Extensive and Divergent Effects of Sleep and Wakefulness on Brain Gene Expression

Report

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Summary

Sleep is present in all species where it has been studied, but its functions remain unknown. To investigate what benefits sleep may bring at the cellular level, we profiled gene expression in awake and sleeping rats by using high-density microarrays. We find that ~10% of the transcripts in the cerebral cortex change their expression between day and night and demonstrate that half of them are modulated by sleep and wakefulness independent of time of day. We also show that molecular correlates of sleep are found in the cerebellum, a structure not known for generating sleep rhythms. Finally, we show that different functional categories of genes are selectively associated with sleep and wakefulness. The ~100 known genes whose expression increases during sleep provide molecular support for the proposed involvement of sleep in protein synthesis and neural plasticity and point to a novel role for sleep in membrane trafficking and maintenance.

Introduction

Sleeping animals are impaired in their ability to monitor their environment, yet all animals studied so far sleep (Tobler, 2000). Moreover, the need for sleep is tightly regulated (Borbély and Achermann, 1999), and sleep deprivation leads to overwhelming sleep pressure, cognitive impairment, and physiological deficits (Horne, 1988; Van Dongen et al., 2003). In rats (Rechtschaffen, 1998) and flies (Shaw et al., 2002), prolonged sleep deprivation is fatal. Thus, compelling evidence suggests that sleep serves one or more fundamental functions, but what these functions might be is unclear (Rechtschaffen, 1998).

It is generally thought that the functions of sleep concern the brain (Hobson, 1989). For example, some evidence indicates that sleep may represent a favorable time for brain protein synthesis (Ramm and Smith, 1990; Nakanishi et al., 1997). Another possibility, suggested by behavioral studies (Stickgold et al., 2001; Walker et al., 2002), is that sleep may promote memory consolidation. It is also widely thought that the functions of sleep may ultimately relate to cellular and molecular aspects of neural function (Moruzzi, 1972; Rechtschaffen, 1998; Steriade and Timofeev, 2003; Tononi and Cirelli, 2003). However, to understand what benefits sleep may bring

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at the cellular level, its molecular correlates need to be investigated.

Recently, genome-wide expression profiling has greatly advanced our comprehension of several biological processes at the cellular level. Microarray studies have identified hundreds of transcripts that cycle with circadian time in the brain and peripheral tissues of mice (Akhtar et al., 2002; Panda et al., 2002; Storch et al., 2002; Ueda et al., 2002a) and flies (Claridge-Chang et al., 2001; McDonald and Rosbash, 2001; Ceriani et al., 2002; Lin et al., 2002; Ueda et al., 2002b). Cycling genes are involved in many cellular functions, from host defense to metabolism, and may thus play a role in biological processes that change between day and night, including wakefulness and sleep. However, studies in rats (Rhyner et al., 1990; Cirelli and Tononi, 2000a; Cirelli, 2002; Petit et al., 2002; Terao et al., 2003) and flies (Shaw et al., 2000) have shown that the expression of specific transcripts can increase during wakefulness relative to sleep irrespective of day or night. Thus, it is not known to what extent changes in gene expression between day and night depend on circadian time or on behavioral state. Most importantly, it is not known whether there are genes whose expression is increased during sleep, and which these genes might be. Finally, it is not clear whether molecular correlates of sleep, if present, are specific to the brain, and whether they occur only in brain structures that generate sleep rhythms, such as the cerebral cortex, or may also occur in the absence of electrographic signs of sleep.

In order to address these questions, we report here a comprehensive microarray analysis of gene expression in the cerebral cortex of spontaneously awake, sleep deprived, and sleeping rats. The cerebral cortex was chosen because it generates the characteristic electrical rhythms of sleep (Steriade and Hobson, 1976; Steriade and Timofeev, 2003), it responds to prolonged wakefulness with increasing sleep pressure (Borbély and Achermann, 1999), it is responsible for the cognitive defects observed after sleep deprivation (Horne, 1988; Van Dongen et al., 2003), and it is at the center of most hypotheses concerning the functions of sleep (Moruzzi, 1972; Horne, 1988; Krueger et al., 1995; Maquet, 1995; Steriade and Timofeev, 2003; Tononi and Cirelli, 2003).

Results and Discussion

The Effects of Sleep and Wakefulness Can Be Dissociated from Those of Day and Night

To identify gene expression changes related to behavioral state per se as opposed to diurnal fluctuation, rats were polygraphically recorded and assigned to three groups. S rats (spontaneously asleep) were killed at 6 pm during their usual sleep period; SD rats (sleep deprived) were killed at the same time of day after having been kept awake by gentle handling for 8 hr; and W rats (spontaneously awake) were killed at 6 am during their
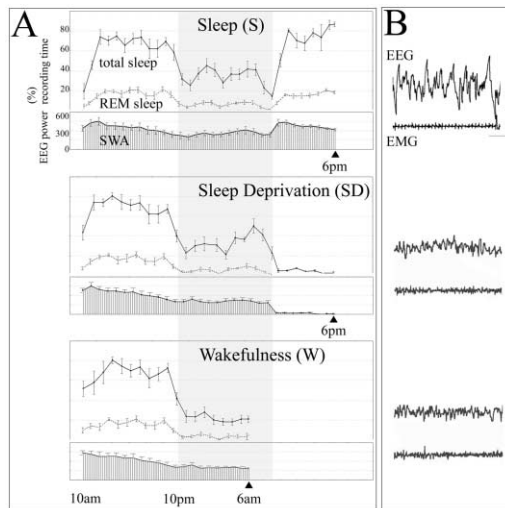
Figure 1. The Three Experimental Groups Selected to Identify Gene Expression Changes Associated with Behavioral State as Opposed to Time of Day

(A) Total sleep (rapid eye movements sleep [REM] + non-REM sleep [NREM]), REM sleep, and slow wave activity (SWA) in S, SD, and W rats during the last 20–32 hr before sacrifice (indicated by an arrowhead). Represented values are mean ± SEM for the 6 rats/group used for microarray analysis. The additional 4 rats/group used for subsequent confirmation experiment with PCR showed similar percentages of behavioral states before sacrifice (data not shown). SWA, an index of NREM sleep intensity (Borbély and Achermann, 1999), is expressed as electroencephalographic (EEG) power (μV^2) in the 1–4 Hz band. As expected, SWA was high at the beginning of the sleep period and declined thereafter. Rats are nocturnal and therefore spontaneously asleep for most of the light period and spontaneously awake for most of the dark period. S rats were sacrificed 8 hr after light on (6 pm, ZT8) at the end of a long period of sleep (>45 min, interrupted by periods of wakefulness <2 min) and after spending at least 75% of the previous 8 hr asleep. W rats were killed 8 hr after light off (6 am, ZT20) after a long period of continuous wakefulness (>1.5 hr, interrupted by periods of sleep <5 min) and after spending at least 70% of the previous 8 hr awake. SD rats were killed at 6 pm (ZT8) as S rats, but they were kept awake for the previous 8 hr by introducing novel objects in their recording cages. Every new object was delivered just following the first signs of synchronization in the frontal EEG signal.

(B) Representative examples of the prevailing EEG activity during the last 8 hr before sacrifice. The low-voltage fast activity cortical EEG of SD and W rats is associated with high electromyographic (EMG) activity, while the slow waves in the cortical EEG of S rats are associated with low EMG activity. Scale bars: x = 1 s, y = 50 μV.

usual waking period (Figure 1A). As expected, the electrocorticogram (EEG) of awake rats (W and SD groups) was characterized by low-voltage/high-frequency patterns. By contrast, the EEG of S rats was dominated by higher voltages/lower frequencies (slow wave activity, Figure 1A) and characteristic sleep rhythms such as spindles and slow waves (Figure 1B). Percentages of behavioral states (mean ± SEM) for the last 8 hr before sacrifice were as follows: S rats, wakefulness = 23.4 ± 1.4, NREM sleep = 60.7 ± 1.2, REM sleep = 15.9 ± 0.7; SD rats, wakefulness = 95.9 ± 0.4, NREM sleep = 4.1 ± 0.4, REM sleep = 0.0 ± 0.0; W rats, wakefulness = 75.8 ± 3.2, NREM sleep = 20.3 ± 2.6, REM sleep = 3.9 ± 0.8. Thus, at the time of sacrifice, S rats had been predominantly asleep, while W and SD rats had been

predominantly awake for several hours. Since S and SD rats were sacrificed at the same time of day but in opposite behavioral state, and since SD and W rats were sacrificed 12 hr apart but in the same behavioral state, day/night and sleep/wakefulness effects could be dissociated.

Microarray Analysis Is Validated through Positive Controls and Quantitative PCR

Total RNA extracted from the cerebral cortex of S, SD, and W rats (n = 6 rats/group) was analyzed using the full set of Affymetrix high-density oligonucleotide arrays (GeneChips RGU34 A, B, and C) to screen for the expression of more than 7,000 annotated sequences and 17,000 expressed sequence tags (ESTs). The analysis was performed using a set of statistical and empirical parameters aimed at minimizing the total number of differentially expressed transcripts while maximizing the number of positive controls (MicroArray Suite 5.0, Affymetrix; see also Experimental Procedures). For any given comparison (S versus SD, S versus W, or SD versus W), the transcripts whose expression level was too low in both chips were called "absent" and removed from the analysis. Approximately 64% of the transcripts remained after this step (15,459/24,000). To control for technical variability, we pooled RNA from 6 S, 6 SD, and 6 W rats and performed 3–5 independent replicas of all the steps of the experiments, from first-strand cDNA synthesis to chip hybridization. Outlier effects with pooled RNA are unlikely in this study because of the homogeneity of cortical tissue and the choice of inbred WKY rats fully adapted to the recording environment and carefully selected for behavioral state (see Experimental Procedures). Moreover, as shown in our previous differential display studies (Cirelli and Tononi, 2000a), interindividual differences in cortical gene expression patterns among animals belonging to the same group are minimal. For each intergroup comparison (S versus SD, S versus W, or SD versus W), significant expression changes were determined with paired nonparametric tests (MicroArray Suite 5.0, Affymetrix; Bolstad et al., 2003). Sleep-related transcripts were required to increase in at least half of all S versus SD *and* S versus W comparisons. Similarly, wakefulness-related transcripts were required to decrease in at least half of all S versus SD *and* S versus W comparisons. A technique called Discrete Bayesian Approach (DBA, SBI-Moldyn, Inc.) was also used as an independent method for the identification of wakefulness-related transcripts. DBA, which is based on Bayesian nonlinear statistical analysis and extensive Monte Carlo crossvalidation, confirmed >90% of the wakefulness-related genes identified by MicroArray Suite 5.0 (DBA analysis was performed on GeneChip U34A only).

To validate the microarray results and control for biological variability, we first examined positive controls, defined as those behavioral state-related transcripts that had been identified in previous reports (Cirelli, 2002) using differential display, in situ hybridization, and quantitative PCR (qPCR) on independent sets of S, SD, and W rats. Of the 43 previously identified positive controls, 25 were represented on the chips and the expression of 19 of them (76%) was confirmed as state dependent

according to our microarray analysis. They included 1 sleep-related gene (AI013911) and 24 wakefulness-related genes (e.g., those coding for Fos, Arc, BDNF, BiP, arylsulfotransferase, mitochondrial proteins, and heat shock proteins). The amplitude change for confirmed positive controls ranged between 25% and 300%. Second, we used real-time qPCR on 3 independent groups of S, SD, and W rats (n = 4 rats/group) to evaluate the expression of 52 additional transcripts (not corresponding to positive controls) identified by the microarray analysis as "increased in S rats," "increased in W and SD rats," "increased in W rats," or "not changing." Since the positive controls were almost all upregulated during wakefulness, we focused on transcripts upregulated during sleep. In 41/52 cases (S, 32/40; SD and W, 2/2; W, 1/1; not changing, 6/9), qPCR expression analysis confirmed the microarray profiling analysis. Altogether, ~80% of the results of microarray analysis examined were validated by qPCR experiments performed on two independent sets of rats (see Supplemental Tables at http://www.neuron.org/cgi/content/full/41/1/35/DC1). Confirmation rate was ~90% when the same 52 transcripts were measured by qPCR using pooled RNA from the same sets of animals used in the microarray experiments. Thus, based on qPCR results, ~20% of our selected genes can be ascribed to noise related to biological variability, similar to what has been described in other studies (Storch et al., 2002). Because in our hands real-time qPCR had a global absolute error of ~20%, we only report transcripts for which the average signal ratio across all comparisons was at least 0.19. Amplitude changes for the majority of state-dependent transcripts were between 20% and 60%, in agreement with what has been reported for mammalian transcripts modulated by circadian time (Akhtar et al., 2002; Panda et al., 2002; Storch et al., 2002; Ueda et al., 2002a).

Behavioral State and Time of Day Affect Brain Gene Expression to a Similar Extent

When we compared S rats, killed during the day, with W rats, killed during the night, we found that 1,564 transcribed sequences out of 15,459 present in the cerebral cortex were differentially expressed between day and night. This result is consistent with previous reports in mice showing that the expression of 1%–10% of the transcripts in the brain and in peripheral tissues oscillate according to circadian time (Akhtar et al., 2002; Panda et al., 2002; Storch et al., 2002; Ueda et al., 2002a). However, we found that half of these transcribed sequences were differentially expressed also between S and SD rats, which had been killed at the same time of day but in opposite behavioral states. Thus, only 808 transcribed sequences (5.2% of 15,459) were modulated by time of day irrespective of whether the animal was awake or asleep. Among these was the canonical circadian gene *Bmal1,* whose expression was higher during the night than during the day (Figure 2), as already reported in other tissues (Akhtar et al., 2002; Storch et al., 2002).

Most importantly, we found that a comparable number of cortical transcribed sequences (752, 4.9% of 15,459) changed their expression as a function of behavioral state independent of time of day. Among these was
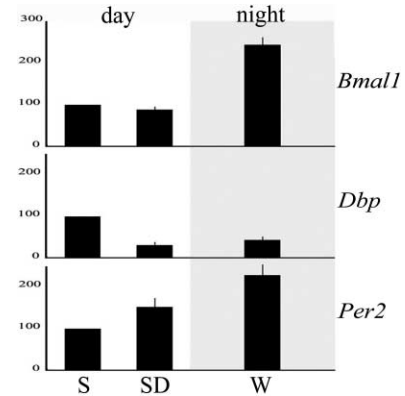

Figure 2. Effects of Behavioral State on the Cortical Expression of Three Circadian Genes

All values are expressed as % relative to S = 100 (mean ± SEM, real-time qPCR). *Bmal1:* SD = 87.1 ± 6.0, W = 251.4 ± 29.3; *Dbp:* SD = 29.8 ± 8.2, W = 42.0 ± 6.3; *Per2:* SD = 157.1 ± 18.1, W = 234.5 ± 27.8. Per2 was one of 121 genes whose levels changed in all three comparisons: S/W, S/SD, and W/SD (data not shown). This heterogeneous group probably includes genes that, while state dependent, are additionally affected by circadian factors or by the gentle handling procedure employed for sleep deprivation. Expression levels following SD > W > S or S > W > SD may be related to the increased amount of wakefulness in the SD group compared to the W group (Figure 1).

Dbp, a gene coding for a transcription factor whose expression cycles according to circadian time in the suprachiasmatic nucleus and in peripheral tissues (Wuarin and Schibler, 1990) but whose cortical transcript levels were >100% higher in S than in W or SD rats (Figure 2). The expression of other genes, including the canonical circadian gene *Per2*, was modulated by both time of day and behavioral state (Figure 2). In summary, these data indicate that day/night time and sleep/wakefulness influence gene expression in the cerebral cortex to a similar extent. Moreover, they show that the cortical expression of some canonical circadian genes, such as *Dbp,* can be markedly affected by behavioral state. The expression of other genes, such as *Per2*, depends both on time of day and on behavioral state, the latter consistent with other reports in rats (Masubuchi et al., 2000) and mice (Wisor et al., 2002). These results contribute to a growing body of evidence pointing to a close interaction between the circadian mechanisms and the homeostatic regulation of sleep (Shaw et al., 2002; Wisor et al., 2002).

Sleep and Wakefulness Affect Cortical Gene Expression to a Similar Extent

The current gene expression profiling analysis identified 95 known genes and 395 ESTs whose mRNA levels increased during wakefulness (Supplemental Tables at http://www.neuron.org/cgi/content/full/41/1/35/DC1). Most importantly, in addition to *Dbp*, we identified here for the first time 106 known genes and 155 ESTs whose mRNA levels were specifically increased during sleep (Supplemental Tables). Thus, despite behavioral quiescence, sleep is associated with the increased expression of at least as many known transcripts as wakefulness, just as it is accompanied by comparable levels

of neuronal activity (Steriade and Hobson, 1976; Steriade and Timofeev, 2003). These findings suggest that sleep and wakefulness, the two main behavioral states, are associated not only with different electrophysiological states in the cerebral cortex, but also with different states at the molecular level. Note that the majority of state-dependent transcripts identified here—essentially all sleep-related transcripts and ∼90% of wakefulness-related transcripts—had not been recognized in previous work (Cirelli and Tononi, 2000a). The present approach using microarrays permits a more comprehensive screening and, more importantly, has higher sensitivity to low-abundance transcripts than mRNA differential display.

There Is Substantial Overlap between Gene Expression Changes in the Cerebral Cortex and in the Cerebellum

The distinctive electrographic correlates of NREM sleep, spindles and slow waves, are generated within the thalamocortical system (Steriade and Timofeev, 2003). These large-scale electrical phenomena are due to the synchronization of a slow oscillation of the membrane potential that occurs in all cortical neurons during sleep and is mediated by cortico-cortical connections (Steriade and Timofeev, 2003). Do brain structures not known to generate endogenous sleep rhythms or intracellular slow oscillation, such as the cerebellum (Hobson and McCarley, 1972), show signs of sleep at the molecular level? To address this question, we examined state-dependent gene expression in cerebellar tissue from the same S, SD, and W animals. In the RGU34A chip hybridized with cerebellar RNA (chips B and C were not used), 4350 transcripts were called present, of which 106 (including 7 ESTs) were sleep-related transcripts and 117 (including 46 ESTs) were "wakefulness" transcripts (Supplemental Tables S2A and S2B at http://www.neuron.org/cgi/content/full/41/1/35/DC1). Thus, as in the cerebral cortex, ∼5% of all transcripts were differentially expressed between sleep and wakefulness. Moreover, when considering the transcribed sequences that were called present in both the cerebral cortex and the cerebellum, there was substantial overlap between state-dependent transcripts: 51% of sleep-related genes in the cerebral cortex were sleep-related in the cerebellum, and 40% of wakefulness-related genes in the cerebral cortex were wakefulness-related in the cerebellum (Figure 3A). Thus, cellular functions associated with sleep can occur in brain regions where electrographic signs of sleep are scarce or absent.

Sleep and Wakefulness Are Associated with the Increased Expression of Different Functional Categories of Genes

The importance of a genome-wide analysis lies in suggesting whether sleep and wakefulness may favor *different* cellular processes or functions. Indeed, we found that sleep and wakefulness are associated with the increased expression of different functional categories of genes (Figure 3B).

A first category of genes whose mRNA levels were increased during wakefulness included mitochondrial genes involved in oxidative phosphorylation and the main astroglial glucose transporter (*GLUT1*). Cerebral
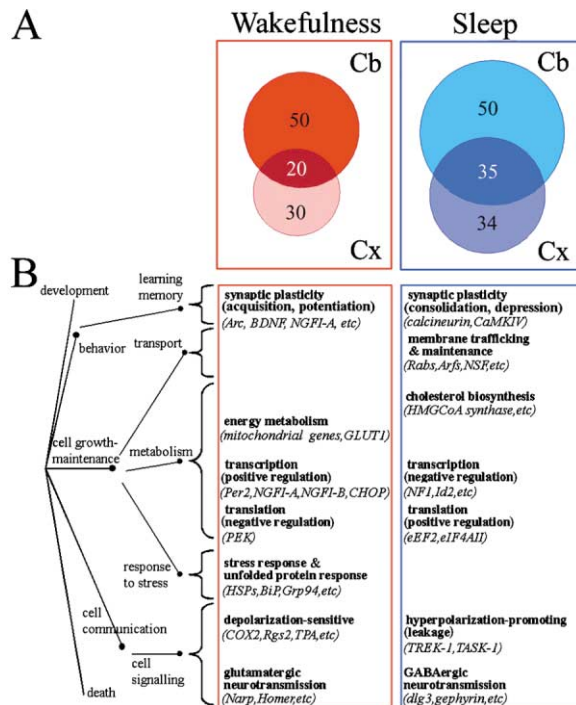

Figure 3. Rat Cortical and Cerebellar Genes whose mRNA Levels Are Modulated by Sleep and Wakefulness Independent of Time of Day

(A) State-dependent transcripts (GeneChip RGU34A) in cerebral cortex (Cx) and cerebellum (Cb) and their overlap. Numbers do not include ESTs or transcripts called present in only one tissue.

(B) Biological functions associated with transcripts with higher expression in wakefulness (red box) and sleep (blue box). The tree on the left (dots and connecting paths) represents biological processes; annotations according to the gene ontology hierarchy.

glucose metabolism is higher in wakefulness than in NREM sleep (Maquet, 2000). Moreover, brain energy expenditure is tightly controlled by brain activity, mainly because of the high metabolic cost of ion pumping by the Na^+/K^+ ATPase to counteract membrane depolarization (Ereciñska and Silver, 1989). Thus, these data are consistent with the elevated metabolic requirements during wakefulness, when neurons are steadily depolarized (Steriade and Timofeev, 2003). Wakefulness and response readiness are also associated with increased firing of monoaminergic systems, such as the noradrenergic (Aston-Jones and Bloom, 1981) and serotoninergic systems. At a molecular level, wakefulness was associated with the increased expression of minoxidil (aryl)sulfotransferase, the main enzyme responsible for the catabolism of catecholamines in rodents (Cooper et al., 1996).

Wakefulness was also associated with higher mRNA levels of several genes coding for stress proteins, such as small heat shock proteins with actin-stabilizing properties (*HSP27, α-B crystallin*), molecular chaperones (*HSP70, HSP60*), and chaperones associated with the unfolded protein response in the endoplasmic reticulum (*BiP, Grp94*). Cerebellar transcript levels of *PEK*, the gene encoding the EIF2a kinase that mediates the inhibition of protein synthesis during the unfolded protein

response (Kaufman et al., 2002), were also higher in wakefulness than in sleep. The unfolded protein response may also be an index of calcium depletion from intracellular stores (Li et al., 1993). Notably, genes whose mRNA levels were higher in sleep included key regulators of Ca^{2+} release from intracellular stores, such as *calcineurin, FK506 binding protein 12*, and *inositol 1,4,5-trisphosphate receptor* (Cameron et al., 1995).

Other genes with higher mRNA levels during wakefulness were those involved in the synthesis of the excitatory neurotransmitter glutamate from glutamine (*glutamine synthase, glutaminase*) and in the clustering of glutamatergic receptors (*Homer/Vesl, Narp*). By contrast, genes related to inhibitory neurotransmission, such as those coding for GABA receptor subunits (*GABAB_{1c}* and *GABAB_{1d}*) and those mediating the clustering of GABA and glycine receptors (*dlg3, gephyrin*), were expressed at higher levels during sleep. Wakefulness was also associated with increased levels of transcripts sensitive to membrane depolarization and synaptic activity, such as *RGS2, Homer/Vesl, tissue-type plasminogen activator*, *casein kinase 2, cyclooxygenase 2, cpg2*, and *connexin 30*. Finally, certain genes involved in activity-dependent neural plasticity and long-term potentiation, such as *Arc, BDNF, Homer/Vesl*, and *NGFI-A*, were selectively induced during wakefulness. As previously shown, the expression of these genes during wakefulness depends on the increased firing of the noradrenergic system during this behavioral state (Cirelli et al., 1996; Cirelli and Tononi, 2000b) and may be related to memory acquisition (Tononi and Cirelli, 2001, 2003).

While during wakefulness neurons are steadily depolarized, during the slow oscillations of NREM sleep, neurons alternate between depolarization and hyperpolarization phases, the latter leading to a reduction of energy needs. Slow oscillations of membrane potential are brought about by an increased leakage current mediated by potassium ions (McCormick and Bal, 1997). Although the responsible channels have not yet been identified electrophysiologically, the finding of increased mRNA levels during sleep of *TREK-1* in the cerebral cortex and of *TASK-1* in the cerebral cortex and cerebellum suggests that two-pore domain potassium channels may be involved (Goldstein et al., 2001; Meuthw et al., 2003). Consistent with this role, TREK-1-mediated potassium currents are blocked by phosphorylation through acetylcholine (Goldstein et al., 2001), the same neuromodulator that can produce behavioral and brain activation by reducing leakage currents (McCormick and Bal, 1997).

Many positive regulators of transcription were upregulated during wakefulness. Sleep was associated instead with increased mRNA levels of key components of the translational machinery, such as the eukaryotic translation elongation factor 2 and the initiation factor 4AII. These findings offer molecular support for the observation that brain protein synthesis, as measured by leucine incorporation in both rats and monkeys, is increased during NREM sleep (Ramm and Smith, 1990; Nakanishi et al., 1997).

While wakefulness is the appropriate time for memory acquisition, recent behavioral and physiological evidence suggests that sleep may represent a favorable time for memory consolidation (Stickgold et al., 2001;

Walker et al., 2002). Our data show a sleep-related increase in the mRNA levels of *calmodulin-dependent protein kinase IV*, a gene that has been specifically involved in synaptic depression as well as in the consolidation of long-term memory (Ahn et al., 1999; Kang et al., 2001). Other sleep-induced genes that may play a role in some aspects of plasticity include *calcineurin, FK506 binding protein 12, inositol 1,4,5-trisphosphate receptor*, and *amphiphysin II*, which have been associated with synaptic depression and depotentiation, as well as *NCS-1, Dpp6, Ash/GRB2,* the gene coding for the phosphatidylinositol 3-kinase p55 regulatory subunit, and *protein kinase C-ζ-interacting protein (ZIP)*. Thus, sleep and wakefulness may be associated with different aspects of neural plasticity (Figure 3B; Tononi and Cirelli, 2003).

Among the most intriguing findings of this study is the sleep-related increase of many transcripts involved in membrane trafficking at several different levels (Gruenberg, 2001): exocytosis and neurotransmitter release (*SV2B, complexin II, Rab3a, neuronal calcium sensor-1*), synaptic vesicle recycling (*Rab5, amphiphysin II, endophilin I*), tethering/docking of vesicles to their target organelles (*Rab4, Rab5, Rab11, Rab14, Rab GDI*), dissociation of the SNARE core complex (*NSF*), recruitment of coat proteins (*ARF1, ARF3, α-centaurin*), and cycling between trans-Golgi network and plasma membrane (*MG160, TGN38*). Another large group of genes with higher mRNA levels during sleep are important for the synthesis/maintenance of membranes in general and of myelin in particular (Kramer et al., 2001), such as oligodendrocitic genes coding for myelin structural proteins (*MOBP, MAG, plasmolipin, CD9*), myelin-related receptors (*insulin-like growth factor binding protein 2*), and enzymes (*2':3'-cyclic nucleotide-3'-phosphodiesterase, Na/K ATPase subunit α2, methionine adenosyltransferase, carbonic anhydrase II*). Another sleep-related gene found in myelin and in other membranes is *glutathione S-transferase*. The gene encoding transketolase, an enzyme of the pentose phosphate pathway that provides NADPH for the synthesis of fatty acids and cholesterol, was also expressed at higher levels during sleep. Finally, sleep-related transcripts also code for enzymes involved in the synthesis and transport of cholesterol, a major constituent of myelin and other membranes (*thiolase, 3-hydroxy-3-methylglutaryl-Coenzyme A synthase, squalene synthase, lanosterol 14 α-demethylase*). In agreement with our results, studies in flies and mice have found that the expression of several genes related to the synthesis of cholesterol (Ceriani et al., 2002; Panda et al., 2002) and to synaptic vesicle recycling (Claridge-Chang et al., 2001) peaks during the resting phase. Since we found no evidence that transcripts related to cholesterol synthesis and membrane trafficking were modulated by day and night independently of behavioral state (data not shown), it may be that sleep provides especially favorable conditions for these cellular processes. Sleep is especially abundant early in life, at a time in which synaptogenesis and myelinization are prominent, and it appears to be important for circuit formation and plasticity (Frank et al., 2001; Shaffery et al., 2002). Recent evidence shows that glia-derived cholesterol may be the limiting factor for synapse formation and maintenance (Mauch et al., 2001). Thus, the associa-

tion between sleep and membrane trafficking, cholesterol synthesis, protein synthesis, and neural plasticity may not be unrelated.

The Expression of Sleep-Related Transcripts Is Brain Specific

Since sleep is thought to be "by the brain and for the brain" (Horne, 1988; Hobson, 1989), it is important to determine to what extent sleep-related changes in gene expression are specific to the neural tissue. For this purpose, we used qPCR to examine the expression of brain sleep-related genes in liver and skeletal muscle of S, SD, and W rats (n = 6 rats/group). Measured transcripts, selected to be representative of all the major functional categories of sleep-related genes, were those coding for Dbp, NF1-X1, eukaryotic translation elongation factor 2, calcineurin, squalene synthase, NSF, ARF1, ARF3, and glutathione S-transferase. We found that none of these transcripts showed a sleep-related increase in expression in the liver or skeletal muscle. In the liver, all brain sleep-related transcripts were expressed at higher levels in SD and W rats relative to S rats (mean % increase in SD, W rats relative to S rats: Dbp = 331, NF1-X1 = 627, eukaryotic translation elongation factor 2 = 1028, calcineurin = 602, squalene synthase = 1201, NSF = 9313, ARF1 = 308, ARF3 = 684, glutathione S-transferase = 1369). Two brain wakefulness-related genes coding for Per2 and minoxidil sulfotransferase were also examined and showed a similar rise in awake animals (mean % increase in SD, W rats relative to S rats = 1037 and 290, respectively). The wakefulness-related increase of all examined transcripts in liver tissue is probably related to feeding. Eating and drinking occur mostly at night in rats kept on a 12:12 light/dark schedule (our own calculations show that 85% ± 11% of food intake occurs at night), and food intake increases during sleep deprivation (Rechtschaffen, 1998). Moreover, the peak of Dbp expression (Wuarin and Schibler, 1990) and of liver cholesterol synthesis (Jurevics et al., 2000; Yamada et al., 2000) occurs at night in rats kept on a 12:12 light/dark schedule and with food available at all time but shifts to the light period if feeding is only allowed during the day (Ogawa et al., 1997). In the skeletal muscle, the expression of the same genes was either not changing between S, SD, and W rats (Dbp, NF1-X1, NSF, ARF1) or was modulated by time of day and not by behavioral state (mean % increase in S, SD rats relative to W rats: eukaryotic translation elongation factor 2 = 90, glutathione S-transferase = 66; mean % increase in W rats relative to S, SD rats: calcineurin = 62, ARF3 = 240).

Conclusions

Gene expression studies have certain shortcomings. The kinetics of trancriptional changes is difficult to determine based on a few time points. Trancriptional changes do not map directly to functional changes, which are determined by translational and posttranslational events. Also, the evidence they provide is correlative. Despite these limitations, several conclusions can be drawn from the present study. First, half of the ~1500 genes that are differentially expressed in the cerebral cortex between day and night do so because of changes in sleep and wakefulness rather than in time of day. Thus, approximately the same number of cortical genes is sensitive to modulation by time of day and by behavioral state. A direct implication of these results is that changes in behavioral state should be taken into account in gene expression studies.

Second, the finding that molecular correlates of sleep and wakefulness are found in the cerebellum indicate that cellular processes associated with sleep may occur in brain structures that are not known for generating sleep rhythms. This suggests that, at the cellular level, functions associated with sleep may take place whether or not electrographic signs of sleep can be recorded. Several mechanisms could explain these molecular changes. For example, cerebellar neurons could undergo slow oscillations characteristic of sleep, even if such oscillations may not synchronize on a large scale due to the absence of associative fibers. Alternatively, oscillations of membrane potential may be imposed onto cerebellar neurons through indirect cortico-cerebellar connections. Molecular changes associated with sleep and wakefulness could also result from the action of neuromodulatory systems whose firing rate is state dependent.

Third, the present results demonstrate that, although sleep is a state of behavioral inactivity, it is associated with the increased expression of many genes in the brain. Indeed, the number of known genes whose expression in the brain increases during sleep is roughly the same as the number of genes whose expression increases during wakefulness. Moreover, the increased expression in the brain during sleep is specific, since transcripts that are sleep-related in the brain are not sleep-related in other tissues such as liver and muscle.

Finally, a functional analysis of transcripts modulated by behavioral state suggests that sleep and wakefulness may favor different cellular processes. Wakefulness-related transcripts may help the brain to face high energy demand, high synaptic excitatory transmission, high transcriptional activity, and the need for synaptic potentiation in the acquisition of new information, as well as the cellular stress that may derive from one or more of these processes. An analysis of brain sleep-related transcripts supports an involvement of sleep in protein synthesis and in complementary aspects of neural plasticity such as synaptic depression and suggests, for the first time, that sleep may play a significant role in membrane trafficking and maintenance. In line with recent intracellular recording studies (Steriade and Timofeev, 2003), our findings suggest that sleep, far from being a quiescent state of global inactivity, may actively favor specific cellular functions.

Experimental Procedures

Animal Groups and Sleep Recording

Male inbred WKY rats under pentobarbital anesthesia (60–75 mg/kg, i.p.) were implanted with screw electrodes in the skull to record the electroencephalogram (EEG) and silver electrodes in the neck muscles of both sides to record the electromyogram. After surgery, rats were housed individually in sound-proof recording cages where lighting and temperature were kept constant (LD 12:12, light on at 10 a.m., 24°C ± 1°C, food and drink ad libitum). Immediately after recovery from anesthesia, rats were connected by means of a flexible cable and a commutator (Airflyte) to a Grass electroencephalo-

graph (mod. 15LT) and recorded continuously for 2–3 weeks (Kissei Comtec). Each day from 10 to 10:30 a.m. all rats were gentle handled to become familiar with the sleep deprivation procedure. Gentle handling included stimulating the rats with a light puff of air and exposing them to novel objects. Thirty rats were used: 10 S rats, 10 SD rats, and 10 W rats. For each experimental group, microarrays analysis was performed on pooled RNA from 6 rats, while subsequent real-time qPCR was performed both on pooled RNA from the same 6 rats (technical verification) as well as on pooled RNA from independent groups of 4 animals (biological verification). Animal protocols followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were in accordance with institutional guidelines.

It should be noted that, despite the use of three experimental groups (S, SD, W), our protocol cannot detect genes whose expression changes depending on multiple factors or on interactions among factors. For example, although our protocol rules out direct effects of light (S and SD rats had light on, W rats had light off), a "sleep-related" gene would be missed if its expression were increased by sleep but suppressed by light. Note also that our protocol was not designed to distinguish between the effects on gene expression of different sleep stages. As expected, however, NREM sleep episodes made up the majority of sleep time.

Microarray: Labeling, Hybridization, and Data Analysis
Rats were deeply anesthetized with isoflurane (within 2 min) and decapitated. The head was cooled in liquid nitrogen and the whole brain was removed. Cerebral cortex, hippocampus, and cerebellum were dissected, while the rest of the brain was left intact. Liver and skeletal muscle (hind limb) were also removed. Samples were immediately frozen on dry ice and stored at $-80°C$. Total RNA was isolated from right cerebral cortex, cerebellum, liver, and skeletal muscle of each animal by using Trizol (GIBCO-BRL, Gaithersburg, MD) according to the manufacturer's instructions. Final RNA concentrations were determined spectrophotometrically.

An equal mass amount of total RNA from the right cerebral cortex or cerebellum of each animal was pooled from 6 rats within each experimental group (S, SD, W; 20 μg/pool) and converted into first-strand cDNA using Superscript II RNase H⁻ reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA), and the second strand was synthesized, all according to the Affymetrix Gene Expression manual (Affymetrix, Inc., Santa Clara, CA). cDNA was then converted to biotinylated cRNA using the ENZO BioArray High Yield In Vitro Transcription kit (Enzo Life Sciences, Farmingdale, NY) according to the manufacturer's instructions. The cRNA was fragmented at 0.5 μg/μl final concentration in $1\times$ fragmentation buffer (40 mM Tris-acetate [pH 8.1], 100 mM potassium acetate, 30 mM magnesium acetate). The size range of cRNA before (0.5 kb and longer) and after (35–200 base fragments) fragmentation was checked by denaturing agarose electrophoresis. The hybridization reaction and the automated hybridization procedure were performed according to Affymetrix instructions. Briefly, the procedure involved a 16 hr hybridization followed by two washes and staining with Streptavidin-Phycoerythrin (SAPE, Affymetrix, Inc.) stain solution. The staining was followed by another wash, antibody amplification with biotinylated antibody, a second SAPE staining, and a final wash (Affymetrix GeneChip Expression Analysis Technical Manual). Each sample was hybridized to Affymetrix GeneChip U34A, B, or C, scanned, and normalized according to the Affymetrix GeneChip Expression Analysis Technical Manual (see https://www.affymetrix.com/Download/manuals/expression_ever_manual.pdf). Quality of the cDNA and cRNA syntheses was determined by the 3'/5' ratio of housekeeping genes within the array (ubiquitin, rat glyceraldehyde 3-phosphate dehydrogenase, β-actin, and hexokinase). For each comparison (e.g., S relative to SD) and for each cDNA represented in the array, a ratio of intensities and its statistical significance were calculated. Statistical analysis was performed using paired nonparametric tests (MicroArray Suite 5.0, Affymetrix; Bolstad et al., 2003). For wakefulness-related transcripts on chip A, results were confirmed using a Discrete Bayesian Approach (DBA, SBI-Moldyn Inc.).

Real-Time qPCR
Real-time qPCR was performed as already described (Cirelli and Tononi, 2000a; Sequence Detection System 5700, Perkin Elmer).

Briefly, reverse transcription reactions were carried out in parallel on DNase I digested pooled total RNA from S, SD, and W rats. Prior to reverse transcription, total RNA was confirmed to be free of contaminating DNA sequences by PCR using rat β-actin specific primer pairs designed to differentiate between cDNA, genomic DNA, and pseudogene genomic DNA. Eight reverse transcription reactions were performed for each experimental group (S, SD, and W). Reverse transcription reactions were as follows: 100 ng total RNA, 2.5 μl oligo dT$_{16}$ (500 μg/ml), 5 μl dNTPmix (10 mM each dNTP), 1 pg artificial transcript (IDT, Inc., Coralville, IA), H$_2$O to 29.75 μl. Samples were incubated at 70°C for 10 min, put briefly on ice, and then incubated at 42°C for 2–5 min. Mix #2 (10 μl 5$\times$ Superscript II First Strand Buffer, 5 μl 0.1 M DTT, 4 μl 25 mM MgCl$_2$, and 1.25 μl Superscript II RNase H⁻ Reverse Transcriptase 200 U/μl) was added and mixed, and samples were immediately returned to incubate at 42°C for 1 hr. Reactions were stopped by incubation at 70°C for 15 min. PCR reactions to measure levels of artificial transcript were done to confirm uniformity of reverse transcription within sample groups and between samples. Comparable reverse transcription reactions within a sample group were pooled. Each PCR reaction contained specific forward and reverse primers (200–750 nM final concentration), 2$\times$ SYBR Green Master Mix (used at 3.2$\times$), 5 μl of a 1:10 dilution of pooled reverse transcription product, and H$_2$O to a total volume of 25 μl. A two-step PCR profile was used: 10 min at 95°C denaturation and Amplitaq gold activation, followed by 40 cycles alternating between 95°C for 15 s and 60°C for 60 s. Dilution series (1:2, 1:10, 1:50, 1:250, 1:1250) standard curves were performed in quadruplicate for each primer pair using reverse transcription products from generic rat brain total RNA. PCR was done in quintuplicate for each sample condition assayed and relative quantities determined based on the equation of the line of best fit derived from the standard curve ($R^2 \geq 0.985$).

References

Ahn, S., Ginty, D.D., and Linden, D.J. (1999). A late phase of cerebellar long-term depression requires activation of CaMKIV and CREB. Neuron *23*, 559–568.

Akhtar, R.A., Reddy, A.B., Maywood, E.S., Clayton, J.D., King, V.M., Smith, A.G., Gant, T.W., Hastings, M.H., and Kyriacou, C.P. (2002). Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic nucleus. Curr. Biol. *12*, 540–550.

Aston-Jones, G., and Bloom, F.E. (1981). Activity of norepinephrine-containing locus coeruleus neurons in behaving rats anticipates fluctuations in the sleep-waking cycle. J. Neurosci. *1*, 876–886.

Bolstad, B.M., Irizarry, R.A., Astrand, M., and Speed, T.P. (2003). A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics *19*, 185–193.

Borbély, A.A., and Achermann, P. (1999). Sleep homeostasis and models of sleep regulation. J. Biol. Rhythms *14*, 557–568.

Cameron, A.M., Steiner, J.P., Roskams, A.J., Ali, S.M., Ronnett, G.V., and Snyder, S.H. (1995). Calcineurin associated with the inositol

1,4,5-trisphosphate receptor-FKBP12 complex modulates Ca^{++} flux. Cell *83*, 463–472.

Ceriani, M.F., Hogenesch, J.B., Yanovsky, M., Panda, S., Straume, M., and Kay, S.A. (2002). Genome-wide expression analysis in *Drosophila* reveals genes controlling circadian behavior. J. Neurosci. *22*, 9305–9319.

Cirelli, C. (2002). How sleep deprivation affects gene expression in the brain: a review of recent findings. J. Appl. Physiol. *92*, 394–400.

Cirelli, C., and Tononi, G. (2000a). Gene expression in the brain across the sleep-wakefulness cycle. Brain Res. *885*, 303–321.

Cirelli, C., and Tononi, G. (2000b). Differential expression of plasticity-related genes in waking and sleep and their regulation by the noradrenergic system. J. Neurosci. *20*, 9187–9194.

Cirelli, C., Pompeiano, M., and Tononi, G. (1996). Neuronal gene expression in the waking state: a role for the locus coeruleus. Science *274*, 1211–1215.

Claridge-Chang, A., Wijnen, H., Naef, F., Boothroyd, C., Rajewsky, N., and Young, M.W. (2001). Circadian regulation of gene expression systems in the *Drosophila* head. Neuron *32*, 657–671.

Cooper, J.R., Bloom, F.E., and Roth, R.H., eds (1996). The Biochemical Basis of Neuropharmacology (Oxford: Oxford University Press).

Erecińska, M., and Silver, I.A. (1989). ATP and brain function. J. Cereb. Blood Flow Metab. *9*, 2–19.

Frank, M.G., Issa, N.P., and Stryker, M.P. (2001). Sleep enhances plasticity in the developing visual cortex. Neuron *30*, 275–287.

Goldstein, S.A., Bockenhauer, D., O'Kelly, I., and Zilberberg, N. (2001). Potassium leak channels and the KCNK family of two-P-domain subunits. Nat. Rev. Neurosci. *2*, 175–184.

Gruenberg, J. (2001). The endocytic pathway: a mosaic of domains. Nat. Rev. Mol. Cell Biol. *2*, 721–730.

Hobson, J.A. (1989). Sleep (New York: Scientific American Library HPHLP).

Hobson, J.A., and McCarley, R.W. (1972). Spontaneous discharge rates of cat cerebellar Purkinje cells in sleep and waking. Electroencephalogr. Clin. Neurophysiol. *33*, 457–469.

Horne, J.A. (1988). Why We Sleep. The Functions of Sleep in Humans and Other Mammals (Oxford: Oxford University Press).

Jurevics, H., Hostettler, J., Barrett, C., Morell, P., and Toews, A.D. (2000). Diurnal and dietary-induced changes in cholesterol synthesis correlate with levels of mRNA for HMG-CoA reductase. J. Lipid Res. *41*, 1048–1054.

Kang, H., Sun, L.D., Atkins, C.M., Soderling, T.R., Wilson, M.A., and Tonegawa, S. (2001). An important role of neural activity-dependent CaMKIV signaling in the consolidation of long-term memory. Cell *106*, 771–783.

Kaufman, R.J., Scheuner, D., Schroder, M., Shen, X., Lee, K., Liu, C.Y., and Arnold, S.M. (2002). The unfolded protein response in nutrient sensing and differentiation. Nat. Rev. Mol. Cell Biol. *3*, 411–421.

Kramer, E.M., Schardt, A., and Nave, K.A. (2001). Membrane traffic in myelinating oligodendrocytes. Microsc. Res. Tech. *52*, 656–671.

Krueger, J.M., Obal, F., Jr., Kapas, L., and Fang, J. (1995). Brain organization and sleep function. Behav. Brain Res. *69*, 177–186.

Li, W.W., Alexandre, S., Cao, X., and Lee, A.S. (1993). Transactivation of the grp78 promoter by Ca^{++} depletion. J. Biol. Chem. *268*, 12003–12009.

Lin, Y., Han, M., Shimada, B., Wang, L., Gibler, T.M., Amarakone, A., Awad, T.A., Stormo, G.D., Van Gelder, R.N., and Taghert, P.H. (2002). 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.

Maquet, P. (1995). Sleep function(s) and cerebral metabolism. Behav. Brain Res. *69*, 75–83.

Maquet, P. (2000). Functional neuroimaging of normal human sleep by positron emission tomography. J. Sleep Res. *9*, 207–231.

Masubuchi, S., Honma, S., Abe, H., Ishizaki, K., Namihira, M., Ikeda, M., and Honma, K. (2000). Clock genes outside the suprachiasmatic nucleus involved in manifestation of locomotor activity rhythm in rats. Eur. J. Neurosci. *12*, 4206–4214.

Mauch, D.H., Nagler, K., Schumacher, S., Goritz, C., Muller, E.C., Otto, A., and Pfieger, F.W. (2001). CNS synaptogenesis promoted by glia-derived cholesterol. Science *294*, 1354–1357.

McCormick, D.A., and Bal, T. (1997). Sleep and arousal: thalamocortical mechanisms. Annu. Rev. Neurosci. *20*, 185–215.

McDonald, M.J., and Rosbash, M. (2001). Microarray analysis and organization of circadian gene expression in *Drosophila*. Cell *107*, 567–578.

Meuthw, S.G., Budde, T., Kanyshkova, T., Broicher, T., Munsch, T., and Pape, H.C. (2003). Contribution of TWIK-related acid-sensitive K+ channel 1 (TASK1) and TASK3 channels to the control of activity modes in thalamocortical neurons. J. Neurosci. *23*, 6460–6469.

Moruzzi, G. (1972). The sleep-waking cycle. Ergeb. Physiol. *64*, 1–165.

Nakanishi, H., Sun, Y., Nakamura, R.K., Mori, K., Ito, M., Suda, S., Namba, H., Storch, F.I., Dang, T.P., Mendelson, W., et al. (1997). Positive correlations between cerebral protein synthesis rates and deep sleep in *Macaca mulatta*. Eur. J. Neurosci. *9*, 271–279.

Ogawa, A., Yano, M., Tsujinaka, T., Morimoto, T., Morita, S., Taniguchi, M., Shiozaki, H., Okamoto, K., Sato, S., and Monden, M. (1997). Modulation of circadian expression of D-site binding protein by the schedule of parenteral nutrition in rat liver. Hepatology *26*, 1580–1586.

Panda, S., Antoch, M.P., Miller, B.H., Su, A.I., Schook, A.B., Straume, M., Schultz, P.G., Kay, S.A., Takahashi, J.S., and Hogenesch, J.B. (2002). Coordinated transcription of key pathways in the mouse by the circadian clock. Cell *109*, 307–320.

Petit, J.M., Tobler, I., Allaman, I., Borbely, A.A., and Magistretti, P.J. (2002). Sleep deprivation modulates brain mRNAs encoding genes of glycogen metabolism. Eur. J. Neurosci. *16*, 1163–1167.

Ramm, P., and Smith, C.T. (1990). Rates of cerebral protein synthesis are linked to slow wave sleep in the rat. Physiol. Behav. *48*, 749–753.

Rechtschaffen, A. (1998). Current perspectives on the function of sleep. Perspect. Biol. Med. *41*, 359–390.

Rhyner, T.A., Borbely, A.A., and Mallet, J. (1990). Molecular cloning of forebrain mRNAs which are modulated by sleep deprivation. Eur. J. Neurosci. *2*, 1063–1073.

Shaffery, J.P., Sinton, C.M., Bissette, G., Roffwarg, H.P., and Marks, G.A. (2002). Rapid eye movement deprivation modifies expression of long-term potentiation in visual cortex of immature rats. Neuroscience *110*, 431–443.

Shaw, P.J., Cirelli, C., Greenspan, R.J., and Tononi, G. (2000). Correlates of sleep and waking in *Drosophila melanogaster*. Science *287*, 1834–1837.

Shaw, P.J., Tononi, G., Greenspan, R.J., and Robinson, D.F. (2002). Stress response genes protect against lethal effects of sleep deprivation in *Drosophila*. Nature *417*, 287–291.

Steriade, M., and Hobson, J.A. (1976). Neuronal activity during the sleep-waking cycle. Prog. Neurobiol. *6*, 155–376.

Steriade, M., and Timofeev, I. (2003). Neuronal plasticity in thalamocortical networks during sleep and waking oscillations. Neuron *37*, 563–576.

Stickgold, R., Hobson, J.A., Fosse, R., and Fosse, M. (2001). Sleep, learning, and dreams: off-line memory reprocessing. Science *294*, 1052–1057.

Storch, K.F., Lipan, O., Leykin, I., Viswanathan, N., Davis, F.C., Wong, W.H., and Weitz, C.J. (2002). Extensive and divergent circadian gene expression in liver and heart. Nature *417*, 78–83.

Terao, A., Steininger, T.L., Hyder, K., Apte-Deshpande, A., Ding, J., Rishipathak, D., Davis, R.W., Heller, H.C., and Kilduff, T.S. (2003). Differential increase in the expression of heat shock protein family members during sleep deprivation and during sleep. Neuroscience *116*, 187–200.

Tobler, I. (2000). Phylogeny of sleep regulation. In Principles and Practice of Sleep Medicine, M.H. Kryger, T. Roth, and W.C. Dement, eds. (Philadelphia: W.B. Saunders), pp. 72–81.

Tononi, G., and Cirelli, C. (2001). Some consideration on sleep and neural plasticity. Arch. Ital. Biol. *139*, 221–241.

Tononi, G., and Cirelli, C. (2003). Sleep and synaptic homeostasis: a hypothesis. Brain Res. Bull. *62*, 143–150.

Ueda, H.R., Chen, W., Adachi, A., Wakamatsu, H., Hayashi, S., Takasugi, T., Nagano, M., Nakahama, K., Suzuki, Y., Sugano, S., et al. (2002a). A transcription factor response element for gene expression during circadian night. Nature *418*, 534–539.

Ueda, H.R., Matsumoto, A., Kawamura, M., Iino, M., Tanimura, T., and Hashimoto, S. (2002b). Genome-wide transcriptional orchestration of circadian rhythms in *Drosophila*. J. Biol. Chem. *277*, 14048–14052.

Van Dongen, H.P.A., Maislin, G., Mullington, J.M., and Dinges, D.F. (2003). The cumulative cost of additional wakefulness: dose-response effects on neurobehavioral functions and sleep physiology from chronic sleep restriction and total sleep deprivation. Sleep *26*, 117–126.

Walker, M.P., Brakefield, T., Morgan, A., Hobson, J.A., and Stickgold, R. (2002). Practice with sleep makes perfect: sleep-dependent motor skill learning. Neuron *35*, 205–211.

Wisor, J.P., O'Hara, B.F., Terao, A., Selby, C.P., Kilduff, T.S., Sancar, A., Edgar, D.M., and Franken, P. (2002). A role for cryptochromes in sleep regulation. BMC Neurosci. *3*, 20.

Wuarin, J., and Schibler, U. (1990). Expression of the liver-enriched transcriptional activator protein DBP follows a stringent circadian rhythm. Cell *63*, 1257–1266.

Yamada, M., Nagatomo, J., Setoguchi, Y., Kuroki, N., Higashi, S., and Setoguchi, T. (2000). Circadian rhythms of sterol 12alpha-hydroxylase, cholesterol 7alpha-hydroxylase and DBP involved in rat cholesterol catabolism. Biol. Chem. *381*, 1149–1153.