

# *Drosophila* Olfactory Response Rhythms Require Clock Genes but Not Pigment Dispersing Factor or Lateral Neurons

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**Abstract** Odors elicit a number of behavioral responses, including attraction and repulsion in *Drosophila*. In this study, the authors used a T-maze apparatus to show that wild-type *Drosophila melanogaster* exhibit a robust circadian rhythm in the olfactory attractive and repulsive responses. These responses were lower during the day and began to rise at early night, peaking at about the middle of the night and then declining thereafter. They were also independent of locomotor activity. The olfactory response rhythms were lost in *period* or *timeless* mutant flies (*per*<sup>0</sup>, *tim*<sup>0</sup>), indicating that clock genes control circadian rhythms of olfactory behavior. The rhythms in olfactory response persisted in the absence of the pigment-dispersing factor neuropeptide or the central pacemaker lateral neurons known to drive circadian patterns of locomotion and eclosion. These results indicate that the circadian rhythms in olfactory behavior in *Drosophila* are driven by pacemakers that do not control the rest-activity cycle and are likely in the antennae.

**Key words** circadian rhythm, *Drosophila*, olfactory, attractive, repulsive, pigment-dispersing factor, lateral neurons

Diverse life forms from fly to human show circadian (about 24 h) rhythms in their biochemistry, physiology, and behavior. These rhythms are controlled by self-sustaining circadian oscillators or biological clocks, which may impart an advantage to the organisms by temporally orchestrating biochemical, physiological, and behavioral processes to allow them to better adapt to the predictable daily change in the environment (Panda et al., 2002). The molecular underpinnings of the clocks have been studied in detail by genetic analysis in *Drosophila*. Several specific clock genes, including *period* (*per*), *timeless* (*tim*), *Clock* (*Clk*), and *cycle* (*cyc*), form interlocked feedback loops, con-

stituting a core mechanism of circadian oscillators (Glossop et al., 1999; Allada, 2003).

Circadian oscillators are present in different tissues of *Drosophila*, which are divided into peripheral and central oscillators (Plautz et al., 1997; Giebultowicz, 2001; Glossop et al., 2002; Myers et al., 2003; Tanoue et al., 2004). Peripheral oscillators are present in many peripheral tissues, including the gut, excretory system, testes (Beaver et al., 2002), and olfactory organs, and they are light sensitive. This property determines their independence and is different from that of mammalian peripheral oscillators, such as those in the liver, heart, and lung, which are not light entrainable

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and thus dependent on neuronal and humoral signals from central oscillators directly entrained by light (Herzog and Huckfeldt, 2003). In *Drosophila*, central oscillators include visual photoreceptors, several subsets of brain neurons, and groups of glia cells. Lateral neurons (LNs), located in the central brain, are considered as central circadian pacemakers. The LNs consist of dorsal and ventral LNs (LN<sub>d,s</sub> and LN<sub>v,s</sub>), and only the LN<sub>v,s</sub> express the pigment-dispersing factor (PDF) neuropeptide (Helfrich-Forster, 1995; Stoleru et al., 2004; Grima et al., 2004). The LNs are required for locomotor activity rhythms and for controlling the timing of eclosion (Helfrich-Forster, 1998; Renn et al., 1999; Myers et al., 2003; Stoleru et al., 2004; Grima et al., 2004). The PDF neuropeptide is a circadian effector from central to peripheral oscillators in locomotion and eclosion rhythms. The PDF is rhythmically released into the dorsal protocerebrum, which has anatomical connections to most sites of the brain and furthermore houses much of the neurosecretory system for *Drosophila* (Helfrich-Forster, 2003).

Olfaction is a very important sensory modality in flies, which is essential for food acquisition, mating activity, and detection of predators (Carison, 1996; Krishnan et al., 1999; Vosshall, 2000). Previous investigators (Krishnan et al., 1999) have demonstrated a robust circadian rhythm in *Drosophila* in electrophysiological responses to 2 classes of olfactory stimuli by measuring electroantennogram (EAG) amplitude, and they further provided evidence that peripheral circadian oscillators are necessary for circadian rhythm in olfactory responses. Recent investigators (Tanoue et al., 2004) further confirmed that the LNs as central oscillators are not required for olfactory rhythms, and the antennal neurons as peripheral oscillators are necessary and sufficient for olfactory rhythms. Moreover, there is evidence that olfactory cues can also entrain circadian behavior (Levine et al., 2002), which is a demonstration of circadian modulation of olfactory function. However, to date, there have been no reports about circadian rhythms in the olfactory behavior of *Drosophila*. In this study, we used an olfactory T-maze assay to test if olfactory attractive and repulsive behaviors of *Drosophila melanogaster* exhibit rhythmicity; if so, we wanted to further confirm whether these rhythms are under the control of clock genes and the central circadian oscillators, as well as whether the PDF is involved in the control of rhythmic behavior. The results further provided evidence that the olfactory behavior is under circadian

regulation and that the LNs as central circadian oscillators are not necessary for the behavioral rhythms.

## MATERIALS AND METHODS

### Fly Strains

The following fly strains were used in this study: standard wild-type *Canton-S* (CS), *yellow white* (*yw*), null clock mutants *period<sup>0</sup>* (*per<sup>0</sup>*), *timeless* (*tim<sup>0</sup>*), *pigment-dispersing factor* (*pdf<sup>0</sup>*), and *disconnected*, a neuroanatomical mutant missing most of the fly central circadian pacemaker neurons. These strains were grown on cornmeal, sugar, yeast, and agar medium (Xia et al., 1997). Flies were raised in an incubator at 25 °C, 60% relative humidity, in 12-h light/12-h dark (LD) cycles. Lights-on occurred at ZT 0, and lights-off occurred at ZT 12.

### Fly Collection

Adult flies were collected within 8 h after eclosion. Virgin females were sorted out and counted under CO<sub>2</sub> treatment and then separately loaded into a number of plastic 2.5 × 10-cm culture vials, each containing a population of 50 individuals with food. The CO<sub>2</sub> treatment time was limited below 3 min to reduce the effect of CO<sub>2</sub> treatment on neural activity. Finally, these females were entrained to 12-h/12-h LD cycles for 3 days in an incubator at 25 °C, 60% relative humidity. Behavioral assays were made on day 4 of LD, as well as on day 2 and sometimes day 3 of DD. The populations tested for olfactory behavior were respectively maintained in clean vials without medium for 2 h before testing.

### Olfactory Behavior Assay

Olfactory behavior assays were carried out in a dark room at 25 °C, 60% relative humidity, by using a T-maze apparatus that was described in detail previously (Wang et al., 2003). The apparatus was connected to 2 constant airflows that were set at 500 mL/min, one of which carried the test odorants, the other pure air. Fifty females were loaded into the apparatus and then allowed to choose between air and odor for 1 min. The flies trapped in both tubes were counted, and a response index was calculated by the following for-

mula:  $(N_{air} - N_{odor}) / (N_{air} + N_{odor}) \times 100$ , with  $N_{air}$  and  $N_{odor}$  being the number of flies inside the air and odor tubes, respectively. A score of 0 indicates neutrality, while a +1 or -1 means that flies are repulsed or attracted, respectively, by an odor. Odorants tested were 4-methylcyclohexanol (MCH; Fluka, Steinheim, Switzerland) and 3-octanol (OCT; Aldrich, Milwaukee, WI); the 2 kinds of odorants were diluted in heavy mineral oil (Fisher, Fair Lawn, NJ).

### *Drosophila* Population Activity Monitor

The fly population was monitored for 3 to 4 days in 12-h/12-h LD conditions, followed by 5 to 6 days in DD, using Trikinetics *Drosophila* Population Monitors (Waltham, MA). Calculations of actogram and period were performed using the ClockLab analysis software package (Actimetrics, Evanston, IL).

### Statistics

All values indicate mean behavioral responses ( $\pm$  standard error of the mean [SEM]), and each point data was obtained from 10 populations. Data were analyzed by one-way analysis of variance (ANOVA). All significant differences in the graphs passed post hoc (Student-Newman-Keuls [SNK]) analysis. Different letters in the graphs represent different SNK mean groupings.

## RESULTS

### Circadian Rhythms of Olfactory Behavior Responses

We first examined olfactory behavioral responses of wild-type flies under different odorant concentrations and obtained dose-response curves (Fig. 1). We found that the olfactory responses of flies to MCH exhibited repulsion between  $10^{-5}$  and  $10^0$  dilution. The repulsive response was more than 90% and saturated at  $10^{-2}$  MCH dilution, as well as about 10% at  $10^{-5}$  MCH dilution. However, the olfactory attractive responses to MCH appeared at lower than  $10^{-6}$  dilution. According to this dilution's response curves, we chose the appropriate dilution for behavioral experiments. Moreover, in our experiment, fewer than 6 flies

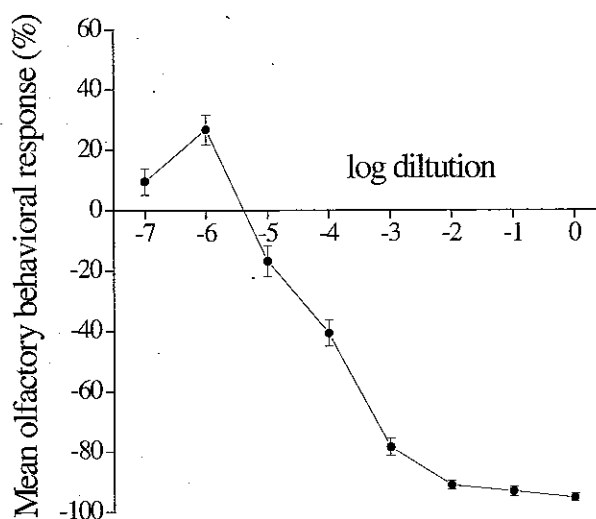
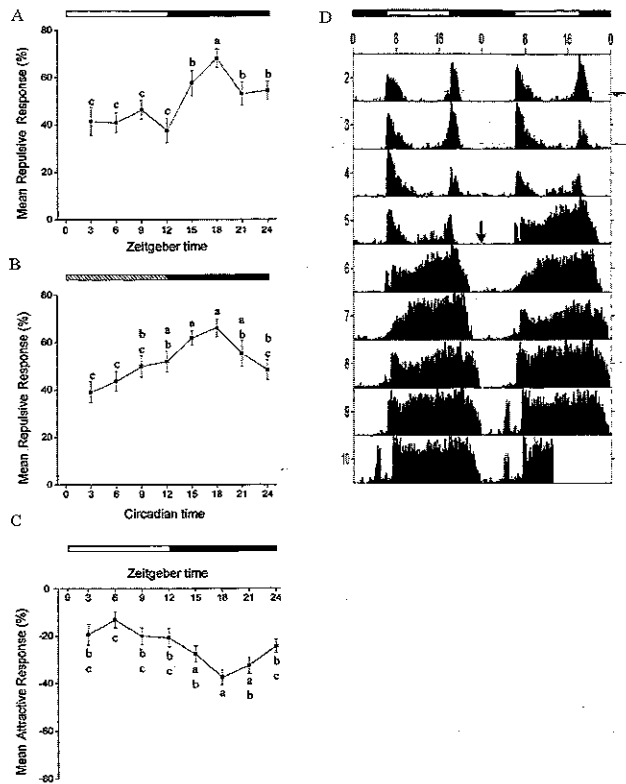


Figure 1. Dose-response curves of the behavioral responses to 4-methylcyclohexanol (MCH) in *Canton-S* (CS) flies. CS populations display repulsive responses to MCH at higher than  $10^{-5}$  dilution and reached saturation at  $10^{-2}$  dilution while exhibiting attractive responses to MCH at lower than  $10^{-6}$  dilution.  $n = 10$  for each point.

remained in the T-maze chamber, but a large part of flies made a choice.

To examine whether the olfactory behavioral responses of *Drosophila* fluctuate throughout the day, we tested 2 types of flies, CS and *yw*, raised in 12-h/12-h LD cycles (Fig. 2A; data from *yw* population not shown). We found that the olfactory repulsive responses of the 2 strains to the MCH odorant at the higher concentration ( $3 \times 10^{-4}$ ) showed a circadian fluctuation with a similar temporal pattern under LD cycles, which was lower during the day and began to rise at early night. It peaked at about the middle of night and then declined during the rest of the night. In addition, a similar pattern was observed in the olfactory repulsion responses to another odorant, OCT (data not shown). We confirmed that the olfactory attractive responses of CS flies to MCH at the lower concentration ( $2 \times 10^{-6}$ ) exhibited a rhythm similar to that of the repulsive responses (Fig. 2C). To further investigate whether these rhythms are under the regulation of an endogenous clock, we tested the olfactory repulsive responses of CS flies on day 2 of DD after 3 days of entrainment in LD cycles. A similar response rhythm was seen (Fig. 2B), indicating that the olfactory response rhythms are controlled by an endogenous clock.



**Figure 2.** Olfactory behavioral responses at different times of day and locomotor activity actogram of *Canton-S* (CS) populations. (A) Daily changes in mean olfactory repulsive responses of CS populations to 4-methylcyclohexanol (MCH;  $3 \times 10^{-4}$  dilution) on day 4 under LD cycles. The white and black bars above the graph represent when lights were on and off, respectively. Mean repulsive responses of CS populations increased during early night, peaked at ZT 18, and declined thereafter. One-way analysis of variance (ANOVA) revealed that the effect of time of day was statistically significant ( $p < 0.0001$ ). (B) Daily changes in mean olfactory repulsive responses of CS populations to MCH ( $3 \times 10^{-4}$  dilution) on day 2 of DD. Mean repulsive responses of CS populations increased at CT 9, peaked at CT 15-18, and declined thereafter. The pattern resembled that under LD, indicating circadian regulation. The hatched part of the bar above the graph represents subjective day, while the black part of the bar represents subjective night. One-way ANOVA revealed that the effect of time of day was statistically significant ( $p < 0.0001$ ). (C) A similar temporal pattern in mean olfactory attractive responses of CS populations to MCH ( $2 \times 10^{-6}$  dilution) was seen on day 4 under LD cycles. One-way ANOVA revealed that the effect of time of day was statistically significant ( $p < 0.001$ ). (D) A representative double-plotted actogram of a CS population with 50 individuals. The population was first monitored for 4 days under LD cycles and subsequently monitored for 5 days under DD. The activity of the population was organized in pronounced morning and evening peaks under LD cycles by visual inspection, which occurred at the lights-on-to-off transition. After transfer into DD, the population still displayed a robust activity rhythm with a period of 23.92 h and an acrophase at about CT 10. The arrow in this actogram denotes light regime transition from LD to DD.

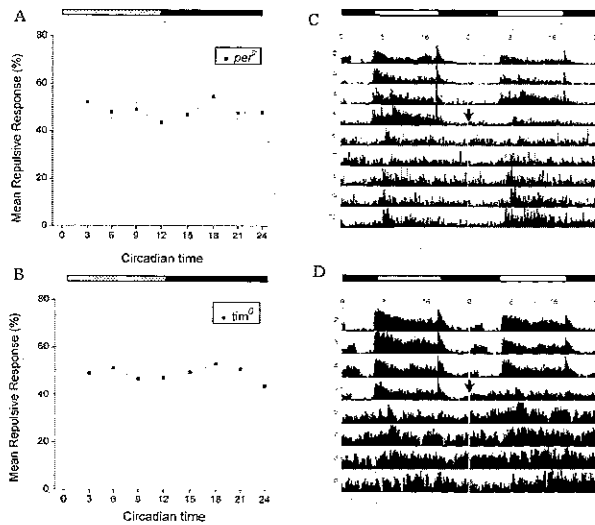
To know whether the olfactory responses are independent of fly locomotor activity, we monitored population locomotor activity by using a *Drosophila* population monitor. Six CS populations, each with 50 virgin females, were entrained for 4 days in LD cycles and then kept under DD cycles for 5 days. These CS populations were well entrained during LD cycles with 2 activity peaks; 1 was the morning peak at  $ZT 0.4 \pm 0.1$ , and the other was the evening peak at  $ZT 12.2 \pm 0.1$ . The free-running behavior of wild-type populations from LD into DD kept its rhythmicity (Fig. 2D). By ClockLab software analysis, the mean period was  $23.89 \pm 0.05$ , and the mean activity peak was at CT  $15.39 \pm 0.20$ . These observations were consistent with previous reports from individual monitoring, although there is some subtle difference in activity onset between populations and individuals. These suggest that the wild-type CS populations retained their well-rhythmic behavior in locomotor activity, and the olfactory rhythms in attractive and repulsive behavior, which appear to be in phase with each other, are in a different phase to locomotor activity.

#### Olfactory Behavioral Rhythms Are under Regulation of Circadian Clock Genes

To determine whether the olfactory response rhythms of the *Drosophila* population are controlled by circadian clock genes, we tested the olfactory repulsive responses and population locomotor activity of *per<sup>0</sup>* and *tim<sup>0</sup>* mutant populations that lose rhythms in eclosion and individual locomotor activity. We found that neither of the 2 clock mutants increased their olfactory repulsive responses during early night and thus lost their olfactory response rhythms (Fig. 3A,B). At the same time, we monitored the population activity of the 2 clock mutants. Neither of the 2 mutant populations showed obvious activity rhythms under DD cycles (Fig. 3C,D), although they were rhythmic under LD cycles, which was consistent with previous reports from individual monitoring. These results indicate that core clock genes regulate the olfactory response rhythms.

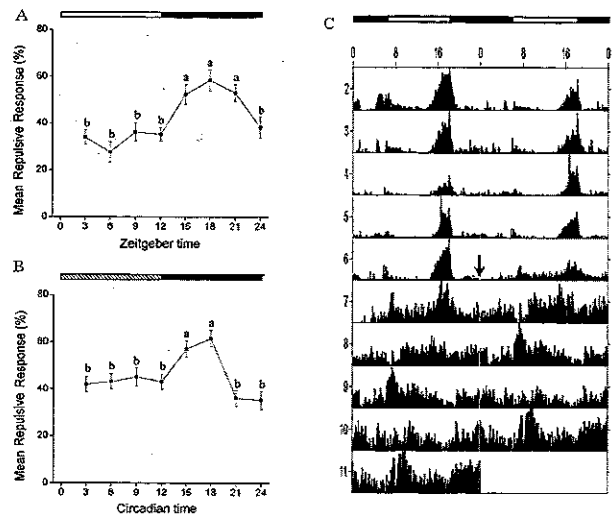
#### The Circadian Central Pacemakers Are Not Necessary for Olfactory Behavioral Rhythms

To determine whether the olfactory response rhythms are under the control of central circadian



**Figure 3.** Olfactory repulsive responses at different times of day and locomotor activity in rhythmic mutant populations. (A, B) Circadian changes in olfactory repulsive responses of *per*<sup>0</sup> and *tim*<sup>0</sup> populations to 4-methylcyclohexanol (MCH;  $3 \times 10^{-4}$  dilution) on day 2 of DD. One-way analysis of variance (ANOVA) revealed that the effect of time of day was not statistically significant ( $p > 0.05$ ). (C, D) A representative double-plotted actogram of *per*<sup>0</sup> and *tim*<sup>0</sup> populations. The 2 mutant populations were still rhythmic in LD cycles but lost rhythmicity after transfer into DD.

pacemakers in *Drosophila*, we examined the locomotor activity and olfactory repulsive responses of the *pdf*<sup>0</sup> and *disconnected* mutant populations. The *pdf*<sup>0</sup> populations were entrained normally during LD cycles, notwithstanding a damped morning peak, but their activity rhythms were severely disrupted during DD cycles (Fig. 4C). However, the olfactory repulsive responses of the *pdf*<sup>0</sup> populations did not lose rhythmicity either in LD cycles or in DD cycles (Fig. 4A,B). The responses remained on a lower level during the day and went up at dusk. They peaked at about midnight and then went down after midnight. This result indicates that the PDF neuropeptide is not necessary for driving the olfactory response rhythms. We further examined the *disconnected* mutant population. The *disconnected* populations could be entrained in LD cycles, only lacking an obvious morning peak. After being transferred into DD cycles, the populations did not immediately display arrhythmicity but kept rhythmicity for 1 day and then lost their rhythmicity (Fig. 5C). However, the *disconnected* population kept a temporal pattern similar to that of the CS population in olfactory behavior throughout the day (Fig. 5A,B).



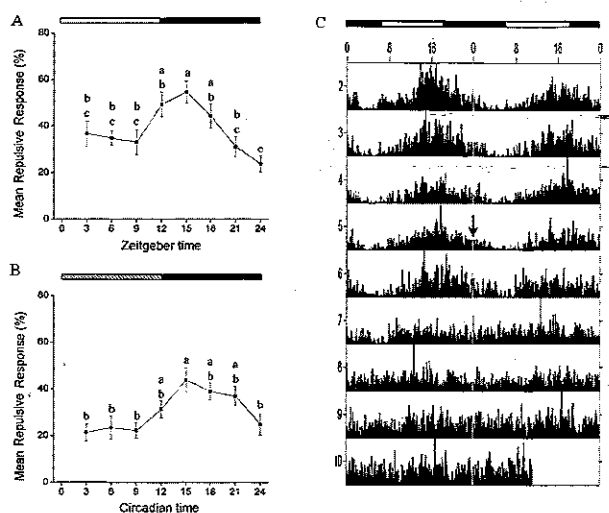
**Figure 4.** Olfactory behavioral responses at different times of day and locomotor activity actogram of *pdf*<sup>0</sup> populations. (A, B) A circadian pattern in mean olfactory repulsion responses of *pdf*<sup>0</sup> populations to 4-methylcyclohexanol (MCH;  $3 \times 10^{-4}$  dilution) was similar to that of *Canton-S* (CS) populations either on 4 days of 12:12 LD cycles or on 3 days of DD. One-way analysis of variance (ANOVA) revealed that the effects of time of day were statistically significant ( $p < 0.0001$ ). (C) A representative double-plotted actogram of a *pdf*<sup>0</sup> population with 50 individuals. The evening peak was pronounced, but the morning peak was severely damped under LD cycles. After transfer into DD, the population lost circadian rhythmicity thereafter. The arrow in this actogram denotes light regime transition from LD to DD.

This result suggests that the LNs are not necessary for olfactory behavioral rhythms.

## DISCUSSION

### *Drosophila melanogaster* Exhibit Circadian Rhythms in Olfactory Behavior Responses, and the Rhythms Are Independent of Locomotor Activity

Using an olfactory T-maze, we showed that olfactory behavior responses to odorants, including repulsion and attraction, exhibit a circadian rhythm that maintained a lower level during the day and began to rise at early night. It peaked at about the middle of the night, then declined during late night. The behavioral results were basically consistent with previous electrophysiological results (Krishnan et al., 1999). The electrophysiological responses (EAG) to olfactory stimuli measured here only occur in chemosensory



**Figure 5.** Olfactory behavioral responses at different times of day and locomotor activity actogram of the *disconnected* populations. (A, B) A circadian pattern in mean olfactory repulsive responses of the *disconnected* populations to 4-methylcyclohexanol (MCH;  $3 \times 10^{-4}$  dilution) was similar to that of *Canton-S* (CS) populations either on 4 days of 12:12 LD cycles or on 3 days of DD. One-way analysis of variance (ANOVA) revealed that the effect of time of day was statistically significant ( $p < 0.0001$ ). (C) A representative double-plotted actogram of a *disconnected* mutant population with 50 individuals. The activity was distributed in the several hours before and after lights-off under LD cycles; an evening peak that was still visible occurred at ZT 11.90. After transition into DD, the population did not immediately display arrhythmicity but kept rhythmicity for 1 day and then lost rhythmicity. The arrow in this actogram denotes light regime transition from LD to DD.

cells of the antennae. But in our study, the olfactory behavioral responses in our study involved not only peripheral chemosensory neurons but also central and efferent neurons (Devaud, 2003). However, within a range of odorant concentration, such as higher than  $10^{-5}$  MCH dilution, the olfactory behavior responses in our study invariably displayed repulsion, whereas the responses invariably exhibited attraction at lower than  $10^{-6}$  MCH dilution. The results were also consistent with previous observations (Wang et al., 2003). Moreover, the olfactory behavior rhythm was independent of *Drosophila* population activity. The olfactory behavior responses peaked at about midnight in either LD or DD cycles, whereas CS population activity peaks did not occur at this phase. Finally, neurons from the adult olfactory organs project directly to the antennal lobes of the brain. There are projections to higher centers of the brain from the antennal lobes, including the mushroom body (MB) and lateral horn (LH) (Carison, 1996). There is evidence that MB and LH mediate olfactory

attractive and repulsive behavior, respectively (Wang et al., 2003). In our study, olfactory attractive responses to lower concentration odorants displayed a rhythm similar to that of olfactory repulsive responses to higher concentration odorants, suggesting that circadian regulation of the olfactory behavior is independent of the reflex pathway of olfactory behavior. Therefore, these behavioral results are consistent with regulation within chemosensory neurons.

There are many clock-controlled physiological functions in *Drosophila*, such as sleep and courtship (Hall, 1994; Hendricks et al., 2001; Shaw et al., 2001; Sakai and Ishida, 2001). Rest in *Drosophila* is a sleep-like state and mainly occurs during the dark phase of the LD cycle. Flies in rest state have an increased arousal threshold or a decrease in sensory responsiveness to experimentally induced mechanical stimuli (Hendricks et al., 2001; Shaw et al., 2001). However, our observations indicate that the flies, which were awakened for this study, showed peak olfactory responsiveness at a time when they would normally be resting. One possible explanation is that olfaction as an exception has an important role in the sleep-like state. Therefore, we still agree on the view (Krishnan et al., 1999) that olfaction is very important for *Drosophila*, and high olfactory sensitivity may be a survival advantage to escape from danger when *Drosophila* are in a sleep-like or inactive state. Moreover, the olfactory behavior responses of the wild-type flies peaked at about midnight, but at that time, mating activities remained on a relatively low level (Sakai and Ishida, 2001). It is possible that the mechanism underlying mating activity rhythms is independent of that which underlies olfactory behavior rhythms. The mechanism that generates mating activity rhythms involves a female sex pheromone (volatile) that attracts male courtship, and the courtship song produced by male wing vibration affects female receptivity and direct contact, making the male sense a nonvolatile sex pheromone produced by his mate. There is evidence that a dominant factor that affects mating activity is not olfaction but nonvolatile sex pheromones (Hall, 1994; Coyne et al., 1994).

#### The Role of Clock Genes in Determining the Olfactory Behavioral Rhythm

The circadian clock is composed of 2 interlocked transcriptional-translational feedback loops in which at least 4 core genes are involved, including *per*, *tim*,

*Clk*, and *cyc*. Previous locomotor activity experiments on individuals have demonstrated that *per*<sup>0</sup> and *tim*<sup>0</sup> mutants lose their rhythmicity (Konopka and Benzer, 1971; Sehgal et al., 1994). In this study, we further confirmed that this is also the case for the fly population. In the olfactory behavior responses, neither of the 2 mutants exhibited any circadian fluctuation. This indicates that the olfactory behavior responses, such as locomotion activity and eclosion, are under the control of clock genes.

### Are the Central Circadian Oscillators Necessary for Driving the Olfactory Behavioral Rhythms?

The circadian system consists of central clocks in the brain and peripheral clocks in peripheral tissues. A great deal of evidence indicates that the LNs are the principal circadian pacemakers controlling locomotion and eclosion rhythms in *Drosophila*, and the PDF is a principal circadian transmitter (Renn et al., 1999; Myers et al., 2003; Stoleru et al., 2004; Grima et al., 2004), which is released by the LN<sub>v</sub>s and secreted into the neurosecretory system in *Drosophila* (Helfrich-Forster, 2003). The antennae in *Drosophila* possess peripheral oscillators for the olfactory rhythm. In this study, we investigated whether the PDF participates in transmitting timing information from LNs to antennae oscillators or whether the LNs are required for the olfactory behavior rhythm. Our results suggest that the PDF neuropeptide does not participate in rhythmic signals from central to antennae oscillators. These results are also consistent with recent observations (Tanoue et al., 2004). The PDF is not the only neuropeptide involved in the rhythmic control of behavior because the neurotransmitters of the LNs are still unknown (Taghert et al., 2001). Therefore, this result does not exclude the possibility that other molecules from the LNs or other central pacemakers may be involved. However, it is also possible that the LN<sub>v</sub>s themselves are not required for olfactory behavior rhythms. We then examined *disconnected* mutants, which miss most of their LN<sub>v</sub>s and LN<sub>a</sub>s and most of which are behaviorally arrhythmic (Helfrich-Forster, 1998). We found that the *disconnected* populations were still rhythmic in locomotor activity in LD cycles but lost rhythmicity on day 2 in DD cycles. This observation is consistent with previous reports from individual monitoring and suggests that the LN function

of the mutant is impaired. However, the *disconnected* populations still retain their olfactory behavior rhythms either in LD or DD cycles, which further supports the idea that the LNs are not required for sustaining the olfactory behavioral rhythms. This is not the case for eclosion; the LNs are required but insufficient to drive eclosion rhythms (Myers et al., 2003). But this behavior result does not exclude the possibility that other central oscillators such as visual photoreceptors or dorsal neurons are necessary because the circadian pacemaker in the optic lobes drives the rhythm of the olfactory response in the antennae in cockroaches (Page and Koelling, 2003). However, very recent data that antennal neuron oscillators are sufficient for olfaction (EAG) rhythms in *Drosophila* (Tanoue et al., 2004) do not support this possibility. Therefore, it is very possible that antennal neuron oscillators completely drive the behavioral rhythms in *Drosophila*. This result is also consistent with the recent observations in mammals in which the suprachiasmatic nucleus of the hypothalamus, basically equivalent to the LNs in *Drosophila* to control activity rhythms, is not required to sustain all rhythms in the olfactory bulb (Granados-Fuentes et al., 2004).

In summary, our results showed that *Drosophila* display a robust circadian rhythm in olfactory behavior responses, and clock genes regulate the behavioral rhythm. We also provided behavioral evidence that the rhythms in olfactory response persisted in the absence of the PDF or the LNs. These results indicate that circadian rhythms in the olfactory behavior in *Drosophila* are driven by pacemakers that do not control the rest-activity cycle and are likely in the antennae.

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