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RESEARCH ARTICLE

$GABA_B$ receptors play an essential role in maintaining sleep during the second half of the night in *Drosophila melanogaster*

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SUMMARY

GABAergic signalling is important for normal sleep in humans and flies. Here we advance the current understanding of GABAergic modulation of daily sleep patterns by focusing on the role of slow metabotropic $GABA_B$ receptors in the fruit fly *Drosophila melanogaster*. We asked whether $GABA_B$-R2 receptors are regulatory elements in sleep regulation in addition to the already identified fast ionotropic *Rdl* $GABA_A$ receptors. By immunocytochemical and reporter-based techniques we show that the pigment dispersing factor (PDF)-positive ventrolateral clock neurons (LN_v) express $GABA_B$-R2 receptors. Downregulation of $GABA_B$-R2 receptors in the large PDF neurons (l-LN_v) by RNAi reduced sleep maintenance in the second half of the night, whereas sleep latency at the beginning of the night that was previously shown to depend on ionotropic *Rdl* $GABA_A$ receptors remained unaltered. Our results confirm the role of the l-LN_v neurons as an important part of the sleep circuit in *D. melanogaster* and also identify the $GABA_B$-R2 receptors as the thus far missing component in GABA-signalling that is essential for sleep maintenance. Despite the significant effects on sleep, we did not observe any changes in circadian behaviour in flies with downregulated $GABA_B$-R2 receptors, indicating that the regulation of sleep maintenance *via* l-LN_v neurons is independent of their function in the circadian clock circuit.

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INTRODUCTION

The fruit fly *Drosophila melanogaster* has become a well-accepted model for sleep research (reviewed by Cirelli, 2009; Minot et al., 2011). As in mammals, it has been shown that the sleep-like state of *Drosophila* is associated with reduced sensory responsiveness and reduced brain activity (Nitz et al., 2002; van Swinderen et al., 2004), and is subject to both circadian and homeostatic regulation (Hendricks et al., 2000; Shaw et al., 2000). Similarly to in humans, monaminergic neurons (specifically dopaminergic and octopaminergic neurons) enhance arousal in fruit flies (Andretic et al., 2005; Kume et al., 2005; Lebestky et al., 2009; Crocker et al., 2010), whereas GABAergic neurons promote sleep (Agosto et al., 2008). As in humans, GABA advances sleep onset (reduces sleep latency) and prolongs total sleep (increases sleep maintenance) (Agosto et al., 2008). Brain regions possibly implicated in the regulation of sleep in *D. melanogaster* are the pars intercerebralis (Foltenyi et al., 2007; Crocker et al., 2010), the mushroom bodies (Joiner et al., 2006; Pitman et al., 2006; Yuan et al., 2006) and a subgroup of the pigment dispersing factor (PDF)-positive neurons called the l-LN_v neurons (Parisky et al., 2008; Sheeba et al., 2008a; Chung et al., 2009; Lebestky et al., 2009; Shang et al., 2011). The l-LN_v belong to the circadian clock neurons, indicating that in flies, as in mammals, the sleep circuit is intimately linked to the circadian clock and that the mechanisms employed to govern sleep in the brain are evolutionarily ancient.

The l-LN_v are conspicuous clock neurons with wide arborisations in the optic lobe, fibres in the accessory medulla – the insect clock centre – and connections between the brain hemispheres (Helfrich-Förster et al., 2007a). Thus, the l-LN_v neurons are anatomically well suited to modulate the activity of many neurons. In addition, their arborisations overlap with those of monaminergic neurons (Hamasaka and Nässel, 2006). Several studies show that they indeed receive dopaminergic, octopaminergic and GABAergic input and that they control the flies' arousal and sleep (Agosto et al., 2008; Parisky et al., 2008; Kula-Eversole et al., 2010; Shang et al., 2011). Furthermore, the l-LN_v are directly light sensitive and promote arousal and activity in response to light, especially in the morning (Shang et al., 2008; Sheeba et al., 2008a; Sheeba et al., 2008b; Fogle et al., 2011).

A part of the sleep-promoting effect of GABA on the l-LN_v has been shown to be mediated *via* the fast ionotropic $GABA_A$ receptor *Rdl* (*Resistance to dieldrin*) (Agosto et al., 2008). *Rdl* Cl^- channels are expressed in the l-LN_v (Agosto et al., 2008) and, similar to mammalian $GABA_A$ receptors, they mediate fast inhibitory neurotransmission (Lee et al., 2003). As expected, GABA application reduced the action potential firing rate in the l-LN_v, whereas application of picrotoxin, a $GABA_A$ receptor antagonist, increased it (McCarthy et al., 2011). Furthermore, an *Rdl* receptor mutant with prolonged channel opening and consequently increased

channel current significantly decreased sleep latency of the flies after lights-off, whereas the downregulation of the *Rdl* receptor *via* RNAi increased it (Agosto et al., 2008).

Nevertheless, the manipulation of the *Rdl* receptor had no effect on sleep maintenance. Because the latter is significantly reduced after silencing the GABAergic neurons (Parisky et al., 2008), other GABA receptors must be responsible for maintaining sleep. Suitable candidates are slow metabotropic $GABA_B$ receptors that are often co-localised with ionotropic $GABA_A$ receptors (Enell et al., 2007). In *Drosophila*, like in mammals, the metabotropic $GABA_B$ receptors are G-protein-coupled seven-transmembrane proteins composed of two subunits, $GABA_B$-R1 and $GABA_B$-R2 (Kaupmann et al., 1998; Mezler et al., 2001). The $GABA_B$-R1 is the ligand binding unit and $GABA_B$-R2 is required for translocation to the cell membrane and for stronger coupling to the G-protein (Kaupmann et al., 1998; Galvez et al., 2001). In this study we show that the l-LN_v do express metabotropic $GABA_B$-R2 receptors and that these receptors are relevant for sleep maintenance but not for sleep latency. Thus, metabotropic and ionotropic GABA receptors are cooperating in sleep regulation.

MATERIALS AND METHODS

Fly strains and rearing

Oregon R was used as a wild-type strain for $GABA_B$-R2, PDF and GAD1 immunohistochemistry. For visualizing $GABA_B$-R2 receptors we also used a *$GABA_B$-R2-GAL4* line (Root et al., 2008) (kindly provided by Jing Wang, University of California, San Diego) to express green fluorescent protein (GFP) with the binary UAS-GAL4 system (using *UAS-s65tGFP*, stock 1522, Bloomington Stock Center, Bloomington, IN, USA). The specificity of the *$GABA_B$-R2-GAL4* line for *$GABA_B$-R2*-expressing neurons has been demonstrated previously for the adult olfactory sensory neurons (OSNs) (Root et al., 2008) and seems to be correct also for most the other labelled neurons, judging from a wide overlap between immunostaining with a *$GABA_B$-R2* antiserum and *$GABA_B$-R2-GAL4*-driven GFP (Hamasaka et al., 2005). In order to downregulate the $GABA_B$-R2 receptor specifically in the PDF neurons (s-LN_v and l-LN_v), we used *Pdf-GAL4* (Park et al., 2000) to either express *UAS-$GABA_B$-R2-RNAi* (Root et al., 2008) (provided by Jing Wang) alone, or to simultaneously express *UAS-$GABA_B$-R2-RNAi* and *UAS-Dicer2* (no. 60012, Vienna *Drosophila* RNAi Center, Wien, Austria). In the first experiment, the *Pdf-GAL4* driver and *UAS-$GABA_B$-R2-RNAi* effector lines crossed to white^{1118} were taken as controls, and in the second experiment, the *Pdf-GAL4* driver and *UAS-$GABA_B$-R2-RNAi* effector lines crossed to *UAS-Dicer2* were taken as controls. In a second set of experiments, we drove an independent *UAS-Trip-$GABA_B$-R2-RNAi* line (no. 27699; Bloomington Stock Center) together with *dicer2* under control of *Pdf-GAL4* in order to downregulate $GABA_B$-R2. All flies were raised on *Drosophila* food (0.8% agar, 2.2% sugar-beet syrup, 8.0% malt extract, 1.8% yeast, 1.0% soy flour, 8.0% corn flour and 0.3% hydroxybenzoic acid) at 25°C under a 12 h:12 h light:dark (LD) cycle and transferred to 20°C at an age of ~3 days.

Immunohistochemistry

For PDF and GFP co-labelling, whole-mount brains were dissected in PBS-TX (0.01 mol l^{-1} phosphate-buffered saline with 0.5% Triton X-100, pH 7.2) before fixing them in ice-cold 4% paraformaldehyde (in 0.1 mol l^{-1} phosphate buffer, pH 7.2) for 1 h. For $GABA_B$-R2 immunolabelling, entire fly heads were fixed for 3 h prior to brain dissection. The fixed and dissected brains were washed in PBS-TX, blocked for 2 h in 5% normal goat serum (NGS, in PBS-TX) and then incubated with primary antibodies at 4°C overnight or for 48 h. Primary antibodies were used in the following concentrations in PBS-TX with 5% NGS: anti-PDF 1:2500 and anti-$GABA_B$-R2 1:10,000 (for description of primary antibodies, see below). After washing several times in PBS-TX, the brains were incubated in secondary fluorochrome-conjugated antibodies in PBS-TX with 5% NGS either overnight at 4°C or for 2 h at room temperature. We used Alexa Fluor antibodies (Molecular Probes, Carlsbad, CA, USA) of 488 nm (goat anti-mouse) or 546 nm (goat anti-rabbit) and Cy2- or Cy3-tagged IgGs (goat anti-mouse or anti-rabbit; Jackson ImmunoResearch, West Grove, PA, USA) at a dilution of 1:1000. After incubation with the secondary antibodies, brains were washed in PB-TX and subsequently mounted in Vectashield medium (Vector Laboratories, Burlingame, CA, USA) on glass slides, all in the same orientation.

$GABA_B$-R2 antiserum

Production and characterization of antiserum (code B7873/3) to $GABA_B$-R2 was described previously (Hamasaka et al., 2005). In western blots of brain tissue from *Drosophila* the antiserum detected a band of appropriate size (Hamasaka et al., 2005), and in $GABA_B$-R2 immunohistochemistry on *Drosophila* brains the antiserum labelled neurons that were in close proximity to neural elements immunopositive for a vesicular GABA transporter, GAD and a $GABA_A$ receptor (RDL), strongly indicating that the $GABA_B$-R2 antiserum is reliably detecting a $GABA_B$ receptor (Enell et al., 2007). The anti-$GABA_B$-R2 specificity was further established by diminished $GABA_B$-R2 immunolabelling after $GABA_B$-R2 RNAi in specific neurons (OSNs) in the antenna/antennal lobe (Root et al., 2008). Furthermore, physiological/pharmacological evidence (using Ca^{2+} imaging) for $GABA_B$-R2 expression has been produced for the larval s-LN_v and the adult OSN (Hamasaka et al., 2005; Root et al., 2008). Recently, physiological evidence for metabotropic GABA signalling was also provided for the adult s-LN_v using cAMP imaging (Lelito and Shafer, 2012).

PDF antiserum

The mouse anti-PDF was provided by Justin Blau (obtained from the Developmental Studies Hybridoma Bank).

Microscopy and image analysis

We performed laser scanning confocal microscopy (Leica TCS SPE, Leica, Wetzlar, Germany) to analyse immunofluorescent brains. To avoid bleeding through, we used sequential scanning (laser lines 488 and 532 nm). Confocal stacks of 1.5 µm thickness were acquired. For quantifying intensity of $GABA_B$-R2 immunostaining, the settings of the confocal microscope (laser power, gain and contrast) were kept the same for all preparations. The staining intensity of $GABA_B$-R2 labelling was then quantified on single confocal images by two different methods. To compare labelling intensity between $GABA_B$-R2 immunostaining and $GABA_B$-R2 GFP reporter staining, we coded the images and judged by eye whether the intensity in each single PDF-positive neuron was strong, weak or completely absent. Subsequently, we calculated the percentage of l-LN_v and s-LN_v with strong, weak and no staining (Fig. 1J,K). To judge the degree of $GABA_B$-R2 downregulation in the l-LN_v and s-LN_v by RNAi, entire confocal stacks were imported into ImageJ (Fiji distribution; http://fiji.sc/wiki/index.php/Fiji or http://rsb.info.nih.gov/ij/). Individual somata of l-LN_v and s-LN_v were selected using the magic wand selection tool on single confocal stacks. The average staining intensity of the selected area was calculated (grey value 0: no staining; grey value 255: maximal

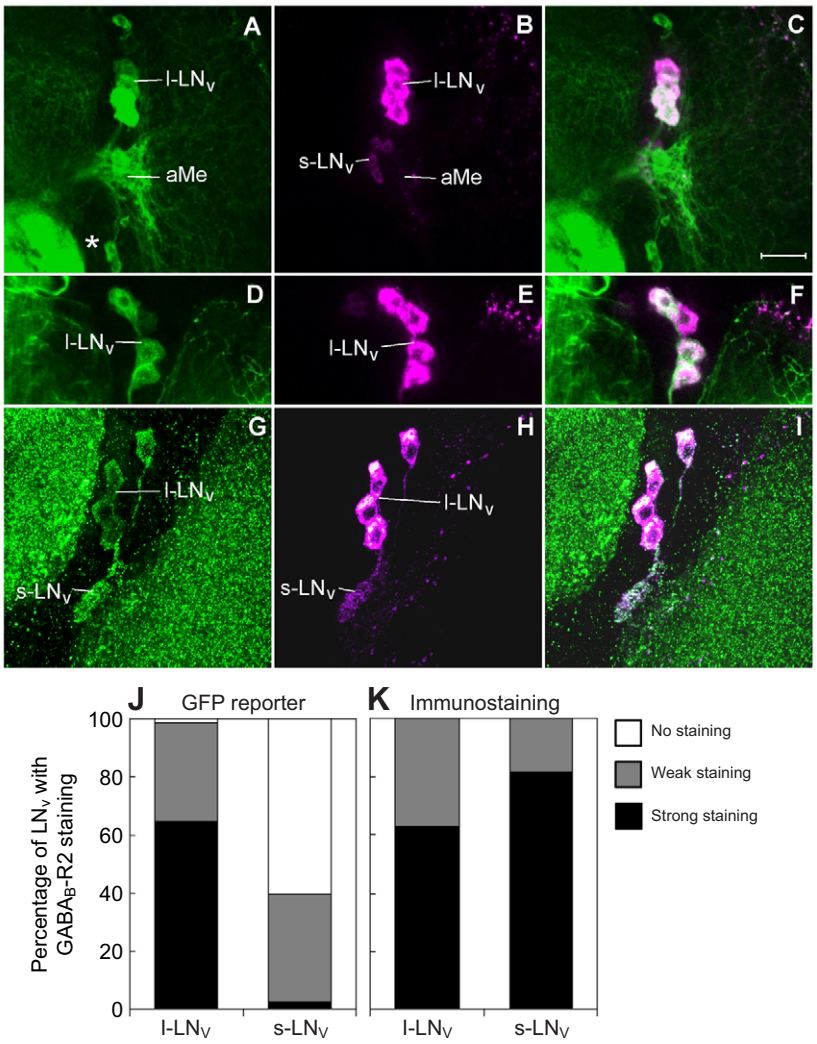

Fig. 1. The PDF-positive lateral neurons (l-LN_v and s-LN_v) express the $GABA_B$ receptor 2 ($GABA_B$-R2). (A–I) All images are projections of ~10 confocal stacks of the anterior region of the right brain hemispheres of whole-mount brains. GABA-receptor-expressing neurons are depicted in green and the PDF-positive lateral neurons in magenta. The right column shows overlays of both labelling. Magnification is the same in all pictures (scale bar in C, 20 µm). (A–F) Neurons expressing $GABA_B$-R2 visualized by green fluorescent protein (GFP) (in a *$GABA_B$-R2-GAL4;UAS-gfp* line). Strong expression is found in the accessory medulla (aMe), in one to three l-LN_v (A,D) and in few non-LN_v neurons (asterisk in A). The s-LN_v are only weakly marked or not at all. (G–I) Neurons expressing $GABA_B$-R2 visualised by the $GABA_B$-R2-antiserum. Both the l-LN_v and the s-LN_v show punctate staining, which is more pronounced in the s-LN_v (G). (J,K) Judgement of the $GABA_B$-R2 staining intensity (no, weak, strong) in the l-LN_v and the s-LN_v. Overall, GFP staining was rated in 22 brain hemispheres (85 l-LN_v and 73 s-LN_v) and $GABA_B$-R2-antibody staining in 11 brain hemispheres (43 l-LN_v and 44 s-LN_v).

staining), and the same was done for the neighbouring area outside the neurons to obtain the staining intensity of the background. The background staining was subtracted from the value gained for the soma. An average staining intensity was calculated for all l-LN_v and s-LN_v of one brain hemisphere, and finally the values of all 11–13 brain hemispheres were averaged. After testing for normal distribution, a one-way ANOVA was used to test for significant staining differences between control flies and flies in which $GABA_B$-R2 was downregulated.

Locomotor activity recording and activity analysis

The locomotor activity of single male control flies and flies with downregulated $GABA_B$-R2 was recorded using the TriKinetics DAM2 System (TriKinetics, Waltham, MA, USA). Monitors were put in light-tight boxes (recording units) with white light LED illumination of 47.6 µW cm^{-2} (Lumitronix LED-Rechnik GmbH, Jungingen, Germany) during the light phase. These recording units were placed in a climate chamber with a constant temperature of 20±0.5°C and 60±1% relative humidity. Flies were exposed to 12 h:12 h LD cycles for 11 days and subsequently kept in constant darkness (DD) for another 10 days. Only male flies with an age of 3 to 6 days were taken for the experiments. Flies that died during the experiment were excluded from the calculations.

Raw data actograms were created using ActogramJ for ImageJ (Schmid et al., 2011). The average activity levels of all individual flies were calculated for the entire LD and DD period. To do so, the beam crosses per minute from days 2–11 (LD) and days 12–21 (DD) were first averaged for each single fly and then the activity values of all flies of one genotype were averaged. This calculation was possible during DD, because the periods of the individual flies were close to 24 h. During the LD period, average activity levels were additionally determined during the first and second halves of the day and the night, respectively, to be compared with the sleep values during the same time periods (see below). To see whether $GABA_B$-R2 downregulation affects the speed of the circadian clock, we determined the free-running periods of all individual flies during days 12 to 21 in DD by periodogram analysis (Sokolove and Bushell, 1978) combined with a chi-square test with a 5% significance level (Schmid et al., 2011).

Sleep analysis

The sleep analysis was only performed during the LD cycles (from day 2 to day 11). Calculations of total sleep were performed for each hour of the day using a macro written in Microsoft Excel 2007. Average sleep bout durations were calculated for 6 h intervals using the very same macro. In previous studies sleep was defined as period of inactivity longer than 5 min (Hendricks et al., 2000; Shaw et al., 2000; Andretic and Shaw, 2005; Ho and Sehgal, 2005). At first we used the same criterion, but then we switched to a more stringent one (a period of inactivity longer than 10 min as definition of sleep)

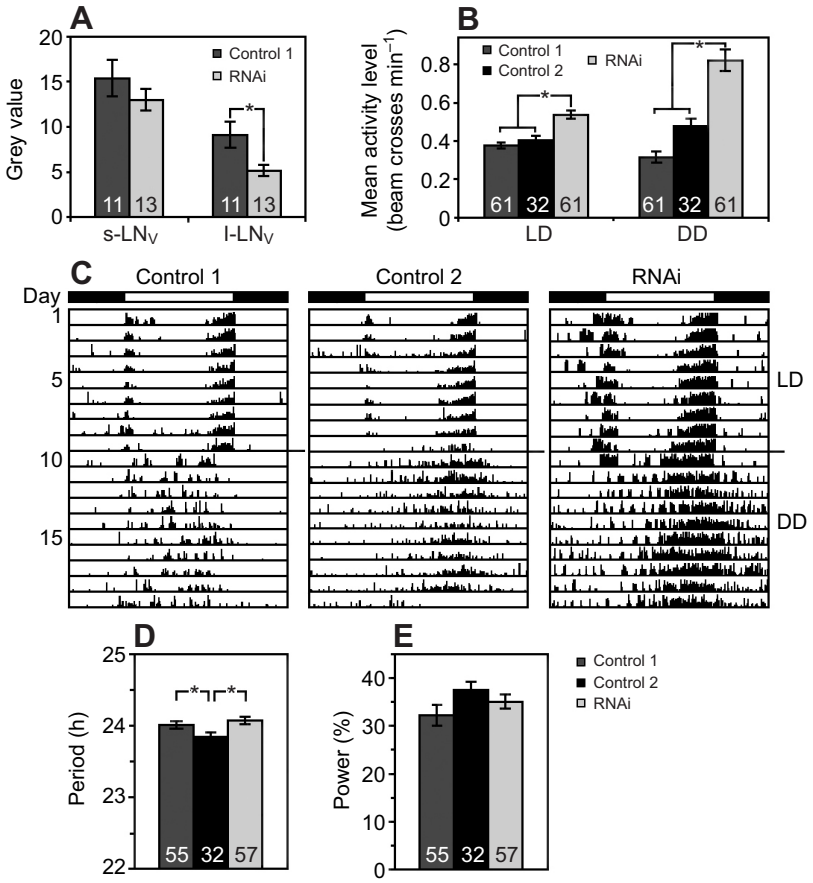

Fig. 2. Downregulation of $GABA_B$-R2 in the l-LN_v increases the activity level in light:dark (LD) cycles and constant darkness (DD) but does not affect circadian rhythmicity. (A) $GABA_B$-R2 immunolabelling is significantly reduced in the l-LN_v (ANOVA: $F_{1,21}$=7.409, P=0.0128), but not in the s-LN_v (ANOVA: $F_{1,21}$=0.292, P=0.5947) of *UAS-Dicer2;Pdf-GAL4/UAS-GABA$_B$-R2-RNAi* flies (RNAi) as compared with *UAS-Dicer2;UAS-GABA$_B$-R2-RNAi* flies (Control 1). (B) Flies with downregulated $GABA_B$-R2 (RNAi) significantly increase their activity level in LD and DD as compared with Control 1 and Control 2 (*UAS-Dicer2;Pdf-GAL4/+*) flies. (C) Typical actograms of individual control flies and a fly with downregulated $GABA_B$-R2 (RNAi). The black/white bars on top of the actograms indicate the light:dark cycle during LD. ANOVA followed by a *post hoc* test showed that period length (D) depended on the strain (ANOVA; power: $F_{1,141}$=4.587, P=0.0118) and was significantly shorter in Control 2 flies than in Control 1 and RNAi flies. Nevertheless, the percentage of rhythmic flies and the power (E) of the free-running rhythm was independent of fly strain (ANOVA; power: $F_{1,141}$=2.678, P=0.0722). Numbers in the columns indicate the number of tested flies; asterisks indicate significant differences. Error bars represent s.e.m.

in order to make very sure that periods of inactivity that resemble rest rather than sleep are not included. An average sleeping curve was calculated for each genotype out of the hourly sleep duration determined for each individual fly. Furthermore, average total sleep and average sleep bout duration were calculated in 6-h intervals for each genotype for the first and second halves of the day and night, respectively. Sleep latency after lights-off was determined as the time in minutes after lights off until the first sleep bout of at least 10 min occurred. To do so, we imported the TriKinetics file into Excel and searched manually for the first occurrence of 10 zeros in a row after lights-off for each individual fly.

RESULTS

Previous studies have shown that there are large numbers of GABA-producing neurons in the adult brain of *Drosophila* (Enell et al., 2007; Okada et al., 2009). The processes of some of these arborise in the region of the accessory medulla that contains presumed dendrites of the PDF neurons (Hamasaka et al., 2005). Thus GABAergic neurons may converge on PDF neurons.

$GABA_B$-R2 receptors are located on the PDF cells

To identify whether PDF neurons express metabotropic $GABA_B$ receptors, we used two different markers: we expressed GFP with a $GABA_B$-R2-specific GAL4 driver line (Root et al., 2008) and we used an antiserum raised against the $GABA_B$-R2 protein (Hamasaka et al., 2005). In both cases we co-stained with anti-PDF to judge whether the two signals overlap (Fig. 1A–I). We found that the PDF-positive l-LN_v neurons are reliably marked by both methods. $GABA_B$-R2-GAL4-driven GFP was present in 98.8% of the l-LN_v labelled by anti-PDF (Fig. 1J) and in 100% of the l-LN_v that were labelled with the $GABA_B$-R2 antiserum (Fig. 1K). Whereas the $GABA_B$-R2-antibody labelling was similarly strong in all four l-LN_v neurons (Fig. 1G), GFP was strongly expressed in only two to three of the four l-LN_v neurons and weak in the remaining one to two cells (Fig. 1A,D). The PDF-positive s-LN_v were not reliably stained by $GABA_B$-R2-GAL4-driven GFP (Fig. 1A): only 39.7% of the s-LN_v showed a GFP signal at all and just 2.7% of them revealed a strong signal (Fig. 1J). Nevertheless, the $GABA_B$-R2 antibody revealed the s-LN_v reliably (Fig. 1G,K): 100% of the s-LN_v showed a prominent punctuate staining. We conclude that the $GABA_B$-R2 receptor is expressed on all PDF neurons (the l-LN_v and the s-LN_v), but that $GABA_B$-R2-GAL4 drives only weakly in the s-LN_v.

Downregulation of $GABA_B$-R2 receptors in PDF neurons increases the activity level but has little effect on free-running period

To investigate whether $GABA_B$ receptors on the PDF neurons might be relevant for locomotor activity rhythms and the general activity level, we downregulated the receptors by expressing two different constructs of UAS-RNAi for $GABA_B$-R2 (with or without *dicer2*) in the PDF neurons (using *Pdf-GAL4*). First, we measured the intensity of immunolabelling in the PDF neurons in comparison to control flies (that carried only the RNAi construct). We observed a significant reduction in staining intensity only for one of the RNAi constructs that had previously been employed successfully (Root et al., 2008), and only if it was combined with *dicer2*. Furthermore, the knock-down was only significant in the l-LN_v but not in the s-LN_v neurons (Fig. 2A). The latter observation is in agreement with our findings that $GABA_B$-R2 antibody staining revealed stronger signals in the s-LN_v than in the l-LN_v and that *Pdf-GAL4* usually drives less strongly in the s-LN_v as compared with the l-LN_v (Renn et al., 1999).

After we had made sure that the $GABA_B$-R2 downregulation is preferentially working in the l-LN_v, we monitored locomotor activity of the flies for 11 days in 12 h:12 h LD followed by 10 days of DD, both at 20°C. We found that the flies with significantly downregulated $GABA_B$-R2 in the l-LN_v were significantly more active than the control flies, and this was true during both LD and DD (Fig. 2B,C). No significant differences in the activity level were present between controls and RNAi flies in the two lines, in which no $GABA_B$-R2 knock-down was detectable by antibody staining (data not shown). The activity pattern of the flies was in principle similar for all strains, showing bimodal patterns with activity bouts in the morning and evening under LD conditions and more unimodal patterns under DD conditions. $GABA_B$-R2 downregulation had no effect on the activity rhythms under DD conditions; the great majority of flies (86–100%) were rhythmic, free-ran with a period of approximately 24 h (Fig. 2D), which was of wild-type-like power (Fig. 2E).

Downregulation of $GABA_B$-R2 receptors in PDF neurons reduces sleep maintenance

Next, we analysed the effects of $GABA_B$-R2 downregulation on sleep. Again, we did not see any significant differences in sleep between controls and RNAi flies of the two lines, in which the $GABA_B$-R2 knock-down appeared inefficient (data not shown). But, we found that the flies with significantly downregulated $GABA_B$-R2 receptors in the l-LN_v showed a largely reduced amount of nighttime sleep during LD (Fig. 3). This difference was mainly due to a reduced amount of sleep in the second half of the night (Fig. 3A). During the 6-h interval in the late night, the experimental flies showed 30 min less sleep in total as compared with the controls (Fig. 3C). Simultaneously, they decreased their average sleep bout duration significantly (to 108 min in the 6-h interval, in contrast to 135 and 160 min, respectively, of the control strains) (Fig. 3D). Obviously, the flies with downregulated $GABA_B$-R2 receptors were clearly unable to maintain sleep in the second half of the night. They woke up early and displayed a high amount of locomotor activity. This is also clearly visible in the actograms of the flies (Fig. 2C).

No effects on sleep were visible during the day or the first half of the night. Downregulation of $GABA_B$-R2 receptors in the l-LN_v did also not increase sleep latency. After lights-off, the experimental flies fell asleep as fast as flies of Control 2 (*UAS-dicer2;Pdf-GAL4/+*) (Fig. 3B). Just the flies of Control 1 (*UAS-dicer2;UAS-$GABA_B$-R2-RNAi*) fell asleep faster.

As stated above, the expression of the $GABA_B$-R2 RNAi in the PDF neurons also influenced the overall activity level of the flies, but in contrast to sleep, activity level was increased throughout the entire 24-h day (all four 6-h intervals; Fig. 3E). Thus the regulation of the overall activity level cannot be by the same mechanism as sleep regulation.

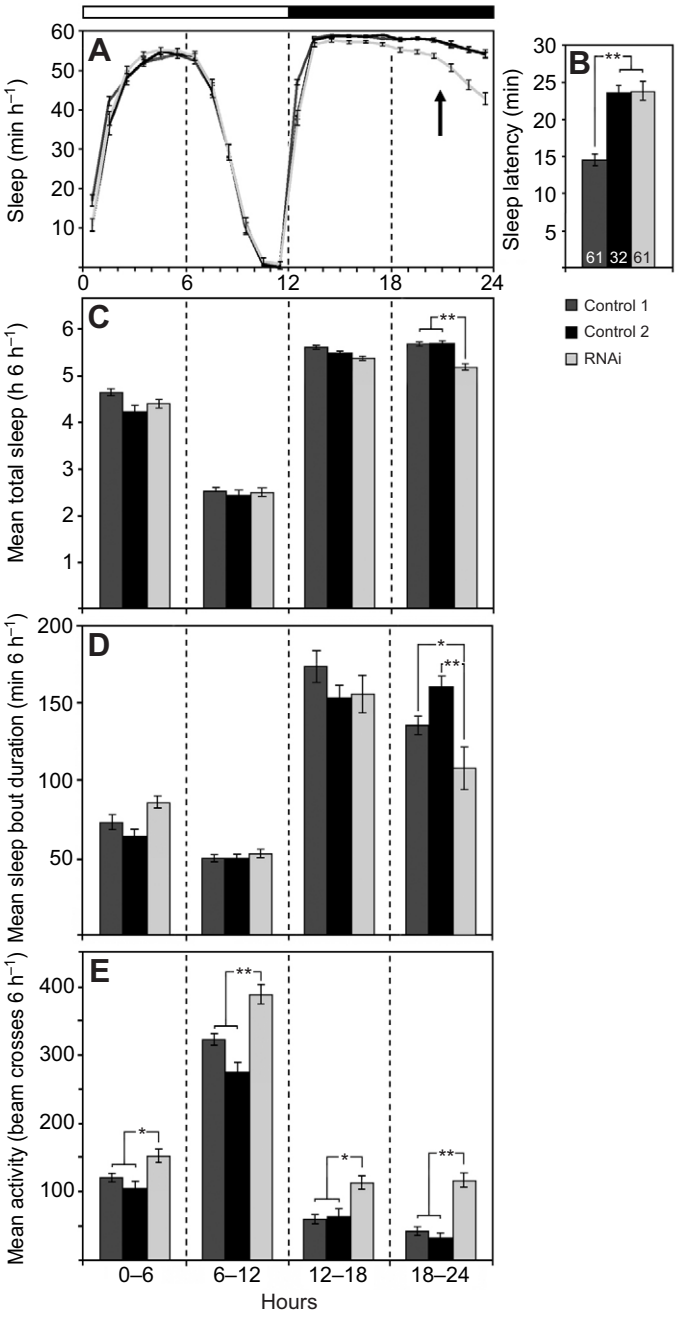

Fig. 3. Downregulation of $GABA_B$-R2 in the l-LN_v decreases sleep maintenance in the second half of the night. (A) Average sleep curves during the 10 days of LD shown in Fig. 2C for flies with $GABA_B$-R2 downregulated (RNAi) and the two controls (fly strains and number of flies are as in Fig. 2). The arrow points to the reduced sleep of the RNAi flies during the second half of the night. (B) Sleep latency after lights-off is unusually short in flies of Control 1, but not different between RNAi flies and flies of Control 2. (C–E) The 24-h day is divided in 6-h intervals (broken lines) to show mean total sleep (C), mean sleep bout duration (D) and mean activity (E) during these intervals. ANOVA followed by a *post hoc* test revealed that downregulation of $GABA_B$-R2 affects the sleep parameters during the last 6-h interval (second half of the night), whereas it increases activity at all times. Significant differences are marked by asterisks (*P<0.05; **P<0.0005). Error bars represent s.e.m.

DISCUSSION

Here we show that metabotropic $GABA_B$-R2 receptors are expressed on the PDF-positive clock neurons (LN_v neurons), and that their downregulation in the l-LN_v by RNAi results in: (1) a higher activity level throughout the day and night and (2) reduced sleep maintenance in the second half of the night. Neither sleep onset nor circadian rhythm parameters were affected by the downregulation. We conclude that GABA signalling *via* metabotropic receptors on the l-LN_v is essential for sustaining sleep throughout the night and for keeping activity at moderate levels throughout the 24-h day (preventing flies from hyperactivity). A major caveat of RNAi is off-target effects, particularly when Gal4 drivers are expressed in large numbers of non-target neurons. Though we have downregulated $GABA_B$-R2 in only eight neurons per brain hemisphere and were careful to correlate the behavioural effects of our knockdown experiments with observation and measures of

$GABA_B$-R2 immunostaining in the s-LN_v and l-LN_v, it is still possible that some effects were due to off-target knockdown of other membrane proteins. Nevertheless, given the fact that no such effects have been reported in the previous paper that used the same $GABA_B$-R2 RNAi line (Root et al., 2008), we think it is unlikely that the behavioural effects described here were due to off-target knockdown of other genes.

Our results are in line with a former study describing the location of $GABA_B$ receptors in *D. melanogaster* (Hamasaka et al., 2005). The ionotropic $GABA_A$ receptor *Rdl* has also been identified on the l-LN_v neurons and has been shown to regulate sleep, but its downregulation delayed only sleep onset and did not perturb sleep maintenance (Parisky et al., 2008). In contrast, silencing GABAergic signalling influenced sleep onset and sleep maintenance, indicating that GABA works through the fast *Rdl* receptor, and also implying a longer-lasting signalling pathway. $GABA_B$ receptors are perfect candidates in mediating slow but longer-lasting effects of GABA. Often, $GABA_A$ and $GABA_B$ receptors cooperate in mediating such fast and slow effects. For example, in the olfactory system, $GABA_A$ receptors mediate the primary modulatory responses to odours whereas $GABA_B$ receptors are responsible for long-lasting effects (Wilson and Laurent, 2005).

In *D. melanogaster*, $GABA_B$ receptors consist of the two subunits $GABA_B$-R1 and $GABA_B$-R2, and only the two units together can efficiently activate the metabotropic GABA signalling cascade (Galvez et al., 2001; Mezler et al., 2001). In our experiments, we downregulated only $GABA_B$-R2, but this manipulation should also have decreased the amount of functional $GABA_B$-R1/$GABA_B$-R2 heterodimers and, therefore, reduced $GABA_B$ signalling in general. Taking into account that sleep maintenance in the second half of the night was already significantly impaired by an ~46% reduction in detectable $GABA_B$-R2 immunostaining intensity in the l-LN_v clock neurons, it can be assumed that $GABA_B$ receptors account for an even larger portion of the sleep maintenance than detected in our experiments. Thus $GABA_B$ receptors play a crucial role in mediating GABAergic signals to the l-LN_v neurons, which are needed to sustain sleep throughout the night. This is mainly due to the maintenance of extended sleep bout durations in the second half of the night. When signalling by the $GABA_B$ receptor is reduced, sleep bouts during this interval are significantly shortened, leading to less total sleep.

Most importantly, we confirm the l-LN_v as important components in regulation of sleep and arousal (Agosto et al., 2008; Parisky et al., 2008; Kula-Eversole et al., 2010; Shang et al., 2011). In contrast, the s-LN_v seem to be not involved in sleep–arousal regulation but are rather important for maintaining circadian rhythmicity under DD (reviewed by Helfrich-Förster et al., 2007b). One caveat in clearly distinguishing the function of s-LN_v and l-LN_v is the fact that both cell clusters express the neuropeptide PDF and, as a consequence, *Pdf-GAL4* drives expression in both subsets of clock neurons. Though we did not see a significant $GABA_B$-R2 knock-down in the s-LN_v, we cannot completely exclude that $GABA_B$-R2 was slightly downregulated in these clock neurons and that this knock-down contributed to the observed alterations in sleep. To restrict the knock-down to the s-LN_v we used the *R6-GAL4* line that is expressed in the s-LN_v but not in the l-LN_v (Helfrich-Förster et al., 2007a). We observed neither a reduction in $GABA_B$-R2 staining intensity in the s-LN_v nor any effects on sleep in the second half of the night (F.G. and C.H.-F., unpublished observations). The lack of any visible $GABA_B$-R2 downregulation in the s-LN_v with *R6-GAL4* is in agreement with observations of Shafer and Taghert (Shafer and Taghert, 2009), who could completely downregulate PDF in the s-LN_v using *Pdf-GAL4* but not using *R6-GAL4*. Thus, *R6-GAL4* is a weaker driver than *Pdf-GAL4* and is obviously not able to influence $GABA_B$-R2 in the s-LN_v. Nevertheless, in our experiments the *R6-Gal4*-driven $GABA_B$-R2 RNAi led to flies that had slightly higher diurnal activity levels and less diurnal rest than the control flies (F.G. and C.H.-F., unpublished observations; supplementary material Fig. S1). This suggests that $GABA_B$-R2 was downregulated somewhere else. When checking the *R6-GAL4* expression more carefully we found that it was not restricted to the brain, but was also present in many cells of the thoracic and especially the abdominal ganglia (F.G. and C.H.-F., unpublished observations). Given the broad expression of $GABA_B$-R2, a putative knock-down in the ventral nervous system is likely to affect locomotor activity.

Our results on the l-LN_v certainly do not exclude a role of GABA in the circadian clock controlling activity rhythms under DD conditions (here represented by the s-LN_v). In mammals, GABA is the most abundant neurotransmitter in the circadian clock centre in the brain – the suprachiasmatic nucleus (van den Pol and Tsujimoto, 1985). GABA interacts with $GABA_A$ and $GABA_B$ receptors, producing primarily but not exclusively inhibitory responses through membrane hyperpolarisation (Wagner et al., 1997; Choi et al., 2008). GABA signalling is important for maintaining behavioural circadian rhythmicity, it affects the amplitude of molecular oscillations and might contribute to synchronisation of clock cells within the suprachiasmatic nucleus (Liu and Reppert, 2000; Albus et al., 2005; Aton et al., 2006; Ehlen et al., 2006). The same seems to be true for fruit flies. The s-LN_v neurons of adults alter cAMP levels upon GABA application on isolated brains *in vitro* (Lelito and Shafer, 2012). Hyperexcitation of GABAergic neurons disrupts the molecular rhythms in the s-LN_v and renders the flies arrhythmic (Dahdal et al., 2010). Thus, GABA signalling affects the circadian clock in the s-LN_v. We found that flies with downregulated $GABA_B$-R2 receptors had slightly longer free-running periods than the control flies, but this turned out to be only significant in comparison with Control 2 and not to Control 1 (Fig. 2D). Dahdal et al. (Dahdal et al., 2010) found similar small effects on period after downregulating $GABA_B$-R2 receptors, but a significant period lengthening after downregulating $GABA_B$-R3 receptors. This indicates that GABA signals *via* $GABA_B$-R3 receptors to the s-LN_v and was confirmed *in vitro* in the larval *Drosophila* brain by Ca^{2+} imaging (Dahdal et al., 2010). Nevertheless, the study of Dahdal et al. (Dahdal et al., 2010) does not rule out that GABA signals *via* $GABA_B$-R3 plus $GABA_B$-R2 receptors on the adult s-LN_v. We found a rather strong expression of $GABA_B$-R2 receptors in these clock neurons, and were not able to downregulate it significantly by RNAi, although we used *dicer2* as amplification. Dahdal et al. (Dahdal et al., 2010) did not use *dicer2*, and they also did not measure the effectiveness of the downregulation of $GABA_B$-R2 by RNAi immunocytochemically directly in the s-LN_v. Thus, the exact $GABA_B$ receptors that mediate GABA responses in the adult s-LN_v need still to be determined.

In summary, we conclude that the l-LN_v subgroup of the PDF-positive clock neurons is a principal target of sleep-promoting and activity-repressing GABAergic neurons and sits at the heart of the sleep circuit in *D. melanogaster*. Thus, the sleep circuitry of flies is clearly more circumscribed and simpler than that of mammals. Mammals have many targets of sleep-promoting GABAergic neurons, and the circadian clock seems to have a mainly modulatory and less direct influence on sleep (Mistlberger, 2005). The fly sleep circuitry may therefore have condensed the mammalian arousal and sleep stimulating systems (e.g. monaminergic, cholinergic, peptidergic and GABAergic systems) into a simpler and more compact region, which seems to largely coincide with the eight PDF-positive l-LN_v cells of the circadian circuit.

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AUTHOR CONTRIBUTIONS

F.G. performed and analysed the sleep experiments, immunostained the brains with the $GABA_B$-R2 antiserum, quantified the staining intensity before and after RNAi, designed the figures and provided a first draft of the paper; A.K. performed the GFP reporter-based stainings; T.Y. wrote the Excel-based software for analyzing sleep, helped with the analysis and provided advice during the experiments; D.R. performed activity recordings and analyzed the circadian behaviour of the flies; D.R.N. and C.H.-F. designed the experiments; and C.H.-F. wrote the final version of the paper and revised it.

COMPETING INTERESTS

No competing interests declared.

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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.

Albus, H., Vansteensel, M. J., Michel, S., Block, G. D. and Meijer, J. H. (2005). A GABAergic mechanism is necessary for coupling dissociable ventral and dorsal regional oscillators within the circadian clock. *Curr. Biol.* **15**, 886-893.

Andretic, R. and Shaw, P. J. (2005). Essentials of sleep recordings in *Drosophila*: moving beyond sleep time. *Methods Enzymol.* **393**, 759-772.

Andretic, R., van Swinderen, B. and Greenspan, R. J. (2005). Dopaminergic modulation of arousal in *Drosophila*. *Curr. Biol.* **15**, 1165-1175.

Aton, S. J., Huettner, J. E., Straume, M. and Herzog, E. D. (2006). GABA and Gi/o differentially control circadian rhythms and synchrony in clock neurons. *Proc. Natl. Acad. Sci. USA* **103**, 19188-19193.

Choi, H. J., Lee, C. J., Schroeder, A., Kim, Y. S., Jung, S. H., Kim, J. S., Kim, Y., Son, E. J., Han, H. C., Hong, S. K. et al. (2008). Excitatory actions of GABA in the suprachiasmatic nucleus. *J. Neurosci.* **28**, 5450-5459.

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.

Cirelli, C. (2009). The genetic and molecular regulation of sleep: from fruit flies to humans. *Nat. Rev. Neurosci.* **10**, 549-560.

Crocker, A., Shahidullah, M., Levitan, I. B. and Sehgal, A. (2010). Identification of a neural circuit that underlies the effects of octopamine on sleep:wake behavior. *Neuron* **65**, 670-681.

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.

Ehlen, J. C., Novak, C. M., Karom, M. C., Gamble, K. L., Paul, K. N. and Albers, H. E. (2006). $GABA_A$ receptor activation suppresses *Period* 1 mRNA and *Period* 2 mRNA in the suprachiasmatic nucleus during the mid-subjective day. *Eur. J. Neurosci.* **23**, 3328-3336.

Enell, L., Hamasaka, Y., Kolodziejczyk, A. and Nässel, D. R. (2007). γ-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.

Fogle, K. J., Parson, K. G., Dahm, N. A. and Holmes, T. C. (2011). CRYPTOCHROME is a blue-light sensor that regulates neuronal firing rate. *Science* **331**, 1409-1413.

Foltenyi, K., Greenspan, R. J. and Newport, J. W. (2007). Activation of EGFR and ERK by rhomboid signaling regulates the consolidation and maintenance of sleep in *Drosophila*. *Nat. Neurosci.* **10**, 1160-1167.

Galvez, T., Duthey, B., Kniazeff, J., Blahos, J., Rovelli, G., Bettler, B., Prézeau, L. and Pin, J. P. (2001). Allosteric interactions between GB1 and GB2 subunits are required for optimal $GABA_B$ receptor function. *EMBO J.* **20**, 2152-2159.

Hamasaka, Y. and Nässel, D. R. (2006). Mapping of serotonin, dopamine, and histamine in relation to different clock neurons in the brain of *Drosophila*. *J. Comp. Neurol.* **494**, 314-330.

Hamasaka, Y., Wegener, C. and Nässel, D. R. (2005). GABA modulates *Drosophila* circadian clock neurons via $GABA_B$ receptors and decreases in calcium. *J. Neurobiol.* **65**, 225-240.

Helfrich-Förster, C., Shafer, O. T., Wülbeck, C., Grieshaber, E., Rieger, D. and Taghert, P. (2007a). Development and morphology of the clock-gene-expressing lateral neurons of *Drosophila melanogaster*. *J. Comp. Neurol.* **500**, 47-70.

Helfrich-Förster, C., Yoshii, T., Wülbeck, C., Grieshaber, E., Rieger, D., Bachleitner, W., Cusamano, P. and Rouyer, F. (2007b). The lateral and dorsal neurons of *Drosophila melanogaster*: new insights about their morphology and function. *Cold Spring Harb. Symp. Quant. Biol.* **72**, 517-525.

Hendricks, J. C., Finn, S. M., Panckeri, K. A., Chavkin, J., Williams, J. A., Sehgal, A. and Pack, A. I. (2000). Rest in *Drosophila* is a sleep-like state. *Neuron* **25**, 129-138.

Ho, K. S. and Sehgal, A. (2005). *Drosophila melanogaster*: an insect model for fundamental studies of sleep. *Methods Enzymol.* **393**, 772-793.

Joiner, W. J., Crocker, A., White, B. H. and Sehgal, A. (2006). Sleep in *Drosophila* is regulated by adult mushroom bodies. *Nature* **441**, 757-760.

Kaupmann, K., Malitschek, B., Schuler, V., Heid, J., Froestl, W., Beck, P., Mosbacher, J., Bischoff, S., Kulik, A., Shigemoto, R. et al. (1998). $GABA_B$-receptor subtypes assemble into functional heteromeric complexes. *Nature* **396**, 683-687.

Kula-Eversole, E., Nagoshi, E., Shang, Y., Rodriguez, J., Allada, R. and Rosbash, M. (2010). Surprising gene expression patterns within and between PDF-containing circadian neurons in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **107**, 13497-13502.

Kume, K., Kume, S., Park, S. K., Hirsh, J. and Jackson, F. R. (2005). Dopamine is a regulator of arousal in the fruit fly. *J. Neurosci.* **25**, 7377-7384.

Lebestky, T., Chang, J. S., Dankert, H., Zelnik, L., Kim, Y. C., Han, K. A., Wolf, F. W., Perona, P. and Anderson, D. J. (2009). Two different forms of arousal in *Drosophila* are oppositely regulated by the dopamine D1 receptor ortholog DopR via distinct neural circuits. *Neuron* **64**, 522-536.

Lee, D., Su, H. and O'Dowd, D. K. (2003). GABA receptors containing Rdl subunits mediate fast inhibitory synaptic transmission in *Drosophila* neurons. *J. Neurosci.* **23**, 4625-4634.

Lelito, K. R. and Shafer, O. T. (2012). Reciprocal cholinergic and GABAergic modulation of the small ventrolateral pacemaker neurons of *Drosophila*'s circadian clock neuron network. *J. Neurophysiol.* **107**, 2096-2108.

Liu, C. and Reppert, S. M. (2000). GABA synchronizes clock cells within the suprachiasmatic circadian clock. *Neuron* **25**, 123-128.

McCarthy, E. V., Wu, Y., Decarvalho, T., Brandt, C., Cao, G. and Nitabach, M. N. (2011). Synchronized bilateral synaptic inputs to *Drosophila melanogaster* neuropeptidergic rest/arousal neurons. *J. Neurosci.* **31**, 8181-8193.

Mezler, M., Müller, T. and Raming, K. (2001). Cloning and functional expression of $GABA_B$ receptors from *Drosophila*. *Eur. J. Neurosci.* **13**, 477-486.

Minot, S., Sinha, R., Chen, J., Li, H., Keilbaugh, S. A., Wu, G. D., Lewis, J. D. and Bushman, F. D. (2011). The human gut virome: inter-individual variation and dynamic response to diet. *Genome Res.* **21**, 1616-1625.

Mistlberger, R. E. (2005). Circadian regulation of sleep in mammals: role of the suprachiasmatic nucleus. *Brain Res. Brain Res. Rev.* **49**, 429-454.

Nitz, D. A., van Swinderen, B., Tononi, G. and Greenspan, R. J. (2002). Electrophysiological correlates of rest and activity in *Drosophila melanogaster*. *Curr. Biol.* **12**, 1934-1940.

Okada, R., Awasaki, T. and Ito, K. (2009). Gamma-aminobutyric acid (GABA)-mediated neural connections in the *Drosophila* antennal lobe. *J. Comp. Neurol.* **514**, 74-91.

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, J. H., Helfrich-Förster, C., Lee, G., Liu, L., Rosbash, M. and Hall, J. C. (2000). Differential regulation of circadian pacemaker output by separate clock genes in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **97**, 3608-3613.

Pitman, J. L., McGill, J. J., Keegan, K. P. and Allada, R. (2006). A dynamic role for the mushroom bodies in promoting sleep in *Drosophila*. *Nature* **441**, 753-756.

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.

Root, C. M., Masuyama, K., Green, D. S., Enell, L. E., Nässel, D. R., Lee, C. H. and Wang, J. W. (2008). A presynaptic gain control mechanism fine-tunes olfactory behavior. *Neuron* **59**, 311-321.

Schmid, B., Helfrich-Förster, C. and Yoshii, T. (2011). A new ImageJ plug-in 'ActogramJ' for chronobiological analyses. *J. Biol. Rhythms* **26**, 464-467.

Shafer, O. T. and Taghert, P. H. (2009). RNA-interference knockdown of *Drosophila* pigment dispersing factor in neuronal subsets: the anatomical basis of a neuropeptide's circadian functions. *PLoS ONE* **4**, e8298.

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

Shang, Y., Haynes, P., Pírez, N., Harrington, K. I., Guo, F., Pollack, J., Hong, P., Griffith, L. C. and Rosbash, M. (2011). Imaging analysis of clock neurons reveals light buffers the wake-promoting effect of dopamine. *Nat. Neurosci.* **14**, 889-895.

Shaw, P. J., Cirelli, C., Greenspan, R. J. and Tononi, G. (2000). Correlates of sleep and waking in *Drosophila melanogaster*. *Science* **287**, 1834-1837.

Sheeba, V., Fogle, K. J., Kaneko, M., Rashid, S., Chou, Y. T., Sharma, V. K. and Holmes, T. C. (2008a). Large ventral lateral neurons modulate arousal and sleep in *Drosophila*. *Curr. Biol.* **18**, 1537-1545.

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

Sokolove, P. G. and Bushell, W. N. (1978). The chi square periodogram: its utility for analysis of circadian rhythms. *J. Theor. Biol.* **72**, 131-160.

van den Pol, A. N. and Tsujimoto, K. L. (1985). Neurotransmitters of the hypothalamic suprachiasmatic nucleus: immunocytochemical analysis of 25 neuronal antigens. *Neuroscience* **15**, 1049-1086.

van Swinderen, B., Nitz, D. A. and Greenspan, R. J. (2004). Uncoupling of brain activity from movement defines arousal states in *Drosophila*. *Curr. Biol.* **14**, 81-87.

Wagner, S., Castel, M., Gainer, H. and Yarom, Y. (1997). GABA in the mammalian suprachiasmatic nucleus and its role in diurnal rhythmicity. *Nature* **387**, 598-603.

Wilson, R. I. and Laurent, G. (2005). Role of GABAergic inhibition in shaping odor-evoked spatiotemporal patterns in the *Drosophila* antennal lobe. *J. Neurosci.* **25**, 9069-9079.

Yuan, Q., Joiner, W. J. and Sehgal, A. (2006). A sleep-promoting role for the *Drosophila* serotonin receptor 1A. *Curr. Biol.* **16**, 1051-1062.

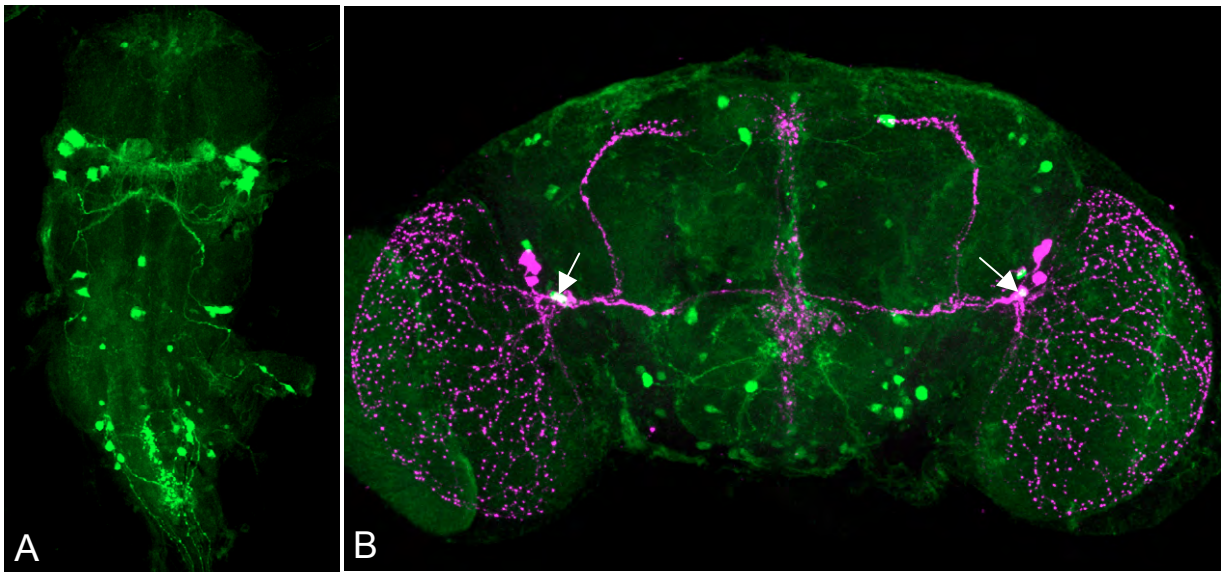

Fig. S1. GFP driven by the *R6-GAL4* driver in the thoracic-abdominal nervous system (A) and in the brain (B). The brain is double-labelled by anti-PDF. The arrows point to the s-LN_v.