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Multiple mechanisms limit the duration of wakefulness in *Drosophila* brain

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Zimmerman JE, Rizzo W, Shockley KR, Raizen DM, Naidoo N, Mackiewicz M, Churchill GA, Pack AI. Multiple mechanisms limit the duration of wakefulness in *Drosophila* brain. *Physiol Genomics* 27: 337–350, 2006. First published September 5, 2006; doi:10.1152/physiolgenomics.00030.2006.—The functions of sleep and what controls it remain unanswered biological questions. According to the two-process model, a circadian process and a homeostatic process interact to regulate sleep. While progress has been made in understanding the molecular and cellular functions of the circadian process, the mechanisms of the homeostatic process remain undiscovered. We use the recently established sleep model system organism *Drosophila melanogaster* to examine dynamic changes in gene expression during sleep and during prolonged wakefulness in the brain. Our experimental design controls for circadian processes by killing animals at three matched time points from the beginning of the consolidated rest period [Zeitgeber time (ZT) 14)] under two conditions, sleep deprived and spontaneously sleeping. Using ANOVA at a false discovery rate of 5%, we have identified 252 genes that were differentially expressed between sleep-deprived and control groups in the *Drosophila* brain. Using linear trends analysis, we have separated the significant differentially expressed genes into nine temporal expression patterns relative to a common anchor point (ZT 14). The most common expression pattern is a decrease during extended wakefulness but no change during spontaneous sleep ($n = 114$). Genes in this category were involved in protein production ($n = 47$), calcium homeostasis, and membrane excitability ($n = 5$). Multiple mechanisms, therefore, act to limit wakefulness. In addition, by studying the effects of the mechanical stimulus used in our deprivation studies during the period when the animals are predominantly active, we provide evidence for a previously unappreciated role for the *Drosophila* immune system in the brain response to stress.

sleep deprivation; temporal regulation; stress

SLEEP HAS BEEN OBSERVED in animal species ranging from insects to humans. We know that total sleep deprivation results in animal death (3, 69, 70), but the biological function of sleep remains poorly understood. With a rising prevalence of sleep restriction in our society (2, 6, 26), it becomes increasingly important to understand the function and regulation of sleep as well as the consequences of sleep deprivation on the brain.

A popular conceptual framework to understand the regulation of sleep is called the two-process model. According to this model, a circadian process and a sleep-promoting (homeostatic) process, which are mechanistically distinct, interact to regulate sleep (7, 8). While the cellular and molecular basis for the circadian process has been largely delineated, the homeostatic process remains poorly understood. A key feature of the homeostatic process is that the drive for sleep is proportional to the prior duration of wakefulness and that the restorative function of sleep is related to sleep time. Therefore, to understand the molecular underpinnings of the homeostatic process, wakefulness and sleep cannot be treated as single static behavioral states but, rather, as dynamic processes.

To gain insight into the dynamic molecular processes that are altered during prolonged wakefulness and during sleep, we designed a brain microarray experiment using the fruit fly *Drosophila melanogaster* as our animal model. Rest in *Drosophila* is most profound during the night-time period, is associated with a higher sensory arousal threshold (38), and is homeostatically regulated (35, 77). Pharmacological interventions that increase or decrease sleep in mammals have similar affects on rest in *Drosophila* (35, 77). Thus *Drosophila* is a powerful model to elucidate the molecular mechanisms regulating sleep and the molecular functions of sleep.

We performed an RNA expression profiling study examining temporal changes in the brain of *Drosophila* in relationship to the duration of prior sleep or wakefulness. Our experimental design allowed us to determine whether genes identified as differentially regulated between sleep and wakefulness were up- or downregulated in these states.

Because stimulation of the experimental animal during the normal sleep period is used to prolong wakefulness in most experimental paradigms, the interpretation of the effects of prolonged wakefulness is confounded by the effect of the perturbation stimulus itself on the animal's biology. We controlled for this effect in our experimental paradigm by examining changes in gene expression in response to identical stimulation but during a time when the animals are predominantly awake. The design of our study also allowed us to control for circadian variation in gene expression, since we compared sleeping and sleep-deprived flies at the same diurnal time.

We show that expression of many genes involved in the *Drosophila* innate immune response increases in the brain in response to stimulation during a period when the animals are predominantly awake. The increase is equal to or greater than the response to stimulation during a period when the animals are sleeping. This finding suggests a role for the immune system in the brain in this aspect of the stress response. The largest number of genes differentially expressed between prolonged wakefulness and sleep were genes whose expression was downregulated by sleep deprivation. These genes were involved in many steps of protein transcription/translation as well as both presynaptic and postsynaptic neuronal excitability processes. Thus, multiple mechanisms seem to be employed to limit the duration of wakefulness.

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MATERIALS AND METHODS

Animals. Drosophila melanogaster adult females of the wild-type strain Canton-S were collected within 24 h after eclosion and kept at 25°C under 12:12-h light-dark cycle conditions either in population vials that housed 50 flies and contained 5 ml of 5% sucrose 1% agar or in individual monitor tubes that housed a single fly and contained 150 μl of 5% sucrose 1% agar for the duration of the experiment. Individual tubes (32 total) were placed in locomotion monitors with a single infrared beam, and population vials were placed in population activity monitors that have three rings of infrared beams located at three positions and infrared beam breaks were recorded (Trikinetics, Waltham, MA).

Behavioral monitoring. Individual flies were acclimated for 4 days before beam break data were collected on *day 5*. Populations of flies were acclimated for 4 days in the monitors before death on *day 5*. Activity data were recorded on *day 4* to determine whether the population behavior of flies in an individual monitor was normal. We established the normative behavior of flies in these monitors by looking at average beam breaks for 1 h from Zeitgeber time (ZT) 11 to ZT 12 during the normal active period and the average beam breaks for 2 h during the consolidated rest period from ZT 14 to ZT 16 (Fig. 1) for all three rings of infrared beams for each population. An initial sample of 30 population vials was evaluated with the Mahalanobis D2 (60) distance metric to identify individual observations of population behavior that were outliers in the multivariate space defined on the basis of six variables (one activity and one rest variable for each ring of the three infrared beams) characterizing rest/activity behavior. When observations follow a multivariate normal distribution, the D2 values are χ^2 distributed with six degrees of freedom. We evaluated the plot of percentiles of the χ^2 against the empirical percentiles of the ordered D2 values. This plot should be a straight line assuming multivariate normality. Outliers were defined as observations with χ^2 percentiles values >0.999 (i.e., $P < 0.001$). The algorithm was designed to remove the largest outlier and then to sequentially reevaluate the reference. Outlier observations ($P < 0.001$) were not used for tissue collection. Flies from populations that were found to be normative were placed on dry ice. Behavioral data from nonoutlier populations were added to the reference with the goal of obtaining at least 100 observations. The final reference sample size was $n = 103$. No additional nonoutlier observations were added to avoid an over homogenous reference.

Sleep deprivation paradigm. Flies were killed at the beginning of the consolidated rest period (ZT 14) for the 0-h control group (Fig. 1). Flies were sleep deprived for 2, 4, or 6 h (killed at ZT 16, ZT 18, or ZT 20, respectively) by tapping on the vial as necessary to keep the flies moving as determined by visual observation of fly behavior under red light conditions. Time-matched controls were maintained in the monitors without intervention until death at ZT 16, ZT 18, and ZT 20. Three biological replicates were collected for all time points and each replicate study was performed from 139–193 pooled brains dissected as described (94).

Control experiment involving mechanical stimulation during the active period. To identify genes whose regulation might be altered by the mechanical stimulation that was used to achieve sleep deprivation, brains were collected from a second set of animals stimulated from ZT 10 to ZT 14, a period in which the animals were predominantly awake. Animals were stimulated at intervals of 60–90 s for 4 h (Fig. 1). The spacing of the mechanical stimulation approximates the stimulation used in the sleep derivation paradigm. A time-matched control group (ZT 14) was also collected. Three biological replicates ($n = 169$–196 brains per replicate) were collected for all time points of the mechanical stimulation experiment dissected as described (94).

RNA isolation. Flies were killed by immersion in boiling water to instantly inactivate RNases and facilitate tissue recovery. Animals were transferred onto absorbent paper and quickly placed at −70°C until dissection. Brains were extracted as described previously (94). Extracted brains were immediately placed into 1 ml of TRIzol solution (Invitrogen, Carlsbad, CA), and total RNA was isolated from the pooled tissue following manufacturer's instructions. A sample was removed, and the quality of the RNA was determined by standard methods (74). The rest of the sample RNA was stored at −70°C until delivery on dry ice to the Microarray Core Facility of the University of Pennsylvania.

Microarray. All protocols were performed by the Microarray Core Facility of the University of Pennsylvania using GeneChip *Drosophila* Genome arrays as described in the Affymetrix GeneChip Expression Analysis Technical Manual (http://www.med.upenn.edu/microarr/). We used 5 μg of total RNA for first-strand cDNA synthesis.

Data normalization. Ten time groups were used in this study, each with three biological replicates. Seven time groups were used to determine differential gene expression between normal and sleep-deprived flies, whereas three time groups were used to test for differential gene expression due to the effect of mechanical stimulation. Probe intensity data from all 30 arrays were read into the R software environment (http://www.R-project.org) directly from .CEL files using the R/*affy* package (32). R/*affy* was also used to extract and manipulate probe level data to assess data quality and to create expression summary measures. Normalization was carried out using the robust multiarray average (RMA) method (40) on all probe intensity data sets together to form one expression measure for each gene on each array. In brief, the RMA method adjusted the background of perfect match (PM) probes, applied a quantile normaliza-

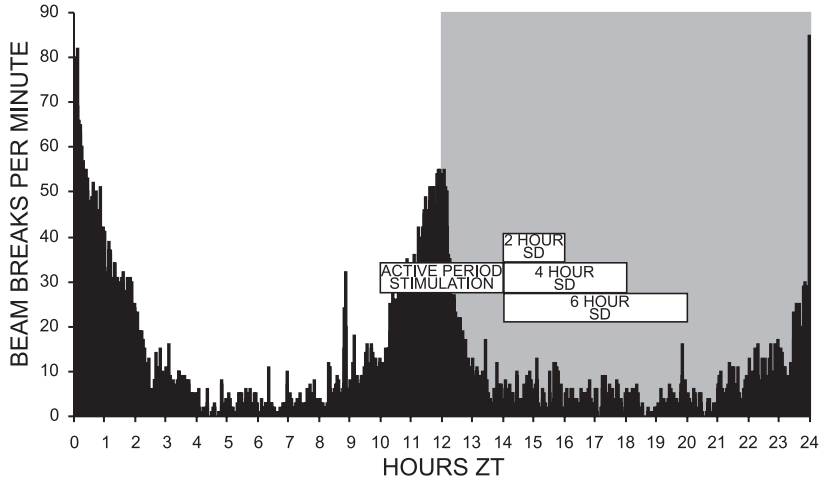

Fig. 1. Experimental design. Rest/activity of a population of flies across the day starting at lights-on at Zeitgeber time (ZT) 0. The *y*-axis is total number of beam crossings at *channel 2* in the middle of the activity monitor. Sleep deprivations of 2-h, 4-h, and 6-h duration (2 HOUR SD, 4 HOUR SD, and 6 HOUR SD, respectively) were performed during the consolidated sleep period beginning at ZT 14. The effect on gene expression of manual stimulation alone was measured in an additional experiment that involved stimulation of the flies during a predominantly active period from ZT 10 to ZT 14 (labeled "Active Period Stimulation").

tion of the corrected PM values, and calculated final expression measures using the median polish algorithm.

Statistical approaches for differential gene expression. Differential gene expression between sleep-deprived and sleeping flies was assessed by gene-specific fixed-effect analysis of variance (ANOVA) models. A similar approach was used to assess the active period stimulation experiment. RMA expression measures (Y_i) were input into R/*maanova* (91) and analyzed using a one-way layout. The gene-specific fixed model used to fit the data was $Y_i = \mu + T_k + \epsilon_i$, where Y_i represents the expression measure of probe set i, μ is the mean for each array, T_k is the fixed effect for a time group k ($k = 1, \ldots, 7$), and ϵ_i captures random error. Since the Affymetrix platform is a one-color system, array and biological error components are absorbed in ϵ_i. *P* values were calculated through permutation analyses in which model residuals were shuffled 1,000 times and *t*-statistics were pooled across genes. All analyses were based on the *F*s statistic, a modified *F* statistic incorporating shrinkage estimates of variance components (19). To address the problem of multiple testing common to microarray analyses, the permutation *P* values were adjusted by the false discovery rate (FDR) method of Storey (82). The resulting "*q* values" estimated the proportion of false positives in lists of differentially expressed genes and were used to generate suitable candidate gene lists used for subsequent pathway analyses.

Statistical approach for trend over time. In addition to detecting differential gene expression between the control and sleep-deprived samples, it was also of interest to detect trends in gene expression across time. This question was addressed through a linear multiple regression analysis with a common intercept (α_0) at the time = 0 group. The model used to fit the residuals after mean centering for each array was $Y_i = \alpha_0 + \beta_1 t_1 + \beta_2 t_2 + \epsilon_i$, where the expected value of the centered expression measures followed a linear trend through time in normal resting or sleep-deprived flies. This multiple regression equation included a common intercept (α_0) and a unique slope that described the progression of gene expression in both the undisturbed (β_1) and sleep-deprived (β_2) flies. Trends over time were evaluated in R/*maanova* by testing the following null hypotheses: *1*) H^0: $\beta_1 = 0$, *2*) H^0: $\beta_2 = 0$, and *3*) H^0: $\beta_1 = \beta_2$. The presence of differential gene expression over time was indicated by whether a slope was significantly different from zero (that is, by testing H^0: $\beta_1 = 0$ or H^0: $\beta_2 = 0$). The direction of change was calculated based on differences in gene-specific expression estimates derived from the ANOVA models. The third null hypothesis (H^0: $\beta_1 = \beta_2$) allowed the assessment of time group effects and served as a confirmation of the results obtained in the first ANOVA analysis. Unadjusted *P* values and *q* values were obtained from permutation-based tests in the same manner as described in the ANOVA analysis for detecting changes in gene expression between normal and sleep-deprived flies described above.

Application of statistical approaches to data analysis. The data from the Affymetrix arrays were processed in a stepwise fashion to derive the identification of the classes of gene expression in this study (Fig. 2). The raw signal data were normalized by the RMA method (40). Differences between the sleep and sleep-deprived groups at 2, 4, and 6 h were analyzed by a one-way ANOVA based on the *F*s statistic (19), *P* values were calculated by permuting model residuals 1,000 times and subsequently adjusted with an FDR algorithm (82), At an FDR of 10%, 578 genes were determined to be differentially expressed between sleep and sleep-deprived groups, and at an FDR of 5%, 252 genes were differentially expressed. We used the more stringent 5% FDR for subsequent analysis (Fig. 2).

In our study of effects of stimulation during the period when flies are predominantly awake, we chose to focus on genes differentially expressed by analyzing only a subset of expression data from this experiment. Only the expression data for the 252 genes identified in the sleep deprivation experiment were used for the determination of the stimulation-responsive genes since this was a secondary analysis (see Fig. 2). Here, we used the ANOVA analysis without correcting for multiple testing due to the small number of tests (252) ($P < 0.01$) and to avoid underestimating the effects of manual stimulation (Fig. 2). Genes whose expression was altered by mechanical stimulation during the predominantly active period were then excluded from the final trends analysis of genes identified as being significantly differentially expressed between sleep deprivation and sleep groups.

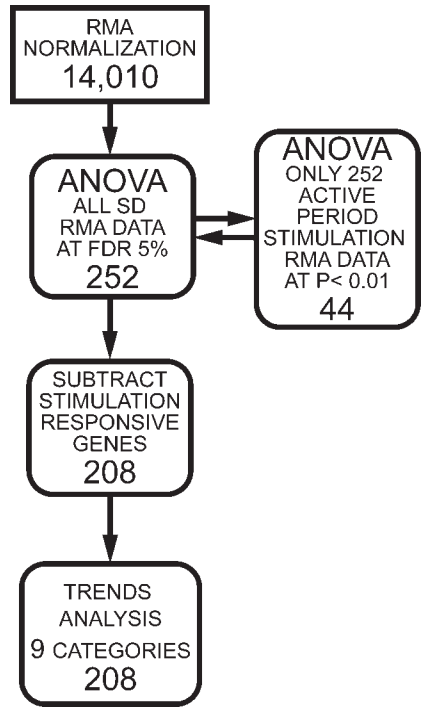

Fig. 2. Flow chart of data analysis. Expression of the 14,010 probes on the Affymetrix array were compared between sleep and sleep deprivation at 2, 4, and 6 h and analyzed using a false discovery rate (FDR) of 5%. For the 252 genes identified in this manner as differentially regulated between sleep and sleep deprivation, the robust multiarray average (RMA) normalized active period stimulation data were analyzed by ANOVA at a $P < 0.01$ significance level to determine genes differentially expressed in this secondary study. The 44 genes found to be differentially expressed where flies were stimulated during the predominantly active period were removed from the 252 differentially expressed data set, and the remaining 208 were then separated into 9 categories of expression using linear regression trend analysis. (For details about 9 categories, see text.)

The microarray data are deposited at the National Center for Biotechnology Information Gene Expression Omnibus database as a data series under the accession number GSE4174.

RESULTS

Behavioral data. Although activity data collected in population monitors can be used to determine whether a population of flies is normative in its behavior (see MATERIALS AND METHODS), such data cannot be used to determine sleep or wakefulness of individual flies. The behavioral data obtained from population monitors are similar at all three levels of beams assessed within the monitor (see Fig. 3). There are, as expected, less beam crossings at the top of the monitor (*channel 3*) than nearer the food (*channel 1*) during the nighttime inactive period. The behavior observed in population monitors is similar to that found when individual flies are monitored. If the beam break data for 50 individual female Canton-S flies of 5 days of age studied in individual behavioral monitors are summed, the resulting data are similar to the data collected from a single channel of the population monitor for 50 flies

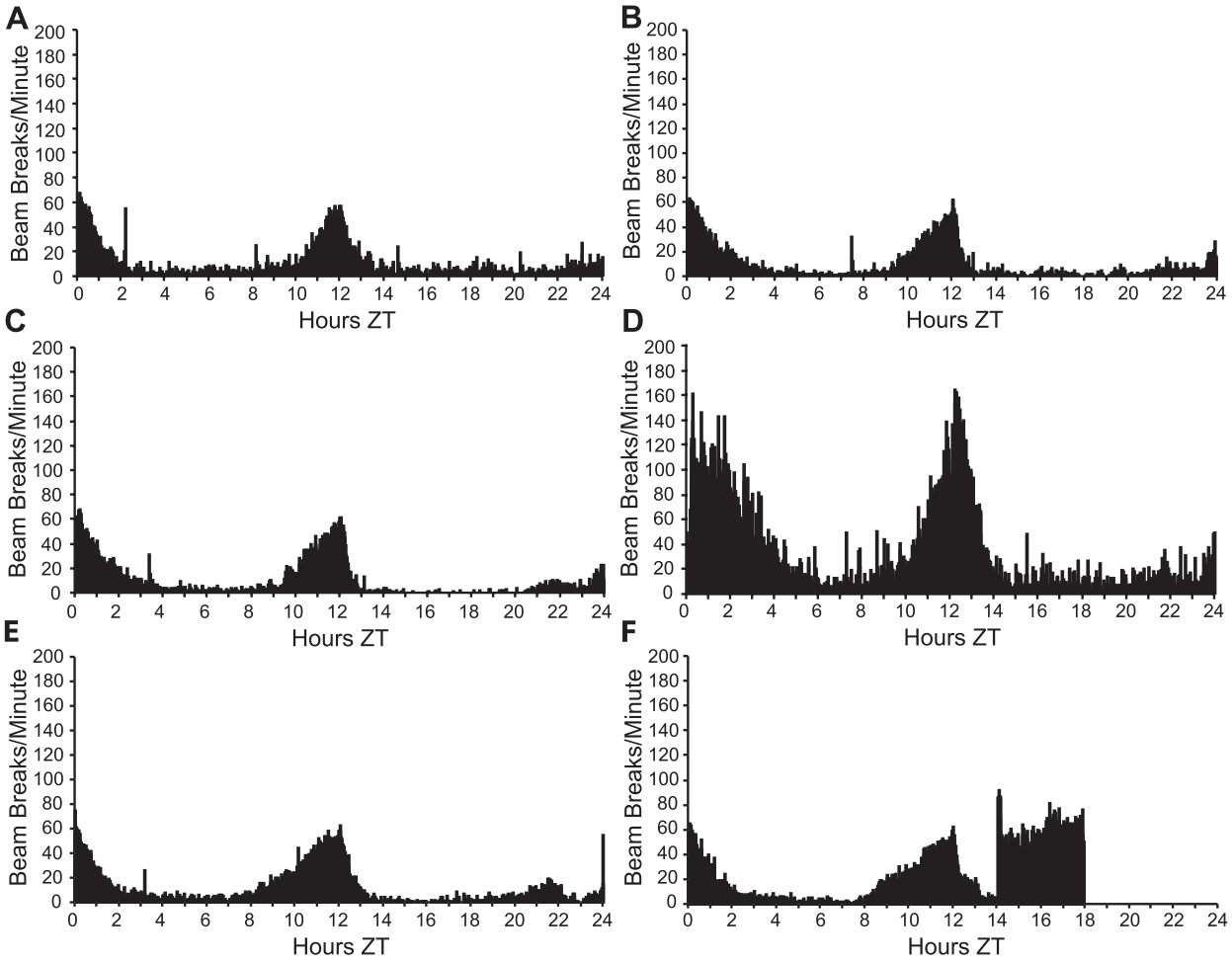

Fig. 3. Beam break activity data from a population monitor of 50 flies and the sum of 50 individual monitors. The beam break activity data of a population of 50 5-day-old female Canton-S flies are shown at 3 positions in the population monitor: *channel 1* is closest to the food at the bottom of the monitor (*A*); *channel 2* is halfway between the surface of the food and the top of the monitor (*B*); *channel 3* is closest to the top of the monitor (*C*). The total of all beam breaks/min summed across data from 50 individual 5-day-old Canton-S female flies monitored in individual behavior monitors (*D*). There were fewer beam breaks/min in the population monitor than when summed across individual flies because in the population monitor when multiple flies cross the beam simultaneously, only one beam break would register. The beam breaks for *channel 2* are shown for a population of the day before sleep deprivation (*E*) and on the day of a 4-h sleep deprivation beginning at ZT 14 until time of death at ZT 18 (*F*).

(Fig. 3). Using beam break data from three sets of 50 individual 5-day-old female Canton-S flies, we can estimate the amount of sleep during the 2, 4, and 6 h of the undisturbed sleep groups, i.e., from ZT 14 to ZT 20. We estimated sleep and wakefulness for 150 flies because this is close to the number of flies used for brain collection for a single biological replication (range $n = 139–193$ brains) and represents three population monitors. The means and SD of the total amount of sleep for the three sets of 50 flies from ZT 14 to ZT 16 were 95 ± 10%, 93 ± 9%, and 93 ± 11%; from ZT 14 to ZT 18 were 91 ± 7%, 91 ± 9%, and 94 ± 10%; and from ZT 14 to ZT 20 were 93 ± 5%, 93 ± 7%, and 94 ± 9%. Thus, during these time periods, the undisturbed flies are predominately asleep, and this is a highly reproducible behavior. In contrast, the 4-h period from ZT 10 to ZT 14 encompasses the longest activity bout for 5-day-old Canton-S females (Figs. 1 and 3). There is not, however, totally consolidated wakefulness during this period. In the three sets of 50 flies studied in individual behavior monitors, the average total wakefulness from ZT 10 to ZT 14 was 64 ± 18%, 63 ± 20%, and 62 ± 19% (means ± SD). Therefore, mechanical stimulation of flies during the interval from ZT 10 to ZT 14 will result in some degree of sleep deprivation albeit much less sleep deprivation than during the period ZT 14 to ZT 20. The stimulated flies, both during the predominantly wake period (ZT 10 to ZT 14) and consolidated sleep period (ZT 14 to ZT 20), were monitored visually in population monitors to ensure that flies did not sleep. Their response to stimulation was confirmed by data from population monitors (Fig. 3, *E* and *F*).

Manual stimulation may trigger an immune response in the brain. At an FDR of 5%, 252 genes were differentially expressed between sleep and sleep deprivation groups. Of the 252 genes differentially expressed between sleep and sleep-deprivation groups, 44 were also differentially expressed between brains of flies that were stimulated and those that were undisturbed from ZT 10 to ZT 14 (Table 1). For 24 of these 44 genes, the change in expression by stimulation from ZT 10–14 was as large as or larger than the change caused by stimulation during sleep, from ZT 14–18 (Table 1). This finding suggests the possibility that differential expression of these 44 genes is

partially or wholly due to the stimulation paradigm used to produce sleep deprivation. Thus, these 44 stimulation-responsive genes were not considered as being differentially expressed between sleep and sleep deprivation. Of the 44 genes, 39 were upregulated during stimulation and the remaining 5 genes were downregulated (Table 1). We show in Fig. 4 examples of changes in expression of one upregulated gene, Cecropin A2, and one downregulated gene, heat shock protein 83 (*Hsp83*). The temporal changes in expression between sleep-deprived and stimulated conditions were similar for the other up- and downregulated genes.

Strikingly, 22 of the 39 genes upregulated by mechanical stimulation between ZT 10 and ZT 14 (Table 2) play a role in the *Drosophila* immune response (9, 22, 23, 41, 66, 79). These 22 genes represent approximately one-fifth of the 103 genes previously shown to be upregulated during the *Drosophila* immune response (22, 23). We show the fold changes from stimulation during ZT 10 to ZT 14 for all 103 immune genes in Fig. 5. Genes detected as being significantly upregulated in our study are indicated. As anticipated, the genes that were detailed as being significantly upregulated had, in general, the largest change. This figure shows that this stimulation affects the expression of some but not all immune genes. The 44 genes we have identified as partially or wholly responding to manual stimulation were removed from subsequent analysis of the remaining 208 differentially expressed genes (see Fig. 2)

Table 1. *The 44 genes differentially expressed during sleep deprivation and stimulation from ZT 10 to ZT 14*

Symbol	FlyBase ID	SD	Stim
Aay	FBgn0023129	1.911	1.374
Akap200	FBgn0027932	−1.234	−1.381
AttA	FBgn0012042	2.589	3.554
AttB	FBgn0033959	3.126	4.048
AttC	FBgn0032835	3.147	4.475
CecA1	FBgn0000276	6.055	9.575
CecA2	FBgn0000277	3.350	6.757
CG10383	FBgn0032699	1.321	1.215
CG11089	FBgn0039241	1.398	1.353
CG11686	FBgn0040551	1.301	1.350
CG13315	FBgn0040827	1.984	1.666
CG13999	FBgn0031629	1.361	1.268
CG15065	FBgn0040734	1.643	1.480
CG1572	FBgn0030309	1.602	1.430
CG16772	FBgn0031973	1.591	1.554
CG16978	FBgn0040972	1.644	2.110
CG17198	FBgn0039366	1.075	1.147
CG1887	FBgn0035290	−1.468	−1.437
CG3244	FBgn0025583	1.331	1.341
CG3348	FBgn0040609	1.231	1.491
CG5791	FBgn0040582	1.767	1.553
CG7219	FBgn0031753	1.200	1.672
CG7272	FBgn0036501	−1.153	−1.151
CG7530	FBgn0038256	1.287	1.205
CG8147	FBgn0037699	−1.426	−1.458
cher	FBgn0014141	1.449	1.496
Dpt	FBgn0003499	3.110	5.252
DptB	FBgn0034407	2.169	3.273
dro5 (CG10812)	FBgn0035434	1.707	1.845
Fs	FBgn0040746	1.176	1.214
h	FBgn0001168	1.297	1.411
Hsp83	FBgn0001233	−2.109	−1.885
IM1	FBgn0034329	2.724	2.356
IM10 (CG18279)	FBgn0033835	1.585	1.864
IM2	FBgn0014865	1.941	2.090
IM23 (CG15066)	FBgn0034328	1.336	1.792
IM3 (CG16844)	FBgn0040736	1.918	1.754
Mtk	FBgn0004240	2.460	2.252
PGRP-LB	FBgn0037906	1.244	1.283
PGRP-SB1	FBgn0036658	1.575	2.249
Rel	FBgn0014018	1.466	1.363
Side	FBgn0032741	1.211	1.186
sr	FBgn0003499	2.813	2.226
Tsp42EI	FBgn0033134	1.324	1.320

Symbol refer to the gene symbol. Flybase ID is the number assigned to the gene by the Flybase curators. SD is the relative fold change of the expression of the gene at 4 h of sleep deprivation vs. 4 h of sleep. Stim is the relative fold change of the expression of the gene after 4 h of mechanical stimulation from Zeitgeber time (ZT) 10 to ZT 14 vs. the undisturbed control animals killed at the same diurnal time.

Gene expression patterns fall into nine temporal categories. A significant difference in RMA values between sleep and sleep-deprived animals' brains as determined by ANOVA can be explained by an increase in one condition, a decrease in the other, both an increase in one and a decrease in the other, or a change in the same direction in both but a greater change in one condition compared with the other. Each of these different explanations would have a different implication regarding the underlying transcriptional regulation of a particular gene.

To differentiate among these possibilities, we analyzed the temporal trend of expression using the data from *time zero* at ZT 14 in addition to the data from 2, 4, and 6 h that was used in the primary analysis to identify differentially expressed genes (Fig. 1). This analysis was only done for genes that were found to be differentially expressed in our primary ANOVA analysis and not to be differentially expressed during the stimulation study in the predominantly wakeful period (n = 208). Trend analysis involves the examination of slopes over time using least-squares linear regression, anchored by a common intercept. We performed a trend analysis separately for sleep deprivation and sleep using the same *time zero* (ZT 14) data as the anchor for both groups. A positive slope indicates that the gene increased expression over time, whereas a negative slope indicates that the gene decreased expression over time. A slope that is not significantly different from zero indicates that although the RMA values at one or more individual time points were significantly different between the two groups as determined in the primary analysis ANOVA, the overall linear trend during the 6 h was neutral.

On the basis of this analysis, we divided the 208 differentially expressed genes into nine categories (Table 3), described in detail in the following paragraphs. At the stringent FDR of 1% for the trend analysis, the majority of genes were assigned to *category 5*, indicating that there was no directionality to either sleep deprivation or sleep conditions. Relaxing the FDR allowed more genes to be assigned to other categories. Because this was a secondary analysis of genes already identified as differentially expressed on the basis of the initial ANOVA using a stringent FDR of 5%, we chose to relax the stringency of our FDR to 20% FDR for trend analysis to minimize the number of genes in the less informative *category 5*.

There is only one gene in each of *categories 1* and *9*, the categories where the temporal change was in the same direction for both the sleep deprivation and sleep groups. These two genes are differentially regulated during sleep deprivation but in addition have a strong circadian response (see Supplemental

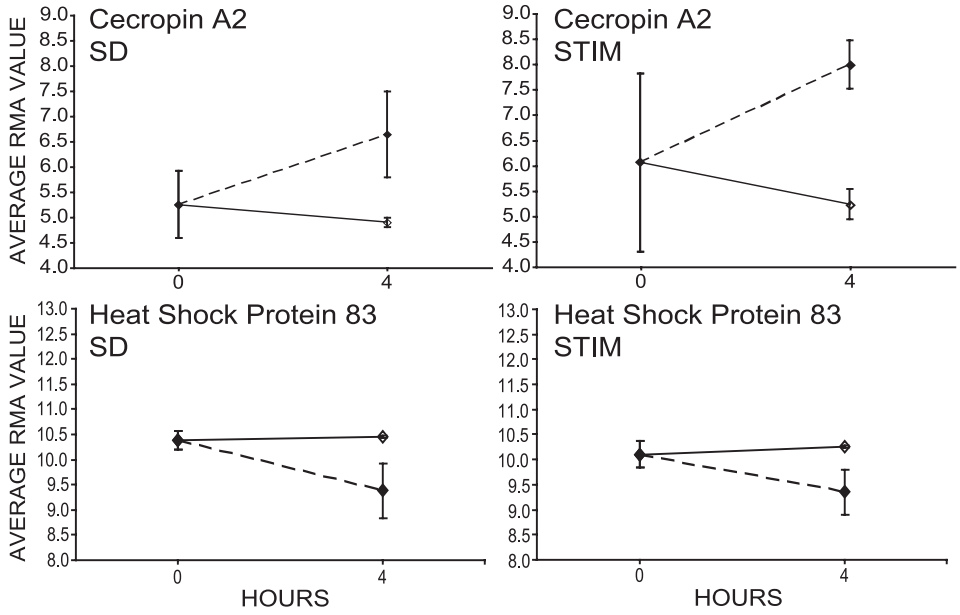

Fig. 4. *Cecropin A2* and *Heat shock protein 83* are regulated as a consequence of stimulation both during the predominantly active period ZT 10–ZT 14 (*right* panels, STIM) and the consolidated sleep period ZT 14–ZT 18 (*left* panels, SD). For all graphs the solid line represents the unperturbed groups and the broken line represents the stimulated groups. Graphs from the sleep deprivation studies (SD, *left* panels) show gene expression when stimulated during sleep, and *hour zero* corresponds to ZT 14. To allow direct comparison between sleep deprivation studies and stimulation from ZT 10 to ZT 14 during the predominantly active period, only the RMA values for 4 h of sleep deprivation are shown. For the stimulation from ZT 10 to ZT 14 (STIM, *right* panels), *hour zero* corresponds to ZT 10. Shown are the average RMA values and error bars representing standard deviations.

Table 1; the online version of this article contains supplemental material).

Gene regulation during sleep deprivation is more complex than during sleep. Genes assigned to *categories 4* and *6* are ones whose expression changes only during sleep deprivation and not during sleep, whereas genes assigned to *categories 2* and *8* are ones whose expression changes only during sleep and not during sleep deprivation (Table 3). The number of genes regulated by sleep deprivation but not sleep, 169, was far greater than the number regulated by sleep but not by sleep deprivation, 12. This finding suggests that gene regulation at the transcriptional level is more complex during sleep deprivation than during sleep.

A large class of genes is downregulated during sleep deprivation. Of the 169 genes whose expression changed during sleep deprivation but not during sleep, 114 genes were downregulated, and 55 were upregulated. Among this large group of downregulated genes, 78 have known or proposed functions.

Table 2. *Immune genes responding to mechanical stimulation from ZT 10 to ZT 14*

Name	Gene Function	ID	Immune Pathway
Fs	immune response, activin inhibitor activity	A	-
Peptidoglycan recognition protein LB	defense response; peptidoglycan recognition activity	B	Imd
Relish	antibacterial polypeptide induction; antifungal polypeptide induction; transcription factor activity	C	Toll
CG15065	Immune response	D	Toll
CG5791	Immune response	E	Toll
CG7219	serine-type endopeptidase inhibitor activity	F	Toll
Immune induced molecule 3	antibacterial humoral response	G	-
Immune induced molecule 23	antibacterial humoral response	H	Toll
drosomysin-5	defense/immunity protein activity	I	Both
Immune induced molecule 10	antibacterial humoral response	J	-
Immune induced protein 2	defense/immunity protein activity	K	Toll
CG16978	Immune response	L	Toll
PGRP-SB1	defense response; peptidoglycan recognition activity	M	Imd
Metchnikowin	gram-positive antibacterial peptide activity; antifungal peptide activity	N	Both
Immune induced protein 1	defense/immunity protein activity	O	Toll
Diptericin B	antibacterial peptide activity	P	Both
Attacin-A	gram-negative antibacterial peptide activity	Q	Both
Attacin-B	antibacterial peptide activity	R	Both
Attacin-C	defense/immunity protein	S	Both
Diptericin	gram-negative antibacterial peptide activity	T	-
Cecropin A2	gram-negative antibacterial peptide activity; gram-positive antibacterial peptide activity; antifungal peptide activity	U	Both
Cecropin A1	gram-negative antibacterial peptide activity; gram-positive antibacterial peptide activity; antifungal peptide activity	V	Both

Immune genes are upregulated during stimulation in both ZT 10–ZT 14 and ZT 14–ZT 18 (4 h SD). The Gene Function assignment is based on gene ontology terms from Ref. 24. ID refers to the label for the specific bar of the gene fold change shown in Fig. 4. Immune Pathway (from Ref. 23) "Toll" and "Imd" indicate regulation by their respective pathways. "Both" indicates regulation by both pathways, and a dash indicates no data.

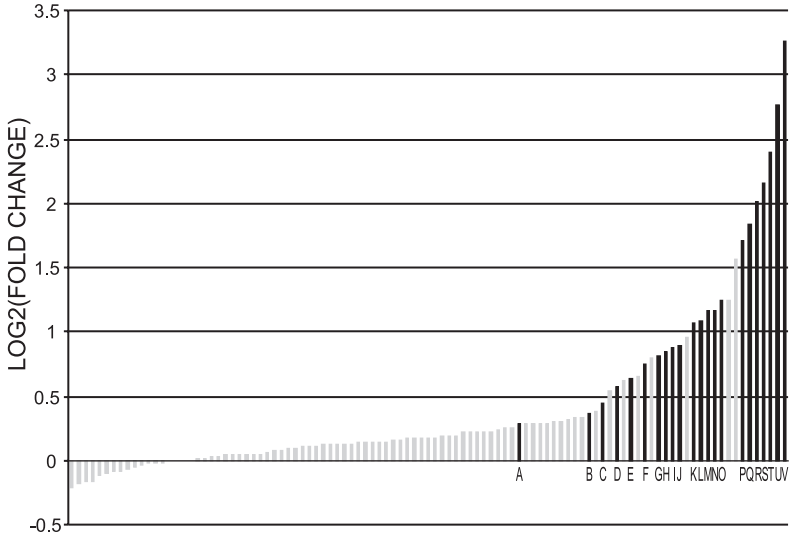

Fig. 5. The majority of immune-responsive genes show an upward trend in expression during stimulation. The log_2 (fold change) of groups stimulated during the predominantly active period relative to undisturbed controls is shown for 103 genes that have previously been shown to be upregulated during immune challenge (22, 23). Log_2 (fold change) for individual genes is indicated by bars. Gray bars indicate genes that were not identified as significant in our analysis. Genes with significant increased expression during both sleep deprivation and control stimulation experiments are indicated by black bars and identified by letter (see names of genes in Table 2).

Among these 78 genes, 47 are involved with different aspects of protein synthesis and degradation (Fig. 6 and Table 4). The genes downregulated are involved in chromatin modification, transcription, RNA processing, translation, aminoacylation of tRNA, protein folding, nuclear pores, and protein ubiquitination. Therefore, reduction in protein synthesis in brain may be a key part of the response to extended wakefulness.

Eight chaperone proteins are downregulated with sleep deprivation; *CG5525*, *CG8258*, *Hsp60*, *CG8531*, *CG8863*, *DnaJ-1*, *Hsp70/Hsp90 organizing protein* (*Hop*), and *Cdc37* (Table 4). These eight genes fall into four groups: J-domain containing *CG8531*, *CG8863,* and *DnaJ-1*; GroEL-like chaperone ATPase-domain containing *Hsp60*, *CG8258,* and *CG5528* (57); *Hop*; and *Cdc37* (20). These chaperones play a role in the folding of newly synthesized proteins (11, 30, 43, 52, 65).

There is downregulation of five genes controlling membrane excitability and calcium homeostasis. The *nicotinic Acetylcholine Receptor alpha 18C* was downregulated during sleep deprivation. In *Drosophila*, cholinergic synapses are limited to the central nervous system (76, 93), and acetylcholine is the main excitatory brain neurotransmitter (48, 73). Also involved in synaptic transmission and downregulated by sleep deprivation is the synaptic vesicle protein *AP-47* (50, 81). Neuropeptide mechanisms of synaptic transmission may also be affected since CG7415, which encodes a protein predicted to process neuropeptides (54), has reduced expression with sleep deprivation.

The downregulation of these genes will likely lead to reduced synaptic transmission and neuronal excitability, which will directly impact the mechanisms that control wakefulness and sleep in the fly. Evidence that the level of neuronal excitability can impact the duration of sleep and wakefulness has been provided by the observation that mutations in the Shaker potassium channels result in reduced sleep in *Drosophila* (14).

We also found the gene *homer* to be downregulated with sleep deprivation. Homer is an adapter protein that connects the group I metabotropic glutamate receptors to inositol triphosphate (IP3) receptors (87), thereby affecting calcium release. In one study in rats the inhibitory isoform homer1a was found to be upregulated during sleep deprivation (84). A second study, again in rats, found that although both homer1a and homerbc isoforms were upregulated during sleep deprivation, homer1a was significantly more upregulated than the homerbc isoform (64). Downregulation of *homer* in the fly or upregulation of homer1a in the rat with sleep deprivation would result in the same outcome, a less effective coupling of

Table 3. *Expression patterns of 208 differentially expressed genes*

Category	SD vs. 0 h	Sleep vs. 0 h	Genes in Category, *n*			
			FDR 1%	FDR 5%	FDR 10%	FDR 20%
1	up	up	0	0	1	1
2	equal	up	0	1	7	7
3	down	up	0	0	1	6
4	up	equal	6	39	50	55
5	equal	equal	174	83	42	13
6	down	equal	27	83	101	114
7	up	down	0	1	1	6
8	equal	down	1	1	4	5
9	down	down	0	0	1	1

The nine possible categories of gene expression were defined by a trend analysis of the SD or sleep group relative to the expression data of that gene at *time zero* (ZT 14). Up indicates that gene expression shows a trend with a significant positive slope, Down indicates a significant negative slope, and equal indicates a nonsignificant slope for that trend. The differentially expressed genes were analyzed using false discovery rate (FDR) ranging from 1 to 20% for the trend analysis.

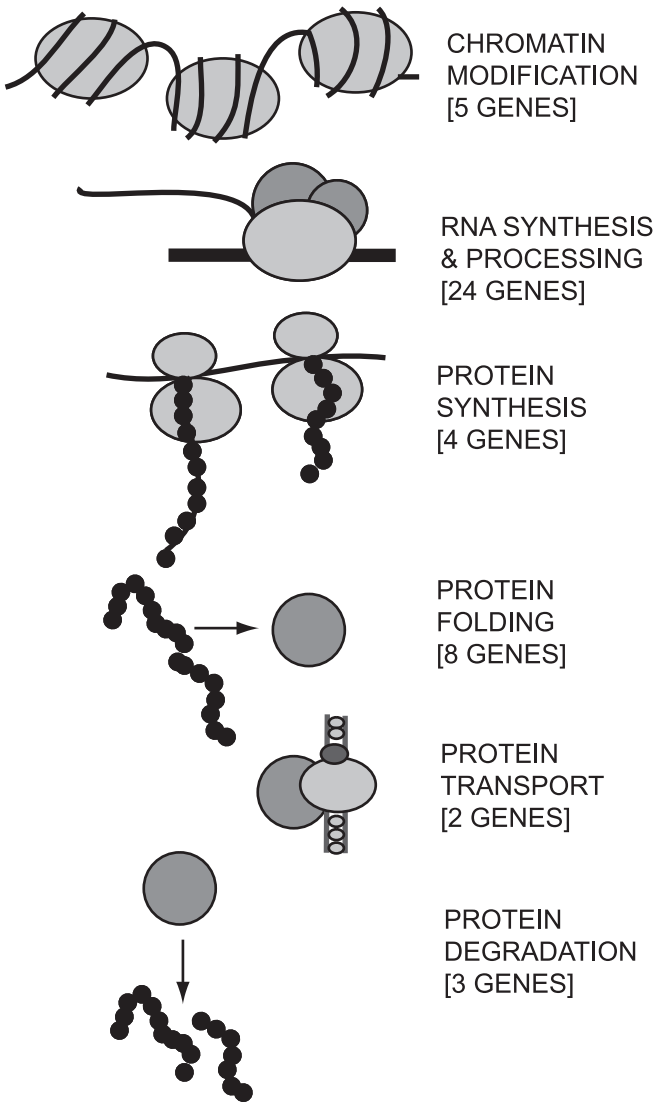

Fig. 6. Genes downregulated with extended wakefulness affect multiple steps in protein metabolism. Genes downregulated in sleep deprivation are involved in 6 separate steps of protein production and subsequent fate. The number of genes is listed in parentheses below each step. The specific genes at each of these steps are given in Table 4.

the metabotropic glutamate receptors to their downstream targets with extended wakefulness and, therefore, reduced calcium release. Another gene involved in regulating calcium levels that is downregulated during sleep deprivation encodes calreticulin. Calreticulin, which resides in the endoplasmic reticulum (ER), has been implicated in IP3 receptor-mediated Ca^{2+} release (reviewed in Ref. 58).

Genes regulated by sleep can be identified. Genes assigned to *categories 2, 3, 7,* and *8* in Table 3 are ones whose expression changes during sleep. In *categories 2* and *3* (see list in Table 5), gene expression increases during sleep and either does not change (*category 2*) or decreases (*category 3*) during sleep deprivation. In *categories 7* and *8* (see list in Table 6), gene expression decreases during sleep and either does not change (*category 8*) or increases (*category 7*) during sleep deprivation.

A sleep gene has been shown to be part of the circadian clock. Upregulated in sleep is the gene encoding the casein kinase II beta subunit (CKIIβ) (Table 5), which has been shown to be necessary for the translocation of the circadian

Table 4. *Genes in several steps of protein metabolism are downregulated during SD*

Chromatin modification	
Chd1	chromatin binding; helicase activity
Df31	chromatin
brm	chromatin-mediated maintenance of transcription
Top1	DNA topoisomerase type 1 activity
CG6121	histone acetyltransferase activity
RNA synthesis and processing	
Sry-delta	DNA-dependent; transcriptional activator activity
RpII140	DNA-directed RNA polymerase II, core complex
Ssl1	general RNA polymerase II transcription factor activity; transcription factor TFIIH complex
sqd	mRNA catabolism, nonsense-mediated; mRNA localization, intracellular; mRNA-nucleus export
CG6015	mRNA splicing; pre-mRNA splicing factor activity; spliceosome complex
CG1646	mRNA splicing; small nuclear ribonucleoprotein complex; snRNP U1
CycK	nuclear cyclin-dependent protein kinase holoenzyme complex
CG31388	nucleic acid binding; nucleus
d4	nucleus; regulation of transcription; transcription factor activity
sage	nucleus; regulation of transcription; transcription factor activity
zf30C	nucleus; transcription factor activity
mub	poly(rC) binding
her	positive regulation of transcription from Pol II promoter
lola	regulation of transcription from Pol II promoter
elav	RNA binding
CG6995	RNA binding
CG7879	RNA binding
CG9373	RNA binding
Hrb98DE	RNA binding; heterogeneous nuclear ribonucleoprotein complex
nonA-I	RNA binding; pre-mRNA splicing factor activity; spliceosome complex
rin	RNA binding; RAS protein signal transduction; SH3-domain binding; cytosol
CG10077	RNA helicase activity
mbf1	transcription coactivator activity; methyl-CpG binding
Meics	transcription factor activity
Protein synthesis	
Aats-gly	glycine-tRNA ligase activity
Aats-lys	lysine-tRNA ligase activity
EfTuM	translation elongation factor activity; mitochondrion
Rbp2	translation initiation factor activity; RNA binding; cytosol
Protein folding	
CG8531	chaperone activity
CG8863	chaperone activity
Cdc37	chaperone activity; co-chaperone activity
DnaJ-1	chaperone activity; cytoplasm; heat shock protein activity
CG5525	chaperonin ATPase activity; chaperonin-containing T-complex
CG8258	chaperonin ATPase activity; chaperonin-containing T-complex
Hsp60	de novo' protein folding; mitochondrial matrix
Hop	Hsp70/Hsp90 organizing protein activity; chaperone activity
Protein transport	
Mtor	nuclear pore; nucleus
Nup358	RAN protein binding; nuclear pore
Protein degradation	
CG7656	ubiquitin conjugating enzyme activity
UbcD4	ubiquitin conjugating enzyme activity; ubiquitin cycle
CG9153	ubiquitin-protein ligase activity

The general categories are from Fig. 6. The gene symbol is in the left column, and the specific function is listed in the right column.

Table 5. *Genes upregulated by sleep*

Category	Gene	Gene Function
2	bubblegum	long-chain-fatty-acid-CoA ligase activity
2	casein kinase II beta subunit	protein kinase CK2 activity; circadian rhythm; eclosion rhythm; mushroom body development
2	esterase-6	carboxylesterase activity; courtship behavior; pheromone biosynthesis; regulation of female receptivity, post-mating
3	found in neurons	RNA binding
3	minidiscs	amino acid transporter activity
3	retinoid- and fatty-acid binding protein	fatty acid binding; microtubule binding; retinoid binding; transport
3	RhoGAP102A	Rho GTPase activator activity
2	CG5618	sulfinoalanine decarboxylase activity
3	CG9220	acetylglucosaminyltransferase activity; chondroitin sulfate biosynthesis
3	CG31638	myosin
2	CG32666	protein amino acid phosphorylation; protein serine/threonine kinase activity
2	CG11887	unknown
2	CG18542	unknown

The category column lists the gene expression pattern category determined by the linear regression trend analysis. Gene lists the gene name. The gene function assignment is based on gene ontology terms from Ref. 24.

clock proteins PER and TIM to the nucleus (1, 63). The transcriptional repression activity of PER within the nucleus may also be dependent on phosphorylation by CKIIβ (63). Protein levels for CKIIβ increase from ZT 12 to ZT 24, and the PER and TIM proteins become primarily nuclear at ZT 22 and ZT 23, respectively (1). In a CKIIβ mutant this entry into the nucleus is delayed by 2 h (1). Although PER and TIM are only two of many substrates for this enzyme (56), the differential regulation of this gene between sleep and sleep deprivation demonstrates a novel mechanism to potentially connect the homeostatic and circadian regulatory processes for sleep.

Genes regulated by sleep affect cytoskeleton structure and function. Three genes regulated by sleep have an effect on actin organization. RhoGAP102A and CG32666 are upregulated, whereas PDGF- and VEGF-receptor related (*Pvr*) is downregulated with sleep (Tables 5 and 6). The human homolog of *RhoGAP102A* inactivates the RhoA isoform involved with cytoskeleton reorganization and in addition affects actin independently of RhoA (67). *CG32666* encodes a structural component of the cytoskeleton (57) and affects cell morphology (44). *Pvr* induces actin polymerization, which leads to changes in cell morphology through activation of the jun kinase (JNK) pathway (42). The JNK pathway also has a role in a mammalian stress response that is conserved in *Drosophila* (reviewed in Ref. 33). Therefore, the pattern of temporal regulation we have observed for *Pvr*, downregulation in sleep and upregulation in sleep deprivation, may be explained by a stress response. There is no significant change in gene expression of *pvr* when flies are stimulated during the predominantly wake period. The finding that actin reorganization is directed by different genes during sleep and wake in *Drosophila* is consistent with microarray experimental results obtained in the rat (15). These results are also compatible with previous studies of neuronal activation demonstrating morphological changes in axonal processes (45, 86) and postulated synaptic downscaling occurring during sleep (85).

Genes involved in membrane recycling and lipid synthesis are upregulated during sleep. Another gene upregulated during sleep may be involved in membrane recycling; *CG1887* has sequence homology to *CD36,* a member of the class B receptor scavenger proteins (57). Mammalian class B receptor scavenger proteins are expressed in the brain by astrocytes (39) and may contribute to an increase in trafficking of lipids necessary for membrane reorganization (71). Other genes involved in lipid metabolism were also found to be upregulated during sleep, i.e., *retinoid- and fatty acid-binding protein* (25) and *bubblegum* (59, 80). Recycling of membranes has been previously proposed to be one of the functions of sleep in mammals (15) and *Drosophila* (16).

Genes with a role in nervous system development are upregulated during sleep. Three of the genes upregulated during sleep have been shown to be involved in *Drosophila* nervous system development (Table 5). *Found in neurons* is a member of an RNA binding protein family and is expressed in neurons

Table 6. *Genes downregulated by sleep*

Category	Gene	Gene Function
7	Cyp4ac3	cytochrome P450 activity; membrane; microsome
7	PDGF- and VEGF-receptor related	actin cytoskeleton organization and biogenesis
7	Tetraspanin 42Ed	integral to membrane
8	CG3066	monophenol monooxygenase activator activity; proteolysis and peptidolysis; serine carboxypeptidase activity; trypsin activity
8	CG7675	oxidoreductase activity, acting on CH-OH group of donors
7	Obp56d	unknown
7	CG11293	unknown
7	CG30069	unknown
8	CG16926	unknown
8	CG9338	unknown

The category column lists the gene expression pattern category determined by the linear regression trend analysis. Gene lists the gene name. The gene function assignment is based on gene ontology terms from Ref. 24.

during embryogenesis (75). *CG9220* is an enzyme involved in the biosynthesis of chondroitin sulfate (90), an extracellular matrix molecule that promotes neurite outgrowth (29). The gene *minidisks* is necessary for imaginal disc and central nervous system development (53). The function of these three genes in the adult nervous system is largely unknown, but gene products that promote axon growth and other nervous system developmental processes are hypothesized to also be used in the synaptic homeostasis of mature neurons (51).

Genes previously identified as being regulated by the circadian clock are differentially expressed between sleep and wakefulness. Microarray experiments to determine circadian regulation of gene expression in *Drosophila* have been performed with whole heads (13, 18, 55, 89). We found that 14 genes (Supplemental Table 2), which had previously been identified as regulated in a circadian fashion (13, 18, 55, 89), were differentially expressed between sleep and wakefulness. This observation, also made by others (15, 16), suggests that behavioral state rather than clock function is the main variable regulating transcription of these genes. In future studies, the conclusion that a diurnal or a circadian clock controls a gene found to be differentially expressed across the day must be made with caution, since the behavioral state of the animal (sleep/wake) rather than clock function may be playing a role in the gene's regulation.

Other studies to identify circadian gene regulation made use of the *Drosophila clock* (*clk*) mutant, in which circadian regulation of behavior is disrupted (13, 18, 55, 89). Among the 267 genes identified as differentially expressed between *clk* mutants and wild-type flies, 33 were differentially regulated between sleep and sleep deprivation in our analysis (see Supplemental Table 2). The alterations in gene expression in clk^{Jrk} mutants could be caused by an altered sleep/wake pattern, by the removal of a key circadian regulator, or by both. Of these 33 genes, 13 are upregulated when flies are stimulated during their predominantly wakeful period, suggesting that clk^{Jrk} flies are under stress, possibly due to a lack of consolidated sleep.

Our trend analysis was used to identify temporal gene expression patterns in the genes already identified as being differentially expressed by ANOVA. Genes with circadian regulation but without behavioral state regulation would be excluded from this initial analysis. As a supplemental analysis we also did the temporal trend analysis for all 14,010 array elements. We identified 25 genes that increase and 28 genes that decrease expression over time in both sleep and sleep-deprivation groups (Supplemental Table 3). Of these 53 genes, 14 have been previously identified as circadian genes based on microarray studies from whole heads (Supplemental Table 3). We did not expect to find the same number of genes as previous studies of circadian variation, since we are only looking at a 6-h time window and our statistical analysis was not designed to detect circadian oscillation of gene expression. However, trends analysis did identify a component of the molecular clock of *Drosophila*, *vrille* (21), as a *category 9* gene, indicating that this gene was downregulated in both sleep deprivation and sleep, a finding that is consistent with previous studies (5, 16). Also identified as a *category 1* gene was the glucuronosyltransferase *Ugt35b,* which has been found to be a circadian regulated gene in a number of studies (13, 18, 49, 55).

DISCUSSION

A current model, the two-process model (7, 8), maintains that mechanistically distinct circadian and sleep homeostatic processes interact to regulate sleep. Although sleep homeostasis is a fundamental process common to many organisms (reviewed in Ref. 12), the molecular processes underlying this process are unknown. *Drosophila* has been recently shown to be a good model organism for sleep research (14, 16, 35, 36, 77, 78, 94). Therefore, we chose to determine gene regulation during sleep deprivation in the brain of *Drosophila*. At a 5% FDR, 252 genes were found to be significantly differentially expressed between sleep and sleep-deprivation groups ($n = 9$ for each). Of these differentially expressed genes, 44 were found to also be significantly different between flies mechanically stimulated during the predominantly wakeful period from ZT 10 to ZT 14 and undisturbed controls. Half of the genes differentially expressed during stimulation between ZT 10 and ZT 14 have been shown to have a role in the *Drosophila* immune response (9, 22, 23, 41, 66, 79).

In our study we chose to use population monitors to assess behavior rather than monitoring behavior of individual flies. Our study design called for investigating changes in gene expression in the fly brain rather than whole head; the latter has been done in other microarray studies studying circadian (13, 18, 49, 55, 89) or sleep (16) effects. The organ of interest is, however, is the brain. One cannot directly extrapolate results from studies of the whole head to brain since the head contains not only the brain but also eyes, muscles, etc. Studies of glycogen reveal differences with respect to sleep/wake effects when the whole head is studied compared with the brain (94). The decision to study the brain necessitated pooling brains from 139–193 flies to obtain sufficient RNA for one expression profile. Population monitors are an ideal way to do this and have the advantage that all flies in a pool are killed at the same moment. Obtaining flies from the large number of individual monitors that would have been required would have resulted in variable time of death, adding an additional source of variability. We further took the step of ensuring that behavior of flies in a given population monitor conformed to previously established norms. The approach of using population monitors is feasible since sleep/wake behavior is stereotypical in a given genotype of *Drosophila*, at a given age and sex. All of our studies were done using 5-day-old females of the Canton-S wild-type strain. These flies have very consolidated sleep between ZT 14 and ZT 20, the primary period of study. Moreover, summing behavioral data from 50 flies in individual monitors reproduces the data obtained in a population monitor (see Fig. 3). Thus there is no doubt that for our primary analysis there is a major difference in prior behavior between flies that were sleep deprived and those allowed to sleep and killed at the same diurnal time (see Fig. 3, *E* and *F*). By comparing expression between groups killed at the same diurnal time, our study design controlled for any circadian effect on gene expression.

Because we chose to kill a normally behaving population of animals at a particular time point rather than in response to the sleep history of individual flies, it was important to perform the sleep deprivation during a period of consolidated sleep. By analyzing the sleep patterns of individual flies ($n = 150$), we found that in the period of ZT 14 to ZT 20 the flies spent an

average of 93 ± 7% (mean ± SD) of the time asleep. We then made the assumption that a population of flies in a vial would sleep to the same extent and chose to kill animals at ZT 16, ZT 18, and ZT 20. While this assumption cannot be formally tested, it is supported by the fact that the overall activity pattern of a population of flies in a vial is very similar to the summed activity of 50 individual flies of the same age, strain, and sex (Fig. 3) and reproducible across groups of flies.

We also performed an ancillary study that is not typically done in this area of investigation to address the possibility that the changes we saw with sleep deprivation might, at least in part, be due to the mechanical stimuli we employed. We therefore used mechanical stimulation during the period from ZT 10 to ZT 14 when flies are much more active. On the basis of the pooled data of individual fly behavior during this time, this is not a perfect control, since even during this active period flies are asleep for on average 37 ± 19% of the time. Thus, flies mechanically stimulated during this period will also be sleep deprived but to a lesser extent. This is similar to the yoked-control paradigm used in rodents where the disc is moved whenever the experimental animal falls asleep, thereby awakening both the experimental animal and the yoked control animal, if the latter happens to be sleeping at this time (4). Thus, as in our study, yoked control animals are partially sleep deprived. We performed this aspect of our study as a secondary study to determine whether genes identified in the major analyses might be affected by the stimulus used to keep flies awake. For the majority of genes we identified, this was not the case. This supports the conclusion that their differential expression is due to loss of sleep.

There were, however, some genes, particularly immune response genes, which were also differentially, expressed when mechanical stimulation was applied between ZT 10 and ZT 14. There are two potential explanations for this finding. First, such genes are particularly sensitive to sleep deprivation, having their expression altered even by lesser amounts of deprivation. In support of this explanation is the finding that genes involved in an immune response are regulated by wakefulness (16). An alternative explanation is that the change in expression of these genes is largely due to the mechanical stimulation used to keep flies awake. Our data support the latter explanation, since the degree of change in expression is either the same or greater (see Table 1) as a consequence of stimulation during the period when they are more active (ZT 10 to ZT 14) compared with that during their consolidated rest period (ZT 14 to ZT 18). Thus, we question whether sleep deprivation in flies increases expression of immune response genes in brain.

In a previous study of the effect of sleep deprivation on gene expression in *Drosophila* heads, RNA expression after 8 h of sleep deprivation or after spontaneous wakefulness was compared with RNA expression in sleeping flies; only changes >1.5-fold were considered significant (16). The difference in the specific results between our two studies might be partially explained by the different analysis methods used in the two studies. We used an FDR algorithm and therefore identified many genes with <1.5-fold change as significant. The different results might also be explained by the tissue source: we used only brains, whereas in the prior study whole heads were used.

In our main analysis comparing expression of genes between sleep-deprived and sleeping flies at ZT 16, ZT 18, and ZT 20, we initially collapsed data across all time points and did a one-way ANOVA analysis with permutation (82). We used a stringent FDR of 5% to determine genes that were differentially expressed. This strategy maximized the power of our study since the primary question is whether there is a group difference, i.e., sleep group compared with sleep deprived. Such an analysis would not be expected to detect genes whose expression is altered only by circadian influence and not by behavioral state, since expression of such genes would change equally in both the sleep and sleep-deprived groups. In support of this, we did not find any of the canonical clock genes in the differentially expressed group. While time point information was not used in the first step of the analysis, we used this in the next step of our analysis to determine temporal trends for genes that were found to be differentially expressed in the main analysis. Thus we used data at all time points separately as well as data at the additional time zero point (ZT 14). For our temporal trend analysis, we used linear regression. Our goal was not to evaluate specific slopes nor determine whether a linear fit was the best fit to our data. Rather, we were simply interested in defining direction of change within the sleep and sleep-deprived groups separately (i.e., did expression of a specific gene go up or down or stay the same within each group). Given our goal, we used a much more relaxed FDR (20%) to determine whether the linear slopes for a particular gene in the sleep and sleep-deprived groups separately are different from zero.

Stimulation may induce an immune response in brain. Unlike mammals, which have both innate and adaptive immunity, *Drosophila* is believed to have only an innate immune system (reviewed in Ref. 10). Gram-positive bacterial and fungal infections induce the Toll pathway in *Drosophila* immune response (10), whereas gram-negative bacterial infections induce the Imd pathway (reviewed in Ref. 88). Downstream targets of Toll and Imd activation have been determined (9, 22, 23, 41, 79). Manual stimulation during ZT 10–ZT 14 induces genes in brain regulated by both pathways (Table 2). As discussed above, this induction may represent a response to the stress of the deprivation paradigm or to the small degree of sleep deprivation during this period. In mammals, methods to induce deprivation of sleep can themselves induce neuronal stress responses, with different methods of deprivation producing different degrees of response (31). Our results show that in flies, too, there may be a brain stress response and that this may be mediated, at least in part, by transcriptional activation of immune-response genes. Stress response in *Drosophila* has been previously little studied. The *turandot* (*tot*) genes are upregulated in whole flies with the stressors UV exposure, heat shock, paraquat, and bacterial infection (27, 28). The *tot* genes found on the *Drosophila* 1.0 Affymetrix GeneChip used in our experiments were not upregulated by stimulation, suggesting that either there is more than one type of transcriptional stress response or that the brain is not a site for the response observed in whole body regulation.

Of the 44 genes responding to mechanical stimulation during the predominantly active period, only five were downregulated (Table 1). One of these downregulated genes encodes *Hsp83* (Fig. 4). The degree of reduction in *Hsp83* in response to ZT 10–14 stimulation was slightly less (−1.9-fold) than the degree of change in response to sleep deprivation (−2.1). As in the case of the immune genes, this expression change of *Hsp83* may either be caused by the mechanical stimulation itself or be

an early response to even a limited amount of sleep deprivation. This reduction in HSP83 brain expression was surprising given prior reports of a 1.3-fold increase in HSP83 transcripts in whole heads after 3 h of sleep deprivation (78). Since whole heads contain the eyes, head muscles, and other tissues, the different results underscore the importance of examining gene expression specifically in the brain, the key organ involved in sleep regulation. That the brain regulates transcription in a manner different from the general regulation observed in the whole head has precedence. We have previously observed a marked difference in the regulation of glycogen stores between whole head and brain during sleep deprivation (94).

Multiple mechanisms limit prolonged wakefulness. The experimental design we employed allows detection of the direction of gene change relative to an anchor point. We chose the start of the consolidated rest period, ZT 14, in the Canton-S strain of *Drosophila* as the anchor point. Using linear trends analysis the gene expression patterns fall into nine categories. The largest category of expression is no change with sleep and downregulation during sleep deprivation (*category 6*). A significant portion of these genes downregulated with sleep deprivation are involved in protein metabolism.

Therefore, our data indicate that protein production, an energy-consuming process (72), should be significantly reduced by sleep deprivation. Also, neuronal excitability and intracellular calcium release, which also pose an energetic challenge to cells, should be reduced by extended wakefulness. These data support the view that extended wakefulness is a state in which the brain in energetically challenged. During wakefulness there is increased neuronal firing with a concomitant requirement for ATP (47). The reduction in three genes, CG7656, UbcD4, and CG9153, involved in protein degradation can be explained either by additional attempts by brain cells to limit ATP consumption, which is required for protein degradation (reviewed in Ref. 37), or by cellular efforts to limit protein turnover in a situation when little new protein is produced. These results in the fly brain are consistent with prior studies in the rat brain that showed a pattern of gene expression changes that would promote increased protein synthesis during sleep compared with wakefulness (15) and are compatible with earlier findings of increased cerebral protein synthesis during sleep compared with wakefulness in mammals (62, 68).

In mammalian studies 6 h of sleep deprivation leads to an increase in six ER and mitochondrial heat shock proteins (83, 84). One of these, Grp78/BiP, has been found to be upregulated during sleep deprivation in a number of organisms (15, 17, 61, 77, 83, 84), including in *Drosophila* heads (77, 78). This is an indication of the unfolded protein response. In mice after 6 h of sleep deprivation, all the markers of the unfolded protein response are present in cerebral cortex (61). In our study we did not find BiP or other markers of ER stress upregulated in the *Drosophila* brain with sleep deprivation. This could be the result of low levels of expression in brain compared with whole head. Our study design allowed us to estimate the false positive but not the false negative rate (82). Thus, our failure to find increased expression of Grp78/BiP may be a false negative result.

Our study did find eight chaperone proteins that are downregulated with sleep deprivation (Table 4). Such chaperones have a very different function from that of the ER and mitochondria-associated heat shock proteins that are upregulated with sleep deprivation in mammals. The chaperone genes that we found to be downregulated with sleep deprivation are all part of the "de novo" protein folding machinery (11, 43, 65). The GroEL domain containing proteins use energy from ATP to fold native proteins (11) and the J-domain containing family co-chaperones with Hsp70 facilitating the transfer of energy from ATP (43). Therefore, the downregulation of these genes would lead to reduction in energy expenditure. As the chaperone genes that are downregulated with sleep deprivation are involved in de novo protein folding primarily (11, 43, 65), our data are not contradictory to the previously observed upregulation of chaperone proteins, which have proven roles in dealing with misfolded or aggregated proteins (46, 92).

The finding that multiple genes involved with energy requiring processes are downregulated during sleep deprivation is complemented by the finding of an upregulation of three genes involved with release of energy stores from fat via β-oxidation. Two lipases, CG5966 and CG6113, and one fatty acid CoA ligase, CG12512, are upregulated during sleep deprivation. Interestingly, CG6113, a lysosomal lipase, as well as Bzd, a gene that encodes a zinc finger protein with unknown function, were both previously shown to increase in expression in whole animals in response to starvation in *Drosophila* (95). This finding suggests an overlap in the physiological consequences of sleep deprivation and starvation and supports the idea that brain energy stores are being depleted during prolonged wakefulness. We should note that many other starvation-responsive genes identified by Zinke et al. (95) were not identified in our study, but they studied whole body while we specifically studied brain gene expression.

Multiple mechanisms are likely to limit wakefulness and hence promote sleep. Taken together, our data suggest that extended wakefulness is a time of increased energy expenditure in the brain and that multiple mechanisms are employed to limit this energy expenditure. These multiple mechanisms will likely also act to limit the duration of wakefulness. The search for molecular markers of process S, the homeostatic drive for sleep (7), have traditionally focused on chemicals that increase in concentration during prolonged wakefulness to promote sleep and that subsequently decrease in concentration during sleep. Our data suggest an alternative explanation for the homeostatic regulation of sleep and wakefulness. During wakefulness, there is a reduction in multiple cellular processes that likely act together to limit the duration of wakefulness. Therefore, a decrease rather than an increase in a chemical may be a more likely consequence of extended wakefulness. A main mechanism for regulating sleep and wakefulness may be a progressive decline in a process that promotes wakefulness. That this process is genetically controlled is supported by observations that reductions in activity of the transcription factor cAMP response element binding protein leads to reduced wakefulness in both the fly (36) and mouse (34). Given the multiple mechanisms that appear to be employed to limit wakefulness in the fly brain, it is doubtful that a single chemical will be identified as the regulator of this process. This molecular redundancy serves to insure that sleep is not bypassed.

In conclusion, we have shown that the method of sleep deprivation may induce significant changes in gene expression, in particular immune response genes, which may reflect a

central nervous system stress response. Among genes differentially regulated between sleep and wakefulness, the largest class of genes is that of genes downregulated by sleep deprivation. The genes downregulated during extended wakefulness encode for proteins that control multiple steps in protein metabolism and affect calcium homeostasis and neuronal excitability. Thus, multiple mechanisms seem to be employed to limit the duration of wakefulness.

GRANT

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