Insulin-Producing Cells Monitor the Temperature and Compensate for Cold-Induced Sleep in *Drosophila*

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Running title: IPCs Compensate for Cold-Induced Sleep

Abstract

It is widely recognized that sleep can be regulated by the environmental temperature, but the underlying neural mechanism remains unclear. Here, we examined the neural mechanism underlying cold-induced sleep pattern changes in *Drosophila* by behavior tracking and optogenetics. *Drosophila* exhibit a longer sleep duration, shorter sleep latency, and deeper sleep status in lower temperatures. We also demonstrated that insulin-producing cells (IPCs) can be activated in a cold environment, potentially via an indirect functional connection with the cold-sensing neurons, the 11216 neurons. Elevation of the IPCs sensitivity to cold impairs the sleep-promoting effect of cold, while blocking of IPCs' activity primarily enhances the effect on the sleep circadian rhythm, suggesting that the cold-activated IPCs have a compensatory role in sleep regulation. Our finding revealed a potential neural circuit that could aid the maintenance of the sleep circadian rhythm at different temperatures and may offer novel insights into the mechanisms of sleep regulation.

Keywords: Insulin-Producing Cells, Sleep, Cold, *Drosophila*

Acknowledgements

We thank Zhefeng Gong for supporting and advising this research. We also thank Weiqiao Zhao, Caixia Gong, Jie Wang, and Kun Li for advice and assistance. We acknowledge the Bloomington Drosophila Stock Center for providing the fly stocks.

Introduction

Sleep is a universal behavior that is presented by nearly all animals, including a genetically well-established model organism, *Drosophila melanogaster* (Cirelli 2009). In recent years, *Drosophila* has been widely utilized to propel the field of sleep research. The dorsal neuron (DN), lateral ventral neuron (LN_v), and dorsolateral neuron (LD_d) in adult *Drosophila* were found to be circadian neurons, which express clock genes and control the rhythm of sleep (Helfrich-Forster 2003). There is another type of less-known neurons that contribute to the accumulation and release of sleep pressure, and balance the homeostasis of waking and sleeping (Donlea et al. 2014). It has been presumed that environmental factors such as light, sound, and temperature can regulate sleep via these two mechanisms, but the details of these processes are not clear (Busza et al. 2007; Cantero et al. 2002; Dubruille and Emery 2008).

Temperature-induced changes in sleeping patterns can be observed widely across phyla. However, studies on the impact of low temperature on the sleeping pattern of *Drosophila* have reported conflicting results. With decreasing temperature, flies have been observed to either reduce their daytime sleep but prolong their nighttime sleep, or prolong their sleep both during the day and at night (Lamaze et al. 2017; Parisky et al. 2016; Shih et al. 2011). Therefore, the exact effect of cold on sleep still needs verification.

Several different molecular mechanisms of thermoregulation of sleep have been reported. For instance, the important circadian proteins, PER and TIM, are thermosensitive, and were found to mediate the thermoregulation of the circadian rhythm (Majercak et al. 1999). Neural circuitry mechanisms have not been well examined, but the DN1 neuron has been demonstrated to monitor the cold and set sleep timing (Yadlapalli et al. 2018). Previous work in our lab confirmed that insulin-producing cells (IPCs) in the larva brain can sense cold via synaptic connections with the cold-sensing neuron, named the 11216 neuron (Li

and Gong 2015). IPCs are located in the pars intercerebralis, which is considered the homolog of the hypothalamus in mammals due to its similar neuroendocrine and sleep regulation functions (Cavanaugh et al. 2014; de Velasco et al. 2007). Four of eight *Drosophila* insulin-like peptides (DILPs) are secreted by IPCs, which regulate growth, metabolism, longevity, fecundity, and maturation (Nassel et al. 2015). Crocker et al. reported that IPCs mediate octopamine regulation of sleep with a wake-promoting role via the octopamine receptor and cAMP pathway. They also reported that DILPs are not involved in this process, while another group reported that mutations in DILPs or insulin receptors resulted in an abnormal sleep phenotype (Cong et al. 2015; Crocker et al. 2010). Given these results, it is reasonable to connect the cold sensation and sleep regulation function of IPCs and consider the role that IPCs may play in the effect of cold on sleep.

Materials and Methods

Fly strains and conditions

Flies were reared on a standard cornmeal-yeast-agar medium at 25°C in a 12 h light/12 h dark cycle. Stocks of *WTB*, *Canton-S*, *dilp2-Gal4*, *LexAop-mCD4-spGFP11*, *UAS-mCD4::spGFP1-10*, *UAS-mCD8-GFP*, *UAS-GCAMP6.0*, *UAS-Chrimson*, *UAS-NaChBac*, *LexAop-CD8-GFP-2A-CD8-GFP*;*UAS-mLexA-VP16-NFAT* were purchased from the Bloomington Stock Center. w^{1118} was provided by Dr Xiaohang Yang (Zhejiang University), *UAS-Kir2.1* was gifted from Dr Liming Wang (Zhejiang University), and *11216-Gal4* and *dilp2-LexA* were from our lab (Li and Gong 2015).

Flies used for optogenetic stimulation and calcium imaging were obtained by crossing *11216-Gal4*, *dilp2-LexA* flies with *UAS-Chrimson*, *LexAop-GCaMP6.0* flies, and raised at 25°C in constant darkness on food supplemented with or without 500 nM or 1 mM all-trans-retinal after eclosion experimental group.

Sleep analysis

Female flies (2-3 days old) were housed in 65 × 5 mm tubes containing 5% sucrose and 2% agar and kept in an incubator (Chichi Electric, China) at 18°C or 25°C and a relative humidity of 75% (Hendricks et al. 2000). Flies were enriched in the relevant temperature for 24-36 h before being placed in the incubator. All flies were kept under a 12:12 light:dark cycle and the zeitgeber (ZT) 0 was set at 9 am. Sleep was monitored using the *Drosophila* Activity Monitoring System (TriKinetics, USA). Sleep was defined as a 5 min bout of inactivity. The data were analyzed with the Tracker program in Matlab (Donelson et al. 2012). The temperature compensation index, Q10, of sleep latency was calculated as $Q_{10} = \left(\frac{R_2}{R_1}\right)^{10^{\circ}/(T_2-T_1)}$ (Reyes et al. 2008).

Immunohistology and fluorescence quantification

The brains , wings, and antennae of the flies were dissected in phosphate-buffered saline (PBS), and fixed in PBS containing 4% formaldehyde for 60 min at 22±2°C and washed three times for 30 min each in PBS containing 0.5% Triton X-100 (PBT). If an antibody was used, the tissue was blocked for 1 h in PBT containing 10% goat serum, and then incubated with primary antibodies (rabbit anti-Dilp2, 1:1,000 or rabbit anti-CD4, 1:200, Epitomics Inc., Burlingame, CA, USA) overnight at 4°C, followed by four 30 min washes in PBT. The samples were then incubated with a secondary antibody (TRITC-conjugated goat anti-rabbit, 1:100, Molecular Probes Inc., Grand Island, NY, USA) for 2 h at 22±2°C. The samples were mounted and imaged. Images were acquired using an Olympus FV1000 confocal laser scanning microscope.

To quantify the green fluorescent protein (GFP) levels in Calcium-dependent Nuclear Import of LexA (CaLexA) imaging, confocal Z series of the cell bodies of IPCs were

acquired. ImageJ was used to generate a sum-intensity Z stack projection and measure the total fluorescence intensity.

Adult Drosophila fixation and dissection for in vivo imaging

All Adult *Drosophila* used in all calcium imaging experiments were fixed using Scotch® Transparent Tape (Cat. 600). Flies anesthetized by CO_2 were transferred to a segment of tape (approximately 6 cm in length) with their wings stuck to the sticky surface of the tape. While the flies were under anesthesia, short (1.5 cm in length) thin (1.5 mm in width) strips of tape were applied above the wings and mouth piece of *Drosophila* with gentle pressure to fully fix them to the tape at the base. Flies with the basal tape can be transferred to the 3D-printed fly supporter (See Supplementary Fig. 1). The fly was hanged within the inner hole of the supporter. Before dissection, the supporter together with the fly was inverted, and adult-hemolymph like (AHL) saline was applied above the central area of the basal tape, above the head of fly. Fine dissection forceps were then used to carefully remove the basal tape and then the cuticle and fat cells above the brain tissue. After the brain tissue was exposed in the AHL saline, the fly along with the supporter was then carefully transferred to the microscope for imaging.

Optogenetic stimulation and calcium imaging

During the optogenetic stimulation-calcium imaging experiments, light-emitting-diode-emitted red light (620±15 nm) was applied to one fixed and dissected fly to stimulate its *11216-Gal4* neurons. The application of red light is illustrated in Supplementary Fig. 2. The application period of red light varied for each fly tested during each experiment. GCaMP 6.0 was used for the calcium imaging experiments, with an excitation wavelength of 910 nm.

Images were acquired using an Olympus FV1200MPE two-photon microscope with a resolution of 800 × 800 pixels. Specially, if the image was acquired with time-lapse and z-stack (hereafter referred to as ZT images), the resolution was set to 512 × 512 pixels, and the Z-axis step size was set to 5 or 10 µm, depending on the volume of the whole IPCs (the slice number of each scanning volume was controlled to be <8, because the imaging speed would otherwise be too slow to capture the neural activity). The objective used in all imaging experiments was the Olympus LUMPlanFL N 40x, water dipping, NA: 0.80.

The acquired images were processed with ImageJ. Specially, for images acquired with time-lapse *and* Z-stack, a MATLAB program was used to calculate the fluorescence intensity projection over the Z axis for each volume (see Supplementary code).

Results

Cold promotes sleep in wild type Drosophila

Several studies on the effect of temperature on sleep have been previously reported, but their paradigms and temperature difference varied widely (Lamaze et al. 2017; Parisky et al. 2016; Shih et al. 2011). Here, we defined the cold condition as 18°C, which is the lower limit of *Drosophila*'s temperature preference (Hong et al. 2006). To confirm the effect of cold temperature on sleep, we measured the sleep pattern of female flies from three commonly used control lines at a constant 18°C or 25°C environment with a standard 12:12 h light:dark cycle.

Although these three lines have different sleep patterns, all of them exhibited a consistent change in sleep duration and structure under cold conditions (Fig. 1a). At 18°C, the flies exhibited a robust increase in sleep duration (Fig.1b) and decrease in sleep latency (Fig.1c) both during the day and at night, which is presented as higher value and phase

lag in Fig.1a. This suggests that flies in the cold condition generally slept longer and required less time to fall asleep. In addition, flies kept at 18°C had fewer sleep episodes (Fig. 1d) but significantly longer sleep time in each episode (Fig. 1e) at night, which led to an overall greater sleep duration. This suggests that it was more difficult to interrupt sleep during the night under the cold condition. These data suggest that cold has a consistent promoting effect on both sleep quantity and quality.

IPCs are activated under the cold condition

To investigate whether IPCs in adult flies can sense the cold and respond to it, we used an NFAT-based neural tracing method, CaLexA, in IPC neurons to test the response of the IPCs to the cold condition (Masuyama et al. 2012). After treatment at 18°C for 24 h, the IPCs in the cold-treated adult flies exhibited prominent GFP expression compared to nearly no GFP expression observed in the flies treated at 25°C (Fig. 2a). The morphology of the IPCs can also clearly be observed in the image, suggesting that IPCs can be activated by long-term cold treatment in adult flies.

IPCs and 11216 neurons have no direct synaptic connections in adult Drosophila

As reported previously, in *Drosophila* larva, IPCs respond to cold via the input of the cold-sensing 11216 neurons via direct synaptic connections (Li and Gong 2015). To examine whether IPCs in adult flies also receive cold signal input from the 11216 neurons, we first assessed the expression pattern of *11216-Gal4* in adult flies by expressing GFP under *11216-Gal4* drivers. In our results, no GFP signal was observed in the central brain, but some GFP signals were observed in the peripheral organs (Fig. 2b-d). We then used GFP Reconstitution across Synaptic Partners (GRASP) to evaluate the presence of any direct synaptic connection between them in adult flies (Feinberg et al. 2008; Gong et al. 2010). Consistent with our assumption based on their expression patterns, no GRASP signal was

observed in any of our samples, indicating that there was no direct synaptic connection between the 11216 neurons and the IPCs in adult *Drosophila* (Fig. 2e).

Optogenetic stimulation of the 11216 neurons results in inhomogeneous activity of IPCs

To investigate the functional relationship between 11216 neurons and IPCs, we optogenetically stimulated the 11216 neurons while recording from IPCs expressing GCaMP6.0. In the time-lapse imaging results, we observed a significant increase in the calcium signal in the experimental group (fed with retinol) after we stimulated the 11216 neurons via red light illumination. Whereas in the control group, which lacked retinal supplementation, no significant increase in the calcium imaging fluorescence signal was observed (Fig. 3a, b).

The time-lapse scanning result seems robust, but without z-stacking, the time-lapse scanning results can only record the fluorescence signal of a part of IPCs (those in the focus plane) during optogenetic stimulation, and thus can be biased. To further investigate the response of all IPCs, we then performed z-stack time-lapse scanning (i.e., ZT scanning) for calcium imaging during optogenetic stimulation. For each trial, the Z dimension range was set to fully cover all visible IPCs. Unexpectedly, the ZT scanning results revealed that, although some IPCs can be activated by the activation of the 11216 neurons, other IPCs tend to be inhibited by the activation (Fig. 3c, d). Furthermore, this activation/inhibition pattern of IPCs remained consistent across trials for each fly. Taken together, these results suggest that, in adult *Drosophila*, the 11216 cold-sensing neuron has a certain but complex influence on different subgroups of IPCs, which may imply a possible mechanism for neural coding.

Manipulating IPCs excitability alters the effect of cold on sleep

As we confirmed that cold can induce sleep in adult *Drosophila* and that IPCs can be activated by long-term cold treatment and by the cold-sensing 11216 neurons, we then attempted to determine the role of IPCs in thermoregulation of sleep. We used *UAS-NaChBac* and *UAS-Kir2.1* to express NaChBac and Kir2.1 to manipulate the excitability of IPCs. NaChBac drives a bacterial sodium ion channel, which can reduce the depolarizing threshold of the target neuron (Nitabach et al. 2006), whereas *UAS-Kir2.1* drives an inwardly rectifying potassium channel, which can reduce IPCs excitability (Baines et al. 2001; Doring et al. 2002). We used IPC-targeting *dilp2-Gal4* to drive the expression of *UAS-NaChBac* at 18°C in order that the IPCs were more easily activated by cold, while *UAS-Kir2.1* was used to increase the difficulty of cold-induced activation.

IPCs have been reported to be wake-promoting (Crocker et al. 2010). Our results indicated that cold consistently promoted sleep in both the IPC-manipulated and control flies (Fig. 4a), in the same manner as in wild type lines (Fig. 1). There was a significant difference in sleep duration between flies treated at 25°C and those treated at 18°C, but there was little difference between the IPC-manipulated lines and the control line at 18°C (Fig. 4b). This suggests that manipulation of IPCs cannot override the effect of cold on the total sleep duration. However, the detailed sleep structure and state in IPC-manipulated flies were different from those in control flies. Both manipulated fly lines exhibited fewer sleep episodes during the day, but slept longer on average in a single episode (Fig. 4c, d). The opposite trend of the three lines in the two indices may have contributed to the lack of difference in their total sleep duration. It also suggests that blocking IPCs under the cold condition may result in an even deeper sleep state than the normal cold-induced sleep.

The basic sleep pattern plot (Fig. 4a) demonstrates a phase lag among the three lines at 18°C. The time at which the flies fell asleep during the day, but not during the night, was

postponed when the IPCs were more sensitive to the cold, while blocking of IPCs always resulted in an advanced sleep onset time compare to the control or activated line, both during the day and at night (Fig. 4e). The time (bin) when daytime sleep reaches its peak during the day, termed the *siesta*, was robustly delayed when the IPCs were activated and advanced when the IPCs were blocked (Fig. 4f). We characterized the ratio of change that cold exerted on sleep latency for each line using the temperature coefficient index, Q10. The result indicated that elevation of IPCs excitability made the cold temperature less effective in the initiation of sleep, while blocking of IPCs prominently reinforced the sleep-promoting effect of cold (Fig. 4g).

Discussion

Many physiological processes are mutually antagonistic in the maintenance of homeostasis. Ectotherms such as *Drosophila*, whose body temperature varies according to the ambient temperature, also have strategies to adapt to or compensate for temperature fluctuations and thus maintain key processes (Garrity et al. 2010). One example of the maintenance of sleep homeostasis in response to a temperature shift is the homeostatic restoration observed in previous *Drosophila* research, as the differences between sleep duration at the experimental and normal temperatures during the day were reversed at night. Such restoration was found when the temperature difference was as subtle as 4-5°C, but disappeared when the temperature difference gradually increased (Lamaze et al. 2017; Parisky et al. 2016; Shih et al. 2011). In our research, the sleep patterns changed uniformly under the 7°C difference and no homeostatic restoration was observed. We conclude that cold has a strong effect of increasing the sleep duration and decreasing the time required to fall asleep both during the day and at night.

We determined the cold response of IPCs in adult *Drosophila* using the CaLexA system. We did not observe any direct synapses between the 11216 neurons and IPCs in the adult fly brain, as found in larva with the GRASP tool, suggesting a developmental plasticity. However, using optogenetics tools, we found that the 11216 neurons can activate some IPCs while inhibiting other IPCs. This observation confirmed the functional correlation between these two groups of neurons and revealed the inhomogeneous activity of IPCs in response to cold stimulation. This could be a component of the markedly more complicated neural coding inside the *Drosophila* brain.

IPCs have been reported to promote wakefulness in the downstream pathway of sleep regulation by octopamine (Crocker et al. 2010). However, manipulation of IPCs excitability under cold stimulation generated limited impact on sleep duration, while a prominent effect was demonstrated on sleep onset and maintenance. Sleep latency and siesta time robustly decreased when the IPCs could not be activated by cold, and increased when the IPCs were depolarized by cold. The number of sleep episodes and average duration of each sleep episode were inversely affected when the IPCs were blocked, with both trending towards a deeper sleep state. Therefore, instead of mediating the sleep-promoting effect of cold, IPCs act to diminish this effect while being activated by cold.

Sleep latency indicates the accumulation of sleep pressure and initiation of sleep. The fact that IPCs elicit a stronger effect on sleep initiation under the cold condition indicates that IPCs play a more important role in the output of the circadian pacemakers. Recent work has demonstrated that the DN1 neuron, one of the circadian neurons, has both synaptic and functional connections with IPCs and consequently generates a metabolism rhythm via this connection (Barber et al. 2016). The DN1 neuron has also been reported to be activated by cold via peripheral neurons and to affect sleep onset. Here, we provided

further evidence to support the functional role of IPCs as a circadian output. Sleep regulation via IPCs may constitute the underlying pathway of octopamine, and may also be involved in the circadian mechanism of sleep.

Thermoregulation of sleep has been demonstrated to impact the initiation of sleep in both humans and animals, and has implications in several sleep disorders such as insomnia (Strogatz et al. 1987; Van der Heijden et al. 2005). By partially attenuating the cold-induced circadian shift and deep sleep state, IPCs function as a compensatory mechanism in the detrimental environment. Although its effect is relatively small compared to that of the cold, it could help animals to retain some of the physiological and behavioral processes and maintain the possibility of survival. The relatively small change by manipulation of IPCs also suggests that IPCs may function lower downstream of the compensatory mechanism. Our work may provide novel insights into the complicated and interactive sleep regulation mechanism in the brain.

Author Contributions

Conceptualization: Xu Zhang; Behavior assays, CaLexA imaging, *11216-Gal4* expression pattern identification, and GRASP imaging: Xu Zhang; Optogenetics and *in vivo* calcium imaging: Xinyue Cui; *Drosophila* husbandry: Xu Zhang, Xinyue Cui, and Dikai Zhu; Writing of the optogenetics-related section: Xinyue Cui; Writing of the other sections: Xu Zhang. Review and editing: Xu Zhang and Xinyue Cui.

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Figure

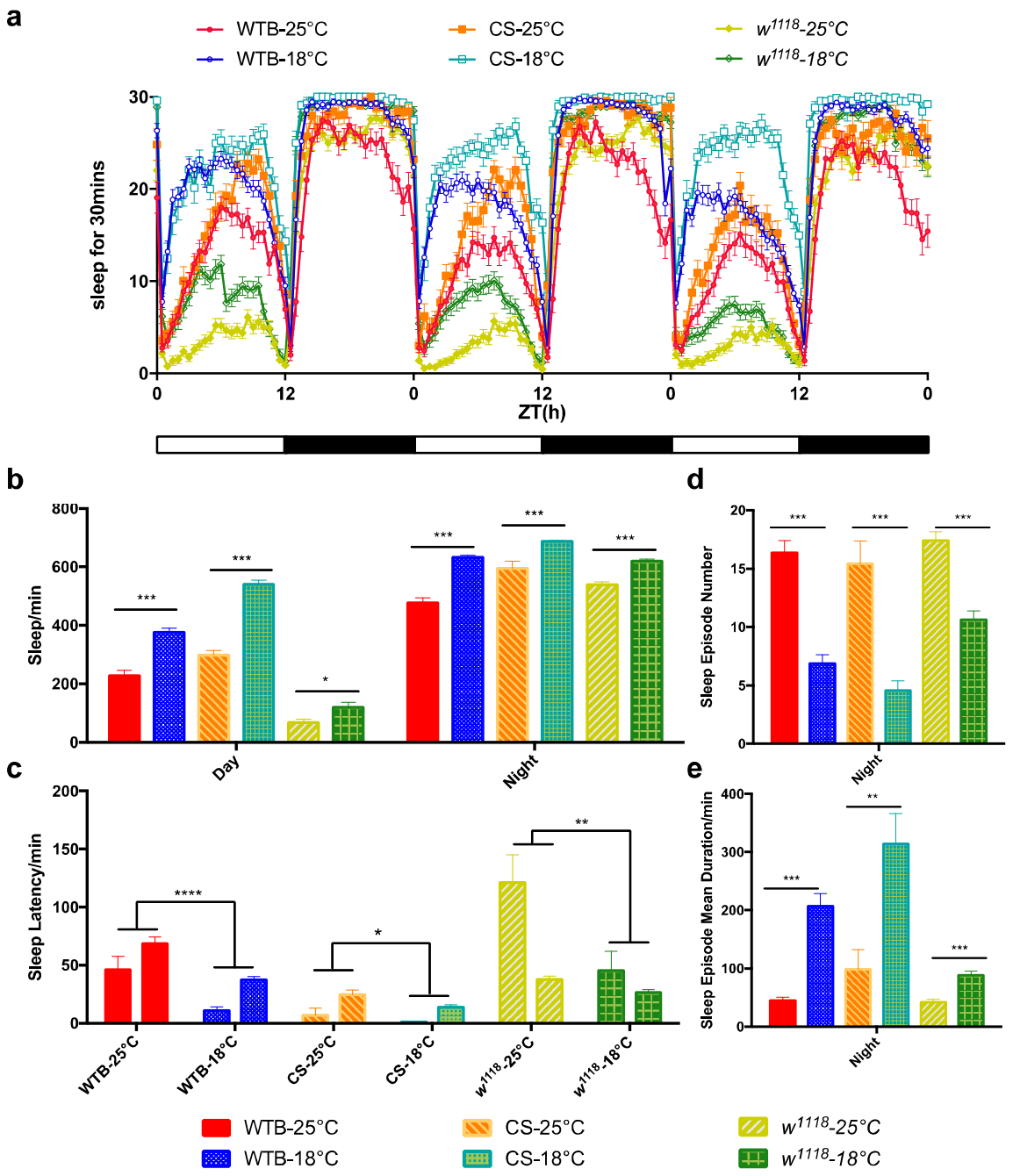


Fig. 1 Effect of cold on sleep in wild type flies
a. Sleep of three wild type flies from days 4-6 after eclosion. **b.** Total sleep duration during the day and at night of three lines on day 6 after eclosion. **c.** Sleep latency on day 6 after eclosion. For each group, the left bar is the daytime data, and the right bar is the night-time data. **d.** Sleep episodes on day 6 after eclosion. **e.** Average sleep episode duration on day 6 after eclosion. WTB, $n_{18°C}$=77, $n_{25°C}$=55; WTCS, $n_{18°C}$=23, $n_{25°C}$=22; w1118, $n_{18°C}$=85, $n_{25°C}$=84; Error bars: SEM; **b, d, e,** t test; **c**, two-way ANOVA; *p<0.05, **p<0.01, ***p<0.001.

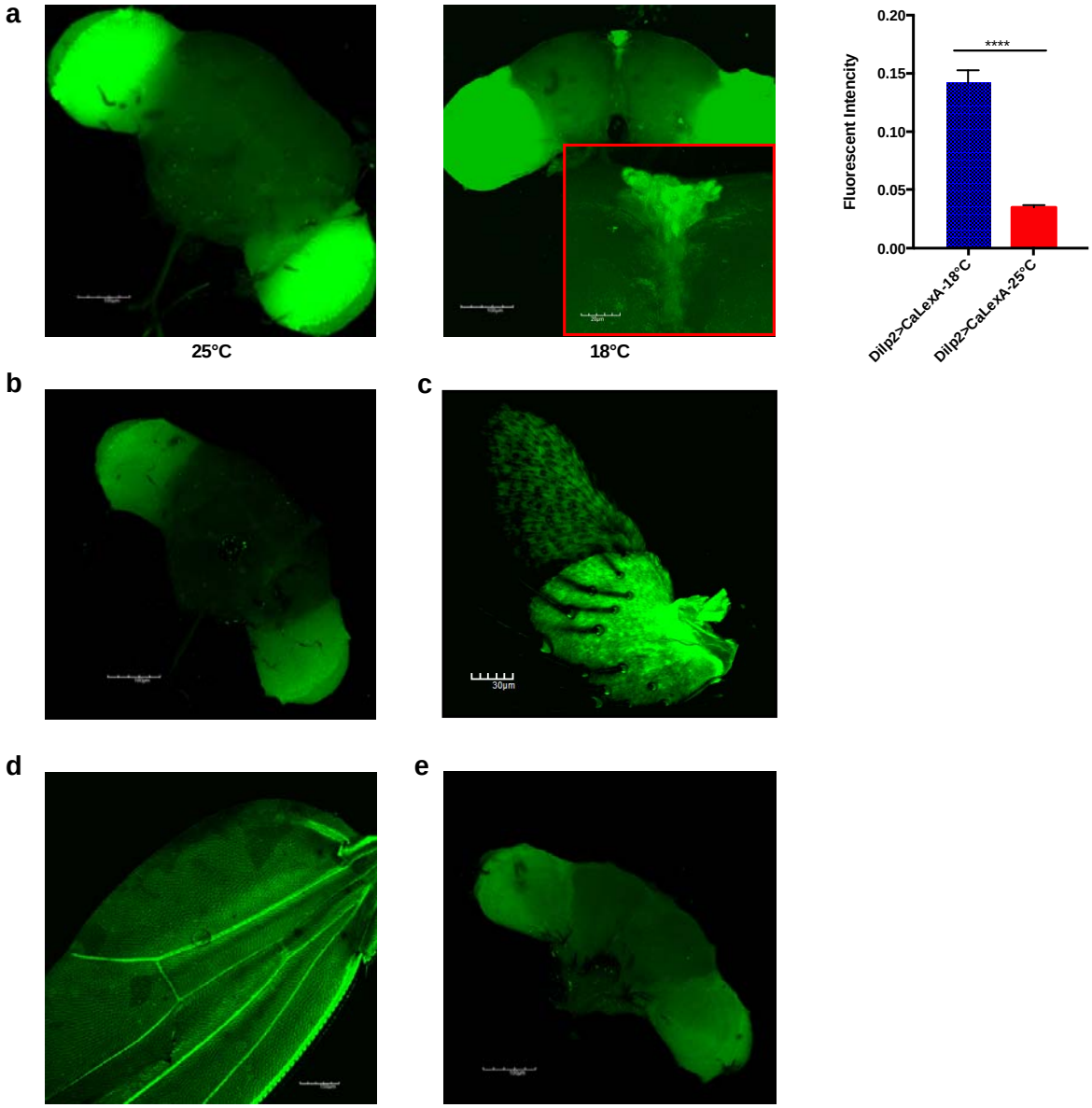


Fig. 2 Insulin-producing cells are activated under cold conditions
a. Calcium-dependent Nuclear Import of LexA-based (CaLexA) imaging and fluorescent intensity of the insulin-producing cells at 25°C and 18°C, for 24 h, n=4 for each group. Details of the expression are presented in the red square with a shape highly similar to that of the IPCs. Error bars: SEM; t test, ****$p<0.0001$. **b-e.** Expression pattern of the 11216 neurons in adult flies. **b.** The brain. **c.** The antenna. **e.** The wings. **d.** No GRASP signal was found between the 11216 neurons and the IPCs in adult flies.

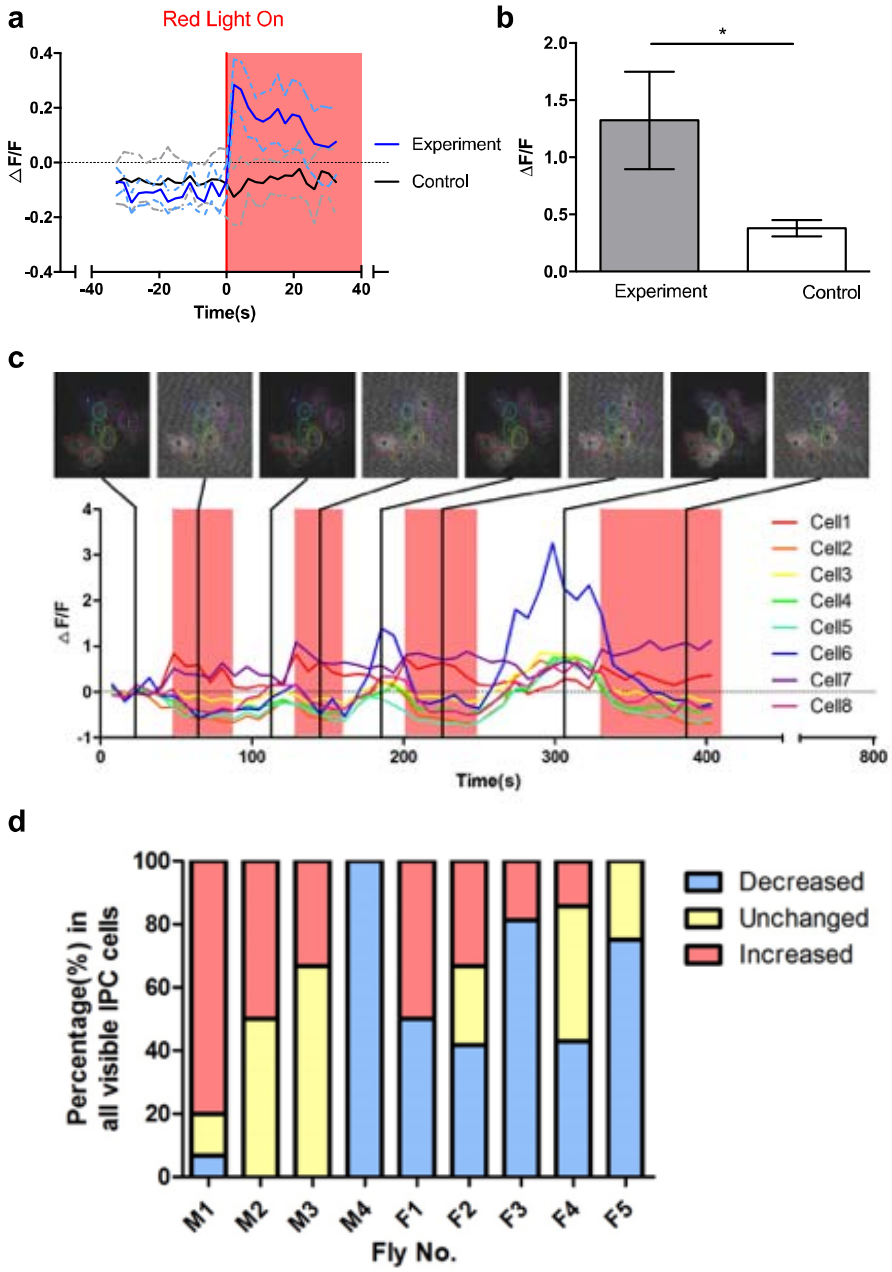


Fig. 3 Optogenetic stimulation of the 11216 neurons causes partial activation/inhibition of the insulin-producing cells

a. Initial time-series scanning calcium imaging data of the *11216>Chrimson; dilp2>GCaMP6* flies. Illumination of 620 nm red light is indicated by the red block. Only time series scanning data are included in this panel. Dotted lines: SEM. **b.** Average maximum increase of the fluorescence intensity in the experimental and control samples. Error bars: SEM; t test, *p<0.05. **c.** One example of the ZT scanning results. Eight sample frames from these data are provided above, and their frame # is indicated by black lines. Each color represents an individual cell. Note that cell 1 (red) and cell 7 (purple) were both activated during red light illumination, which is indicated by red blocks, while the other cells were inhibited. The background intensity was subtracted in the calculation of ΔF/F of each cell. **d.** The percentage of cells that decreased (average ΔF/F during illumination - average ΔF/F without illumination, or, change in ΔF/F of <-0.1), increased (change in ΔF/F of >0.1), or did not change with regard to calcium imaging fluorescence intensity (-0.1 <= change in ΔF/F <= 0.1) during red light illumination of all nine sample flies (M: male, F: female).

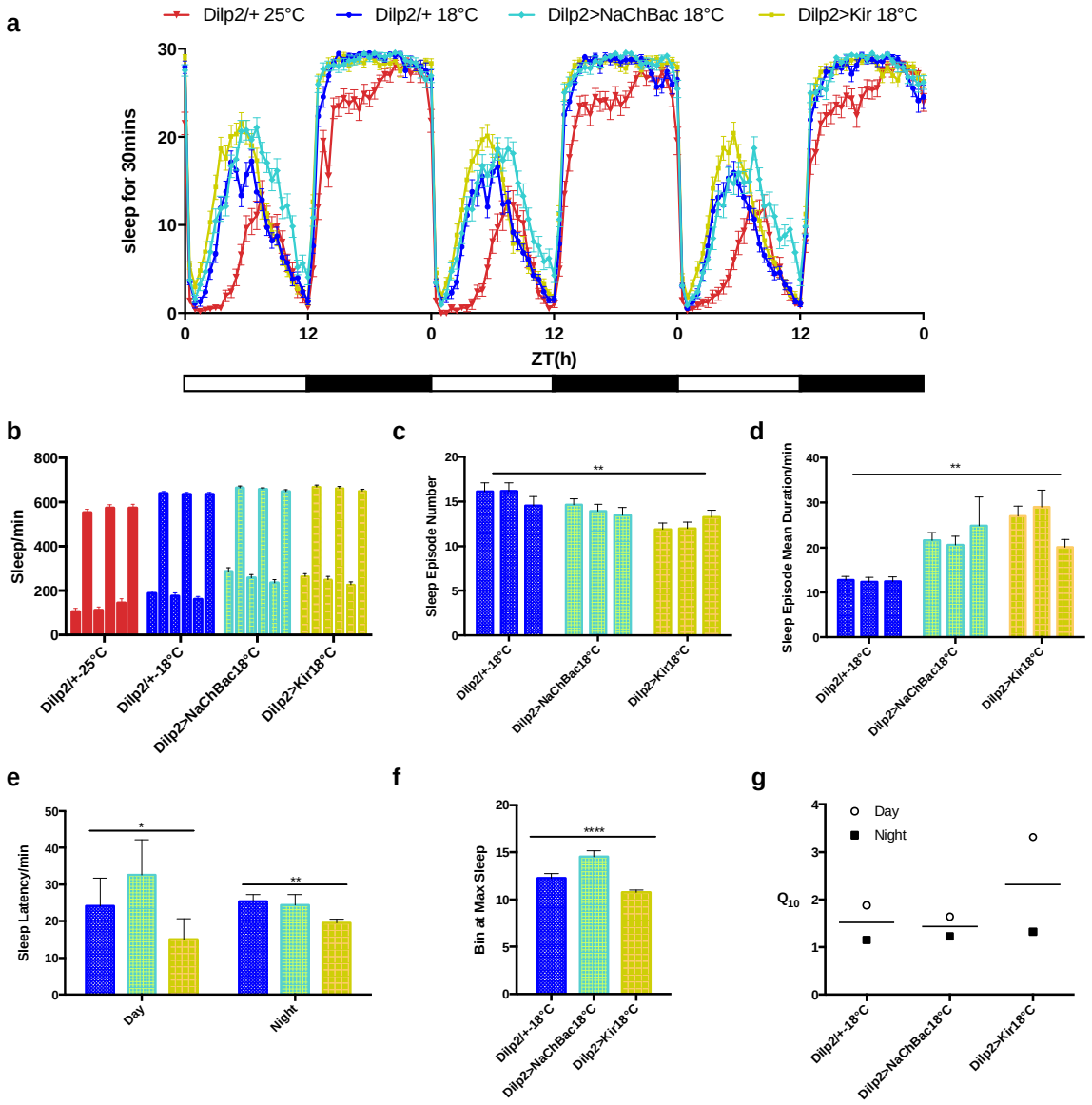
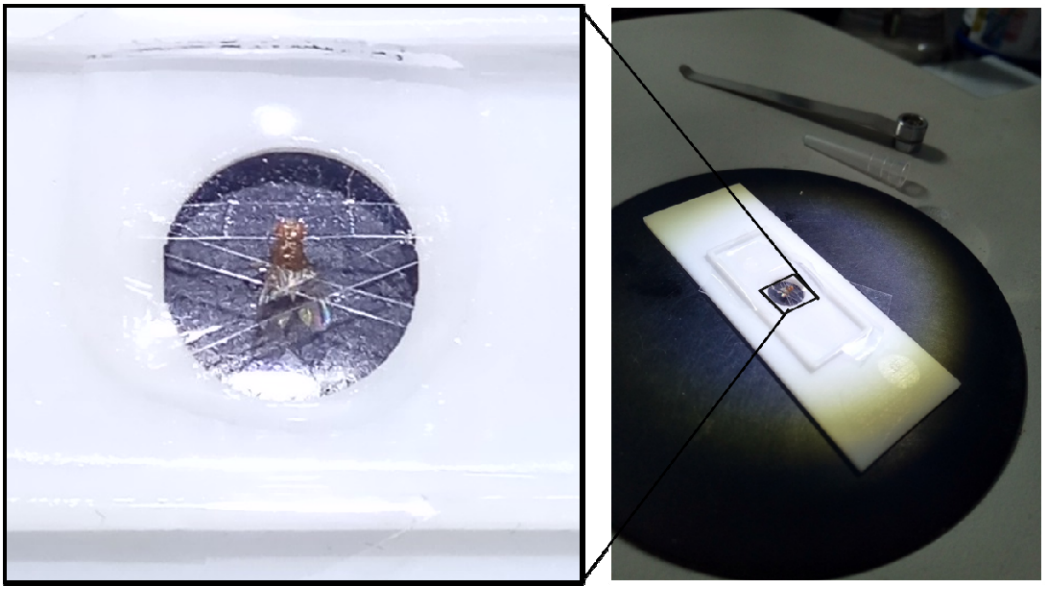
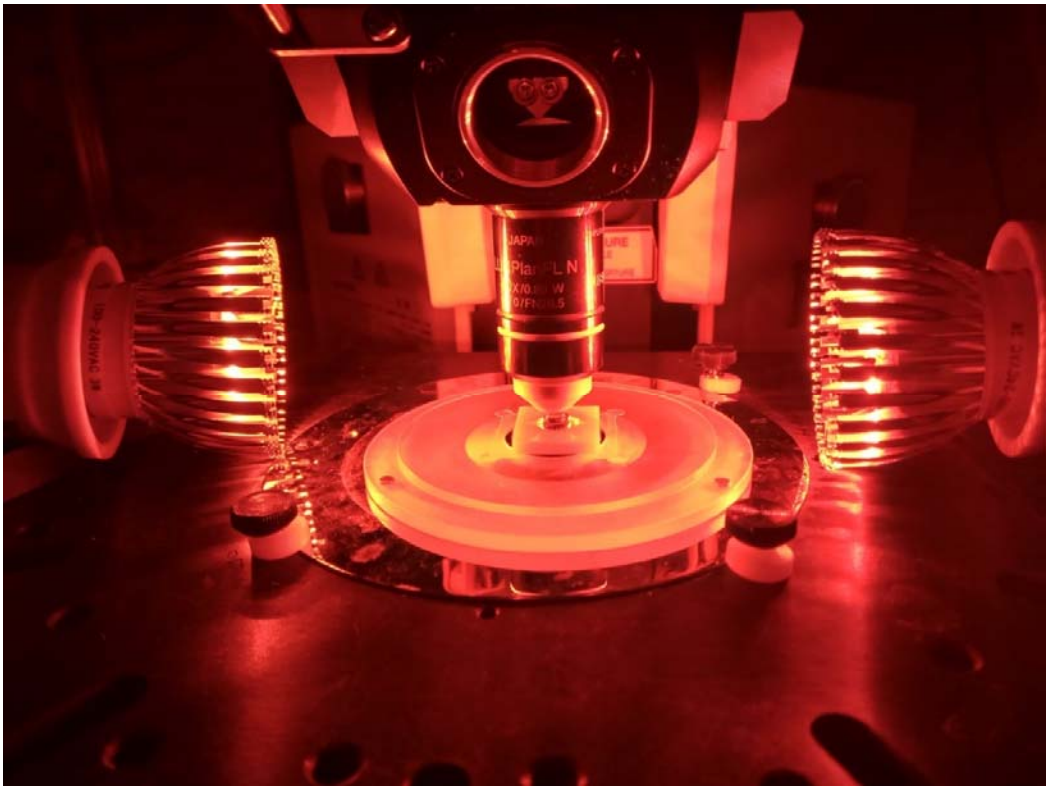


Fig. 4 Manipulation of insulin-producing cells under cold conditions changes sleep architecture **a.** Sleep pattern of insulin-producing cell-manipulated flies at 18°C and control lines at 25°C and 18°C from days 4-6 after eclosion. **b.** Total sleep duration during the day and at night at 25°C and 18°C on the 6th day after eclosion. **c.** Sleep episodes from days 4-6 after eclosion. **d.** Average sleep duration of each episode from the days 4-6 after eclosion. **e.** Sleep latency on the 6th day after eclosion. **f.** The average time (bin) when flies underwent max daytime sleep from days 4-6 after eclosion. One bin is 30 min long. **g.** The temperature coefficient index (Q10) of the sleep latency from days 4-6 after eclosion. Dilp2/+, $n_{18°C}$=57, $n_{25°C}$=55; Dilp2>NaChBac, $n_{18°C}$=68; Dilp2>Kir, $n_{18°C}$=85; Error bars: SEM; **c-g**, ANOVA, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Supplementary figures



Supplementary Fig. 1 Fixation of Adult *Drosophila Melanogaster*



Supplementary Fig. 2 Application of red light stimulation (fly supporter used here is different from that used in Supplementary Fig. 1)

Supplementary Code (MATLAB):

```
%-------Merge the result of all scanning planes into one image for each scanned volume-----

clear all;
clc;
imname = dir('.\*.png'); %read all png images under current folder
im_num = length(imname);
im_temp = imread(imname(1).name,'png');
[height,width] = size(im_temp);
DB(:,:,:) = zeros(height,width,im_num,'uint8'); %the result images were previously exported as gray scale .png files using Olympus FV10-ASW 3.1 Viewer.

prompt = {'Z number?:','T number?:'}; %Z number and T number were manually inputted for each file
dlg_title = 'Input';
num_lines = 1;
answer = inputdlg(prompt,dlg_title,num_lines);
znumber=str2num((answer{1}));
tnumber=str2num((answer{2}));

if im_num~=znumber*tnumber;
```

```
    h=warndlg('ZxT is not equal to total image number, some image
missing?','WARNING','modal')
else
    for i1 = 1:im_num
        DB(:,:,i1) = imread(imname(i1).name,'png');
    end
    imshow(DB(:,:,1));

    im_num_zmerged=im_num/znumber
    im_zmerged(:,:,:)=zeros(height,width,im_num_zmerged,'uint8');
    for i2=1:im_num_zmerged
        for i3=1:znumber
            im_zmerged(:,:,i2)=im_zmerged(:,:,i2)+DB(:,:,i2+(i3-1)*tnumber);
        end
    end

    mkdir zmerged;
    directory=[cd,'\zmerged\']
    for i4=1:im_num_zmerged
        imwrite(im_zmerged(:,:,i4),[directory,strcat('T',num2str(i4),'.png')]);
        disp(i4);
    end
end
```