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Interactive report

Gene expression in the brain across the sleep–waking cycle[1]

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Abstract

Sleep and waking differ significantly in terms of behavior, metabolism, and neuronal activity. Recent evidence indicates that sleep and waking also differ with respect to the expression of certain genes. To systematically investigate such changes, we used mRNA differential display and cDNA microarrays to screen ~10 000 transcripts expressed in the cerebral cortex of rats after 8 h of sleep, spontaneous waking, or sleep deprivation. We found that 44 genes had higher mRNA levels after waking and/or sleep deprivation relative to sleep, while 10 were upregulated after sleep. Known genes that were upregulated in waking and sleep deprivation can be grouped into the following categories: immediate early genes/transcription factors (*Arc*, CHOP, *IER5*, *NGFI-A*, *NGFI-B*, *N-Ras*, *Stat3*), genes related to energy metabolism (*glucose type I transporter Glut1*, *Vgf*), growth factors/adhesion molecules (*BDNF*, *TrkB*, *F3 adhesion molecule*), chaperones/heat shock proteins (*BiP*, *ERP72*, *GRP75*, *HSP60*, *HSP70*), vesicle- and synapse-related genes (*chromogranin C*, *synaptotagmin IV*), neurotransmitter/hormone receptors (*adrenergic receptor* α_{1A} and β_2, $GABA_A$ *receptor* β_3, *glutamate NMDA receptor 2A*, *glutamate AMPA receptor GluR2* and *GluR3*, *nicotinic acetylcholine receptor* β_2, *thyroid hormone receptor TR*β), neurotransmitter transporters (*glutamate/aspartate transporter GLAST*, Na^+/Cl^- *transporter NTT4/Rxt1*), enzymes (*aryl sulfotransferase*, *c-jun N-terminal kinase 1*, *serum/glucocorticoid-induced serine/threonine kinase*), and a miscellaneous group (*calmodulin*, *cyclin D2*, *LMO-4*, *metallothionein 3*). Several other genes that were upregulated in waking and all the genes upregulated in sleep, with the exception of the one coding for membrane protein E25, did not match any known sequence. Thus, significant changes in gene expression occur across behavioral states, which are likely to affect basic cellular functions such as RNA and protein synthesis, neural plasticity, neurotransmission, and metabolism. © 2000 Elsevier Science B.V. All rights reserved.

Theme: Neural basis of behavior

Topic: Biological rhythms and sleep

Keywords: Cerebral cortex; Plasticity; Rat; Sleep deprivation

1. Introduction

The functions of sleep remain obscure despite its ubiquitous occurrence [75] and its exquisite homeostatic regulation [6]: in virtually all species studied so far, prolonged waking is followed by a compensatory increase in the duration and/or the intensity of sleep [88]. Such homeostatic regulation of sleep suggests that a distinct physiological, biochemical, or molecular process may build up beyond its usual level if sleep initiation is postponed, and that uncovering such processes may provide critical clues as to the functions of sleep.

Over the past few years, evidence has been collected indicating that several compounds accumulate in the brain during short periods of spontaneous waking or sleep deprivation, potentially leading to a need for restoration. For example, the neurotransmitter and metabolic by-product adenosine has been shown to increase in proportion to the amount of waking in the basal forebrain region [74]. Recent studies have provided evidence that the expression of certain genes changes as a function of behavioral state. In the rat cerebral cortex, the expression of *tumor necrosis factor* [94], *interleukin 1* [48,86], *cortistatin* [19] and *BDNF* [70] increases after prolonged waking or sleep deprivation relative to sleep, while the expression of *neurogranin* and *dendrin* decreases after 24 h of sleep deprivation [61,62,76]. For a few other genes, such as *tyrosine hydroxylase* [4,73], *growth hormone-releasing hormone*, *somatostatin* [91], and *galanin* [90], localized changes have been demonstrated after total or selective REM deprivation in certain hypothalamic or brainstem

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nuclei. Finally, several laboratories have shown that the transition from sleep to waking is accompanied by the activation of the expression of immediate-early genes, such as *c-fos* and *NGFI-A*, in many brain regions (see Refs. [13,14,16]). The finding of a significant change in the expression of these transcription factors between sleep and waking suggests that other 'late' genes, in addition to those already identified, may in turn be activated or deactivated in relation to the behavioral state of the animal.

While targeted experiments aimed at studying specific genes have proved very useful, it is unlikely that they will ever offer an exhaustive picture of the regulation of gene expression by sleep and waking. An alternative to candidate gene approaches is the systematic investigation of all the genes whose expression in the brain changes in relation to different behavioral states. As a first step in this direction, in a recent study we used mRNA differential display to screen for changes in gene expression in the cerebral cortex of rats after short periods (3 h) of sleep, spontaneous waking, and sleep deprivation [12]. It was found that several immediate early genes, as well as mitochondrial genes encoded by the mitochondrial genome, are differentially expressed across the sleep–waking cycle.

The present paper continues and extends previous work, with the goal of systematically establishing the differences in gene expression that occur between sleep and waking. To this end, we analyzed the expression of ~10 000 genes in the cerebral cortex of rats after sustained periods (8 h) of sleep, spontaneous waking, and sleep deprivation. We have used two complementary approaches: mRNA differential display, which allowed us an unbiased screen for random mRNAs without prior assumptions as to which transcripts might change, and cDNA microarray technology, which allowed us to screen 1176 known mRNAs, most of which are specifically expressed in the rat brain. We focused on the cerebral cortex as it appears to be the structure most significantly affected by sleep deprivation in humans [34] and is the main target of the restorative effects of sleep according to several influential hypotheses about the functions and the local mechanisms of sleep [41,50].

2. Materials and methods

2.1. Recordings

Male Wistar WKY rats (Charles River, 300–350 g) were anesthetized with pentobarbital (65–75 mg/kg) and implanted with stainless steel, round-tipped miniature screw electrodes in the skull to record the electroencephalogram (EEG), and with silver electrodes in the neck muscles of both sides to record the electromyogram. EEG electrodes were located over frontal cortex (2 mm anterior to the bregma and 2 mm lateral to the midline) and over occipital cortex (4 mm posterior to the bregma and 3.8 mm lateral to the midline). After surgery, rats were housed individually in sound-proofed recording cages. Lighting and temperature were kept constant (LD, 12:12, light on at 10:00, ~150 lux; 24±1°C, food and drink ad libitum). One week after surgery the rats were connected by means of a flexible cable and a commutator (Airflyte, Bayonne, NJ) to a Grass polygraph (Quincy, MA; model 78), and recorded continuously until the percentages and distributions of sleep and waking were normal and within published values [72]. Each day from 10:00 to 10:30 the rats were allowed to play with a new object introduced into their cage to familiarize them with the sleep deprivation procedure. After adaptation, the rats were recorded for as many days as required to satisfy the criteria for any of three experimental groups. Sleeping (S, $n=6$) rats were sacrificed during the light hours (around 18:00) at the end of a long period of sleep (at least 45 min, interrupted by periods of waking no longer than 2 min) after spending at least 75% of the previous 8 h asleep. Sleep deprived (SD, $n=6$) rats were sacrificed during the light period (around 18:00) after 8 h of total sleep deprivation. SD rats were kept awake by introducing new objects into the cage and later by moving them to a new cage, by tapping on the cage, and exposing them to new odors. Each stimulus was delivered whenever a slowing of the EEG was noted. Spontaneously awake (W, $n=5$) rats were sacrificed during the dark phase (around 6:00) after a long period of continuous waking (1 h, interrupted by periods of sleep not longer than 4 min), and after spending at least 75% of the previous 8 h awake. Animal care was in accordance with institutional guidelines, and the experiments were conducted under protocols approved by The Neurosciences Institute.

2.2. Tissue and RNA preparation

Rats were deeply anesthetized with isoflurane (within 2 min) and decapitated. The head was cooled in liquid nitrogen and the whole brain was removed. The right cerebral cortex and hippocampus were dissected, while the rest of the brain was left intact for in situ hybridization experiments. Samples were immediately frozen on dry ice and stored at −80°C. A few additional S, SD and W rats were used only for in situ hybridization experiments. In these cases, the brain was frozen without dissection. Total RNA was isolated from the right cerebral cortex of each animal by using Trizol (Gibco-BRL, Gaithersburg, MD) according to the manufacturer's instructions. Final RNA concentrations were determined spectrophotometrically.

2.3. mRNA differential display

mRNA differential display was performed as in Ref. [12]. Briefly, total RNA (2 μg) from the right cerebral cortex of each animal was individually reverse transcribed using Superscript II reverse transcriptase (Gibco-BRL) and

one of three 3′ composite anchor primers E1T_{12}M (E1= 5′CGGAATTCGG, M=A, C, or G). Each reverse transcription reaction was then amplified by PCR in the presence of [α-^{33}P]dATP (New England Nuclear–Du Pont, Natick, MA). The primers used were one of the three 3′ anchor primers used for the reverse transcription and one of 35 E2-AP 5′ arbitrary primers, where E2= CGTGAATTCG and AP is a sequence of 10 bp with a presence of 50% G+C and A+T and an absence of uninterrupted self-complementarity of more than two nucleotides. PCR conditions and specific primers for *c-fos, NGFI-A, cytochrome c oxidase subunit IV* mRNAs were as in Ref. [12]. Radiolabeled PCR products were separated on 6% denaturating polyacrylamide gels (Genomyx, Foster City, CA) and visualized by autoradiography. Individual animals were not pooled but run in parallel, and PCR reactions were performed in duplicate for each animal. Differentially expressed bands were quantified with a phosphorimager (Molecular Dynamics, Sunnivale, CA) by measuring the average volume of each band for each lane and subtracting the background. The mean density within and among conditions was calculated and significant differences among conditions were evaluated by ANOVA followed by Bonferroni correction. Differentially expressed bands were recovered from dried gels, reamplified by PCR using the same primers and subcloned into pCR™II vectors using the TA cloning kit (Invitrogen, San Diego, CA). Plasmid DNA sequencing of cloned cDNAs was carried out using the Cy5™ AutoRead sequencing kit (Pharmacia Biotech, Sweden). Sequences were screened using the BLAST program and the EMBL and GenBank databases.

2.4. cDNA microarray

Total RNA from the right cerebral cortex of the same animals used for mRNA differential display was pooled from all the rats within each experimental group (S, SD, W; 2 μg/pool), converted into ^{32}P-labeled first-strand cDNA, and used to hybridize to rat cDNA microarrays (rat Atlas cDNA array 1.0 and 1.2, Clontech, Palo Alto, CA). Each membrane was prehybridized for 1 h at 71°C in 10 ml of hybridization solution (ExpressHyb, Clontech) with continuous agitation. Hybridization with radiolabeled cDNA probes (~1×10^6 cpm/ml) was carried out overnight in 5 ml of ExpressHyb at 71°C. Membranes were washed with continuous agitation at 71°C in 2× SSC, 1% SDS (4×30 min) and then in 0.1× SSC, 0.5% SDS (2×30 min). The membranes were mounted on Whatman paper, plastic-wrapped, and exposed to a phosphorimager screen for 2–7 days. All hybridization experiments were repeated three times (with new sets of microarrays) using independent reverse transcription reactions. Signals were normalized using five housekeeping genes within the array (ubiquitin, glyceraldehyde 3-phosphate dehydrogenase, α-tubulin, β+γ-actin, ribosomal protein S29) as well as using the lowest signals from four cDNAs close to the one of interest (AtlasImage 1.0, Clontech). For each comparison (e.g., S relative to SD), and for each cDNA represented in the array, a ratio (S/SD) and an absolute difference (of intensities for S and SD) were calculated. Based on pilot experiments, a difference was considered eligible for further confirmation if the ratio was >1.3 or <0.7 and the absolute difference >30 (as measured by the phosphorimager in arbitrary signal intensity units) in at least two out of three hybridization experiments. Specific primers (Clontech) were used to amplify each cDNA of interest from rat cerebral cortex. PCR products were subcloned into pCR™II vectors and partially sequenced to verify their identity.

2.5. Ribonuclease protection assays (RPA), quantitative PCR, in situ hybridization

For RPA, antisense RNA probes were synthesized by run-off transcription from a linearized DNA template using the MAXIscript in vitro transcription kit (Ambion, Austin, TX) and [α-^{32}P]UTP (New England Nuclear–Du Pont). RPA was performed using the RPAIII™ kit (Ambion). DNAse I-digested total RNA from the right cerebral cortex was hybridized with an excess of [α-^{32}P]UTP-labeled riboprobe. To normalize the amount of sample RNA, a β-actin riboprobe was used to measure β-actin mRNA.

For real time quantitative PCR, 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 done for each experimental group (S, SD, and W), for a total of 24 reactions. 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, Coralville, IA), H_2O 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× 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, mixed, and samples were immediately returned to incubate at 42°C for 1 h. 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. PCR reactions were performed using a Sequence Detection System 5700 (Perkin Elmer, Norwalk, CT). Each reaction contained specific forward and reverse primers (200–750 nM final concentration), 2× SYBR Green Master Mix (used at 3.2×), 5 μl of a 1:10 dilution

of pooled reverse transcription product and H_2O 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, 1:100) standard curves were performed in triplicate 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 ($R^2 >= 0.99$).

In situ hybridization was performed on frontal sections as in Ref. [11] using the same antisense RNA probes used for RPA. Pretreatment of tissue sections with RNAse eliminated true hybridization signals. Hybridization with sense RNA probes showed no specific hybridization signal. Slides were scanned with a phosphorimager (Molecular Dynamics) and then exposed to Biomax film (Eastman Kodak, New Haven, CT). The riboprobe used to detect *F1-ATPase subunit α* mRNA was the same as in Ref. [12]. RPA and in situ hybridization signals were quantified densitometrically with a phosphorimager as in Ref. [12].

3. Results

3.1. Sleep percentages

Rats kept in a 12:12 light/dark cycle are asleep for most of the light period and awake for most of the dark period. We selected rats that had been asleep for the first 8 h of the light period, rats that had been spontaneously awake for the first 8 h of the dark period, and rats that had been sleep deprived during the light period for 8 h (see Section 2.1 for details). The three experimental conditions were chosen to distinguish between changes in gene expression related to sleep and waking per se and changes related to circadian time or to the sleep deprivation procedure (Fig. 1). Table 1 shows percentages of waking, non-REM sleep, and REM sleep during the final 8 recording hours prior to sacrifice for the three experimental groups of rats. The values for spontaneous waking and sleep are in agreement with baseline standard values (see, e.g., Refs. [12,72]). In sleep-deprived rats, short episodes (<30 s) of NREM sleep could not be avoided in the last 2–3 h of the deprivation without causing substantial stress, while REM sleep was completely suppressed.

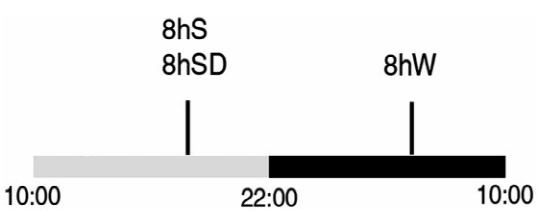

Fig. 1. Schematic drawing showing the experimental conditions chosen to distinguish changes associated with sleep, spontaneous waking, circadian time, and sleep deprivation procedure.

Table 1
Percentages of waking, non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep for the last 8 recording hours before sacrifice in the three groups of rats

	Waking	NREM	REM
S[a]	24.4±1.4	60.7±1.2	14.9±0.7
SD[a]	95.9±0.4	4.1±0.4	0.0
W[a]	78.4±2.2	19.5±1.6	2.1±0.7

[a] S, rats sacrificed after spontaneous sleep during the light period ($n=6$); SD, rats sacrificed after being sleep deprived during the light period ($n=6$); W, rats sacrificed after spontaneous waking during the dark period ($n=5$). Values are expressed as percentages of the recording time (mean±S.E.M.).

3.2. mRNA differential display

The primary mRNA differential display screening used 105 primer combinations. Thirty-two bands were identified by visual inspection as differentially expressed between sleep, sleep deprivation, and waking. Of these, 22 were higher in sleep deprivation and waking than in sleep and 10 were higher in sleep. All bands were excised from the gel, reamplified, cloned, and sequenced. Partial DNA sequence analysis revealed flanking sites complementary to the PCR primers in all cases. All species were 200–700 bp in size.

The 22 bands with higher expression levels in spontaneous waking and/or sleep deprivation relative to sleep corresponded to 19 genes, of which 10 were identified rat genes (*aryl sulfotransferase*, *BDNF*, *BiP*, *bone morphogenic protein 2*, *CHOP*, *GRP75*, *IER5*, *metallothionein 3*, *NGFI-A*, *synaptotagmin IV*). BiP was isolated using three different sets of primers. Another transcript was found to be the rat homologue of the mouse gene *LMO-4* [40,85]. The other eight transcripts with higher expression levels in spontaneous waking and/or sleep deprivation relative to sleep could not be assigned to any previously reported gene. Among the 10 transcripts with higher expression in sleep relative to spontaneous waking and sleep deprivation, one corresponded to the gene encoding the membrane protein E25 [20], while the other nine had no matches in the public sequence databases.

Confirmation of the results obtained with mRNA differential display was performed for each differentially expressed cDNA by using RPA and/or real time quantitative PCR. In situ hybridization experiments were also carried out to study the regional distribution of the transcripts. With the exception of *bone morphogenic protein 2*, all results obtained with mRNA differential display were confirmed. Examples of the differential expression of *IER5*, *BiP*, and *aryl sulfotransferase* are given in Figs. 2–4.

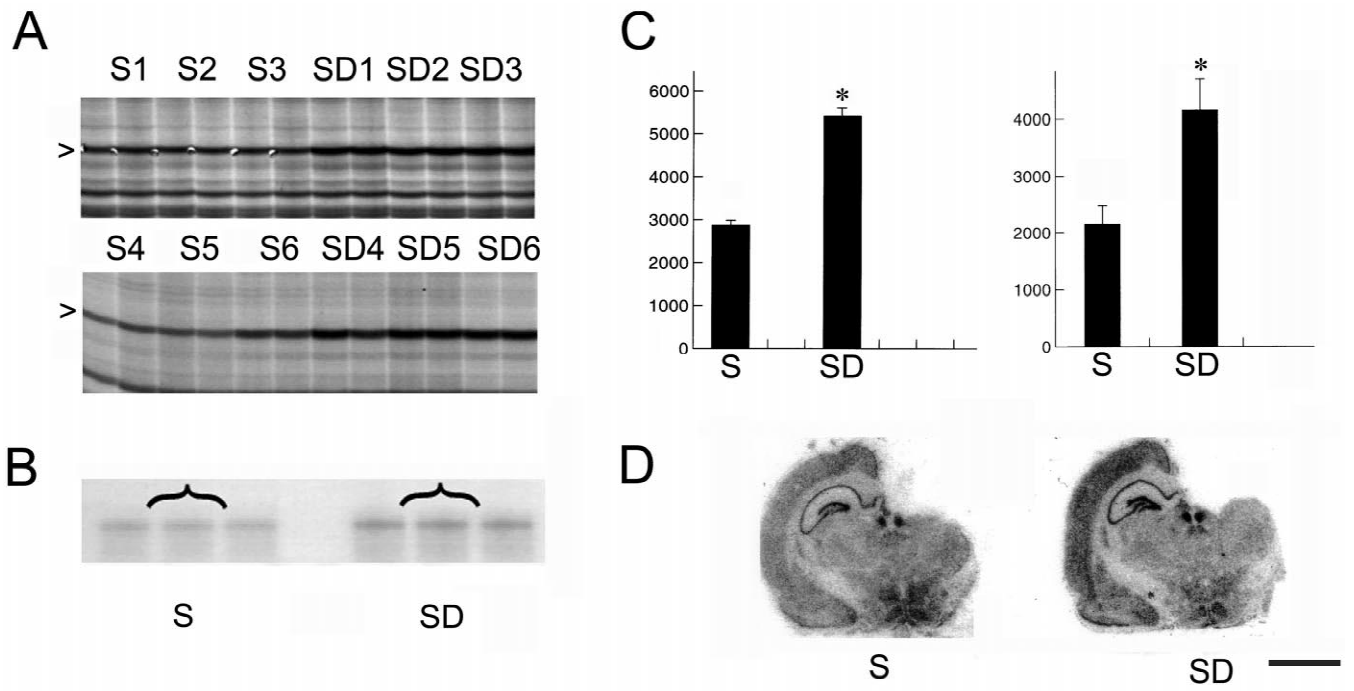

Fig. 2. Differential expression of *IER5*. (A) mRNA differential display. A band corresponding to *IER5* mRNA (arrowhead) is expressed at higher levels in the cerebral cortex of rats that were sleep deprived for 8 h (SD1–SD6) than in the cerebral cortex of rats that were asleep for 8 h (S1–S6). Pairs 1–3 and 4–6 of S and SD rats were run in different gels. The figure only shows a portion of these gels. (C, left panel) Densitometric analysis performed by scanning the two gels with a phosphorimager (*$P<0.01$, ANOVA). (B) An RPA experiment in which an equal amount of pooled total RNA from the cerebral cortex of S and SD rats was hybridized with a riboprobe specific for *IER5*. (C, right panel) Densitometric analysis performed by scanning the RPA gel with a phosphorimager (*$P<0.01$, ANOVA). (D) In situ hybridization experiments show *IER5* mRNA levels in the cerebral cortex after 8 h of sleep and sleep deprivation. Scale bar = 500 μm.

3.3. cDNA microarrays

The rat cDNA microarrays used for this study contain 1176 cDNAs representing transcription factors, intracellular signal transduction modulators, receptors for neurotransmitters and growth factors, genes involved in apoptosis, metabolism, protein turnover, and cell–cell communication. In agreement with the results obtained with mRNA differential display, the great majority of genes were expressed at the same level after 8 h of sleep, sleep deprivation and spontaneous waking. However, several genes were found to be upregulated in spontaneous waking and/or sleep deprivation relative to sleep, while no gene was found to be upregulated in sleep relative to spontaneous waking and/or sleep deprivation.

The genes whose expression was increased during spontaneous waking and/or sleep deprivation could be grouped into the following categories: immediate early genes/transcription factors (*Arc*, CHOP, *NGFI-A*, *NGFI-B*, *N-Ras*, *Stat3*), genes related to energy metabolism and energy balance (*glucose type I transporter Glut1*, *Vgf*), growth factors/adhesion molecules (*TrkB*, *F3 adhesion molecule*), chaperones/heat shock proteins (*BiP*, *ERP72*, *GRP75*, *HSP60*, *HSP70*), vesicle- and synapse-related genes (*chromogranin C*, *synaptotagmin IV*), genes for neurotransmitter/hormone receptors (*adrenergic receptor* α_{1A} and β_2 *subunits*, *GABA*$_A$ *receptor* β_3 *subunit*, *glutamate NMDA receptor 2A subunit*, *glutamate AMPA receptor GluR2* and *GluR3 subunits*, *nicotinic acetylcholine receptor* β_2, *thyroid hormone receptor TR*β), neurotransmitter transporters (*glutamate/aspartate transporter GLAST*, *Na*$^+$*/Cl*$^-$ *transporter NTT4/Rxt1*), enzymes (*c-jun N-terminal kinase 1*, *serum/glucocorticoid-induced serine/threonine kinase*), and other genes (*calmodulin*, *cyclin D2*). The differential expression of these genes was confirmed in all cases using RPA and/or real time quantitative PCR, and in situ hybridization experiments were performed to study the regional distribution of the transcripts (e.g., Fig. 5).

Table 2 summarizes all the known transcripts that were found to be upregulated after 8 h of spontaneous waking and/or sleep deprivation relative to 8 h of sleep using either mRNA differential display or cDNA microarray technology and whose differential expression was independently confirmed with other techniques. Fig. 6 shows the differential expression of these transcripts as measured by RPA or real time quantitative PCR. In most cases, the increase in mRNA levels relative to sleep was more marked after sleep deprivation than after spontaneous waking. In a subset of cases (*Stat3*, *ERP72*, *GRP75*, *HSP70*, *adrenergic receptor* α_{1A} and β_2 *subunits*, *thyroid hormone receptor TR*β, *aryl sulfotransferase*, *SGK1*,

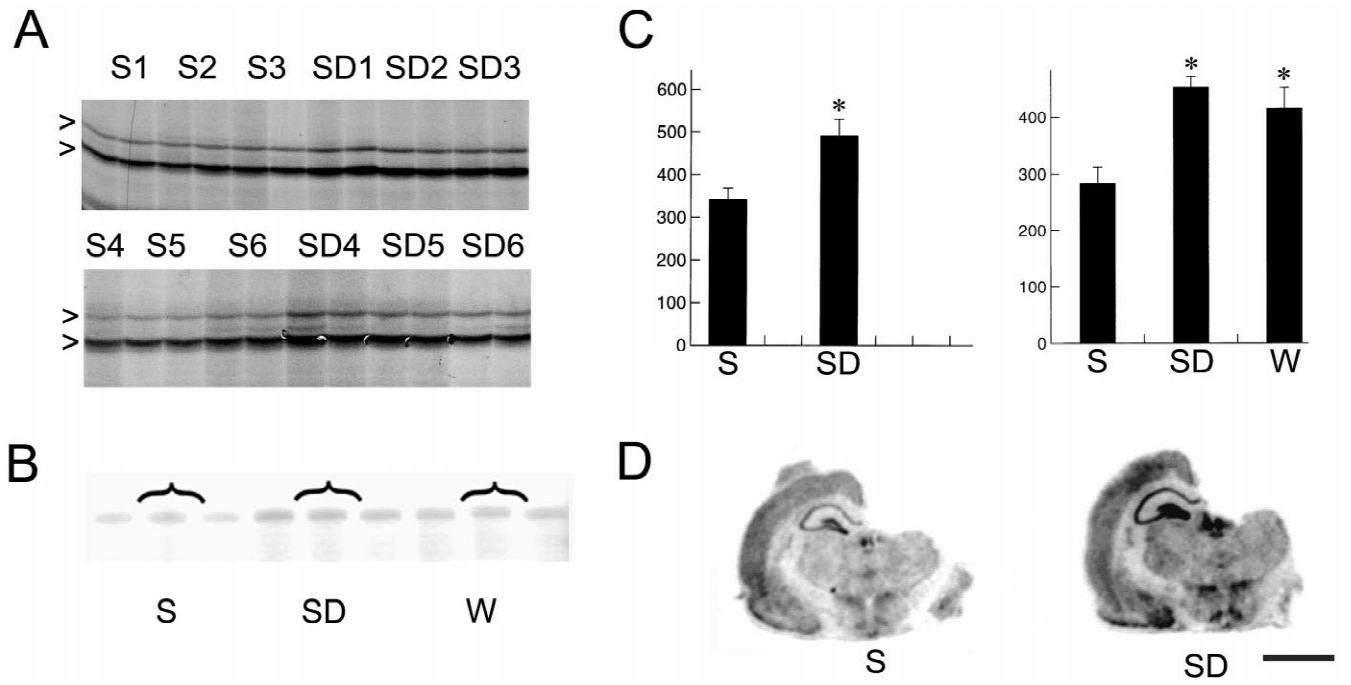

Fig. 3. Differential expression of *BiP*. (A) mRNA differential display. Two bands corresponding to *BiP* mRNA (arrowheads) are expressed at higher levels in the cerebral cortex of rats that were sleep deprived for 8 h (SD1–SD6) than in the cerebral cortex of rats that were asleep for 8 h (S1–S6). Pairs 1–3 and 4–6 of S and SD rats were run in different gels. The figure only shows a portion of these gels. (C, left panel) Densitometric analysis performed by scanning the two gels with a phosphorimager (*$P<0.01$, ANOVA). (B) An RPA experiment in which an equal amount of pooled total RNA from the cerebral cortex of S, SD, and W rats was hybridized with a riboprobe specific for *BiP*. (C, right panel) Densitometric analysis performed by scanning the RPA gel with a phosphorimager (*$P<0.01$, ANOVA; comparison between S and SD and between S and W). (D) In situ hybridization experiments show *BiP* cortical mRNA levels after 8 h of sleep and sleep deprivation. Scale bar = 500 μm.

MT3), only sleep deprived but not spontaneously awake rats showed significantly higher mRNA levels relative to sleep.

3.4. Expression of transcripts upregulated after 3 h of waking

Our previous study [12] found two main groups of known genes to be upregulated after 3 h of spontaneous waking and sleep deprivation relative to sleep: immediate early genes/transcription factors (including *c-fos* and *NGFI-A*) and mitochondrial genes encoded by the mitochondrial genome (*cytochrome oxidase subunit I, NADH dehydrogenase subunit 2* and *12S rRNA*).

In this study, mRNA differential display showed changes in the expression of *NGFI-A* after 8 h of spontaneous waking and sleep deprivation relative to sleep, but not in the expression of *c-fos*, nor in the expression of *cytochrome oxidase subunit I, NADH dehydrogenase subunit 2* and *12S rRNA*. RPA (Fig. 7) and in situ hybridization confirmed that RNA levels of the three mitochondrial transcripts were similar after 8 h of sleep, spontaneous waking, and sleep deprivation. *Cytochrome oxidase subunit IV* (Fig. 7) and *F1-ATPase subunit α*, two nuclear-encoded mitochondrial genes, also did not increase their expression after 8 h of spontaneous waking and/or sleep deprivation relative to sleep.

4. Discussion

4.1. General findings

The present study provides a comprehensive evaluation of changes in gene expression in the cerebral cortex as a function of behavioral state. The results obtained after sustained periods of sleep, sleep deprivation, and spontaneous waking (8 h) complement and extend previous findings from a screening of gene expression after shorter periods of sleep and waking (3 h [12–14]). Several general conclusions can be drawn from these experiments. First, only a small minority of the genes screened change their expression after both short and long periods of physiological sleep or waking. A previous study that examined changes in gene expression after 24 h of sleep deprivation by subtractive cDNA cloning reached similar conclusions: of the 4000 clones screened, only 10 transcripts were found to be differentially expressed in the rat forebrain [76]. Screening studies employing less physiological experimental paradigms have yielded different results. For example, hundreds of genes change their expression in the

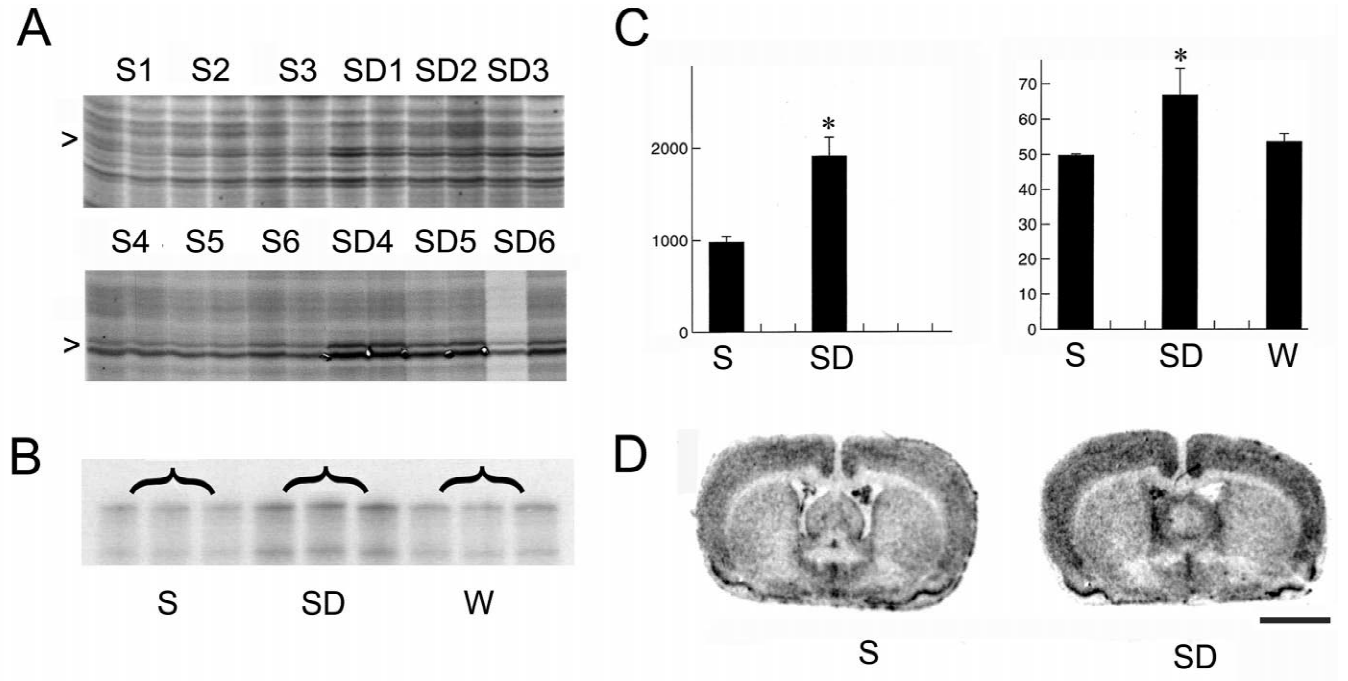

Fig. 4. Differential expression of *aryl sulfotransferase*. (A) mRNA differential display. A band corresponding to *aryl sulfotransferase* mRNA (arrowhead) is expressed at higher levels in the cerebral cortex of sleep deprived rats (SD1–SD6) than in sleeping rats (S1–S6). Pairs 1–3 and 4–6 of S and SD rats were run in different gels. The figure only shows a portion of these gels. (C, left panel) Densitometric analysis performed by scanning the two gels with a phosphorimager. The difference in signal intensity between S and SD rats was statistically significant ($*P<0.01$, ANOVA). (B) An RPA experiment in which an equal amount of pooled total RNA from the cerebral cortex of S, SD, and W rats was hybridized with a riboprobe specific for *aryl sulfotransferase*. (C, right panel) Densitometric analysis performed by scanning the RPA gel with a phosphorimager ($*P<0.01$, ANOVA; comparison between S and SD and between S and W). (D). In situ hybridization experiments show *aryl sulfotransferase* mRNA levels after 8 h of sleep and sleep deprivation. Scale bar = 500 μm.

dentate gyrus a few hours after systemic application of kainate (e.g., Ref. [31]). However, most of the 'activity-regulated' genes identified after seizures or electroconvulsive shock have not been isolated under the milder stimulation conditions used for LTP [53].

A second observation is that the majority of differentially expressed genes are upregulated in spontaneous waking and/or sleep deprivation rather than in sleep. Whether this finding indicates that waking is more conducive than sleep to the activation of gene transcription remains to be determined. In this context, it is relevant that the induction of at least some of the genes that are expressed at higher levels during waking is controlled by the noradrenergic system of the locus coeruleus, a system that fires at higher rates during waking than during sleep [1]. Specifically, the noradrenergic system is responsible for the increased expression during waking of *c-fos*, *NGFI-A*, *P-CREB* [9], *BiP* [10], *Arc*, and *BDNF* (Cirelli and Tononi, submitted). It should be cautioned, however, that the finding of higher mRNA levels in one behavioral state relative to another may reflect an increase in transcription rate, a reduced degradation of mRNA, or a combination of both factors. In the case of *c-fos*, previous findings support increased transcription [3]. On the other hand, the increase in mRNA levels of the glucose transporter *Glut1* observed in many experimental conditions is due to mRNA stabilization, rather than to an increased transcription rate [5].

A third observation is that only a minority of the genes that are upregulated in spontaneous waking and/or sleep deprivation but almost all the genes that are upregulated in sleep do not match any known sequence. This asymmetry may be explained in part by the fact that differential cloning techniques have generally been used to identify genes that are rapidly induced by depolarizing stimuli, pharmacological agents, or pathological conditions, but not by conditions that more closely resemble physiological sleep, such as, for example, anesthesia. The characterization of the transcripts that are upregulated in sleep is in progress and will be the subject of a future publication.

A final observation is that, while most genes upregulated by sleep deprivation were also upregulated by spontaneous waking, the increase in mRNA levels was generally more marked in the former condition. In addition, a few genes were upregulated by sleep deprivation but not by spontaneous waking. In interpreting this result, it should be pointed out that sleep deprived rats were prevented from sleeping for 8 h starting from light on, after a 12-h dark period during which they were mostly awake. This means that these rats had generally been awake for considerably more than 8 h, with little intervening sleep. By contrast,

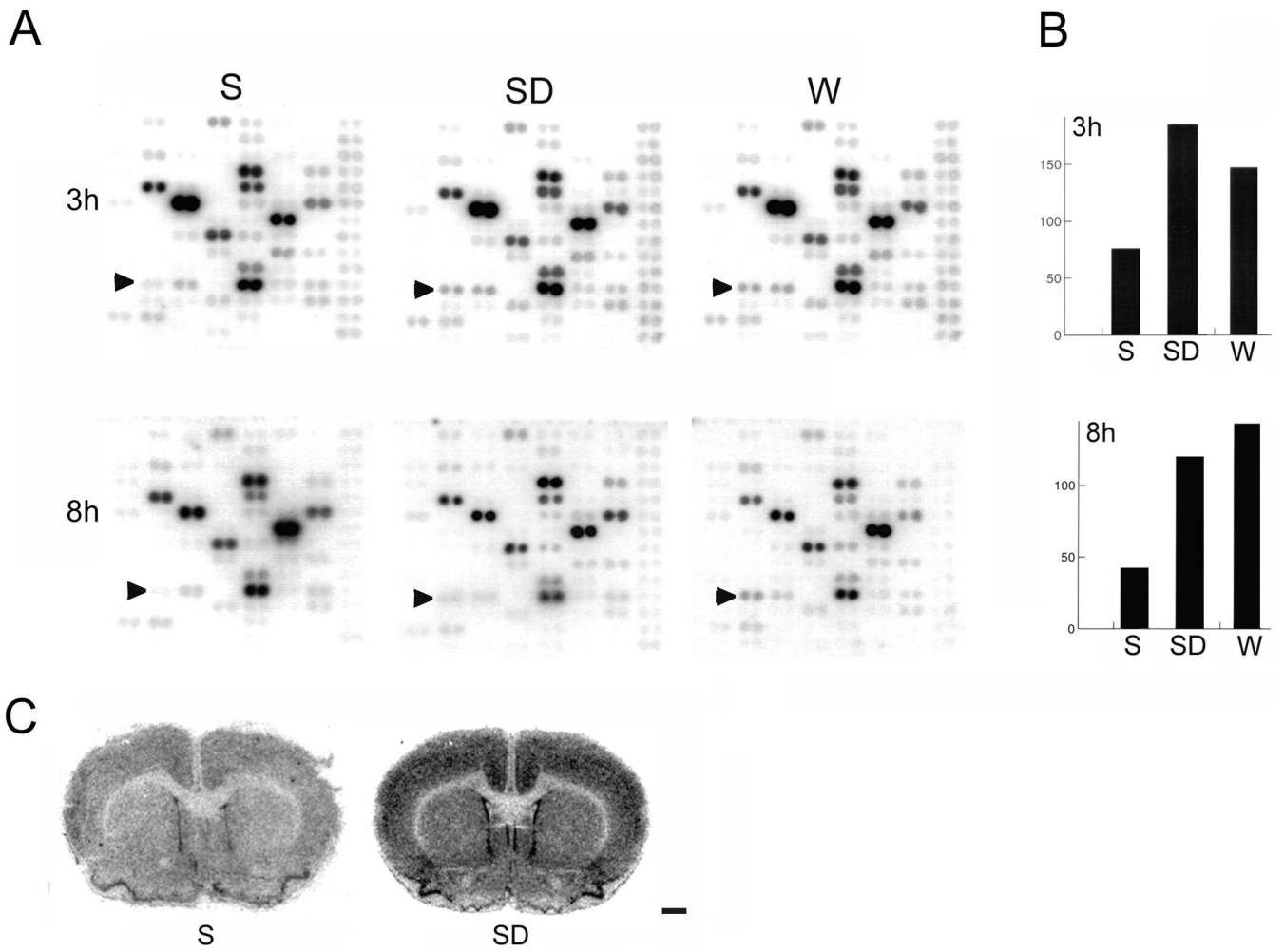

Fig. 5. Differential expression of *NGFI-B*. (A) Rat cDNA microarray. *NGFI-B* mRNA levels (arrowhead) are higher in the cerebral cortex after 3 or 8 h of either sleep deprivation (SD) or spontaneous waking (W) than after 3 or 8 h of spontaneous sleep (S). The figure only shows a portion of the cDNA microarray. Signal quantification using a phosphorimager is shown in (B). In situ hybridization experiments show *NGFI-B* mRNA levels after 8 h of sleep and sleep deprivation. Scale bar = 1 mm.

rats sacrificed after 8 h of spontaneous waking starting from light off were mostly asleep during the previous light period.

In our previous screening, performed after 3 h of sleep and waking, we identified two main groups of known genes that are upregulated during spontaneous waking and/or sleep deprivation relative to sleep [12]: immediate early genes/transcription factors and mitochondrial genes encoded by the mitochondrial genome. The present study demonstrates that the genes induced by 8 h of spontaneous waking and/or sleep deprivation encompass a much wider spectrum. This includes a large number of immediate early genes/transcription factors, genes related to energy metabolism (but not mitochondrial genes), growth factors/adhesion molecules, molecular chaperones, vesicle and synapse-related genes, genes for neurotransmitter receptors and transporters, and genes coding for enzymes involved in the catabolism of monoamines or in signal transduction pathways. Such diversity is to be expected because it includes 'late' genes, which are induced after the immediate early genes, and which are therefore more likely to be isolated after 8 h rather than 3 h of sleep deprivation or waking.

It is not precisely known how many genes are expressed in the rat cerebral cortex. However, it has been estimated that there are somewhere between 5000 and 30 000 different mRNAs in the rat brain [56]. The rat cDNA microarrays used in this study permit the screening of 1176 known genes. mRNA differential display was performed with 105 combinations of primers, with an average of 150 different bands per gel. If each PCR product visualized in a gel corresponds to a different gene, over 15 000 RNA species have been screened. However, nearby bands in a gel often correspond to the same gene [12]. Thus, the actual number of genes screened in this study is more likely to be ~10 000, making the screening extensive, but not exhaustive. This may explain why some transcripts induced by a few hours of sleep deprivation according to recent studies (e.g., cortistatin [19]) were not identified in

Table 2
Known genes expressed at higher levels in the rat cerebral cortex after 8 h of sleep deprivation or spontaneous waking relative to sleep[a]

Immediate early genes and/or transcription factors
Arc (*Arg 3.1*)
CHOP
IER5
NGFI-A
NGFI-B
N-Ras
Stat3
Genes related to energy metabolism and energy balance
Glucose type I transporter (*Glut1*)
Vgf
Growth factors and adhesion molecules
Brain-derived neurotrophic factor (*BDNF*)
Tyrosine kinase receptor B (*TrkB*)
F3 adhesion molecule
Chaperones, heat shock proteins
BiP (glucose regulated protein 78 (*GRP78*)
Endoplasmic reticulum protein 72 (*ERP72*)
Glucose-regulated protein 75 (*GRP75*)
Heat shock protein 60 (*HSP60*)
Heat shock protein 70 (*HSP70*)
Vesicle- and synapse-related genes
Chromogranin C (*secretogranin II*)
Synaptotagmin IV
Neurotransmitter and hormone receptors
Adrenergic receptor α_{1A}
Adrenergic receptor β_2
GABA$_A$ *receptor* β_3
Glutamate NMDA receptor 2A
Glutamate AMPA receptor GLUR2
Glutamate AMPA receptor GLUR3
Nicotinic acetylcholine receptor β_2
Thyroid hormone receptor TRβ
Transporters
Glutamate/aspartate transporter (*GLAST*)
Na^+/Cl^-*-dependent neurotransmitter transporter* (*NTT4/Rxt1*)
Enzymes
Aryl sulfotransferase
c-Jun N-terminal kinase 1 (*JNK1*)
Serum/glucocorticoid-induced serine/threonine kinase (*SGK1*)
Other genes
Calmodulin
Cyclin D2 (*Vin-1*)
LMO-4
Metallothionein 3

[a] Genes were identified using mRNA differential display and cDNA microarray technology and differential expression was confirmed using ribonuclease protection assay and/or real-time quantitative PCR.

our study. A recent comprehensive mRNA differential display screening designed to identify long term potentiation (LTP)-related genes isolated 10 differentially expressed transcripts, but failed to detect other genes known to be upregulated following LTP induction [53]. As mentioned by Matsuo et al. [53], false-negatives can be due to the fact that: (1) low abundance mRNAs may not be sufficiently amplified to be visible in the gel; and (2) changes in gene expression limited to small subgroups of cells can be diluted when screening a brain region as a whole. Interestingly, two of the 10 genes identified in the hippocampus after LTP by Matsuo et al. [53] were *CHOP* and *IER5*, which we also found to be upregulated in the cerebral cortex after spontaneous waking and sleep deprivation.

4.2. Immediate early genes/transcription factors

Immediate early genes are so called because their transcription is induced in response to a stimulus without the need for de novo protein synthesis. Previous studies found that the expression of *c-fos*, *NGFI-A*, *NGFI-B*, *Jun B* and *rfl* is higher after 3–6 h of spontaneous waking and sleep deprivation relative to 3–6 h of sleep (see Refs. [12–14,16] for references). *c-Fos* induction typically occurs when a new stimulus is applied, while *NGFI-A* induction persists throughout the duration of the stimulation (see, e.g., Ref. [38]). In agreement with this evidence, *c-fos* mRNA levels are increased after 3 h of waking but revert to the levels observed during sleep after 8 h of waking (a detailed discussion of the significance of *c-fos* induction during waking can be found in Ref. [16]. By contrast, *NGFI-A* mRNA levels, which increase after 3 h of waking, remain elevated after 8 h of spontaneous waking or sleep deprivation. A similar time course is observed for *NGFI-B* (Fig. 5) and *Arc* (Cirelli and Tononi, submitted). *IER5*, which belongs to a group of immediate early genes that are induced with slower kinetics than *c-fos* [100], was found to increase after 8 but not after 3 h of sleep deprivation.

In contrast to *c-fos* and *jun B*, *c-jun* mRNA and protein levels are not upregulated by membrane depolarization in vitro, nor by increases in electrical activity (e.g., seizures) in vivo (see Ref. [16] for references). It is not surprising, therefore, that *c-jun* expression did not change in the brain after sleep deprivation [16]. However, for its transcription regulating activity *c-jun* needs to be phosphorylated by c-Jun N-terminal kinases (JNK 1, 2, and 3), and JNK activity increases in the brain after seizures, restraint stress, and exposure to a novel environment [8,102]. We show here that the mRNA levels of JNK 1 are increased in spontaneous waking and sleep deprivation relative to sleep. As a next step, it will be necessary to determine whether the increase in mRNA levels is also associated with an increase in JNK protein levels and in JNK enzymatic activity during waking. If this were the case, the ability of *c-jun* to activate gene transcription could be strongly potentiated during waking relative to sleep even in the absence of an increase in c-Jun protein levels.

With the exception of *Arc* (a cytoskeleton associated

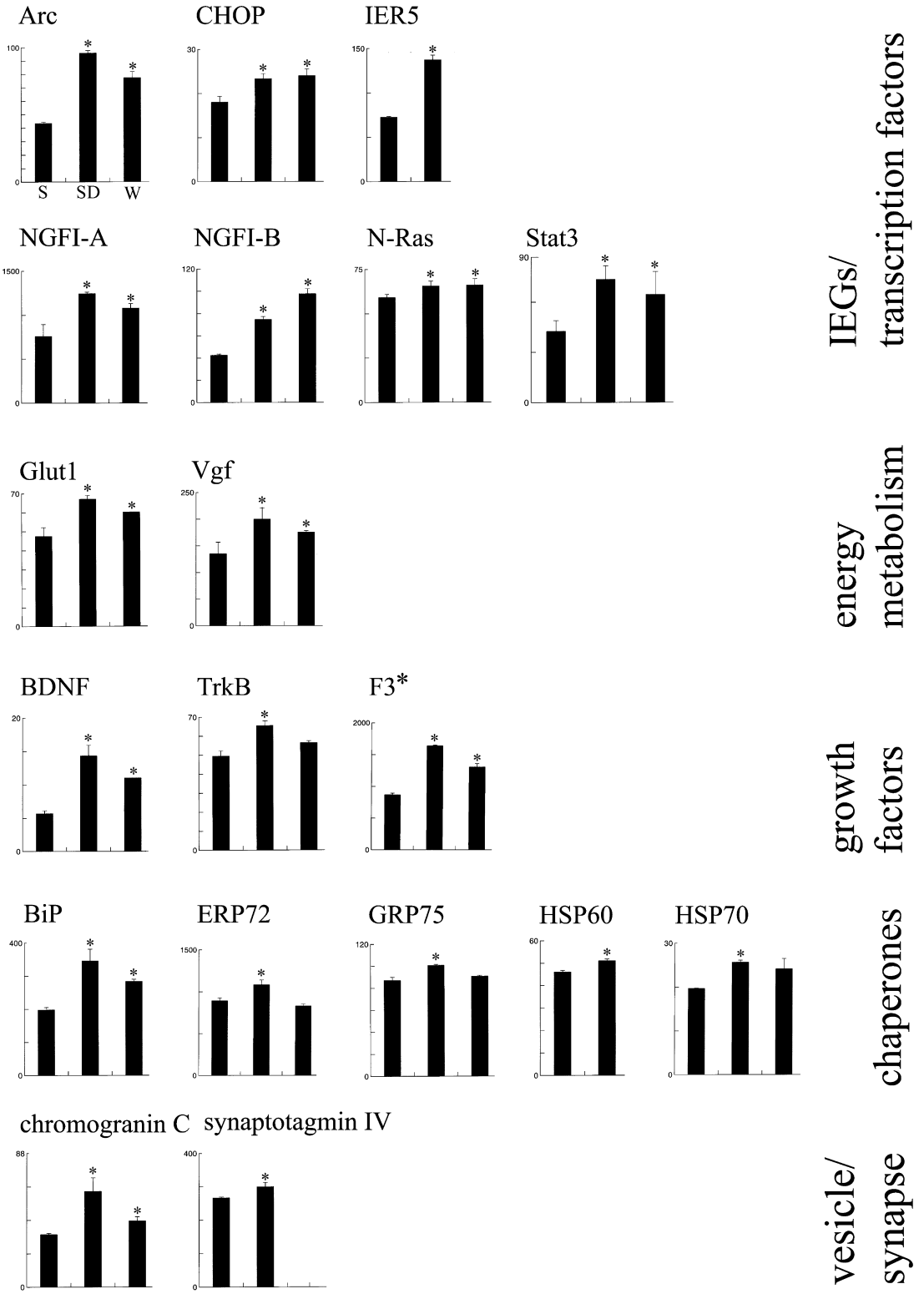

Fig. 6. Differential expression of all the transcripts corresponding to known genes whose mRNA levels were higher after spontaneous waking (W) and/or sleep deprivation (SD) than after sleep (S). Most of the graphs refer to RPA experiments performed to confirm the results previously obtained with mRNA differential display and/or cDNA microarray technology. The graphs show the densitometric analysis performed by scanning the RPA gel with a phosphorimager. The signal intensity units on the ordinate are dependent upon the exposure length of each gel to the phosphorimager screen and are meaningful only for comparisons within the same gel. In some cases (*), the confirmation was performed using real-time quantitative PCR rather than RPA and the values on the ordinate refer to the number of mRNA copies. Results from real-time quantitative PCR are not comparable between genes. *$P<0.05$, ANOVA; comparison between S and SD and between S and W. For the abbreviation used for each gene, see Table 2. For *IER5*, *HSP60*, and *synaptotagmin IV*, results from the W group were not available.

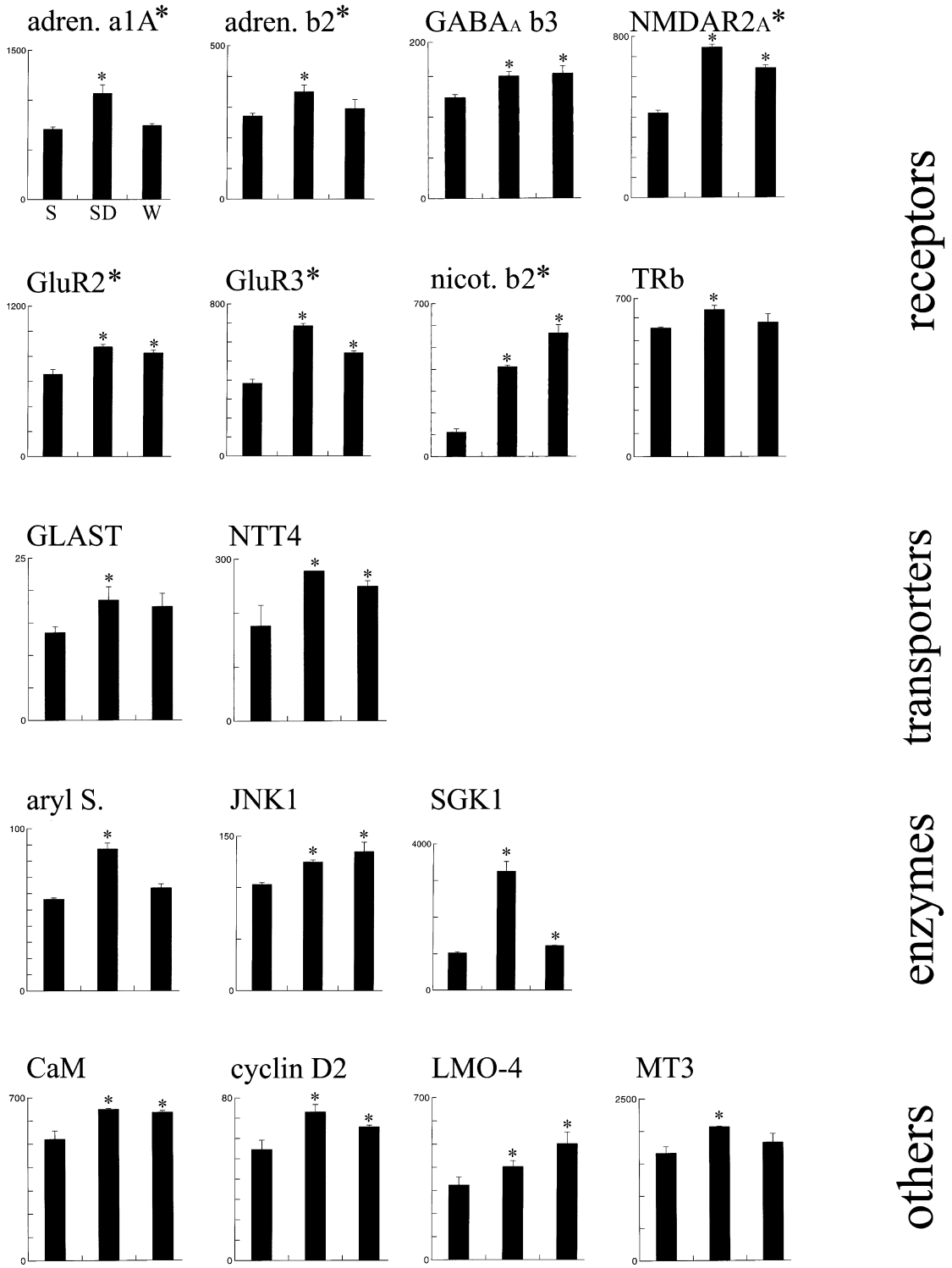

Fig. 6. (*continued*)

protein), *N-Ras* (a proto-oncogene encoding a GTPase) and *IER5* (whose function is still unclear), all immediate early genes whose mRNA levels increase during spontaneous waking and/or sleep deprivation (*c-fos*, *NGFI-A*, *NGFI-B*, *CHOP*, *Stat3*) are transcription factors, i.e., proteins that control the transcription of other 'target' genes by binding to specific DNA sequences. Another gene that seems to be involved in the control of transcription, and whose expression is upregulated in spontaneous waking and sleep deprivation, is *LMO-4*. LMO proteins are a family of coregulators thought to influence gene transcription by associating with DNA-binding proteins (see Ref. [85]).

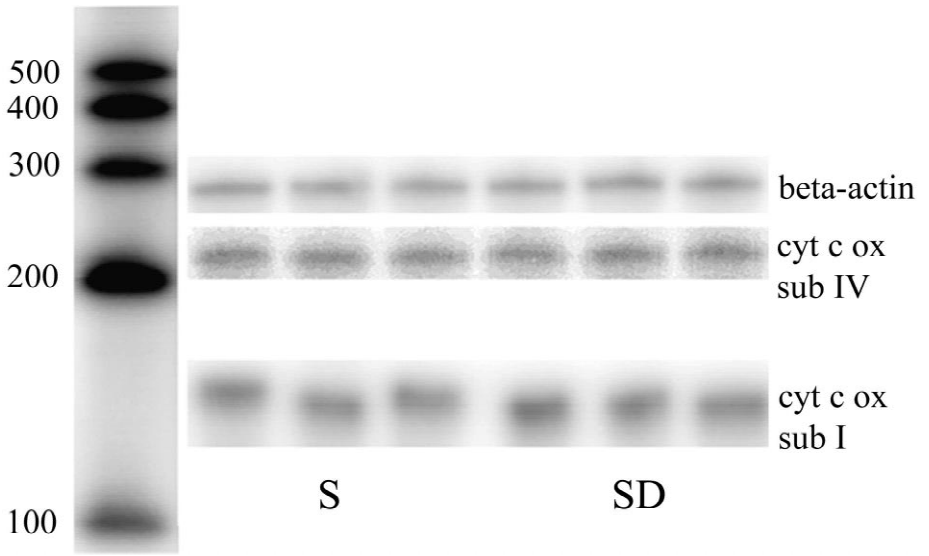

Fig. 7. RPA experiments showing similar cortical mRNA levels of *β-actin*, *cytochrome c oxidase subunit I*, and *cytochrome c oxidase subunit IV* in rats spontaneously asleep (S) or sleep deprived (SD) for 8 h. Total RNA from six S and six SD rats was pooled and run in triplicate. The *β-actin* antisense riboprobe was used to normalize the amount of sample RNA on each gel. The gel was exposed for 1 day for *cytochrome c oxidase subunit IV* and for 5 days for *β-actin*. *Cytochrome c oxidase subunit I* was run on a different gel and exposed for 1 day (*β-actin* not shown). Lane 1 (from left) shows molecular weight markers.

LMO-4 is highly expressed in the cerebral cortex [30] and the expression of *LMO-1, 2,* and *3* is regulated by seizure activity [32]. To our knowledge, the finding that *LMO-4* mRNA levels are increased during spontaneous waking and sleep deprivation relative to sleep is the first demonstration that the expression of this new member of the LMO family is regulated by neuronal activity. In a previous study we found that another transcript that is upregulated after 3 h of spontaneous waking and sleep deprivation relative to sleep is similar to the human homologue of SNF2/SWI2, a global activator of transcription in yeast [13,14]. Thus, the induction of transcription factors, the upregulation of LMO-4, and the increased levels of the transcript related to SNF2/SWI2, suggest that waking may promote the transcriptional activation of a select group of 'target' genes.

Arc [43,47] is unique among activity-regulated genes in that, after induction, its mRNA is selectively transported to activated postsynaptic sites, where it may play a role in activity-dependent synaptic plasticity requiring protein synthesis [84]. *Arc* expression has been shown to be important for the formation of long-term memories in the hippocampus [27]. In addition to *Arc*, several other genes whose mRNA levels where found to be higher in spontaneous waking and sleep deprivation relative to sleep have been implicated in neural plasticity. They include *NGFI-A*, which has already been mentioned, and other genes that will be discussed later, such as *BiP, synaptotagmin IV, chromogranin C, BDNF*, and its receptor *TrkB*. Thus, it would appear from these molecular data that neural plasticity, inasmuch as it requires the induction of gene expression, occurs preferentially during waking. In agreement with this hypothesis, we have recently found that the levels of the phosphorylated form of cAMP responsive element binding protein (P-CREB) are higher in the cerebral cortex of awake rats than in sleeping rats (Cirelli and Tononi, submitted). P-CREB has been tied to the occurrence of plastic changes in several animal models [81].

4.3. Genes related to energy metabolism and energy balance

mRNA levels of the glucose transporter *Glut1* were found to increase by 30–40% during spontaneous waking and sleep deprivation relative to sleep. Brain metabolism is 15–20% higher in waking than in sleep and the brain relies almost exclusively on glucose as its energy substrate. Glucose is transported from blood to neurons and glia by facilitative glucose transporters, six of which have been identified in the brain (see Ref. [21] for references). The predominant forms are Glut1 (in the brain capillary endothelium and in the perivascular endfeet of astrocytes) and Glut3 (in neurons). Increases or decreases in *Glut1* expression correlate with increases or decreases in cerebral glucose utilization, respectively (e.g., Ref. [22]). *Glut1* mRNA and/or protein levels increase in response to kainic acid-induced seizure, K^+-induced depolarization, and chronic administration of nicotine [21] and decrease in visual brain areas after chronic visual deprivation [22]. Thus, *Glut1* induction may represent a mechanism by which the brain responds to the increased energy requirements of waking. According to the traditional view, glucose transport capacity in the brain always exceeds

demand. However, recent work has shown that hippocampal extracellular glucose concentration significantly decreases during a difficult spatial task and that such a decrease can be prevented by systemic administration of glucose [55]. Thus, intense cognitive activity during waking can deplete brain extracellular glucose and may trigger compensatory mechanisms.

Cerebral glucose is almost exclusively metabolized through mitochondrial oxidative phosphorylation. In our previous gene expression screening, we found that, in many cortical and subcortical regions, RNA levels of three mitochondrial genes encoded by the mitochondrial genome — *subunit I of cytochrome c oxidase* (*CO*), *subunit 2 of NADH*, and *12S rRNA* — were uniformly higher after 3 h of waking than after 3 h of sleep, while the expression of the nuclear-coded *CO subunit IV* and *F1-ATPase subunit α* did not change. In the present study, we found that none of these genes was upregulated after 8 h of spontaneous waking or sleep deprivation. Thus, there is no upregulation of nuclear-encoded mitochondrial transcripts after either short (3 h) or sustained (8 h) periods of waking. On the other hand, mitochondrial-encoded transcripts are upregulated shortly after the transition between sleep and waking, but the response is transient and does not persist after sustained periods of waking. In agreement with this conclusion, mRNA levels of *NADH dehydrogenase subunit 2* were found to be similar in rats sleep-deprived for 24 h with respect to their controls [76]. It has recently been shown that mitochondria contain an excess of nuclear-encoded CO subunits, and that it is the synthesis of the mitochondrial-encoded subunits, followed by the CO holoenzyme assembly, that is controlled by local changes in energy demands [101]. This finding suggests that the increased amount of CO holoenzyme assembled shortly after the onset of waking as a result of the rapid upregulation of mitochondrial-encoded transcripts may be sufficient to sustain the energy demand of a prolonged period of waking.

Another gene whose mRNA levels were increased in spontaneous waking and sleep deprivation relative to sleep corresponds to *Vgf*. Vgf is a neuropeptide amply expressed in the developing and adult brain, particularly in hypothalamus and cerebral cortex, where it is localized in synaptic large dense core vesicles and is released through regulated pathways. *Vgf* expression is rapidly induced by growth factors, cAMP, neuronal activity, seizures, and lesions [46,59,82]. Recently, *Vgf* has been implicated in the regulation of energy balance because its expression is upregulated in the hypothalamus during fasting and knockout mice lacking *Vgf* are hypermetabolic and hyperactive [28].

4.4. BDNF, TrkB, and F3

Our results indicate that mRNA levels of *BDNF* are higher in spontaneous waking and sleep deprivation relative to sleep. In addition, mRNA levels of BDNF's specific tyrosine kinase receptor, *TrkB*, are also higher in the waking conditions. *BDNF* expression is increased not only by strong stimuli such as seizures and treatment with depolarizing agents, but also by LTP and, more physiologically, by visual and osmotic stimuli. *BDNF* is considered a good candidate as modulator of activity-dependent plasticity because: (1) its expression is strongly modulated by neuronal activity (at both mRNA and protein levels); (2) it has potent effects on synaptic transmission and signaling properties of neurons; (3) it is able to alter dendritic morphology (reviewed in Ref. [54]). Electrical or pharmacological stimulation also increase *TrkB* mRNA and protein levels and *TrkB*, together with *BDNF*, plays an essential role in hippocampus-mediated learning [57]. mRNA levels of F3/contactin were also higher in spontaneous waking and sleep deprivation relative to sleep. F3/contactin is a cell adhesion molecule specifically expressed in the brain. It can be anchored to the cell membrane of neurons or be present in a soluble form. By binding to different ligands such as other adhesion molecules and extracellular matrix molecules, F3 can act either as a neuronal receptor or as a substrate for neurite growth. During development, F3 expression can control neurite growth and can be upregulated with synaptic contact formation [96]. In the adult, activity-dependent changes in F3 expression have been described in the magnocellular nuclei of the hypothalamus, where F3 colocalizes with vasopressin and oxytocin in neurosecretory granules [87]. The fact that F3 mRNA levels are increased during spontaneous waking and sleep deprivation could be another indication that synaptic plastic changes are occurring during these behavioral states rather than during sleep.

4.5. Molecular chaperones

Molecular chaperones facilitate the folding of most newly synthesized proteins in the cell [25]. The major families of general chaperones are HSP40, HSP60, HSP70 (which includes *Bip* and *Grp75*), and HSP90. In this study, the largest increase in expression after spontaneous waking and sleep deprivation relative to sleep was shown by *BiP*, while the induction of the two mitochondrial chaperones Hs*p60* and G*rp75* was less marked.

BiP is constitutively expressed at high levels in the endoplasmic reticulum (ER), where it assists in the folding and assembly of newly synthesized glycoproteins and secretory polypeptides. Increases in mRNA and/or protein levels of *BiP* have been classically described after heat shock and ischemia, which cause accumulation of unfolded proteins in the ER. In these abnormal conditions BiP may either target unfolded protein for degradation or participate in their refolding. *BiP* is also induced by other conditions that adversely affect ER function, such as anoxia, glucose deprivation (BiP is also called glucose-regulated protein 78) and depletion of ER Ca^{2+}. *BiP* expression increases

during status epilepticus [44] and after axotomy [58] and its induction may impart cellular tolerance to environmental stress (e.g., Ref. [45]).

The increase in *BiP* mRNA levels observed after 8 h of spontaneous waking and sleep deprivation suggests that this gene can be induced in normal cells in the absence of any obvious stress factor such as glucose deprivation or hypoxia. Such physiological, waking-related increase is all the more interesting in view of the recent demonstration that an upregulation of *BiP* also occurs in the brain of *Drosophila*, where *BiP* mRNA levels are significantly higher after periods of waking and sleep deprivation relative to comparable periods of sleep-like behavior [77].

The events responsible for *BiP* induction during waking are unclear at the moment. An increase in protein synthesis, notably of proteins that require assembly in the ER, could play a role. The *Aplysia* homologue of BiP, which is induced after long-term sensitization training, may be instrumental in the folding of newly synthesized proteins involved in synaptic plasticity [42]. Interestingly, preliminary results in our laboratory show that cortical *BiP* mRNA levels in awake rats are significantly decreased after lesion of the noradrenergic system of the locus coeruleus [10], a neuromodulatory system that can gate the triggering of plastic changes [9]. Another possibility is that *BiP* induction during waking is triggered by a depletion of Ca^{2+} in the ER. Although there is no direct evidence that waking causes Ca^{2+} depletion, the finding that prolonged REM sleep deprivation is associated, in most brain areas, with a decrease in synaptosomal Ca^{2+}, is at least consistent with this hypothesis [49].

In addition to triggering the expression of BiP, conditions that perturb ER function and protein folding cause the induction of the transcription factor CHOP (C/EBP homologous protein 10, also called gadd153; [97]) and of the ER protein 72 (e.g., Ref. [69]). Both genes were found in this study to be upregulated during spontaneous waking and/or sleep deprivation.

Metallothioneins (MT) are intracellular zinc-binding proteins whose primary function remains unclear [93]. MT1 and MT2 are found in all organs and are induced by glucocorticoids, cytokines, and metal ions. MT1 and MT2 seem to be important for zinc uptake, distribution, storage, and release. They have been suggested to function as chaperones for the synthesis of metalloproteins [93]. MT3, on the other hand, which is upregulated in sleep deprivation relative to sleep, is unresponsive to most inducers of MT1 and MT2 [67] and might have a function distinct from MT1 and MT2.

4.6. Vesicle- and synapse-related genes

Chromogranins (A, B, and C) are secretory glycoproteins that coexist with peptide transmitters in large dense-core synaptic vesicles of many types of neurons (see references in Ref. [78]). Chromogranin B and C are thought to be released upon depolarization and are sensitive to changes in neuronal activity. mRNA levels of both chromogranin B and C increase in acutely and chronically stimulated neurons, e.g., after kainic acid-induced seizures and cortical spreading depression [78]. We found that spontaneous waking and sleep deprivation increase cortical mRNA levels of chromogranin C (also called secretogranin II) and synaptotagmin IV. Chromogranin B cDNA was not represented in the microarrays.

Synaptotagmin IV is an abundant membrane protein that is localized to synaptic vesicles. Both mRNA [95] and protein levels [23] of synaptotagmin IV increase a few hours after potassium depolarization or kainic acid-induced seizures. In cultured neurons, synaptotagmin IV is present in the Golgi apparatus and in distal parts of growing neurites, where its expression increases in response to membrane depolarization [36]. Because of its activity-dependent expression and its subcellular localization, synaptotagmin IV has been considered by some as a marker of synaptic plasticity. The upregulation of components of the synaptic transmission machinery following sleep deprivation may represent a compensatory response to increased levels of neurotransmission during these behavioral states.

4.7. Neurotransmitter and hormone receptors

The mRNA levels of several receptor subunits of neurotransmitters and neuromodulators were found to be increased in spontaneous waking and/or sleep deprivation relative to sleep. They include the genes for the adrenergic receptor α_{1A} and β_2 subunits, $GABA_A$ receptor β_3 subunit, glutamate NMDA receptor 2A subunit, glutamate AMPA receptor GluR2 and GluR3 subunits, nicotinic acetylcholine receptor β_2, and thyroid hormone T3 receptor β.

Little is known about changes in adrenergic receptor mRNA levels in different experimental conditions, except that cortical spreading depression increases α_{1B} (but not α_{1A} or β_2) adrenoceptor mRNA levels [79]. Noradrenergic neurons, which fire during waking in response to salient events, markedly decrease their firing rate or cease firing altogether during sleep [1]. It has been hypothesized that such changes in locus coeruleus firing during the sleep–waking cycle should be accompanied by a homeostatic regulation of the sensitivity and/or the number of adrenergic receptors [80]. Irrespective of the specific predictions of this hypothesis, the finding of a modulation in the expression of α_{1A} and β_2 adrenoceptors by sleep deprivation could indeed be due to the concurrent changes in the activity of the locus coeruleus, as has been demonstrated for other genes (see above).

The fast component of the GABA-mediated inhibitory transmission is mediated by $GABA_A$ receptors. The β_3 subunit is a major component of the $GABA_A$ receptor, especially in the cerebral cortex and hippocampus (see references in Ref. [33]). Knockout β_3 deficient mice

mostly die as neonates. The few that survive are hyperactive, hyperresponsive to sensory stimuli and display motor problems, myoclonus, and occasional seizures [33]. In monkeys, β_3 mRNA levels in the visual cortex are not affected by monocular deprivation [35], suggesting that they are not responsive to reduced neuronal activity. However, as suggested by our results, they might respond to sustained electrical activity during prolonged periods of either spontaneous or forced waking, with potential functional consequences on neuronal excitability.

The NMDA-type glutamate receptor is involved in synaptic plasticity and excitotoxicity. It is composed of NMDAR1 and NMDAR2 protein subunits. There are four genes encoding variants of NMDAR2 ($NMDAR2_{A-D}$). The composition of NMDAR subunits affects the functional properties of the receptor and different subunits are differentially regulated during development and by various ligands (e.g., Ref. [26]). The finding that the expression of $NMDAR2_A$ is upregulated in waking with respect to sleep suggests that this receptor subunit is regulated under physiological conditions in the adult animal.

The AMPA-type glutamate receptor is the principal mediator of fast excitatory transmission in the brain. AMPA receptors are comprised of different combinations of four subunits, GluR1–4. The GluR2 subunit is expressed in most cortical pyramidal cells and plays a major role in determining calcium permeability of the receptor, which is low when Glur2 is highly expressed (see references in Ref. [17]). Pathological events such as ischemia can cause a switch in subunit composition and a downregulation of GluR2 expression, with subsequent increased Ca^{2+} permeability and cell death (see references in Ref. [17]). The finding of increased GluR2 and GluR3 mRNA levels after spontaneous waking and sleep deprivation relative to sleep raises the question of whether Ca^{2+} permeability of cortical neurons may change across the sleep–waking cycle.

Activation of neuronal nicotinic acetylcholine receptors, which are located on axon terminals, increases the release of several neurotransmitters, including acetylcholine, dopamine, norepinephrine, serotonin, GABA, and glutamate. The predominant nicotinic receptor expressed in the vertebrate brain contains α_4 and β_2 subunits, with the β_2 subunit mediating many of the pharmacological and behavioral effects of nicotine [71]. Except for the effect of nicotine treatment (e.g., Ref. [66]), little is known about changes in mRNA levels of nicotinic receptor subunits. To our knowledge, the significant increase in mRNA levels of the β_2 subunit in spontaneous waking and sleep deprivation relative to sleep reported here is the first demonstration of an activity-dependent regulation in the expression of this gene.

The adult brain is considered to be unresponsive to thyroid hormones based on traditional biochemical criteria such as oxygen consumption. However, thyroid hormones are selectively taken up in the adult brain and the relatively inactive T4 is metabolized to the more active T3 [51]. When complexed with its receptor, T3 acts as transcription factor that binds to DNA and regulate gene expression. However, thyroid hormones are highly concentrated in synaptic terminal fractions and may also function as neuromodulators [51]. There are two main subtypes of T3 receptors, α and β, encoded by two different genes, TRα and TRβ, whose mRNAs are highly expressed in the entire cerebral cortex [7]. To our knowledge, changes in the expression of T3 receptors had been demonstrated so far only in the developing brain, usually in response to hypothyroid conditions. The finding of a small but significant upregulation of TRβ mRNA levels in the cerebral cortex of adult rats after sleep deprivation suggests a possible involvement of this signaling system in the physiological regulation of neural activity, although its functional relevance is at present unknown.

4.8. Transporters

At least five different types of excitatory amino acid transporters (EAATs) have been identified. Two of them, the astroglial transporters GLAST and GLT1, are responsible for most of the functional glutamate transport that maintains the extracellular glutamate concentration at a low level. Several experimental conditions are associated with an increase in extracellular glutamate concentration, including kainic acid-induced seizures, ischemia, and nerve transection. In all cases GLAST mRNA levels increase considerably [65], probably as an attempt to prevent glutamate neurotoxicity. After kainic acid-induced seizures, GLAST mRNA levels reached 150, 200 and 300% of baseline levels 6, 12, and 48 h following treatment, respectively [65]. In the present study, GLAST mRNA levels increased by ~30% after 8 h of spontaneous waking, a completely physiological condition. This result is consistent with an increased release of glutamate in the cerebral cortex during waking relative to sleep. Indeed, cortical glutamate release assessed using a plastic chamber sealed into the skull was found to be higher in waking than in sleep [37]. Subsequent studies of glutamate release in other brain structures have yielded contrasting evidence, possibly due to regional differences. Two microdialysis studies found higher extracellular glutamate levels in spontaneous waking ([2]; anterior hypothalamus) or after modafinil-induced waking ([24]; thalamus and hippocampus) relative to sleep, but a third study obtained opposite results ([39]; thalamus), and two other studies found no changes across the sleep–waking cycle ([63,64]; posterior hypothalamus and locus coeruleus).

Spontaneous waking and sleep deprivation also cause an upregulation of the neurotransmitter transporter NTT4/Rxt1. This is a member of a large family of Na^+/Cl^--dependent transporters that includes GABA, noradrenaline, serotonin, and glycine transporters [52,60]. NTT4 is widely distributed in the brain, with the highest levels of

expression in cerebral cortex and thalamus, where it is associated with synaptic vesicles in nerve terminals of glutamatergic neurons and of some GABAergic neurons. NTT4 is therefore a vesicular transporter, but its substrate has yet to be identified.

4.9. Aryl sulfotransferase and serum/glucocorticoid-induced serine/threonine kinase

Aryl sulfotransferases are a large family of enzymes that catalyze sulfate conjugation of catecholamines, steroids, and drugs. The cDNA that we isolated in the cerebral cortex of sleep-deprived rats encodes an enzyme first cloned from rat liver as phenol sulfotransferase 1 and subsequently identified in the same tissue as aryl sulfotransferase IV, minoxidil sulfotransferase, tyrosine-ester sulfotransferase, and aryl sulfotransferase [99]. In the brain, aryl sulfotransferase is responsible for the sulfonation of norepinephrine, dopamine and, to a lesser extent, serotonin [92]. In rodents, norepinephrine is first metabolized to 3-methoxy-4-hydroxyphenylethylene glycol (MHPG) by catechol-*o*-methyl transferases and monoaminooxidases. Subsequently, MHPG is sulfonated by aryl sulfotransferase. MHPG-sulfate is the major metabolite of norepinephrine and MHPG-sulfate levels are well correlated with the activity of the noradrenergic system of the locus coeruleus [18]. As was mentioned above, the activity of noradrenergic neurons is strictly state-dependent, being high in waking and low in sleep [1]. Preliminary results show that mRNA levels of *aryl sulfotransferase* increase further after long periods (several days) of sleep deprivation [15]. If confirmed, this would be the first demonstration of a molecular response in the brain to prolonged sleep loss and would suggest that the induction of aryl sulfotransferase may constitute a homeostatic response to the uninterrupted activity of the central noradrenergic system during waking. This notion is strengthened by the evidence of converging molecular correlates that was recently obtained in *Drosophila* [77]. Rest deprivation in *Drosophila* is associated with an increased expression of arylalkylamine *N*-acetyltransferase (DAT), an enzyme implicated in the catabolism of monoamines and functionally related to *aryl sulfotransferase*. Moreover, flies mutant or deficient for DAT have an increased rest rebound after rest deprivation, suggesting that the accumulation of monoamines in the brain may trigger sleep homeostasis.

Another enzyme whose mRNA levels are considerably increased (200%) after sleep deprivation is serum/glucocorticoid-induced serine/threonine kinase (*SGK1*; [98]). As with *aryl sulfotransferase*, preliminary results suggest that *SGK1* expression is further increased after long-term with respect to short-term sleep deprivation (Cirelli and Tononi, unpublished results). *SGK1* has been implicated in both cell proliferation and anti-proliferation, as well as in the control of cell volume and sodium homeostasis. However, little is known about its functions and none of its downstream targets have been identified. Like most protein kinases, *SGK1* is regulated at the post-translational level [68]. However *SGK1* is unique in being strongly regulated at the transcriptional level by glucocorticoid, serum, and other signals (see references in Ref. [68]).

4.10. Other genes

Other genes upregulated during spontaneous waking and/or sleep deprivation form a heterogeneous group. These genes include *LMO-4* and *MT3*, which were discussed above, *cyclin D2 (Vin-1)*, which has been involved in oncogenesis [29] and *calmodulin*. Calmodulin is a major Ca^{2+}-binding protein present in all eucaryotic cells and particularly abundant in the brain. Calmodulin modulates the action of numerous Ca^{2+}-dependent enzymes such as adenylate cyclase and Ca^{2+}-ATPase, and it is involved in the synthesis of neurotransmitters, in synaptic function and neurotransmitter release, microtubule function, and Ca^{2+}-mediated regulation of gene expression. Three genes encoding calmodulin have been isolated in the rat: *CaM I*, *CaM II*, and *CaM III*. The three genes produce five different transcripts but the same identical protein because they share the same coding region. The cDNA contained in the microarrays used for this study includes only a portion of the coding region. Thus, we cannot establish which of the three *CaM* genes were differentially regulated across the sleep–waking cycle. *CaM I*, *II*, and *III* expression may be differentially regulated during development and after kainate-induced seizures in the hippocampus [83].

5. Conclusions

The results of the systematic screening of brain gene expression during sleep and waking lead to several conclusions. As shown here, sleep and waking differ not only in terms of behavior, metabolism, and neuronal activity, but they are characterized by the up- or down-regulation of distinctive categories of genes. The few genes that are specifically upregulated during sleep are presently still unidentified and await further characterization. The genes that are upregulated after periods of waking and/or sleep deprivation suggest that several basic cellular functions are affected by the arousal state of the animal. The increase in the expression of genes regulating glucose transport may reflect the elevated metabolic needs of waking. The higher expression during waking of genes related to neural plasticity provides a molecular correlate for the observation that long-term memories are established preferentially or perhaps exclusively during waking [89]. It is well known that neuronal firing patterns and, consequently, the release of several neurotransmitters change significantly between sleep and waking. The present results further

indicate that sleep and waking are associated with marked changes in the expression of neurotransmitter receptors and transporters, which could exert sustained effects on neurotransmission. Another category of genes whose expression is consistently higher in waking than in sleep is that of cellular chaperones, which play a key role in protein folding and ER function. This suggests that waking may place some increased demands on such cellular processes, or that sleep may be a time for their restoration. The delineation of the spectrum of molecular differences between sleep and waking should help directing further work and provide important data to constrain hypotheses on the functions of sleep.

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References

[1] G. Aston-Jones, F.E. Bloom, Activity of norepinephrine-containing locus coeruleus neurons in behaving rats anticipates fluctuations in the sleep-waking cycle, J. Neurosci. 1 (1981) 876–886.
[2] S. Azuma, T. Kodama, K. Honda, S. Inoué, State-dependent changes of extracellular glutamate in the medial preoptic area in freely behaving rats, Neuroscience 214 (1996) 179–182.
[3] R. Basheer, J.E. Sherin, C.B. Saper, J.I. Morgan, R.W. McCarley, P.J. Shiromani, Effects of sleep on wake-induced c-fos expression, J. Neurosci. 17 (1997) 9746–9750.
[4] R. Basheer, M. Magner, R.W. McCarley, P.J. Shiromani, REM sleep deprivation increases the levels of tyrosine hydroxylase and noradrenaline transporter mRNA in the locus coeruleus, Mol. Brain Res. 57 (1998) 235–240.
[5] R.J. Boado, W.M. Pardridge, Glucose deprivation causes post-transcriptional enhancement of brain capillary endothelial glucose transporter gene expression via GLUT1 mRNA stabilization, J. Neurochem. 60 (1993) 2290–2296.
[6] A.A. Borbély, P. Achermann, Concepts and models of sleep regulation: an overview, J. Sleep Res. 1 (1992) 63–79.
[7] D.J. Bradley, W. Scott Young III, C. Weinberger, Differential expression of α and β thyroid hormone receptor genes in rat brain and pituitary, Proc. Natl. Acad. Sci. USA 86 (1989) 7250–7254.
[8] S. Brecht, S. Simler, M. Vergnes, K. Mielke, C. Marescaux, T. Herdegen, Repetitive electroconvulsive seizures induce activity of c-Jun N-terminal kinase and compartment-specific desensitization of c-Jun phosphorylation in the rat brain, Mol. Brain Res. 68 (1999) 101–108.
[9] C. Cirelli, M. Pompeiano, G. Tononi, Neuronal gene expression in the waking state: a role for the locus coeruleus, Science 274 (1996) 1211–1215.
[10] C. Cirelli, G. Tononi, Changes in gene expression in DSP-4 treated animals revealed by mRNA differential display, Soc. Neurosci. Abstr. 23 (1997) 689.
[11] C. Cirelli, M. Pompeiano, G. Tononi, Immediate early genes as a tool to understand the regulation of the sleep-waking cycle: immunocytochemistry, in situ hybridization and antisense approaches, in: R. Lydic (Ed.), Molecular Regulation of Arousal States, CRC Press, Boca Raton, FL, 1998, pp. 45–55.
[12] C. Cirelli, G. Tononi, Differences in gene expression between sleep and waking as revealed by mRNA differential display, Mol. Brain Res. 56 (1998) 293–305.
[13] C. Cirelli, G. Tononi, Differences in brain gene expression between sleep and waking as revealed by mRNA differential display and cDNA microarray technology, J. Sleep Res. 8 (Suppl 1) (1999) 44–52.
[14] C. Cirelli, G. Tononi, Differences in gene expression during sleep and wakefulness, Ann. Med. 31 (1999) 117–124.
[15] C. Cirelli, G. Tononi, Changes in gene expression in the cerebral cortex of rats after short-term and long-term sleep deprivation, Sleep 22 (Suppl. 1) (1999) 113.
[16] C. Cirelli, G. Tononi, On the functional significance of *c-fos* induction during the sleep/waking cycle, Sleep 23 (2000) 453–469.
[17] F. Conti, A. Minelli, N.C. Brecha, Cellular localization and laminar distribution of AMPA glutamate receptor subunits mRNAs and proteins in the rat cerebral cortex, J. Comp. Neurol. 350 (1994) 241–259.
[18] J.R. Cooper, F.E. Bloom, R.H. Roth (Eds.), The Biochemical Basis of Neuropharmacology, 7th Edition, Oxford University Press, New York, 1996, pp. 226–292.
[19] L. De Lecea, J. Criado, M. Calbet, P.E. Danielson, S.J. Henriksen, J.G. Sutcliffe, O. Prospero-Garcia, Sleep regulates accumulation of the cortical neuropeptide cortistatin, Soc. Neurosci. Abstr. 24 (1998) 1429.
[20] W. Deleersnijder, G. Hong, R. Cortvrindt, C. Poirier, P. Tylzanowski, K. Pittois, E. Van Marck, J. Merregaert, Isolation of markers for chondro-osteogenic differentiation using cDNA library subtraction. Molecular cloning and characterization of a gene belonging to a novel multigene family of integral membrane proteins, J. Biol. Chem. 271 (1996) 19475–19482.
[21] S.U. Devaskar, P.A. Rajakumar, R.B. Mink, R.A. McKnight, S. Thamotharan, S.J. Hicks, Effect of development and hypoxic-ischemia upon rabbit brain glucose transporter expression, Brain Res. 823 (1999) 113–128.
[22] R. Duelli, M.H. Maurer, W. Kuschinsky, Decreased glucose transporter densities, rate constants and glucose utilization in visual structures of the rat brain during chronic visual deprivation, Neurosci. Lett. 250 (1998) 49–52.
[23] G.D. Ferguson, D.M. Thomas, L.A. Elferink, H.R. Herschman, Synthesis, degradation, and subcellular localization of synaptotagmin IV, a neuronal immediate early gene product, J. Neurochem. 72 (1999) 1821–1831.
[24] L. Ferraro, T. Antonelli, W.T. O'Connor, S. Tanganelli, F. Rambert, K. Fuxe, The antinarcoleptic drug modafinil increases glutamate release in thalamic areas and hippocampus, Neuroreport 8 (1997) 2883–2887.
[25] A.L. Fink, Chaperone-mediated protein folding, Physiol. Rev. 79 (1999) 425–449.
[26] P. Follesa, M.K. Ticku, NMDA receptor upregulation: molecular studies in cultured mouse cortical neurons after chronic antagonist exposure, J. Neurosci. 16 (1996) 2172–2178.
[27] J.F. Guzowski, G.L. Lyford, G.D. Stevenson, F.P. Houston, J.L. McGaugh, P.F. Worley, C.A. Barnes, Inhibition of activity-dependent Arc protein expression in the rat hippocampus impairs the maintenance of long-term potentiation and the consolidation of long-term memory, J. Neurosci. 20 (2000) 3993–4001.
[28] S. Hahm, T.M. Mizuno, T.J. Wu, J.P. Wisor, C.A. Priest, C.A. Kozak, C.N. Boozer, B. Peng, R.C. McEvoy, P. Good, K.A. Kelley, J.S. Takahashi, J.E. Pintar, J.L. Roberts, C.V. Mobbs, S.R. Salton, Targeted deletion of the *Vgf* gene indicates that the encoded secretory peptide precursor plays a novel role in the regulation of energy balance, Neuron 23 (1999) 537–548.

[29] Z. Hanna, M. Jankowski, P. Tremblay, X. Jiang, A. Milatovich, U. Francke, P. Jolicoeur, The Vin-1 gene, identified by provirus insertional mutagenesis, is the cyclin D2, Oncogene 8 (1993) 1661–1666.
[30] O. Hermanson, T.M. Sugihara, B. Andersen, Expression of LMO-4 in the central nervous system of the embryonic and adult mouse, Cell. Mol. Biol. 45 (1999) 677–686.
[31] D. Hevroni, A. Rattner, M. Bundman, D. Lederfein, A. Gabarah, M. Mangelus, M.A. Silverman, H. Kedar, C. Naor, M. Kornuc, T. Hanoch, R. Seger, L.E. Theill, E. Nedivi, G. Richter-Levin, Y. Citri, Hippocampal plasticity involves extensive gene induction and multiple cellular mechanisms, J. Mol. Neurosci. 10 (1998) 75–98.
[32] G.L. Hinks, B. Shah, S.J. French, L.S. Campos, K. Staley, J. Hughes, M.V. Sofroniew, Expression of LIM protein genes *Lmo1*, *Lmo2*, and *Lmo3* in adult mouse hippocampus and other forebrain regions: differential regulation by seizure activity, J. Neurosci. 17 (1997) 5549–5559.
[33] G.E. Homanics, T.M. DeLorey, L.L. Firestone, J.J. Quinlan, A. Handforth, N.L. Harrison, M.D. Krasowski, C.E. Rick, E.R. Korpi, R. Mäkelä, M.H. Brilliant, N. Hagiwara, C. Ferguson, K. Snyder, R.W. Olsen, Mice devoid of gamma-aminobutyrate type A receptor beta3 subunit have epilepsy, cleft palate, and hypersensitive behavior, Proc. Natl. Acad. Sci. USA 94 (1997) 4143–4148.
[34] J.A. Horne, in: Why We Sleep. The Functions of Sleep in Humans and Other Mammals, Oxford University Press, Oxford, 1988, 319 pp.
[35] M.M. Huntsman, E.G. Jones, Expression of alpha3, beta3 and gamma1 GABA(A) receptor subunit messenger RNAs in visual cortex and lateral geniculate nucleus of normal and monocularly deprived monkeys, Neuroscience 87 (1998) 385–400.
[36] K. Ibata, M. Fukuda, T. Hamada, H. Kabayama, K. Mikoshiba, Synaptotagmin IV is present at the Golgi and distal parts of neurites, J. Neurochem. 74 (2000) 518–526.
[37] H.H. Jasper, R.T. Khan, K.A.C. Elliott, Amino acids released from the cerebral cortex in relation to its state of activation, Science 147 (1965) 1448–1449.
[38] L. Kaczmarek, A. Chaudhuri, Sensory regulation of immediate-early gene expression in mammalian visual cortex: implications for functional mapping and neural plasticity, Brain Res. Rev. 23 (1997) 237–256.
[39] K.A. Kekesi, A. Dobolyi, O. Salfay, G. Nyitrai, G. Juhasz, Slow wave sleep is accompanied by release of certain amino acids in the thalamus of cats, Neuroreport 8 (1997) 1183–1186.
[40] D.A. Kenny, L.W. Jurata, Y. Saga, G.N. Gill, Identification and characterization of *LMO4*, an LMO gene with a novel pattern of expression during embryogenesis, Proc. Natl. Acad. Sci. USA 95 (1998) 11257–11262.
[41] J.M. Krueger, F. Obal Jr., L. Kapas, J. Fang, Brain organization and sleep function, Behav. Brain Res. 69 (1995) 177–186.
[42] D. Kuhl, T.E. Kennedy, A. Barzilai, E.R. Kandel, Long-term sensitization training in *Aplysia* leads to an increase in the expression of BiP, the major protein chaperon of the ER, J. Cell Biol. 19 (1992) 1069–1076.
[43] W. Link, U. Konietzko, G. Kauselmann, M. Krug, B. Schwanke, U. Frey, D. Kuhl, Somatodendritic expression of an immediate early gene is regulated by synaptic activity, Proc. Natl. Acad. Sci. USA 92 (1995) 5734–5738.
[44] E. Little, G. Tocco, M. Baudry, A.S. Lee, S.S. Schreiber, Induction of glucose-regulated protein (glucose-regulated protein 78/BiP and glucose-regulated protein 94) and heat shock protein 70 transcripts in the immature rat brain following status epilepticus, Neuroscience 75 (1996) 209–219.
[45] H. Liu, R.C. Bowes, B. van de Water, C. Sillence, J.F. Nagelkerke, J.L. Stevens, Endoplasmic reticulum chaperones GRP78 and calreticulin prevent oxidative stress, Ca^{2+} disturbances, and cell death in renal epithelial cells, J. Biol. Chem. 272 (1997) 21751–21759.
[46] A. Lombardo, S.A. Rabacchi, F. Cremisi, T. Pizzorusso, M.C. Cenni, R. Possenti, G. Barsacchi, L. Maffei, A developmentally regulated nerve growth factor-induced gene. VGF, is expressed in geniculocortical afferents during synaptogenesis, Neuroscience 65 (1995) 997–1008.
[47] G.L. Lyford, K. Yamagata, W.E. Kaufmann, C.A. Barnes, L.K. Sanders, N.G. Copeland, D.J. Gilbert, N.A. Jenkins, A.A. Lanahan, P.F. Worley, Arc, a growth factor and activity-regulated gene, encodes a novel cytoskeleton-associated protein that is enriched in neuronal dendrites, Neuron 14 (1995) 433–445.
[48] M. Mackiewicz, P.J. Sollars, M.D. Ogilvie, A.I. Pack, Modulation of IL-1 beta gene expression in the rat CNS during sleep deprivation, Neuroreport 7 (1996) 529–533.
[49] B.N. Mallick, S. Gulyani, Alterations in synaptosomal calcium concentrations after rapid eye movement sleep deprivation in rats, Neuroscience 75 (1996) 729–736.
[50] P. Maquet, Sleep function(s) and cerebral metabolism, Behav. Brain Res. 69 (1995) 75–83.
[51] J.V. Martin, D.B. Williams, R.M. Fitzgerald, H.K. Im, P.F. Vonvoigtlander, Thyroid hormonal modulation of the binding and activity of the GABAA receptor complex of brain, Neuroscience 73 (1996) 705–713.
[52] J. Masson, M. Riad, F. Chaudhry, M. Darmon, Z. Aidouni, M. Conrath, B. Giros, M. Hamon, J. Storm-Mathisen, L. Descarries, S. El Mestikawy, Unexpected localization of the Na^+/Cl^--dependent-like orphan transporter, Rxt1, on synaptic vesicles in the rat central nervous system, Eur. J. Neurosci. 11 (1999) 1349–1361.
[53] R. Matsuo, A. Murayama, Y. Saitoh, Y. Sakaki, K. Inokuchi, Identification and cataloging of genes induced by long-lasting long-term potentiation in awake rats, J. Neurochem. 74 (2000) 2239–2249.
[54] A.K. McAllister, L.C. Katz, D.C. Lo, Neurotrophins and synaptic plasticity, Annu. Rev. Neurosci. 22 (1999) 295–318.
[55] E.C. McNay, T.M. Fries, P.E. Gold, Decreases in rat extracellular hippocampal glucose concentration associated with cognitive demand during a spatial task, Proc. Natl. Acad. Sci. USA 97 (2000) 2881–2885.
[56] F.D. Milner, J.G. Sutcliffe, Gene expression in rat brain, Nucleic Acids Res. 11 (1983) 5497–5520.
[57] L. Minichiello, M. Korte, D. Wolfer, R. Kühn, K. Unsicker, V. Cestari, C. Rossi-Arnaud, H.-P. Lipp, T. Bonhoeffer, R. Klein, Essential role for TrkB receptors in hippocampus-mediated learning, Neuron 24 (1999) 401–414.
[58] M.T. Moreno-Flores, U.E. Olazábal, G.W. Kreutzberg, Axotomy increases the expression of glucose-regulated protein 78 kDa in rat facial nucleus, Exp. Neurol. 146 (1997) 10–16.
[59] K. Nagasaki, K. Sasaki, N. Maass, T. Tsukada, H. Hanzawa, K. Yamaguchi, Staurosporine enhances cAMP-induced expression of neural-specific gene VGF and tyrosine hydroxylase, Neurosci. Lett. 267 (1999) 177–180.
[60] N. Nelson, The family of the Na^+/Cl^- neurotransmitter transporters, J. Neurochem. 71 (1998) 1785–1803.
[61] M. Neuner-Jehle, T.A. Rhyner, A.A. Borbély, Sleep deprivation differentially alters the mRNA and protein levels of neurogranin in rat brain, Brain Res. 685 (1995) 143–153.
[62] M. Neuner-Jehle, J.-P. Denizot, A.A. Borbély, J. Mallet, Characterization and sleep deprivation-induced expression modulation of dendrin, a novel dendritic protein in rat brain neurons, J. Neurosci. Res. 46 (1996) 138–151.
[63] D. Nitz, J.M. Siegel, GABA release in posterior hypothalamus across sleep/wake cycle, Am. J. Physiol. 271 (1996) R1707–1712.
[64] D. Nitz, J.M. Siegel, GABA release in the locus coeruleus as a function of sleep/wake state, Neuroscience 78 (1997) 795–801.
[65] M. Nonaka, E. Kohmura, T. Yamashita, S. Shimada, K. Tanaka, T. Yoshimine, M. Tohyama, T. Hayakawa, Increased transcription of glutamate-aspartate transporter (GLAST/GluT-1) mRNA following kainic acid-induced limbic seizure, Mol. Brain Res. 55 (1998) 54–60.

[66] B.F. O'Hara, E. Macdonald, D. Clegg, S.W. Wiler, R. Andretic, V.H. Cao, J.D. Miller, H.C. Heller, T.S. Kilduff, Developmental changes in nicotinic mRNAs and responses to nicotine in the suprachiasmatic nucleus and other brain regions, Mol. Brain Res. 66 (1999) 71–82.
[67] R.D. Palmiter, S.D. Findley, T.E. Whitmore, D.M. Durnam, MT-III, a brain specific member of the metallothionein gene family, Proc. Natl. Acad. Sci. USA 89 (1992) 6333–6337.
[68] J. Park, M.L.L. Leong, P. Buse, A.C. Maiyar, G.L. Firestone, B.A. Hemmings, Serum and glucocorticoid-inducible kinase (SGK) is a target of the PI 3-kinase-stimulated signaling pathway, EMBO J. 18 (1999) 3024–3033.
[69] W. Paschen, C. Gissel, T. Linden, J. Doutheil, Erp72 expression activated by transient cerebral ischemia or disturbance of neuronal endoplasmic reticulum calcium stores, Metab. Brain Dis. 13 (1998) 55–68.
[70] C. Peyron, S.W. Wurts, H.K. Srere, H.C. Heller, D.M. Edgar, T.S. Kilduff, mRNA level of brain-derived neurotrophic factor increases in several brain regions after sleep deprivation, Soc. Neurosci. Abstr. 24 (1998) 1430.
[71] M.R. Picciotto, M. Zoli, R. Rimondini, C. Léna, L.M. Marubio, E.M. Pich, K. Fuxe, J.P. Changeux, Acetylcholine receptors containing the beta2 subunit are involved in the reinforcing properties of nicotine, Nature 391 (1998) 173–177.
[72] M. Pompeiano, C. Cirelli, G. Tononi, Immediate-early genes in spontaneous wakefulness and sleep: expression of c-fos and NGFI-A mRNA and protein, J. Sleep Res. 3 (1994) 80–96.
[73] T. Porkka-Heiskanen, S.E. Smith, T. Taira, J.H. Urban, J.E. Levine, F. Turek, D. Stenberg, Noradrenergic activity in rat brain during rapid eye movement deprivation and rebound sleep, Am. J. Physiol. 268 (1995) R1456–1463.
[74] T. Porkka-Heiskanen, R.E. Strecker, M. Thakkar, A.A. Bjorkum, R.W. Greene, R.W. McCarley, Adenosine: a mediator of the sleep-inducing effects of prolonged wakefulness, Science 276 (1997) 1265–1268.
[75] A. Rechtschaffen, Current perspectives on the function of sleep, Perspect. Biol. Med. 41 (1998) 359–390.
[76] T.A. Rhyner, A.A. Borbély, J. Mallet, Molecular cloning of forebrain mRNAs which are modulated by sleep deprivation, Eur. J. Neurosci. 2 (1990) 1063–1073.
[77] P.J. Shaw, C. Cirelli, R.J. Greenspan, G. Tononi, Correlates of sleep and waking in *Drosophila melanogaster*, Science 287 (2000) 1834–1837.
[78] P.-J. Shen, A.L. Gundlach, Differential increases in chromogranins, but not synapsin I, in cortical neurons following spreading depression: implications for functional roles and transmitter peptide release, Eur. J. Neurosci. 10 (1998) 2217–2230.
[79] P.-J. Shen, A.L. Gundlach, Differential spatiotemporal alterations in adrenoceptor mRNAs and binding sites in cerebral cortex following spreading depression: selective and prolonged upregulation of alpha1B-adrenoceptors, Exp. Neurol. 154 (1998) 612–627.
[80] J.M. Siegel, M.A. Rogawski, A function for REM sleep: regulation of noradrenergic receptor sensitivity, Brain Res. Rev. 13 (1988) 213–233.
[81] A.J. Silva, J.H. Kogan, P.W. Frankland, S. Kida, CREB and memory, Annu. Rev. Neurosci. 21 (1998) 127–148.
[82] S.E. Snyder, H.-W. Cheng, K.D. Murray, P.J. Isackson, T.H. McNeill, S.R.J. Salton, The messenger RNA encoding VGF, a neuronal peptide precursor, is rapidly regulated in the rat nervous system by neuronal activity, seizure and lesion, Neuroscience 82 (1998) 7–19.
[83] C. Sola, J.M. Tusell, J. Serratosa, Differential response of calmodulin genes in the mouse brain after systemic kainate administration, Neuroscience 78 (1997) 155–164.
[84] O. Steward, C.S. Wallace, G.L. Lyford, P.F. Worley, Synaptic activation causes the mRNA for the IEG Arc to localize selectively near activated postsynaptic sites on dendrites, Neuron 21 (1998) 741–751.
[85] T.M. Sugihara, I. Bach, C. Kioussi, M.G. Rosenfeld, B. Andersen, Mouse deformed epidermal autoregulatory factor 1 recruits a LIM domain factor, LMO-4, and CLIM coregulators, Proc. Natl. Acad. Sci. USA 95 (1998) 15418–15423.
[86] P. Taishi, Z. Chen, F. Obál Jr., J. Zhang, M. Hansen, J. Fang, J.M. Krueger, Sleep-associated changes in interleukin-1β mRNA in the brain, J. Interferon Cytokine Res. 18 (1998) 793–798.
[87] D.T. Theodosis, K. Pierre, D.A. Poulain, Differential expression of two adhesion molecules of the immunoglobulin superfamily, F3 and polysialylated NCAM, in hypothalamic magnocellular neurones capable of plasticity, Exp. Physiol. 85 (2000) 187S–196S.
[88] I. Tobler, Phylogeny of sleep regulation, in: M.H. Kryger, T. Roth, W.C. Dement (Eds.), Principles and Practice of Sleep Medicine, Saunders, Philadelphia, PA, 2000, pp. 72–81.
[89] G. Tononi, C. Cirelli, Some consideration on sleep and neural plasticity, Arch. Ital. Biol., in press.
[90] J. Toppila, D. Stenberg, L. Alanko, M. Asikainen, J.H. Urban, F.W. Turek, T. Porkka-Heiskanen, REM sleep deprivation induces galanin gene expression in the rat brain, Neurosci. Lett. 183 (1995) 171–174.
[91] J. Toppila, L. Alanko, M. Asikainen, I. Tobler, D. Stenberg, T. Porkka-Heiskanen, Sleep deprivation increases somatostatin and growth hormone-releasing hormone messenger RNA in the rat hypothalamus, J. Sleep Res. 6 (1997) 171–178.
[92] G.M. Tyce, J.M. Messick, T.L. Yaksh, D.E. Byer, D.R. Danielson, D.K. Rorie, Amine sulfate formation in the central nervous system, Fed. Proc. 45 (1986) 2247–2253.
[93] M. Vasak, D.W. Hasler, Metallothioneins: new functional and structural insights, Curr. Opin. Chem. Biol. 4 (2000) 177–183.
[94] S.C. Veasey, M. Mackiewicz, P. Fenik, M. Ro, M.D. Ogilvie, A.I. Pack, IL1β knockout mice lack the TNFα response to sleep deprivation but have normal sleep and sleep recovery, Soc. Neurosci. Abstr. 23 (1997) 792.
[95] L. Vician, I.K. Lim, G. Ferguson, G. Tocco, M. Baudry, H.R. Herschman, Synaptotagmin IV is an immediate early gene induced by depolarization in PC12 cells and in brain, Proc. Natl. Acad. Sci. USA 92 (1995) 2164–2168.
[96] D. Virgintino, M. Ambrosini, P. D' Errico, M. Bertossi, C. Papadaki, D. Karagogeos, G. Gennarini, Regional distribution and cell type-specific expression of the mouse F3 axonal glycoprotein: a developmental study, J. Comp. Neurol. 413 (1999) 357–372.
[97] X.Z. Wang, M. Kuroda, J. Sok, N. Batchvarova, R. Kimmel, P. Chung, H. Zinszner, D. Ron, Identification of novel stress-induced genes downstream of CHOP, EMBO J. 17 (1998) 3619–3630.
[98] M.K. Webster, L. Goya, Y. Ge, A.C. Maiyar, G.L. Firestone, Characterization of *sgk,* a novel member of the serine/threonine protein kinase gene family which is transcriptionally induced by glucocorticoids and serum, Mol. Cell Biol. 13 (1993) 2031–2040.
[99] R.M. Weinshilboum, D.M. Otterness, I.A. Aksoy, T.C. Wood, C. Her, R.B. Raftogianis, Sulfotransferase molecular biology: cDNAs and genes, FASEB J. 11 (1997) 3–14.
[100] M. Williams, M.S. Lyu, Y.L. Yang, E.P. Lin, R. Dunbrack, B. Birren, J. Cunningham, K. Hunter, Ier5, a novel member of the slow-kinetics immediate-early genes, Genomics 55 (1999) 327–334.
[101] M.T.T. Wong-Riley, M.A. Mullen, Z. Huang, C. Guyer, Brain cytochrome oxidase subunit complementary DNAs: isolation, subcloning, sequencing, light and electron microscopic in situ hybridization of transcripts, and regulation by neuronal activity, Neuroscience 76 (1997) 1035–1055.
[102] X. Xu, J. Raber, D. Yang, B. Su, L. Mucke, Dynamic regulation of c-Jun N-terminal kinase activity in mouse brain by environmental stimuli, Proc. Natl. Acad. Sci. USA 94 (1997) 12655–12660.