosophila. Nature *431*, 862-868.

Talay, M., Richman, E.B., Snell, N.J., Hartmann, G.G., Fisher, J.D., Sorkac, A., Santoyo, J.F., Chou-Freed, C., Nair, N., Johnson, M., *et al.* (2017). Transsynaptic Mapping of Second-Order Taste Neurons in Flies by trans-Tango. Neuron *96*, 783-795 e784.

Wescott, D.L., Soehner, A.M., and Roecklein, K.A. (2020). Sleep in seasonal affective disorder. Curr Opin Psychol *34*, 7-11.

Wood, S., and Loudon, A. (2014). Clocks for all seasons: unwinding the roles and mechanisms of circadian and interval timers in the hypothalamus and pituitary. J Endocrinol *222*, R39-59.

Yoshii, T., Todo, T., Wulbeck, C., Stanewsky, R., and Helfrich-Forster, C. (2008). Cryptochrome is present in the compound eyes and a subset of Drosophila's clock neurons. J Comp Neurol *508*, 952-966.

Zhang, H.G., ffrench-Constant, R.H., and Jackson, M.B. (1994). A unique amino acid of the Drosophila GABA receptor with influence on drug sensitivity by two mechanisms. J Physiol *479 (Pt 1)*, 65-75.

Zhang, Y.Q., Rodesch, C.K., and Broadie, K. (2002). Living synaptic vesicle marker: synaptotagmin-GFP. Genesis *34*, 142-145.

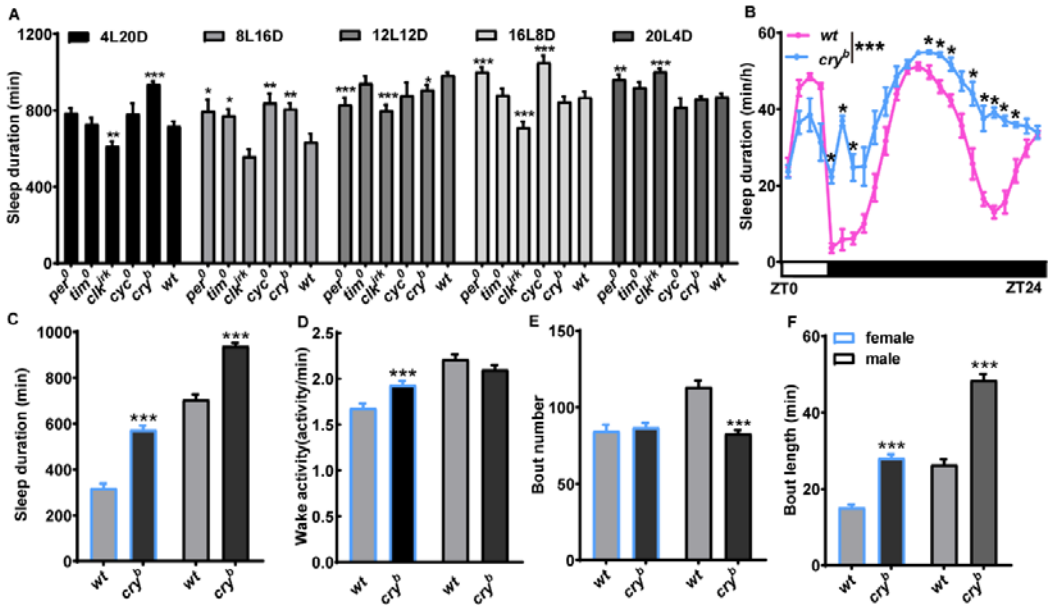
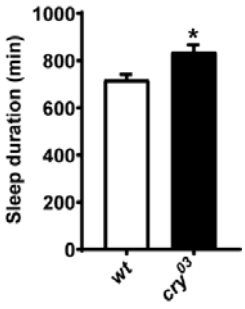


Figure 1. *cry* mutation increases sleep duration selectively under short photoperiods. (A)Daily sleep duration in male circadian clock gene mutants per^0, tim^0, clk^{jrk}, cyc^0, and cry^b compared to wild-type (WT) males under 4L20D, 8L16D, 12L12D, 16L8D, 20L4D (12L12D, n = 86, 63, 91, 14, 91, 53 flies; 8L16D, n = 21, 31, 31, 10, 32, 31 flies; 12L12D, n = 31, 14, 32, 14, 27, 32 flies; 16L8D, n = 36, 30, 33, 17, 36, 30 flies; 20L4D, n = 42, 55, 83, 26, 91, 89 flies).(B)Average sleep traces of cry^b and WT male flies under 4L20D. (C-F) The daily sleep duration (C), waking activity (D), sleep bout number (E), average sleep bout length (F) for cry^b and WT female and male flies under 4L20D (n = 56, 88, 58, 91 flies). For (B), statistical differences between cry^b and WT is determined by paired two-tailed *t*-test, $*P < 0.001$. For A and C-E, statistical differences is determined by two-tailed Student's *t*-test, $***P < 0.001$. Error bars represent standard error of the mean (SEM). ZT, Zeitgeber Time (ZT0 is the time of lights on).



628

629 **Figure 1—figure supplement 1. *cry* knock-out mutation lengthens sleep duration under**
630 **4L20D.** Daily sleep duration of male WT flies and *cry* knock-out flies, monitored under 4L20D (n
631 = 53, 35, 47). Student's *t*-test: $*P < 0.05$. Error bars represent SEM.

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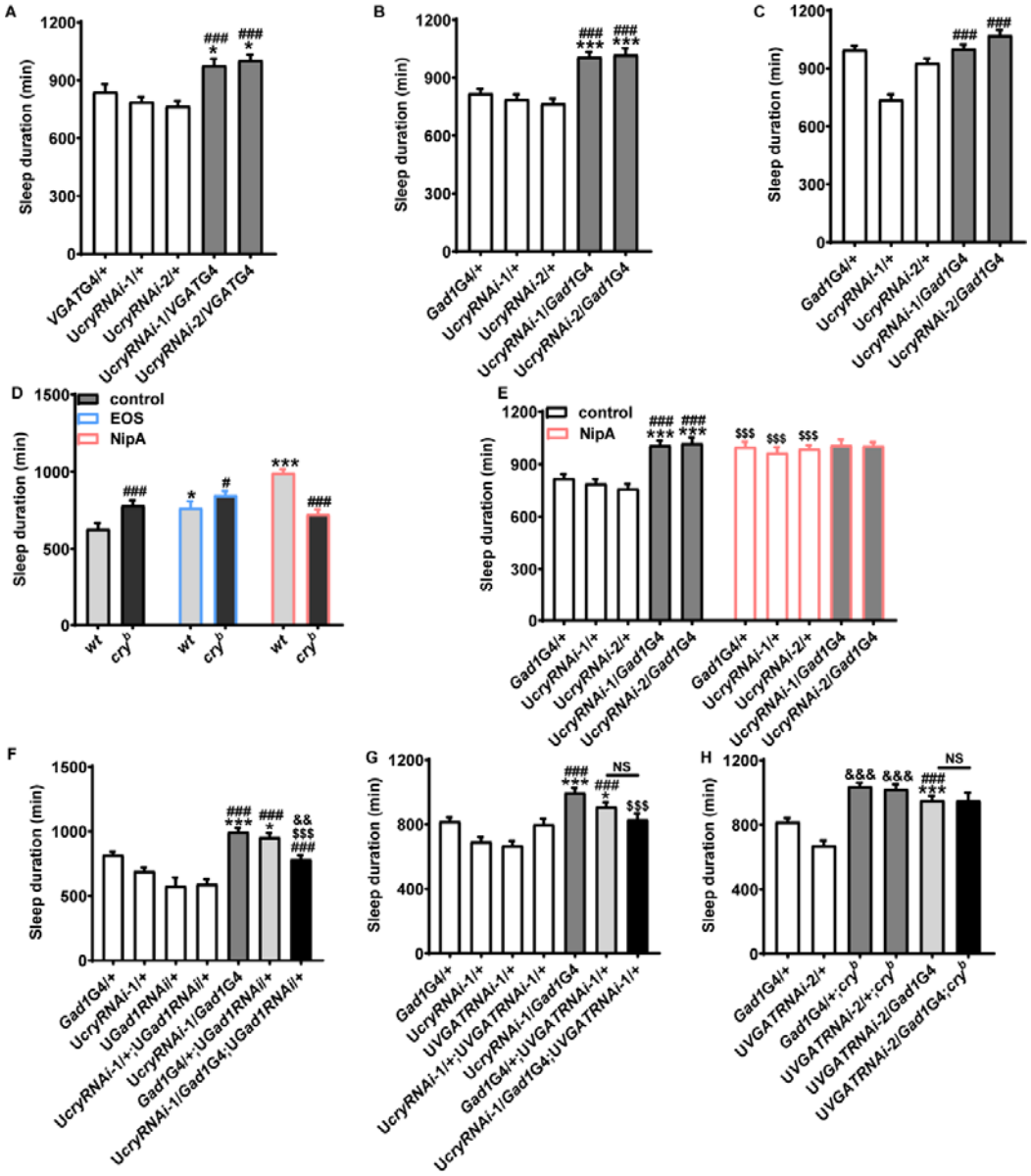


Figure 2. CRY functions in GABAergic neurons and promotes wakefulness via GABA signaling. (A, B) Daily sleep duration of male flies with *cry* knocked down in GABAergic neurons by *VGAT*GAL4 (A) (n = 72, 74, 96, 49, 54 flies) or *Gad1*GAL4 (B) (n = 34, 31, 31, 25, 23 flies) monitored under 4L20D.(C) Daily sleep duration of *cry* knocked down in GABAergic neurons by *Gad1*GAL4 monitored under 12L12D (n = 20, 19, 30, 26, 32, 20 flies). For A-C, One-way ANOVA with Bonferroni multiple comparison test: compared to GAL4 control, ***P < 0.001; compared to UAS control, #P < 0.05, ###P < 0.001.(D) Daily sleep duration of male WT and cry^b flies fed with EOS or NipA under 4L20D (n = 31, 32, 30, 30, 25, 32 flies). Two-tailed Student's t-test: compared to WT, ###P < 0.001; compared to vehicle control, *P < 0.05, ***P < 0.001.(E) Daily sleep duration of *cry* RNAi and control flies fed with NipA under 4L20D (n = 72, 74, 95, 49, 54, 23, 24, 26, 15, 24 flies). For comparison between RNAi flies vs. UAS/GAL4 controls, one-way ANOVA with Bonferroni multiple comparison test was used: compared to GAL4 control, ***P < 0.001; compared to UAS control, ###P < 0.001. For comparing to vehicle

control, two-tailed Student's *t*-test was used, $$$*P* < 0.001.(F) Daily sleep duration of male flies with *cry* and *gad1* knocked down in GABAergic neurons monitored under 4L20D (n = 72, 73, 24, 33, 45, 33, 27 flies). For comparison between RNAi flies vs. UAS/GAL4 controls, one-way ANOVA with Bonferroni multiple comparison test was used: compared to GAL4 control, ***P* < 0.01, ****P* < 0.001; compared to UAS control, ###*P* < 0.001. For comparing to RNAi control, two-tailed Student's *t*-test was used: compared to UAS*cry*RNAi/GAL4 control, $$$*P* < 0.001; compared to UAS*Gad1*RNAi/GAL4 control, &&&*P* < 0.001.(G) Daily sleep duration of male flies with *cry* and *VGAT* knocked down in GABAergic neurons monitored under 4L20D (n = 72, 73, 46, 41, 45, 66, 34 flies). For comparison between RNAi flies vs. UAS/GAL4 controls, one-way ANOVA with Bonferroni multiple comparison test was used: compared to GAL4 control, ****P* < 0.001; compared to UAS control, #*P* < 0.05, ### *P* < 0.001. For comparing to RNAi control, two-tailed Student's *t*-test was used: compared to UAS*cry*RNAi/GAL4 control, $$$*P* < 0.001; compared to UAS*VGAT*RNAi/GAL4 background, not significant. (H) Daily sleep duration of *cry* mutant flies with *VGAT* knocked down in GABAergic neurons monitored under 4L20D (n = 72, 59, 20, 23, 69, 22 flies). For comparison between RNAi flies vs. UAS/GAL4 controls, one-way ANOVA with Bonferroni multiple comparison test was used: compared to GAL4 control, ***P* < 0.01, ****P* < 0.001; compared to UAS control, #*P* < 0.05, ###*P* < 0.001. For comparison between mutant vs. control, two-tailed Student's *t*-test was used: compared to WT background, &&&*P* < 0.001; compared to U*VGAT*RNAi/GAL4, not significant. Error bars represent SEM; G4, GAL4; U, UAS; NS, not significant.

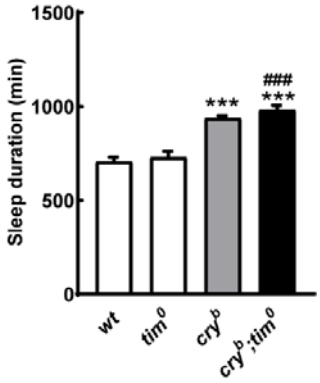
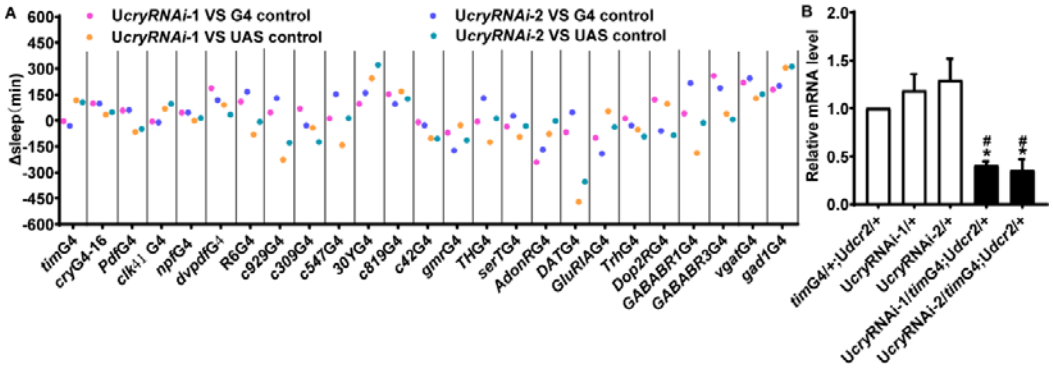


Figure 2—figure supplement 1. CRY does not regulate sleep duration via TIM. Daily sleep duration of male WT flies and flies mutant for *cry* and/or *tim*, monitored under 4L20D (n = 58, 63, 91, 33 flies). Two-tailed Student's *t*-test: compared to WT background, ***$P < 0.001$; compared to tim^0, ###$P < 0.001$. Error bars represent SEM.



674

675 **Figure 2—figure supplement 2. Screening for anatomical substrates that mediate the effects**
676 **of CRY on sleep/wakefulness.** (A) Difference in sleep duration between male *cry* RNAi flies
677 compared to GAL4 and UAS controls under 4L20D (n = 20-92). (B) Plots of relative mRNA
678 abundance for cry determined by qRT-PCR in whole-head extracts of *cry*RNAi and control flies
679 under 4L20D (n = 4). The value of the GAL4 control group was set to 1. Mann-Whitney test:
680 compared to GAL4 control, *$P < 0.05$; compared to UAS control, #$P < 0.05$. G4, GAL4; U, UAS.

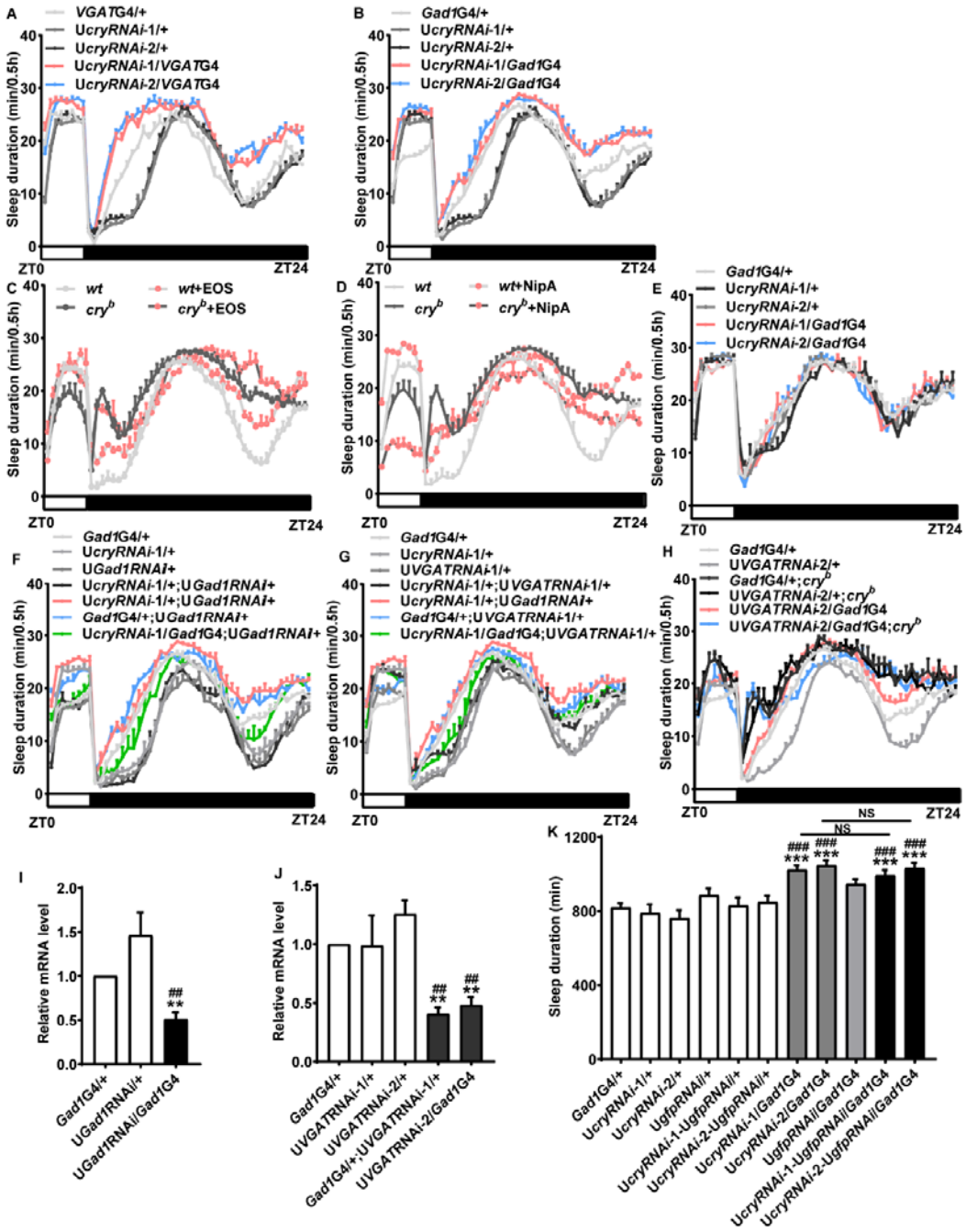


Figure 2—figure supplement 3. CRY promotes wakefulness via GABA signaling. (A, B) Sleep profile of male flies with cry knocked down in GABAergic neurons by *VGAT*GAL4 (A) or *Gad1*GAL4 (B) monitored under 4L20D. (C, D) Sleep profile of male WT and *cry* mutant flies fed with NipA (D) or EOS (E) under 4L20D in Fig 2D. White box indicates light period while black box indicates dark period. (E) Sleep profile of *cry* RNAi and control flies fed with NipA under 4L20D in Fig 2E. (F) Sleep profile of male flies with cry and *gad1* knocked down in GABAergic neurons monitored under 4L20D in Fig 2F. (G) Sleep profile of male flies with *cry* and *VGAT* knocked down in GABAergic neurons monitored under 4L20D in Fig 2G. (H) Sleep profile of *cry* mutant flies with *VGAT* knocked down in GABAergic neurons monitored under

4L20D in Fig 2H. (I, J) Plots of relative mRNA abundance for *Gad1* (J) and *VGAT* (K) determined by qRT-PCR in whole-head extracts of RNAi and control flies under 4L20D (n = 6). The value of the GAL4 control group was set to 1. Mann-Whitney test: compared to GAL4 control, $^{**}P < 0.01$; compared to UAS control, $^{\#\#}P < 0.01$. (K) Daily sleep duration of male flies with *cry* and *gfp* knocked down in GABAergic neurons by *Gad1*GAL4 monitored under 4L20D (n = 72, 26, 29, 20, 30, 31, 68, 32, 31, 29, 29). One-way ANOVA with Bonferroni multiple comparison test: compared to GAL4 control, $^{***}P < 0.001$; compared to UAS control, $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$. Error bars represent SEM; G4, GAL4; U, UAS; NS, not significant.

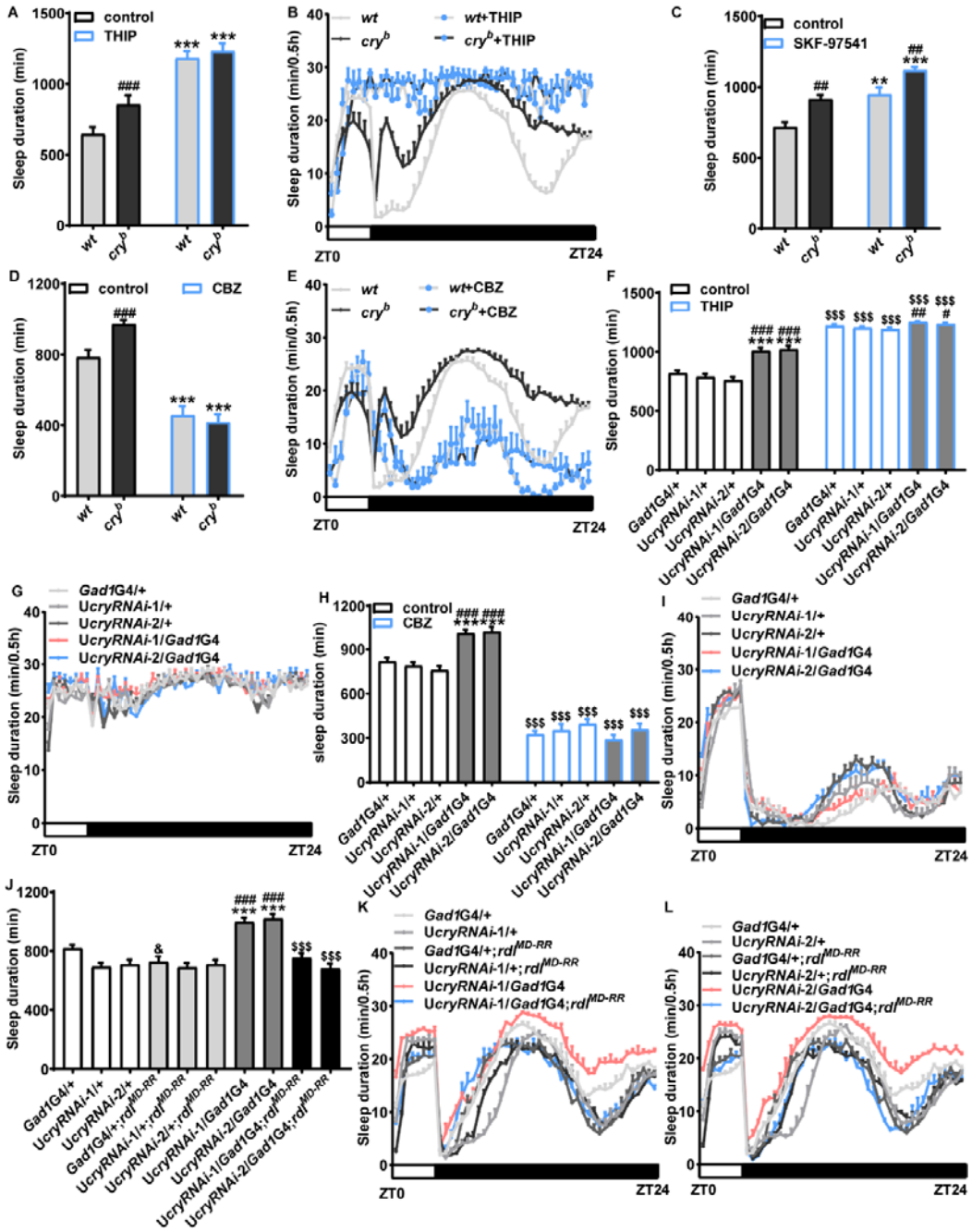


Figure 3. GABA-A receptor mediates the effects of CRY on sleep/wakefulness. (A, C, D) Daily sleep duration of male WT and *cry* mutant flies fed with THIP (A) (n = 28, 29, 16, 19 flies), SKF-97541 (C) (n = 25, 21, 23, 18 flies) or CBZ (D) (n = 28, 25, 31, 29 flies) under 4L20D. Two-tailed Student's *t*-test: compared to WT, ###$P < 0.001$; compared to vehicle control, ***$P < 0.001$.(B, E) Sleep profile of male WT and *cry* mutant flies fed with THIP (B) or CBZ (E) under 4L20D in A and D, respectively. White box indicates light period while black box indicates dark period. (F, H) Daily sleep duration of male *cry* RNAi and control flies fed with THIP (F) (n = 72, 74, 95, 49, 54, 23, 30, 27,27, 20 flies) or CBZ (H) (n = 72, 74, 95, 49, 54, 18, 22, 26, 30, 24 flies) under 4L20D. For comparison between RNAi flies vs. UAS/GAL4 controls, one-way ANOVA with Bonferroni multiple comparison test was used: compared to GAL4 control, ***$P < 0.001$;

compared to UAS control, ##$P < 0.01$, ###$P < 0.001$. For comparing to vehicle control, two-tailed Student's *t*-test was used, \$\$\$$P < 0.001$. (G, I) Sleep profile of male *cry* RNAi and control flies fed with THIP (G) or CBZ (I) under 4L20D in F and H, respectively. White box indicates light period while black box indicates dark period. (J) Daily sleep duration of male $rdl^{MD\text{-}RR}$ flies with *cry* knocked down in GABAergic neurons under 4L20D, along with relevant controls (n = 72, 73, 66, 44, 45, 33, 45, 54, 38, 33 flies). For comparison between RNAi flies vs. UAS/GAL4 controls, one-way ANOVA with Bonferroni multiple comparison test was used: compared to GAL4 control, ***$P < 0.001$; compared to UAS control, ###$P < 0.001$. For comparing to WT or UAS*cry*RNAi/GAL4 control, two-tailed Student's *t*-test was used: compared to WT, &$P < 0.05$; compared to UAS*cry*RNAi/GAL4 control, \$\$\$$P < 0.001$. (K, L) Sleep profile of male $rdl^{MD\text{-}RR}$ flies with *cry* knocked down in GABAergic neurons using *cry*RNAi-1 (K) or *cry*RNAi-2 (L) under 4L20D in J. Error bars represent SEM; G4, GAL4; U, UAS.

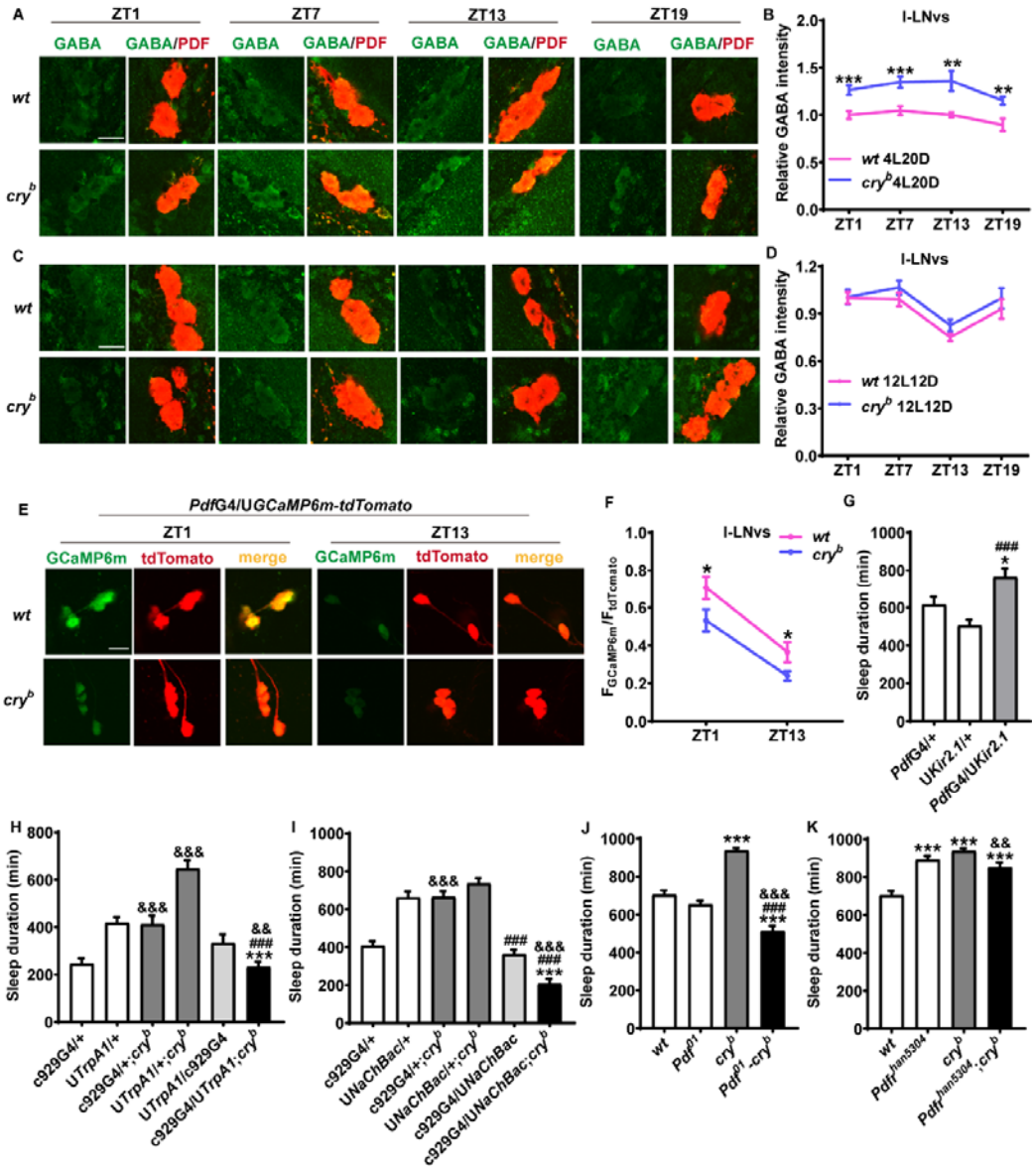


Figure 4. CRY increases the neural activity of the l-LNvs to promote wakefulness. (A-D) Brains from male WT and *cry* mutant flies dissected at ZT1, 7, 13, 19 under 4L20D (A) or 12L12D (C) are immunostained with GABA (green) and PDF (red) antisera, and representative l-LNvs are displayed. Merged signal is shown as yellow. Bar graphs represent normalized GABA intensity in the l-LNvs under 4L20D (B) (n = 26-56 cells) and 12L12D (D) (n = 32-128 cells). The average value of the control group at ZT1 is set to 1. (E) Representative live image of the l-LNvs expressing GCaMP6m and tdTomato using *Pdf*GAL4.Brain samples are dissected at the indicated time points under 4L20D. (F) Quantification of GCaMP6m signal intensity normalized to that of tdTomato (n = 25-54 cells). Student's *t*-test: **P* < 0.05, ***P* < 0.01, ****P* < 0.001. (G) Daily sleep duration of male flies expressing Kir2.1 in PDF neuron using *Pdf*GAL4 and controls, monitored under 4L20D (n = 32, 42, 30 flies). One-way ANOVA with Bonferroni multiple comparison test: compared to GAL4 control, **P* < 0.05; compared to UAS control, ###*P* < 0.001. (H) Daily sleep duration of male *cry* mutant flies expressing TrpA1 in the l-LNvs using c929GAL4 and relevant

controls, monitored under 4L20D and 29℃ to activate TrpA1 (n = 42, 42, 38, 35, 45, 37 flies). For comparison with UAS/GAL4 controls, one-way ANOVA with Bonferroni multiple comparison test was used: compared to GAL4 control, ***$P < 0.001$; compared to UAS control, ###$P < 0.001$. For comparison between mutant vs. control, two-tailed Student's *t*-test was used: &$P < 0.05$, &&&$P < 0.001$. (I) Daily sleep duration of *cry* mutant flies expressing NachBac in the l-LNvs using c929GAL4 and relevant controls, monitored under 4L20D (n = 45, 30, 32, 48, 55, 48 flies). For comparison with UAS/GAL4 controls, one-way ANOVA with Bonferroni multiple comparison test was used: compared to GAL4 control, *$P < 0.05$, ***$P < 0.001$; compared to UAS control, ###$P < 0.001$. For comparison between mutant vs. control, two-tailed Student's *t*-test was used: &$P < 0.05$, &&&$P < 0.001$. (J, K) Daily sleep duration of male Pdf^{01}-cry^{b} (J) (n = 58, 91, 89, 31 flies) and $Pdfr^{han5304}$;cry^{b} (K) (n = 58, 91, 31, 31 flies) mutants along with relevant controls, monitored under 4L20D. Two-tailed Student's *t*-test: compared to WT background, ***$P < 0.001$; compared to Pdf^{01}or $Pdfr^{han5304}$, ###$P < 0.001$; compared to cry^{b}, &&$P < 0.01$, &&&$P < 0.001$. The scale bar represents 15 μm. Error bars represent SEM. G4, GAL4; U, UAS.

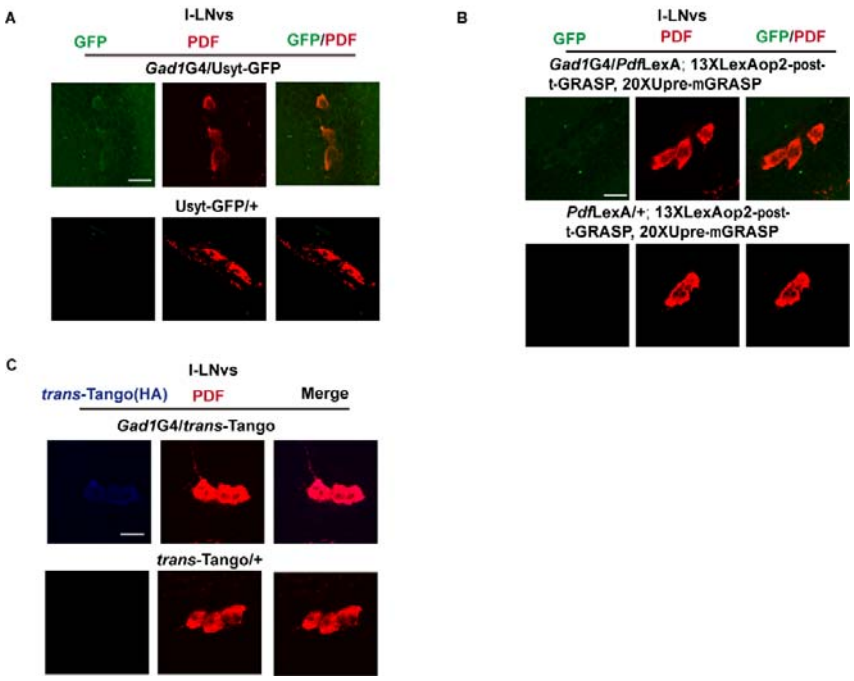


Figure 4—figure supplement 1. GABAergic neurons project to and form synapses with the l-LNvs. (A) l-LNvs of flies expressing syt-GFP in GABAergic neurons using *Gad1*Gal4 maintained under 4L20D and immunostained with PDF antisera. (B) l-LNvs of flies expressing Pre-mGRASP in GABAergic neurons using *Gad1*Gal4 and t-GRASP in the l-LNvs using *Pdf*LexA maintained under 4L20D. Brains are immunostained with PDF antisera. (C) l-LNvs of flies expressing trans-Tango in GABAergic neurons using *Gad1*Gal4 maintained under 4L20D and immunostained with HA and PDF antisera. G4, GAL4; U, UAS; LexAop, LexA operator.

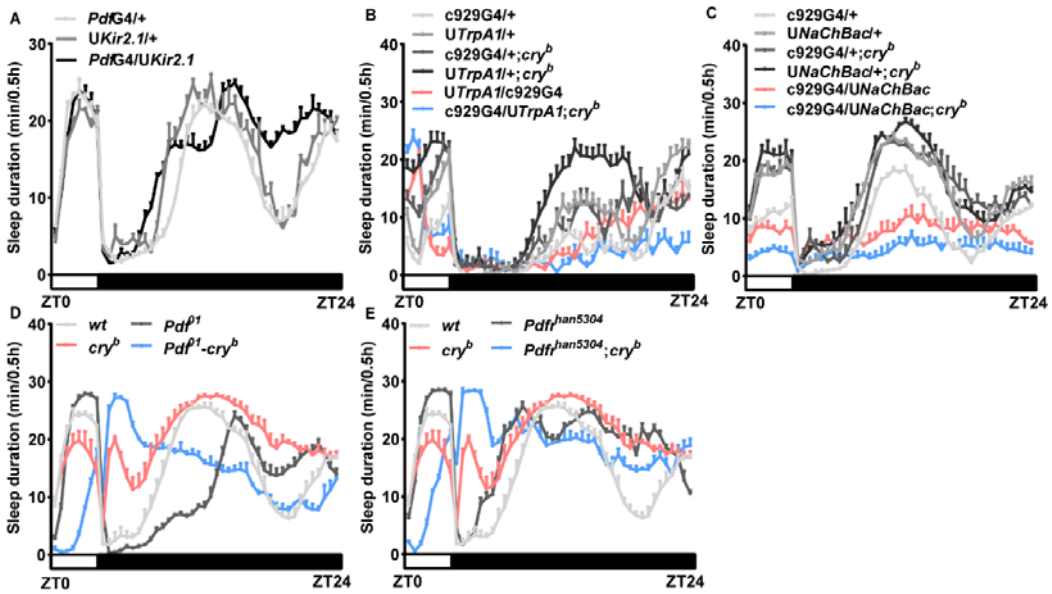


Figure 4—figure supplement 2. CRY promote wakefulness by impinging on the excitability of l-LNv and PDF signaling. (A) Sleep profile of male flies expressing Kir2.1 in PDF neuron using *Pdf*GAL4 and controls, monitored under 4L20D in Fig 4G. White box indicates light period while black box indicates dark period. (B) Sleep profile of male cry mutant flies expressing TrpA1 in the l-LNvs using c929GAL4 and relevant controls in Fig 4H, monitored under 4L20D and 29°C to activate TrpA1. (C) Sleep profile of cry mutant flies expressing NachBac in the l-LNvs using c929GAL4 and relevant controls in Fig 4I, monitored under 4L20D. (D, E) Daily sleep duration of male Pdf^{01}-cry^{b} (D) and $Pdfr^{han5304}$; cry^{b} (E) mutants along with relevant controls in Fig 4J and K, respectively, monitored under 4L20D.

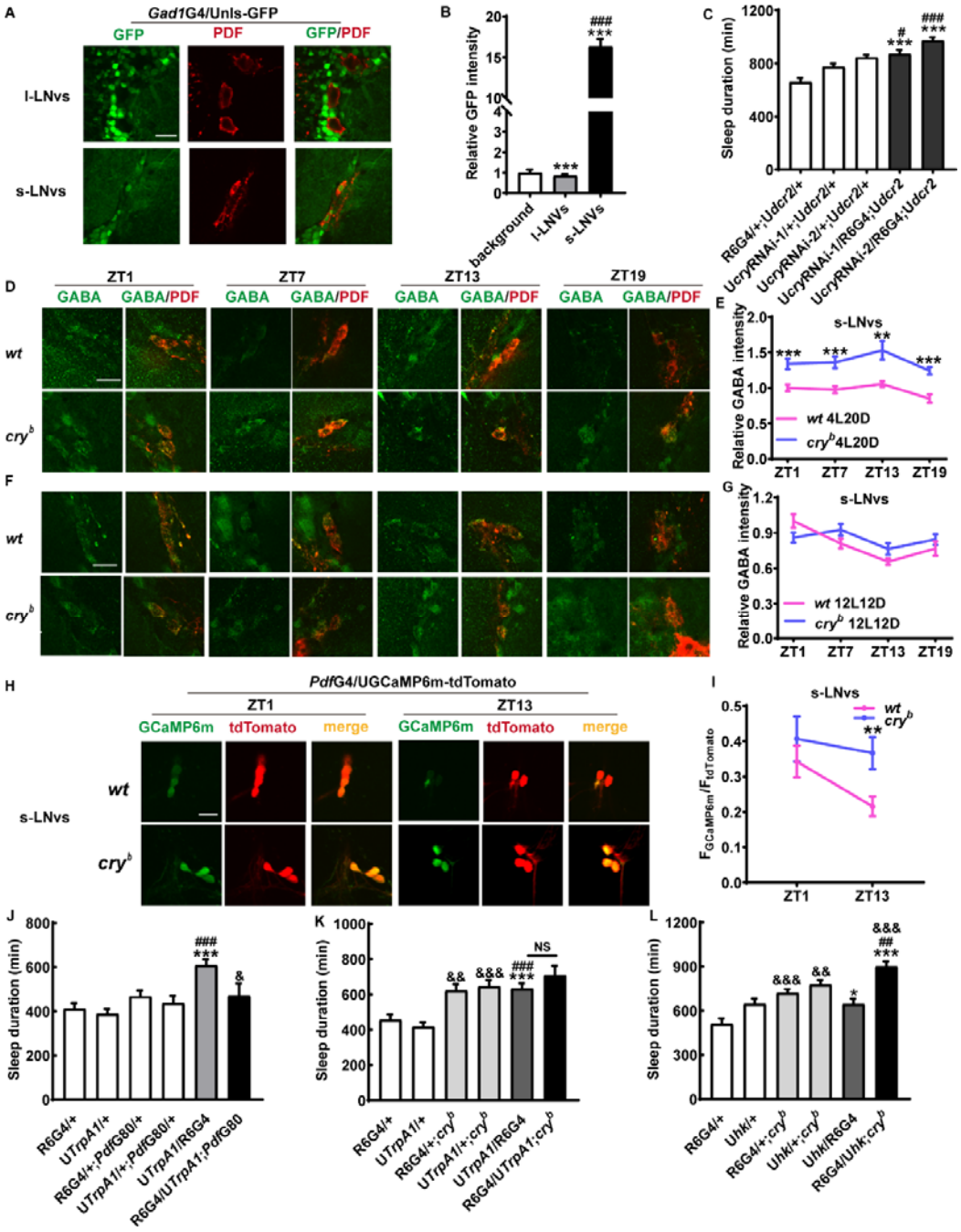


Figure 5. CRY acts in the s-LNvs to inhibit their neural activity and promote wakefulness. (A) Brains of male flies expressing nls-GFP driven by *Gad1*GAL4 maintained under 4L20D and immunostained with PDF antisera. Representative l-LNv and s-LNv are displayed. (B) Quantification of GFP signal intensity of PDF neuron (n = 20, 49, 43 cells). Two-tailed Student's *t* test: ***$P < 0.001$. (C) Daily sleep duration of flies with *cry* knocked down in the s-LNvs using R6GAL4 and relevant controls, monitored under 4L20D (n = 45, 60, 63, 34, 39 flies). (D-G) Brains from male WT and *cry* mutant flies dissected at ZT1, 7, 13, 19 under 4L20D (D) or 12L12D (F) are immunostained with GABA (green) and PDF (red) antisera, and representative s-LNvs are displayed. Merged signal is shown as yellow. Bar graphs represent normalized GABA

783 intensity in the s-LNvs under 4L20D (E) (n = 20-53 cells) and 12L12D (G) (n = 29-67 cells). The
784 average value of the control group at ZT1 is set to 1. (H) Representative live image of the s-LNvs
785 expressing GCaMP6m and tdTomato using *Pdf*GAL4. Brain samples are dissected at the indicated
786 time points under 4L20D. (I) Quantification of GCaMP6m signal intensity normalized to that of
787 tdTomato (n = 29-47 cells). Two-tailed Student's *t*-test: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (J)
788 Daily sleep duration of male flies expressing TrpA1 in the s-LNvs using R6GAL4 and relevant
789 controls, monitored under 4L20D and 29℃ to activate TrpA1 (n = 45, 54, 29, 22, 56, 22 flies). (K)
790 Daily sleep duration of male *cry* mutant flies expressing TrpA1 in the s-LNvs using R6GAL4 and
791 relevant controls, monitored under 4L20D and 29℃ to activate TrpA1 (n = 33, 42, 23, 35, 36, 20
792 flies). (L) Daily sleep duration of male *cry* mutant flies over-expressing HK in the s-LNvs using
793 R6GAL4 and relevant controls, monitored under 4L20D (n = 24, 29, 23, 30, 37, 22 flies). For
794 comparison with UAS/GAL4 controls, one-way ANOVA with Bonferroni multiple comparison
795 test was used: compared to GAL4 control, $*P < 0.05$, $***P < 0.001$; compared to UAS control,
796 $##P < 0.01$, $###P < 0.001$. For comparison between mutant vs. control, two-tailed Student's *t*-test
797 was used: compared to WT background, $\&\&P < 0.01$, $\&\&\&P < 0.001$. The scale bar represents 15
798 μm. Error bars represent SEM. G4, GAL4; U, UAS; NS, not significant.
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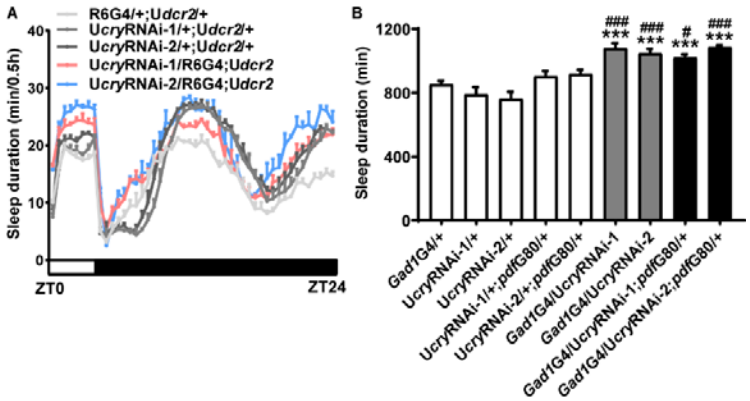

Figure 5—figure supplement 1. *cry* expression in the s-LNvs is necessary but not sufficient for maintaining normal sleep/wakefulness. (A) Sleep profile of *cry* knocked down in s-LNvs by R6GAL4 monitored under 4L20D in Fig 5C. White box indicates light period while black box indicates dark period. (B) Daily sleep duration of cry knocked down in PDF- GABAergic neurons using *Gad1*GAL4 and *Pdf*GAL80, monitored under 4L20D (n =101, 26, 29, 27, 31, 23, 32, 73, 63). One-way ANOVA with Bonferroni multiple comparison test: compared to GAL4 control, ***$P < 0.001$; compared to UAS control, #$P < 0.05$, ###$P < 0.001$. Error bars represent SEM. G4, GAL4; G80, GAL80; U, UAS.

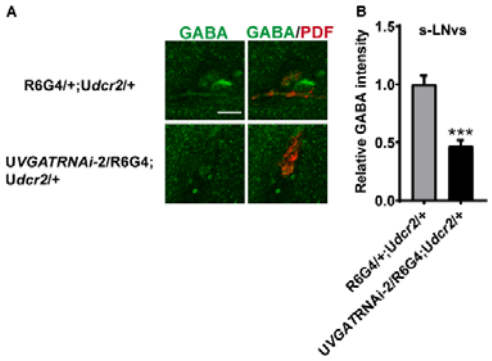

Figure 5—figure supplement 2. s-LNvs appear to be GABAergic neurons. (A) Brains from male flies with *VGAT* knocked down in the s-LNvs using R6GAL4 and control maintained under 4L20D and immunostained with GABA (green) and PDF (red) antisera. Representative s-LNvs are displayed. Merged signal is shown as yellow. (B) Bar graphs represent normalized GABA intensity in the s-LNvs with *VGAT* knocked down using R6GAL4 (n = 25, 32 cells). The average value of the control group is set to 1. Two-tailed Student's *t*-test, ***$P < 0.001$. The scale bar represents 15 µm. Error bars represent SEM. G4, GAL4; U, UAS.

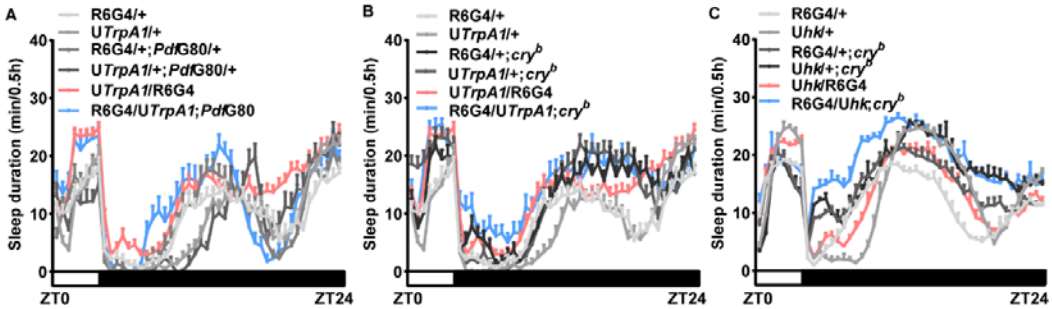

Figure 5—figure supplement 3. CRY promotes wakefulness by impinging on the excitability of the s-LNvs. (A) Sleep profile of male flies expressing TrpA1 in the s-LNvs using R6GAL4 and relevant controls in Fig 5J, monitored under 4L20D and 29°C to activate TrpA1. White box indicates light period while black box indicates dark period. (B) Sleep profile of male *cry* mutant flies expressing TrpA1 in the s-LNvs using R6GAL4 and relevant controls in Fig 5K, monitored under 4L20D and 29°C to activate TrpA1. (C) Sleep profile of male *cry* mutant flies over-expressing HK in the s-LNvs using R6GAL4 and relevant controls in Fig 5L, monitored under 4L20D. Error bars represent SEM. G4, GAL4; G80, GAL80; U, UAS.

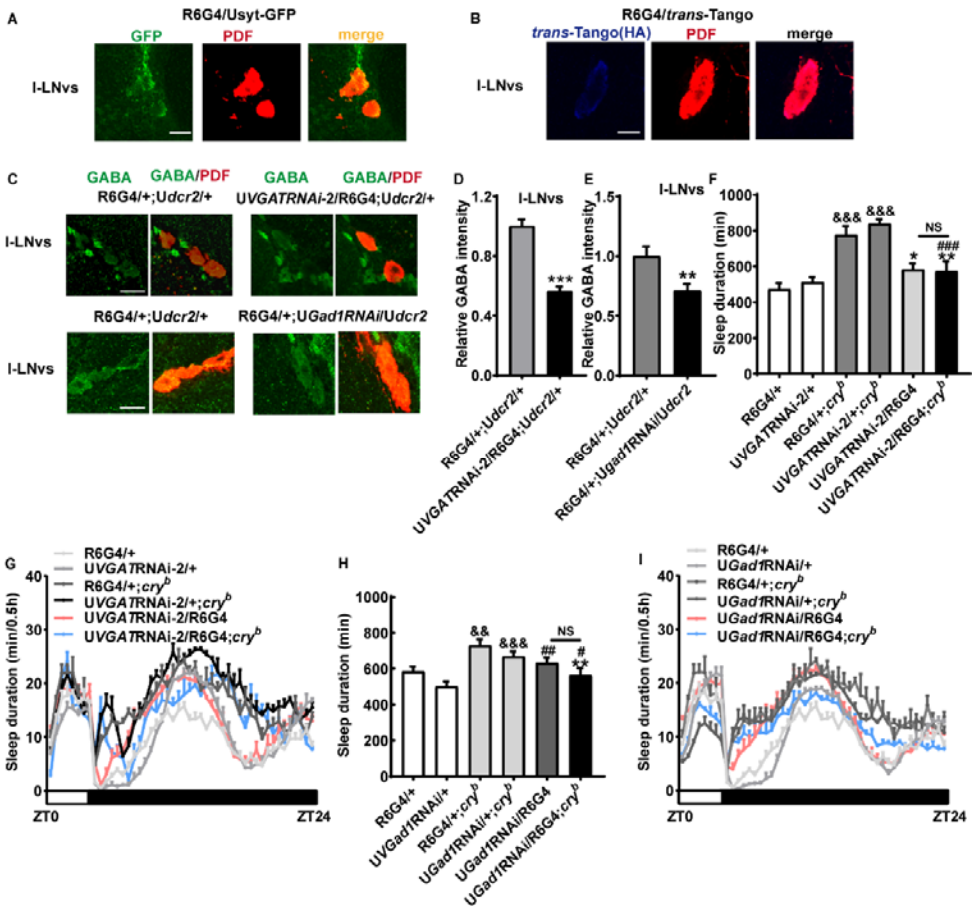

Figure 6. s-LNvs release GABA onto the l-LNvs. (A) l-LNvs of male flies expressing syt-GFP in the s-LNvs using R6GAL4 maintained under 4L20D and immunostained with PDF antisera. (B) l-LNvs of male flies expressing trans-Tango in the s-LNvs using R6GAL4 maintained under 4L20D and immunostained with HA and PDF antisera. (C) Brains from male flies with *VGAT* (top) or *Gad1* (bottom) knocked down in the s-LNvs using R6GAL4 and controls maintained under 4L20D and immunostained with GABA (green) and PDF (red) antisera. Representative l-LNvs are displayed. Merged signal is shown as yellow. (D, E) Bar graphs represent normalized GABA intensity in the l-LNvs of flies with *VGAT* (D) (n = 29, 37 cells) or *Gad1* (E) (n = 37, 51 cells) knocked down in the s-LNvs using R6GAL4, monitored under 4L20D. The average value of the control group is set to 1. Two-tailed Student's *t*-test: $^{**}P < 0.01$, $^{***}P < 0.001$. (F) Daily sleep duration of male *cry* mutant flies with *VGAT* knocked down in the s-LNvs using R6GAL4 and relevant controls, monitored under 4L20D (n = 31, 31, 32, 29, 43, 19 flies). For comparison between RNAi flies vs. UAS/GAL4 controls, one-way ANOVA with Bonferroni multiple comparison test was used: compared to GAL4 control, $^{*}P < 0.05$, $^{**}P < 0.01$; compared to UAS control, ###$P < 0.01$. For comparison between mutant vs. control, two-tailed Student's *t*-test was used: compared to WT background, &&&P < 0.001. (G) Sleep profile of male *cry* mutant flies with *VGAT* knocked down in the s-LNvs using R6GAL4 and relevant controls in F, monitored under 4L20D. (H) Daily sleep duration of male *cry* mutant flies with *Gad1* knocked down in the s-LNvs using R6GAL4 and relevant controls, monitored under 4L20D (n = 59, 59, 53, 60, 62, 45 flies). For comparison between RNAi flies vs. UAS/GAL4 controls, one-way ANOVA with

Bonferroni multiple comparison test was used: compared to GAL4 control, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; compared to UAS control, #$P < 0.05$, ##$P < 0.01$. For comparison between mutant vs. control, two-tailed Student's *t*-test was used: compared to WT background, &&&$P < 0.001$. (I) Sleep profile of male *cry* mutant flies with *Gad1* knocked down in the s-LNvs using R6GAL4 and relevant controls in H, monitored under 4L20D.The scale bar represents 15 μm. Error bars represent SEM. G4, GAL4; U, UAS; NS, not significant.

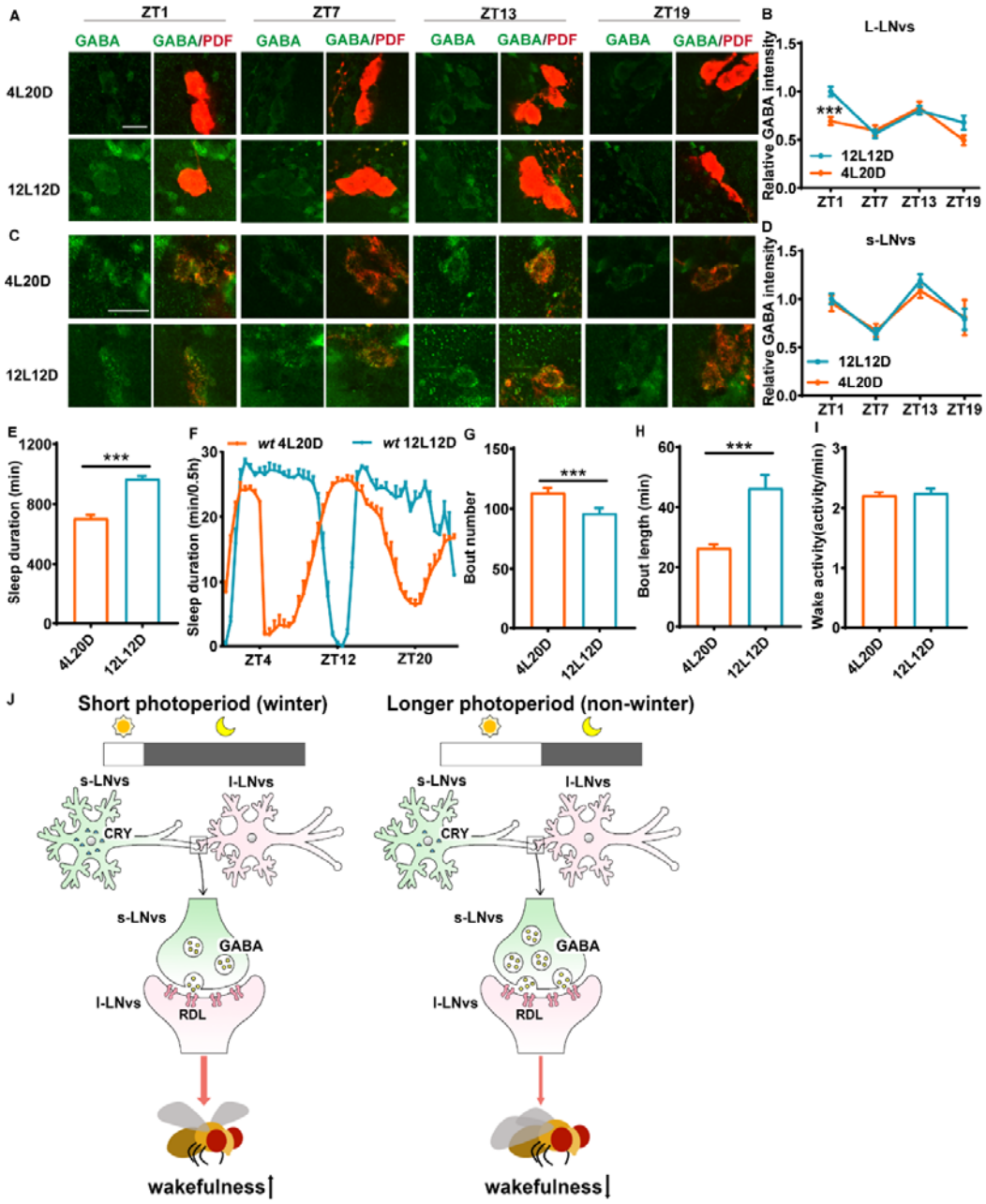

Figure 7. Short photoperiod reduces GABA level at the l-LNvs and increases sleep duration. (A-D) Brains from male WT flies dissected at ZT1, 7, 13, 19 under 4L20D or 12L12D are immunostained with GABA (green) and PDF (red) antisera. Representative l-LNvs (A) and s-LNvs (C) are displayed. Merged signal is shown as yellow. Bar graphs represent normalized GABA intensity in the l-LNvs (B) (n = 28-55 cells) and s-LNvs (D) (n = 21-59 cells). The average value of the control group at ZT1 is set to 1. Student's *t*-test: ***$P < 0.001$. (E-I) The daily sleep duration (E), sleep profile (F), sleep bout number (G), average sleep bout length (H) and waking activity (I) of male WT flies under 4L20D and 12L12D (n = 91, 32 flies). Two-tailed Student's *t*-test: ***$P < 0.001$. The scale bar represents 15 μm. Error bars represent SEM. (J) A model demonstrating how CRY regulates the GABAergic s-LNv/l-LNv circuitry under short vs. longer photoperiods to promote arousal.