Modafinil Maintains Waking in the Fruit Fly Drosophila Melanogaster

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Abstract: Fruit flies exhibit a sleep-like rest state that shares behavioral characteristics with mammalian sleep, including a homeostatic increase in rest after deprivation by mechanical methods. We tested the effect of modafinil, a novel wake-promoting agent, to discover whether its effect is conserved.

Flies fed various concentrations of modafinil were compared to groups of control flies fed diluent only. Flies were also tested for a homeostatic response to the modafinil-related rest deprivation by examining rest and activity during recovery after 48H modafinil administration, compared to rest deprivation alone and to both treatments combined.

The duration and consolidation of rest, and the duration, intensity, and circadian rhythms of activity were measured.

Modafinil significantly and dose-dependently decreased rest when fed at concentrations from 2.5 mg/ml to 0.3125 mg/ml. Activity intensity was not increased, and circadian timing was unchanged, although the 2.5 mg/ml dose blunted the amplitude of overt circadian locomotor rhythms. Compared to controls, the duration of rest bouts was decreased in flies fed 2.5 mg/ml, and waking was frequently interrupted by 5-min periods of immobility. A rest rebound (significant increase in rest) followed withdrawal of either 2.5 mg/ml or 0.625 mg/ml modafinil after 48H. When directly compared to 6H total rest deprivation, the increase after withdrawal was briefer, reminiscent of the attenuated rest rebound seen in mammals, including humans, after modafinil. However, modafinil withdrawal combined with 6H total rest deprivation significantly enhanced the rebound, suggesting that a rest debt is accumulating during modafinil.

We conclude that modafinil affects states of arousal in Drosophila in the same direction as it does in mammals. This discovery provides a tool for searching for conserved molecular mechanisms by which modafinil regulates rest and waking.

Key words: Drosophila, state regulation, modafinil, wake-promoting, sleep-like rest

Citation: Hendricks JC, Kirk D, Panckeri K, et al. Modafinil maintains waking in the fruit fly drosophila melanogaster. SLEEP 2003;2:139-146.

INTRODUCTION

TWO RECENT STUDIES HAVE CONCLUDED THAT THE LABORATORY FRUIT FLY, DROSOPHILA MELANOGASTER, HAS A BEHAVIORAL REST STATE SIMILAR TO MAMMALIAN SLEEP.1,2 As in mammals, sleep-like rest is regulated by both circadian and homeostatic influences, with the latter being demonstrated by increased rest after deprivation. Modafinil (diphenylmethyl-sulfanyl-2 acetamide) is a novel pharmacological agent that reduces sleep without the negative effects of methamphetamine.3-7 Recovery sleep after sleep deprivation with modafinil is more similar to subjects given placebo than after sleep deprivation with methamphetamine,4,5,8 and the duration of recovery sleep following modafinil is generally reduced compared to sleep deprivation by other means.4,5,8 While its mode of action is unclear, it does not act through any known receptor.9 A role for the dopamine transporter has been suggested.9 However, this proposal is difficult to reconcile with previous studies, where the wake-promoting effects of modafinil were found not to be mediated by dopaminergic mechanisms,10,11 and see Jasinski and Kovacevic-Ristanovic6 for review. As judged by c-fos expression, modafinil increases neuronal activity only in a limited number of brain areas.12,13 These are mostly those involved in maintaining wakefulness.13 Demonstrating that modafinil decreases rest in fruit flies would provide another tool to dissect its molecular mechanisms and provide additional evidence that state regulation is fundamentally conserved. We therefore investigated the effect of modafinil on rest and activity in the fruit fly. We first noted behavioral response to modafinil. In studies conducted in constant conditions, we evaluated the total daily duration of consolidated (>30 min duration) rest bouts, the mean and maximal duration of rest bouts, the intensity of locomotion, and the timing of activity as determined by the endogenous circadian clock. Finally, we quantified the homeostatic response in animals that were allowed to recover after being rest-deprived for 48H using modafinil and compared this response to the response to 6H total rest deprivation with mechanical stimulation and both treatments combined.

METHODS

Animals

All flies were 3-day-old Canton Special (CS) adults. Only males were used for the locomotor assay, as females produce larvae that interfere with the assay. Both genders were used for initial trials and for tissue analysis. Flies were housed in well-humidified incubators at 25° C in a 12h L:D cycle (light cycle 2800 lux) in 175 ml bottles or 40-ml vials and fed a standard food.

Behavioral Observations

Groups of 40 CS flies (20 males, 20 females) were placed in vials containing diluent, 2.5 mg/ml modafinil, or 0.625 mg/ml modafinil. The flies were returned to the 25° incubator in LD conditions for 24H. An observer blinded to the experimental conditions then observed the flies during their usual active time in light conditions from zeitgeber time (zt) 1 to zt 5. We noted the following: the distribution in the vial; spontaneous locomotion and interactions with other flies; and response to mechanical stimulation (tapping the vials so that all of the flies dropped to the bottom of the vial).
Groups of 16 flies were briefly sedated with carbon dioxide and placed individually into 60 x 3 mm glass tubes supplied with enough food for 2 weeks. Each tube was placed in a slot of the Trikinetics locomotor monitor (Waltham, MA), with a centrally located infrared beam. For routine studies, beam breaks were automatically collected at 30-minute intervals by the Trikinetics software. To more precisely evaluate the effect of modafinil on the duration of rest bouts, activity counts were collected in 5-min time bins. All locomotor studies were conducted in constant darkness (D:D) at 25 °C. 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 at ct 12. This allowed us to obtain information about circadian timing as well as total daily measures of rest and activity.

Drug Preparation and Administration

Modafinil (provided by Cephalon, Inc., West Chester, PA) was prepared at Cephalon, dissolved in cyclic oligosaccharides with 5% sucrose (to increase palatability) at 20 mg/ml. Preliminary studies were conducted as described previously, with flies placed individually into wells of a 96-well microtiter plate and deprived of food for 6 H. To establish a dose range, flies were initially fed 20, 10, or 5 mg/ml modafinil, in the diluent with 5% sucrose; observed to drink immediately; and videotaped for the subsequent 48 H. Flies fed 20 mg/ml died after a few hours of constant activity. Flies fed ≤10 mg/ml modafinil had near-constant locomotion during the observation period but no abnormal behaviors. The locomotor assay was thus initiated with modafinil at doses of 0.3125, 0.625, 2.5, 5, and 10 mg/ml made by mixing the solution with agar cooled to <50°C. Controls (0mg/ml modafinil) were fed agar made with the diluent and 5% sucrose. Thus, the animals could not eat without ingesting modafinil (or diluent for the controls) throughout the locomotor study.

Tissue Drug Concentrations

To compare tissue levels in fruit flies to mammalian plasma levels and ensure that the drug reached the flies’ central nervous system (CNS), 60 flies fed either diluent or 2.5 mg/ml modafinil for 48H were collected on dry ice. Heads (approx. 90% CNS) were separated from bodies by sieving on dry ice and stored at −70°C. The tissue was homogenized in a fixed volume (300 ul) of acetonitrile, centrifuged, and duplicate 50ul aliquots of the supernatant analyzed. Modafinil was quantified by HPLC with mass spectrometry detection. The standard curve was generated by adding 50ul of acetonitrile to correct for the sample volume. The average tissue levels were 5.6 ug/g wet weight in heads and 140ug/g wet weight in bodies. The high level in the bodies is likely due to modafinil in the gastrointestinal tract. Modafinil was undetectable (<5ug/ml extract) in flies fed diluent. These head (CNS) levels can be compared to effective human plasma levels of 2-8ug/ml.14

STUDY DESIGN

Rest/activity

Following our standard protocol1 we allowed a day for recovery and then collected activity data for 6 days. Flies were left in the monitor for an additional day to ensure that they remained healthy. No flies fed 5 or 10 mg/ml modafinil survived to the 8th day. The rest and activity patterns for these flies were inspected but not statistically analyzed. Thus, the groups analyzed were flies fed 0, 0.3125, 0.625, or 2.5 mg/ml modafinil. We have previously used 30-minute collection bins with no activity counts as an estimate of 30-minute consolidated rest bouts in normal flies. This criterion was used for all studies unless otherwise specified.

Duration of Rest Bouts

To more precisely identify the onset and termination of rest bouts to measure the effect of 2.5mg/ml modafinil on rest-bout duration, data from two groups of 32 flies were downloaded in 5-minute windows. These data were analyzed using a custom-designed software based on Microsoft-Excel Visual Basic (kindly provided by Dr. Kazuhiko Kume, Kamamoto University). Because of the limits of the buffer size in the Trikinetics system, only 3 days of activity counts from each fly could be analyzed. We chose to analyze data from days 3-6 in the assay. Several measures were calculated. Each 5-min window with 0 counts was defined as 5 min of rest. Consecutive 5-min periods were identified, and the total duration defined as the duration of the rest bout. This software enabled us to examine the number of rest bouts when the criterion for rest was varied from ≥5 to ≥30 min. The software also calculated the frequency, mean, and maximum duration of bouts of each duration (≥5 or ≥30 min) for each animal over the 3-day period. Finally, the mean and...
standard deviations of control and drug groups were calculated for each measure.

Effect of Modafinil Withdrawal

To study the behavior of flies during withdrawal from modafinil, groups of 32 flies were provided with 2.5 mg/ml or 0.625 mg/ml modafinil and compared to 32 flies fed agar made with diluent in the locomotor assay for two days. All flies were then transferred in the dark to tubes containing diluent, using a dim red light invisible to flies. For flies fed modafinil, this represented an abrupt switch to diluent only. The rest, activity, and circadian measures were compared for the 6 days after transfer.

Comparison of Rest Deprivation and Withdrawal From Modafinil

Flies were placed in the locomotor assay in tubes containing food made with either 0.625 mg modafinil (n=64) or diluent (n=64). After 3 days in the assay (allowing the collection of 2 complete days of baseline data), flies were removed from the locomotor assay apparatus and placed on the platform of our automated deprivation device described previously by us. Through the administration of small abrupt movements randomly timed at approximately 15 second intervals by a computer, this method prevents flies from resting for a 6H period. We used this duration of deprivation as we have extensive experience with this paradigm in thousands of animals in our previous studies. The stimulation was initiated at ct18 and terminated at ct24. Flies were then transferred to tubes containing diluent only or to tubes containing 0.625 mg/ml modafinil. The two initial groups of 64 flies—64 with modafinil and 64 with diluent—were subdivided into groups of 32 to create 4 groups: (1) 32 flies initially fed diluent were transferred from diluent to diluent (D-D), acting as controls for the deprivation and transfer process; (2) 32 flies initially fed modafinil were transferred from tubes containing modafinil to tubes containing diluent (M-D), to test the hypothesis that modafinil administration combined with rest deprivation would increase the rest debt produced by either manipulation alone; (3) 32 flies that had been fed diluent were transferred to tubes containing modafinil after the sleep; and finally (4) 32 flies that had been fed modafinil were transferred to new tubes containing modafinil. Groups 3 and 4 were included to test the hypothesis that the maintenance of wakefulness produced by modafinil might be overwhelmed by the homeostatic need to rest after mechanical rest deprivation (Group 3) or after combined withdrawal from modafinil and rest deprivation (Group 4).

DATA ANALYSIS

Daily Rest and Activity

Unless otherwise specified, a 30-min period with 0 activity counts was defined to represent 30 min of consolidated rest. This criterion was validated previously by comparison of activity counts in the Trikinetics assay with directly visualized bouts of immobility. The total daily rest duration (H/24H) was then calculated for each day for each animal. As an overall measure of daily activity, the total daily activity (counts/24H) was obtained for each day for each animal. The peak daily activity (maximal activity counts in a single 30-min period on each 24-H day) was used to describe locomotor ability, independent of any effect of rest. To measure the daily waking activity rate, activity counts for every 30-min period when the fly was active were averaged for the day (waking activity counts/24H). For analysis, each of these daily measures (rest, peak activity, waking activity rate, and total activity) was averaged over the 6 days of the study period to obtain a mean for each animal.

Response to Modafinil Withdrawal and/or Rest Deprivation

To evaluate the effect of modafinil withdrawal and/or total rest deprivation, the duration of rest during each 6-hour blocks, i.e., quarter of each day, before and after withdrawal and/or deprivation was calculated for each animal. For the first 6H period, following intervention, the amount of rest, in shorter 1.5H epochs, was also calculated for each animal.

Circadian Rhythm Parameters

The Clocklab software package (The Mathworks, Natick, MA) was used to calculate circadian parameters. The amplitude of the circadian rhythm (relative power at 24H using the Fast Fourier Transform (fft)) was calculated for 6 days (the same days analyzed to obtain rest and activity measures). Wildtype animals generally have an fft of >0.06, while arrhythmic clock mutants have fft values <0.04, and usually <0.02 (unpublished observations, Joan Hendricks, VMD, PhD). In assessing rhythmicity, we also inspected the actograms and in addition required that the χ2 periodogram indicate a period at 23-25H with 95% confidence to determine which animals with an fft >0.02 and <0.06 were rhythmic. For rhythmic animals, we then assessed the circadian timing parameters (phase and period) using the Clocklab package. We used both the χ2 periodogram and thefft analyses to determine period. To quantify the circadian rhythm after 48H of modafinil, the phase, amplitude, and period were analyzed for the 6 days of data obtained after withdrawal from modafinil and compared to flies fed diluent only.

Statistical Approach

The rest and activity values for the 6-day study (total daily rest, peak daily activity, waking activity rate, and total daily activity) were subjected to a mixed-model ANOVA, with dose level as the between-subjects factor and day of study as the within-subject factor. Where this analysis showed an overall effect significant at <0.05, individual groups were compared using Dunnett’s post-hoc adjustment for multiple comparisons. Circadian rhythm measures were evaluated using a one-way ANOVA for the effect of dose. Where this analysis showed an overall effect significant at <0.05, individual groups were compared using Dunnett’s post-hoc adjustment for multiple comparisons. For the evaluation of rest bout duration and homeostatic response, we conducted comparisons between groups of flies fed modafinil and controls fed diluent using Student’s unpaired two-tailed t-test. A p<0.05 was accepted as significant.

RESULTS

Behavioral Observations

We conducted simple observations to discover whether behavioral abnormalities were apparent in flies fed doses of modafinil that decreased rest without increasing mortality. In two replicates, groups of flies fed 2.5 mg/ml modafinil were observed to behave differently from flies fed diluent or flies fed 0.625 mg/ml modafinil. Throughout the 4H observation, the majority of flies fed the higher dose of modafinil were at the bottom of the container while the other groups distributed themselves equally between the top and bottom of the container when undisturbed. The nature and coordination of behaviors of undisturbed flies (eating, grooming, walking, courting) were not distinguishable among the 3 groups (control, 0.625mg/ml modafinil, 2.5mg/ml modafinil). When the containers were tapped so that all flies fell to the bottom of the container, controls and flies fed 0.625 mg/ml modafinil exhibited the normal response: a jump or startle, followed by negative geotaxis, i.e., running rapidly to the top of the container. By contrast, flies fed 2.5 mg/ml modafinil exhibited a marked startle response and walked on the bottom of the container or began to ascend the sides of the tube after tapping, but rarely completed the ascent to the top of the tube, coming to rest instead on the side or floor of the container.
Effect of Modafinil on Rest and Activity

Overall, groups of flies fed modafinil had significantly reduced daily rest (F=9.28, p<0.0001 compared to diluent) (Figure 1A). Post-hoc comparisons revealed that 2.5 mg/ml (n=14) and 0.625 mg/ml (n=12) produced the same level of daily rest suppression, to a mean of 1.5H/24H (p<0.0001 vs. diluent). Flies fed 0.3125 mg/ml (n=14) were able to rest for 3H/24H (p=0.048 vs. diluent). Hyperactivity was not found. In fact, we found an overall reduction in the peak daily activity (F=11.39, p<0.0001). Dunnett post-hoc contrasts revealed significant reductions in flies fed 2.5 mg/ml (p<0.0001 vs. diluent) and 0.625 mg/ml (p=0.034 vs. diluent) (Figure 1B). Waking activity rates were reduced by modafinil (F=2.90, p=0.044 overall), but only the 2.5 mg/ml dose (p=0.021) was significant as compared to diluent in the post-hoc comparisons (see Figure 1C). The total daily activity was statistically the same in all groups (F=1.46, p=0.237) (Figure 1D). No activity measure was altered by the 0.3125 mg/ml dose. There was no evidence of tolerance or compensation. That is, the degree of rest suppression and activity measures did not show a progressive change over the period of the study at any concentration of modafinil (data not shown).

Duration and Frequency of Rest and Activity Bouts in Flies Fed Modafinil

In the studies reported above, as in previous studies\(^1\) we collected data in 30-min bins and defined a period to be rest if the animal did not cross the central beam at any time in the 30-min data collection period. This was based on data obtained by direct visualization of the animal.\(^1\) Having found that modafinil reduced the daily duration of such consolidated rest periods, we performed a second study to discover whether modafinil affects the length and frequency of rest bouts. In groups of flies fed 2.5 mg/ml modafinil (n=24), and control flies fed diluent only (n=31) for 8 days, we analyzed data from days 3-6 in the assay (3 days is the limit of the buffer on the data collection system) using custom-designed software to assess the consolidation of bouts of rest and activity (see Methods). When we inspected the patterns of activity in all of the individuals, we observed that flies fed modafinil exhibited prolonged bouts of activity only rarely interrupted by consolidated rest bouts lasting ≥30 minutes. The longest bout of activity without consolidated rest (≥30 minutes) averaged 22±19H in flies fed modafinil, compared to 3±3H for controls, and one fly was continuously active for the entire 3-day (72H) period. However, we also noted that the bouts of activity were punctuated by frequent pauses (rest) lasting on the order of 5 minutes in flies fed modafinil. To quantify this pattern of frequent state transitions and short, but not consolidated, rest periods, we used a custom-designed software package to look at rest and activity parameters (see Methods). We varied the criterion for rest from 30 minutes to 5 minutes. In controls (wildtype flies fed the diluent), changing the criterion to >5 minutes only slightly changed the calculated daily duration of rest, (11.7H±2.1H using the 30-min criterion vs 13.0H±1.9H using the 5-min criterion, p<0.0001). This is consistent with our previous findings that, once normal flies initiate a rest bout, the vast majority of bouts last ≥30 min,\(^1\) so that briefer bouts of rest normally contribute little to total daily rest. We note that the daily rest average in this control group is longer than that of the control group displayed in Figure 1A. While we see some variability in the rest in groups of animals studied at different times, an important reason for this difference is that the 5-min analysis considered rest only on days 3-6, discarding the first few days when animals are generally more active and have less rest. The simultaneously studied flies fed modafinil rested much less than controls when bouts of consolidated rest (≥30 minutes) were considered (5.4±5.4H, p<0.0005). However, when all ≥5-minute bouts of immobility were considered, the total amount of “rest” increased to 11.0±5.4H, not significantly different from controls (p=0.18). Regardless of the criterion used, flies fed modafinil had significantly shorter rest bouts than controls (46.7±13min for modafinil and 131.9±58.3min, for controls when only bouts lasting ≥30-min were considered, p<0.0001; 17.8±11.7min for flies on modafinil and 63.8±28.1min when all bouts lasting ≥5 min were included, p<0.0001). In summary, flies fed 2.5mg/ml modafinil initiate rest bouts at least as frequently as controls but do not sustain long consolidated periods of rest. Their active periods are fragmented by multiple brief periods of immobility.
Rest Decrease Is Independent of Effects on the Circadian Rhythm

Visually scanning the 6-day group activity patterns suggested an absence of circadian modulation in flies fed 2.5 mg/ml, but a rhythm was discernible at lower doses. The average activity patterns for groups given all doses is shown in Figure 2A. Overall circadian rhythm amplitude measured by fft was significantly decreased by modafinil (F=3.18, p=0.0356), as illustrated in Figure 2B. Post-hoc comparisons showed the suppression to be significant only at 2.5 mg/ml (relative power at 24H decreased from 0.125 to 0.026, p=0.02) (Figure 2B). Phase and period were measured only in rhythmic flies (12/14, diluent; 5/14 2.5 mg/ml; 10/12, 0.625 mg/ml, 12/14, 0.3125 mg/ml), determined as described in Methods. The period was unchanged at any dose (F=2.05, p=0.12 for \( \chi^2 \) method; F=.56, p=0.648 for fft method). Overall, phase was changed in flies fed modafinil (F=3.25, p=0.033), but this was not a dose-related effect. Rather, post-hoc comparisons showed a phase delay by a mean of 5.7H (p=0.006) only in flies fed the intermediate (0.625 mg/ml) dose and no phase shift in flies fed either the higher or the lower dose. Thus, modafinil did not change timing parameters in a dose-dependent fashion. The highest dose suppressed circadian amplitude of activity while lower doses suppressed rest without reducing the amplitude of the circadian rhythm.

To determine whether the internal clock continues to keep time when the overt rhythm is reduced by 2.5mg/ml modafinil, we administered 2.5mg/ml modafinil for 48H to a group of 32 flies and diluent only to another group of 32. We then transferred all animals to fresh tubes containing food made with diluent only and collected another 6 days of data. The initial placement and the transfer were both made at ct 7.5-8.5 (that is, 7.5 to 8.5H after expected dawn). Examples of actograms of this full study for 3 flies fed modafinil and 3 flies fed diluent are shown in Figure 2C. Inspection of the actograms indicated that flies fed modafinil exhibited a constant level of activity during the 2 days when they received modafinil, just as we had noticed at the same dose in the group of flies reported above (Figure 1A). Flies transferred from diluent promptly resumed their previous activity patterns. The 6 days of data collected after withdrawal were analyzed for all flies that survived the full study (n=30 for modafinil and n=27 for diluent) to obtain circadian amplitude, period, and phase. None of these parameters was significantly different in flies withdrawn from modafinil compared to flies transferred from diluent (data not shown).

Recovery After Withdrawal From Modafinil and Rest Deprivation

To discover whether an increase in rest follows withdrawal from modafinil, we analyzed rest in the flies described above. Flies withdrawn from 2.5mg/ml modafinil rested significantly more than controls during the first 6H of recovery. When this 6H time period was broken down into 1.5H bins, we found an initial 1.5H of hyperactivity with no rest in both groups; thereafter, flies withdrawn from modafinil rested significantly more than control flies that were simply transferred from tubes containing diluent into new tubes containing diluent in each time bin (Figure 3A, left panel). During the remainder of the 3-day recovery period, however, flies withdrawn from 2.5mg/ml modafinil rested the same as control flies (data not shown). This suggested to us that 48H of modafinil, with its attendant disruption of consolidated rest, might increase the homeostatic drive for rest. However, as noted above, flies fed this higher dose of modafinil showed reduced waking activity rates and reduced circadian rhythm amplitude. Since the 0.625mg/ml dose reduced consolidated rest to the same degree (see Figure 1) without these other behavioral changes, we repeated the withdrawal study with this lower dose. When we examined 6H time periods, we could not demonstrate that these animals exhibited a significant increase in rest after withdrawal compared to controls at any time. However, when we examined briefer time periods, calculating the rest during each 1.5H of transfer, we found an initial 1.5H of hyperactivity with no rest in both groups; thereafter, flies withdrawn from modafinil rested significantly more than control flies that were simply transferred from tubes containing diluent into new tubes containing diluent in each time bin (Figure 3A, right panel). On the right, the duration of baseline rest is shown for both groups for each 6H time of day for the day prior to deprivation. On the right, the duration of rest for each 6H time of day after deprivation is shown for both groups. *p value as shown comparing controls (D-D) to combined modafinil withdrawal (M-D). Y-axis: Mean ± SEM hours of rest in each 6H quarter of the day X-axis: circadian time (ct) 0-6, ct 6-12, ct 12-18, ct 18-24 C. The patterns of rest in 3 groups (deprivation only: 0.625mg/ml modafinil withdrawal only; and 0.625mg/ml modafinil combined with deprivation) are shown compared to the pattern of rest for "handled control," i.e., flies that were simply transferred to new tubes containing food made with diluent (HC, same group as in figure 3A, right panel). X-axis: time after transfer Y-axis: Mean ± SEM H rest/1.5H moving window.
clearly different from those in our previous studies of rest deprivation. To more definitively address the question of whether maintaining wakefulness with modafinil contributes to a rest debt in Drosophila, we directly compared the effects of 6H of total rest deprivation, to the effects of 6H total rest deprivation combined with withdrawal from modafinil. We hypothesized that modafinil administration might alter CNS function such that the recovery functions of rest can be attained without overt evidence of consolidated rest. If so, then withdrawal from modafinil combined with deprivation should have no greater effect than deprivation alone. However, if a rest debt accumulates during prolonged waking in animals fed modafinil, then the additional insult of 6H complete rest deprivation should lead to an enhanced rebound.

At the same time we also studied two additional groups that were fed modafinil after the 6H rest deprivation. One group of flies was deprived of rest and then fed modafinil, and one group was fed modafinil both before and after rest deprivation. We speculated that the pressure to rest after 6H complete rest deprivation would be able to overwhelm the ability of the animals receiving modafinil to sustain wakefulness. We hypothesized that such a rest debt would be further enhanced in animals fed modafinil before initial deprivation.

The effect of feeding modafinil after deprivation, whether or not the flies had received modafinil before the deprivation, was to significantly decrease total daily rest by a mean of 4.4 to 5.2H/24H (p<0.003) compared to flies that were fed diluent throughout all 3 days of recovery (data not shown). Thus, feeding modafinil to flies after deprivation, even if they had already been subjected to days of modafinil administration, reduced the flies’ ability to manifest an increase in consolidated rest during recovery.

Most interestingly, flies that were treated with 0.625mg/ml modafinil and then rest deprived and allowed to recover after withdrawal from modafinil clearly exhibited a greater increase in rest (p=0.015) compared to that seen in flies that were simply rest deprived without receiving modafinil. In Figure 3B, the mean duration of consolidated (>30 min) rest during each 6H quarter of the day before (left) and after (right panel) is shown. The control deprived group, while resting more than handled controls studied in other trials (see Figures 3A and 3C), did not exhibit a rebound when compared to baseline levels. We speculate that the activating effect of the transfer, which prevented rest in all groups for at the first 1.5H (see Figure 3A and 3C), prevented the flies from exhibiting a more substantial rebound. The increase in rest for flies that were withdrawn from modafinil and rest-deprived was statistically significant only in the first 6H of the first day of recovery, with no rebound being manifest during days 2 and 3 of recovery (data not shown).

To allow a direct visual comparison of the pattern of increased rest among these 3 groups (flies withdrawn from 0.625 mg/ml modafinil, flies that were fed diluent and rest deprived, and flies that were both withdrawn from 0.625mg/ml modafinil and deprived of rest.), the moving window of rest during the 6H after transfer is displayed in Figure 3C for all 3 groups and for handled controls (flies fed diluent and transferred at the same time as the flies fed 0.625mg/ml were transferred, as shown in Figure 3A, right panel). The duration of the rebound was briefest in the modafinil withdrawal group and longest in the combined modafinil and deprivation group.

There were no differences in survival among the groups, with virtually all flies surviving the full study.

**DISCUSSION**

**Modafinil Alters States of Arousal in Drosophila**

The effects of modafinil on rest and activity in flies were generally consistent with reports of the effect of modafinil on sleep and activity in mammals. There were three main features of modafinil that were similar:

1. Modafinil increased the duration of waking. Modafinil significantly reduced consolidated daily rest and rest bout duration and increased the duration of waking. Consistent with results in mammals, we did not find hyperactivity by any measure. Instead, modafinil damped waking activity rates and peak activity at higher doses (see further below).

2. Rest suppression was independent of changes in the circadian locomotor rhythm. Circadian rhythm amplitude was decreased in flies fed 2.5mg/ml. However, daily rest was significantly decreased at doses of modafinil (0.3125 and 0.625mg/ml) that did not alter the locomotor circadian rhythm. Even in flies fed 2.5 mg/ml, circadian rhythms resumed with normal timing and amplitude after withdrawal. This indicates that the clock continued to keep time even when modafinil prevented overt circadian activity rhythms. Other than a slightly blunted nadir of core temperature rhythms, modafinil has little or no effect on circadian rhythms in man, including melatonin. This should not be surprising, as a recent quantitative study found that modafinil did not change SCN neural activity (measured by c-fos induction) in rats. Thus, our results support that any effect on overt rhythms is mediated through mechanisms downstream of the clock itself, in Drosophila as in mammals.

3. Modafinil withdrawal led to a brief rest rebound. Studies of modafinil and sleep deprivation have clearly shown in both rats and humans that sleep rebound after withdrawal is significantly decreased compared to the sleep rebound after amphetamines and sleep deprivation. By contrast, a recent study showed comparable rebound in mice given modafinil and mice kept awake using novel stimuli. Further, there is some evidence that sleep deprivation together with modafinil administration produces less of a homeostatic response than sleep deprivation alone with no drug treatment. In one study of 6H of wakefulness, subjects given modafinil exhibited a slightly shorter duration of non-REM rebound during the subsequent night of recovery sleep than subjects provided placebo. A recent study in which cats were given modafinil for 8H after 18H of sleep deprivation using the pedestal-over-water method concluded that modafinil did not further increase recovery sleep, suggesting that the prolonged wakefulness that accompanies modafinil administration does not contribute to a sleep debt. We found that withdrawal from either 2.5mg/ml or 0.625mg/ml led to only a brief increase in rest, which could be interpreted as supporting the idea that rest debt accumulation is minimal during modafinil administration. This may be attributable to the fact that the decrease in rest in flies fed modafinil is far from identical to complete rest deprivation. There is still residual consolidated rest (approximately 50% of controls), and there is also an increase in frequency of brief not sustained periods of rest when flies are given 2.5mg/ml modafinil. Conceivably these brief rest periods allow the fly to accomplish the recovery functions of the normal consolidated rest. It is unclear whether any equivalent changes (e.g., “microsleeps”) might occur in mammals, as no detailed studies of the electroencephalogram (EEG) or of states of consciousness of subjects chronically administered modafinil have been published. However, a recent study in mice shows modafinil induced waking to have a different EEG spectral frequency profile compared to mice subjected to novel stimuli.

By directly comparing the effects of modafinil withdrawal to rest deprivation and to the effects of both treatments combined, we found clear evidence that a rest debt does accumulate during 0.625mg/ml modafinil. Flies fed modafinil and then also subjected to total rest deprivation for 6H had a greater and more prolonged rebound compared to flies subjected only to 6H rest deprivation. Thus, even though the minimal rebound after withdrawal may be seen as evidence of good compensation for days of disrupted rest, the additional challenge of complete rest deprivation unmasks an accumulated rest debt.

Finally, we note that administering modafinil after rest deprivation completely abolishes the animal’s ability to exhibit a rest rebound. This is evidence of the power of this agent to prevent consolidated rest, even when there is considerable pressure to rest. This result is consistent with the findings of Lin et al in cats, where modafinil administration after sleep deprivation prevented any rebound sleep for a further 8H.
An Effect of Modafinil on Waking?

While we were struck by the similarities between the wake-promoting effects of modafinil in Drosophila and in mammals, it is also important to note that we found some effects that have not been reported in mammals, including death at very high doses and reduced rates of locomotor activity and circadian amplitude at the highest nonlethal dose (2.5mg/ml). Perhaps the least interesting explanation for all of the effects we have noted might be that modafinil produces some pathology in flies that leads to changes in rest and activity. Since flies fed highly concentrated modafinil (10- to 40-fold the doses used for our analyses) do not survive beyond a few days, one might speculate that, even at doses where there was no apparent ill effect in terms of survival, modafinil might have a nonspecific toxic effect. This explanation might indeed account for some of the changes in flies fed the 2.5-mg/ml dose. The fact that the rest suppression and evidence of an accumulated rest debt are also seen at the 0.625 mg/ml dose, where no ill effects of modafinil were identified, renders this less likely. We note also that, even at the 2.5-mg/ml dose, the levels of tissue modafinil are not particularly high in the CNS, being comparable to effective plasma levels in human.  

Further, the response to modafinil, i.e. decreased rest maintained over a period of days, is distinct from flies’ response to other stressful or toxic manipulations, including administering high doses of caffeine and subjecting flies to starvation or heat. Flies fed caffeine at high doses exhibit high rates of activity and die within a few hours. Flies subjected to starvation exhibit initially increased and then decreased activity. Flies subjected to caloric restriction increase their rest, while flies studied at elevated temperatures (29°C) do not significantly change their 24H rest (Hendricks, unpublished observations).

One difference between our study and those in mammals is that our study used a different protocol to deliver modafinil. Certainly the chronic administration of high concentrations of modafinil in food over a period of days (approximately 10-20% of the mean 45-day lifespan of fruit flies) might be expected to have effects that are not seen with the single doses that have usually been administered to human subjects and experimental animals. One study of repeated daily dosing of modafinil in monkeys reported a decrease in activity over time, suggesting that it is possible that such effects might also be identified in mammals if regimes for administration of modafinil were more similar to the protocol we used. If this protocol resulted in cumulative rest deprivation over 6 days, then the decrease in activity might be due to simple fatigue. However, progressive changes in rest and activity did not occur. That is, there was no main effect of day of study in our analysis. We also found that flies fed 2.5mg/ml modafinil showed an increase in the number of transitions into brief (<5 min) bouts of rest. Several interpretations of this pattern are possible. The increase in transitions into brief bouts of immobility could represent simply brief pauses to allow the flies to recover from the fatigue resulting from long bouts of locomotion, rather than truly constituting a state change. We cannot rule this possibility out. It is also possible that modafinil changes the mechanisms regulating rest, making the state unstable, and resulting in multiple short sleep-like rest periods. Finally, it could be that the increase in brief rest periods represents a compensatory response to the modafinil treatment, which allowed the flies to achieve some restorative function that made up for the lost consolidated rest. The latter two possibilities are not mutually exclusive.

An interesting possibility would be that modafinil alters wakefulness, such that it attenuates the peaks as well as the nadirs of alertness. If this is true, perhaps sleep loss from modafinil in mammals does not provoke a dramatic sleep rebound because the modafinil-induced state of waking is altered in some fashion analogous to the fragmented waking that we found in flies fed high doses of modafinil. It is obviously difficult to study the quality of consciousness objectively, and we found very few studies in the literature aimed at such an analysis of modafinil, either acutely or chronically administered, in normal subjects. One study of human volunteers found altered perception in subjects receiving modafinil (deficient “self-monitoring”). Another possibly relevant report used spectral EEG analysis in conscious rats to show that acute modafinil specifically antagonizes prefrontal cortical activation due to an atypical antipsychotic agent. Additional studies of chronically administered modafinil might reveal some subtle or complex effect on the cognitive abilities and/or waking CNS function that would parallel the marked attenuation of peak locomotor activity that we noted in fruit flies. Specifically, a quantitative analysis of the EEG during behavioral wakefulness for animals given modafinil as a means of prolonged sleep deprivation might detect microsleeps and perhaps a shift toward lower frequency waveforms of the EEG if modafinil does, indeed, permit some “restful” CNS activity that replaces consolidated sleep bouts. Such an analysis might also provide some clues to the relative lack of a homeostatic rebound when modafinil is given during sleep deprivation in mammals. Indeed, the EEG’s of mice acutely given modafinil were different from those of mice kept awake with novel stimuli. Perhaps it is possible to achieve some of the restorative functions of sleep, even while maintaining behavioral evidence of waking.

Future Studies

This study supports the use of the fruit fly to understand the mechanism of modafinil, which remains elusive. We do not know at present how or where modafinil acts in Drosophila. While the change in rest and the evidence of an accumulating rest debt in flies fed modafinil for days is not likely to be a nonspecific effect of modafinil, clearly this question must be resolved by establishing a specific molecular mechanism to explain the changes in rest. Modafinil does not bind directly to any known receptor but has been shown to interact with many neurotransmitter systems in numerous pharmacological and physiological studies. In mammalian in vivo studies, such effects of modafinil may be indirect, or even compensatory. The complexity of conducting and interpreting sleep studies in mammals is highlighted by a recent study that concluded that that mice lacking the dopamine transporter (DAT) are profoundly deficient in responding to modafinil. Interestingly, DAT-knockout mice were grossly hypersensitive to the effects of caffeine and appeared to have a period of significant hypersomnolence at 7-12H after either a selective DAT inhibitor or modafinil (see their Figure 6). These anomalies suggest pleiotropic effects of the mutation or perhaps compensatory responses during development. Such compensation is rare in fruit flies, perhaps because of their smaller genome, and tools for conditional and localized gene expression are well developed. The excellent conservation of the genome from fruit flies to mammals generally and of neurotransmitter systems specifically should allow unbiased mutagenesis screens to identify the molecular mechanism(s) underlying the wake-promoting effects of modafinil in this simple model organism. Toward this end, we have initiated a screen of mutant flies in an effort to discover flies with mutations that render them unresponsive to the effects of modafinil. As with all of our studies of rest in Drosophila, we hope to find conserved mechanisms that help further our understanding of how and where modafinil acts in mammals and, more broadly, of the molecular basis of states of vigilance.

ACKNOWLEDGEMENTS

The authors wish to thank Drs. Joe Herman and Lisa Aimone for quantifying modafinil, Dr. Jacqueline Cater for expert statistical assistance, and Dr. Amita Sehgal for insightful and enthusiastic input throughout. Supported in part by a gift from Cephalon, Inc., and grants from the University of Pennsylvania Research Foundation, NIA (P01 AG-17628 [JH, KP, AIP]) and NHLBI (R01 HL-59496 [JH, KP] and SCOR (HL-60287 [JH, AIP]).

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