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Rest in *Drosophila* Is a Sleep-like State

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Summary

To facilitate the genetic study of sleep, we documented that rest behavior in *Drosophila melanogaster* is a sleep-like state. The animals choose a preferred location, become immobile for periods of up to 157 min at a particular time in the circadian day, and are relatively unresponsive to sensory stimuli. Rest is affected by both homeostatic and circadian influences: when rest is prevented, the flies increasingly tend to rest despite stimulation and then exhibit a rest rebound. Drugs acting on a mammalian adenosine receptor alter rest as they do sleep, suggesting conserved neural mechanisms. Finally, normal homeostatic regulation depends on the *timeless* but not the *period* central clock gene. Understanding the molecular features of *Drosophila* rest should shed new light on the mechanisms and function of sleep.

Introduction

The universality of a basic circadian rest–activity cycle in the animal kingdom is almost unquestioned (Drucker-Colin, 1995). In mammals and birds, a prominent manifestation of this underlying cycle is sleep, a behavioral syndrome of inactivity and reduced sensory responsiveness with correlated changes in the electroencephalogram (EEG) (Campbell and Tobler, 1984). Although EEG recording requires a mammalian-like brain structure, fundamental behavioral features of sleep are likely to be conserved, perhaps even in simpler, more genetically tractable organisms (Hendricks et al., 2000). *Drosophila*, a virtually ideal organism for behavioral genetics, is an obvious choice and has never been studied. In order to be considered sleep-like, an inactive state should have the following features (Campbell and Tobler, 1984; Hendricks et al., 2000): (1) consolidated circadian periods of immobility, (2) a species-specific posture and/or resting place, (3) an increased arousal threshold (although the state can be reversed by intense stimulation), and (4) a homeostatic regulatory mechanism. In addition, we felt it would be important to document that a sleep-like state is related to changes in central neural function, whether physiological, pharmacological, or molecular, in order to provide a basis for comparisons with mammalian sleep mechanisms. In the present study, we present evidence that, according to these criteria, rest in *Drosophila* is a sleep-like state. We also initiated studies to elucidate the relationship of rest behavior to the central clock genes *period* and *timeless*.

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Results

Rest Behavior Is a Circadian Syndrome of Prolonged Immobility and Sporadic Small Movements

In order to address whether the features of a sleep-like state, as outlined above, exist in *Drosophila*, flies were studied in a standard locomotor assay (Hamblen et al., 1986). In addition to recording activity counts to monitor the basic rest–activity cycle, flies were observed individually on videotape recordings. Observation of individual animals revealed that *Drosophila* rest behavior consisted of relaxed immobility in a preferred posture and location in the activity tube (Figure 1A). Prior to resting, the flies turned away from the food, walked a few millimeters and then adopted a supported position, usually prone. To observe even greater details of the behavior, five flies were videotaped at high magnification for 24–48 hr (Figure 1B). Complete immobility, with only respiratory abdominal pumping movements, could last up to 26 min. Usually, however, flies showed small sporadic movements of the proboscis (protrusion and retraction), caudal abdominal twitches, or, rarely, tremors or twitches of the extremities. These intermittent movements were not part of any recognizable coordinated motor behavior and did not appear to have any relationship to the environment. In the subsequent discussion, we use the term "rest" to include both immobility with these apparently purposeless movements and complete immobility.

We next quantified these rest periods in *Drosophila* in terms of epochs and duration to determine the extent to which "rest" could qualify as a consolidated circadian-controlled behavior. In the 11 flies videotaped in continuous darkness while in the circadian locomotor assay, such epochs of visually identified rest lasting $\geq$1 min occupied 11.0 $\pm$ 1.17 hr, or about 48% of the 24 hr day. Not surprisingly, the occurrence of rest was inversely related to the number of activity counts recorded each half hour (compare Figure 1C with 1D, top). For each 24 hr record, the major rest period (mean duration 7.39 $\pm$ 1.96 hr), which consisted of rest more than 80% of the time, generally occurred in the middle of the subjective night (Figure 1D). The single longest bout of rest occurred in the first half of this maximal rest period in 8 of 11 animals and lasted 105.1 $\pm$ 38.65 min. These quiet behaviors contrasted with the full repertoire of behaviors that included running and climbing when activity counts were high. Because most rest occurs in bouts lasting $>$30 min (see Figure 1E), we sought to determine whether short ($\leq$1 min) periods of immobility should qualify as rest. We found that immobile periods lasting $\leq$1 min were relatively unusual, averaging only 16.6 $\pm$ 7.74 total min in a 24 hr day and were associated with circadian activity peaks rather than nadirs. The studies described below therefore excluded such brief pauses in activity from the definition of "rest."

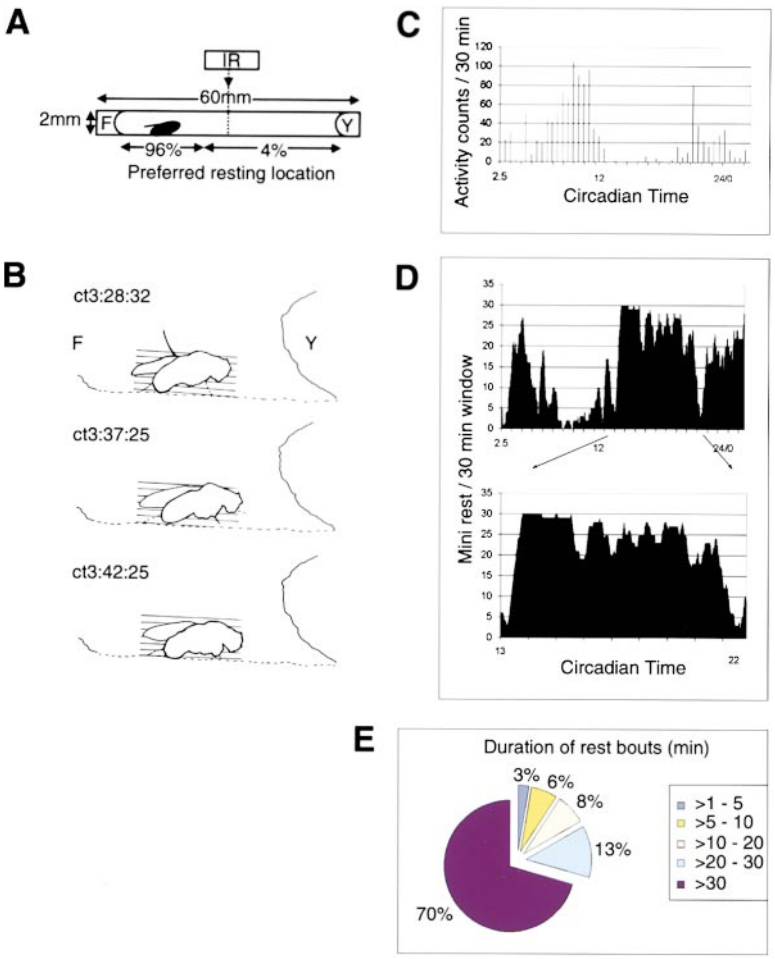

Figure 1. Rest Behavior Is a Circadian Syndrome of Prolonged Immobility and Sporadic Small Movements

Flies placed in standard locomotor assay tubes (A) rested near the food in 96% of all rest bouts. Tracings from a videotape (B) illustrate the typical rest behavior. The animal first moved away from the food and became prone on the floor of the tube at CT 3.28:32 (top). After a 9 min immobile period with four proboscis extension/retraction movements, it shifted to a more supported position (middle) and then relaxed for the next 5 min, exhibiting only respiratory movements (bottom). The relationship between activity counts and rest during 24 hr in continuous conditions (D:D) is shown in a typical fly (C and D). In (C), activity counts were measured in 30 min bins using the standard locomotor activity assay. Peak activity occurred during the latter half of the subjective day (y axis, activity counts; x axis, circadian time). (D) shows the directly observed rest pattern in the same fly. Minutes of rest per 30 min moving window as scored by videotape analysis (see Experimental Procedures) are displayed on the y axis; on the x axis is circadian time. Each recorded rest value is the sum of all minutes of rest at the indicated circadian time and the subsequent 29 min. During a 24 hr recording period (top), rest was most consolidated when activity counts were minimal (compare with 1C). For this record, this was CT 13.5–22.5 (expanded at bottom). Over 74% of this 9 hr period was rest, accounting for 54% of the day's total. The longest continuous rest bout (157 min) occurred at the onset of this 9 hr, and was followed by repeated rest cycles (37 total, averaging 11.32 min in duration). Finally, this major rest period was terminated with a burst of activity. For all 11 flies studied in this fashion, the distribution of rest bouts is shown in (E). The vast majority of the major rest period was comprised of >30 min bouts. Abbreviations: F, food; Y, yarn; IR, infrared beam for recording activity counts. Dotted lines in (B) denote structures out of the plane of focus.

Sensory Responsiveness Is Reduced in Resting Flies

In order to address whether rest in the flies further fulfilled the criteria by having an increased "arousal threshold" or a decrease in sensory responsiveness, we performed two series of experiments. First, while we were able to establish that consolidated periods of rest occurred in the isolated conditions of the standard circadian rhythm assay, we sought to determine whether flies behaved in the same manner in a group setting. *Drosophila* are normally social animals (Hay, 1973), and resting flies might be expected to be stimulated by active flies in a group. We therefore recorded the activity of flies in group conditions as shown in Figure 2A. As many as 60% of the flies in the group rested at once (Figure 2B). Before resting, flies moved away from the food, where social interactions and courting behaviors predominated, sometimes climbing vertically into the space between the cover and bottom of the dish. Active flies frequently approached and even apparently collided with immobile flies. The level of the stimulation could not be quantified, but at times it appeared intense, with up to three active flies congregating around a resting fly for several seconds. Resting flies never responded to mere approaches. Even when they were directly contacted, resting flies made no response or rejected the approach by turning, moving away, or flicking a wing and continued resting 95% of the time (Figure 2C). In contrast, active flies responded by increasing their locomotion or joining in courtship (see also Manning, 1959). In addition, when a simple mechanical stimulus—tapping the container once (see Hay, 1973, for reliability of such stimuli)—was introduced at 1–2 hr intervals, the stimulus never produced a reaction in a fly that was resting ($n = 25$). Consistent with a previous report (Hay, 1973), the active flies jumped or flew and then paused or resumed locomotion.

The second experiment was designed to examine the arousal threshold of resting flies. Arousing stimuli were applied to the second group of flies studied at the same time as the flies described above. Whenever any fly remained immobile for >1 min, a minimal stimulus (tapping the container) was applied and then increased in intensity until all flies were active (see Experimental Procedures). During the initial 2 hr, the rate and intensity of stimulation was relatively low, and the maximal stimulus—lifting the dish and tapping it forcefully—was necessary only once. However, during the subsequent 6.5 hr, the level of stimulation necessary to arouse flies increased significantly, and on several occasions the

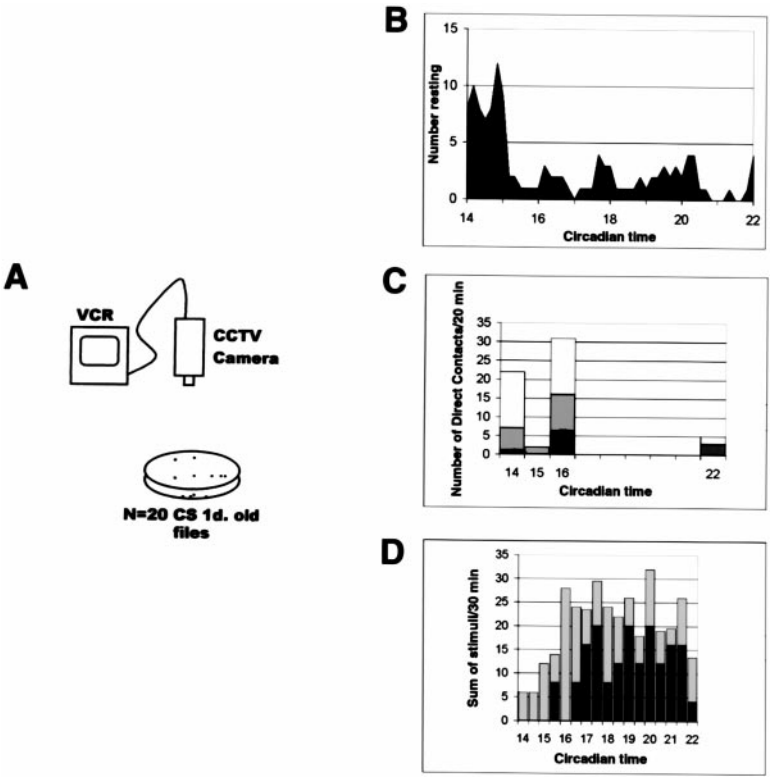

Figure 2. Sensory Responsiveness Is Reduced in Resting Flies

(A) A group of 20 entrained flies was placed in a Petrie dish for observation and videotape recorded in constant darkness using a low-light-sensitive CCTV camera.

(B) Number of flies resting in the dish at given circadian times. Peak resting was from CT 14 to CT 16 during the subjective night, and peak activity was at CT 22, just prior to subjective dawn.

(C) Responses of resting flies to natural contacts were recorded for 20 min in the middle of each indicated hour. The vast majority (95%) of direct contacts produced no detectable response in the resting fly (white), or elicited minimal responses (gray). The $<5\%$ of contacts that resulted in gross arousal are in black.

(D) A second group of flies was subjected to a series of graded stimuli applied when any fly in the dish was observed to rest for ≥ 1 min. Stimulation was repeated, if necessary, at increasing levels every 15 s until all 20 flies were active. The gray bars represent on the y axis the total level of stimulation (number $\times$ intensity grade)/30 min necessary to disrupt rest in all 20 flies. The maximal stimuli are shown in black. The total level of stimulation increased from the first 2.5 hr to the last 6.5 hr ($p < 0.006$). See the Experimental Procedures for details.

maximal stimulus had to be repeated up to five times in 30 min to arouse all the flies. The increasing tendency to rest as the night wore on was in marked contrast to the rest pattern of the control group shown in Figure 2B. The maintenance of rest in the face of intense natural or artificial stimulation provides evidence of decreased responsiveness in resting flies.

Rest Deprivation Produces a Rest Rebound during Recovery

One of the most intriguing and well-studied phenomena in sleep research is the rebound of sleep after sleep deprivation (Parmeggiani et al., 1980; Tobler et al., 1983; Horne and McGrath, 1984; Horne, 1985; Trachsel et al., 1986; Borbely et al., 1989; Lancel et al., 1991; Achermann et al., 1993; Dijk and Czeisler, 1995; Rechtschaffen, 1998; Schwierin et al., 1999). We sought to identify whether a rest rebound occurred after disruption of rest in *Drosophila*. We first conducted trials with mechanical stimuli applied manually to disrupt rest in flies. Multiple trials with 10–50 flies were conducted, in both light:dark (L:D) and dark:dark (D:D) conditions, in groups and in locomotor assay tubes. The proportion of flies resting increased significantly after rest deprivation in every trial. The most extensive such trial is illustrated in Figure 3A. The 20 rest-deprived flies rested significantly more after rest deprivation despite the fact that this time period, from circadian time (CT) 22 to CT 10, was a period of sustained activity in controls.

In order to conduct longer studies in a large number of animals, and to eliminate stimulus variability or observer bias, we developed methods to investigate rest and rest rebound automatically. We confirmed that a 30 min interval with no activity counts on the standard locomotor assay provided an accurate predictor of rest (see Experimental Procedures; also described above). We also automated the rest-depriving stimulus by programming a stepper motor to apply a computer-controlled complex mechanical stimulus at random intervals averaging 1 min (see Experimental Procedures). Our testing indicated that this stimulation prevented rest in 100% of flies for 6 hr.

For this initial study of the rest rebound response in *Drosophila*, we wished our findings to be broadly applicable, rather than limited to a specific set of laboratory conditions. Therefore, the study was carried out as a series of seven trials in a total of >200 flies of two genotypes at random ages. In addition to the rest-deprived group ($n = 96$), the study included both a rested control group ($n = 75$) and a handled control group ($n = 45$) that was removed from the incubator but not rest deprived (see Experimental Procedures). Data from the locomotor assay was used to determine whether each animal was resting every 30 min for 2 baseline days and 3 postdeprivation days. Inspection of the records suggested that handling did not markedly alter the rest pattern (Figure 3B), whereas a rest rebound after deprivation could be identified in individual animals (Figure 3C). For statistical analyses, each 24 hr day was divided into four 6 hr time periods, representing subjective morning, afternoon, early night, and late night and the hours of rest/6 hr were calculated. Rest levels for all animals in the three groups (undisturbed rested control group, handled control group, and rest-deprived groups) were analyzed and compared using a mixed-model analysis of variance (SAS PROC MIXED [Littell et al., 1996]).

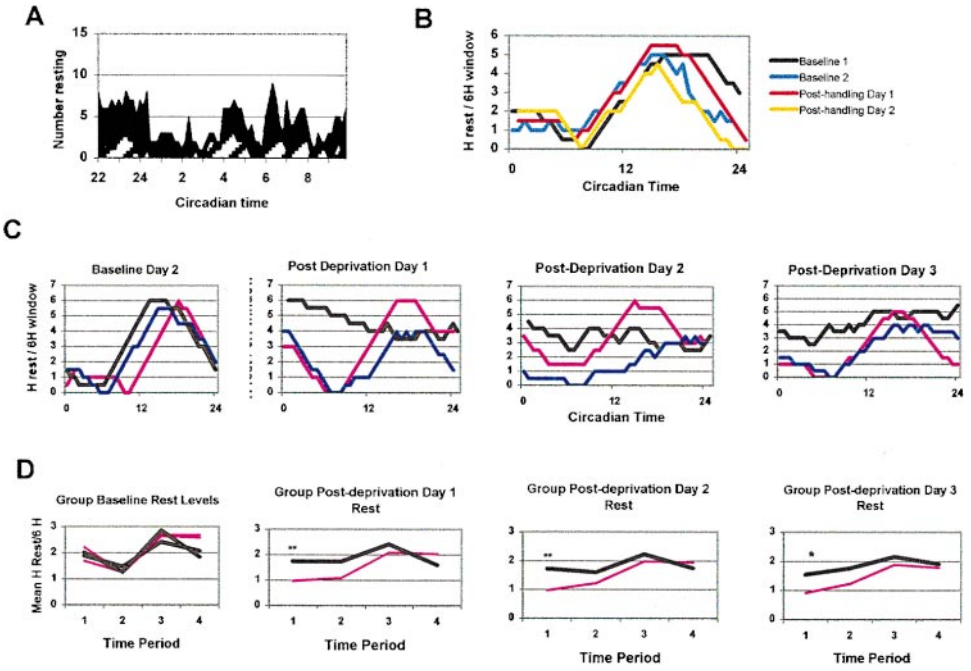

Figure 3. Rest Deprivation Produces a Rest Rebound during Recovery

(A) shows rest rebound after deprivation in a social situation. At the conclusion of the rest deprivation described in Figure 2, the rested (white striped) and rest-deprived (black) groups were left undisturbed. From CT 22 to CT 10 the flies' behavior was videotaped and later scored to measure the number of flies resting (defined as >5 min of immobility) in each group. On the y axis is the number of flies resting; on the x axis is circadian time. The rest-deprived group rested significantly more for the entire period ($p < 0.000001$). For other experiments (B–D), the deprivation was automated and rest was measured using the standard locomotor assay (see Experimental Procedures for details). Examples of rest patterns in a control fly (B) and rest-deprived animals (C) are shown. In (B), the 24 hr rest patterns in a control fly during 2 baseline days and for 2 days after handling are superimposed. The rest pattern was not obviously altered by handling. In (C), the rest patterns of three flies with different degrees of rest rebound are illustrated before (far left) and after rest deprivation (successive panels to the right). One animal (black line) has a very marked rest rebound, such that daytime rest actually exceeds nighttime rest for the first 2 days after deprivation and is grossly increased for all 3 days. A typical fly (pink) has an obvious increase above baseline during the mornings of 2 postdeprivation days but clearly retains the normal circadian rest pattern and appears normal by the last day of the study. A fly with a minimal rebound, with an obvious morning increase above baseline for only the first postdeprivation day, is shown in blue. On the y axis are the total hours of rest during a 6 hr moving window. Each point represents the sum of that 30 min measurement period at the indicated circadian time and the 11 subsequent 30 min periods. (D) shows mean rest levels for handled controls (pink, $n = 45$) and rest-deprived flies (black, $n = 96$) during each 6 hr time period of each day (1, subjective morning; 2, afternoon; 3, early night; 4, late night). Rest levels in the groups were identical during baseline days (left), but the rest-deprived group rested significantly more than controls on all 3 postdeprivation days, as shown in the successive panels to the right ($F[8,3967] = 2.92$, $p = 0.003$ for the day × group interaction). The first 6 hr of the daily analysis (Time 1, subjective morning) was the only time of day when significant differences were seen ($F[8,835] = 3.38$, $p = 0.0008$ for time × day × group interaction). The mean hours of rest/6 hr for each time period in each group is shown on the y axis, with the day of the study on the x axis; $^{**}p < 0.01$, $^{*}p < 0.04$.

This sophisticated analytical approach allowed consideration of multiple factors at once. In addition to between-group factors (effect of rest deprivation or handling), this analysis allowed us to assess within-group factors: day of study and time of day (see Experimental Procedures).

The major finding was that, while the rest in all three groups was the same during the baseline days, flies that had been rest deprived rested significantly more than controls during the 3 postdeprivation days. In contrast to the rest-deprived group, daily rest decreased in both the rested and the handled control groups over the period of the study, perhaps due to the assay conditions.

To determine whether the rebound varied with time of day, group rest levels were compared across the days during each 6 hr time block. This analysis showed that that a significant rest rebound—an increase in rest levels in deprived compared to control flies—occurred only during the morning of all 3 postdeprivation days, waning only slightly by the third postdeprivation day (Figure 3D). Rest durations through the subjective afternoon and night time periods were statistically identical among the groups. That is, while the control animals continued to exhibit decreases in rest during the early morning of the subjective day, flies subjected to rest deprivation exhibited a significant increase in rest during the same time period. A possible interpretation of this pattern is that the homeostatic rest rebound, considered as an increase in rest after rest deprivation, was modulated by circadian influences. One might also describe the rest pattern after deprivation by noting that the day–night differences in the circadian rest pattern appeared reduced, and conclude that recovery rest is characterized by a decrease in circadian influences on rest behavior. However, a significant circadian pattern of rest persisted after rest deprivation, with a peak in the early subjective

night. The study was not designed to characterize the amplitude of the circadian rhythm, but the timing of the rest–activity cycle was not altered. Neither handling nor rest deprivation reset the circadian clock compared to the rested controls (see Experimental Procedures).

If rest rebound results from a homeostatic mechanism, the duration of rest rebound should be affected by the degree of rest deprivation (cf. Parmeggiani et al., 1980). We examined the degree of rebound during the first 6 hr of recovery in relationship to the duration of rest for each fly during the same time period on the last baseline day before deprivation. We found that a significant rest rebound ($p = 0.03$) occurred only when flies were deprived of $\geq$1.5 hr rest.

Stress has been an important confounding factor in the analysis of sleep deprivation in mammals (Horne and McGrath, 1984). Although increased rather than decreased activity appears to result from stress in insects (Brady, 1967; Tobler, 1983), we wished to test directly whether an increase in rest could result from stimulation without rest deprivation. We therefore applied the automated stimulus to flies ($n = 37$) during the highly active 6 hr period from CT 0 to CT 6. We could not find any change in recovery rest compared to controls using the same study design and statistical approach used to study rest deprivation. We conclude that the rest rebound we documented is a specific effect of rest deprivation and not a nonspecific response to stimulation or stress.

Evidence for Shared Neural Mechanisms

Based on the fact that many neurotransmitter systems are evolutionarily conserved from *Drosophila* to mammals (Nassel, 1991, 1993; Saudou and Hen, 1994), we tested whether the rest state might be maintained by neural mechanisms analogous to those involved in sleep in mammals. Considerable evidence implicates adenosinergic mechanisms in sleep regulation (Porkka-Heiskanen et al., 1997). However, no adenosine receptor has been described in invertebrates, and none could be found in the portion of the *Drosophila* genome sequenced as of this writing. Caffeine, through its antagonism of adenosine A1 receptors, is an effective somnolytic agent (Choi et al., 1988). We therefore supplied caffeine at three concentrations to individual entrained flies. Their rest behavior for the subsequent 8 hr was videotaped and analyzed (Figure 4A). Seven flies were studied in each group (except for the 5 mg/ml group, where two flies died). The mean rest levels were decreased by caffeine in a dose-dependent fashion. While these data could suggest conserved neural mechanisms for rest and sleep, the decrease in rest might be nonspecific or stress-related, especially since the 5 mg/ml concentration was lethal to two of seven animals. Further, caffeine is not highly selective for A1 over A2 receptors and has additional actions (Choi et al., 1988). We therefore tested whether an increase in rest would result from the highly selective A1 agonist cyclohexyladenosine (CHA). Rest was significantly increased in flies fed CHA (Figure 4B). These effects on *Drosophila* rest parallel the effects by these same agents on sleep in mammals.

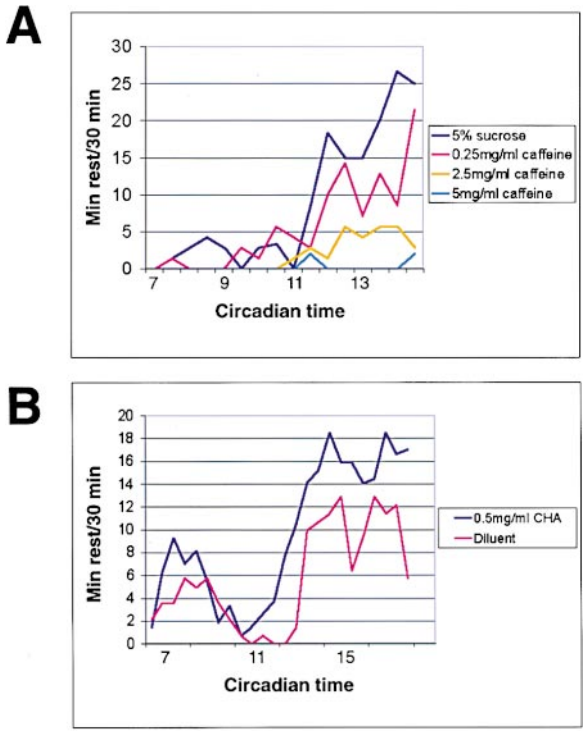

Figure 4. Drugs Acting on the A1 Adenosine Receptor Affect Rest

(A) Individual flies were provided with caffeine in 5% sucrose solutions of different concentrations, and the behavior was recorded on videotape and then analyzed in 10 min intervals for the following 8 hr. Mean group rest values are shown for each 30 min. Caffeine significantly reduced rest ($p = 0.0017$) in a dose-dependent manner. See text for details.

(B) Flies fed 0.5 mg/ml cyclohexyladenosine (CHA), a specific A1 receptor agonist, were studied in the same fashion, and the effect on rest was monitored for the subsequent 12 hr. Flies that ingested 0.5 mg/ml CHA rested more than controls ($p = 0.020$).

On the y axis are the average rest rates for each group for each hour; on the x axis is circadian time.

Rest Rebound Was Affected Differently by Null Mutations of Two Central Clock Genes

The nature of the link between the circadian and the homeostatic systems that modulate sleep-like rest should be relatively efficient to investigate at a molecular level in *Drosophila*. As an initial step, we studied the response to rest deprivation in mutants lacking each of the canonical central clock genes, *timeless* and *period* (both in a *yw* genetic background). The rest levels of mutant flies lacking the *timeless* gene (tim^0) and the *period* gene (per^0) were studied in D:D exactly as described above for wild-type flies. Figure 5 illustrates rest patterns for representative flies (A) and mean 24 hr rest values for the complete study (B). Baseline rest patterns were arrhythmic and the mean rest levels did not differ, although maximum locomotor activity levels were lower in per^0 than in tim^0 flies ($p = 0.008$), consistent with previous observations that per^0 flies are relatively inactive (J. C. H. and A. S., unpublished data). After deprivation at CT 18–24, tim^0 flies showed a significant decrease in rest, similar to handled control wild-type flies. per^0 flies were significantly different, exhibiting an increase

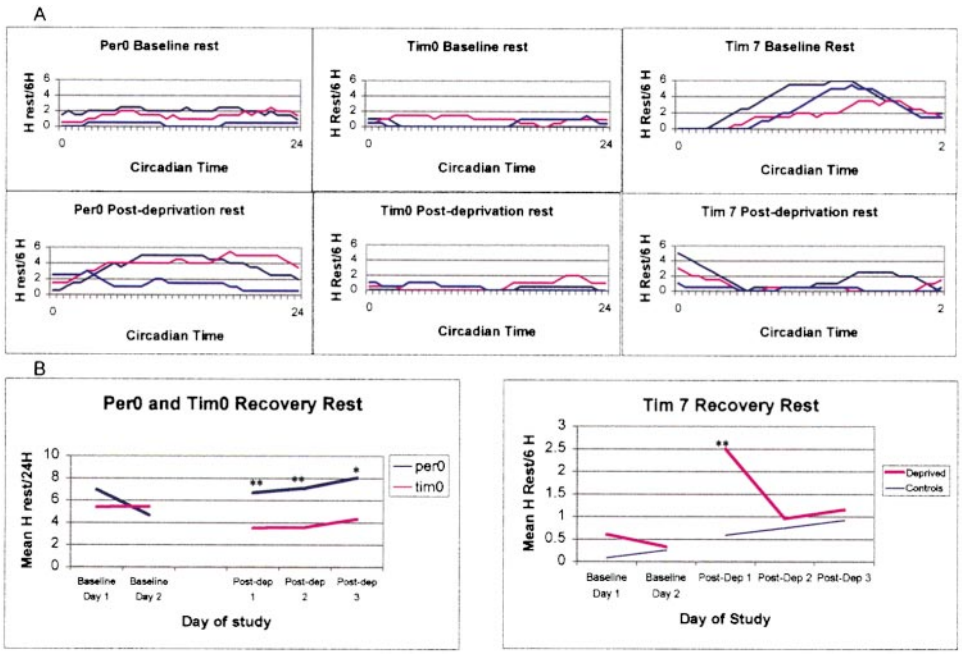

Figure 5. The Roles of the *period* Gene and the *timeless* Gene in Homeostatic Rest Regulation Are Different

Flies lacking a functional *period* gene (*per*^{0} flies) exhibited an increase in rest after deprivation, whereas flies lacking a functional *timeless* gene (*tim*^{0} flies) failed to increase rest. The abnormal phenotype was rescued in transgenic *tim*^{0} flies that were transformed with a construct containing the full-length *timeless* gene and *timeless* promotor (*tim*^{7} flies).

(A) Upper panels show baseline rest patterns of three representative *per*^{0} (left), *tim*^{0} (middle), and *tim*^{7} (right) flies. Lower panel shows rest patterns on the first postdeprivation day in the same flies for each genotype. No circadian pattern was observed for the baseline or the recovery rest in the arrhythmic mutants. The circadian rest pattern was restored in *tim*^{7} flies, and the rest rebound was limited to the first 6 hr quarter of recovery rest. The y axis shows a 6 hr moving window of rest, as described in Figures 3B and 3C.

(B) Left panel shows the mean 24 hr rest levels for *per*^{0} ($n = 18$) and *tim*^{0} ($n = 17$) flies for each day of the study. Rest levels were identical during baseline days, but *per*^{0} flies rested significantly more than *tim*^{0} flies during all 3 postdeprivation days. Right panel shows the mean rest levels of *tim*^{7} flies for the first quarter (CT 0–6) of each day of the study. A significant rest rebound occurred during the first quarter of the first postdeprivation day, indicating rescue of the homeostatic component of rest regulation; $^{**}p < 0.01$, $^{*}p < 0.02$.

in rest that persisted into the third postdeprivation day, similar to the rest rebound of wild-type flies. Rest levels in *per*^{0} flies were significantly greater than those of *tim*^{0} flies until the last quarter of the third postdeprivation day. As would be expected in the absence of a clock, there was no circadian aspect to the rest rebound. This difference between the genotypes was not due to a difference in amount of rest during the period of deprivation, as the mean baseline levels of rest during the 6 hr time period were statistically identical (2.94 versus 2.55 hr, $p = 0.61$). The difference between the genotypes was confirmed in a second trial comparing *tim*^{0} ($n = 12$) and *per*^{0} ($n = 12$) flies to handled controls. *tim*^{0} flies significantly decreased rest after deprivation compared to handled *tim*^{0} controls ($p < 0.02$), whereas rest in *per*^{0} flies again was significantly increased compared to *tim*^{0} flies for all 3 postdeprivation days ($p < 0.02$ for all 3 days; data not shown). Baseline rest patterns in both groups of arrhythmic flies were fragmented with few periods lasting >30 min. Rest was more consolidated in *per*^{0} flies after deprivation, with the longest continuous period of rest increasing from 3 hr during baseline rest to 8.5 hr on the first postdeprivation day. The proportion of rest episodes lasting 2 or more hr also increased significantly in *per*^{0} flies from 0% during baseline to 12.3% on the first recovery day ($p < 0.001$), whereas no change was found in *tim*^{0} flies ($p > 0.99$).

In order to map this defect in rest homeostasis to the *timeless* gene, we rest deprived *tim*^{7} flies, a line of *tim*^{0} flies transformed with a construct containing the *timeless* gene including the *timeless* promoter. During baseline days, *tim*^{7} flies ($n = 11$) exhibited normal circadian rest patterns, as would be expected. Most importantly, the *tim*^{7} flies exhibited a significant rest rebound compared to *tim*^{7} handled controls, while the *tim*^{0} flies exhibited a decrease compared to handled *tim*^{0} controls. As with wild-type flies, the significant increase in rest in *tim*^{7} flies was limited to the first 6 hr of recovery. In this group of animals, the rest rebound did not persist beyond the first postdeprivation day. As can be seen in the examples in Figure 3C, a proportion of wild-type flies also exhibited a similarly prompt recovery from rest deprivation, so that it is not clear at present whether the brief rest rebound duration is related to the transgene or reflects normal variation in the population. In addition to the significant first-quarter rest increase above handled controls and above baseline levels, approximately half of the deprived flies and of the handled controls exhibited either a circadian rhythm shift or a decrease in amplitude during the last 3 days of the study. Unlike the rest rebound, this change appeared to be a nonspecific response to stimulation rather than a specific rest deprivation response. Representative examples of rest patterns in individual flies (Figure 5A) and mean values

for the first quarter of each study day (Figure 5B) are illustrated in Figure 5.

Discussion

Rest Is a Sleep-like Behavioral State in *Drosophila*

Based upon the observations described above, we conclude that rest in *Drosophila* fulfills the criteria for a sleep-like state. First, flies exhibited, both in isolated and in social conditions, periods of immobility that lasted up to 2.5 hr, with the majority of these rest periods occurring during the first half of the subjective night. There were times when only respiratory movements occurred, but more commonly sporadic small skeletal muscle movements were seen every 4–5 min. No temporal or behavioral pattern that would clearly distinguish substates of rest was identified. We cannot rule out the possibility that the twitching movements are the external manifestation of a distinct CNS rest state, but invertebrate rest may be a unitary state.

A second feature of rest behavior was that the flies exhibited a characteristic posture and resting location. In particular, most flies rested on the floor of the tube near the food in isolated conditions, and in a more secluded area away from the food when placed in a group situation.

Third, we found that the animals were less responsive during rest to natural stimulation from other flies or to experimentally induced mechanical stimuli. Further, the level of stimulation required to prevent rest increased during an 8.5 hr period, indicating an increased arousal threshold during prolonged rest deprivation.

Finally, we found evidence for a homeostatic regulation. Whether studied in groups or individually, in L:D or in D:D, flies displayed an increase in rest after a period of rest disruption. We analyzed a large group of animals of different ages and of both the *yw* and CS genotypes for an extended period after deprivation in D:D and found that the rebound persisted for 3 days. The degree of rebound (change from baseline rest levels) was not constant within each 24 hr period. Rather, the homeostatic rest rebound was modulated so that a significant increase above controls was evident only during the morning 6 hr period of each day. With this analysis, we have shown that *Drosophila* rest is regulated by homeostatic factors as well as the well-known circadian control of rest–activity patterns. This is consistent with the well-established observations in mammals that sleep rebound after deprivation is modulated by both circadian and homeostatic influences (Parmeggiani et al., 1980; Tobler et al., 1983; Trachsel et al., 1986; Borbely et al., 1989; Lancel et al., 1991; Achermann et al., 1993; Dijk and Czeisler, 1995; Schwierin et al., 1999).

In addition to the extensive analysis described above, it is equally important to emphasize that simply observing the animals and moving the containers to prevent rest was sufficient to produce an obvious and easily quantified rest rebound (Figure 3A). Such simple measures may be useful to produce a rest rebound in large groups for genetic screens or analyses of changes in gene expression. Along these lines, we have used differential display PCR to determine whether rest behavior was associated with changes in gene expression and to screen for genes that were specific for rest or active states. Flies were manually rest deprived en masse during their usual maximal rest period, while controls were allowed to rest without being disturbed. We have isolated candidates that were upregulated during both rest and rest rebound, or during spontaneous activity and rest deprivation (J. A. W. et al., unpublished data). While none of these candidates are yet fully characterized, this provides early evidence that rest deprivation achieved by simple means can change gene expression and that genes may be upregulated specifically during rest.

Neurochemical Mechanisms of Rest

As a first step in determining whether neuronal or neurotransmitter mechanisms are conserved between flies and mammals, we analyzed the effect on rest of drugs known to increase or decrease sleep through their action on the A1 adenosine receptor in mammals. The fact that the effects on rest paralleled the effects on mammalian sleep reveals a conserved behavioral effect of these drugs from *Drosophila* to mammals. The mechanism(s) mediating these effects may include a conserved G protein–coupled receptor, as has been found for other systems (Saudou and Hen, 1994). However, at the present time, given that adenosine receptors have not yet been found in *Drosophila*, we cannot exclude other possibilities. If a *Drosophila* adenosine receptor can be identified, adenosine might be further studied for its role in rest regulation in *Drosophila*. This is of specific interest because adenosine is currently an important candidate as a natural sleep-promoting substance that accumulates during prolonged waking (Benington and Heller, 1995), but definitive studies have proved difficult in mammals (Porkka-Heiskanen et al., 1997).

Rest and Central Clock Genes

The results of our study of null mutants for the *timeless* (tim^0) and *period* (per^0) genes led us to conclude that the role of the two genes in rest regulation might be different. If one considers the null mutants as molecular lesions of the clock equivalent to suprachiasmatic nucleus lesions in mammals, one would expect that rest and rest rebound would be normal (Edgar et al., 1993). This is the result we found for animals lacking the *period* gene and is consistent with a role for *period* as a central clock gene regulating the timing of rest rather than the level of rest. The decrease in maximal activity, however, might be consistent with a role in enhancing activity levels. This may be analogous to a role in promoting consolidated waking that has been suggested for the mammalian clock (Edgar et al., 1993). In contrast, we found that tim^0 flies lacked a rest rebound. In view of the fact that this abnormality was rescued in tim^7 flies, we propose that the *timeless* gene has a function beyond its role in the central clock: *timeless* may be linked to the rest homeostatic mechanism. The duration of the rest deprivation response in tim^7 flies was briefer than the average in wild-type flies, although a wide range of individual responses was observed in both backgrounds (see Figures 3C and 5A). This could indicate that something other than *tim* in the tim^0 background contributes to the homeostatic phenotype but does not, of course,

negate a role for *tim*. Interestingly, Andretic and coworkers (Andretic et al., 1999) have reported that *period* and *timeless* have differential effects on a behavioral output. Sensitization to cocaine administration in *Drosophila* is abnormal in per^0 flies but not in tim^0 flies. If *timeless* is indeed related to the homeostatic control of rest, this would be a novel *per*-independent role for *timeless*.

We conclude that rest in *Drosophila* shares with sleep the intriguing features of prolonged immobility, lack of sensory responses, and homeostatic rebound in response to rest deprivation in addition to the well-known circadian regulatory influences on activity. Rest in these simple animals may be considered as a primordial form of the sleep state. Although Tobler and others have noted some sleep-like features of rest in invertebrates including insects (Kaiser and Steiner-Kaiser, 1983; Tobler, 1983; Campbell and Tobler, 1984; Kaiser, 1988; Tobler and Neuner-Jehle, 1992), this is a novel comprehensive description of a sleep-like state in a genetically tractable simple organism. The utility of *Drosophila* for genetic dissection of complex behaviors has a long history. Among recent successes in using *Drosophila* are new information about the molecular basis of long-term memory consolidation (reviewed by Carew, 1996) and of circadian rhythms (Dunlap, 1999), both behaviors that could be relevant to the function and regulation, respectively, of sleep. The gene responsible for inherited narcolepsy (*hypocretin/orexin 2 receptor*) in dogs has just recently been identified (Lin et al., 1999) and shown in knockout mice to play a role in REM sleep regulation (Chemelli et al., 1999). This finding may herald a new era for the field of sleep research. We anticipate that the study of sleep-like rest and rest rebound in *Drosophila* will complement mammalian studies by using rest and rest rebound assays to investigate the role of potentially rest-related transgenics and mutants and help identify new rest-relevant genes. We expect that this will lead to new insights into the molecular basis of sleep function and control.

Experimental Procedures

Animals

We used both Canton S (CS), a standard wild-type strain, and yellow-white (*yw*) flies that are commonly used for transgenics and immunocytochemistry and have wild-type circadian rhythms. Mutations used in this study are in a *yw* background. tim^7 flies were generated by injecting a *tim* construct, composed predominately of genomic sequences. The transgene rescued rhythmicity in 92.6% of tim^0 flies, with a 23.69 $\pm$ 0.69 hr period (G. W. Wang, A. Ousley, L. J. Hickman, and A. S., unpublished data). Unless otherwise noted, flies were of random age and both sexes were studied.

Flies were housed in well-humidified incubators at 25°C in a 12 hr L:D cycle (light cycle 2800 lux) in 175 ml bottles or 40 ml vials and fed a standard food. Behavioral studies were done in constant darkness (D:D). In these conditions, the animals' subjective time of day is determined by the circadian clock and is termed "circadian time" (CT). The time of expected lights on was at CT 0 and the time of expected lights off was at CT 12. When necessary, CO_2 was used for sedation and flies were allowed to recover before studies.

Behavioral Observations

All observations of rest were conducted in constant (D:D) conditions. Dim red light that we verified does not reset the circadian clock was used for visualization or videotaping at room temperatures averaging 23°C. For detailed observations, flies were briefly sedated to allow them to be placed individually in the same glass 2 $\times$ 60 mm tubes that are used for locomotor assays. Only male flies were used, as females produce larvae that obscure the adult fly's movements within 24–48 hr. To observe details of movements at high magnification, tubes were removed after 1–5 days in D:D in the Trikinetics circadian rhythm monitor, placed in dim red light under a NOVA FST652 trinocular dissecting microscope, and videotaped by a Cohu High Performance 4915-2000/0000 CCTV camera for 48 hr on a Panasonic AG-6124 time-lapse VCR at 24 hr speed. The fly was confined to a 4 mm length of tube by moving the yarn further into the tube. After videotaping, the yarn was retracted and the tube was replaced in the locomotor monitor.

During analysis of the videotapes, the fly's location, position, posture, and activity during each minute were noted. A minute with any coordinated behavior was scored as "active." Full minutes without any coordinated movement were scored as "rest." If the animal could not be seen clearly ($<$1% of minutes) the activity was "unknown." Five flies survived the 48 hr in the tightly confined space, but three of five had fragmented rest–activity pattern periods different from their usual consolidated circadian rest–activity as measured in the locomotor assay, perhaps due to stress from the confinement. Rest behavior, posture, and position data were similar among all five flies, but the duration of immobility was assessed only in two animals (one CS and one *yw*) with normal, consolidated rest–activity patterns.

To observe rest in less confined conditions and to compare rest behavior with simultaneous activity counts, 11 male flies (8 CS, 3 *yw*) were videotaped in the 60 mm $\times$ 2 mm tubes in the Trikinetics monitor (Trikinetics, Waltham, MA). After 1–5 days in D:D, the monitors were moved out of the incubator for 24 hr of videotaping in dim red light. For analysis, each minute was scored as resting (immobile for a full minute) or active (any coordinated activity). The rest position and posture were also noted, as was the specific nature of any coordinated behavior that occurred between rest periods. Behavior was scored as "unknown" if the fly could not be visualized ($<$5% of the maximal rest periods for all animals). Unlike the flies studied in confined spaces, all 11 flies had normal rest–activity patterns throughout. These data were used for quantitative analyses of rest duration and for validation of the automated measure of rest.

To study rest behavior in groups, 20 CS flies (10 male, 10 female) at 1 day posteclosion were sedated, placed in 8 cm Petrie dishes, and allowed to recover for 24 hr. Standard food diluted 1:1 with distilled water was provided on a 1.5 $\times$ 1.5 cm square of filter paper. At CT 12.5, flies were moved into dim red light. Recording began at CT 13.5. One group of 20 flies was rest deprived for 8.5 hr, and one was a control.

The videotapes were reviewed to describe rest behavior, defined as $\geq$5 min without activity. As described in the Results, resting flies were sometimes contacted by active flies, and made rejecting movements that were not scored as "activity." Rest was measured for the control group from CT 13.5 to CT 10 (a total of 20.5 hr) and for the rest deprivation group for 12 hr after the end of the rest deprivation (CT 22–10). The number of flies that were resting was noted each 5 min for the first 30 min of each hour. Thus, for each hr, six consecutive 5 min measures were made.

To describe responsiveness to natural stimuli, tapes of the controls were reviewed in slow motion for 20 consecutive min during CT 14, 15, and 16, when most rest occurred, and during CT 22, when most flies were active. Each approach (active fly passing within 2 mm) or direct contact with a resting fly was noted. Responses were scored as none (immobile for $>$1 min after contact), rejecting (flicks of the wing or twisting away), or arousal (gross locomotor activity).

Rest Disruption

To disrupt rest manually, mechanical stimuli were applied whenever $\geq$1 fly was immobile for $\geq$1 min. A more intense stimulus was applied after 15 s if needed. The stimuli were graded as 1 (one tap), 2 (two taps), 3 (move dish 1 mm), and 4 (lift dish and tap forcefully). The grade of the stimulus required was noted, and the total of all stimuli (number $\times$ intensity grade) was summed for every 30 min for the 8.5 hr rest disruption period CT 13.5 to CT 22. Rest during recovery from CT 22 to CT 10 was monitored as described above for both the rest-deprived and the control group.

Automated Deprivation Stimulus

A motion controller was programmed to move the drive shaft of a synchronous/stepping motor (SM091-FF-206T, Applied Controls) at a rate of 1440°/s in a loop (40° clockwise, 10 ms pause; 10° counterclockwise, 10 ms pause) repeated six times. The total sequence lasted a total of ≤0.5 s. A computer triggered the stimulus at random intervals (range, 30–90 s; mean, 1 min) in alternating directions. In initial trials, flies responded to this automated stimulus for 6 hr, but up to 50% failed to respond from 6 to 12 hr when the stimulation was prolonged. Perhaps rest deprivation, combined with habituation, allowed the animals to rest despite ongoing stimulation. The 6 hr stimulus was used in all reported studies.

Population Study of Rest Rebound

Male flies were monitored in the Trikinetics assay. In each trial, animals were distributed into three groups: rested controls, handled controls, and rest deprived flies. After 3 days, flies in handled control and deprivation groups were removed from the monitor in dim red light. Both groups were wrapped in aluminum foil to prevent light contamination and attached to the platform of the stepper motor (rest deprivation) or placed next to the motor (handled controls) for 6 hr (CT 18–24/0) and then replaced in the monitor. In a preliminary trial to study the effect of handling, we monitored the behavior of flies removed from the incubator while still in the locomotor monitor. We found that transient increases in activity followed both removal from and replacement into the incubator, but that the rest pattern while next to the motor was the same as that on baseline days. Movement artifact contaminated the data collected at CT 0–0.5 when flies were replaced. This single data point was eliminated from the analysis for all animals. Rest was then recorded continuously for 3 postdeprivation days.

Data Analysis

Rest Rebound

Flies were in the monitors for 8 days, but only 2 baseline days and 3 postdeprivation (or posthandling) days were used for statistical comparisons. An initial day was allowed for recovery from sedation. Data were incomplete on the day when flies were handled or deprived. Monitoring on the eighth day ensured that flies remained active and healthy.

Automated Measure of Rest

A 30 min data collection with 0 activity counts was defined to represent 30 min of rest. The validity of this estimate was analyzed for sensitivity, specificity, and predictive value. Briefly, a measure of 0 activity counts correctly predicted that a full 30 min of immobility was actually observed over 80% of the time (sensitivity of 80.8%). On average, only 3.22 ± 3.26 min with any activity were seen when the locomotor assay recorded 0 counts. The likelihood ratio for detecting 30 min of continuous rest was 3.51:1 for 0 counts compared to >0 counts.

The data analyzed statistically were the number of 30 min rest periods during each of four 6 hr time blocks in each 24 hr day. During baseline, Time 1 was CT 0–6, Time 2 was CT 6.5–12, Time 3 was CT 12.5–18, and Time 4 was CT 18.5–24. After handling or deprivation, because of the elimination of the first 30 min data collection period at CT 0–0.5, these time periods were offset by 30 min.

Circadian Phase

To identify whether the forced locomotion or handling reset the internal clock, we calculated the median phase defined as the circadian time of activity offset in a subset of animals ($n = 26$ rested, 26 handled, 32 rest deprived). The changes from the last baseline to the first postdeprivation day, and for all of the postdeprivation days, were nonsignificant ($p > 0.10$) among the three groups or between groups.

Drug Administration

CS flies aged 1 day posteclosion were briefly sedated and placed separately in 28 wells of a 96-well microtiter plate, each with a 0.5 × 0.5 cm square of laboratory tissue paper. The plate was then covered with transparent adhesive tape with 21 gauge needle holes for ventilation, and the flies starved for 6 hr. The paper was saturated with drug or placebo at CT 6, and the flies were observed to drink. Recording began at CT 7. Rest for each fly was scored in 10 min intervals. For each hour, a scorer blind to the experimental conditions calculated the mean proportion of time spent resting in the flies in each group. Interobserver correlation was 0.89.

Statistical Methods

To compare responses to sensory stimulation, manual rest deprivation, or drug administration, we used Student's t tests for pairwise comparisons and an ANOVA for multiple groups when data were normally distributed and of equal variance. For nonparametric data, we used the Mann-Whitney U rank-sum test for pairwise comparisons and the Kruskal-Wallis test for comparisons of multiple groups (Sigma Stat).

For the population study of rest rebound, a mixed model analysis of variance approach was used that allowed between- and within-group comparisons. Three groups (rested, handled, and rest deprived) were analyzed and compared using SAS PROC MIXED (Littell et al., 1996). First, an appropriate covariance structure for the within-subject effects was determined using a series of models including only random effects. The repeated measurements consisted of time periods within a day, as well as sequentially over days. An autoregressive covariance structure that posits an increased correlation between measurements that are temporally contiguous best fit the data. Next, an overall model including group, day, and time period was computed to examine the main effects and higher order interactions.

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