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Circadian modulation of light-induced locomotion responses in *Drosophila melanogaster*

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The relationship between light and the circadian system has long been a matter of discussion. Many studies have focused on entrainment of light with the internal biological clock. Light also functions as an environmental stimulus that affects the physiology and behaviour of animals directly. In this study, we used light as an unexpected stimulus for flies at different circadian times. We found that wildtype flies showed circadian changes in light-induced locomotion responses. Elevation of locomotor activity by light occurred during the subjective night, and performance in response to light pulses declined to trough during the subjective day. Moreover, arrhythmic mutants lost the rhythm of locomotion responses to light, with promotion of activity by light in *timeless*^{01} mutants and inhibition of activity by light in *Clock*^{ar} mutants. However, neither ablation of central oscillators nor disturbance of the functional clock inside compound eyes was sufficient to disrupt the rhythm of light responses. We show that, compound eyes, which have been identified as the control point for normal masking (promotion of activity by light), are sufficient but not necessary for paradoxical masking (suppression of activity by light) under high light intensity. This, taken together with the clear difference of light responses in wildtype flies, suggests that two different masking mechanisms may underlie the circadian modulation of light-induced locomotion responses.

Keywords: Arrhythmic mutants, circadian rhythm, *Drosophila*, light, locomotion responses, normal masking, paradoxical masking

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Circadian rhythms are endogenously generated daily oscillations of biological processes (Reppert & Weaver 2002). Endogenous pacemakers can be entrained by environmental cues that are transferred from peripheral tissues to form a 24-h periodic pattern. In turn, entrained pacemakers synchronize the phase of circadian oscillation of peripheral tissues and allow animals to adapt more readily to predictable daily changes in the environment (Panda *et al.* 2002).

In fruit flies, *Drosophila melanogaster*, circadian clocks consist of multiple oscillators (Bell-Pedersen *et al.* 2005; Glossop & Hardin 2002). The small ventral lateral neurons (sLN$_v$s) in each hemisphere of the brain govern locomotor activity and eclosion rhythms and act as central oscillators (Blanchardon *et al.* 2001; Renn *et al.* 1999). However, a growing number of circadian phenomena have been shown to be non-LN$_v$s dependent. Malpighian tubules, non-innervated epithelial tubes, can maintain circadian clock gene expression even in headless flies (Giebultowicz & Hege 1997). The molecular clock system inside antennal neurons is also thought to be necessary and sufficient to regulate the electroantennogram response rhythm to odour (Krishnan *et al.* 1999; Tanoue *et al.* 2004). Some circadian behaviours are also non-LN$_v$s dependent, for example olfactory attractive and repulsive responses (Zhou *et al.* 2005), responsiveness to the dopamine receptor agonist in decapitated flies (Andretic & Hirsh 2000) and egg-laying behaviour (Howlader & Sharma 2006; Howlader *et al.* 2006).

Light is an important environmental cue for animals. Besides the entrainment of circadian rhythms, other physiological functions can be affected directly by light, such as suppression of locomotor activity in nocturnal animals or promotion of activity in diurnal animals. These physiological changes, directly driven by light, often mask the activity controlled by the endogenous pacemaker and are referred to as 'masking effects of light' (Mrosovsky 1999; Rieger *et al.* 2003). Fruit flies are considered to be diurnal animals and favour dim light (Rieger *et al.* 2007). In contrast, some mutant strains of flies show inhibited activity by lights-on, as is the case in nocturnal animals. The *glass*^{60j} *cry*^{b} double mutants that are completely blind to circadian entrainment show marked inhibition of activity under high light intensity (Helfrich-Forster *et al.* 2001). Two kinds of *Clock* mutants, *Clk*^{Jrk} and *Clk*^{ar}, having defects in the transcription of core circadian genes, lose their normal ability to react to lights-on transitions (Allada *et al.* 1998, 2003) and are preferentially nocturnal in light/dark cycles (Kim *et al.* 2002). Additionally, *narrow abdomen*, a putative ion channel mutant, shows an abnormal circadian output phenotype and repressed locomotor activity during the light phase (Lear *et al.* 2005; Nash *et al.* 2002). Although it is generally thought that masking works independently of the circadian clock (Helfrich-Forster *et al.* 2001; Rieger *et al.* 2003), the above studies suggest that disturbance of the circadian system may affect light masking in flies.

The *Drosophila* larval visual system, Bolwig's organ (BO), mediates larval photophobic behaviour (Sawin-McCormack

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et al. 1995). Mazzoni *et al.* (2005) found that ablation of larval pacemaker neurons [lateral neurons (LNs)] abolished light avoidance behaviour and that clock mutants showed different light responses in larval photophobic behaviour. These studies imply a connection between light masking in larvae and the endogenous clock system. Consequently, we investigated the light responses of adult flies at different circadian times (CTs). Our purpose was to clarify the relationship between masking and the circadian system, specially focusing on adaptation of adult flies to sudden changes in environment.

Materials and methods

Fly strains

The following fly strains were used in this study: standard wildtype *Canton-S* (*CS*), *yellow white* (*y w*), *period*^{01} (*y w per*^{01}), *timeless*^{01} (*y w; tim*^{01}), *pigment dispersing factor*^{01} (*y w;; pdf*^{01}), *Clock*^{ar}(*y w;; Clk*^{ar}), *cryptochrome*^{baby} (*y w;;cry*^{b}), *eyes absent*^{2}(*eya*^{2}), *Clock*^{Jrk} (*Clk*^{Jrk}), *cycle*^{01} *rosy*^{506} (*cyc*^{01} *ry*^{506}), *pdf-GAL4*, *p[GMR-GAL4]*, *UAS-reaper* (*UAS-rpr*), *UAS-head involution defective* (*UAS-hid*), *per*^{01}*;Rh1-per*, *UAS-CYCΔ*, *UAS-CLKΔ*. The double-mutant *eya*^{2}*;Clk*^{Jrk} was generated from *eya*^{2} (located on the second chromosome) and *Clk*^{Jrk} (located on the third chromosome) with the balancer *y w;Adv/Cyo;Sb/TM6B*. These strains were grown on cornmeal, sugar, yeast and agar medium as previously described (Guo *et al.* 1996). Flies were raised at 25°C, 60% relative humidity in 12-h light/12-h dark (LD) cycles, with lights-on at zeitgeber time (ZT) 0 and lights-off at ZT 12.

Light-induced behaviour experiment

Locomotor activity was continuously monitored and recorded in 1 min bin by placing individual adult flies (3- to 7-day-old males) in glass tubes and monitoring using a Trikinetics (Waltham, MA, USA) system. Black boxes were fixed with light-emitting diodes. The light intensity inside boxes was adjusted using an Atten (Shenzhen, China) regulated DC power supply and detected with a Trikinetics environmental monitor. Before being placed in the locomotor monitor, adult flies were entrained in LD cycles in the same incubator, with lights-on at 0600 h (ZT0). After entrainment, the test group and the control group were transferred to constant darkness (DD) conditions in the black box for 3 days. DD day 1 was the adaptive day. The second day of DD was considered the baseline day, with locomotor activity on DD day 2 considered the baseline activity. Subsequently, during day 3, flies were exposed to light (1-h intervals) at different CTs and their locomotor activity was recorded. The 0 lux groups served as the dark control for relative changes in locomotor activity to baseline day.

Data analysis

Results for light-induced locomotion responses are given as percentages of the total activity of the baseline day (DD day 2). Locomotor activity during the light phase (P_{test}) was compared with the activity recorded at the same CT in the previous baseline day ($P_{baseline}$). DELTA means the change in locomotor activity relative to the baseline day (DELTA $= P_{test} - P_{baseline}$).

Data were analysed by one-way analysis of variance (ANOVA) with *post hoc* tests for multiple comparisons and Student's *t*-test for two samples. All data are given as mean behavioural responses (±SEM). The level of significance was set at $P < 0.05$.

Results

Light evokes locomotion responses in wildtype flies in a circadian manner

To evaluate the direct effect of light (masking effects of light) at different CTs, we first examined the responses to light of wildtype *CS* flies. Locomotor activity was clearly enhanced under 1 h of light exposure (5 lux) when the lights-on time-point was CT18 (Fig. 1a). However, a similar phenomenon was not observed when the lights-on time-point was at CT6. *Canton-S* flies did not show any obvious response to light during the hour of light exposure, only the notable lights-off response (Fig. 1b). Light-induced locomotion responses were then measured at 12 CTs and results were given as the change in locomotion relative to baseline day. We found that light-induced locomotion responses in *CS* flies were a function of the time of day (Fig. 1c and Figure S1). Elevation of locomotor activity by light occurred when the light pulse was administered during the subjective night when flies showed relatively low locomotor activity. Locomotor performance declined to baseline level, however, when the light treatment was administered during the subjective day.

In addition, lights-off evoked obvious locomotion responses. We observed different post-pulse responses after 1 h of light treatment by examining the 24-h locomotion pattern of *CS* flies. The amplitude of the lights-off response changed in a daily bimodal manner (Figure S1). Significantly longer bouts of activity were observed at CT10 and CT22 that corresponded to the evening peak and morning peak, respectively.

The *y w* flies also showed daily oscillations in light-induced locomotion responses (Fig. 2a and Figure S2). The influence of genetic background on this behaviour was evident. Suppression of locomotor activity by light in *y w* flies was particularly marked during the subjective day compared with *CS* flies (Figure S2). When light intensity was raised to 200 or 500 lux, however, suppression also occurred in *CS* flies (Fig. 1d). When entrained *CS* flies were exposed to light for 1 h at CT18, light still promoted locomotor activity at 200 and 500 lux. However, flies showed inhibited activity under high light intensity at CT6. These data suggest that, while the oscillation of locomotion responses induced by light is not dependent on genetic background, light sensitivity is obviously influenced by genetic background.

A countercurrent apparatus (Benzer 1967) was used to test phototactic behaviour of the flies. In contrast to photophobic behaviour in larvae (Sawin-McCormack *et al.* 1995), adult flies exhibit a strong tendency to move towards light (Benzer 1967). No obvious differences were observed in phototaxis performance of *CS* flies and *y w* flies at the two time-points, CT6 and CT18 (Figure S7). However, flies from both genetic backgrounds showed distinct locomotion responses to unexpected light treatments at these two time-points. We believe the reason for this difference in phototactic and locomotion responses may be because of experimental procedures: the unavoidable disturbance of flies during the experimental procedure before the phototactic test caused flies to enter a prewarning state before treatment. However, the locomotor test detected differences in responses effectively as light treatments administered in this case were truly unexpected.

Light-induced locomotion responses are clock molecule dependent

To determine whether light-induced locomotion response rhythms of *Drosophila* were controlled by circadian clock

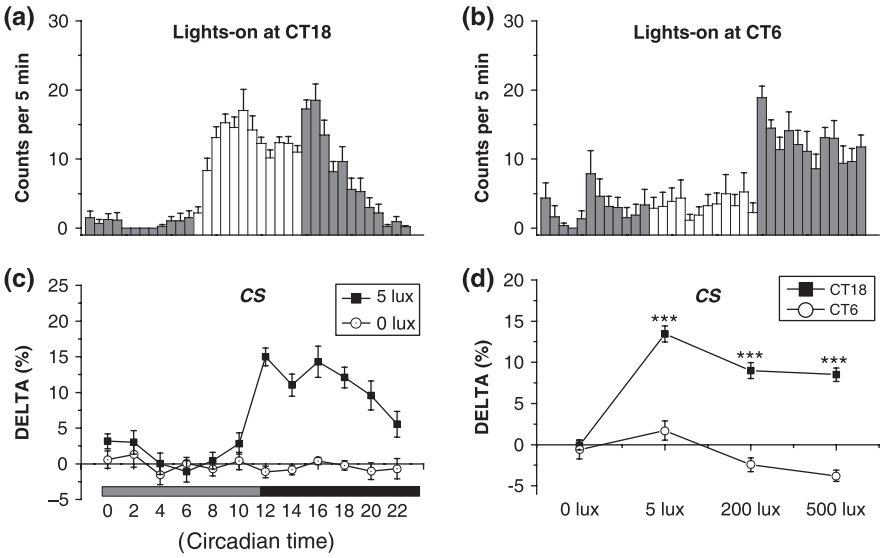

Figure 1: Light can evoke locomotion responses of wildtype *CS* flies in a circadian manner. (a) *CS* flies showed elevated locomotor activity (5-lux light exposure, 1 h, lights-on time-point: CT18). Activity records are shown on a 3-h scale. Each vertical bar represents average counts of recorded activity within 5 min. (b) The *CS* flies did not respond significantly to light under 1-h light exposure, except for the notable lights-off response (lights-on time-point: CT6). To facilitate comparisons, activity during the light treatment is represented white bars and 1-h light exposure is administered at CT18 and CT6, respectively, in (a) and (b). (c) *CS* flies showed circadian modulation of locomotion responses to light at different CTs, with elevated locomotor activity during the subjective night (grey horizontal bar: CT12–24) and baseline level locomotion responses during the subjective day (light grey horizontal bar: CT0–12). The relative changes in locomotor activity (DELTA) are given as the percentages of the total activity of the baseline day. Filled squares represent the relative response values at different CTs under 5-lux light exposure; open circles denote the dark control group (0 lux) at the same time. The effect of time of day was significant under the circumstance of 5 lux ($P < 0.0001$, ANOVA; control group: $P = 0.458$, ANOVA; $n = 16$ for each time-point). (d) The response patterns to light across a range of light intensities at CT18 (filled square) and CT6 (open circle). Light promoted locomotor activity at CT18 from 5 to 500 lux (one-sample *t*-test. $P < 0.0001$, $n = 16$, respectively, test value = 0). The effect of light was not significant at CT6 under 5 lux (one-sample *t*-test, $P = 0.161$, $n = 16$, test value = 0). However, under higher light intensity, flies showed observably inhibited locomotor activity at CT6 (one-sample *t*-test, test value = 0, 200 lux: $P = 0.007$, $n = 16$; 500 lux: $P < 0.0001$, $n = 16$). The locomotion responses to light at CT6 and CT18 were significantly different under 5, 200 and 500 lux treatments ($P < 0.0001$, respectively, Student's *t*-test).

genes, we tested the light responses of per^{01} mutants. These mutants have been shown to lose circadian rhythms in eclosion as well as locomotor activity (Baylies *et al.* 1987; Konopka & Benzer 1971; Yu *et al.* 1987). Our study indicated that per^{01} mutants with a *y w* genetic background did not show fluctuation in light-induced locomotion responses: weak light-driven locomotion responses were not significantly different from the 0 lux control group (Fig. 2b and Figure S3). Wijnen *et al.* (2006) recently reported a systematic analysis of light-driven transcription in some arrhythmic mutants, showing that light-regulated transcription was altered in the mutant studies. So, we tested light-induced locomotion responses of other arrhythmic mutants and found that tim^{01} and Clk^{ar} mutants also lost the oscillation of light-induced locomotion responses (Fig. 2c,d). However, while tim^{01} mutants always showed elevated locomotion responses to light, regardless of CT, Clk^{ar} mutants showed inhibited locomotor activity under 5-lux light exposure (Figure S3). These results indicate that core clock genes can influence the rhythm of light-induced locomotion responses. Furthermore, the light response pattern (suppression or activation of locomotor activity) may be affected by clock genes.

Circadian central oscillators are not necessary for light-induced locomotion response rhythms

As previously mentioned, some circadian behaviours are non-LN_vs dependent. We questioned, therefore, whether circadian central oscillators were necessary for light-induced locomotion rhythms. Pigment dispersing factor (PDF) is thought to be a circadian signal secreted from the LN_vs to regulate behaviour output (Helfrich-Forster *et al.* 2000; Renn *et al.* 1999). We tested light responses of pdf^{01} mutants and found that they still retained rhythmic fluctuation in light-induced locomotion responses (Fig. 3a). Next, we ablated the central pacemaker neurons by using *pdf-GAL4* to drive expression of apoptosis-promoting genes in PDF-positive LN_vs (Renn *et al.* 1999). *UAS-reaper* (*rpr*) (White *et al.* 1996) and *UAS-head involution defective* (*hid*) (Grether *et al.* 1995) were used to activate ectopic apoptosis in *Drosophila*. Our data suggest that these ablation lines also show daily

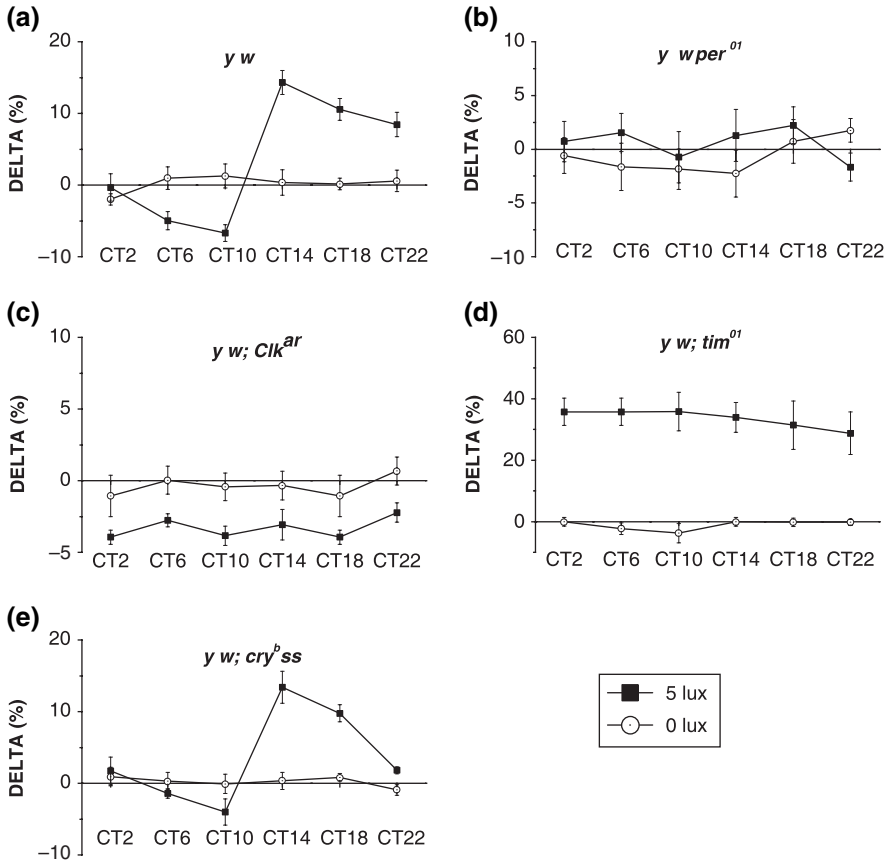

Figure 2: Light-induced locomotion rhythms are clock gene dependent. Light responses were tested in arrhythmic mutants with a *y w* genetic background. Each strain was exposed to 1-h light pulses at six CT during the test day. (a) *y w* flies showed a rhythmic oscillation in light-induced locomotion responses when 1-h light exposure of 5 lux was given at different CT ($P < 0.0001$, ANOVA; control group: 0 lux, $P = 0.761$, ANOVA; $n = 16$ for each time-point), with clearly enhanced locomotion responses occurring during the subjective night and depressed responses to light occurring during the subjective day. Arrhythmic mutants lost the circadian rhythm of light-induced locomotion responses: (b) weak light-driven locomotion responses in per^{01} flies ($P = 0.661$, ANOVA; control group: 0 lux, $P = 0.308$, ANOVA; $n = 16$ for each time-point), (c) suppression of locomotor activity by light in Clk^{ar} flies ($P = 0.485$, ANOVA; control group: 0 lux, $P = 0.532$, ANOVA; $n = 16$ for each time-point), (d) promotion of locomotor activity by light in tim^{01} flies ($P = 0.921$, ANOVA; control group: 0 lux, $P = 0.608$, ANOVA; $n = 16$ for each time-point). (e) cry^{b} flies still showed a circadian oscillation in light-induced locomotion responses when 1 h of light exposure was given at different CTs ($P < 0.0001$, ANOVA; control group: 0 lux, $P = 0.954$, ANOVA; $n = 16$ for each time-point). Filled squares represent the relative response values at different CTs under 5-lux light exposure; open circles denote the dark control group (0 lux) at the same time.

oscillation in light-induced locomotion responses (Fig. 3b,c). Unlike the photophobic behaviour observed in larvae (Mazzoni *et al.* 2005), the absence of PDF-positive LN_vs did not abolish the light responses in adult flies (Figure S4). Although we cannot exclude the potential effect of LN_vs on the regulation of light responses, our observations suggest that LN_vs and PDF are not necessary for the rhythms of light-induced locomotion responses.

A functional clock inside compound eyes is not necessary for light-induced locomotion rhythms

Compound eyes are necessary for the normal masking effects of light (provoking activity by lights-on) (Rieger *et al.* 2003), and photoreceptors inside the compound eyes of flies have been shown to contain tissue autonomous circadian oscillators (Cheng & Hardin 1998). So, we questioned whether an eye-specific functional clock underlies the circadian light response. We disrupted the normal molecular clock in compound eyes by *p[GMR-GAL4]*-driven expression of dominant-negative versions of CYCLE or CLOCK (Tanoue *et al.* 2004). Unexpectedly, *pGMR>UAS-CLKΔ* flies died before eclosion. So the response of *pGMR>USA-CYCΔ* flies was detected with only two independent *UAS-CYCΔ* lines, *UAS-CYCΔ103* and *UAS-CYCΔ24*. These fly lines, with targeted disruption of the eye-specific functional clock, showed normal oscillation in light-induced locomotion responses (Fig. 3d and Figure S4). Light responses were then tested in

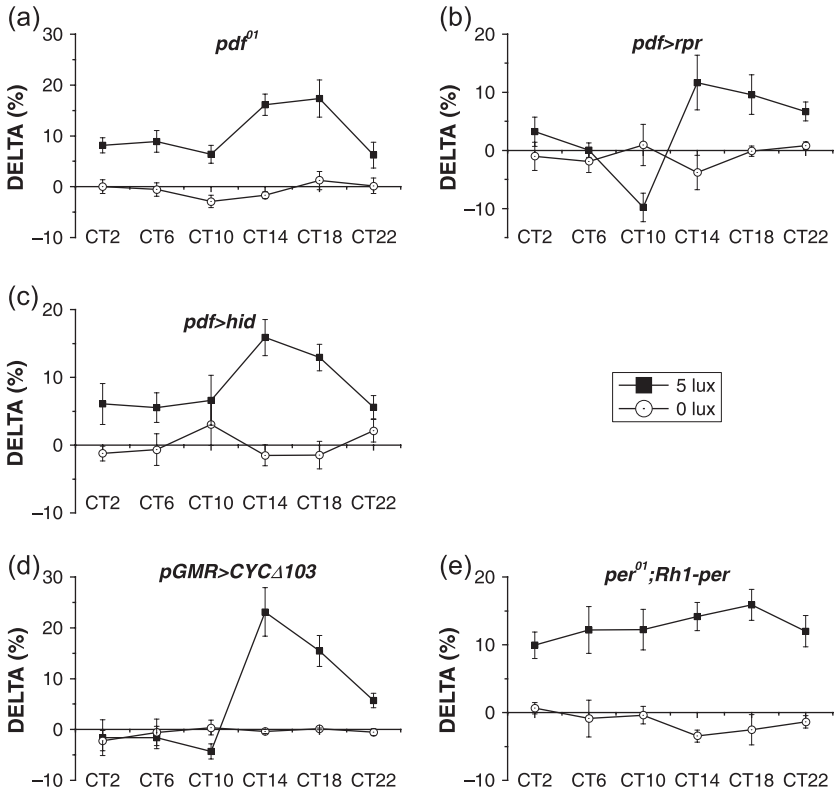

Figure 3: Circadian central pacemakers and functional clock inside compound eyes are not necessary for the rhythm of light-induced locomotion responses. (a) The pdf^{01} mutant flies showed rhythmic oscillation in light-induced locomotion responses ($P = 0.001$, ANOVA; control group: 0 lux, $P = 0.330$, ANOVA; $n = 16$ for each time-point). (b) The *pdf>rpr* flies, with genetic ablation of the LNs by *reaper*, retained circadian oscillation in light-induced locomotion responses ($P = 0.001$, ANOVA; control group: 0 lux, $P = 0.657$, ANOVA; $n = 16$ for each time-point). (c) *hid*-ablation of PDF-positive ventral LNs also showed light response rhythms ($P = 0.015$, ANOVA; control group: 0 lux, $P = 0.552$, ANOVA; $n = 16$ for each time-point). (d) Disruption of the functional clock inside compound eyes by the dominant-negative version of CYC did not block circadian behaviour in light-induced locomotion responses ($P < 0.0001$, ANOVA; control group: 0 lux, $P = 0.887$, ANOVA; $n = 16$ for each time-point). (e) No oscillation was detected in per^{01};*Rh1-per* flies, which specifically rescue *per* oscillation in photoreceptors under a per^{01} background ($P = 0.650$, ANOVA; control group: 0 lux, $P = 0.541$, ANOVA; $n = 16$ for each time-point). Filled squares represent the relative response values at different CTs under 5-lux light exposure; open circles denote the dark control group (0 lux) at the same time.

per^{01};*Rh1-per* flies, in which oscillation of PER is restored specifically in photoreceptors (Cheng & Hardin 1998). However, there was no oscillation in light-induced locomotion responses in per^{01};*Rh1-per* flies (Fig. 3e). These results strongly suggest that circadian modulation of light-induced locomotion responses dose not depend on the functional clock inside the photoreceptors of compound eyes. Compound eyes may just serve as a light input pathway to transfer light signals to the masking control centre.

As mentioned above, *Clk* mutants showed paradoxical masking responses (suppression activity by lights-off) (Mrosovsky 1999; Rieger *et al.* 2003). We wondered whether compound eyes also play a major role in paradoxical masking. Double-mutant flies eya^2;Clk^{Jrk} were constructed to detect the light responses. These double-mutant flies had phenotypes of eya^2 and Clk^{Jrk}, no compound eyes and loss of circadian rhythm. However, the flies also displayed paradoxical masking behaviour under LD conditions (500 lux light intensity), with suppression of activity by lights-on and promotion of activity by lights-off (Fig. 4). When light intensity was reduced to 5 lux, however, no obvious changes were detected in the double mutants under 1 h of light exposure (data not shown). This result indicates that compound eyes are essential for perception of weak light. However, eya^2 mutants also showed paradoxical masking under 1 h high intensity light exposure during the subjective day (Figure S6). Thus, we propose that compound eyes are sufficient but not necessary for paradoxical masking under high light intensity.

CRYPTOCHROME is not necessary for light-induced locomotion response rhythm

The CRYPTOCHROME (CRY) is a critical circadian photoreceptor (Emery *et al.* 2000; Stanewsky *et al.* 1998) and controls light-dependent sequestration of TIMELESS (TIM) (Ceriani *et al.* 1999). cry^b mutants, with a point mutation in the

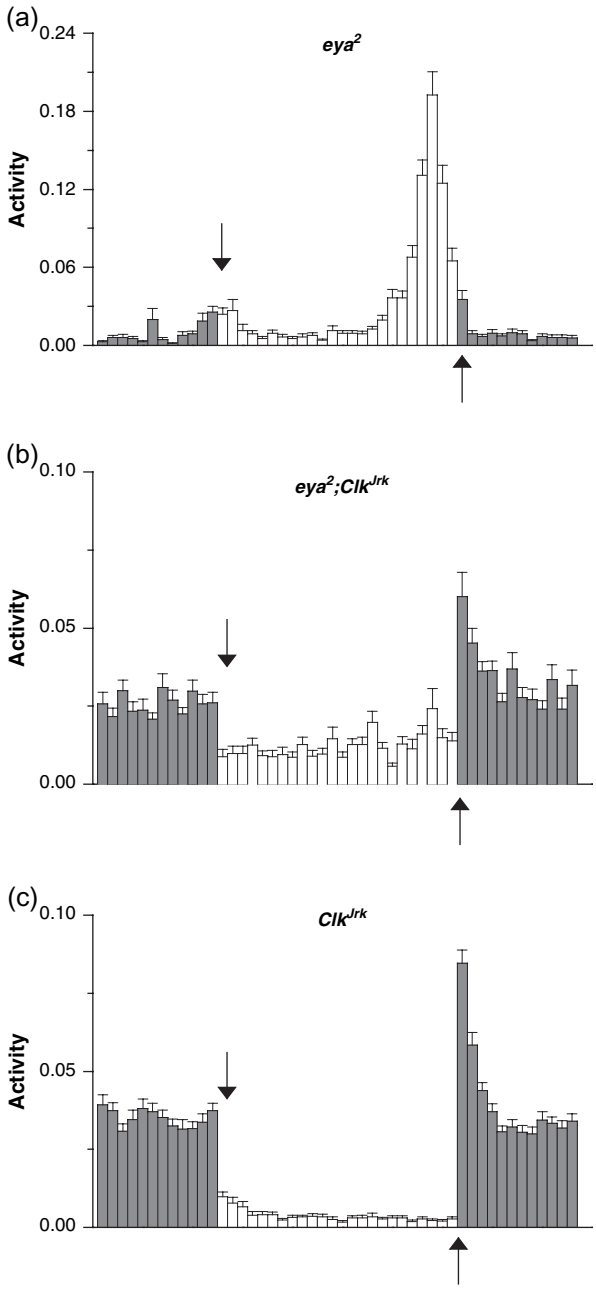

Figure 4: Compound eyes are not necessary for paradoxical masking under 500-lux light intensity during LD conditions. (a) *eya*^{2} flies showed normal morning and evening peaks under LD conditions (12 h light: 12 h dark, 500-lux light intensity), but lost lights-on and lights-off peaks. Activity records are shown on a 24-h scale. Each vertical bar represents relative activity recorded over a 30-min period. White bars represent the light phase of the LD regime and black bars denote the dark phase. Arrows denote time-point of lights-on and lights-off. *eya*^{2};*Clk*^{Jrk} double mutants (b) and *Clk*^{Jrk} flies (c) showed obvious nocturnal behaviour under LD conditions.

flavin-binding site of the CRY protein (Stanewsky *et al.* 1998), showed a normal light response rhythm (Fig. 2e and Figure S3), suggesting that CRY was not necessary for establishing the rhythm of light-induced locomotion responses, although CRY has been shown to be a clock component in olfaction output rhythms in the antenna (Krishnan *et al.* 2001) and the circadian transcriptional repressor in peripheral oscillators (Collins *et al.* 2006). Consequently, we suggest that the role of CRY in peripheral circadian systems might be tissue specific.

Discussion

In this study, we report that light can evoke circadian locomotion responses in adult flies. It has been shown that light masking is present in arrhythmic mutants of flies (Wheeler *et al.* 1993) and in mammals with suprachiasmatic nucleus lesions (Redlin & Mrosovsky 1999), and it is widely agreed that masking is independent of the circadian clock (Helfrich-Forster *et al.* 2001; Rieger *et al.* 2003). However, the current study shows that light masking (light-induced locomotion response) is modulated by the circadian system in adult flies.

Do our results reflect rest/activity differences?

The results presented here may reflect distinct responses under different physiological conditions such as sleep and wake cycles. The rest state in flies is a sleep-like state characterized by an increased arousal threshold and function of homeostatic regulation (Hendricks *et al.* 2000; Shaw *et al.* 2000). The increased arousal threshold is associated with decreased responsiveness to stimuli. The arousal threshold to mechanical stimuli has been shown to gradually increase during the dark phase of LD conditions (Hendricks *et al.* 2000). Consequently, we tried to observe the sleep-like state of flies in DD conditions. As expected, the long-lasting rest state occurred mainly during the subjective night (data not shown). In other words, the arousal threshold during this period remained at a high level. However, our observations indicate that the locomotor activity of wildtype flies can be significantly promoted by light during this relatively high arousal threshold period. One possibility is that the high perception sensitivity of flies to light is a necessary survival strategy when flies are in an inactive state. Thus, the circadian response to light may be distinguished from the potential influence of rest/activity cycles.

Light masking, light resetting and post-pulse activity

A short light pulse during the subjective night can reset the endogenous clock and cause obvious phase shifting (Pittendrigh 1993). The phase shifting of clock resetting by light was also detected in our experiments (lights-on at CT20 in Figure S1 or lights-on at CT18 in Figure S2). It seems that there is no direct connection between light resetting and masking. Although *cry*^{b} mutants lose phase shifting in response to a light pulse (Stanewsky *et al.* 1998), the flies still showed normal light-induced locomotion rhythms in our experiments. Conversely, loss of function in eyes does not block resetting

of the circadian clock (Helfrich-Forster *et al.* 2001) although light masking disappears in eyeless flies (Rieger *et al.* 2003). Therefore, masking and resetting can both be evoked by light signals and are regulated by different mechanisms.

Kim *et al.* (2002) reported that short duration light pulses (10 min) could elicit time-of-day differences in amplitude of the activity bursts (Kim *et al.* 2002). The induced activity bursts mentioned by Kim *et al.* (2002) consist of lights-on activity and lights-off activity, with lights-off activity predominating. Our results support the finding of Kim *et al.* (2002) that post-pulse activity or lights-off responses, change in a circadian manner.

Different response patterns to light in arrhythmic mutants

Circadian modulation of light-induced locomotion responses is lost in some clock mutants. Interestingly, arrhythmic mutants showed different locomotion responses to light in our experimental conditions. CLOCK (CLK) and CYCLE (CYC) are the critical components of the circadian transcriptional feedback loop and heterodimerize to activate transcription of *per* and *tim* (Allada *et al.* 1998; Rutila *et al.* 1998). Paradoxical masking in *Clk* mutants was independent of genetic background under the conditions used, while *cyc* mutants showed a slightly inhibited light response under high light intensity (Figure S5). It is not sufficient to identify the phenotype of *cyc* mutants in one kind of genetic background. Rutila *et al.* (1998) also reported that *cyc* mutants have difficulty with light perception. The mammalian homologue of CYC (MOP3) has also been shown to affect light masking in mice (Bunger *et al.* 2000). Allada *et al.* (1998) have discussed a possible function of the PAS domain in CLK light responses. We consider the abnormal light response observed in our experiments may depend on CLK/CYC-related transcription. But there is still no evidence to connect light with CLK/CYC transcription directly. Research from mammals implies that a potential signal pathway in light masking, which is mediated by the dopamine D2 receptor (Doi *et al.* 2006), links light with CLOCK/BMAL1-dependent transcription (Yujnovsky *et al.* 2006).

The PER/TIM heterodimer is the core of the circadian transcriptional feedback loop, inhibiting transcriptional activity of the CLK/CYC heterodimer (Panda *et al.* 2002). If *Clk* or *cyc* play a critical role in light-induced locomotion responses, *per* or *tim* may have a related function in this behaviour. In the current study, while tim^{01} mutants consistently showed elevated locomotor activity when exposed to light, per^{01} mutants did not exhibit strong responses compared with the tim^{01} mutation in a *y w* background. We suggest that genetic background is involved in this behaviour pattern because locomotor activity of $per^{01};ry^{506}$ flies can be activated by light (data not shown). How genetic background affects light-triggered behaviour output is not clear. However, there is substantial evidence showing clear physiological differences between *per* and *tim* null mutants (Andretic *et al.* 1999; Mehnert *et al.* 2007; Sakai *et al.* 2004). Anatomical evidence from neuromuscular terminals indicates that the tim^{01} mutation causes a hyperbranching phenotype and per^{01} mutants have fewer branches than wildtype flies (Mehnert *et al.* 2007). This may underlie the different light responses of *per* and *tim* null mutants in our experimental conditions.

Function of central oscillators in light responses

Small ventral LNs in each hemisphere of the brain are considered to be the central oscillators in flies (Glossop & Hardin 2002). The relationship between circadian pacemaker neurons and light-induced responses has been explored in larval flies. As Mazzoni *et al.* (2005) reported, LNs are required for photophobic behaviour in larvae. However, Hassan *et al.* (2005) found that ablation of circadian neurons did not significantly disrupt lights ON/OFF responses (Hassan *et al.* 2005). These results suggest that light-induced behaviour may be divided into several steps. Initiation of light-triggered behaviour (the ON/OFF assay) is LNs independent and light avoidance (photophobic behaviour) requires the normal function of circadian pacemaker neurons in larvae.

Our study suggests that PDF-positive neurons (or PDF) are not necessary for light response rhythms. However, either pdf^{01} or *hid*-ablation line altered the light response pattern during the subjective day in the *y w* genetic background (Figure S4). Anatomical results suggest a potential or direct connection between LNs and photoreceptors (Helfrich-Forster 2003). Unlike the simple BO light receptors in larval flies, however, the photoreceptor system of adult flies leads to greater complexity in light detection. Consequently, further work is necessary to clarify how LNs affect light responses.

The contribution of photoreceptors

Photoreceptors inside the compound eyes of flies contain tissue autonomous circadian oscillators (Cheng & Hardin 1998). Additionally, expression of some components involved in visual processes was found to be under the control of the circadian clock (Claridge-Chang *et al.* 2001; Wijnen *et al.* 2006). Anatomical results reported by Pyza and colleagues (Gorska-Andrzejak *et al.* 2005; Pyza & Meinertzhagen 1999) also provided a physical analysis of circadian changes in the visual system of flies. However, disturbance of the local functional clock in compound eyes had no impact on the oscillation of light-induced locomotion responses. This result suggests that the functional clock inside compound eyes is not necessary for the rhythm of light-induced locomotion responses.

Previous studies suggest that normal masking effects of light have a direct relationship with compound eyes (Rieger *et al.* 2003). But paradoxical masking occurs in $glass^{60j}$ cry^{b} double-mutant flies, in which all known photoreceptors have been eliminated (Helfrich-Forster *et al.* 2001). There are obviously different mechanisms between normal and paradoxical masking (Rieger *et al.* 2003). The responses of *y w* flies to weak light intensity include promotion of activity during the subjective night and suppression of activity during the subjective day. This is an interesting functional difference in the response to light. Circadian modulation of light-induced locomotion responses presumably results from the interaction of normal masking with paradoxical masking. While normal masking predominated during the subjective night,

paradoxical masking counteracted the light promotion of activity during the subjective day. Different responses between *CS* and *y w* flies suggest that lack of normal eye pigment and body pigmentation in *y w* flies might help the paradoxical masking centre more easily perceive light. It would appear that *y*- or *w*- related biochemical processes (Drapeau *et al.* 2006; Zhang & Odenwald 1995) may affect light responses.

In summary, the current study used light to simulate an unexpected environmental change and investigated the light locomotion responses of flies. Our results, of flies exhibiting circadian light responses in locomotion output, show that light masking (light-induced locomotion response) is modulated by the circadian system in adult flies. Moreover, this circadian behaviour is distinct from the rhythm of spontaneous locomotion activity, especially when flies are in an inactive state, in that high perception sensitivity is still maintained. Olfaction rhythms in flies observed in physiological and behavioural research (Krishnan *et al.* 1999; Zhou *et al.* 2005) also support our conclusion. We propose that these physiological phenomena are essential survival strategies for animals, such as the detection of predators or opportunistic feeding. To understand the relationship of circadian perception and the environment more fully, further research is needed to clarify the role of other perception systems and the potential molecular and cellular mechanisms involved.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Figure S1. Actograms of light-induced locomotion responses in *CS* flies. *Canton-S* flies were exposed to 1-h light pulses at 12 CTs during the test day (DD day 3). The time indicated denotes when the 1-h light pulse was administered. Activity records are shown on a 24-h scale. Vertical bars represent activity recorded over a 30-min period and are shown as fractions of the total activity of baseline day. To facilitate comparisons, activity during the light treatment, subjective day and subjective night is represented by white bars, light grey bars and grey bars, respectively. Note that during the subjective night, light triggered high levels of locomotion; however, no obvious locomotion responses were detected, except the notable lights-off response, when light was delivered during the subjective day. Also note that the lights-off response, or the post-pulse activity, was different when light treatments ended at different CTs. Clearly extended lights-off responses occurred at CT22 or CT10, when the morning and evening activity peaks were taking place.

Figure S2. Actograms of light-induced locomotion responses in *y w* flies. The *y w* flies were exposed to 1-h light pulses at six CTs during the test day (DD day 3). Vertical bars represent activity recorded over a 30-min period and are shown as fractions of the total activity of baseline day. The greatest difference with *CS* flies was that *y w* flies showed clearly inhibited light-induced locomotion responses to weak light intensity during the subjective day (CT6 or CT10).

Figure S3. Actograms of light-induced locomotion responses in arrhythmic mutant flies. Light responses of clock mutants are shown at two CTs, CT18 (left column) and CT6 (right column). Actograms of *per^{01}*, *tim^{01}*, *Clk^{ar}* and *cry^{b}ss* mutants are shown (top to bottom). All mutants have a *y w* genetic background. Responses to light shown in these mutants are clearly different: promotion of activity by light occurs in *tim^{01}*, suppression of activity by light occurs in *Clk^{ar}* and there are weak responses to light in *per^{01}*. Only the *cry^{b}ss* mutant shows clear behavioural oscillation in light responses,

with enhanced locomotor activity when the light pulse was administered at CT18 and inhibited locomotor activity when the light pulse was administered at CT6.

Figure S4. (a) Actograms of light-induced locomotion responses in pdf^{01} and *pdf>rpr* flies. Behavioural oscillation of light responses is clearly shown at CT18 (left column) and CT6 (right column) in flies lacking pigment dispersing factor (pdf^{01}, upper row) or ventral LNs ablated by *reaper* (*pdf>rpr*, bottom row). Note that pdf^{01} flies have a *y w* genetic background, but showed an altered response to light when it was administered at CT6 compared with *y w* flies (Figure S2; CT6). (b) Actograms of light-induced locomotion responses in *hid*-ablation related lines. Light-induced locomotion responses in *hid*-ablation of the PDF-positive neurons line and its controls are shown. *pdf>hid* (top row) flies showed enhanced locomotion responses to light at CT16 (left column) as do *pdf-GAL4* flies (middle row) and *UAS-hid* flies (bottom row). But when light treatment began at CT10 (right column), distinct responses from control groups were observed in *pdf>hid* flies, with changed light responses compared with suppression of locomotion responses by light in *pdf-GAL4* flies and *UAS-hid* flies. (c) Actograms of light-induced locomotion responses in flies with disruption or rescue of the functional clock in compound eyes. Two independent lines with disruption of the functional clock in compound eyes show enhanced locomotor activity at CT18 and inhibited locomotor activity at CT6 (top row: *pGMR>CYCΔ103*; middle row: *pGMR>CYCΔ24*). per^{01}*;Rh1-per* flies (bottom row) showed similar light response patterns when light was administered at CT18 (left column) or CT6 (right column). Activity records are all shown on a 24-h scale. Vertical bars represent activity recorded over a 30-min period and are shown as fractions of the total activity of baseline day.

Figure S5. Light responses of cyc^{01} flies. (a) cyc^{01} flies lost the circadian rhythm of light-induced locomotion responses when 1-h light exposure of 5 lux was given at different circadian times ($P = 0.536$, ANOVA; control group: 0 lux, $P = 0.291$, ANOVA; $n = 16$ for each time-point). The light responses of cyc^{01} flies were tested at six circadian times. The relative changes in locomotor activity (DELTA) are given as the percentages of the total activity of the baseline day. Filled squares represent the relative response values at different circadian times under 5-lux light exposure; open circles denote the dark control group (0 lux) at the same time. Actograms of light-induced locomotion responses in cyc^{01} flies are shown on a 24-h scale (b, c). (b) No obvious light responses were detected when light of 5-lux light intensity was administered at CT2. (c) When light intensity was raised to 1000 lux, a slightly inhibited locomotion response to light and an obvious lights-off response were detected in cyc^{01} flies.

Figure S6. Compound eyes were not necessary for paradoxical masking under high light intensity. Light treatment of 1000 lux intensity triggered suppression of locomotor activity in eya^2 flies (a), eya^2*;*Clk^{Jrk} flies (b) and Clk^{Jrk} flies (c) at CT10. Vertical bars represent activity recorded over a 30-min period and are shown as fractions of the total activity of baseline day.

Figure S7. Phototactic behaviour in *CS* and *y w* flies. (a) There was no significant difference in the phototaxis index of *CS* or *y w* flies between CT6 and CT18 (*CS*: $P = 0.745$; *y w*: $P = 0.518$; six groups for each bar, Student's *t*-test). (b) Experiments were carried out with the light source in the side of distal tubes (to light/towards light: upper row) or start tubes (from light/away from light: lower row). The proportion of flies distributed in the collecting tubes is shown. Filled squares and open circles denote CT6 and CT18, respectively. *Canton-S* (left column) or *y w* (right column) flies showed similar distributions in the collecting tubes at CT6 and CT18.

Appendix S1. Phototactic behaviour experiment.

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