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Rapid Alterations in Cortical Protein Profiles Underlie Spontaneous Sleep and Wake Bouts

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ABSTRACT

Existing data indicate that sleep–wakefulness is an essential behavior. The biological function(s) of sleep, however, remains unknown, due, in part, to the lack of information available at the intracellular level. Preliminary microarray analyses show that changes in behavioral state influence regional mRNA profiles; however, the impact of sleep on protein signatures is virtually unexplored. In these studies, cortical protein profiles were examined after timed bouts of spontaneous sleep–wakefulness. Within minutes of each behavioral state examined, a small number of spots showing unique expression were detected. Mass spectroscopy analyses of sleep- and wake-related spots identified proteins associated with multiple functional categories. Two sleep-associated proteins were further validated using a sleep deprivation paradigm. We found preliminary evidence for two different post-transcriptional mechanisms—one (GAPDH) in which the amount of protein was increased in the recovery sleep following prolonged waking, while the other (actin) suggested that post-translational modifications may underlie sleep. The similarities between the effects of sleep on both protein and mRNA profiles indicate that dynamic intracellular changes underlie sleep–wake states and are consistent with roles for sleep in multiple biological functions. J. Cell. Biochem. 105: 1472–1484, 2008. © 2008 Wiley-Liss, Inc.

KEY WORDS: TWO-DIMENSIONAL ELECTROPHORESIS (2DE); MASS SPECTROMETRY; SPONTANEOUS SLEEP–WAKE BOUTS; SLEEP-ASSOCIATED PROTEIN EXPRESSION

Sleep–wake behavior is a complex and tightly regulated [Borbely and Achermann, 1999, 2000] amalgam of physiological processes that involves reciprocal interactions between numerous brain regions that is coordinated by multiple neurotransmitter/peptide systems [Lin, 2000; Jones, 2004]. Sleep is exhibited by all animals studied thus far [Campbell and Tobler, 1984; Tobler, 2000] and constitutes a significant percentage of a 24 h period [Carskadon and Dement, 2000; Ohayon et al., 2004]. Alternations between sleep and wakefulness are regulated by a combination of circadian (sleep timing) [Edgar, 1995] and homeostatic factors (sleep need) [Borbely, 1982]. The homeostatic regulation of sleep is based on the observation that following sleep deprivation (SD), there is an increase in sleep time and intensity that is proportional to the sleep lost, suggesting that a need for sleep accumulates during waking in both rodents and humans [Tobler and Borbely, 1986; Riedner et al., 2007; Vyazovskiy et al., 2007]. In humans, SD results in marked cognitive and physiological impairments [Drummond and Brown, 2001; Durmer and Dinges, 2005], while prolonged SD in rats [Rechtschaffen, 1998] and flies [Shaw et al., 2002] is fatal. The biological function(s) of sleep, however, remains poorly characterized, though most agree that sleep serves a restorative function [Benington and Heller, 1995]. Proposed roles for sleep include the maintenance of body temperature [McGinty and Szymusiak, 1990; Wehr, 1992], energy homeostasis [Adam, 1980; Walker and Berger, 1980; Benington and Heller, 1995], immune function [Majde and Krueger, 2005; Opp, 2005], synaptic plasticity [Tononi and Cirelli, 2006] and memory consolidation/reconsolidation [Stickgold and Walker, 2005; Born et al., 2006]. The amount of time devoted to sleep, the deleterious effects that accompany SD, its' proposed roles in the maintenance of a variety of biological functions and the apparent conservation in animals are consistent with the idea that sleep–wakefulness is a fundamental behavior, similar to eating and drinking.

Though it has long been hypothesized that the impact of sleep on the intracellular milieu affects neural function [Rechtschaffen, 1998; Steriade and Timofeev, 2003; Tononi and Cirelli, 2003], relatively little is known of the intracellular consequences of sleep due to its' complex underpinnings and the lack of appropriately sensitive high throughput technologies. Early studies showed increased regional protein and RNA contents during sleep [Giuditta et al., 1980; Ramm and Smith, 1990; Nakanishi et al., 1997], consistent with the stimulation of protein biosynthesis by

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transcriptional activation. Subsequent studies that focused on the expression of immediate early gene mRNAs, provided additional evidence implicating a nuclear response during sleep [Cirelli and Tononi, 2001; Cirelli et al., 2005]. Most of these studies monitored expression following SD and recovery sleep (RS) to distinguish circadian (time of day) from homeostatic influences. The stress produced from SD, however, is well documented [Meerlo et al., 2002; Machado et al., 2004]. Spontaneous sleep–wakefulness or natural sleep is less well studied, based on the short, rapid cycling of sleep–wake bouts in rodents, the requirement to dissociate circadian and homeostatic influences, and the possible effects on cellular activities resulting from the practical difficulty of sacrificing a sleeping animal without first waking him up. Such technical considerations further elaborate the complexities involved in studies designed to understand the intracellular response to sleep.

More recent microarray analyses showed that a small percentage of genes (i.e., 1–5%) were differentially expressed across sleep and waking states, independent of the time of day (TOD) or the brain region studied [Cirelli et al., 2004; Terao et al., 2006]. These patterns were conserved across rodent species [Terao et al., 2006] and encoded proteins associated with a variety of housekeeping functions, including energy metabolism, synaptic plasticity, membrane trafficking and maintenance, and cholesterol biosynthesis [Cirelli et al., 2004; Terao et al., 2006]. In addition, a subset of state-dependent mRNAs was common to two or more brain regions [Cirelli et al., 2004; Terao et al., 2006], suggesting that sleep influences global as well as regional brain function(s). While these studies provided valuable insights regarding the intracellular impact of sleep at the nucleic acid level, alterations in mRNA levels often do not reflect corresponding protein expression [Anderson and Seilhamer, 1997; Abbott et al., 1999; Gygi et al., 1999; Ideker et al., 2001; Takahashi, 2004], a more direct functional indicator of the cellular response to sleep. Additionally, few sleep studies have correlated mRNA and protein expression [Neuner-Jehle et al., 1995; Greco et al., 1999; Guzman-Marin et al., 2006]; thus, the temporal relationship between sleep and protein expression is also unclear. The identification and characterization of putative protein correlates of sleep is therefore key to understanding the cellular response to sleep.

To examine the temporal relationship between behavioral state and protein expression, rats were sacrificed after timed bouts of spontaneous sleep and wakefulness within the same circadian time frame. To identify putative protein correlates of sleep and wakefulness, we used two-dimensional electrophoresis (2DE) to create regional protein expression maps [Fountoulakis, 2004]. Two expression maps were generated: one that reflected total protein expression and a second that monitored phosphorylated protein expression as an indicator of total cellular activity [Krebs, 1994; Sun and Tonks, 1994; Hunter, 1995; Graves and Haystead, 2002]. State-related spots were identified by a combination of mass spectrometry (MS) analyses. State-associated metabolic pathways were inferred from the function of each identified protein. To confirm the 2DE results and begin examination of cellular mechanisms associated with sleep, the expression of two sleep-related proteins was monitored following SD by mRNA and Western analyses. Frontal cortex tissue was analyzed, as this region exhibits the electrical activities characteristic of spontaneous sleep–wakefulness [Steriade and Timofeev, 2003] and SD [Borbely and Achermann, 1999], mediates some of the cognitive deficits associated with SD [Rogers et al., 2003; Van Dongen et al., 2003] and shows alterations in mRNA profiles after periods of spontaneous sleep–wakefulness [Cirelli et al., 2004] and SD [Cirelli et al., 2004; Terao et al., 2006].

EXPERIMENTAL PROCEDURES

ANIMALS AND SURGERY

Male Sprague Dawley rats (250–350 g; n = 17) were anesthetized and implanted with sleep recording electrodes to monitor electroencephalographic (EEG) and electomyographic (EMG) activities. A femoral vein catheter was also implanted and dorsally extended subcutaneously to exit at the posterior end of the EEG/EMG implant. The rats were individually housed in a controlled environment (12 h lights on/off; lights on, 8 am) with access to food and water ad libitum. The animals were allowed to recover for approximately 7–10 days following surgery and habituated to the recording chambers. Just prior to the initiation of the experiment, an extension of PE 50 polyurethane tubing was attached to the femoral vein catheter and affixed to the outside of the cage, to permit sacrifice by pentobarbital administration (200 mg/kg, i.v.) without handling the animal. Baseline behavior was continuously recorded for 48–60 h prior to the day of sacrifice and throughout the day of sacrifice. All procedures using animals were approved by the Institutional Animal Care and Use Committee at SRI International and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, 7th edition [Council, 1996]. Every effort was made to minimize animal discomfort throughout the experimental protocol.

SLEEP SCORING AND ANALYSIS

EEG/EMG activities during wakefulness, slow wave sleep (SWS), and rapid eye movement (REM) sleep were captured digitally and stored to disk. Wakefulness was defined by the presence of low amplitude, high frequency EEG waves with high EMG activity. SWS was identified by high amplitude slow waves in conjunction with a decreased level of EMG activity compared to waking. REM sleep was characterized by the presence of rhythmic theta EEG waves and no EMG activity. Behavioral states were scored manually in 10 s epochs and the percents of wakefulness, SWS, and REM and total sleep time (the sum of the percent SWS sleep and percent REM sleep; %TST) were calculated.

TISSUE COLLECTION

Frontal cortex was dissected (5.2–4.2 mm anterior to Bregma) [Paxinos and Watson, 1982], immediately frozen and stored at −80°C. For 2DE separations, the tissue was bisected. A portion of the remaining tissue was used to extract total RNA.

SAMPLE LYSIS AND PROTEIN SEPARATION

Tissue samples were lysed in buffer with containing 7 M urea, 2 M thiourea, 2% CHAPS, 2% ASB-14, 20 mM Tris, 1% DTT, 0.2% 3/10 ampholytes, and 1% protease inhibitors. All reagents, supplies and equipment were obtained from BioRad unless otherwise indicated. The total protein concentration was determined using the *RC DC*™

protein assay kit. Approximately 7.5 mg total protein was obtained from 120 mg tissue. Protein aliquots (100 μg/gel) were first separated according to isoelectric point on strips (pH 3–10 NL; 11 cm), reduced in the presence of DTT, and acetoacetamide, then separated in the 2nd dimension according to molecular mass (10.5–14% Tris–HCl polyacrylamide gradient gels; 11 cm). The gels were first stained with ProQ Diamond to detect phosphorylated proteins. After imaging, this stain was removed and the gels were re-stained with SYPRO Ruby to visualize total protein. For the identification of selected spots, a second separation was performed on large (17 cm) pI strips and pre-cast gels (12% Tris–HCl). For these analyses, samples within each behavioral state were pooled (1 mg/sample) and stained with GelCode Blue® (Pierce Biotechnology). A second subtractive analysis was performed. Spots unique to waking and SWS were excised and processed for mass spectroscopy analyses [Boonjakuakul et al., 2007].

SPOT DETECTION AND WHOLE GEL ANALYSES

Proteins separated by 2DE were scanned and analyzed with PDQuest 2D Image Analysis Software. Spots were identified as the sum of the intensities of the image pixels within a boundary, where spot height or peak value on an *x*- and *y*-axis were measured in ODs or counts/image units^2 and fitted to the scanned spots using Gaussian curves. This Gaussian modeling allows for the identification of overlapping spots, spots in gel streaks, and multiple spots in dense clusters and was used for all subsequent analyses. Duplicate samples from each animal (i.e., a total of 30 gels from 15 rats) were analyzed to reduce spot detection errors. Errors detected after the automated matching of spots were manually inspected. Spot intensities of equal to or less than fourfold were considered artifacts and/or background staining. Individual gels from each rat were then grouped according to state of sacrifice, spot matched, and normalized to one another. Gel composites were used to generate a Master image of all spots present within an experimental condition, thus minimizing variance between gels. Averaging parameters (criteria to assist with group comparisons) across experimental groups were set to equal to or greater than 70% consistent with instrument specifications [Zhan and Desiderio, 2003a,b]. Master gel images for each stain were subsequently generated by combining all spots identified in all gels across all experimental groups. Spots from each behavioral state were compared and matched to ProQ Diamond and SYPRO Ruby Master images. Qualitative analysis sets were generated by subtractive (Boolean) analyses to identify spots common to all experimental groups, those common to two of three states, and spots unique to one behavioral state.

PREPARATION OF PEPTIDES

Spots selected for protein identification were excised from GelCode Blue® stained gels followed by in-gel tryptic digestion and peptide extraction as previously published [Boonjakuakul et al., 2007]. Peptides were submitted to the Biomolecular Resource Center Mass Spectrometry Facility, UCSF, San Francisco, CA for protein identification.

MASS SPECTROMETRY

Protein identification was accomplished by using a combination of peptide mass fingerprinting (PMF) and high performance liquid chromatography (HPLC) electrospray ionization (ESI) tandem mass spectrometry (MS/MS) as described [Boonjakuakul et al., 2007]. For PMF protein identification, monoisotopic mass peak lists were submitted to the Aldente (http://expasy.org/tools/aldente/) search engine to facilitate protein identification. Searches were performed that interrogated the SwissProt rodent protein database. Acceptance criteria for protein identification was based on the top hit score, a requirement for high precision of mass measurement, defined as a low standard deviation (<25 ppm for internal calibration) of mass assignment errors for all matching peptide masses detected within the sample spot and a minimum of 25% sequence coverage. Protein identification by HPLC/MS/MS employed either ProteinPilot™ (Applied Biosystems) or Mascot (Matrix Science) search engines; rodentia taxonomy in NCBInr database was interrogated. ProteinPilot peptide acceptance criteria required the confidence level of ≥98% and ≥99% for protein identifications based upon the multiple and single peptide, respectively. Match quality at P Value <0.05 was required to accept MASCOT-identified peptides. All MS/MS spectra derived from proteins identified by a single confidently matched peptide were manually verified.

WESTERN ANALYSIS

Frozen tissue was homogenized in lysis buffer (25 mM Tris, pH 7.5, 250 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10% glycerol, 2 mM PMSF, 1 mg/ml leupeptin and 2 mg/ml aprotinin), separated (by conventional SDS–PAGE or 2DE) and transferred to PVDF membranes in 20 mM Tris (pH 8), 150 mM glycine and 0.1% SDS (100 v, 1 h). The membranes were blocked in 10 mM Tris/150 mM sodium chloride (pH 8) containing 0.1% Tween-20 (TBST) and 10% nonfat milk (1 h at room temperature). Glyceradehyde phosphate dehydrogenase (GAPDH; 1:20,000 dilution, Chemicon International) and actin primary antibodies (1:20,000 dilution, Sigma–Aldrich) were incubated for 1 h at room temperature in TBST-5% nonfat milk. After washing, the membranes were incubated in secondary antibody (1:100,000 in TBST-5% nonfat milk; 1 h, room temperature). The antigen–antibody complexes were visualized with an ECL kit (GE Healthcare), imaged, and quantified using Quantity One software (BioRad).

RNA EXTRACTION AND RT-PCR ANALYSES

Total RNA was extracted with Ultraspec RNA reagent (Biotecx Labs), treated with RNAse free DNAse and quantitated spectrophotometrically. The RNA (50 ng) was reverse transcribed and amplified by RT/PCR using a TaqMan One Step RT/PCR kit and pre-developed assay kits for *18S rRNA*, *gapdh* and *actin* (ABI). Reverse transcription (48°C, 30 min) and the analysis of fluorescent product formation in real time were performed using an ABI Prism 7500 Sequence Detection System. The PCR conditions were: 95°C, 10 min for one cycle, then 95°C for 15 s and 60°C for 1 min for a total of 35 cycles. All samples were analyzed in duplicate. *18S rRNA* was used to control for differences in relative amount of total RNA. Relative gene expression was quantified using a comparative concentration threshold (C_t) method (ABI). A control for DNA contamination

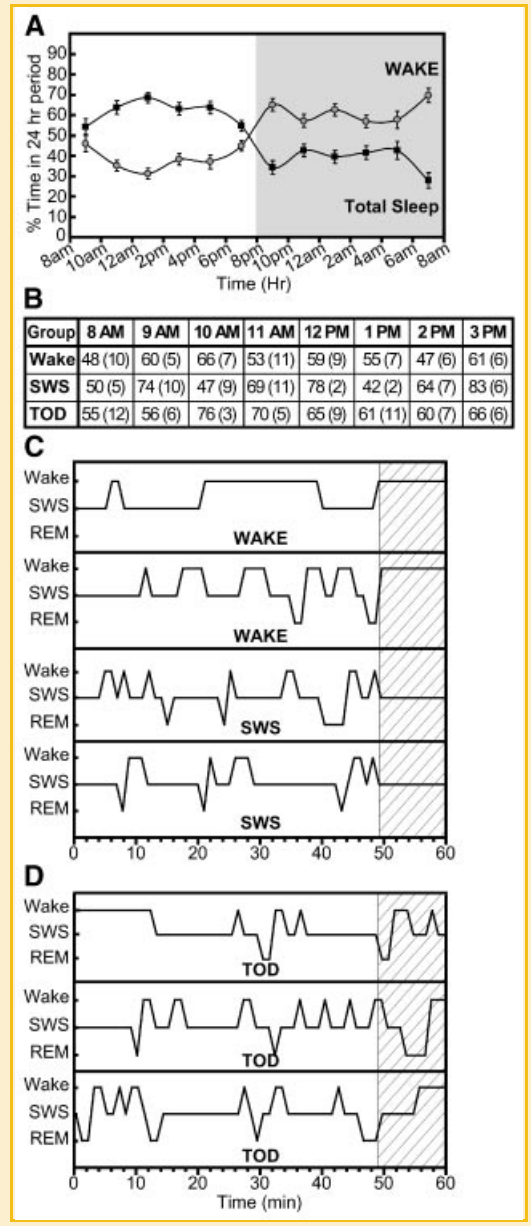
(RNAse treated sample) and a sample containing no RNA template were routinely monitored to ensure the validity of the results.

RESULTS AND DISCUSSION

THE EFFECTS OF SLEEP AND WAKING ON PROTEIN PROFILES ARE DETECTED WITHIN MINUTES OF SPONTANEOUS SLEEP–WAKE BOUTS

To ascertain whether changes in whole protein expression occurred as a function of behavioral state in a time frame consistent with rodent sleep architecture, rats were sacrificed under the following conditions: (1) after 10 min of continuous waking (n = 4); (2) after 10 min of continuous SWS (n = 7); or (3) as a function of the TOD (n = 4), independent of the behavioral state. All sacrifice took place 7–8 h into the lights-on period (i.e., 3–4 pm), a time the rats are predominantly asleep [Tobler, 2000]. Animals were sacrificed by the intravenous administration of pentobarbital; death occurred within seconds. Thus, none of the rats were awakened by human handling. Baseline behavioral recordings (Fig. 1A) were consistent with those reported in the literature [Timo-Iaria et al., 1970; Van Gool and Mirmiran, 1983; Tobler and Borbely, 1986], indicating that the animals were not sleep deprived. The %TST for each hour of the lights-on period on the day of sacrifice was also similar between experimental groups (Fig. 1B). Representative hypnograms (Fig. 1C,D) of the hour immediately prior to sacrifice revealed individual variability in behavioral states between and within experimental groups in the first 50 min (non-shaded areas of hypnograms). The experimental groups therefore differed from one another only in the 10 min immediately prior to sacrifice (shaded area, Fig. 1C,D). Wake and SWS groups were sacrificed only after 10 min of continuous behavior (Fig. 1C), while rats sacrificed as a function of TOD exhibited a heterogeneous combination of Wake, SWS and REM sleep in the 10 min prior to sacrifice (shaded area, Fig. 1D). TOD rats thus constituted an experimental group independent of continuous sleep–wake behavior and served as controls for the states analyzed. The heterogeneity of states that underlie this behavioral group (Fig. 1D, shaded area) make TOD an important control for assessing alterations in protein expression as a consequence of specific wake or sleep states.

Frontal cortex lysates were separated by 2DE and sequentially stained with SYPRO Ruby (Fig. 2) and ProQ Diamond (Fig. 3) to visualize cytosolic and phosphorylated protein profiles, respectively. Comparisons between waking, SWS and TOD rats were determined by subtractive analyses of Master gel images generated by the input of spots from all animals. The numerical analyses of SYPRO Ruby spots (Fig. 2A) were based on a total of 717 protein spots identified in the Master gel image (Fig. 2B). The total number of spots visualized were consistent with previous reports of 2DE separations of cortical tissue [Castegna et al., 2003; Shin et al., 2004a,b; Poon et al., 2006a,b]. Representative raw data from each experimental group used to create the Master gel image are shown in Figure 2C–E. Seventy-eight percent of the spots (n = 560) were present in any two of the three states monitored and 14% (n = 98) were present in all states examined. The majority of spots (92%; n = 658) were thus present in two or more conditions. Spots showing unique expression across state were detected throughout the gel



Group	8 AM	9 AM	10 AM	11 AM	12 PM	1 PM	2 PM	3 PM
Wake	48 (10)	60 (5)	66 (7)	53 (11)	59 (9)	55 (7)	47 (6)	61 (6)
SWS	50 (5)	74 (10)	47 (9)	69 (11)	78 (2)	42 (2)	64 (7)	83 (6)
TOD	55 (12)	56 (6)	76 (3)	70 (5)	65 (9)	61 (11)	60 (7)	66 (6)

Fig. 1. The three experimental animal groups selected to assess changes in protein expression across spontaneous sleep–wake bouts. Rats were sacrificed between 3 and 4 pm under the following conditions: (1) after 10 min continuous waking (n = 4); (2) following 10 min continuous SWS (n = 7); and (3) as a function of the TOD (n = 4), independent of behavioral state. Rats were euthanized with an overdose of pentobarbital delivered via the femoral catheter. EEG/EMG activities ceased within 1–2 s after initiation of the pentobarbital administration. A: Baseline EEG recordings over 24 h (lights-off period is gray shaded area of graph). The percentages of waking, SWS and REM were similar to that reported in the literature [Timo-Iaria et al., 1970; Van Gool and Mirmiran, 1983; Tobler and Borbely, 1986]. B: %TST for the 8 h period prior to sacrifice. No significant differences were noted in %TST for each experimental group/hour. Numbers in parentheses denote standard deviation. C: Individual EEG recordings show the last hour of the sleep–wake cycle of four animals sacrificed between 3 and 4 pm, after either 10 min of continuous waking or after 10 min of continuous SWS (shaded area of hypnograms). D: Representative hypnograms of TOD rats. Graphs depict the distribution of wake, SWS and REM sleep in rats (n = 3) in the hour immediately prior to sacrifice. Behavior in the TOD rats varied in the 10 min immediately prior to sacrifice (shaded area of graphs).

A

Behavioral State	# of SYPRO Spots (n = 717)	% Total
Any 2 states	560	78
All 3 states	98	14
SWS	23	3
Wake	14	2
TOD	19	3

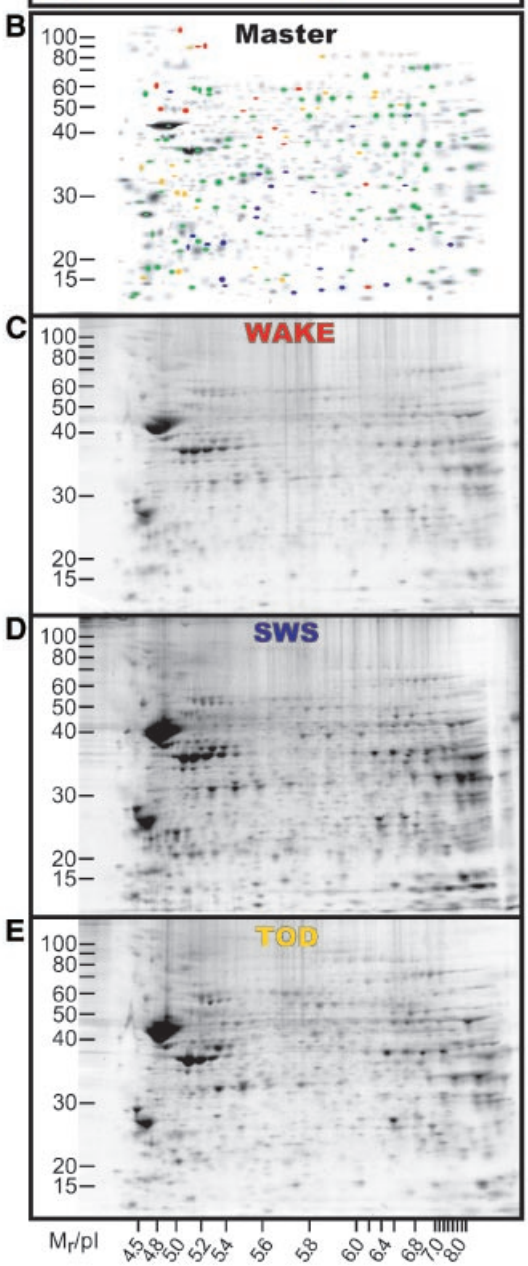


Fig. 2. SYPRO Ruby stained gels show state-related protein expression across spontaneous sleep–wake bouts. A: Quantitation of SYPRO ruby stained spots. B: The Master gel image was compiled from the input of all spots from all gels across all conditions followed by subtractive analysis as described in Experimental Procedures Section. Protein spots are colored-coded as follows: green spots, proteins detected in all states; blue spots, proteins expressed only during SWS; red spots, proteins expressed only during waking; yellow spots, proteins expressed only as a function of the TOD. Representative raw images of (C) waking, (D) SWS, and (E) TOD profiles.

A

Behavioral State	# of Phos Spots (n = 194)	% Total
Any 2 states	137	71
All 3 states	10	5
SWS	24	12
Wake	5	3
TOD	18	9

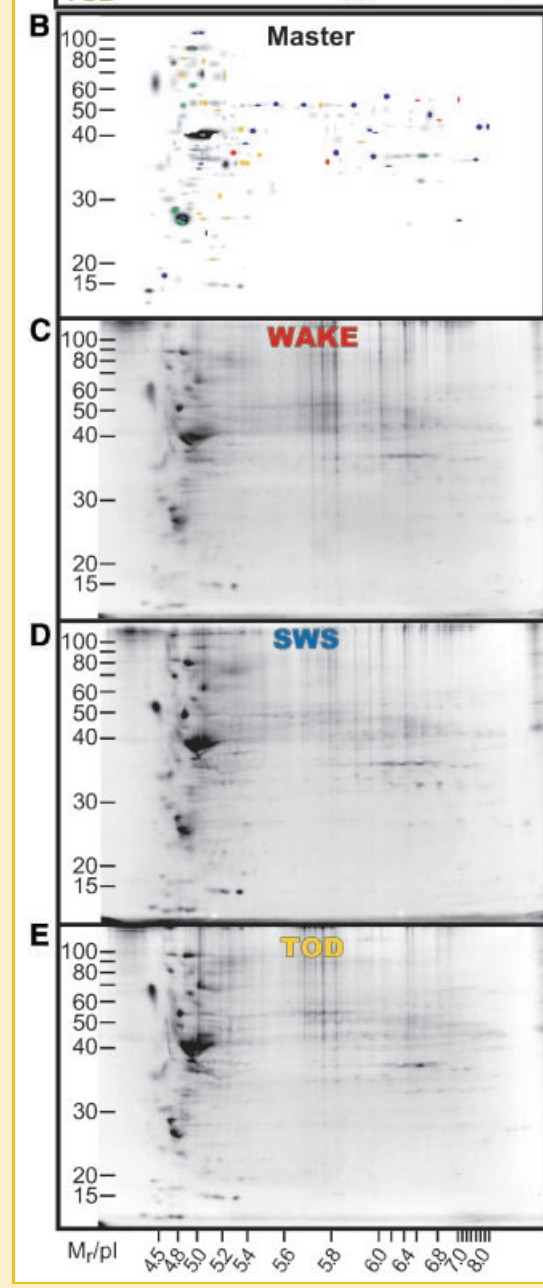


Fig. 3. ProQ Diamond staining shows unique phosphoprotein expression across spontaneous sleep–wake states. A: Quantitation of phosphorylated protein spots. B: The Master gel image was compiled from the input of all spots from all gels across all conditions followed by subtractive analysis as described in Experimental Procedures Section. Protein spots are colored-coded as follows: green spots, proteins detected in all states; blue spots, proteins expressed only during SWS; red spots, proteins expressed only during waking; yellow spots, proteins expressed only as a function of the TOD. Representative raw images of (C) waking, (D) SWS, and (E) TOD profiles.

(Fig. 2B) and accounted for approximately 8% of the total number of spots visualized. Approximately 3% spots were present only in rats sacrificed during SWS (Fig. 2A; n = 23); ~2% spots were unique to waking (Fig. 2A; n = 14); and ~3% spots were uniquely expressed as a function of the TOD (n = 19; Fig. 2A). The detection of unique spots in each of the experimental groups provides the first evidence that alterations in protein profiles underlie spontaneous sleep–wake bouts. In addition, the percentages of state-related SYPRO Ruby expression were comparable to those reported for cortical mRNA profiles from rats sacrificed after several hours of spontaneous sleep [albeit, in the same portion of the lights-on period as our studies; Cirelli et al., 2004], suggesting that the intracellular response to sleep and waking impacts small subsets of both total cellular protein and mRNA.

The analysis of phosphorylated spots suggested that cellular activities also varied across spontaneous sleep–wake states (Fig. 3A). A total of 194 spots were identified in the ProQ Diamond Master gel image (Fig. 3B), corresponding to 27% of the total number of SYPRO Ruby spots monitored, a percentage consistent with estimates of cellular phosphoprotein expression [Schulenberg et al., 2004]. Representative raw data from individual gels used to produce the Master gel image are shown in Figure 3C–E. Seventy-one percent of the spots (n = 137) were detected in two of the three states, with 5% (n = 10) present in all experimental groups (Fig. 3A). State-related spots accounted for ~24% of the total number visualized: 12% (n = 24) were unique to SWS; ~3% of the spots (n = 5) were unique to waking; and 9% spots (n = 18) were unique to the TOD (Fig. 3A). The percentage of spots unique to SWS and TOD were similar, parallel to the microarray data showing that behavioral state and TOD affected gene expression to a similar extent [Cirelli et al., 2004]. The number of phosphorylated spots unique to waking, however, was low compared to SWS and TOD conditions. The reason for this is unclear. One possibility is that several Wake proteins of similar molecular mass and charge could be present within a single spot, as was detected in one of the spots chosen for MS analyses (see Fig. 4B). Alternatively, the low number of wake-specific spots may be a consequence of sacrifice during the lights-on period when sleep predominates [Tobler, 2000]. Nonetheless, our data show that alterations in both protein expression (Fig. 2) and phosphorylation (Fig. 3) were detected in experimental groups that differed behaviorally from one another only in the 10 min immediately prior to sacrifice (shaded area, Fig. 1C,D). As changes in protein phosphorylation occur within minutes and are associated with alterations in cellular activities [Shaw et al., 1995; Asthagiri et al., 1999; King et al., 2006; Morii et al., 2006; Nunez Rodriguez et al., 2006], our results provide evidence that such alterations occur within the time frames associated with individual sleep–wake bouts in rats [Timo-Iaria et al., 1970; Tobler and Borbely, 1986]. In addition, these data are consistent with previous reports that examined regional phosphorylated protein expression by immunohistochemical methods [Cirelli and Tononi, 1998], levels of specific phosphoproteins [Guan et al., 2004; Basheer et al., 2005; Naidoo et al., 2005; Bandyopadhya et al., 2006; Davis et al., 2006], or enzymatic activities [Ramanathan et al., 2002; Mackiewicz et al., 2003; Naidoo et al., 2005] following SD.

IDENTIFICATION OF STATE-RELATED PROTEINS

To identify state-related spots, lysates from waking or SWS animals were pooled, separated on large format gels (17 cm × 17 cm), and stained with GelCode Blue®. Representative spots associated with each behavioral group were arbitrarily chosen for MS analyses (Fig. 4A). Nineteen proteins were identified from a total of 24 spots (Fig. 4B). One spot often corresponded to a single protein (68%; n = 13); however, in one case, multiple proteins were present within a single spot (i.e., spot 12 W contained 4 wake-unique proteins), likely reflective of the relatively small gels and wide range of charge and molecular mass used in our 2DE separations. In other instances, a single protein was identified in two or more spots (i.e., actin, creatine kinase B chain, mitochondrial aconitase and phosphoglycerate kinase). Nevertheless, the final number of spots in the groups was similar: 7 (~37%) were unique to SWS and 7 (~37%) were present only during waking, a result analogous to the microarray study [Cirelli et al., 2004]. Thus the percentages of state-associated proteins monitored by SYPRO ruby and by GelCode Blue® staining were comparable to one another and to the mRNA profiling results, providing additional evidence that the protein and mRNA pools are similarly affected by behavioral state.

The state-related proteins were associated with four cellular functions (Fig. 4B). The majority of proteins were associated with energy metabolism (~58%; n = 11). SWS-unique proteins were involved in glycolysis (GAPDH), oxidative phosphorylation (NADH dehydrogenase 1β subcomplex 10), and in creatine kinase B chain, a cytoplasmic protein involved in generation of ATP from phosphocreatine. Another SWS-unique protein, ES1 protein, is a mitochondrial protein of unknown function described in zebrafish, bacteria and humans [Nagamine et al., 1996; Shin et al., 2004a,b]. Wake-related proteins included pyruvate dehydrogenase, involved in ATP production via oxidative phosphorylation and the mitochondrial form of creatine kinase. Another wake-specific protein, glutamine synthetase, is involved in both the detoxification of ammonia in the brain [Suarez et al., 2002] and the synthesis of glutamate, a neurotransmitter known for its role in waking [Lin, 2000]. Aconitase, another Kreb's cycle protein, was present in one wake-related spot and one SWS-related spot. The aconitase spots differed in both molecular mass and charge, suggesting that this protein may be post-translationally modified across behavioral states.

Collectively, these results suggest that waking and sleep may differentially affect proteins involved in ATP production through cellular mechanisms that include multiple pathways (i.e., Kreb's cycle and glycolysis) and/or components of a single metabolic pathway (i.e., Kreb's cycle proteins NADH dehydrogenase and pyruvate dehydrogenase). In addition, the identification of SWS- and Wake-unique creatine kinase expression suggests that ATP production and utilization within specific cellular compartments (i.e., cytosol and mitochondria) may vary across behavioral state [Burklen et al., 2006]. Changes in creatine kinase and mitochondrial aconitase also occur after cortical injury [Jenkins et al., 2002] and hypoxia [Gozal et al., 2002]. The observation that these proteins are differentially expressed across spontaneous sleep indicates the sensitivity of these proteins to changes in extracellular effectors

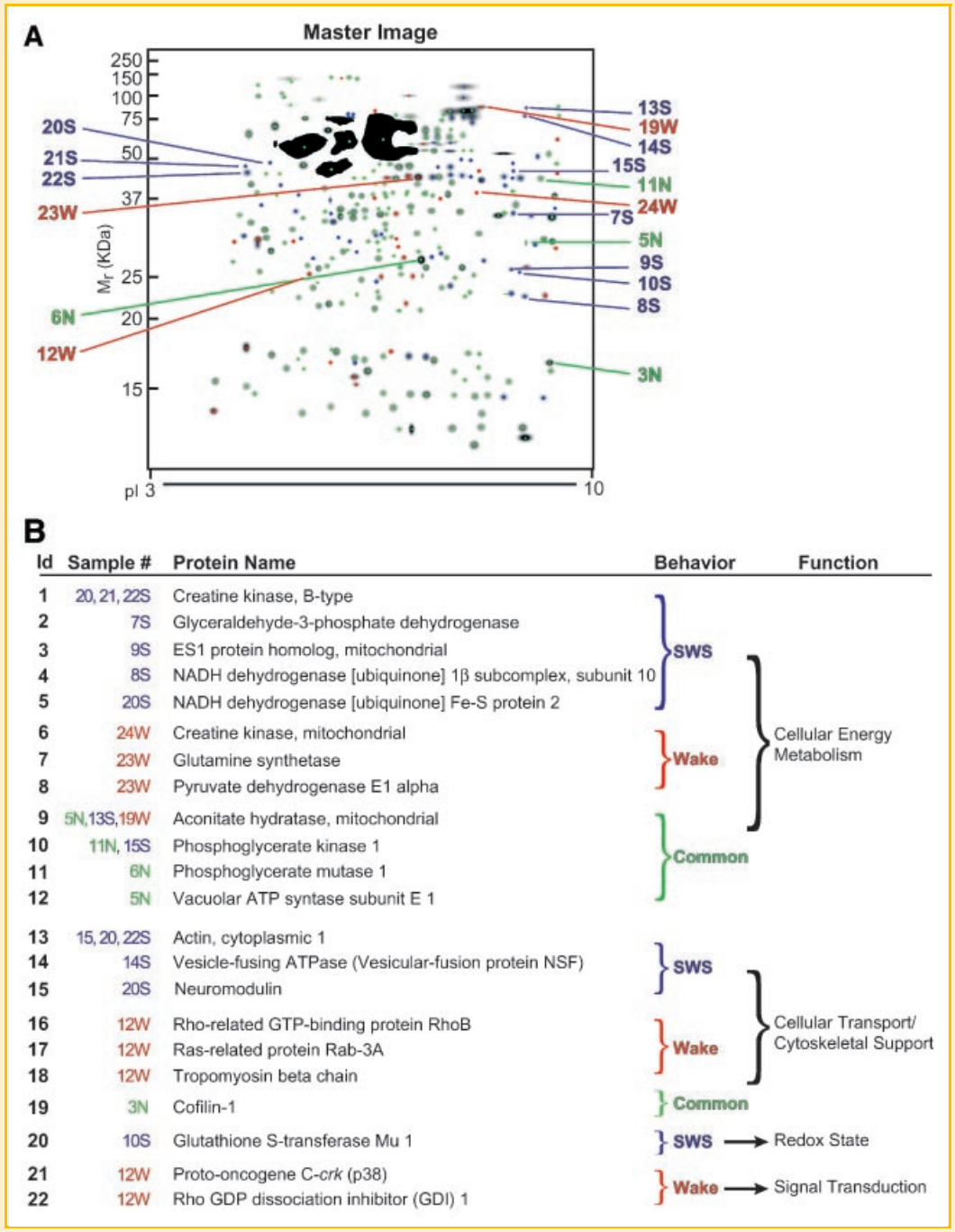


Fig. 4. Identification of state-related proteins. A: Digitized Master image composite showing spots chosen for MS analyses. Red spots were specific to waking, blue spots were unique to SWS, and green spots were common to both behavioral states. B: Results of MS analyses. Functional categories of proteins identified included those associated with energy metabolism, cytoskeletal support/cellular transport, the oxidation reduction state and signal transduction. SWS-specific spots (shown in blue) were associated with cytoskeletal structure/support, energy metabolism (ATP production/storage) and the re-dox state of the cell. C: [1]Only peptides identified with a confidence of 99% and 98% were considered; they contribute 2.0 and ~1.7, respectively, to the score. [2]Score above random score is statistically significant. [3]Unused score is a sum of contributions of all peptides unique for a given protein. For the highest scoring protein in the group, unused and total scores are identical. For the lower scoring protein within a group, only contribution of peptides that were not used for the higher scoring hit is calculated and hence, the unused score is lower than total score. [4]No rat equivalent was found by performing NCBI BLASTP 2.2.16 and interrogating 98157 rodent sequences from UniProt Knowledgebase Release 12.1. [5]Search engine reported mouse species. Blast analysis (see above) was performed on confidently identified peptide sequences to identify equivalent rat species. [6]Analyzed by Mascot utilizing NCBI 20050118, Taxonomy Rodentia, 124005 sequences, score >35 denotes identity or extensive homology at $P < 0.05$. [7]Data do not differentiate between cytoplasmic actin 1 and 2. [8]Peptide unique for cytoplasmic actin 1 (beta) was detected.

C

Proteins identified in the selected 2D gel spots.

ID #	Sample #	Accession|Entry name	Protein name	Theor MW (Da)	Theor pI	MS/MS Evidence[1] #pept>98%	MS/MS Evidence[1] Unused[3] score	PMF Evidence[2] Detected	PMF Evidence[2] Sequence Coverage
1	20S	P07335|KCRB_RAT	Creatine kinase B-type (EC 2.7.3.2)	42725	5.39	9	23.68	yes	36%
1	21S	P07335|KCRB_RAT	Creatine kinase B-type (EC 2.7.3.2)	42725	5.39	MS/MS not performed		yes	53%
1	22S	P07335|KCRB_RAT	Creatine kinase B-type (EC 2.7.3.2)	42725	5.39	2	23.68	yes	30%
2	7S	P04797|G3P_RAT	Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)	35810	8.43	9	19.15	yes	16%
3	9S	P56571|ES1_RAT	ES1 protein homolog, mitochondrial precursor	28172	9.13	5	11.06	no	
4	8S	Q9DCS9|NDUBA_MOUSE[4]	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 10	20892	8.35	5	12.88	yes	36%
5	20S	Q641Y2|Q641Y2_RAT[4]	NADH dehydrogenase (Ubiquinone) Fe-S protein 2	52561	6.52	2	4.08	no	
6	24W	Q5BJT9|Q5BJT9_RAT[4]	Creatine kinase, mitochondrial 1, ubiquitous	46961	8.57	2	5.03	yes	33%
7	23W	P09606|GLNA_RAT	Glutamine synthetase (EC 6.3.1.2) (GS)	42136	6.68	6	18.28	yes	42%
8	23W	P26284|ODPA_RAT	Pyruvate dehydrogenase E1 component alpha (EC 1.2.4.1)	40298	6.51	3	8.77	yes	26%
9	19W	Q9ER34|ACON_RAT	Aconitate hydratase, mitochondrial precursor (EC 4.2.1.3)	82461	7.14	8	19.57	yes	35%
9	5N	Q9ER34|ACON_RAT	Aconitate hydratase, mitochondrial precursor (EC 4.2.1.3)	82461	7.14	15	35.65	no	
9	13S	Q9ER34|ACON_RAT	Aconitate hydratase, mitochondrial precursor (EC 4.2.1.3)	82461	7.14	0	1.2	yes	21%
10	11N	P16617|PGK1_RAT	Phosphoglycerate kinase 1 (EC 2.7.2.3)	44407	8.02	14	31.21	yes	53%
10	15S	P16617|PGK1_RAT	Phosphoglycerate kinase 1 (EC 2.7.2.3)	44407	8.02	12	28.42	yes	55%
11	6N	P25113|PGAM1_RAT	Phosphoglycerate mutase 1 (EC 5.4.2.1) (EC 5.4.2.4) (EC 3.1.3.13)	28700	6.76	14	32.55	yes	67%
12	5N	Q6PCU2|VATE1_RAT[5]	Vacuolar ATP synthase subunit E 1	26128	8.43	15	35.65	yes	40%
13	15S	P60711|ACTB_RAT[5,7]	Actin, cytoplasmic 1 (Beta-actin)	41605	5.3	3[6]	14[6]	no	
13	20S	P60711|ACTB_RAT[7]	Actin, cytoplasmic 1 (Beta-actin)	41605	5.3	3	36.95	yes	44%
13	22S	P60711|ACTB_RAT[8]	Actin, cytoplasmic 1 (Beta-actin)	41605	5.3	12	36.95	yes	47%
14	14S	Q9QUL6|NSF_RAT	Vesicle-fusing ATPase (EC 3.6.4.6)	82651	6.55	2	5.75	yes	25%
15	20S	P07936|NEUM_RAT	Neuromodulin (Axonal membrane protein GAP-43)	23603	4.61	10	21.8	no	
16	12W	P62747|RHOB_RAT	Rho-related GTP-binding protein RhoB precursor	22123	5.34	3	5.82	no	
17	12W	P63012|RAB3A_RAT	Ras-related protein Rab-3A	24969	4.85	1	2	no	
18	12W	P58775|TPM2_RAT	Tropomyosin beta chain	32836	4.65	not detected in MS/MS		yes	35%
19	3N	P45592|COF1_RAT	Cofilin-1 (Cofilin, non-muscle isoform)	18401	8.27	10	21.27	yes	70%
20	10S	P04905|GSTM1_RAT	Glutathione S-transferase Mu 1	25782	8.43	MS/MS not performed		yes	68%
21	12W	Q63768|CRK_RAT	Proto-oncogene C-crk (p38) (Adapter molecule crk)	33844	5.39	1	2.74	no	
22	12W	Q5XI73|Q5XI73_RAT[6]	Rho GDP dissociation inhibitor (GDI) alpha	23407	5.12	6	13.4	yes	37%

Fig. 4. (*Continued*)

under natural (baseline) as well as stressful conditions. Our data are consistent with the concept that biological oscillations like sleep–wakefulness, circadian rhythms and hibernation evolved such that the temporal compartmentalization of metabolism allows the organism to optimize the utilization of ATP for survival [Tu and McKnight, 2006].

A smaller subset of differentially expressed proteins was involved with cytoskeletal support and/or cellular transport (~26%; n = 5, Fig. 4B). SWS-specific proteins included actin (n = 3 spots) and the vesicular *N*-ethylmaleimide fusion (NSF) protein [Stenbeck, 1998; Valentijn et al., 1999; Paavilainen et al., 2004; Wheeler and Ridley, 2004]. RhoB and GTP binding protein rab3D, involved in cytoskeletal transport, were identified within a single wake-unique spot that also contained two other wake-specific proteins (C-crk and RhoGDI), components of signal transduction pathways implicated in the maintenance of cytoskeletal integrity [Olofsson, 1999; Hennige et al., 2000]. Cofilin, involved in actin polymerization/depolymerization, was present in both waking and SWS states. Proteins in this functional category have been implicated in the maintenance of neuronal plasticity [Matus, 2000; Ramakers, 2002; Ethell and Pasquale, 2005], cognition [Ramakers, 2002; Rex et al., 2007], memory [Lin et al., 2005] and mental retardation [Ramakers, 2002]. These results thus provide additional evidence that waking and sleep differentially affect proteins involved with similar cellular and/or biological functions at multiple behavioral levels, consistent with higher-order functions of the frontal cortex.

The Yb-1 subunit of glutathione *S*-transferase (GST) was also unique to SWS (Fig. 4B). GSTs are a multi-gene family of proteins that are associated with the maintenance of cellular oxidation–reduction [Pocernich et al., 2000; Zhu et al., 2006]. The failure to maintain the cellular oxidation state has been proposed to account for some of the deleterious effects of SD [D'Almeida et al., 1998; Ramanathan et al., 2002] and has long been associated with the detrimental effects of aging [Zhu et al., 2006]. It is interesting to note that an increase in *gst pi 2 subunit* mRNA was also reported following spontaneous sleep [Cirelli et al., 2004]. Sleep may therefore play a role in the maintenance of the cellular redox state through the differential expression of proteins/mRNAs involved in the clearance of reactive oxygen species.

SD DIFFERENTIALLY AFFECTS GAPDH AND ACTIN EXPRESSION

To verify the state-associated protein expression during spontaneous sleep, tissue from rats subjected to SD was analyzed. As described above, this paradigm is classically used to differentiate sleep timing (circadian) from sleep need (homeostatic regulation) and is based on the observation that following SD, there is an increase in sleep time and intensity that is proportional to the amount of sleep lost. Thus a need for sleep progressively accumulates during waking [Tobler and Borbely, 1986, 1990; Franken et al., 1991]. This need is measured in rodents and humans by EEG power density (0.1–4 Hz), also called slow wave activity (SWA) [Borbely and Achermann, 1999; Riedner et al., 2007; Vyazovskiy et al., 2007].

In these experiments, a separate group of rats were kept awake for 6 h by gentle handling. Animals (n = 16) were randomly chosen for sacrifice after SD or after 1–2 h RS (RS1, RS2, respectively). A group of freely behaving animals (n = 18) not handled in any manner, was sacrificed as a function of the TOD. EEG power density (0.1–4 Hz) was monitored as an indicator of sleep need [Borbely and Achermann, 1999]. Figure 5A shows the gradual decrease in SWA during RS relative to SD, consistent with results in both rats and humans [Tobler and Borbely, 1986, 1990; Franken et al., 1991; Borbely and Achermann, 1999; Cirelli et al., 2004; Vyazovskiy et al., 2007, 2008]. GAPDH levels began to increase within 2 h RS relative

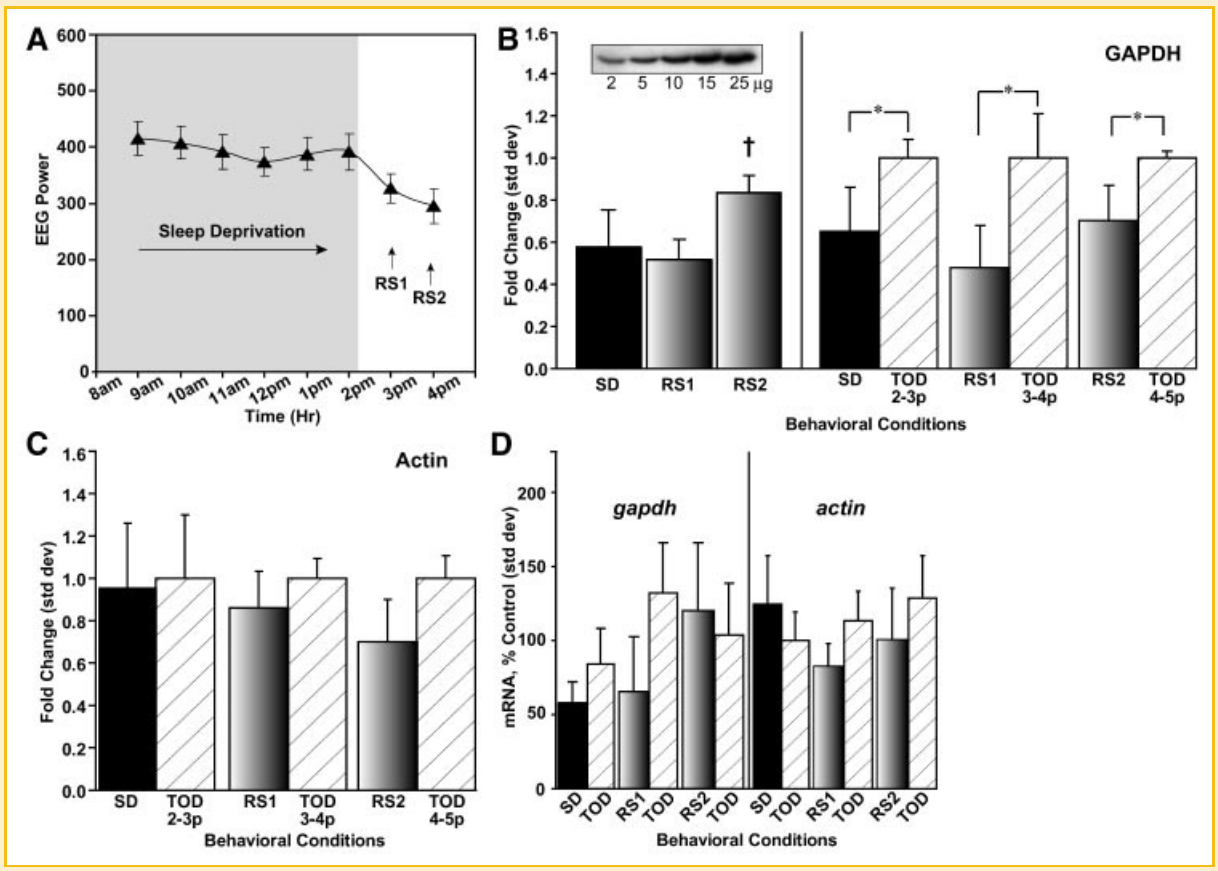


Fig. 5. Effects of SD/RS on cortical GAPDH and actin expression. Animals were randomly chosen for sacrifice after 6 h of SD and RS. TOD rats were not behaviorally manipulated and sacrificed in the same hour time frames. A: SWA during 6 h of SD, RS1, and RS2. Data are expressed as 1 h meaned values (±SEM) of SWA for every animal (n = 16) as a percentage of baseline conditions (data not shown). B: GAPDH levels showed expression patterns characteristic of homeostatic regulation. Dagger (†) indicates statistical differences ($P < 0.05$) in protein levels following 2 h RS compared to 6 h SD and 1 h RS. Inset shows GAPDH immunoreactivity as a function of protein concentration. GAPDH levels following SD/RS were also significantly different compared to the corresponding TOD controls. Asterisk (*) indicates statistical differences ($P < 0.05$) in protein levels between experimental conditions. C: Actin protein levels showed no change following SD/RS. D: Real-time RT/PCR analysis of *gapdh* and *actin* mRNA levels showed no statistical differences in SD and RS rats. For each marker, results were compared relative to the TOD 2–3 pm value. All data were analyzed using GBStat v8. ANOVA analyses used behavioral state as factor, followed by post hoc Fisher's LSD procedure to adjust for multiple comparisons among behavioral groups. Number of rats analyzed per behavioral condition was as follows: SD (n = 5); TOD 2-3p (n = 7); RS1 (n = 5); TOD 3-4p (n = 6); RS2 (n = 6); TOD 4-5p (n = 5).

to SD ($^{\dagger}P < 0.05$, Fig. 5B, left panel). GAPDH levels after SD, RS1 and RS2, however, were still decreased relative to their respective TOD controls, suggesting that a return to baseline protein levels takes more than 2 h (* in Fig. 5B, right panel, $P < 0.05$). The magnitude of these changes was small yet significant (~1.4-fold increase in RS2 over SD, left panel, Fig. 5B, a ~0.6-fold decrease for SD/TOD2-3p, ~0.5 decrease for RS1/TOD3-4p, and ~0.7 decrease for RS2/TOD4-5p, right panel, Fig. 5B). *gapdh* mRNA levels did not change across the conditions examined (Fig. 5D, left panel), in accordance with the use of this marker as a control for sleep studies [Terao et al., 2003]. These data are consistent with a sleep-dependent regulation of GAPDH protein levels by post-transcriptional mechanism(s).

In contrast, no changes in actin expression were detected across SD/RS at either the protein or mRNA levels (Fig. 5C,D, respectively). Previous studies show that actin is phoshorylated [Gettemans et al., 1992; Waelkens et al., 1995]. To assess whether alterations in actin phosphorylation [Agnew et al., 1995; Waelkens et al., 1995; Baba et al., 2003; Barda-Saad et al., 2005] could account for the detection of SWS-unique spots, actin was monitored in spontaneous sleep (Fig. 6A,B) and SD/RS extracts (Fig. 6C) by 2DE Western analysis. The actin antibody cross-reacted with several spots across both spontaneous states (Fig. 6A, right panels), though more actin immunoreactive spots were visualized during SWS (n = 5) compared to waking (n = 4). This staining pattern, or "train," results from the binding of small molecules, which alters the net charge and migration of the protein [Packer et al., 1998]. A train of similar mass and charge was also visualized in phospho-stained gels (Fig. 6A, left panels). Overlay of the actin (SWS) Western onto the phospho Master image indicated that the phospho-stain contained actin immunoreactivity (Fig. 6B). The additional actin spot associated with SWS was basic relative to W (boxed region in Fig. 6A,B), suggesting that actin may undergo dephosphorylation during sleep. Further, in the sleep that follows prolonged waking, there was an apparent shift in actin immunoreactivity from an acidic to a more basic net charge, suggesting that actin is also dephosphorylated during RS (Fig. 6C, hatched region). It is also possible that the shift in

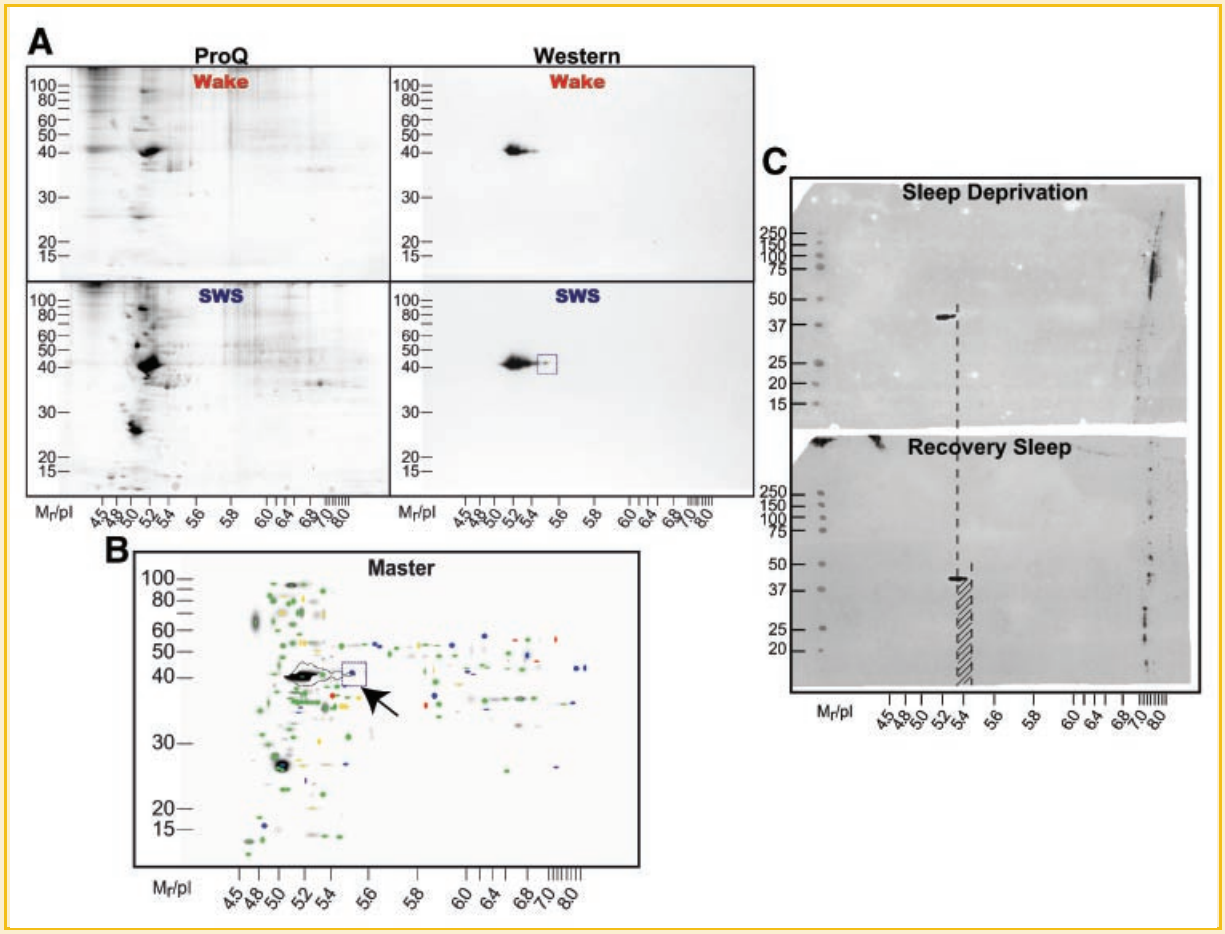


Fig. 6. Actin expression across spontaneous sleep and after SD/RS. A: ProQ staining of Wake and SWS phosphoproteins is shown in left panels. Corresponding actin Westerns are in (A), right panel. B: Overlay of SWS Western 2DE profile onto phospho-Master image. Protein standards were used to align and compare the actin Western images to one another across state, to generate a Master actin Western image (not shown), and then to overlay the actin Western Master gel image onto the phospho-Master image. Coincident Western and phosphorylated proteins are outlined in black (molecular mass, ~40 kDa; pI range of 5.1–5.5). Green spots represent phosphoproteins common to all behavioral states; blue spots, unique to SWS; red spots, unique to waking; yellow spots, unique to TOD. C: Comparison of actin staining following SD and RS showed a shift in net charge from a more acidic (SD; top panel) to a more basic pI (RS1; bottom panel).

actin is also due to aceytlation [Kim et al., 2006]. Taken together, these results are consistent with a state-dependent control of actin via post-translational modifications. The GAPDH and actin data suggest a number of post-transcriptional mechanisms are active during sleep and highlight the impact of sleep on cortical protein expression.

CONCLUSIONS

Identification of the cellular underpinnings of sleep–wake behavior have been hampered by complexities present at every level of its' hierarchical organization. We screened hundreds of proteins by 2DE and MS to identify putative protein correlates of sleep and wakefulness. Our data are the first to show that a small subset of proteins exhibit specific state-related expression and are detected within the minute time frames of spontaneous sleep–wake bouts characteristic of rodents. In addition, state-related differences in protein phosphorylation indicate that the electrophysiological signaling used to distinguish spontaneous sleep from wake states impacts cellular activities. Thus, while sleep is often associated with quiescence at the behavioral and electrophysiological levels [Lin, 2000], at the cellular level, sleep appears to be quite dynamic. This novel finding suggests that behavioral state should be carefully considered when assessing protein levels and/or activities in animal models.

Our results suggest that subtle changes in the expression and/or phosphorylation of proteins associated with common metabolic pathways occur across spontaneous sleep–wake states. These results are in apparent contrast to the idea that different functional categories of genes are selectively associated with sleep and wakefulness [Cirelli et al., 2004]. It is difficult to reconcile whether these discrepancies are due to state-related differences between mRNA and protein expression or to limitations of the respective technologies used. On the one hand, though microarrays allow investigation of nearly the entire genomic complement, it is well

known that mRNA levels often do not correlate with protein levels and/or activities [Graves and Haystead, 2002], particularly so since the temporal relationship between behavior and mRNA profiles was so broadly defined in the microarray studies [Cirelli et al., 2004; Terao et al., 2006]. On the other hand, proteins expressed in relatively low abundance (<0.01% of the total protein) would likely not be detected in our 2DE system and in addition, not all state-related spots were subjected to MS analyses. Thus, it is not possible to determine whether more selective 2DE conditions and/or more MS analyses would have provided evidence that different functional categories of proteins are selectively associated with sleep and wakefulness. There is, however, some overlap in the state-related functional categories identified in our studies, compared to microarray analyses of spontaneous [Cirelli et al., 2004] and SD/RS tissue [Cirelli et al., 2004] and protein profiles following SD/RS [Basheer et al., 2005], though the specific proteins differ. Collectively, the protein and mRNA data implicate roles for sleep in essential cellular functions like energy metabolism, maintenance of oxidation state and cellular transport. Maintenance of these cellular functions, in turn, may allow the brain to maintain higher level functions like plasticity, alertness and cognition.

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