Macromolecule biosynthesis: a key function of sleep

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¹Center for Sleep and Respiratory Neurobiology, ²Division of Sleep Medicine/Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania; ³The Jackson Laboratory, Bar Harbor, Maine; ⁴University of Pennsylvania Microarray Facility, and ⁵The Wharton School, University of Pennsylvania, Philadelphia, Pennsylvania

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Mackiewicz M, Shockley KR, Romer MA, Galante RJ, Zimmerman JE, Naidoo N, Baldwin DA, Jensen ST, Churchill GA, Pack AI. Macromolecule biosynthesis: a key function of sleep. *Physiol Genomics* 31: 441–457, 2007. First published August 14, 2007; doi:10.1152/physiolgenomics.00275.2006.—The function(s) of sleep remains a major unanswered question in biology. We assessed changes in gene expression in the mouse cerebral cortex and hypothalamus following different durations of sleep and periods of sleep deprivation. There were significant differences in gene expression between behavioral states; we identified 3,988 genes in the cerebral cortex and 823 genes in the hypothalamus with altered expression patterns between sleep and sleep deprivation. Changes in the steady-state level of transcripts for various genes are remarkably common during sleep, as 2,090 genes in the cerebral cortex and 409 genes in the hypothalamus were defined as sleep specific and changed (increased or decreased) their expression during sleep. The largest categories of overrepresented genes increasing expression with sleep were those involved in biosynthesis and transport. In both the cerebral cortex and hypothalamus, during sleep there was upregulation of multiple genes encoding various enzymes involved in cholesterol synthesis, as well as proteins for lipid transport. There was also upregulation during sleep of genes involved in synthesis of proteins, heme, and maintenance of vesicle pools, as well as antioxidant enzymes and genes encoding proteins of energy-regulating pathways. We postulate that during sleep there is a rebuilding of multiple key cellular components in preparation for subsequent wakefulness.

gene expression; microarray; cholesterol; protein synthesis

SLEEP IS A REGULARLY OCCURRING, stereotypical, and reversible behavior that is disengaged from and unresponsive to the environment (9). Although unambiguous in its everyday meaning, sleep is an intricate physiological and behavioral process. There are two fundamental processes that underlie the control of sleep: 1) a homeostatic process that determines drive to sleep in relationship to the duration of prior wakefulness and 2) a circadian process that defines rhythmic periods of low and high sleep propensity across the 24-h day (5). Implicit in current models of the control of sleep and wakefulness is the idea that sleep provides key restorative function(s) whose nature is currently unknown. This question is amenable to study by microarrays.

Microarray-based expression is widely used to quantify gene expression levels on a high-throughput basis (74). Genomic approaches have been successfully applied to the study of the

spatial and temporal patterns of gene expression in complex biological structures such as the central nervous system (39, 47). Microarray studies have identified numerous genes with broad physiological functions whose transcripts exhibit diurnal or circadian rhythmicity in the brain and peripheral tissue (1, 10, 38, 45, 57). Microarray studies have also been applied to the study of sleep, primarily comparing gene expression in wakefulness to that during sleep, treating them as binary states (12, 78). There is, however, a distinct temporal dimension to these processes. As wakefulness is prolonged, there is a corresponding increase in the drive for sleep, while with increasing sleep there is a progressive restoration of function. We, therefore, sought to identify dynamic changes in gene expression associated with different durations of sleep and sleep deprivation. We proposed that determining genes which change expression during sleep can point to a possible function(s) of sleep. Our study design allowed us to determine not only which genes change expression, but also the direction of change, i.e., the increase or decrease during sleep, or increase or decrease during sleep deprivation.

We, therefore, report the results from a microarray study on temporal changes in gene expression during spontaneous sleep and sleep deprivation in the mouse cerebral cortex, a neuronal target for processes that control sleep, and the hypothalamus, an important site of sleep regulatory processes (55, 63). We determined these changes by comparing expression in sleeping animals killed at different times during the lights-on period to that in animals sleep deprived and killed at the same diurnal time. We found that there are remarkable changes in the steady-state level of various transcripts during sleep as ~2,000 genes changed their expression during sleep. The categories of genes that were differentially upregulated during sleep were multiple genes encoding proteins involved primarily in biosynthetic pathways.

MATERIALS AND METHODS

Animal Handling

Experiments were performed on male mice (C57BL/6J) 10 wk of age $\pm$ 1 wk. Animals were housed in a light/dark cycle of 12 h, in a pathogen-free, temperature- and humidity-controlled room (22°C and 45–55%, respectively) with water available ad libitum. Food was accessible for 12 h only during the active period (15). Animals were subjected to 14 days of acclimatization during which a nighttime feeding pattern was established. This was done to avoid differential food intake between mice that were subsequently sleep deprived during the lights-on period and those allowed to sleep. During the acclimatization process, all animals were given time to become accustomed to the extended presence of an experimenter, repeated gentle touching, and presence in the cage of such objects as fragments

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of cotton or paper towels. This likely reduced, but not fully excluded, the stress involved with the subsequent sleep deprivation procedure. Mice were killed following 3, 6, 9, and 12 h of total sleep deprivation ($n = 5$ at each time point). Deprivation was initiated at lights-on and performed through gentle handling (36). Sleeping animals, which were left undisturbed, were killed at the same diurnal time points as sleep-deprived mice ($n = 5$ at each time point). An additional control group of mice ($n = 5$) was killed at time zero, i.e., at the time of lights-on at 7:00 AM. All mice were behaviorally monitored with the AccuScan infrared monitoring system that detects movement when the mouse crosses electronic beams (Columbus Instruments). For each animal, descriptive statistics such as average activity (beam breaks) for each hour (and then averaged across 12 h of light and dark) and estimated sleep amounts were computed. Sleep was considered to be present when there were ≥ 40 s of continuous inactivity (56). In addition, plots of activity and sleep graphed as moving averages broken down by light/dark cycle per day were generated for visual inspection.

Tissue Sampling and RNA Isolation

Mice were killed by cervical dislocation. Brain sectioning was performed according to the mouse brain atlas of Franklin and Paxinos (22). The sample of cerebral cortex was composed largely of the primary and secondary motor areas (M1 and M2) but also included the midline-located cingulate cortex areas 1 and 2 (Cg1 and Cg2), as well as parts of the barrel field of the somatosensory area 1 (S1FL). In the hypothalamus, broadly defined regions and zones were sampled. Total RNA was isolated with TRIzol (Invitrogen) and further purified using RNeasy columns (Qiagen) as per the manufacturer's instructions.

Microarrays

Transcript levels were assayed using the GeneChip Mouse Genome 430 2.0 array (Affymetrix), comprising $>45,000$ probe sets and covering $\sim 39,000$ transcripts and variants, including $\sim 34,000$ well-defined genes. Target preparation, which included hybridization and posthybridization procedures, was performed as described by the

Affymetrix GeneChip Expression Analysis Technical Manual (<http://www.affymetrix.com>) and was conducted at the University of Pennsylvania Microarray Core Facility.

Data Analysis

Probe intensity data from all arrays were read into the R software environment (<http://www.R-project.org>) directly from .cel files using the R/affy package (23). R/affy was also used to extract and manipulate probe level data to assess data quality and to create summary measures of expression. Normalization was carried out using the robust multiarray average (RMA) method to form one expression measure for each gene on each array (31). RMA processing was performed on all probe intensity data sets together. In brief, the RMA method was used to adjust the background of perfect match (PM) probes, apply a quantile normalization of the corrected PM values, and calculate final expression measures by the Tukey median polish algorithm.

Data analysis proceeded in a staged approach (see flow diagram in Fig. 1). The first step was an analysis of variance (ANOVA)-based approach that was used to statistically resolve and categorize gene expression differences between behavioral states (sleep or sleep deprived). The data from the animals at the anchor point (time zero) were not used in this first step of the analysis. Overall contrasts across conditions were considered by implementing gene-specific, fixed-effect ANOVA models (11) using the R/maanova package (81). Specifically, the model $Y_i = \mu + \text{STATE} + \epsilon_i$ was used to fit mean centered gene expression measures within each brain region, where μ is the mean for each array, STATE is the effect for each behavioral state (awake or sleeping), and ϵ_i captures random error. The first analysis looked for a main effect of state, comparing expression levels between all sleep-deprived (awake) and sleeping (sleep) animals ($n = 20$ for each behavioral state).

A second stage of analysis used the subset of differentially expressed genes returned from the ANOVA between states, which were then tested for temporal changes in expression within the sleep and sleep-deprived states separately. This secondary trend analysis uti-

Fig. 1. Experimental design and data analysis flow chart. *A*: the experiment comprised a $2 \times 2 \times 5$ full factorial experimental design with 2 behavioral states (sleep-deprived ○ and sleeping ●), 2 brain regions (cerebral cortex and hypothalamus), and 5 time points. Each circle represents 5 independent mice. The gray circles represent control animals killed at *time 0*, the time point of lights-on; this time point is referred to as the anchor time point. *B*: all 45,101 probe sets on the entire set of 90 arrays were normalized together using the robust multiarray average (RMA) procedure. An analysis of variance (ANOVA) approach was used to test for overall differences between “awake vs. sleeping” states in each brain region. Using a false discovery rate (FDR) threshold of 1%, 3,988 and 823 differentially expressed transcripts were found in the cortex and hypothalamus, respectively. These differentially expressed transcripts were subjected to a linear trends analysis to find genes that showed significantly changed expression during sleep (FDR $< 5\%$). In this way, a total of 2,090 and 409 probe sets were found to be sleep specific with increased or decreased expression during sleep. Note that *A* shows the experimental design but does not describe the analysis steps. The analysis steps are described in *B*. The anchor point was not involved in the first step of the analysis (sleep deprived vs. sleep) but was used in the trends analysis as an “anchor,” or time = 0, point for regression.

lized the additional set of data obtained from mice killed at lights-on (time zero at 7:00 AM). These trend tests were performed through linear regression analyses in which time was treated as a continuous variable. Linear regression is a convenient tool, but the analysis was not focused on whether the changes with time were linear or not. Rather, this step in the analysis was used to estimate direction of change i.e., increase, decrease, or no change within the sleeping and sleep-deprived groups separately, but only for genes found to be differentially expressed in the main analysis. Therefore, within each brain region, mean centered expression measures were fitted to the model $Y_{i(t)} = \alpha_{0(j)} + \beta_{j(t)}t + \epsilon_{ijk}$, which included common intercepts ($\alpha_{0(j)}$) and unique slopes over time ($\beta_{j(t)}$) for each condition. Here, the temporal pattern of differential gene expression over time was determined according to whether a slope (β_j) (positive or negative) was significantly different from zero.

For both statistical procedures, critical P values were calculated through permutation analyses incorporating 1,000 sample shuffles and pooling t -statistics. To adjust for multiple testing, q -values were generated from the unadjusted permutation P values by the false discovery rate (FDR) adjustment of Storey (73). For the main analysis (first stage, ANOVA), an FDR threshold of 1% was used to determine differential gene expression. For the secondary analysis of temporal pattern of change in these genes within sleep or sleep-deprived groups, which was done on only the genes that were found to be differentially expressed in the primary analysis, an FDR threshold of 5% was employed.

For those differentially expressed genes with linear trends that were not statistically significant in either condition (i.e., sleep deprivation or sleep), we used (as a tertiary analysis) an area-between-curves statistic to provide an additional examination of the temporal pattern of expression. Cubic curves were fit to the expression pattern over time for each gene, with separate curves used for the sleep-deprived vs. sleep-allowed data points. The baseline (time zero) data points were included in the fit for both the sleep-deprived and sleep-allowed curves. The total area between the sleep-deprived and sleep-allowed cubic curves was then calculated using integration. The area between curves was assigned a positive value when expression during sleep was higher than in the sleep-deprived and negative values when expression during wakefulness was higher than during sleep. This area-between-curves statistic reflects nonlinear time trends in the subset of genes that were not detected by fitting just a linear trend to the data.

Functional categories of overrepresented genes were then identified by the one-tailed Fisher's exact test implemented in the Expression Analysis Systematic Explorer (EASE) (16, 29). The EASE score is a conservative adjustment to the one-tailed Fisher's exact probability and represents the upper bound of the distribution of jackknife Fisher's exact probabilities (29). In addition to EASE scores, for each overrepresented category of genes we calculated the within-system FDR to assess the impact of multiple testing on determining which categories were overrepresented; the "FDR by EASE" phrase is used throughout the text to indicate this.

RESULTS AND DISCUSSION

Sleep and Wakefulness Were Assessed Noninvasively

We estimated sleep and wakefulness in experimental animals through noninvasive monitoring of locomotor activity with an infrared monitoring system. Sleep was defined as a bout of continuous inactivity of ≥ 40 s. The average agreement across 8,640 10-s epochs in 24 h in 7 mice between this definition of sleep and that from simultaneous electroencephalographic/electromyographic (EEG/EMG) assessment of sleep in C57BL/6J mice of the age used in our study is 92% (56). To further demonstrate the agreement between the assessment of sleep in our experiments and previously published data on

C57BL/6J mice, we compared our results with previously published data for this inbred mouse strain of the same sex and age. For convenience, we report amounts of sleep in the 3 h prior to death. The percentage of sleep was as follows: group killed at 10:00 AM, 57% (range: 41–67%); group killed at 1:00 PM, 76% (range: 71–79%); group killed at 4:00 PM, 79% (range: 77–81%); and group killed at 7:00 PM, 64% (range: 44–80%) (see Fig. 2). The percentage of sleep in each experimental group closely corresponds with normative data for C57BL/6J male mice of this age; the average percentage of time spent asleep in the time intervals listed above based on EEG/EMG recording is 58, 75, 78, and 65%, respectively (56). However, it should be noted that we did not take percentages of sleep per se in different experimental groups into consideration while searching for genes differentially expressed between sleeping and sleep-deprived animals. The key statistical test (i.e., ANOVA) used expression measures from both groups of mice across four experimental time points (40 animals in total) and evaluated for the main effect of group, rather than relating to amounts of sleep etc. The amount of sleep in spontaneously sleeping animals estimated by an infrared monitoring system in each hour across 24-h day is shown in Fig. 2. In addition, Table 1 provides the total amount of sleep in four groups of spontaneously sleeping animals i.e., in the group sleeping for 3 h, the amount of sleep between 7:00 AM and 10:00 AM; in the group sleeping for 6 h, the amount of sleep between 7:00 AM and 1:00 PM etc. Thus, the percentage of time spent asleep in the group of animals designated as spontaneously sleeping for 3, 6, 9, and 12 h is estimated at 57.3, 66.5, 70.9, and 69.0%, respectively. To assess changes in the temporal pattern of gene expression, a group of animals was killed at lights-on (7:00 AM). This group is referred to as time "0" or the "anchor" group. Table 1 gives the amount of sleep in the time "0" group of animals in blocks of time 3, 6, 9, and 12 h before death.

There are Profound Differences in Gene Expression Between Sleep and Wakefulness

There were 90 microarrays utilized, i.e., 45 arrays to assess gene expression in the cerebral cortex and an equal number in

Fig. 2. Total sleep time in minutes (means $\pm$ SD) in each hour across 24-h day in C57BL/6J mice used in this study as estimated by electronic beam breaking. Graph shows data starting at 7:00 AM (lights-on), i.e., beginning of the inactive period. Lights-off (7:00 PM) marks the beginning of the activity period. Sleep is defined as continuous inactivity of ≥ 40 s.

Table 1. Total amount of sleep in blocks of time preceding death in different experimental groups

	Spontaneous Sleep in the Block of Time Prior to Death				Spontaneous Sleep in the Block of Time Prior to Death at 7:00 AM			
	3 h	6 h	9 h	12 h	3 h	6 h	9 h	12 h
% of sleep	57.3	66.5	70.9	69.0	35.6	43.8	36.2	32.3
Min of sleep	103.1	239.4	382.2	496.8	64.2	157.6	195.4	230.4

Groups of mice were killed after 3 h of spontaneous sleep (column labeled 3 h); 6 h of spontaneous sleep (column labeled 6 h) etc. For each group, data are given from time of lights-on until time of death. A group of animals killed at 7:00 AM served as an anchor point to assess the temporal pattern of gene expression. The total amount of time sleeping in this group of animals is provided in blocks of 3, 6, 9, and 12 h prior to death at 7:00 AM.

the hypothalamus. Overall, $49.5 \pm 2.5\%$ probe sets were classified as having detectable expression levels (present call), $48.5 \pm 2.5\%$ probe sets were called absent (undetectable expression level), with $1.9 \pm 0.09\%$ probe sets classified as having marginal expression using the presence/absence call algorithm available in the Affymetrix MAS 5.0 microarray software package.

We found that there were remarkable differences in the steady-state level of various transcripts between sleep and sleep deprivation: we identified 3,988 genes in the cerebral cortex and 823 genes in the hypothalamus that exhibited differences in their expression profiles between behavioral states. The list of all probe sets that define the genes that were found (i.e., 3,988 genes in the cerebral cortex and 823 genes in the hypothalamus) is provided in Supplemental Table S1_cerebral cortex and Supplemental Table S1_hypothalamus.¹ The RMA expression values for all probe sets differentially expressed between sleep and sleep deprivation in the cerebral cortex and hypothalamus are provided in Supplemental Table S2_cerebral cortex and Supplemental Table S2_hypothalamus, respectively. The complete expression data sets are available at <http://www.ncbi.nlm.nih.gov/geo> (GEO record number: GSE6514).

Genes that reached a statistically significant difference in expression between sleeping mice and animals deprived of sleep exhibited only subtle differences in mRNA levels between behavioral states. In the cerebral cortex and hypothalamus, ~90% of differentially expressed genes modulated their expression by <40%. The magnitude of change in the transcript level between behavioral states observed in this study is comparable to data previously reported in the rat (12).

Using the Gene Ontology database, we assigned differentially expressed genes functional roles within three gene ontology systems: biological process, molecular function, and cellular component. Although many genes with altered expression could be assigned at least one ontology term, a significant fraction, i.e., 36.0% of all differentially expressed genes in the cerebral cortex and 35.6% of genes in the hypothalamus, were unclassified, as their function(s) remains to be determined.

Gene Expression Patterns Fall Into Different Temporal Classes

To classify the expression profile as state specific, i.e., changing expression during sleep or sleep deprivation, the temporal pattern of genes that ANOVA determined to have significant differences between behavioral states was further assessed across various durations of sleep or sleep deprivation as described above. There were nine temporal classes of genes

that emerged from the linear trend analysis; the number of genes in each class is shown in Table 2. A number of differentially expressed genes (i.e., 307 genes in the cerebral cortex and 55 genes in the hypothalamus) did not demonstrate any significant slope with time for a trend analysis and could not be categorized into a specific temporal class. This is class 5 in Table 2. The temporal pattern of these genes was assessed by the area between curves. The majority of genes in temporal class 5 exhibit sleep-specific expression patterns, i.e., increased their transcript levels during sleep relative to sleep deprivation. The largest temporal class of genes in both the cerebral cortex and hypothalamus was those that decreased expression during sleep deprivation and did not change expression during sleep (temporal class 6). The information on the temporal pattern of gene expression for all probe sets for differentially expressed genes in the cerebral cortex and hypothalamus is provided in Supplemental Table S1_cerebral cortex and Supplemental Table S1_hypothalamus, respectively.

The sleep-specific temporal pattern of expression was defined as those differentially expressed genes that increase or decrease transcript levels during sleep regardless of their tem-

Table 2. The temporal classes and the number of genes in each class in the cerebral cortex and hypothalamus

Class	Expression Pattern		Cerebral Cortex Genes	Hypothalamus Genes
	Sleep	Wake		
1	increase	increase	179	41
2	increase	no change	979	186
3	increase	decrease	285	79
4	no change	increase	326	85
5	no change	no change	307	55
6	no change	decrease	1,039	268
7	decrease	increase	91	11
8	decrease	no change	466	85
9	decrease	decrease	316	13

The classes were created using a set of genes that emerged from the ANOVA test at a false discovery rate (FDR) of <1% and that were further subjected to trend analyses (with FDR of <5%) to examine changes in expression with time from the time point of lights-on (7:00 AM) as an "anchor." Due to the differences in the statistical power between the ANOVA and linear trend analysis tests, a number of genes (i.e., 307 genes in the cerebral cortex and 55 genes in the hypothalamus) did not demonstrate a slope significantly different from zero for the linear trend analyses; these genes are listed in class 5. The classification of genes in temporal class 5 as sleep or wakefulness specific was accomplished by "area-between-curves" statistics (see details in text). The largest temporal class is class 6, i.e., genes whose expression declines during sleep deprivation. In each temporal class the number of probe sets (i.e., Affymetrix identification numbers) is higher than the number of genes. For example, the temporal class 2 contains 1,129 probes sets that define 979 genes in the cerebral cortex; supplemental data tables contain information on expression of all probe sets.

¹ The online version of this article contains supplemental material.

poral changes during sleep deprivation. This definition does not rely on the magnitude of change in the transcript levels, but only the direction of change (i.e., an increase or decline) during sleep relative to the anchor point (7:00 AM). Thus, sleep-specific genes belonged to temporal classes 2, 3, 7, and 8 (see Table 2). There were 1,264 genes in the cerebral cortex (256 genes in hypothalamus) that increased their transcript levels during sleep (classes 2 and 3) and 557 genes in the cerebral cortex and 96 in hypothalamus that showed a decrease in mRNA during sleep (classes 7 and 8). In addition, the area-between-curves statistics that was applied to temporal class 5 demonstrated that 269 genes in the cerebral cortex and 48 genes in hypothalamus (87.6 and 87.2% of all genes present in the temporal category 5 in the cerebral cortex and hypothalamus, respectively) exhibited sleep-specific patterns of expression with the majority of genes being upregulated during sleep. Thus, 2,090 genes in the cerebral cortex and 409 genes in the hypothalamus were classified as sleep specific (52.9 and 49.6% of all differentially expressed in the cerebral cortex and hypothalamus, respectively).

Sleep Affects Steady-State Level of Various Transcripts for Genes of Diverse Functions

Using the Fisher's exact test (implemented in the EASE software; Refs. 16, 29), we compared the proportion of genes in specific temporal classes that were differentially expressed to their overall proportion present on Affymetrix GeneChip Mouse Genome 430 2.0 array. Sleep-specific genes upregulated in the cerebral cortex formed 22 overrepresented categories in the gene ontology of biological process, 26 gene categories in molecular function, and 26 categories in cellular component. In the hypothalamus there were respectively 2, 4, and 4 overrepresented categories in these gene ontology systems. The complete list of overrepresented functional categories in the cerebral cortex and hypothalamus is presented in Supplemental Table S3_cerebral cortex and Supplemental Table S3_hypothalamus.

The analysis of overrepresented categories of differentially expressed genes in the cerebral cortex and hypothalamus during sleep indicated that at the FDR level by EASE of <1%, most overrepresented processes were biosynthesis and intracellular transport, which were significantly augmented during sleep. Surprisingly, the mRNA level of genes encoding components of the neuronal machinery involved in electrical signaling (e.g., components of ion channels or pumps) was only negligibly affected during sleep. The most overrepresented categories of genes increasing expression during sleep encoded proteins localized in the cellular compartments of cytoplasm, mitochondrion, ribosome, lysosome, and vacuole (all classified as overrepresented at the FDR level of <1% by EASE).

Genes Upregulated During Sleep

Biosynthesis of macromolecules is the most common process affected during sleep. In the cerebral cortex, and to the lesser extent in the hypothalamus, there were a number of upregulated genes that encoded enzymes of various, predominantly intermediary, biosynthetic pathways. Among different biosynthetic pathways upregulated during sleep were heme and porphyrin protein and cholesterol biosynthesis (see Supplemental Table S3_cerebral cortex and Supplemental Table

S3_hypothalamus). A summary of functional categories of genes upregulating their transcripts during sleep in the cerebral cortex and hypothalamus is given in Fig. 3.

HEME AND PORPHYRIN BIOSYNTHESIS. We identified heme/porphyrin biosynthesis as an overrepresented gene ontology category (FDR <1% by EASE) in the sleep-upregulated class of genes in the cerebral cortex. Genes that were upregulated during sleep include *Alas1*, *Alad*, *Hmbs*, *Ppox*, and *Fech*. This upregulation of the enzymes of the heme synthesis pathway is specific to the cerebral cortex since none of the heme biosynthesis genes were significantly differentially expressed in the hypothalamus. There was also upregulation during sleep in the cerebral cortex of two genes encoding proteins that regulate excess heme levels: heme-binding protein 1, which is a cytosolic heme binding protein purported to function in the removal of excess heme and its metabolic intermediates (32), and heme oxygenase (decycling) 2, which codes for an enzyme that also sequesters heme (44). In the cerebral cortex there are a number of genes coding for proteins containing heme that are also upregulated during sleep, i.e., proteins of cytochrome P450 complex (*Cyp1b1*, *Cyp2d22*, *Cyp7b1*, and *Cyp46a1*), fatty acid desaturases (*Fads1*, *Fads2*, and *Fads3*), and nitric oxide synthase 3 (*Nos3*). Only the *Fads1* gene was found to be significantly differentially expressed in the hypothalamus, and it was also upregulated during sleep. The upregulation of six of the seven genes involved in heme synthesis only in the cerebral cortex, and a subset of heme containing proteins also being upregulated in the cerebral cortex, would suggest that an increase in heme synthesis may be for the sleep dependent production of these heme containing proteins.

PROTEIN BIOSYNTHESIS. Among the overrepresented categories of genes that were upregulated during sleep in the cerebral cortex were those involved in protein biosynthesis (FDR <2% by EASE). Further analysis subdivided this overrepresented category into "subcategories": the structural constituents of the ribosomes, translation regulation activity, amino acylation of tRNA for translation, and ribosome biogenesis. In the subcategory of structural constituents of the ribosomes, there were 22 genes encoding components of both cellular and mitochondrial ribosomes. This increase in ribosomal protein transcripts indicates a build up of structural elements of ribosomes and suggests that ribosome assembly and biogenesis takes place during sleep. This is supported by the observation that a number of genes upregulated during sleep are in the overrepresented subcategory of ribosome biogenesis and assembly.

There were also a number of genes encoding translation initiations factors (eIF4b, eIF5, eIF3 subunits 7, 8, and 12) and the translation elongation factor (eEF2) that were among those upregulated in the overrepresented subcategory of translation regulation. Translation control is regulated primarily at the initiation step through factors such as eIF2 and eIF4. Upregulation during sleep of a gene for translational factor eIF4b suggests that active translation takes place during sleep. Upregulation during sleep of transcript levels of eIF5 and all subunits of eIF3 indicates that "preloading" of the 40S ribosome, which occurs early during initiation, is further evidence of translation taking place during sleep. The key regulation step during translation elongation is that involving eEF2, which we observe to increase during sleep. eEF2 catalyses the translocation of peptidyl-tRNA on the ribosome and facilitates the movement of the ribosome relative to mRNA and process-

Fig. 3. The top overrepresented functional categories in the gene ontology system of biological process that are upregulated (A) and downregulated (B) in the cerebral cortex (light gray bars) and hypothalamus (dark gray bars) during sleep. In the cerebral cortex the functional categories 17 and 18 have an FDR estimated between 1 and 2%, and categories 21–22 have an FDR of >2%. All other categories in the cerebral cortex and hypothalamus in A and B have an FDR estimated at <1%. The y-axis represents $-\log_{10}$ of P values determined by Expression Analysis Systematic Explorer (EASE). Functional categories of genes present in the gene ontology system of cellular component and molecular function along with the relevant P values by EASE, as well as the corresponding FDR are provided in the Supplemental Tables S3_cerebral cortex and S3_hypothalamus.

ing of tRNA. In addition to genes that have translation regulator activity, a small subset of transcripts involved in tRNA activation were found to be upregulated during sleep; these were the prolyl and asparaginyl tRNA synthase. The tRNA synthase attach amino acids to the correct tRNA, and the upregulation of genes encoding tRNA synthase serves as another indicator of active translation occurring during sleep. The genes involved in protein synthesis are not, however, differentially expressed in the hypothalamus.

These findings can be related to early experiments that examined global changes in protein synthesis during specific states. Bobillier et al. (3) reported a generalized decrease in [^3H]amino acid incorporation into proteins of the telencephalon and the brainstem of rats with 3 h of sleep deprivation. Conversely, rats that had 1.5 h of recovery sleep following 1.5 h of sleep deprivation exhibited increased labeled proteins. Richardson and Rose (61) also demonstrated an increased incorporation of labeled lysine into rat brain regions during the

lights-on period that corresponds to the inactive period. Increased protein synthesis during nonrapid eye movement (NREM) sleep has also been shown in the rat (60) and rhesus monkey (50). Also, a recent preliminary proteomic study using mass spectrometry found a general decrease in protein expression in the cerebral cortex of sleep-deprived mice relative to that in sleeping mice (17). These findings support the view coming from our microarray studies that synthesis of proteins is a key function of sleep and that changes in the steady-state transcript levels of genes encoding proteins involved in these processes play an important role.

CHOLESTEROL METABOLISM. The Fisher's exact test identified cholesterol metabolism as an overrepresented category of genes increasing expression during sleep (FDR by EASE <2 and 8% in the cerebral cortex and hypothalamus, respectively). This category of genes was also found in the overrepresented category of genes that decreased expression during sleep deprivation in both the cerebral cortex and hypothalamus; this was at an FDR <1% by EASE. The Fisher's exact test performed on genes that were common to both cerebral cortex and hypothalamus also identified cholesterol metabolism as a category of overrepresented genes. Differentially expressed transcripts included genes encoding synthetic enzymes of cholesterol, proteins involved in cholesterol trafficking, and the relevant specific transcription factors. All of these genes were upregulated during sleep and did not change expression or were downregulated during wakefulness (see further below).

Cholesterol synthesis is a multistage process in which isopentenyl pyrophosphate (IPP) serves as a building block. In Fig. 4 we show the steps in cholesterol synthesis and indicate those genes that are upregulated during sleep in the cerebral

cortex and in hypothalamus. Its synthesis begins with condensation of acetyl-CoA and acetoacetyl to hydroxy-methylglutaryl-CoA (HMG-CoA) catalyzed by HMG-CoA synthase (*Hmgcs*) and is followed by the reduction of HMG-CoA to mevalonate by HMG-CoA reductase (*Hmgcr*). *Hmgcr* is the key regulatory enzyme that catalyzes an irreversible step in the pathway of cholesterol synthesis. Subsequently, in consecutive steps involving mevalonate kinase (*Mvk*) and mevalonate (diphospho) decarboxylase (*Mvd*), mevalonate is converted into IPP. The cyclization of squalene, demethylation, and subsequent reduction of the double bonds performed respectively by lanosterol synthase (*Lss*), NADP-dependent steroid dehydrogenase (*Nsdhl*), and 7- and 24-dehydrocholesterol reductase (*Dhcr7* and *Dhcr24*) are the final steps in cholesterol synthesis. Genes for all of these enzymes were differentially expressed between sleep and sleep-deprived groups and upregulated during sleep. Genes for five of the 10 enzymes were upregulated in both cortex and hypothalamus, while three were upregulated only in the cerebral cortex and two only in the hypothalamus. In the majority of cases where these genes were not found to be differentially expressed in one tissue, the FDR in this tissue just missed the 1% FDR threshold for calling a gene as being differentially expressed in the main ANOVA. Data on the differential expression of selected genes encoding proteins of the cholesterol synthesis pathway in the cerebral cortex and hypothalamus are illustrated in Fig. 5, A and B, and presented numerically in Table 3. Although cholesterol synthesis is under complex multilevel regulatory control, results of our analysis strongly suggest that there is an increase in cholesterol synthesis during sleep.

Fig. 4. The cholesterol synthesis pathway and its major intermediaries. Enzymes that are upregulated during sleep as determined by the genes expression data are listed on the right side of the diagram. *Genes known to be regulated the sterol regulatory element binding factor (*Srebf*); ^Hgenes that were upregulated during sleep in hypothalamus; ^Cgenes upregulated in cortex.

Fig. 5. Examples of the temporal changes in the expression of genes encoding enzymes of the cholesterol synthesis pathway (A and B), the sterol regulatory factors (C and D), and in raft-associated proteins (E and F). A: hydroxymethyl glutaryl-CoA synthase (*Hmgcs*); B: mevalonate (diphospho) decarboxylase (*Mvd*); C: sterol regulatory element binding factor 1 (*Srebf1*); D: Srebf cleavage activating protein (*Scap*), a regulatory binding protein that activates the transcription factor Srebf1 and Srebf2; E: lipid raft-associated proteins - flotillin 1 (*Flot1*); and F: stomatin (*Stom*) in the cerebral cortex (circles) and hypothalamus (triangles). The profiles were established after 3, 6, 9, and 12 h of spontaneous sleep (black circles or triangles) or sleep deprivation (white circles or triangles). Transcript abundance change is expressed in arbitrary units and is the log₂ of mean fluorescence signal for each time point minus that of the control animals killed at time 0.

Probe sets associated with genes encoding the sterol regulatory element binding factors (*Srebf*), i.e., *Srebf1* and *Srebf2* in the cerebral cortex and *Srebf1* in the hypothalamus, were also differentially expressed between sleeping and sleep-deprived mice. These genes were upregulated during sleep and did not change expression or were downregulated during sleep deprivation (Fig. 5C and Table 3). Moreover, in both the cerebral cortex and hypothalamus, there was upregulation during sleep of the *Srebf1* cleavage activating protein (*Scap*), a regulatory binding protein that activates the transcription factors Srebf1 and Srebf2 (18) (Fig. 5D and Table 3). These changes in *Srebf* and *Scap* likely contribute to the increased expression of genes encoding enzymes of cholesterol synthesis. The sterol regulatory element binding factors that affect genes encoding proteins involved in cholesterol synthesis include *Hmgcs1*, *Hmgcr*, *Mvk*, *Mvd*, *Pmkv*, and *Fdft1* (Fig. 4, marked with asterisk).

In addition to cholesterol synthetic enzymes, there was differential expression of genes encoding components of cholesterol uptake and transport, such as low-density lipoprotein

receptor (*Ldlr*), low-density lipoprotein receptor-related protein 10 (*Lrp10*), low-density lipoprotein receptor-related protein associated protein 1 (*Lrpap1*), as well as StAR-related lipid transfer (START) domain containing 4 (*Stard4*). The *Ldlr*, *Lrpap1*, and *Stard4* genes were differentially expressed in both cerebral cortex and hypothalamus, whereas the *Lrp10* gene was differentially expressed only in the cerebral cortex. All these genes were upregulated during sleep and did not change expression or were downregulated during sleep deprivation. *Ldlr* and *Lrp1* protein are members of the cell-surface density lipoprotein receptor family and the key components of the mechanism of cholesterol uptake. Cholesterol transport can also be mediated by such carriers as the steroidogenic acute regulatory proteins (*Star*). Sleep-specific increases in both the cerebral cortex and hypothalamus in the level of mRNA for *Ldlr*, *Lrp1*, and *Stard4* proteins imply that cholesterol trafficking is also enhanced during sleep. A number of genes involved in cholesterol trafficking that are upregulated during sleep are also targets of the *Srebf* transcription factors.

Table 3. Fold change in transcript level in the cerebral cortex and hypothalamus and the corresponding FDR expressed as a percentage for selected cholesterol or lipid-related genes that increased expression during sleep

Gene	Gene ID	Gene Name/Description	Fold Change		FDR	
			CX	HT	CX	HT
<i>Hmgcs1</i>	208715	3-HMG-CoA synthase 1	1.33	1.29	$<10^{-16}$	7.47×10^{-4}
<i>Hmgcr</i>	15357	3-HMG-CoA reductase	1.24	1.72	8.43×10^{-2}	1.32×10^{-2}
<i>Mvk</i>	17855	mevalonate kinase	1.23	1.14	$<10^{-16}$	5.54×10^{-2}
<i>Mvd</i>	192156	mevalonate (diphospho) decarboxylase	1.52	1.40	4.74×10^{-6}	1.65×10^{-4}
<i>Fdps</i>	110196	farnesyl diphosphate synthase	1.54	1.31	7.81×10^{-6}	1.04×10^{-2}
<i>Fdft1</i>	14137	farnesyl diphosphate farnesyl transferase 1	1.31	1.35	2.04×10^{-5}	1.54×10^{-2}
<i>Lss</i>	16987	lanosterol synthase	1.35	1.65	$<10^{-16}$	1.17×10^{-4}
<i>Dhcr7</i>	13360	7-dehydrocholesterol reductase	1.32	1.25	$<10^{-16}$	2.77×10^{-4}
<i>Ldlr</i>	16835	low-density lipoprotein receptor	1.44	1.49	$<10^{-16}$	$<10^{-16}$
<i>Srebf1</i>	20787	sterol regulatory element binding factor 1	1.29	1.34	4.10×10^{-5}	5.81×10^{-5}
<i>Scap</i>	235623	Srebf1 cleavage activating protein	1.16	1.22	7.81×10^{-6}	1.01×10^{-3}
<i>Flot1</i>	14251	flotillin 1	1.22	1.19	1.08×10^{-6}	2.72×10^{-2}
<i>Stom</i>	13830	stomatin	1.13	1.07	1.93×10^{-4}	1.47×10^{-1}
<i>Gna13</i>	14674	guanine nucleotide binding protein, $\alpha 13$	1.31	1.28	1.31×10^{-4}	6.93×10^{-2}

CX, cerebral cortex; HT, hypothalamus.

The increased expression of genes encoding known elements of the cholesterol synthesis pathway during sleep suggests that this process may be one of the functions of sleep. Cholesterol plays an important role in membrane stability and is the key structural component of membrane microdomains called lipid rafts (see Ref. 71 for review and additional references). There are a number of raft-associated proteins that have been identified by proteomic analysis (52). For example, flotillins are integral membrane proteins that have been shown to be present in lipid rafts. Although the physiological role of flotillins remains uncertain, they are likely involved in the structural organization of lipid rafts (48). Flotillin genes demonstrate a sleep-specific increase in transcript levels (see Fig. 5E and Table 3). In addition to *Flot* genes, there was also an increase during sleep in transcript levels of other raft-associated proteins: guanine nucleotide binding proteins *Gna13*, *Gnb4*, *Gnai1*, stomatin (*Stom*), and stomatin-like 1 and 2 (*Stoml1* and *Stoml2*) (see Fig. 5F and Table 3). Except for the *Gnb4* gene, the augmentation of *Gna13*, *Gnai1*, *Stom*, *Stoml1*, and *Stoml2* transcript levels was restricted to the cerebral cortex. This suggests that lipid rafts are reassembled during sleep. This would likely alter signaling strength for a number of neurotransmitters, including glutamate (7, 19, 28, 66) and may be in preparation for subsequent wakefulness, since there is enhanced release of various neurotransmitters upon arousal from sleep (33). Reassembly of lipid rafts may allow maximal signaling on awakening. The data regarding magnitude of change (i.e., fold change) in expression of genes encoding proteins involved in cholesterol synthesis, its transport, as well as lipid raft proteins, are provided in Table 3.

Intracellular transport is enhanced during sleep. Not only are biosynthetic pathways upregulated during sleep, but also genes for intracellular transport mechanisms. This is not surprising since new macromolecules that are synthesized also need to be transported. At the FDR level of $<1\%$ we identified genes encoding proteins involved in intracellular transport to be overrepresented in the genes upregulated during sleep. This is true in the cerebral cortex but not in the hypothalamus. This includes vesicle-mediated protein trafficking to lysosomal compartments and membrane docking/fusion reactions of late endosomes/lysosomes, likely involving a clathrin-mediated

mechanism. The transcripts that were upregulated during sleep in the cerebral cortex include the vacuolar sorting proteins (*Vps11*, *Vps28*, and *Vps45*), sortin nexin proteins (*Snx1*, *Snx4*, *Snx7*), syntaxin 17 (*Stx17*), coatamer protein (*Copg*), adaptor-proteins (*Ap1b1*, *Ap1g2*, *Ap2m1*, and *Ap4s1*), and ADP-ribosylation factors (*Arf5*, *Arfgap1*, *Arfgap3*, *Arl4a*). The increase in transcript levels of *Scamp4* and *Scamp5* genes during sleep in the cerebral cortex likely impacts trafficking of membrane proteins. There was also an increase during sleep in gene expression for Snap-associate protein (Snapap) in the cerebral cortex. This protein modulates a step between vesicle priming, fusion, and calcium-dependent neurotransmitter release. The increase in expression of the *Snapap* gene may be a preparatory step for future wakefulness by helping rebuild mechanisms for neurotransmitter release. In support of this hypothesis we also find sleep-specific enhancement of mRNA for an ADP-ribosylation factor in the cerebral cortex (*Arl2*) that points toward upregulation of a secretory pathway involved in calcium-dependent neurotransmitter release.

An increase during sleep in expression of importing and exporting genes (*Ipo4* and *Exp6*, respectively) in the cerebral cortex (but not the hypothalamus) suggests that the transport between the nucleus and cytoplasm through nuclear pore complexes is also enhanced during sleep. These genes mediate nuclear transport of cytoskeleton complexes. Nuclear protein import involves GTPase proteins, among them *Ran*. We found that a number of genes encoding GTPase proteins, including *Ran* and *Ran-binding protein* (*Ranbp5*), increased their transcript levels during sleep in the cerebral cortex. These genes were not differentially expressed in the hypothalamus.

In addition to protein transport, there may be, in the cerebral cortex, an enhancement during sleep of transport of proteins and intermediary metabolites between the cytoplasm and mitochondrion. There is an increase during sleep in the transcript levels of the *Mtx1* gene that is a component of the protein translocation apparatus of the outer mitochondrial membrane, suggesting enhanced transport of proteins between these two compartments. In the cerebral cortex (but not the hypothalamus) an increase in mRNA during sleep for the *Slc25a10* gene that plays a role in supplying malate for citrate transport required for fatty acid synthesis adds support to the concept

that there is an increase in lipid synthesis during sleep, whereas the sleep-specific augmentation in the cerebral cortex of transcripts for the *Slc25a4* and *Slc25a5* genes point toward enhancement of the exchange of ADP and ATP across the mitochondrial inner membrane.

In the cerebral cortex, genes of the Ras family are upregulated during sleep. There were a number of genes for which the steady-state level of their transcript was upregulated during sleep in the cerebral cortex that encoded various elements of the second messenger signaling pathways. Among the most intriguing findings was the upregulation of genes in the cerebral cortex encoding proteins of the Ras family. Within the gene ontology of biological process, the gene category, small GTPase-mediated signal transduction, was highly overrepresented (FDR <1% by EASE). For example, there was sleep-specific upregulation of the *Ras* gene (*H-ras*) and Ras-related gene (*Rras2*) that possess GTP-binding and GTPase activity and are involved in signal transduction. In the cerebral cortex, an increase during sleep in the transcript level of *Gna13* guanine nucleotide binding protein gene (*Gna13*) suggests a link between G protein-coupled receptors and the activation of *Ras* gene family members. In addition, there was sleep-specific upregulation of the *Rab1b*, *Rab3a*, *Rab4b*, *Rab7*, *Rab12*, *Rab33a*, *Rab34*, and *Rab40b* genes. The Rab proteins comprise the largest branch of the Ras superfamily of small GTP-binding proteins. Different Rab proteins are specifically localized at the cytoplasmic side of distinct vesicular and organellar membranes and have been implicated in the regulation of various steps along the endocytic and exocytic pathways (for review see Ref. 76). In particular, the Rab proteins are regulators of intracellular vesicular transport, facilitate vesicle formation, vesicle fusion, and release of the vesicle content. For example, *Rab1* is involved in transport steps between the endoplasmic reticulum and Golgi; *Rab3* protein regulates secretory vesicle release; *Rab4* protein mediates recycling from the endosome back to the plasma membrane; and *Rab7* regulates trafficking events related to late endocytotic pathways. Consequently, mice with a targeted loss-of-function mutation in *Rab3a* gene have defects in calcium-dependent synaptic transmission (24). Interestingly, mice with a semidominant mutation in the *Rab3a* gene have a shortened period of circadian locomotor activity and marked reduction in sleep homeostasis, i.e., less recovery sleep following sleep deprivation (34). These data, together with the upregulation of genes for the Snap-associated protein described earlier, suggest that during sleep there is a rebuilding of the endo- and exocytosis machinery. This is likely in preparation for subsequent wakefulness during which there is sudden increased activity of various wakefulness-promoting neurotransmitter systems (for review, see Ref. 33). Given the higher level of neuronal firing in the cerebral cortex during wakefulness, there is likely to be degradation of endo- and exocytosis mechanisms as wakefulness is prolonged. Indeed, it is well established that at many synapses, periods of elevated activity lead to a decline in synaptic efficacy related primarily to a decrease in the release of neurotransmitter due to a depletion of the pool of vesicles with neurotransmitter ready for release (8) (see also Ref. 65 for review and additional references).

An increase in the steady-state transcript levels of genes of the *Ras* family may, however, have other functions. For example, an increase in the transcript level of GTP-binding protein

Centg3 in cerebral cortex during sleep suggests an enhancement of the signaling pathway that involves reactive oxygen species (59). Indeed, there are a number of genes encoding proteins with antioxidant properties such as: glutathione S-transferase (*Gstp1*), peroxidase (*Gpx*), and reductase (*Gsr*), as well as superoxide dismutase (*Sod1*), catalase (*Cat*), methionine sulfoxide reductase (*Msra*), and thioredoxin reductase (*Txnrd2*) that upregulate their transcript levels during sleep. This is supported by the observation that mRNA for transcription factor *Nfe2*, involved in induction of genes encoding antioxidant proteins and certain detoxifying enzymes, increases during sleep. Changes in the expression of genes encoding antioxidant proteins occur predominantly in the cerebral cortex since only *Gstp1*, *Txnrd2*, and in addition *Txnrd3* genes are upregulated during sleep in hypothalamus. Given that cortical neurons are more active in wakefulness and consume more ATP, it has long been proposed that as wakefulness is extended there is more production of reactive oxygen species (30). Thus, sleep could be a time to remove reactive oxygen species and/or to prepare the "antioxidant system" for the heightened level of reactive oxygen species in wakefulness.

Other aspects of the Ras pathway are also differentially regulated. *Spry4* and *Spred2* are inhibitors of Ras activation (37); the level of genes for *Spry4* and *Spred2* proteins in the cerebral cortex declines during sleep, thus further enabling the Ras signaling pathway. Thus, it seems that multiple mechanisms are employed during sleep to activate the pathway.

In addition to the *Ras* family of genes, there was upregulation during sleep, in the cerebral cortex, of genes encoding cyclins (*Ccne1*, *Cnnm3*) and cyclin-dependent protein kinases: *Cdk4*, *Cdk5*, *Cdk11* (the gene encoding the Cdk inhibitor *Cdkn1a* was upregulated during wakefulness). Since cyclin-dependent phosphorylation of proteins is central to regulation of replication and cell division, our studies raise an intriguing question of what processes are affected by changes with sleep and sleep deprivation of genes encoding the Cdk signaling pathway. While neuronal proliferation has been described in the brain, particularly in the hippocampus (25), there is no information on sleep-related neurogenesis in the cerebral cortex. Thus, it seems that these molecules must fulfill different signaling functions in brain that remain to be fully elucidated.

It is also difficult to assess what processes are influenced by upregulation during sleep of the mitogen-activated protein kinases (*Mapk*), which are also widely regarded as activators of cell proliferation and differentiation. Similar to that of the Cdk pathway, the *Mapk* signaling pathway likely fulfills different functions in the mostly postmitotic brain than in a tissue with high activity of cell proliferation.

Steady-state level of transcripts of genes encoding proteins involved in energy metabolism increases during sleep. Among the genes in the cerebral cortex differentially upregulated with sleep are genes involved in the regulation of main pathways of carbohydrate metabolism, oxidative phosphorylation (FDR <1% by EASE), and in general, the energy pathways (FDR <1.8% by EASE). During NREM sleep there is decreased energy need and consumption in the cerebral cortex (6, 35, 42, 54), and hence, upregulation of energy genes during sleep was unexpected. However, the precise meaning of this depends on how quickly proteins are made and, moreover, whether it is the key regulatory enzymes that are upregulated. It seems that sleep may be a stage to rebuild the

systems for producing energy to meet the higher energy demands during wakefulness.

Our data demonstrate that during sleep there are a number of upregulated genes (i.e., *Ldh2*, *Eno3*, *Eno1*, *Aldoa*, *Tpi1*, *Pfkfb*, *Hk1*, and *6330505F04Rik*) that encode proteins involved in glycolysis. Additional genes coding for proteins that are involved in glycolysis also upregulated in sleep were: *G6pc3*, *Bpgm*, *Pgm1*, *Pgm2*, and *Pgm3*. Also there was sleep-specific upregulation of the regulatory (inhibitory) subunit 1a of protein phosphatase 1 and inhibitory subunit 2 of protein phosphatase 1 (*Ppp1r2*), which, when phosphorylated, inhibit the dephosphorylation of glycogen synthase and glycogen phosphorylase leading to the degradation of glycogen (reviewed in Ref. 14). The upregulation of a phosphorylase b kinase alpha regulatory chain as well as two inhibitory subunits of protein phosphatase 1 suggests that with increasing sleep, glycogen degradation is favored in anticipation of energy needs upon awakening. Genes encoding proteins involved in glycolysis/glycogen metabolism were not found to be significantly differentially expressed in the hypothalamus.

In addition, there were five genes in the gene ontology category regulation of energy pathways that encode proteins of the citrate cycle, i.e., *Aco2*, *Acly*, *Idh3g*, *Mdh2*, and *Sdhb* (which is also involved in oxidative phosphorylation). There are three critical points in the citric acid cycle (reviewed in Ref. 75); only one of the enzymes involved in these critical control points is upregulated during sleep, isocitrate dehydrogenase 3 (NAD⁺) alpha. Therefore, the upregulation of these genes would indicate that mRNA (and possibly proteins) is being accumulated during sleep but that an upregulation of the citric acid cycle is not yet occurring. The remaining genes in this category have roles in thiamine, galactose, aminosugar metabolism, pentose phosphate pathway, and the degradation of ketone bodies.

Elements of the oxidative phosphorylation pathway are also upregulated during sleep. There were a number of genes that encode proteins involved in oxidative phosphorylation that were upregulated during sleep. This was unexpected as a link between neuronal activity and upregulation of a key component of complex IV has been shown in several organisms (58, 79, 80). The Affymetrix microarray platform used in this study does not, however, have probes for any of the mouse mitochondrial DNA-encoded proteins. Thus, we cannot comment on changes in any genes encoded by mitochondrial DNA. Therefore, all of the genes we see upregulated in sleep are genes for nuclear-encoded proteins. Seventeen genes encoding for subunits of complex I were upregulated during sleep: *Ndufa6*, *Ndufa7*, *Ndufa8*, *Ndufa10*, *Ndufa11*, *Ndufa12*, *Ndufab1*, *Ndufb4*, *Ndufb7*, *Ndufb11*, *Ndufs1*, *Ndufs2*, *Ndufs3*, *Ndufs7*, *Ndufs8*, *Ndufv1*, and *Ndufv2*. Also upregulated during sleep were genes encoding a single subunit of complex II (*Sdhb*), subunits of complex III (*Uqcr*, *Uqcrc1*, *Uqcrc2*, *Uqcrls1*, *1110020P15Rik*), and subunits of complex IV (*Cox5b*, *Cox7a2l*, *Cox8a*). We propose that the upregulation of the nuclear mRNA encoding oxidative phosphorylation subunits we observe during sleep is a buildup of mRNA and possibly protein in anticipation of energy needs upon waking. Other studies show that mitochondrial encoded components of the oxidative phosphorylation pathway, such as the key catalytic subunit of complex IV, are upregulated during wakefulness (13, 68), as is the activity of this key enzyme (53). Upregulation of mRNA

of subunits from mitochondrial DNA occurs rapidly (46), and thus, it is not surprising that they are upregulated during wakefulness. Transcriptional regulation of mitochondrial- and nuclear-encoded genes for oxidative phosphorylation differs in that the stability of the mitochondrial mRNA encoding key subunits of the oxidative phosphorylation complexes (*Cox I*, *Cox II*, *Cox III*, *Nd5*, *Nd4*, and *CytB*) is significantly less compared with nuclear encoded genes (46).

Genes That Downregulate the Steady-State Level of Their Transcripts During Sleep

There were fewer genes that were downregulated during sleep in the cerebral cortex and hypothalamus than were upregulated in these brain regions (see Table 2). The one clearly overrepresented group was formed by genes encoding proteins involved in RNA processing (see summary, Fig. 3).

RNA processing decreases during sleep. Genes encoding proteins involved in RNA splicing and processing belonged to the overrepresented categories of genes that were downregulated during sleep in the cerebral cortex (FDR <1% by EASE) (Fig. 6). There was a sleep-specific decrease in RNA binding protein (*Rbm3*) and genes encoding RNA splicing factors (*Sfrs1*, *Sfrs6*, *Sfrs7*, *Fusip1*, *Prpf4b*). The processes of capping of the 5'-end of pre-mRNA and mRNA polyadenylation also appear to decrease during sleep since the transcript levels for the RNA guanylyltransferase/5'-phosphatase (*Rngtt*) and polyadenylation factor 2 (*Cpsf2*) declined. Interestingly, the expression of Dicer1 gene (*Dcl1*) increases during sleep; *Dcl1* is the key enzyme in the process of RNA interference, in which small-interfering RNA (produced by Dicer) induce a sequence-dependent degradation of mRNA. Thus, upregulation of Dicer1 during sleep will work in concert with downregulation of other parts of the RNA machinery to slow synthesis of proteins at the RNA stage. Consequently, our studies suggest that while many components of the protein synthesis pathway are upregulated during sleep, RNA processing is downregulated.

Genes That Upregulate the Steady-State Level of Their Transcripts During Sleep Deprivation

There were also a large numbers of genes whose expressions were altered during sleep deprivation; however, in the cerebral cortex there were no overrepresented categories upregulated during sleep deprivation (see Supplemental Table S3_cerebral cortex). Thus, the prevailing view that the major regulatory mechanism for control of sleep is upregulation of multiple sleep-promoting molecules (Process S) during wakefulness is not supported by our data. It is, of course, possible that there is upregulation of expression of key sleep-promoting molecules without these genes constituting a functional gene category based on gene ontology. In the hypothalamus, the one overrepresented category included genes broadly defined as being involved in the regulation of cell growth (FDR <1% by EASE). It is likely that the function(s) of these proteins in the postmitotic brain are different from that in dividing cells (see Supplemental Table S3_cerebral cortex and Supplemental Table S3_hypothalamus).

Sleep deprivation induces an unfolded protein response. In the cerebral cortex and hypothalamus, sleep deprivation up-regulated the transcript levels of a number of chaperone pro-

Fig. 6. The top overrepresented functional categories in the gene ontology system of biological process that are downregulated in the cerebral cortex (light gray bars) and hypothalamus (dark gray bars) during sleep deprivation. These categories have an FDR estimated at $<1\%$. At an FDR of $<1\%$, there were no functional categories upregulated in the cerebral cortex during sleep deprivation. The y-axis represents $-\log_{10}$ of P values determined by EASE. Note that in the hypothalamus, at the FDR level of $<1\%$, the overrepresented functional categories in the gene ontology of biological process contains genes involved in cell growth, cytoskeleton organization, and biogenesis (data not shown). Functional categories of genes present in the gene ontology of cellular component and molecular function along with the P values by EASE, as well as the corresponding FDR are provided in the Supplemental Tables S3_cerebral cortex and S3_hypothalamus. Note that a number of genes involved in cholesterol synthesis and transport that were upregulated during sleep in the cerebral cortex and hypothalamus are downregulated in these brain regions during sleep deprivation.

teins, although these genes did not form an overrepresented category. The fact that sleep deprivation increases expression of molecular chaperones has been described previously in the rat (77), mouse (49), and *Drosophila* (69) led us to examine this group. In the cerebral cortex, the following genes encoding heat shock proteins and molecular chaperones upregulated the steady-state levels of their transcripts during sleep deprivation: *Dnajc3*, *Dnajb5* (*Hsp40*), *Dnajb11*, *Dnajc1*, *Hsp105*, *Hspa1a*, *Hspa1b*, and *Hspa5* (*Bip*), while in the hypothalamus *Dnajc3*, *Dnajb5*, *Dnajb11*, and *Hspa5* genes increased their transcript levels during sleep deprivation [see changes in expression of *Hspa5* (*Bip*) in Fig. 7, left]. Such increases in molecular chaperones suggest that with sleep deprivation there is cellular stress and the unfolded protein response is activated.

The unfolded protein response occurs when proteins are not being properly folded, as occurs during hypoxia, glucose starvation, etc. (for reviews, see Refs. 27, 40, 67). Indeed, all steps in the unfolded protein response occur with 6 h of sleep deprivation in mouse cerebral cortex (49). The unfolded protein response involves facilitatory refolding of misfolded proteins or targeting them for degradation. Moreover, translation of proteins is inhibited, thereby reducing the burden of misfolded proteins. This is, however, likely not the only mecha-

nism that reduces synthesis of proteins with sleep deprivation. Microarray studies in *Drosophila* show that multiple steps in the protein synthesis pathway are downregulated with sleep deprivation (83). In the current study in mice, we also observe downregulation of genes for key components of protein production with extended wakefulness.

Increase in the steady-state level of Homer1 mRNA with sleep deprivation. Another gene that has been described to increase during sleep deprivation in a previous microarray study in the rat is *Homer* (12, 78). *Homer1a*, which is an immediate early gene transcript of *Homer1*, has been shown to be upregulated by sleep loss in the rat cerebral cortex (51). We thus believe that the *Homer1* transcript we observe upregulated in our studies is likely to be *Homer1a* (see expression of *Homer1* gene in Fig. 7, right). Homer proteins are bipartite scaffolding proteins that bind to an array of other proteins, like the metabotropic glutamate receptors (mGluR) and IP3 receptors, and allow them to create multiprotein complexes. *Homer1a* protein lacks the COOH terminal domain required for multimerization and therefore behaves as a dominant negative element. The expression of *Homer1a* gene is dynamically regulated in response to synaptic activity (2, 64, 72, 82), unlike the other members of the Homer family, which are constitutively expressed. *Homer1a* gene has been shown to be rapidly

Fig. 7. Temporal changes in the expression of *Hspa5* (*Bip*, left), and *Homer1* (right) genes in the cerebral cortex (circles) and hypothalamus (triangles), respectively. The profiles were established after 3, 6, 9, and 12 h of spontaneous sleep (● or ▲) or sleep deprivation (○ or △). The mRNA level of *Hspa5* and *Homer1* genes increases during sleep deprivation and does not change during sleep. Transcript abundance change is expressed in arbitrary units and is the $\log_2$ of mean fluorescence signal for each time point minus that of the control animals killed at time 0.

upregulated in multiple models of activity dependent plasticity and is thought to modulate mGluR signaling by uncoupling mGluR-Homer-IP3R complexes. *Homer1a* overexpression has been shown to downregulate synapse formation (62). Consistent with the observation that *Homer1a* gene is upregulated in the mouse cerebral cortex with sleep deprivation are data from a *Drosophila* brain microarray study conducted by our group. The single *Homer* gene in *Drosophila* is upregulated with sleep and downregulated during sleep deprivation (83). Since this single molecule in *Drosophila* is an activator (not a dominant negative), the effect of this change will have the same functional result as in mammals even though the pattern of change is exactly opposite to that for *Homer1a*.

Homer is also one of the genes in the quantitative trait locus region for altered temporal accumulation of sleep drive in different inbred mouse strains (21) and is a likely candidate to explain this phenotype. It is very likely that the upregulation of *Homer1a* serves to terminate wakefulness through disruption of the mGluR-Homer-IP3 complexes, which in turn will attenuate glutamate signaling.

Genes That Downregulate the Steady-State Level of Their Transcripts during Sleep Deprivation

The largest number of genes was those downregulated with sleep deprivation. Indeed, out of all the temporal classes, there were more genes with this response pattern than any other (see Table 2). This is identical to recent data from similar studies in the *Drosophila* brain that also found that the largest class of genes differentially expressed was genes downregulated with sleep deprivation (83). Thus, it appears that if wakefulness is prolonged there are multiple mechanisms to limit it being sustained. In both the cerebral cortex and hypothalamus, there were a number of downregulated genes during sleep deprivation that encoded enzymes of various intermediary biosynthetic metabolic pathways (see Supplemental Tables S3_cerebral cortex and S3_hypothalamus). In general, genes that were upregulated during sleep were downregulated during sleep deprivation. For example, we found that the cholesterol synthesis genes were downregulated with sleep deprivation in both the cerebral cortex and hypothalamus (Fig. 5). Proteins associated with lipid rafts such as flotillin were also downregulated (Fig. 5E). Thus, it appears that during sleep deprivation lipid rafts are disassembled, thereby reducing the strength of cell signaling (for instance glutamate), making wakefulness more difficult to sustain. The reduction during sleep deprivation in expression of genes encoding proteins involved in the main pathways of carbohydrate metabolism, energy metabolism, tricarboxylic acid metabolism, and various metabolic pathways (lipid, aldehyde, amine) also supports the idea that as wakefulness is prolonged there are various mechanisms that limit the ability for wakefulness to be sustained. We postulate that the reduction of cellular metabolism is one such limiting mechanism.

Magnitude of change in gene expression and implications for protein synthesis. The analysis of differential gene expression in this study did not rely on the ability to detect changes at an arbitrary fold-change level. Rather, we used a stringent FDR of 1% to determine differentially expressed genes. While this limits false positives, there could be also false negatives. For example, expression of a number of genes encoding

proteins involved in cholesterol synthesis and transport, relevant transcription factors, or lipid raft proteins that did not pass the 1% FDR cut-off in one or other brain region had an FDR well below 5%. For example, the FDR for the *Gnail* gene encoding lipid raft-associated protein was ~0.8% in the cerebral cortex and 1.2% in the hypothalamus. Thus, the *Gnail* gene failed the 1% cut-off in the hypothalamus.

Genes that did pass the 1% FDR test as being differentially expressed between sleep and sleep deprivation did not, however, show major differences in mRNA levels between behavioral states. In the cerebral cortex ~95% of the differentially expressed genes modulated their expression by <50%. We believe that such modest changes in mRNAs will be typical of changes found with variations in normal behavioral states. The extent of change in the transcript levels between sleep and sleep deprivation observed in this study is comparable to that previously reported in the rat (12). To assess the magnitude of fold change in transcript levels, differences in expression measures between sleep and sleep deprivation were calculated at each time point. The largest difference in gene expression between any time point was used to represent the fold change. We illustrate this here for genes in the cholesterol synthesis pathway. For most genes, degree of change was similar in the cerebral cortex and hypothalamus (Table 3). The degree of change across genes was from a low of 1.07 to a high of 1.65. The statistical significance of these changes was, however, high with an estimated FDR much lower than 1% for each of the genes (for many genes <10⁻¹⁶) (see columns labeled FDR in Table 3).

Our study, as well as other microarray studies, assesses changes in the steady-state level of mRNA for various genes. This will depend on both transcription rates of the relevant genes and stability of their mRNA. There is growing appreciation for the discordance between mRNA levels and protein abundance; the changes in transcriptome may or may not be reflected in the level of relevant proteins (proteome). Assessment of the transcriptome allows one to infer what physiological processes might be impacted in the cell, while the composition of the proteome indicates what processes are actually affected. Attempts to measure the abundance of mRNA bound to polysomes by microarrays may address this discordance (43). However, such studies are beyond the scope of our current research. Our data lead to different possible interpretations. For example, an increase during sleep in the transcript levels of numerous gene encoding proteins involved in cholesterol synthesis and transport, relevant transcription factors, or lipid raft proteins may indicate an enhancement of cholesterol synthesis during sleep or, alternatively, a preparatory step for the augmentation of cholesterol synthesis and transport during upcoming wakefulness. Extensive studies would be required to assess the concordance of mRNA and protein changes in the brain during sleep and sleep deprivation.

Comparison between changes in gene expression in the cerebral cortex and hypothalamus. As described, we identified certain processes that changed similarly in both brain regions such as cholesterol biosynthesis during sleep and increases in molecular chaperones with sleep deprivation. However, for other processes, such as heme synthesis, the changes were limited to the cerebral cortex. A previous microarray study of changes in expression with sleep/wakefulness found largely identical changes in the cerebral cortex and cerebellum, lead-

ing the authors to conclude that there were ubiquitous changes in expression that are found in all brain regions (12). In contrast, Terao et al. (78) found in another microarray study that there were differences in the genes that changed expression in the cerebral cortex and hypothalamus. We would agree with this assertion. However, such differences need to be interpreted with caution. The hypothalamus, in particular, is made up of a number of different neuronal groups with different functions, as well as glia cells. Thus, there could be changes in expression of genes in distinct neuronal populations that are not found when averaged across a larger amount of tissue. Future studies need to move toward expression profiling in specific relevant neuronal populations using laser capture microdissection techniques as has been applied previously to gene expression profiling in nuclei and subnuclei in mammalian brain (4).

Comments on study design. The statistical analyses were performed in a step-wise fashion. The first statistical test identified genes differentially expressed between groups of sleeping and sleep-deprived animals. We were primarily interested in finding differences between behavioral states rather than how different states may differ across time. In *stage 1* of our analysis, we tested whether the time-averaged effect of sleep state (i.e., our main effect) differs between “awake” and “sleeping” mice. At this stage of the analysis, time was taken into account by averaging our main effect levels of state (i.e., “sleep deprived” or “sleeping”) over all the levels of time (i.e., 3, 6, 9, 12 h). Our study had substantial statistical power to detect change in gene expression since for each brain region we used, for comparison in our primary analysis, 20 arrays from sleeping animals and 20 from sleep-deprived animals. Moreover, a robust statistical approach, based on FDR, was used to control the false positive rate at a stringent level of $<1\%$. We also added an anchor group at time zero, so that we could then determine direction of change in steady-state transcript levels. This was performed in the second step of data analysis (see “Trends analysis” in Fig. 1B) and was aimed at interpreting the results from our first step by focusing on how gene expression changes over time. Thus, the second step was to uncover a temporal pattern of gene expression within the sleeping group and sleep-deprived group separately and utilized the 7:00 AM anchor point to facilitate data analyses of temporal trend. The trend analysis was linear regression; it was used simply to determine the direction of change, and we do not interpret magnitude of slope or whether a linear relationship was the optimal one, particularly given the small number of time points assessed (4 in each group). Deviations from linearity could relate to different temporal regulation of expression and/or varying amounts of sleep across the day. For most differentially expressed genes, direction of change could be assessed by the linear trend analysis. However, for a small subset of genes, direction of change could not be determined in this way and we used an area-under-the-curve analysis.

Since mice in each experimental group were killed at the same diurnal time point (e.g., 3-h sleep deprivation was concluded at 10:00 AM, and mice sleeping for 3 h were also killed at 10:00 AM), genes which had a pure diurnal effect on their expression were eliminated at this step. Since the purpose of our study was to identify genes with a sleep-specific pattern of expression, we did not perform comparisons of our results with previously published data on genes with diurnal and/or circa-

dian pattern of expression; we do not believe that comparing our results with previously published data on genes with the diurnal/circadian influences is warranted. However, we appreciate that there are a number of comprehensive microarray studies in various organisms on the impact of diurnal and circadian factors on steady-state mRNA level (1, 10, 38, 45, 57).

In our study, we chose to assess amounts of sleep and wakefulness based on rest/activity measures and a definition of sleep as ≥ 40 s of continuous inactivity. We have recently shown that this provides a highly accurate estimate of sleep and wakefulness amounts in male C57BL/6J mice of the age used in this study (56). As expected, the amounts of sleep and wakefulness in the mice studied here were essentially identical to data that were obtained by EEG/EMG recordings of sleep in male mice of this age and genotype. Our approach has the advantage that it avoids subjecting mice to prior surgery and having chronically implanted electrodes. Such implantation on its own changes the pattern of gene expression [e.g., immediately early gene *Fos* in the rat brain (41)].

We did not estimate sleep in animals during the period of sleep deprivation. The constant and consistent motor activity throughout the deprivation period enforced by the ever-present experimenter assured that mice experienced little if any sleep during this time. It is conceivable that mice had episodes of microsleep that are more likely to occur during longer periods of sleep deprivation. Microsleeps will, however, not influence our results since the goal of this study was to compare gene expression in animals that were allowed to sleep spontaneously and killed at the same time of day as animals that had extensive sleep deprivation prior to death.

Our study involved sleep depriving mice by gentle handling (36). This approach to sleep deprivation produces the least change compared with other techniques for sleep deprivation in expression of the *Fos* gene in brain areas associated with the stress response (36); see also Ref. 41. Nevertheless, we cannot exclude the possibility that the mice that were sleep deprived were stressed. Most of the major conclusions of our study come, however, from analysis within the undisturbed sleeping groups. There is no reason to believe that these mice are stressed, and we conclude that our findings are specific to the sleep state. For analysis of sleeping mice, the sleep-deprived groups serve as controls for diurnal variation, thereby excluding such influences. We cannot exclude, however, that stress effects could, at least in part, be responsible for the changes that we describe that take place during sleep deprivation.

Given our design, it is conceivable that if expression of a particular gene was not regulated by sleep/wake but was affected by the stress associated with sleep deprivation, it would appear as a differentially expressed gene. For it to appear in the genes we are discussing as increasing during sleep, the primary focus of our results, it would also need to have a circadian regulation to explain the temporal change in the sleep groups. Moreover, since we are primarily focusing on genes where expression increased with sleep and either decreased or stayed the same with extended wakefulness, expression of this gene would need to decrease with stress by an amount equal to or greater than the increase produced by the postulated circadian effect during the inactive period. This specific combination, while possible, seems highly unlikely. Further support for this assertion comes from our observations

that we largely replicate previous findings from microarray studies of sleep/wake differences, which used different behavioral approaches to address this issue (see below). We do, however, find that many more genes are differentially expressed with sleep and wakefulness given the power of our study and study design.

Comparison of results presented here to previous microarray studies of sleep and wakefulness. Our results can be compared with previous studies using microarrays to examine changes in gene expression with sleep and wakefulness (12, 78), both of which were conducted in rats. In the study of Terao et al. (78), changes in expression were compared between sleep-deprived and sleeping rats and during recovery sleep following deprivation. The investigators used an Affymetrix Rat Neurobiology U34 GeneChip containing 1,322 elements and hence did not perform as comprehensive an analysis as described here. More importantly, they set an arbitrary threshold of a 50% change in expression to call a gene as being differentially expressed. As shown here, and in the previous study by Cirelli et al. (12), the changes in expression between sleep and wakefulness of the majority of differentially expressed genes are <50%. Thus, this analysis strategy missed the majority of differentially expressed genes being described here. Nevertheless, 36.3% of the genes labeled as being differentially expressed by Terao et al. (78) were also identified by us with a FDR of <1%; at the FDR <5 or 10%, the percentage of genes identified by Terao et al. and found in our study was 50.8 and 61.7%, respectively (Table 4).

The study of Cirelli et al. (12) also had important differences to the design used here. First, sleep and wakefulness were considered binary states such that the direction of change was not assessed. Interpretation of the results assumed that gene expression only changed in one direction, i.e., upwards, and hence genes higher in sleep than wakefulness were called "sleep-specific" genes, while genes expressed higher in wakefulness than sleep were called "wakefulness-specific" genes. In this study we also used terms sleep and wakefulness specific, although we do not imply that these transcripts are restricted to or exclusive to wakefulness or sleep. Unlike in the previous study, the use of the terms sleep and wakefulness specific was based on the temporal pattern of expression across five time points, and we describe genes that both increase or decrease their expression during these states. The study of Cirelli et al. (12) had less power than that reported here since changes between sleep and wakefulness were assessed in three techni-

cal replicates after pooling RNA from six rats; pooling was used to reduce biological variability. In our study, the primary analysis was based on comparison between 20 sleeping mice and 20 sleep-deprived mice without pooling across mice. In Cirelli et al. (12) sleep-related transcripts were required to be greater in at least half of all comparisons of sleep vs. sleep deprivation and also sleep vs. spontaneous wakefulness. Similarly, transcripts called wakefulness-related were required to be greater in at least half of all comparisons to sleep and also sleep deprivation samples (12). Despite these major differences in design, we find that 53% of genes labeled differentially expressed by Cirelli et al. (12) were found to be differentially expressed by us using the stringent FDR of <1% (see Table 4). If we relax our FDR to <5%, we find the number of the genes identified in Cirelli et al. (12) that we call differentially expressed increases to 61.9 and 70.8% for their wakefulness- and sleep-specific genes, respectively. With the FDR of <10%, this percentage increases further to 69.4% for their wakefulness-specific genes and 82.4% for their sleep-specific genes. There were only a few genes found in Cirelli et al. (12) that had a very high FDR, e.g., $\geq 20\%$ (11.3 and 9.3% for their wakefulness- and sleep-specific genes, respectively). These are likely to be true false positives. The largest difference, however, between our results and that of Cirelli et al. (12) is in the number of genes. We found a total of 3,988 genes differentially expressed in the cerebral cortex, whereas Cirelli et al. (12) found only 715. Thus, as anticipated, the major issue with studies with less power is false negative results. Interestingly, despite this difference, we find that similar processes are identified in our studies and that of Cirelli et al. (12). For example, Cirelli et al. reported that cholesterol synthesis increased in brain during sleep on the basis of the observation that the genes encoding Hmgcs and Fdft1 proteins increased during sleep. We confirmed this, but in addition, with our larger power and FDR strategy, found that expression of all genes in the cholesterol synthesis pathway is increased during sleep, as are the relevant transcription factors (see above).

Thus, previous concerns that microarray results cannot be replicated (see for example Ref. 20) are not supported by our data, a conclusion that is in line with recent studies on the topic of reliability of microarray results (26, 70).

Concluding remarks. The data obtained in this study lead to some key conclusions about alterations in gene transcription in sleep and wakefulness. Our data support the assertion of our recent microarray study in *Drosophila* brain (83), that a key regulator of sleep and wakefulness is not simply increasing levels of sleep-promoting molecules with increases in wakefulness but, rather, downregulation of a number of key processes, such as protein synthesis, to limit the duration of wakefulness that can be sustained. As *Drosophila* temporal analysis reveals, the largest group of differentially regulated genes are those that decrease their expression as wakefulness is prolonged through sleep deprivation. During sleep deprivation, there is evidence of cellular stress, and hence, the state needs to be terminated to allow recovery to occur. The most important novel conclusion of our study is, however, that sleep is a stage of biosynthesis of diverse macromolecules. The functions of sleep have long been illusory, and this study lays groundwork for understanding this at a molecular level and leads to a number of testable hypotheses.

Table 4. The percentages of wakefulness- and sleep-specific genes common between results presented here and that of Cirelli et al. (12) (top and middle), and wakefulness-specific of Terao et al. (78) (bottom) at different FDR in our study

	FDR					
	<1%	1–5%	5–10%	10–15%	15–20%	>20%
Wakefulness	53.1	8.8	7.5	6.3	12.6	11.3
Sleep	53.4	17.4	11.6	3.4	4.6	9.3
Wakefulness	36.3	14.5	10.9	9.0	5.4	23.6

Note, that for the FDR of <1% there is ~53% agreement between this study and Cirelli et al. (12) and for FDR $\leq 10\%$, there is ~69% wakefulness-specific and 82% sleep-specific genes that are common between this paper and previous results in the rat. These numbers were obtained by adding percentages in the different columns.

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REFERENCES

- Akhtar RA, Reddy AB, Maywood ES, Clayton JD, King VM, Smith AG, Gant TW, Hastings MH, Kyriacou CP. Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic nucleus. *Curr Biol* 12: 540–550, 2002.
- Ange F, Pin JP, Tu JC, Xiao B, Worley PF, Bockaert J, Fagni L. Dendritic and axonal targeting of type 5 metabotropic glutamate receptor is regulated by homer1 proteins and neuronal excitation. *J Neurosci* 20: 8710–8716, 2000.
- Bobillier P, Sakai F, Seguin S, Jouvet M. Deprivation of paradoxical sleep and in vitro cerebral protein synthesis in the rat. *Life Sci* 10: 1349–1357, 1971.
- Bonaventure P, Guo H, Tian B, Liu X, Bittner A, Roland B, Salunga R, Ma XJ, Kamme F, Meurers B, Bakker M, Jurzak M, Laysen JE, Erlander MG. Nuclei and subnuclei gene expression profiling in mammalian brain. *Brain Res* 943: 38–47, 2002.
- Borbely AA, Achermann P. Sleep homeostasis and models of sleep regulation. In: *Principle and Practice of Sleep Medicine*, edited by Kryger MH, Roth T, and Dement WC. Philadelphia: Elsevier, 2005, p. 405–418.
- Braun AR, Balkin TJ, Wesenten NJ, Carson RE, Varga M, Baldwin P, Selbie S, Belenky G, Herscovitch P. Regional cerebral blood flow throughout the sleep-wake cycle. An H2(15)O PET study. *Brain* 120: 1173–1197, 1997.
- Butchbach ME, Tian G, Guo H, Lin CL. Association of excitatory amino acid transporters, especially EAAT2, with cholesterol-rich lipid raft microdomains: importance for excitatory amino acid transporter localization and function. *J Biol Chem* 279: 34388–34396, 2004.
- Cabin DE, Shimazu K, Murphy D, Cole NB, Gottschalk W, McIlwain KL, Orrison B, Chen A, Ellis CE, Paylor R, Lu B, Nussbaum RL. Synaptic vesicle depletion correlates with attenuated synaptic responses to prolonged repetitive stimulation in mice lacking alpha-synuclein. *J Neurosci* 22: 8797–8807, 2002.
- Carskadon MA, Dement WC. Normal human sleep: an overview. In: *Principles and Practice of Sleep Medicine*, edited by Kryger MH, Roth B, and Dement WC. Philadelphia: Elsevier, 2005, p. 13–24.
- Ceriani MF, Hogenesch JB, Yanovsky M, Panda S, Straume M, Kay SA. Genome-wide expression analysis in *Drosophila* reveals genes controlling circadian behavior. *J Neurosci* 22: 9305–9319, 2002.
- Churchill GA. Using ANOVA to analyze microarray data. *Biotechniques* 37: 173–175, 177, 2004.
- Cirelli C, Gutierrez CM, Tononi G. Extensive and divergent effects of sleep and wakefulness on brain gene expression. *Neuron* 41: 35–43, 2004.
- Cirelli C, Tononi G. Differences in gene expression between sleep and waking as revealed by mRNA differential display. *Brain Res Mol Brain Res* 56: 293–305, 1998.
- Cohen P. The structure and regulation of protein phosphatases. *Annu Rev Biochem* 58: 453–508, 1989.
- Damiola F, Minh NL, Preitner N, Kornmann B, Fleury-Olela F, Schibler U. Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. *Genes Dev* 14: 2950–2961, 2000.
- Dennis G Jr, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, Lempicki RA. DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol* 4: P3, 2003.
- Ding J, Gip P, Franken P, Lomas L, O'Hara B. A proteomic analysis in brain following sleep deprivation suggests a generalized decrease in abundance for many proteins. *Sleep* 27: A391–392, 2004.
- Edwards PA, Tabor D, Kast HR, Venkateswaran A. Regulation of gene expression by SREBP and SCAP. *Biochim Biophys Acta* 1529: 103–113, 2000.
- Eroglu C, Brugger B, Wieland F, Sinning I. Glutamate-binding affinity of *Drosophila* metabotropic glutamate receptor is modulated by association with lipid rafts. *Proc Natl Acad Sci USA* 100: 10219–10224, 2003.
- Etter PD, Ramaswami M. The ups and downs of daily life: profiling circadian gene expression in *Drosophila*. *Bioessays* 24: 494–498, 2002.
- Franken P, Chollet D, Tafti M. The homeostatic regulation of sleep need is under genetic control. *J Neurosci* 21: 2610–2621, 2001.
- Franklin KBJ, Paxinos G. *The Mouse Brain in Stereotaxic Coordinates*. San Diego, CA: Academic, 1996.
- Gautier L, Cope L, Bolstad BM, Irizarry RA. affy-analysis of Affymetrix GeneChip data at the probe level. *Bioinformatics* 20: 307–315, 2004.
- Geppert M, Bolshakov VY, Siegelbaum SA, Takei K, De Camilli P, Hammer RE, Sudhof TC. The role of Rab3A in neurotransmitter release. *Nature* 369: 493–497, 1994.
- Grassi Zucconi G, Cipriani S, Balgouranidou I, Scattoni R. 'One night' sleep deprivation stimulates hippocampal neurogenesis. *Brain Res Bull* 69: 375–381, 2006.
- Guo L, Lobenhofer EK, Wang C, Shippey R, Harris SC, Zhang L, Mei N, Chen T, Herman D, Goodsaid FM, Hurban P, Phillips KL, Xu J, Deng X, Sun YA, Tong W, Dragan YP, Shi L. Rat toxicogenomic study reveals analytical consistency across microarray platforms. *Nat Biotechnol* 24: 1162–1169, 2006.
- Harding HP, Calfon M, Urano F, Novoa I, Ron D. Transcriptional and translational control in the mammalian unfolded protein response. *Annu Rev Cell Dev Biol* 18: 575–599, 2002.
- Hering H, Lin CC, Sheng M. Lipid rafts in the maintenance of synapses, dendritic spines, and surface AMPA receptor stability. *J Neurosci* 23: 3262–3271, 2003.
- Hosack DA, Dennis G, Sherman BT, Lane HC, Lempicki RA. Identifying biological themes within lists of genes with EASE. *Genome Biology* 4: P4, 2003.
- Ikeda M, Ikeda-Sagara M, Okada T, Clement P, Urade Y, Nagai T, Sugiyama T, Yoshioka T, Honda K, Inoue S. Brain oxidation is an initial process in sleep induction. *Neuroscience* 130: 1029–1040, 2005.
- Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* 4: 249–264, 2003.
- Jacob Blackmon B, Dailey TA, Lianchun X, Dailey HA. Characterization of a human and mouse tetrahydropyridine-binding protein. *Arch Biochem Biophys* 407: 196–201, 2002.
- Jones BE. Basic mechanisms of sleep-wake states. In: *Principles and Practice of Sleep Medicine*, edited by Kryger MH, Roth T, and Dement WC. Philadelphia: Elsevier Saunders, 2005, p. 136–153.
- Kapfhammer D, Valladares O, Sun Y, Nolan PM, Rux JJ, Arnold SE, Veasey SC, Bukan M. Mutations in Rab3a alter circadian period and homeostatic response to sleep loss in the mouse. *Nat Genet* 32: 290–295, 2002.
- Kennedy C, Gillin JC, Mendelson W, Suda S, Miyaoka M, Ito M, Nakamura RK, Storch FI, Pettigrew K, Mishkin M, Sokoloff L. Local cerebral glucose utilization in non-rapid eye movement sleep. *Nature* 297: 325–327, 1982.
- Ledoux L, Sastre JP, Buda C, Luppi PH, Jouvet M. Alterations in c-fos expression after different experimental procedures of sleep deprivation in the cat. *Brain Res* 735: 108–118, 1996.
- Leeksa OC, Van Achterberg TA, Tsumura Y, Toshima J, Eldering E, Kroes WG, Mellink C, Spaargaren M, Mizuno K, Pannekoek H, de Vries CJ. Human sprouty 4, a new ras antagonist on 5q31, interacts with the dual specificity kinase TESK1. *Eur J Biochem* 269: 2546–2556, 2002.
- Lin Y, Han M, Shimada B, Wang L, Gibler TM, Amarakone A, Awad TA, Stormo GD, Van Gelder RN, Taghert PH. Influence of the period-dependent circadian clock on diurnal, circadian, and aperiodic gene expression in *Drosophila melanogaster*. *Proc Natl Acad Sci USA* 99: 9562–9567, 2002.
- Luo Z, Geschwind DH. Microarray applications in neuroscience. *Neurobiol Dis* 8: 183–193, 2001.
- Ma Y, Hendershot LM. The unfolding tale of the unfolded protein response. *Cell* 107: 827–830, 2001.
- Maloney KJ, Mainville L, Jones BE. c-Fos expression in dopaminergic and GABAergic neurons of the ventral mesencephalic tegmentum after paradoxical sleep deprivation and recovery. *Eur J Neurosci* 15: 774–778, 2002.

42. Maquet P, Degueldre C, Delfiore G, Aerts J, Peters JM, Luxen A, Franck G. Functional neuroanatomy of human slow wave sleep. *J Neurosci* 17: 2807–2812, 1997.
43. Mazan-Mamczarz K, Kawai T, Martindale JL, Gorospe M. En masse analysis of nascent translation using microarrays. *Biotechniques* 39: 61–62, 64, 66–67, 2005.
44. McCoubrey WK Jr, Huang TJ, Maines MD. Heme oxygenase-2 is a hemoprotein and binds heme through heme regulatory motifs that are not involved in heme catalysis. *J Biol Chem* 272: 12568–12574, 1997.
45. McDonald MJ, Rosbash M. Microarray analysis and organization of circadian gene expression in *Drosophila*. *Cell* 107: 567–578, 2001.
46. Mehrabian Z, Liu LI, Fiskum G, Rapoport SI, Chandrasekaran K. Regulation of mitochondrial gene expression by energy demand in neural cells. *J Neurochem* 93: 850–860, 2005.
47. Mirnics K. Microarrays in brain research: the good, the bad and the ugly. *Nat Rev Neurosci* 2: 444–447, 2001.
48. Morrow IC, Parton RG. Flotillins and the PHB domain protein family: rafts, worms and anaesthetics. *Traffic* 6: 725–740, 2005.
49. Naidoo N, Giang W, Galante RJ, Pack AI. Sleep deprivation induces the unfolded protein response in mouse cerebral cortex. *J Neurochem* 92: 1150–1157, 2005.
50. Nakanishi H, Sun Y, Nakamura RK, Mori K, Ito M, Suda S, Namba H, Storch FI, Dang TP, Mendelson W, Mishkin M, Kennedy C, Gillin JC, Smith CB, Sokoloff L. Positive correlations between cerebral protein synthesis rates and deep sleep in *Macaca mulatta*. *Eur J Neurosci* 9: 271–279, 1997.
51. Nelson SE, Duricka DL, Campbell K, Churchill L, Krueger JM. Homer1a and 1bc levels in the rat somatosensory cortex vary with the time of day and sleep loss. *Neurosci Lett* 367: 105–108, 2004.
52. Nguyen HT, Amine AB, Lafitte D, Waheed AA, Nicoletti C, Villard C, Letisse M, Deyris V, Roziere M, Tchiakpe L, Danielle CD, Comeau L, Hiol A. Proteomic characterization of lipid raft markers from the rat intestinal brush border. *Biochem Biophys Res Commun* 342: 236–244, 2006.
53. Nikonova EV, Vijayasarathy C, Zhang L, Cater JR, Galante RJ, Ward SE, Avadhani NG, Pack AI. Differences in activity of cytochrome C oxidase in brain between sleep and wakefulness. *Sleep* 28: 21–27, 2005.
54. Nofzinger EA, Buysse DJ, Miewald JM, Meltzer CC, Price JC, Sembrat RC, Ombao H, Reynolds CF, Monk TH, Hall M, Kupfer DJ, Moore RY. Human regional cerebral glucose metabolism during non-rapid eye movement sleep in relation to waking. *Brain* 125: 1105–1115, 2002.
55. Pace-Schott EF, Hobson JA. The neurobiology of sleep: genetics, cellular physiology and subcortical networks. *Nat Rev Neurosci* 3: 591–605, 2002.
56. Pack AI, Galante RJ, Maislin G, Cater J, Metaxas D, Lu S, Zhang L, Von Smith R, Kay T, Lian J, Svenson K, Peters LL. Novel method for high-throughput phenotyping of sleep in mice. *Physiol Genomics* 28: 232–238, 2007.
57. Panda S, Antoch MP, Miller BH, Su AI, Schook AB, Straume M, Schultz PG, Kay SA, Takahashi JS, Hogenesch JB. Coordinated transcription of key pathways in the mouse by the circadian clock. *Cell* 109: 307–320, 2002.
58. Porter RH, Greene JG, Higgins DS Jr, Greenamyre JT. Polysynaptic regulation of glutamate receptors and mitochondrial enzyme activities in the basal ganglia of rats with unilateral dopamine depletion. *J Neurosci* 14: 7192–7199, 1994.
59. Qin Q, Inatome R, Hotta A, Kojima M, Yamamura H, Hirai H, Yoshizawa T, Tanaka H, Fukami K, Yanagi S. A novel GTPase, CRAG, mediates promyelocytic leukemia protein-associated nuclear body formation and degradation of expanded polyglutamine protein. *J Cell Biol* 172: 497–504, 2006.
60. Ramm P, Smith CT. Rates of cerebral protein synthesis are linked to slow wave sleep in the rat. *Physiol Behav* 48: 749–753, 1990.
61. Richardson K, Rose SP. A diurnal rhythmicity in incorporation of lysine into rat brain regions. *Nat New Biol* 233: 182–184, 1971.
62. Sala C, Piech V, Wilson NR, Passafaro M, Liu G, Sheng M. Regulation of dendritic spine morphology and synaptic function by Shank and Homer. *Neuron* 31: 115–130, 2001.
63. Saper CB, Chou TC, Scammell TE. The sleep switch: hypothalamic control of sleep and wakefulness. *Trends Neurosci* 24: 726–731, 2001.
64. Sato M, Suzuki K, Nakanishi S. NMDA receptor stimulation and brain-derived neurotrophic factor upregulate homer 1a mRNA via the mitogen-activated protein kinase cascade in cultured cerebellar granule cells. *J Neurosci* 21: 3797–3805, 2001.
65. Schneggenburger R, Sakaba T, Neher E. Vesicle pools and short-term synaptic depression: lessons from a large synapse. *Trends Neurosci* 25: 206–212, 2002.
66. Schratzenholz A, Soskic V. NMDA receptors are not alone: dynamic regulation of NMDA receptor structure and function by neuregulins and transient cholesterol-rich membrane domains leads to disease-specific nuances of glutamate-signalling. *Curr Top Med Chem* 6: 663–686, 2006.
67. Schroder M, Kaufman RJ. The mammalian unfolded protein response. *Annu Rev Biochem* 74: 739–789, 2005.
68. Shaw PJ, Cirelli C, Greenspan RJ, Tononi G. Correlates of sleep and waking in *Drosophila melanogaster*. *Science* 287: 1834–1837, 2000.
69. Shaw PJ, Tononi G, Greenspan RJ, Robinson DF. Stress response genes protect against lethal effects of sleep deprivation in *Drosophila*. *Nature* 417: 287–291, 2002.
70. Shi L, Reid LH, Jones WD, Shippey R, Warrington JA, Baker SC, Collins PJ, de Longueville F, Kawasaki ES, Lee KY, Luo Y, Sun YA, Willey JM, Setterquist RA, Fischer GM, Tong W, Dragan YP, Dix DJ, Frueh FW, Goodsaid FM, Herman D, Jensen RV, Johnson CD, Lobenhofer EK, Puri RK, Schrf U, Thierry-Mieg J, Wang C, Wilson M, Wolber PK, Zhang L, Slikker W Jr. The MicroArray Quality Control (MAQC) project shows inter- and intraplatform reproducibility of gene expression measurements. *Nat Biotechnol* 24: 1151–1161, 2006.
71. Simons K, Toomre D. Lipid rafts and signal transduction. *Nat Rev Mol Cell Biol* 1: 31–39, 2000.
72. Soloviev MM, Ciruela F, Chan WY, McIlhinney RA. Molecular characterisation of two structurally distinct groups of human homers, generated by extensive alternative splicing. *J Mol Biol* 295: 1185–1200, 2000.
73. Storey JD. A direct approach to false discovery rates. *J Royal Stat Soc B* 64: 479–498, 2002.
74. Stoughton RB. Applications of DNA microarrays in biology. *Annu Rev Biochem* 74: 53–82, 2005.
75. Strumilo S. Short-term regulation of the alpha-ketoglutarate dehydrogenase complex by energy-linked and some other effectors. *Biochemistry* 70: 726–729, 2005.
76. Sudhof TC. The synaptic vesicle cycle. *Annu Rev Neurosci* 27: 509–547, 2004.
77. Terao A, Steininger TL, Hyder K, Apte-Deshpande A, Ding J, Rishipathak D, Davis RW, Heller HC, Kilduff TS. Differential increase in the expression of heat shock protein family members during sleep deprivation and during sleep. *Neuroscience* 116: 187–200, 2003.
78. Terao A, Wisor JP, Peyron C, Apte-Deshpande A, Wurts SW, Edgar DM, Kilduff TS. Gene expression in the rat brain during sleep deprivation and recovery sleep: an Affymetrix GeneChip study. *Neuroscience* 137: 593–605, 2006.
79. Vila M, Levy R, Herrero MT, Faucheux B, Obeso JA, Agid Y, Hirsch EC. Metabolic activity of the basal ganglia in parkinsonian syndromes in human and non-human primates: a cytochrome oxidase histochemistry study. *Neuroscience* 71: 903–912, 1996.
80. Wong-Riley M, Carroll EW. Effect of impulse blockage on cytochrome oxidase activity in monkey visual system. *Nature* 307: 262–264, 1984.
81. Wu H, Kerr K, Churchill GA. MAANOVA: a Software package for the analysis of spotted cDNA microarray experiments. In: *The Analysis of Gene Expression Data: an Overview of Methods and Software*, edited by Parmigiani G, Garrett ES, Irizarry RA, and Zeger SL. New York: Springer, 2003, p. 313–431.
82. Xiao B, Tu JC, Petralia RS, Yuan JP, Doan A, Breder CD, Ruggiero A, Lanahan AA, Wenthold RJ, Worley PF. Homer regulates the association of group I metabotropic glutamate receptors with multivalent complexes of homer-related, synaptic proteins. *Neuron* 21: 707–716, 1998.
83. 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.