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The short- and long-term proteomic effects of sleep deprivation on the cortical and thalamic synapses

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ABSTRACT

Acute total sleep deprivation (SD) impairs memory consolidation, attention, working memory and perception. Structural, electrophysiological and molecular experimental approaches provided evidences for the involvement of sleep in synaptic functions. Despite the wide scientific interest on the effects of sleep on the synapse, there is a lack of systematic investigation of sleep-related changes in the synaptic proteome. We isolated parietal cortical and thalamic synaptosomes of rats after 8 h of total SD by gentle handling and 16 h after the end of deprivation to investigate the short- and longer-term effects of SD on the synaptic proteome, respectively. The SD efficiency was verified by electrophysiology. Protein abundance alterations of the synaptosomes were analyzed by fluorescent two-dimensional differential gel electrophoresis and by tandem mass spectrometry. As several altered proteins were found to be involved in synaptic strength regulation, our data can support the synaptic homeostasis hypothesis function of sleep and highlight the long-term influence of SD after the recovery sleep period, mostly on cortical synapses. Furthermore, the large-scale and brain area-specific protein network change in the synapses may support both ideas of sleep-related synaptogenesis and molecular maintenance and reorganization in normal rat brain.

1. Introduction

The idea that sleep contributes to maintain normal brain functions as memory formation and behavior has been developed several decades ago (Bloch et al., 1977, 1979; Blissitt, 2001; Dang-Vu et al., 2006). It is supported by the facts that the majority of brain disorders are accompanied by sleep disturbances (Reynolds et al., 1988; Vitiello et al., 1990, 1991; Starkstein et al., 1991; Wiegand et al., 1991; Donnet et al., 1992; Baker and Richdale, 2015; Murphy and Peterson, 2015) and sleep deprivation causes memory impairment (Youngblood et al., 1997; Ishikawa et al., 2006), perception (Goel et al., 2005; Lei et al., 2015) and mood (Short and Louca, 2015) deficits.

Recently, two dominant ideas are formed concerning the general function of sleep, focusing mainly on the synapses. The synaptic homeostasis hypothesis (Tononi and Cirelli, 2003, 2006) suggests that sleep is necessary to decrease the enhanced synaptic strength that gradually develops during wakefulness to maintain the optimal balance between flexibility and rigidity in synapses which is crucial for normal brain function. This hypothesis predicted and partly proved the weakening of synaptic connection strength during sleep particularly in the cerebral cortex (Watson and Buzsáki, 2015). The synaptic homeostasis hypothesis emphasizes that the continuous molecular adjustment of the synapses during the active period makes them rigid through a "saturation" of the synaptic strength, therefore, decreases the ability of learning novel information. Thus, sleep reduces synaptic strength in general (Tononi

Abbreviations: 2D-DIGE, two-dimensional differential gel electrophoresis; EEG, electroencephalogram; EMG, electromyogram; FFT, Fast Fourier transform; MS/MS, tandem mass spectrometry; LTP, long-term potentiation; RS, recovery sleep; RS, experiment 2: the brains of rats were removed 16 h after the end of deprivation; SD, sleep deprivation; SD, experiment 1: the brains of rats were removed after 8 h of total sleep deprivation performed by gentle handling.

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and Cirelli, 2014). Another theory assumes the formation of novel dendritic spines, and in turn, synapses during slow-wave sleep, suggesting long-term memory inscription via development of novel synapses during sleep (Yang et al., 2014). The hypothesis of enhanced synaptogenesis during sleep points out that long-term memory trace consolidation is an important function of sleep and it is based on the genesis of novel dendritic spines and synapses (Matsuzaki et al., 2004) to change the neuronal connectome (Chow et al., 2013; Picchioni et al., 2013). While the synaptic homeostasis theory attempts to explain the restoration of "printability" of synapses, the intensive learning-induced, sleep-dependent synaptogenesis hypothesis suggests a mechanism which underlies the consolidation of long-term memory traces into the neuronal connectome. Both ideas are based on the fact that long-term memory traces require the ability of generating novel synapses and fundamental maintenance of flexibility in the protein composition of existing synapses (Trachtenberg et al., 2002; Klann and Sweatt, 2008).

Synaptogenesis and adjustment of synaptic strength are the results of molecular changes in synapses due to *de novo* protein synthesis (Martin et al., 2000) and/or incorporation of trafficking proteins into the synapse (Rumpel et al., 2005) during the memory consolidation process. As a model of synaptic plasticity induced by strong stimuli, long-term potentiation (LTP) is a good tool for studying molecular changes in synapses (Abraham and Otani, 1991; Sweatt, 1999). A fast imprinting into the synapse mediated by e.g., short-term kinase activity and protein trafficking is the major mechanism of early-phase LTP, lasting from a few seconds up to several hours after stimulation onset (Huang, 1998). The synaptogenesis and synaptic size increase were shown in the late phase of LTP (Tominaga-Yoshino et al., 2008). Most importantly, LTP is affected by sleep and sleep deprivation (McDermott et al., 2003; Blanco et al., 2015). These results further strengthen the idea that some sort of molecular maintenance and reorganization in synapses in conjunction with memory consolidation and recovery of learning capabilities are major functions of sleep.

There are several molecular changes in sleep and sleep deprivation uncovered by measuring mRNA level changes (Cirelli and Tononi, 1998; Cirelli et al., 2006; Terao et al., 2003a, 2003b; Mackiewicz et al., 2007; Jones et al., 2008; Vecsey et al., 2012) and also some protein level alterations have been revealed (Basheer et al., 2005; Pawlyk et al., 2007; Poirrier et al., 2008). However, focused high-throughput examination of the synaptic proteome is still lacking. The synaptic proteome contains more than 1,000 known proteins, but this number can be higher, since the available literature provides very different numbers of synaptic proteins probably due to the methodological heterogeneity in the field of proteomics (see http://www.synprot.de) (Pielot et al., 2012). Moreover, the majority of the synaptic proteins are also crucial in other cellular compartments of the neurons. Synapses are supplied by proteins from the local protein synthesis (Martin et al., 2000) and also by selection of proteins from axonal and dendritic protein trafficking systems (Vallee and Bloom, 1991). Interestingly, a general increase in the brain tissue protein synthesis has been revealed during sleep (Ramm and Smith, 1990; Nakanishi et al., 1997) but the data are not specific for the synaptic proteome.

The changes specific to the synaptic protein network underlying the sleep-related adjustment of synaptic strength are poorly understood. In this study, we performed a parietal cortical and thalamic synaptosome proteomic study of rats. The parietal cortex receives inputs from the thalamus which is necessary for genesis of synchronous sleep-related activity in the cortex as extensively studied by Steriade and others (for review, see Steriade and Llinás, 1988). It is also known that the detrimental effects of sleep deprivation are particularly pronounced in the thalamus and parietal cortex (among other cortical structures) (Chee and Choo, 2004; Chee et al., 2006). Therefore, the proteomics study was conducted on samples of thalamo-cortical cross-linked areas of the brain highly sensitive to sleep deprivation. Surprisingly, sleep deprivation inversely affects the activation-state of thalamus and the parietal cortex (Tomasi et al., 2009), emphasizing the importance of separately assessing molecular changes in these brain areas. The proteomic changes were characterized in both brain areas after 8 h of total sleep deprivation (SD) and 16 h after the end of deprivation, when recovery sleep (RS) of sleep deprived animals took place. This experimental design enabled monitoring the effects of SD and RS on the synaptic proteome in relevant brain areas.

2. Materials and methods

2.1. Animals

Adult male Sprague-Dawley rats (4 months old, weighing 350–400 g; Charles River Laboratories, Hungary) were used ($n = 24$ for proteomic experiment, $n = 6$ for electron microscopy and sleep deprivation validation). Animals were housed under standard laboratory conditions (lights on at 9:00 AM, lights off at 9:00 PM), with free access to water and food. The care and treatment of all animals were in conform to Council Directive 86/609/EEC, the Hungarian Act of Animal Care and Experimentation (1998, XXVIII), and local regulations for the care and use of animals in research. All efforts were taken to minimize the animals' pain and suffering and to reduce the number of animals used.

2.2. Experimental paradigm

Sleep deprivation started at 9:00 AM (lights on) and lasted 8 h long until 5:00 PM. In the SD experiment, brains of sleep deprived ($n = 6$) and control ($n = 6$) rats were removed right after the ending of the deprivation. In the RS experiment, brains of sleep deprived ($n = 6$) and undisturbed, control ($n = 6$) rats were removed 16 h after finishing the deprivation, at 9:00 AM, on the next day. For the experimental paradigm, see Fig. 1.

2.3. Sleep deprivation procedure and estimation of its effectivity by EEG and EMG

Sleep deprivation was carried out using the gentle handling method which is the least stressful method of total sleep deprivation (Ledoux et al., 1996; Rechtschaffen et al., 1999; Fenzl et al., 2007).

For electroencephalogram (EEG) recordings, rats were implanted with stainless steel screw electrodes (0.8 mm o.d.) and with teflon-coated, stainless steel multiwire muscle electrodes for electromyogram (EMG) recordings. Animals were anesthetized with 1% (v/v) isoflurane. The screw electrodes were implanted into the skull, bilaterally above the occipital, parietal and frontal cortices. Ground and reference electrodes were placed above the cerebellar cortex. The electrodes were fixed on the skull using dentacrylate cement, and were soldered to ten-pin sockets. Sleep deprivation and polygraphic recordings were performed after one week recovery period.

EEG and EMG were recorded by a Grass Model 8B (Grass Instrument Company) electroencephalograph attached to a CED 1401 mkII data capture and analysis device, using Spike 2 software (Cambridge Electronic Design Limited). The bandwidth of the EEG recording was 0.5–70 Hz and 5–300 Hz for the EMG recording. Signals were digitalized at 500 Hz sampling rate for EEG and at 900 Hz for EMG. Power density analysis was performed using Fast Fourier transform (FFT size 8192, Hanning window) in Spike 2. Somnograms were produced by sleep scoring that was made in 30 s epochs by a script provided by Cambridge Electronic Design Limited for the Spike 2 software ("RatSleepAuto" script; Costa-Miserachs et al., 2003).

2.4. Synaptosome preparation

Synaptosome isolation was performed immediately after the brain removal. Quickly removed brains were placed into ice-cold artificial cerebrospinal fluid and brain structures were dissected on a dry ice-cooled plate. Subsequently, parietal cortices and thalami were removed. From

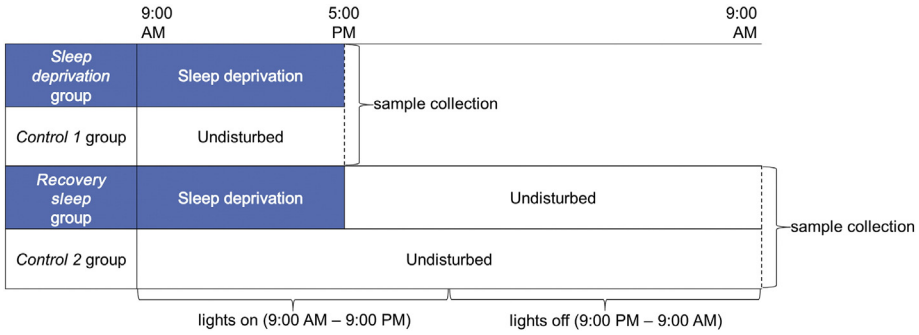

Fig. 1. The experimental paradigm. Rats assigned to the *Sleep deprivation* group were sleep deprived for 8 h from 9:00 AM to 5:00 PM while *Control 1* rats were left undisturbed during the same period. Animals of both groups were sacrificed and their brains were removed immediately after 5:00 PM. Rats assigned to the *Recovery sleep* group were sleep deprived for 8 h from 9:00 AM to 5:00 PM, and then they were left undisturbed for the next 16 h, until 9:00 AM on the following day. *Control 2* rats were left undisturbed during the whole experiment. Animals of the latter two groups were sacrificed at 9:00 AM on the next day.

the cerebral cortices, cortical white matter was separated and the gray matter was further processed.

Synaptosome fractionation was carried out strictly following the protocol published by Bajor et al. (2012). In brief, brain samples were placed into homogenization buffer (320 mM sucrose, 5 mM HEPES, 1 mM $MgCl_2$, pH 7.4) supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich) and homogenized with a Dounce Tissue Grinder manually (40 strokes per sample; Sigma-Aldrich) at 4 °C with pre-cooled equipment. After homogenization, samples were centrifuged at 4 °C with 1000 × *g* for 10 min, the supernatant was gravity filtered through a 5 μm pore-size PVDF membrane (Merck Millipore) and centrifuged at 4 °C with 12,000 × *g* for 30 min. The pelleted synaptosomes purified for electron microscopy examinations were immediately processed as described in Section 2.5, while proteins of the samples obtained for the proteomics study were precipitated with acetone overnight at −20 °C. The protein pellet was resuspended in lysis buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 20 mM Tris, 5 mM magnesium-acetate) and stored at −80 °C.

2.5. Electron microscopy

The synaptosome samples were fixed in 2% formaldehyde (freshly depolymerized from paraformaldehyde), 1% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 for 30 min at room temperature (RT). After extensive washing, samples were postfixed in 0.25% osmium tetroxide, 0.4% potassium hexacyanoferrate for 60 min, and *en bloc* stained with aqueous uranyl acetate for 30 min. Subseqently, synaptosome samples were dehydrated and embedded in LR White resin (Sigma-Aldrich) according to the manufacturer's instructions. Ultrathin sections (70 nm) were collected on 400 mesh copper grids (Sigma-Aldrich) and stained with half saturated aqueous uranyl acetate for 10 min, and lead citrate for 30 s. The grids were examined with a JEM-1011 electron microscope (JEOL) operating at 60 kV. Images were acquired and processed with an 11 megapixel Olympus Morada camera with iTEM software (Olympus Corporation). The images were taken from different mesh along a longitudinal band to cover the entire width of the specimen.

2.6. Two-dimensional differential in-gel electrophoresis (2D-DIGE)

In the proteomics experiment, we compared SD with Control1 and RS with Control2 cortical and thalamic groups separately (4 independent experiments were conducted). The applied protocol was described previously by Szegő et al. (2010). Briefly, pH of the samples was adjusted to 8.5 and the protein concentration was determined. Samples of 50 μg protein content were labeled with CyDye DIGE Fluor Minimal Labeling Kit, according to the manufacturer's instructions (GE Healthcare). Control and sleep deprivation samples were labeled with Cy3 and Cy5 randomly, and a pooled sample, serving as internal standard, was labeled with Cy2. After mixing the labeled samples, isoelectric focusing buffer was added, and the dry strips were rehydrated overnight at room temperature. Isoelectric focusing was performed for 24 h to attain a total of 80 kVh. After isoelectric focusing, the proteins were reduced and carbamidomethylated in equilibrating buffer and the strips were placed onto the top of 10% polyacrylamide gels (24 × 20 cm) casted in the laboratory. Running was conducted using an Ettan DALT System (GE Healthcare) at 2 W/gel for 1 h and at 12 W/gel for 3 h. Finally, gels were scanned with a TyphoonTRIO + scanner (GE Healthcare) and analyzed with DeCyder 2D 7.0 software package (GE Healthcare). Protein spots with significant difference between control and sleep deprivation samples (independent Student's *t*-test, $p < 0.05$) and with higher than ± 1.1-fold changes were selected. For protein identification, a preparative gel containing 800 μg protein was run and stained with Colloidal Coomassie Blue G-250 (Merck Millipore). The spots of interest were manually excised from the preparative gel and stored in 0.5% (v/v) acetic acid solution until the mass spectrometry analysis.

2.7. Protein identification by mass spectrometry and functional classification

Proteins from the excised spots were digested with trypsin using the in-gel digestion protocol without reduction and alkylation of cysteins, as described previously (Szabó et al., 2012). Digested protein samples were analyzed on a Waters NanoAcquity UPLC system coupled with a Micromass Q-TOF premier mass spectrometer (Waters Corporation). Five μL of samples were full-loop injected, and initially transferred with an A eluent to the precolumn at a flow rate of 10 μL/min for 1 min. The column was eluted with a linear gradient of 3–10% B over 0–1 min, 10–30% B over 1–20 min, 30–100% B over 20–21 min, the composition was maintained 100% B for 1 min and then returned to 3% for 1 min. The column was re-equilibrated at initial conditions for 22 min. Mobile phase A was 0.1% formic acid in water, while mobile phase B was 0.1% formic acid in acetonitrile. A 350 nL/min flow rate was applied on a Waters BEH130 C18 75 μm × 150 mm column with 1.7 μm particle size C18 packing (Waters Corporation). The column was thermostated at 45 °C. The mass spectrometer was operated in DDA mode with lockmass correction, with a nominal mass accuracy of 3 ppm. The instrument was operated in positive ion mode, performing full-scan analysis over the *m/z* range 400–1990 at 1/1 spectra/s for MS and 50–1990 in MS/MS. The source temperature was set at 85 °C and nitrogen was used as the desolvation gas (0.5 bar). Capillary voltage and cone voltage were maintained at 3.3 kV and 26 V, respectively.

All acquired data were processed by the WATERS Proteinlynx GlobalServer 2.4 software (Waters Corporation) using default settings.

Database search was performed using Mascot 2.204 (Matrix Science) which was set up to search the latest Swissprot database assuming the digestion enzyme trypsin, allowing 2 missed cleavage sites. The data were searched with 0.15 Da fragment and 60 ppm parent ion mass tolerances. Oxidation of methionine was specified as a variable, and carbamidomethylation of cysteine as a fixed amino acid modification.

Scaffold v 3.09 (Proteome Software) was used to validate MS/MS based peptide and protein identifications. Peptide and protein identifications were accepted if they could be established at greater than 95.0% probability and proteins contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony, in these cases representatives of grouped accession numbers are listed.

Functional classification of the proteins was carried out via thorough literature mining and using the UniProt protein database (http://www.uniprot.org).

2.8. Western blot

Western blot experiments were conducted to validate the synaptosome preparation protocol and our proteomics results. First, protein concentration of samples was determined with 2-D Quant Kit (GE Healthcare). Samples containing equal amounts of proteins were mixed with two-fold concentrated sample buffer (8% (wt/vol) sodium dodecyl sulfate, 3% (wt/vol) dithiothreitol, 24% (vol/vol) glycerol, 0.2% (wt/vol) bromophenol blue, 100 mM Tris–HCl (pH 6.8)) and protein separation was conducted in 10% polyacrylamide gels using Tricine-SDS discontinuous polyacrylamide gel electrophoresis. Subsequently, proteins were transferred onto Hybond-LFP PVDF membranes (GE Healthcare). After blocking of the blots with Tris-buffered saline containing 5% bovine serum albumin, 0.05% Tween-20 for 1 h, membranes were incubated overnight in the blocking buffer with the appropriate primary antibody. For antibody against a well-characterized synaptic marker, we used anti-Psd95 primary antibody (1:1,500 dilution, Thermo Fisher Scientific, catalog number: MA1-046), while anti-Actin antibody (1:1,000 dilution, Abcam, catalog number: ab1801) was used to detect levels of actin as loading control. In order to validate our proteomics data, we used primary antibodies against altered proteins, as follows: anti-Anxa3 (1:800 dilution, Thermo Fisher Scientific, catalog number: PA5-41314), anti-Crmp2 (also known as Dpysl2) (1:5,000 dilution, Abcam, catalog number: ab62661), and anti-Hspa8 (1:500 dilution, Merck Millipore, catalog number: MABE1120). After washing steps, membranes were incubated with appropriate secondary antibodies, as follows: ECL Plex Goat-α-mouse IgG Cy3-and Cy5 (both of them in 1:2,500 dilution; GE Healthcare). Finally, proteins of interest were detected using a TyphoonTRIO + scanner, while the densitometric analyses were performed with the ImageJ image processing program (http://imagej.nih.gov/ij/; Abramoff et al., 2004). Densitometric data of Psd95 protein was normalized to the level of actin. In the course of the densitometric evaluation of Anxa3, Dpysl2, and Hspa8 levels, we used densitometric data normalized to the densities of the total protein amounts of the appropriate lanes using Coomassie Brilliant Blue R-250 (Merck Millipore) staining (according to Eaton et al., 2013). This method was employed because we observed alterations in the level of protein spots of actin in most of our experiments. Statistical analyses were evaluated using independent Student's *t*-test.

3. Results

3.1. Characterization of the sleep deprivation procedure and the synaptosome fraction

In order to test the applied sleep deprivation method, EEG and EMG recordings were carried out on a group of animals. Somnograms, representative EEG and EMG recordings, complemented with the FFT analysis results of the corresponding EEG data from undisturbed, control, and sleep deprived rats, moreover, from rats during the recovery sleep period are shown in Fig. 2. Sleep deprivation procedure was performed with the gentle handling method that kept the rats awake almost entirely during the 8 h long recording, since the sleep deprived rats were awake in 97.3 ± 1.2% of the recorded period (mean ± standard deviation, $n = 6$). Analyzing the polygraphic record of sleep in the different experimental sessions in details also demonstrated that the efficiency of sleep deprivation was appropriate, since very few if any delta activity was recorded in the sleep deprivation group (Fig. 2, middle panels). Sleep deprived rats were mostly characterized by high power of theta frequency. On the other hand, the recovery sleep period after the sleep deprivation procedure was characterized by more slow-wave sleep activity (Fig. 2, bottom panels) than that of the control animals (Fig. 2, upper panels), and an increased delta power was found in their power spectra. Altogether, our physiological data confirmed that the applied sleep deprivation method was efficient and recovery sleep was observed.

Our proteomics study was conducted on a synaptosome fraction, the purity of which was accurately validated. First, Western blot analyses demonstrated the prominent enrichment of the synaptic marker protein Psd95 in the synaptosome fraction prepared from the cerebral cortex in comparison with the whole cortical homogenate (Fig. 3A). Moreover, electron micrographs showed intact synaptosomes, as sealed, synaptic vesicle-filled presynaptic terminals and tightly attached postsynaptic compartments were present (Fig. 3B).

3.2. Widespread proteomic alterations characterize the parietal cortical and thalamic synapses due to sleep deprivation

Gel-based proteomic tools were used to investigate the sleep deprivation-induced short- and longer-term changes in the synaptic proteome of parietal cortices and thalami of rats. A total of ~1600–1700 proteins were detected on every single gel. Thirty-two statistically significantly altered spots were detected in the SD experiment from the parietal cortex, and 7 from the thalamus, while the RS experiment revealed 126 and 34 changed spots in the parietal cortex and thalamus, respectively (Figs. 4 and 5; Suppl. Tables 1–4). One hundred and forty-two different proteins were identified altogether with significantly changed amounts in the SD and RS experiments from the cortical and thalamic samples. The SD experiment revealed 53 altered proteins from the parietal cortex and 19 from the thalamus, while the RS experiment showed 95 proteins from the parietal cortex with changed abundances and 28 from the thalamus (see Tables 1–4 and Suppl. Tables 1–4). The fold changes of the altered spots were in the ranges of −1.1 up to −1.71 and 1.1–1.59 (Fig. 6A–D). From the 125 different cortical proteins, 24 were found to be influenced by both the short- and long-term effects of sleep deprivation, while from the 45 thalamic proteins, 2 overlapping in the SD and RS experiment were observed.

The identified proteins were clustered according to their cellular functions and assigned to cellular localizations, based on the rigorous analysis of the literature and using the UniProt protein database (see Tables 1–4 and Fig. 6E–H). The affected cellular functions were as follows: carbohydrate and energy metabolism (SD cortex: 17 proteins, SD thalamus: 2, RS cortex: 26, RS thalamus: 7), amino acid metabolism (SD cortex: 3 proteins, SD thalamus: 1, RS cortex: 2, RS thalamus: 1), lipid metabolism (SD cortex: 1 protein, SD thalamus: 0, RS cortex: 1, RS thalamus: 1), nucleotide metabolism (SD cortex: 2 proteins, SD thalamus: 1, RS cortex: 1, RS thalamus: 0), synaptic transmission (SD cortex: 5 proteins, SD thalamus: 3, RS cortex: 9, RS thalamus: 4), protein synthesis and folding (SD cortex: 10 proteins, SD thalamus: 7, RS cortex: 11, RS thalamus: 4), proteolysis (SD cortex: 0 protein, SD thalamus: 2, RS cortex: 2, RS thalamus: 2), response to oxidative stress (SD cortex: 1 protein, SD thalamus: 0, RS cortex: 9, RS thalamus: 2), cytoskeletal (SD cortex: 8 proteins, SD thalamus: 3, RS cortex: 14, RS thalamus: 2),

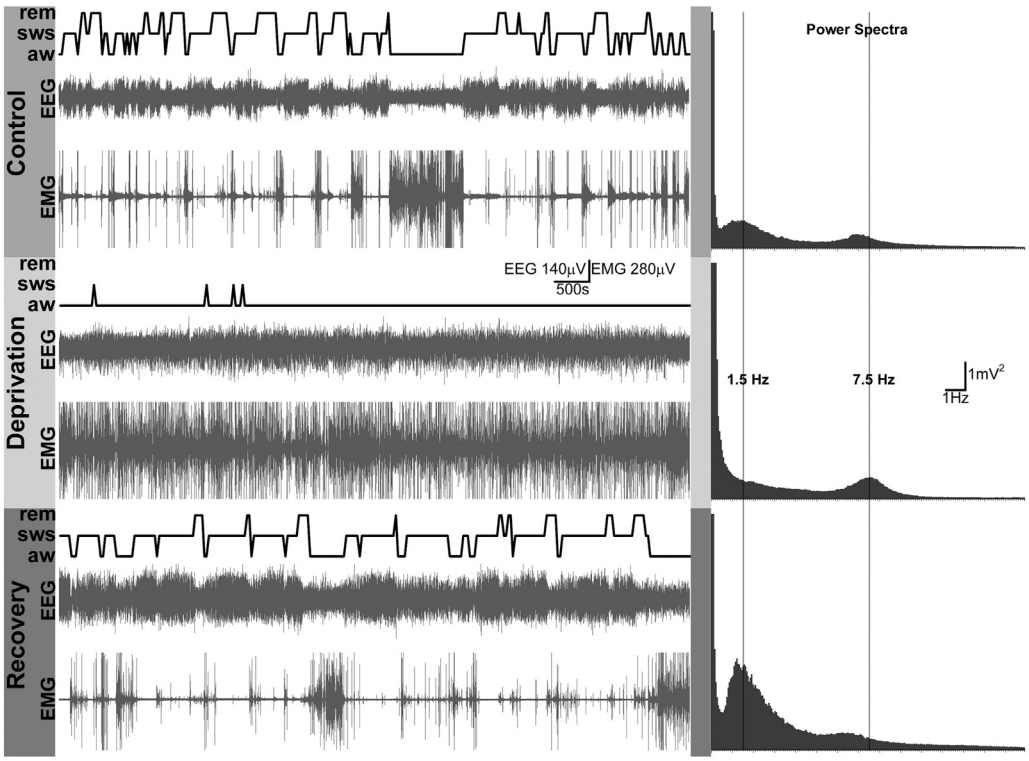

Fig. 2. Representative polygraphic sleep records of the control, sleep deprivation, and recovery sleep period. *Left side*: at the top, the somnogram is shown (*rem* – REM sleep, *sws* – slow-wave sleep, *aw* – awake). The second line is the EEG and the third one is the EMG record. *Right side*: the total EEG power spectra of all of the three conditions which highlight the increase of delta power due to rebound sleep in the recovery period during the first nocturnal period after sleep deprivation.

signal transduction (SD cortex: 1 protein, SD thalamus: 0, RS cortex: 6, RS thalamus: 3) and miscellaneous (SD cortex: 5 proteins, SD thalamus: 0, RS cortex: 14, RS thalamus: 2).

Validation of the proteomics results was conducted using Western blot technique. We have assigned those proteins for validation experiments, the levels of which were showed unidirectional alterations in the proteomics experiments suggesting underlying protein expression changes. In sum, we successfully verified the level changes of selected proteins, i.e., Anxa3, Dpysl2, and Hspa8 (Fig. 7). On the other hand, no net change in the expression level of Dpysl2 was found in the RS parietal cortex and RS thalamus groups (Suppl. Fig. 1), which is in agreement with the observed bidirectional alteration of this protein in the above experiments (Tables 3 and 4, respectively), that points towards differential post-translational modifications in the background.

Moreover, we collected those proteins which are directly implicated in synaptic functions and plasticity (e.g., synaptic vesicle recycling, dendritic outgrowth and synaptogenesis). According to our results, these proteins comprise the 19%, 26%, 23% and 25% of proteins in the SD parietal cortex, SD thalamus, RS parietal cortex and RS thalamus groups, respectively (Fig. 8).

4. Discussion

4.1. Limitations and advantages of using synaptosome proteomics and sleep deprivation in sleep research

Results of our high-throughput proteomics study on synaptic protein level changes induced by sleep deprivation uncovered large-scale molecular alterations in the synapse. The extent of synaptic proteome changes suggests that sleep deprivation elicits a widespread but mild molecular reorganization of the synaptic region including even more proteins than expected. The interpretation of the results is limited because the available proteomic techniques can detect only more abundant proteins in a sample (Chevalier, 2010). Previous sleep-related studies on brain tissue proteome (Basheer et al., 2005; Pawlyk et al., 2007; Poirrier et al., 2008) were not synapse-specific since only a little portion of the brain protein content is synaptic. In addition, several synaptic proteins have wide distribution in other cell compartments and only a fraction of the synaptic proteome is strictly synapse-specific (see http://synprot.de). Therefore, the enrichment of synaptic proteins in the samples was carried out using cell fractionation technique. The applied synaptosome preparation is a good compromise at the actual state of technological development because it can give the widest scope to the synaptic region in its molecular complexity.

Gentle handling was used for sleep deprivation to avoid mixing the effects of sleep deprivation and the stress response. It is widely accepted that gentle handling is the optimal way of total sleep deprivation to minimize stress (Rechtschaffen et al., 1999; Fenzl et al., 2007); however, we cannot state that there is no stress at all after few hours of gentle handling. Our model was close to the clinically applied sleep deprivation method for treatment of depression (Giedke and Schwärzler, 2002), thus, we did not study an extremely severe sleep deprivation that would be very different from the human practice. Therefore, our proteomics data may have some relevance for translational studies as well.

4.2. Differences in the extent of alterations in the synaptic proteome between the parietal cortex and thalamus, moreover, between the SD and RS groups

The neuronal plasticity, learning and cognitive processes extensively use the cerebral cortical synapses during waking. On the other hand, thalamus is a relay structure transmitting information to the cortex in wakefulness and maintains long-loop synchronization via thalamo-cortico-thalamic neuronal circuits (Steriade, 2006). These functional differences in information processing were reflected by our proteomics findings. The number of proteins changed in the parietal cortex was

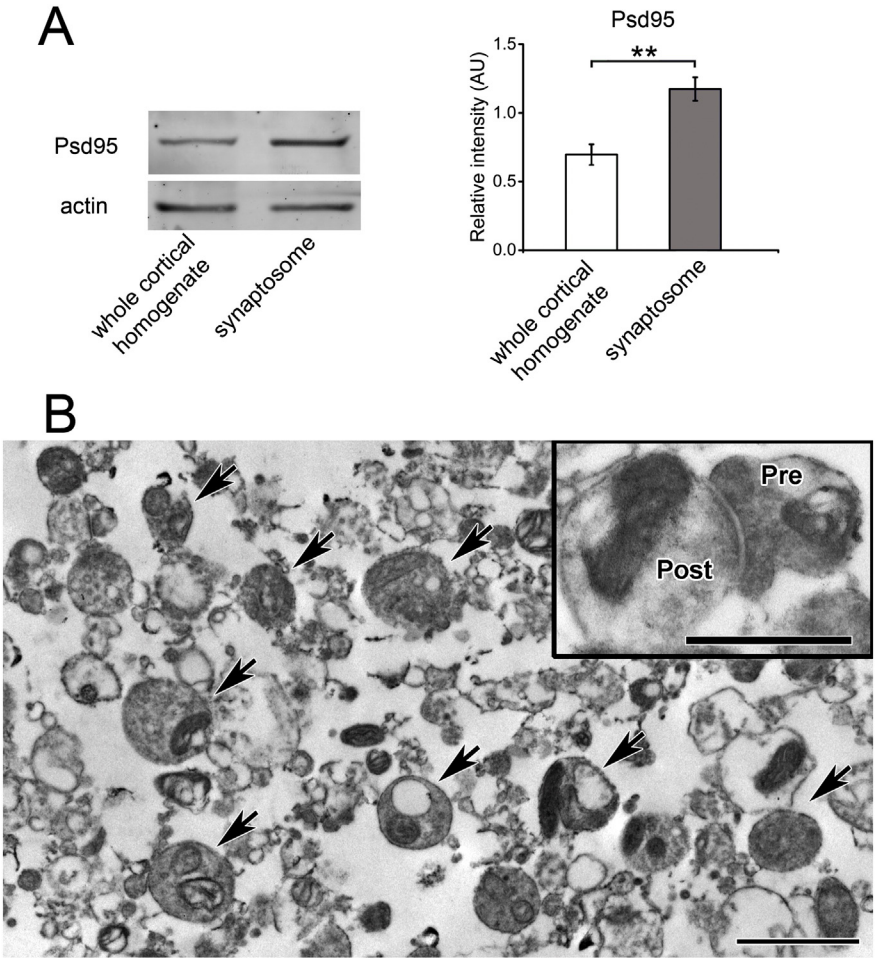

Fig. 3. Validation of the synaptosome preparation protocol. (A) Representative Western blot image of the synaptic marker Psd95 and of the loading control actin in the whole cortical homogenate and synaptosome fraction, with the corresponding total protein labeling gel images. Bar graph demonstrates the enrichment of Psd95 (1.69 ± 0.22-fold increase) in the synaptosome fraction according to the densitometric analysis. (B) Electron micrograph of the synaptosome sample. Arrows show intact synaptosomes consisting presynaptic terminals filled with synaptic vesicles and comprising synaptic mitochondria. Synaptic contacts/junctions are also visible occasionally (*inset*), which are formed by the pre-, and postsynaptic (Pre and Post, respectively) parts. Means ± S.E.M. are presented; $n = 5$ per group; $^{**}p < 0.01$ in (A). Scale bar is 1 μm and 0.5 μm (*inset*) in (B).

higher (SD: 53, RS: 95) than that in the thalamus (SD: 19; RS: 28), suggesting that the higher the plasticity and learning intensity are, the higher the number of affected proteins is influenced by the sleep-wake cycle. It is in agreement with the synaptic homeostasis hypothesis of sleep because it suggests that the extent of proteome changes in sleep deprivation is proportional to the intensity of information processing and learning.

To compare the two investigated time points, we can conclude that larger scale changes are prevalent at 16 h after the sleep deprivation than at the end of the deprivation procedure (Fig. 6A–D), presumably due to the recovery sleep period. On the other hand, this result also suggests that the timing of protein synthesis and degradation is delayed and/or these processes progress after the end of the sleep deprivation. Therefore, our data shows that an unexpectedly long and complex procedure of synaptic proteome adjustment takes place after sleep deprivation, although, we cannot separate the delayed protein synthesis changes induced by sleep deprivation from the long-term effect of the lack of sleep.

4.3. The importance of the most abundant functional clusters of SD and RS proteins

Several categories of cellular functions were revealed with numerous proteins representing them. In most of the experimental situations, the majority of significant protein changes were related to carbohydrate and energy metabolism, protein synthesis and folding and the cytoskeletal functional clusters, besides synaptic transmission (Fig. 6). The enrichment of these general cellular functions suggests that fundamental physiological mechanisms are influenced by sleep deprivation in the synapse.

Sleep serves as a lower energy consuming state, which is demanded after the awake period, characterized as a state with higher metabolic rates (Madsen et al., 1991; Maquet, 1995; Benington and Heller, 1995); and synaptic metabolism is a good index of synaptic molecular reorganization in sleep. Consequently, lower ATP levels in sleep deprivation and waking, and higher in sleep and recovery sleep after deprivation have been demonstrated previously (Dworak et al., 2010). Local metabolic differences between distinct brain areas are known, and the cerebral cortex is one of the regions, which show high fluctuation of metabolic rates throughout the sleep-wake cycle (Braun et al., 1997; Vyazovskiy et al., 2008). In all experimental situations, remarkable changes in the amount of proteins were unveiled which are involved in the carbohydrate and energy metabolism, however the highest number of changes were found in metabolic proteins in the parietal cortex in RS ($n = 26$) and in SD ($n = 17$) (Fig. 6E, G). Interestingly, most of the thalamic proteins implicated in these functions in RS group showed decreased levels (Table 4). Altogether, these data are in accordance with the fact that synapses are extremely energy-sensitive

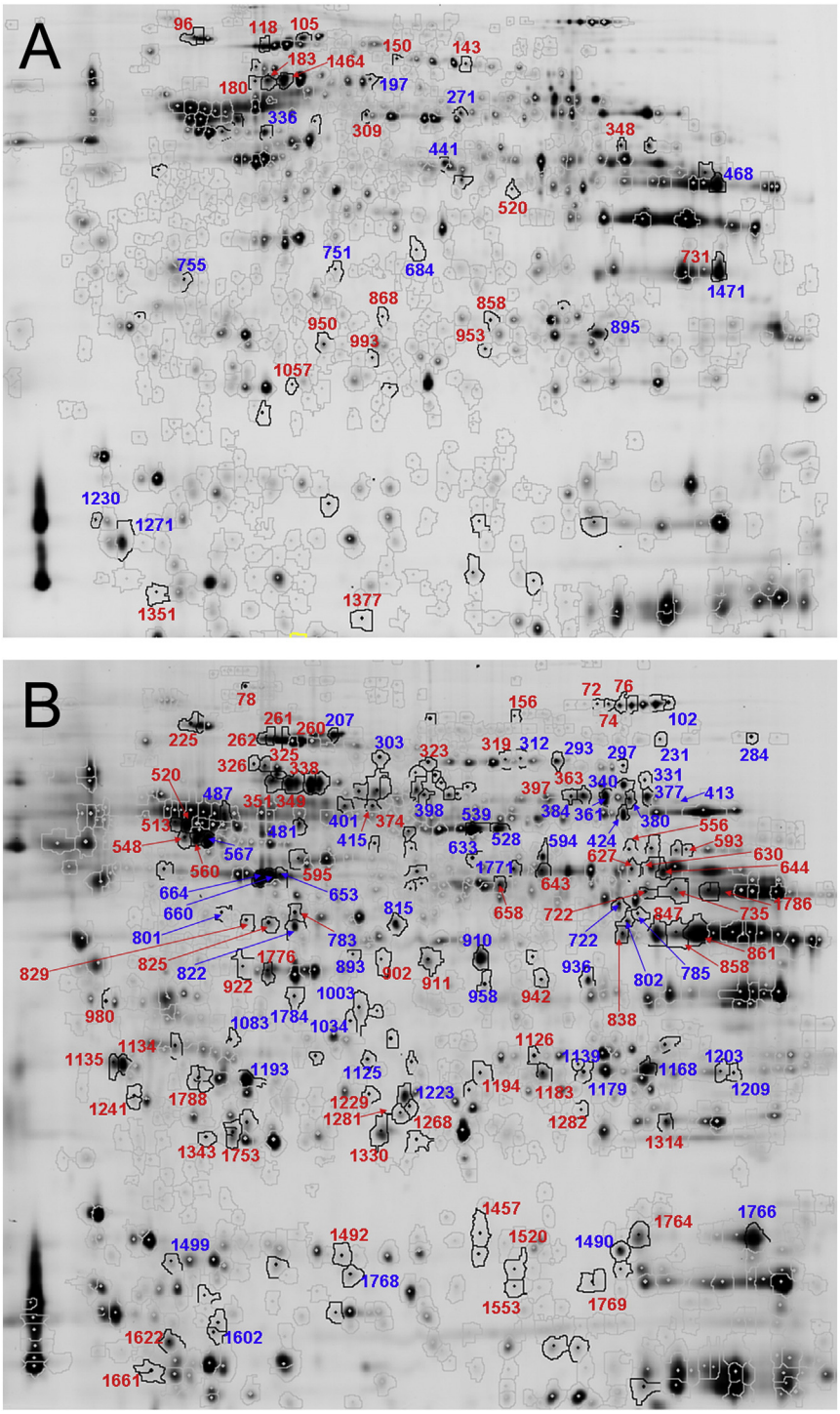

Fig. 4. Representative gel images obtained after the 2D-DIGE analyses of parietal cortical synaptic proteins, supplemented with the presentation of the significantly altered spots. (A) Representative gel image from the SD experiment. (B) Representative gel image from the RS experiment. Red and blue colors show protein level increase and decrease, respectively.

cell compartments and assumes that the functional alternation of synapses in sleep-wake cycle is accompanied by changes in synaptic metabolism.

Sleep is suggested to have a considerable influence on the remodeling of the neural cytoskeleton, supported by the observed alterations in the number and morphology of the dendritic spines (Bushey et al., 2011; Maret et al., 2011). This is in agreement with the fact that sleep promotes neural plasticity (Benington and Frank, 2003) and plasticity requires dynamic morphological changes (Yang et al., 2009; Kasai et al., 2010). Extensive cytoskeletal changes in all experimental groups were demonstrated (Fig. 6). Several major components of the cytoskeleton are influenced (e.g., actin and tubulin) while a huge repertoire of other proteins are mainly associated with the precise regulation of the cytoskeletal structure or related to the transport mechanisms along the cytoskeleton. It should be noted that certain proteins are already revealed as potential regulators of synaptogenesis and the structure of

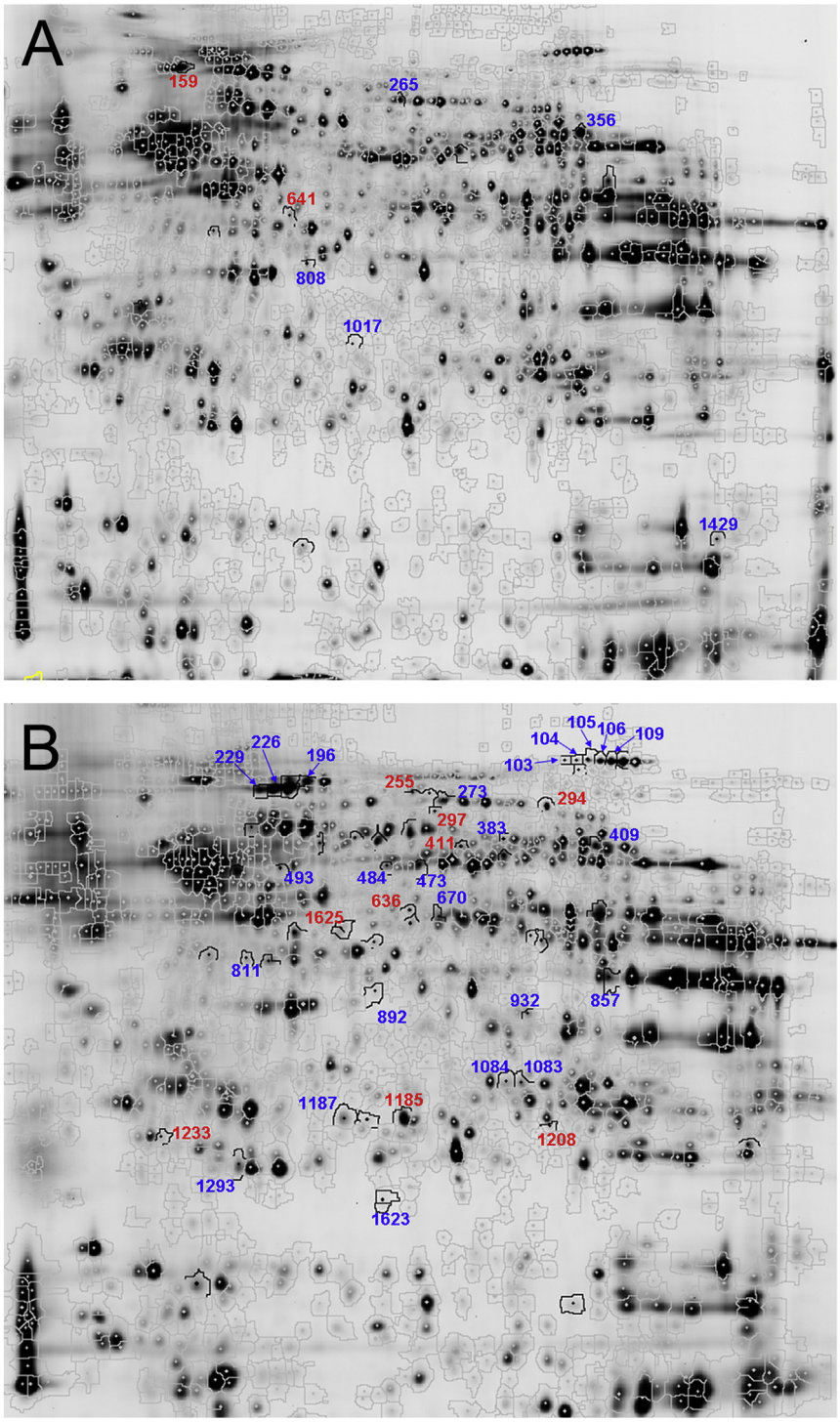

Fig. 5. Representative gel images obtained after the 2D-DIGE analyses of thalamic synaptic proteins, supplemented with the presentation of the significantly altered spots. (A) Representative gel image from the SD experiment. (B) Representative gel image from the RS experiment. Red and blue colors show protein level increase and decrease, respectively. (For interpretation of the references to colors in this figure legend, the reader is referred to the web version of this article.)

existing synapses (Tables 1–4, and Fig. 8). A general decrease was also found in the levels of cytoskeletal and motility-related proteins in the parietal cortex of SD compared to undisturbed rats (Table 1), supporting an inhibited structural remodeling of synapses due to sleep deprivation.

Another hypothesis of sleep function is the "free radical flux theory" which raises that sleep is initiated by free radical level increase, and sleep has a function to eliminate them (Reimund, 1994; Ikeda et al., 2005). The most oxidative stress response-related proteins were found in the parietal cortex of RS group animals, ($n = 9$, Fig. 6) among which 7 protein showed level increase. This is in agreement with previous examinations and theories, that concluded antioxidant responses after sleep deprivation, as a consequence of the higher metabolic rate related to prolonged wakefulness (Reimund, 1994; Brown and Naidoo,

Table 1
List of significantly altered SD synaptic proteins from the parietal cortex, assigned to functional groups.

Protein name	Gene name	Accession number	Up/down regulation[a]	Cellular localization	Cellular function
Carbohydrate and energy metabolism					
Fructose-bisphosphate aldolase A	Aldoa	P05065	↑↓	Cytoplasm	Glycolysis
Fructose-bisphosphate aldolase C	Aldoc	P09117	↑	Cytoplasm	Glycolysis
ATP synthase subunit alpha, mitochondrial	Atp5a1	P15999	↓	Mitochondrion	Energy metabolism
ATP synthase subunit delta, mitochondrial	Atp5d	P35434	↓	Mitochondrion	Energy metabolism
ATP synthase subunit d, mitochondrial	Atp5h	P31399	↑	Mitochondrion	Energy metabolism
Creatine kinase B-type	Ckb	P07335	↓	Cytoplasm	Energy metabolism
Creatine kinase U-type, mitochondrial	Ckmt1	P30275	↓	Mitochondrion	Energy metabolism
Alpha-enolase	Eno1	P04764	↑↓	Cytoplasm	Glycolysis
Electron transfer flavoprotein subunit alpha, mitochondrial	Etfa	P13803	↑	Mitochondrion	Electron transport
Fumarate hydratase, mitochondrial	Fh	P14408	↑	Mitochondrion	Carbohydrate metabolism, involved in tricarboxylic acid cycle
Glyceraldehyde-3-phosphate dehydrogenase	Gapdh	P04797	↓	Cytoplasm	Glycolysis
Malate dehydrogenase, cytoplasmic	Mdh1	O88989	↑	Cytoplasm	Carbohydrate metabolism, involved in malate-aspartate shuttle
Malate dehydrogenase, mitochondrial	Mdh2	P04636	↓	Mitochondrion	Carbohydrate metabolism, involved in tricarboxylic acid cycle and malate-aspartate shuttle
Phosphoglycerate kinase 1	Pgk1	P16617	↓	Cytoplasm	Glycolysis
Pyruvate kinase isozymes M1/M2	Pkm2	P11980	↑	Cytoplasm	Glycolysis
Cytochrome b-c1 complex subunit 1, mitochondrial	Uqcrc1	Q68FY0	↓	Mitochondrion	Electron transport
Cytochrome b-c1 complex subunit 2, mitochondrial	Uqcrc2	P32551	↓	Mitochondrion	Electron transport
Amino acid metabolism					
Aminoacylase-1A	Acy1a	Q6AYS7	↓	Cytoplasm	Deacetylation of amino acids
3-Hydroxyisobutyrate dehydrogenase, mitochondrial	Hibadh	P29266	↑	Mitochondrion	Amino acid metabolism
Isovaleryl-CoA dehydrogenase, mitochondrial	Ivd	P12007	↓	Mitochondrion	Amino acid metabolism
Lipid metabolism					
Enoyl-CoA hydratase, mitochondrial	Echs1	P14604	↑	Mitochondrion	Fatty acid metabolism
Nucleotide metabolism					
2′.3′-cyclic-nucleotide 3′-phosphodiesterase	Cnp	P13233	↑	Cytoplasm	RNA processing, cytoskeletal organization, neurite outgrowth
Guanine deaminase	Gda	Q9WTT6	↓	Cytoplasm	Nucleotide metabolism, regulation of the cytoskeleton, dendritic arborization
Synaptic transmission					
V-type proton ATPase subunit E 1	Atp6v1e1	Q6PCU2	↑	Cytoplasm, membrane of intracellular compartments, mitochondrion	Neurotransmitter uptake into synaptic vesicles, pH regulation in intracellular compartments
Dihydropyrimidinase-related protein 2	Dpysl2	P47942	↑↑↑	Cytoplasm	Neurite outgrowth, synaptic vesicle exocytosis, receptor recycling
Septin-11	Sept11	B3GNI6	↓	Cytoplasm	Regulation of the dendritic arborization and neurite outgrowth, synaptic vesicle trafficking
Neuronal-specific septin-3	Sept3	Q9WU34	↓	Cytoplasm	Synaptic vesicle recycling
Alpha-synuclein	Snca	P37377	↓	Cytoplasm	Modulation of synaptic vesicle exocytosis, receptor recycling, microtubular organization
Protein synthesis and folding					
Endoplasmic reticulum resident protein 29	Erp29	P52555	↑	Endoplasmic reticulum	Processing of secreted and endomembrane proteins, response to stress
78 kDa glucose-regulated protein	Hspa5	P06761	↑	Endoplasmic reticulum	Protein folding
Heat shock cognate 71 kDa protein	Hspa8	P63018	↑↑	Cytoplasm	Protein folding, macromolecular assembly
Stress-70 protein, mitochondrial	Hspa9	P48721	↑	Mitochondrion	Protein folding, macromolecular assembly
60 kDa heat shock protein, mitochondrial	Hspd1	P63039	↑↑↑	Mitochondrion	Folding of mitochondrial proteins
Protein DJ-1	Park7	O88767	↑	Cytoplasm, mitochondrion	Chaperone function, response to oxidative stress, dopaminergic synaptic transmission
Poly(rC)-binding protein 1	Pcbp1	P60335	↑	Cytoplasm, nucleus	RNA processing, regulation of protein synthesis
Protein disulfide-isomerase A3	Pdia3	P11598	↓	Endoplasmic reticulum, nucleus	Protein folding, macromolecular assembly
40S ribosomal protein S16	Rps16	P62250	↓	Cytoplasm	Protein synthesis
Transcription elongation factor B polypeptide 2	Tceb2	P62870	↓	Nucleus	Protein synthesis
Response to oxidative stress					
Peroxiredoxin-6	Prdx6	O35244	↑	Cytoplasm	Response to oxidative stress
Cytoskeletal protein					
Actin, cytoplasmic 1	Actb	P60711	↓	Cytoplasm	Microfilamental component
ADP-ribosylation factor 3	Arf3	P61206	↓	Golgi apparatus, cytoplasm	Vesicle trafficking, exocytosis, organization of the actin cytoskeleton
F-actin-capping protein subunit beta	Capzb	Q5XI32	↓	Cytoplasm	Microfilament organization, synaptic remodelling

Table 1 (*continued*)

Protein name	Gene name	Accession number	Up/down regulation[a]	Cellular localization	Cellular function
Myelin basic protein S	Mbp	P02688	↓↓	Cytoplasm	Regulation of microfilaments and microtubules
Myosin light polypeptide 6	Myl6	Q64119	↓	Cytoplasm	Motor protein function
Profilin-2	Pfn2	Q9EPC6	↑	Cytoplasm	Regulation of microfilaments, synaptogenesis, neurite outgrowth, synaptic vesicle exocytosis, receptor trafficking
Myosin regulatory light chain RLC-A	Rlc-a	P13832	↓	Cytoplasm	Motor protein regulation
Tubulin beta-5 chain	Tubb5	P69897	↑↓	Cytoplasm	Microtubular component
Signal transduction					
Calcineurin subunit B type 1	Ppp3r1	P63100	↓	Cytoplasm	Regulation of several signaling pathways
Miscellaneous					
Annexin A3	Anxa3	P14669	↓	Cytoplasm	Regulation of intracellular calcium levels, signal transduction
Histone H2B type 1	Hist1h2bh	Q00715	↓	Nucleus	Component of the nucleosome
Voltage-dependent anion-selective channel protein 1	Vdac1	Q9Z2L0	↑↑↑↓	Mitochondrion, plasma membrane	Modulation of intracellular calcium levels
Voltage-dependent anion-selective channel protein 2	Vdac2	P81155	↑↑↑↓	Mitochondrion	Modulation of intracellular calcium levels
WD repeat-containing protein 61	Wdr61	Q4V7A0	↓	Cytoplasm, nucleus	Histone methylation

[a] Directions of alterations in the levels of the corresponding protein spots (due to expression changes or post-translational modifications).

2010; Ramanathan et al., 2010; Ramanathan and Siegel, 2011). This result draws attention on a high level of impairment in the oxidative homeostasis which is even more pronounced later after sleep deprivation termination. It is also important to note that besides the defending role of these enzymes, some of them might be involved in synaptic plasticity, due to the fact that reactive oxygen species can serve as messenger

Table 2
List of significantly altered SD synaptic proteins from the thalamus, assigned to functional groups.

Protein name	Gene name	Accession number	Up/down regulation[a]	Cellular localization	Cellular function
Carbohydrate and energy metabolism					
ATP synthase subunit alpha, mitochondrial	Atp5a1	P15999	↓	Mitochondrion	Energy metabolism
Malate dehydrogenase, cytoplasmic	Mdh1	O88989	↓	Cytoplasm	Carbohydrate metabolism, involved in malate-aspartate shuttle
Amino acid metabolism					
Glutamine synthetase	Glul	P09606	↓	Cytoplasm, mitochondrion	Amino acid metabolism
Nucleotide metabolism					
3′(2′).5′-bisphosphate nucleotidase 1	Bpnt1	Q9Z1N4	↑	Cytoplasm	Nucleotide metabolism, sulphate metabolism
Synaptic transmission					
Dihydropyrimidinase-related protein 2	Dpysl2	P47942	↓	Cytoplasm	Neurite outgrowth, synaptic vesicle exocytosis, receptor recycling
Septin-6	Sept6	Q9R1T4	↑	Cytoplasm	Synaptic vesicle recycling, neurite outgrowth
Synapsin-2	Syn2	Q63537	↓	Cytoplasm	Synaptic vesicle docking
Protein synthesis and folding					
Endoplasmic reticulum resident protein 29	Erp29	P52555	↓	Endoplasmic reticulum	Processing of secreted and endomembrane proteins, response to stress
78 kDa glucose-regulated protein	Hspa5	P06761	↑	Endoplasmic reticulum	Protein folding
Mesencephalic astrocyte-derived neurotrophic factor	Manf	P0C5H9	↓	Endoplasmic reticulum, cytoplasm, extracellular space	Protein folding, stress response
Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1	Pin1	Q13526	↓	Cytoplasm, nucleus	Regulation of protein synthesis and cytoskeletal structure
Peptidyl-prolyl cis-trans isomerase A	Ppia	P10111	↓	Cytoplasm	Protein folding, regulation of the cytoskeleton, molecular trafficking
60S acidic ribosomal protein P0	Rplp0	P19945	↓	Cytoplasm, nucleus	Regulation of protein synthesis
Sorting and assembly machinery component 50 homolog	Samm50	Q6AXV4	↓	Mitochondrion	Assembly of mitochondrial proteins
Proteolysis					
Cathepsin D	Ctsd	P24268	↑	Cytoplasm	Lysosomal protein degradation, synaptic transmission
Ubiquitin-conjugating enzyme E2 L3	Ube2l3	P68036	↓	Cytoplasm, nucleus	Ubiquitination
Cytoskeletal protein					
Actin, cytoplasmic 1	Actb	P60711	↑	Cytoplasm	Microfilamental component
F-actin-capping protein subunit alpha-2	Capza2	Q3T1K5	↓	Cytoplasm	Microfilament organization
Destrin	Dstn	Q7M0E3	↓	Cytoplasm	Microfilament organization, receptor trafficking, dendrite formation

[a] Directions of alterations in the levels of the corresponding protein spots (due to expression changes or post-translational modifications).

Table 3
List of significantly altered RS synaptic proteins from the parietal cortex, assigned to functional groups.

Protein name	Gene name	Accession number	Up/down regulation[a]	Cellular localization	Cellular function
Carbohydrate and energy metabolism					
Aconitate hydratase, mitochondrial	Aco2	Q9ER34	↑↑↑↓	Mitochondrion	Involved in tricarboxylic acid cycle
Fructose-bisphosphate aldolase A	Aldoa	P05065	↑↑	Cytoplasm	Glycolysis
Fructose-bisphosphate aldolase C	Aldoc	P09117	↓↓↓	Cytoplasm	Glycolysis
ATP synthase subunit alpha, mitochondrial	Atp5a1	P15999	↓	Mitochondrion	Energy metabolism
ATP synthase subunit beta, mitochondrial	Atp5b	P10719	↓↓↑	Mitochondrion	Energy metabolism
ATP synthase subunit d, mitochondrial	Atp5h	P31399	↑	Mitochondrion	Energy metabolism
Creatine kinase B-type	Ckb	P07335	↑	Cytoplasm	Energy metabolism
Creatine kinase U-type, mitochondrial	Ckmt1	P30275	↑↑	Mitochondrion	Energy metabolism
Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial	Dlat	P08461	↓	Mitochondrion	Carbohydrate metabolism
Dihydrolipoyl dehydrogenase, mitochondrial	Dld	Q6P6R2	↑↓	Mitochondrion	Involved in tricarboxylic acid cycle
Alpha-enolase	Eno1	P04764	↓↓↑	Cytoplasm	Glycolysis
Gamma-enolase	Eno2	P07323	↑↑↓	Cytoplasm	Glycolysis
Fumarylacetoacetate hydrolase domain-containing protein 2	Fahd2	B2RYW9	↓↓↑	Mitochondrion	Carbohydrate and amino acid metabolism
Fumarate hydratase, mitochondrial	Fh	P14408	↑↑↓	Mitochondrion	Carbohydrate metabolism, involved in tricarboxylic acid cycle
Glyceraldehyde-3-phosphate dehydrogenase	Gapdh	P04797	↓↓↓↑	Cytoplasm	Glycolysis
Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial	Idh3a	Q99NA5	↓↓	Mitochondrion	Involved in tricarboxylic acid cycle
Isocitrate dehydrogenase [NAD] subunit beta, mitochondrial	Idh3b	Q68FX0	↑	Mitochondrion	Involved in tricarboxylic acid cycle
Malate dehydrogenase, cytoplasmic	Mdh1	O88989	↑↑↓	Cytoplasm	Carbohydrate metabolism, involved in malate-aspartate shuttle
Malate dehydrogenase, mitochondrial	Mdh2	P04636	↓	Mitochondrion	Carbohydrate metabolism, involved in tricarboxylic acid cycle and malate-aspartate shuttle
Succinyl-CoA:3-ketoacid-coenzyme A transferase 1, mitochondrial	Oxct1	B2GV06	↓	Mitochondrion	Metabolism of ketone bodies
Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial	Pdha1	P35486	↑↑↑↑↓↓	Mitochondrion	Involved in carbohydrate and fatty acid metabolism
Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	Pdhb	P49432	↑↓	Mitochondrion	Involved in carbohydrate and fatty acid metabolism
Phosphoglycerate mutase 1	Pgam1	P25113	↑	Cytoplasm	Carbohydrate metabolism, glycolysis
Phosphoglycerate kinase 1	Pgk1	P16617	↑↑	Cytoplasm	Glycolysis
Pyruvate kinase isozymes M1/M2	Pkm2	P11980	↓↓	Cytoplasm	Glycolysis
Triosephosphate isomerase	Tpi1	P48500	↑↑↓↓	Cytoplasm	Glycolysis
Amino acid metabolism					
Glutamine synthetase	Glul	P09606	↑↑↓↓	Cytoplasm, mitochondrion	Amino acid metabolism
Aspartate aminotransferase, cytoplasmic	Got1	P13221	↑	Cytoplasm	Amino acid metabolism
Lipid metabolism					
Enoyl-CoA hydratase, mitochondrial	Echs1	P14604	↑	Mitochondrion	Fatty acid metabolism
Nucleotide metabolism					
Adenylate kinase isoenzyme 1	Ak1	P39069	↑	Cytoplasm	Nucleotide metabolism, energy metabolism
Synaptic transmission					
V-type proton ATPase subunit B, brain isoform	Atp6v1b2	P62815	↓↓	Cytoplasm, membrane of intracellular compartments, mitochondrion	Neurotransmitter uptake into synaptic vesicles, pH regulation in intracellular compartments
Dihydropyrimidinase-related protein 2	Dpysl2	P47942	↑↑↓	Cytoplasm	Neurite outgrowth, synaptic vesicle exocytosis, receptor recycling
Gamma-soluble NSF attachment protein	Napg	Q9CWZ7	↑↑	Cytoplasm	Vesicular transport, synaptic vesicle exocytosis
Adaptin ear-binding coat-associated protein 1	Necap1	P69682	↓	Cytoplasm	Synaptic vesicle endocytosis
Endophilin-A1	Sh3gl2	O35179	↑	Cytoplasm	Synaptic vesicle recycling
Synaptosomal-associated protein 25	Snap25	P60881	↑↑	Cytoplasm	Synaptic vesicle exocytosis
Synapsin-1	Syn1	P09951	↑↓	Cytoplasm	Synaptic vesicle docking
Synapsin-2	Syn2	Q63537	↓↓↓	Cytoplasm	Synaptic vesicle docking
Thy-1 membrane glycoprotein	Thy1	P01830	↑	Cytoplasm	Synaptic vesicle exocytosis, modulation of the cytoskeleton
Protein synthesis and folding					
Elongation factor 1-beta	Eef1b	O70251	↑	Cytoplasm	Protein synthesis
Eukaryotic translation initiation factor 4H	Eif4h	Q5XI72	↓	Cytoplasm	Protein synthesis
78 kDa glucose-regulated protein	Hspa5	P06761	↑	Endoplasmic reticulum	Protein folding
Heat shock cognate 71 kDa protein	Hspa8	P63018	↑↑↑	Cytoplasm	Protein folding, macromolecular assembly
Stress-70 protein, mitochondrial	Hspa9	P48721	↓	Mitochondrion	Protein folding, macromolecular assembly
60 kDa heat shock protein, mitochondrial	Hspd1	P63039	↑↑↓	Mitochondrion	Folding of mitochondrial proteins
Protein disulfide-isomerase A3	Pdia3	P11598	↑↓	Endoplasmic reticulum, nucleus	Protein folding, macromolecular assembly
Prefoldin subunit 2	Pfdn2	B0BN18	↑	Cytoplasm, mitochondrion,	Posttranslational processing of actin and tubulin

Table 3 (*continued*)

Protein name	Gene name	Accession number	Up/down regulation[a]	Cellular localization	Cellular function
				nucleus	monomers
Peptidyl-prolyl cis-trans isomerase A	Ppia	P10111	↑	Cytoplasm	Regulation of the cytoskeleton, molecular trafficking, protein folding
Peptidyl-prolyl cis-trans isomerase D	Ppid	Q6DGG0	↓	Cytoplasm	Protein folding, regulation of chaperone proteins
Stress-induced-phosphoprotein 1	Stip1	O35814	↓	Cytoplasm	Chaperone function
Proteolysis					
Proteasome subunit beta type-4	Psmb4	P34067	↑	Cytoplasm	Proteolysis
Ubiquitin carboxyl-terminal hydrolase isozyme L1	Uchl1	Q00981	↑↓	Cytoplasm	Proteolysis, regulation of cell morphology (e.g. dendritic arborization), signal transduction
Response to oxidative stress					
Glutathione S-transferase Mu 1	Gstm1	P04905	↓	Cytoplasm	Response to oxidative stress
Glutathione S-transferase P	Gstp1	P04906	↑	Cytoplasm	Response to oxidative stress
Peroxiredoxin-1	Prdx1	Q63716	↑	Cytoplasm	Response to oxidative stress
Peroxiredoxin-2	Prdx2	P35704	↑↓	Cytoplasm	Response to oxidative stress
Thioredoxin-dependent peroxide reductase, mitochondrial	Prdx3	P20108	↑	Cytoplasm	Response to oxidative stress
Peroxiredoxin-5, mitochondrial	Prdx5	Q9R063	↑↑	Cytoplasm	Response to oxidative stress
Peroxiredoxin-6	Prdx6	O35244	↓	Cytoplasm	Response to oxidative stress
Superoxide dismutase [Mn], mitochondrial	Sod2	P07895	↑	Mitochondrion	Response to oxidative stress
Thioredoxin	Txn	P11232	↑	Cytoplasm	Response to oxidative stress
Cytoskeletal protein					
Actin, cytoplasmic 1	Actb	P60711	↑↑↑↓	Cytoplasm	Microfilamental component
Beta-centractin	Actr1b	Q8R5C5	↓	Cytoplasm	Vesicle trafficking
Actin-related protein 2/3 complex subunit 5	Arpc5	Q4KLF8	↓	Cytoplasm	Regulation of actin cytoskeleton
F-actin-capping protein subunit alpha-2	Capza2	Q3T1K5	↓	Cytoplasm	Microfilament organization
F-actin-capping protein subunit beta	Capzb	Q5XI32	↓	Cytoplasm	Microfilament organization, synaptic remodelling
Cofilin-1	Cfl1	P45592	↑↑↓	Cytoplasm	Regulation of microfilaments, synaptogenesis, neurotransmitter receptor localization
Cofilin-2	Cfl2	P45591	↑	Cytoplasm	Regulation of microfilaments, synaptogenesis, neurotransmitter receptor localization
Protein canopy homolog 2	Cnpy2	Q9QXT0	↓	Endoplasmic reticulum, cytoplasm	Neurite outgrowth
Dihydropyrimidinase-related protein 5	Dpysl5	Q9JHU0	↓	Cytoplasm	Neurite outgrowth, regulation of dendritic arborization
Glial fibrillary acidic protein	Gfap	P47819	↓	Cytoplasm	Intermediate filament organization
Alpha-internexin	Ina	P23565	↑↑↑	Cytoplasm	Microfilament organization, neurite outgrowth, regulation of dendrite morphology
Myelin basic protein S	Mbp	P02688	↑↑↓↓	Cytoplasm	Regulation of microfilaments and microtubules
Stathmin	Stmn1	P13668	↑	Cytoplasm	Regulation of microtubules, synaptogenezis
Tubulin alpha-1C chain	Tuba1c	Q6AYZ1	↓	Cytoplasm	Microtubular component
Signal transduction					
Guanine nucleotide-binding protein G(o) subunit alpha	Gnao1	P59215	↓	Cytoplasm	Signal transduction, neurite outgrowth
Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	Gnb1	P54311	↑↑	Cytoplasm	Signal transduction
Growth factor receptor-bound protein 2	Grb2	P62994	↑	Cytoplasm	Signal transduction, synaptic vesicle recycling
Phosphatidylethanolamine-binding protein 1	Pebp1	P31044	↑↓	Cytoplasm	Signal transduction
14-3-3 protein epsilon	Ywhae	P62260	↑	Cytoplasm	Signal transduction
14-3-3 protein zeta/delta	Ywhaz	P63102	↓	Cytoplasm	Signal transduction
Miscellaneous					
Annexin A4	Anxa4	P55260	↓	Cytoplasm, nucleus	Signal transduction, synaptic vesicle exocytosis
Alcohol dehydrogenase [NADP+]	Akr1a1	P51635	↓↓	Cytoplasm	Alcohol and aldehyde metabolism
Flavin reductase	Blvrb	Q923D2	↑	Cytoplasm	Flavin and biliverdin metabolism
N(G),N(G)-dimethylarginine dimethylaminohydrolase 2	Ddah2	Q6MG60	↓	Cytoplasm	Regulation of nitric oxide synthesis
3-Hydroxyacyl-CoA dehydrogenase type-2	Hsd17b10	O70351	↓	Mitochondrion	Steroid metabolism
Latexin	Lxn	Q64361	↓	Cytoplasm	Unknown
Protein NDRG2	Ndrg2	Q8VBU2	↑	Cytoplasm	Synaptogenesis, regulation of neurite outgrowth, signal transduction
Nucleoside diphosphate kinase B	Nme2	P19804	↓	Cytoplasm	Neurite outgrowth, cell differentiation, signal transduction, synaptic vesicle endocytosis
Pyridoxal kinase	Pdxk	O35331	↓	Cytoplasm	Pyridoxal metabolism
Ran-specific GTPase-activating protein	Ranbp1	P34022	↓	Cytoplasm, nucleus	Nuclear transport
Translationally-controlled tumor protein	Tpt1	P63029	↑	Cytoplasm	Regulation of microtubular and microfilamental organization and calcium homeostasis
Transitional endoplasmic reticulum ATPase	Vcp	P46462	↑	Endoplasmic reticulum, cytoplasm	Vesicle trafficking and fusion, regulation of proteolysis
UPF0568 protein C14orf166 homolog	–	Q9CQE8	↑	Unknown	Unknown
ES1 protein homolog, mitochondrial	–	P56571	↓↓	Mitochondrion	Unknown

[a] Directions of alterations in the levels of the corresponding protein spots (due to expression changes or post-translational modifications).

Table 4
List of significantly altered RS synaptic proteins from the thalamus, assigned to functional groups.

Protein name	Gene name	Accession number	Up/down regulation[a]	Cellular localization	Cellular function
Carbohydrate and energy metabolism					
Aconitate hydratase, mitochondrial	Aco2	Q9ER34	↓↓↓↓↓	Mitochondrion	Tricarboxylic acid cycle
Fructose-bisphosphate aldolase A	Aldoa	P05065	↑	Cytoplasm	Glycolysis
Alpha-enolase	Eno1	P04764	↓↓	Cytoplasm	Glycolysis
Fumarylacetoacetate hydrolase domain-containing protein 2	Fahd2	B2RYW9	↓	Mitochondrion	Carbohydrate and amino acid metabolism
Glyceraldehyde-3-phosphate dehydrogenase	Gapdh	P04797	↓	Cytoplasm	Glycolysis
Succinyl-CoA:3-ketoacid-coenzyme A transferase 1, mitochondrial	Oxct1	B2GV06	↓	Mitochondrion	Metabolism of ketone bodies
Phosphoglycerate mutase 1	Pgam1	P25113	↓	Cytoplasm	Carbohydrate metabolism, glycolysis
Amino acid metabolism					
Cytosol aminopeptidase	Lap3	Q68FS4	↓	Cytoplasm	Protein and peptide metabolism
Lipid metabolism					
Enoyl-CoA hydratase, mitochondrial	Echs1	P14604	↓	Mitochondrion	Fatty acid metabolism
Synaptic transmission					
V-type proton ATPase catalytic subunit A	Atp6v1a	P50516	↓	Cytoplasm, membrane of intracellular compartments, mitochondrion	Neurotransmitter uptake into synaptic vesicles, pH regulation in intracellular compartments
Dihydropyrimidinase-related protein 2	Dpysl2	P47942	↑↓	Cytoplasm	Neurite outgrowth, synaptic vesicle exocytosis, receptor recycling
Gamma-soluble NSF attachment protein	Napg	Q9CWZ7	↓	Cytoplasm	Vesicular transport, synaptic vesicle exocytosis
Septin-5	Sept5	Q9JJM9	↑↓	Cytoplasm	Synaptic vesicle exocytosis, neurite outgrowth
Protein synthesis and folding					
T-complex protein 1 subunit beta	Cct2	Q5XIM9	↑	Cytoplasm	Protein (e.g. actin and tubulin) folding
Heat shock cognate 71 kDa protein	Hspa8	P63018	↓↓	Cytoplasm	Protein folding, macromolecular assembly
Heat shock protein beta-8	Hspb8	Q9EPX0	↑	Cytoplasm	Chaperone function
Stress-induced-phosphoprotein 1	Stip1	O35814	↑	Cytoplasm	Chaperone function
Proteolysis					
COP9 signalosome complex subunit 4	Cops4	Q68FS2	↑	Cytoplasm, nucleus	Regulation of protein degradation, modulation of the dendritic arborization
Cathepsin D	Ctsd	P24268	↑	Cytoplasm	Lysosomal protein degradation, synaptic transmission
Response to oxidative stress					
Peroxiredoxin-2	Prdx2	P35704	↓	Cytoplasm	Response to oxidative stress
Peroxiredoxin-6	Prdx6	O35244	↑↓	Cytoplasm	Response to oxidative stress
Cytoskeletal protein					
Dihydropyrimidinase-related protein 3	Dpysl3	Q62952	↑	Cytoplasm	Regulation of the actin cytoskeleton and neurite outgrowth
Glial fibrillary acidic protein	Gfap	P47819	↓	Cytoplasm	Intermediate filament organization
Signal transduction					
Dual specificity protein phosphatase 3	Dusp3	Q9D7X3	↓	Nucleus	Signal transduction, ERK dephosphorylation
Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	Gnb1	P54311	↓	Cytoplasm	Signal transduction
Phosphatidylethanolamine-binding protein 1	Pebp1	P31044	↓	Cytoplasm	Signal transduction
Miscellaneous					
STIP1 homology and U box-containing protein 1	Stub1	Q9WUD1	↓	Cytoplasm	Modulation of chaperone proteins, ubiquitination
Voltage-dependent anion-selective channel protein 2	Vdac2	P81155	↑	Mitochondrion	Modulation of intracellular calcium levels

[a] Directions of alterations in the levels of the corresponding protein spots (due to expression changes or post-translational modifications).

molecules in processes leading to altered synaptic transmission – as described in the case of nitric oxide or superoxide (Böhme et al., 1993; Knapp and Klann, 2002).

There is a hypothesis of sleep function presuming that macromolecule synthesis is generally enhanced in sleep to restore the decreased levels of macromolecules in waking (Mackiewicz et al., 2007; Ramm and Smith, 1990). The majority of protein synthesis- and folding related-proteins showed increased levels in the parietal cortex of SD group ($n = 7$) (Table 1), but in the thalamus, most of the proteins categorized in this group showed opposite direction changes in SD ($n = 6$) (Table 2). The long-term effects of sleep deprivation (RS group) caused bidirectional changes in protein synthesis-related proteins. We have also identified a set of proteins possessing chaperone function necessary for the proper folding of the newly synthesized proteins, and notably, for the proper construction of macromolecular assemblies. These proteins might be associated with the restorative function of sleep (Tables 1–4).

4.4. The role of SD and RS protein changes related to synaptic plasticity

Since one of our main aims was to ask whether the uncovered proteomic changes support the synaptic homeostasis hypothesis (Tononi and Cirelli, 2014), we are discussing that issue. The hypothesis is based on the assumption that the net synaptic strength increases in waking and, in turn, in sleep deprivation, while decreases in sleep. In other words, it claims that waking saturates synaptic plasticity, which

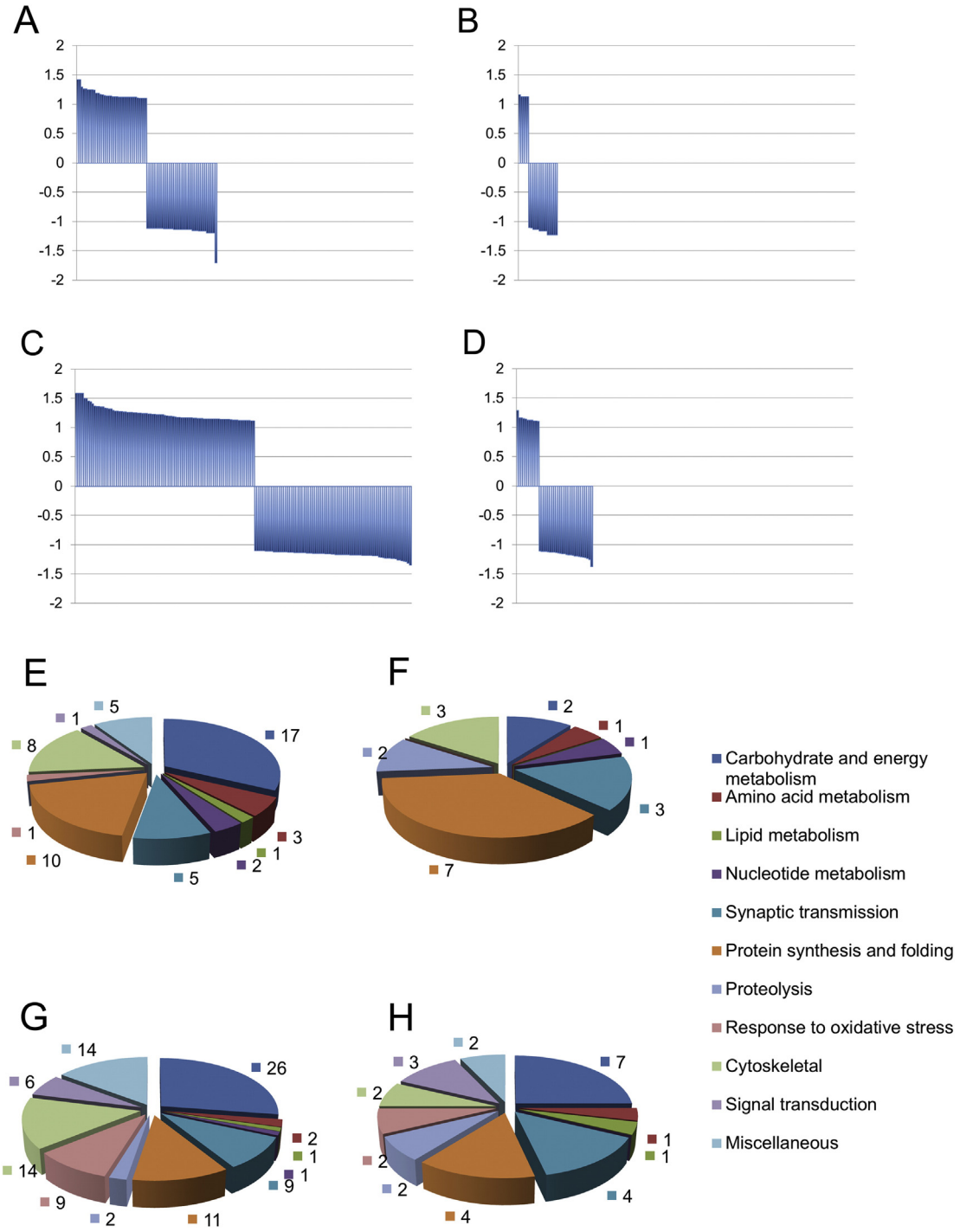

Fig. 6. Protein fold changes and functional classification of the altered proteins. Protein fold changes in the SD cortical experiment (A), SD thalamic experiment (B), RS cortical experiment (C), and RS thalamic experiment (D). Functional classification of SD cortical proteins (E), SD thalamic proteins (F), RS cortical proteins (G) and RS thalamic proteins (H).

is desaturated in sleep. The saturation of synaptic plasticity may weakens learning ability, thus synapses became rigid, which can explain the sleep deprivation-induced impairments in memory consolidation (Stickgold, 2005). The theoretical speculation on synaptic reorganization in sleep and waking predicts that synapses are extensively rebuilt during the sleep-wake cycle depending mostly on the duration of waking and the experiences received during it.

Our proteomics study uncovered that a large group of altered proteins are linked directly to synaptic strength regulation and plasticity via their influence on e.g., synaptic vesicle recycling and synaptogenesis (Fig. 8). All of these proteins participate in various aspects of plasticity in the synaptic machinery. The highest number of synaptic plasticity-related proteins were affected by the long-term influence of sleep deprivation (RS group) in the parietal cortex (Fig. 8). This data points out also that synaptic reorganization is not only apparent 16 h after an 8 h long sleep deprivation, but even more widespread at this time than immediately after the deprivation procedure. Therefore, extensive molecular alterations, predicted by the synaptic homeostasis hypothesis, can be verified.

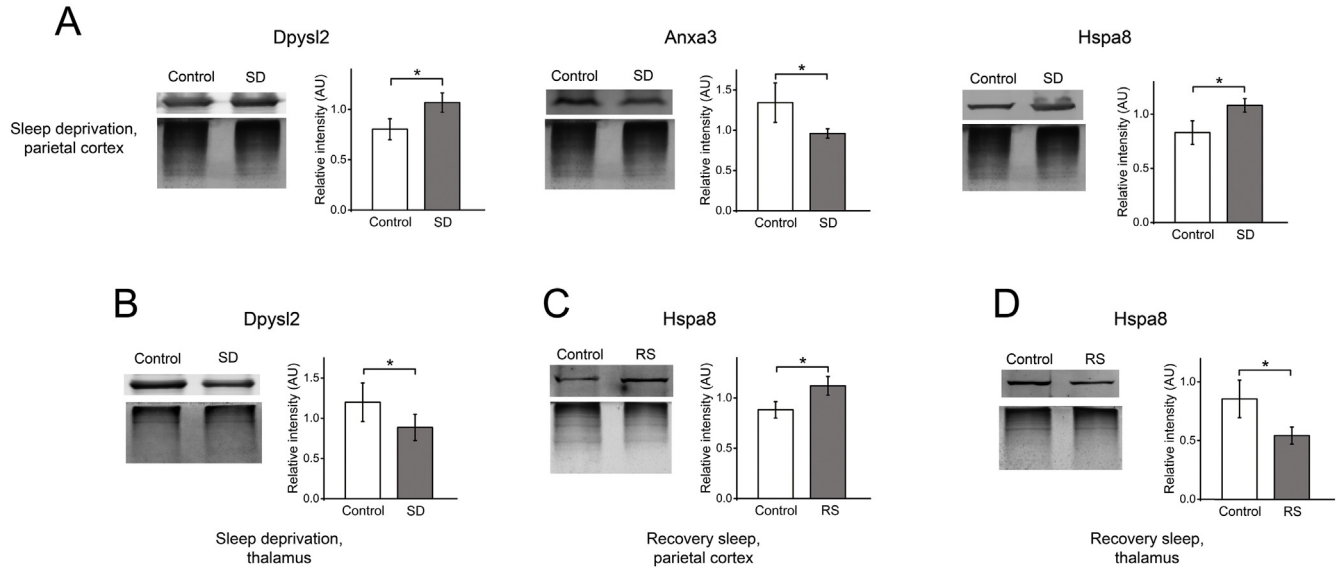

Fig. 7. Validation of the proteomics results using Western blot. Representative Western blot images with the corresponding total protein labeling gel images are shown, in addition to bar graphs demonstrating the results of the densitometric analyses. Verification of the alterations in the levels of Dpysl2 (1.33 ± 0.21-fold increase in SD group), Anxa3 (−1.40 ± 0.27-fold decrease in SD group), and Hspa8 (1.30 ± 0.18-fold increase in SD group) proteins in the "Sleep deprivation (SD), parietal cortex" experiment (A), of Dpysl2 (−1.35 ± 0.37-fold decrease in SD) in the "Sleep deprivation, thalamus" experiment (B), of Hspa8 (1.27 ± 0.16-fold increase in RS group) in the "Recovery sleep (RS), parietal cortex" experiment (C), and of Hspa8 (−1.57 ± 0.36-fold decrease in RS group) in the "Recovery sleep, thalamus" experiment. Means ± S.E.M. are presented; $n = 6$ per experimental group; $^{*}p < 0.05$.

5. Conclusions

Subcellular enrichment technique for the investigation of synaptic protein level alterations induced by the short- and long-term effects of sleep deprivation revealed that the synaptic molecular machinery is extensively impaired. Our results suggest that sleep has a very complex role at the synaptic level, such as the restorative, homeostatic, and plasticity-enhancing functions that is reflected by the detectable changes in synaptic proteome. Therefore, sophisticated alterations of the synaptic protein machinery during sleep are possible which could improve its printability (plasticity) and enhance its stability at the same time. We revealed that sleep deprivation more extensively influences the parietal cortex than the thalamus, and its long-term effects on the molecular level are remarkable – might be due to the recovery sleep period. The most important conclusion is that the revealed widespread synaptic proteome changes support the synaptic homeostasis hypothesis of sleep.

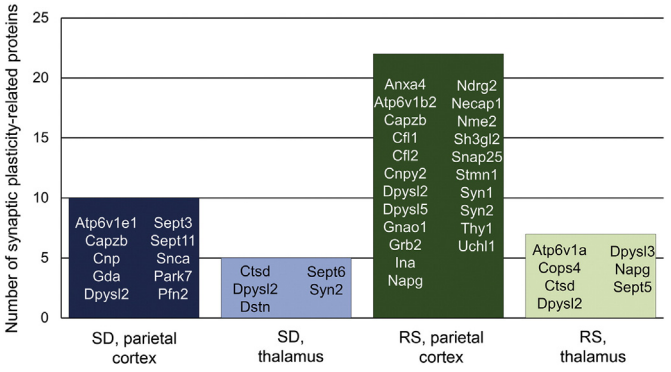

Fig. 8. The list of altered synaptic plasticity-related proteins. We revealed 10, 5, 22 and 7 altered proteins in the SD parietal cortex, SD thalamus, RS parietal cortex and RS thalamus, respectively, which are implicated in the regulation of the plasticity of synaptic transmission.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mcn.2017.01.002.

Conflict of interest statement

All authors declare that there are no conflicts of interest.

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References

Abraham, W.C., Otani, S., 1991. Macromolecules and the maintenance of long-term potentiation. In: Morrell, F. (Ed.), Kindling and Synaptic Plasticity. The legacy of Graham Goddard, Birkhausen, pp. 92–109.
Abramoff, M.D., Magalhaes, P.J., Ram, S.J., 2004. Image processing with ImageJ. Biophoton. Int. 11, 36–42.
Bajor, M., Michaluk, P., Gulyássy, P., Kékesi, A.K., Juhász, G., Kaczmarek, L., 2012. Synaptic cell adhesion molecule-2 and collapsin response mediator protein-2 are novel members of the matrix metalloproteinase-9 degradome. J. Neurochem. 122, 775–788.
Baker, E.K., Richdale, A.L., 2015. Sleep patterns in adults with a diagnosis of high-functioning autism spectrum disorder. Sleep 38, 1765–1774.
Basheer, R., Brown, R., Ramesh, V., Begum, S., McCarley, R.W., 2005. Sleep deprivation-induced protein changes in basal forebrain: implications for synaptic plasticity. J. Neurosci. Res. 82, 650–658.
Benington, J.H., Frank, M.G., 2003. Cellular and molecular connections between sleep and synaptic plasticity. Prog. Neurobiol. 69, 71–101.
Benington, J.H., Heller, H.C., 1995. Restoration of brain energy metabolism as the function of sleep. Prog. Neurobiol. 45, 347–360.
Blanco, W., Pereira, C.M., Cota, V.R., Souza, A.C., Rennó-Costa, C., Santos, S., Dias, G., Guerreiro, A.M., Tort, A.B., Neto, A.D., Ribeiro, S., 2015. Synaptic homeostasis and restructuring across the sleep-wake cycle. PLoS Comput. Biol. 11, e1004241.
Blissitt, P.A., 2001. Sleep, memory, and learning. J. Neurosurg. Nurs. 33, 208–215.
Bloch, V., Hennevin, E., Leconte, P., 1977. Interaction between post-trial reticular stimulation and subsequent paradoxical sleep in memory consolidation processes. In:

Drucker-Colin, M.J. (Ed.), Neurobiology of Sleep and Memory. Academic Press, New York, pp. 255–272.
Bloch, V., Hennevin, E., Leconte, P., 1979. Relationship between paradoxical sleep and memory processes. In: Razier, M. (Ed.), Brain Mechanisms in Memory and Learning: From the Single Neuron to Man. Raven Press, New York, pp. 329–343.
Böhme, G.A., Bon, C., Lemaire, M., Reibaud, M., Piot, O., Stutzmann, J.M., Doble, A., Blanchard, J.C., 1993. Altered synaptic plasticity and memory formation in nitric oxide synthase inhibitor-treated rats. Proc. Natl. Acad. Sci. U. S. A. 90, 9191–9194.
Braun, A.R., Balkin, T.J., Wesenten, N.J., Carson, R.E., Varga, M., Baldwin, P., Selbie, S., Belenky, G., Herscovitch, P., 1997. Regional cerebral blood flow throughout the sleep-wake cycle. An H2(15)O PET study. Brain 120, 1173–1197.
Brown, M.K., Naidoo, N., 2010. The UPR and the anti-oxidant response: relevance to sleep and sleep loss. Mol. Neurobiol. 42, 103–113.
Bushey, D., Tononi, G., Cirelli, C., 2011. Sleep and synaptic homeostasis: structural evidence in *Drosophila*. Science 332, 1576–1581.
Chee, M.W., Choo, W.C., 2004. Functional imaging of working memory after 24 hr of total sleep deprivation. J. Neurosci. 24, 4560–4567.
Chee, M.W., Chuah, L.Y., Venkatraman, V., Chan, W.Y., Philip, P., Dinges, D.F., 2006. Functional imaging of working memory following normal sleep and after 24 and 35 h of sleep deprivation: correlations of fronto-parietal activation with performance. NeuroImage 31, 419–428.
Chevalier, F., 2010. Highlights on the capacities of "gel-based" proteomics. Proteome Sci. 8, 23.
Chow, H.M., Horovitz, S.G., Carr, W.S., Picchioni, D., Coddington, N., Fukunaga, M., Xu, Y., Balkin, T.J., Duyn, J.H., Braun, A.R., 2013. Rhythmic alternating patterns of brain activity distinguish rapid eye movement sleep from other states of consciousness. Proc. Natl. Acad. Sci. U. S. A. 110, 10300–10305.
Cirelli, C., Tononi, G., 1998. Differences in gene expression between sleep and waking as revealed by mRNA differential display. Brain Res. Mol. Brain Res. 56, 293–305.
Cirelli, C., Faraguna, U., Tononi, G., 2006. Changes in brain gene expression after long-term sleep deprivation. J. Neurochem. 98, 1632–1645.
Costa-Miserachs, D., Portell-Cortés, I., Torras-Garcia, M., Morgado-Bernal, I., 2003. Automated sleep staging in rat with a standard spreadsheet. J. Neurosci. Methods 130, 93–101.
Dang-Vu, T.T., Desseilles, M., Peigneux, P., Maquet, P., 2006. A role for sleep in brain plasticity. Pediatr. Rehabil. 9, 98–118.
Donnet, A., Farnarier, G., Gambarelli, D., Aguglia, U., Regis, H., 1992. Sleep electroencephalogram at the early stage of Creutzfeldt-Jakob disease. Clin. Electroencephalogr. 23, 118–125.
Dworak, M., McCarley, R.W., Kim, T., Kalinchuk, A.V., Basheer, R., 2010. Sleep and brain energy levels: ATP changes during sleep. J. Neurosci. 30, 9007–9016.
Eaton, S.L., Roche, S.L., Llavero Hurtado, M., Oldknow, K.J., Farquharson, C., Gillingwater, T.H., Wishart, T.M., 2013. Total protein analysis as a reliable loading control for quantitative fluorescent Western blotting. PLoS One 8, e72457.
Fenzl, T., Romanowski, C.P., Flachskamm, C., Honsberg, K., Boll, E., Hoehne, A., Kimura, M., 2007. Fully automated sleep deprivation in mice as a tool in sleep research. J. Neurosci. Methods 166, 229–235.
Giedke, H., Schwärzler, F., 2002. Therapeutic use of sleep deprivation in depression. Sleep Med. Rev. 6, 361–377.
Goel, N., Rao, H., Durmer, J.S., Dinges, D.F., 2005. Neurocognitive consequences of sleep deprivation. Semin. Neurol. 25, 117–129.
Huang, E.P., 1998. Synaptic plasticity: going through phases with LTP. Curr. Biol. 8, R350–R352.
Ikeda, M., Ikeda-Sagara, M., Okada, T., Clement, P., Urade, Y., Nagai, T., Sugiyama, T., Yoshioka, T., Honda, K., Inoué, S., 2005. Brain oxidation is an initial process in sleep induction. Neuroscience 130, 1029–1040.
Ishikawa, A., Kanayama, Y., Matsumura, H., Tsuchimochi, H., Ishida, Y., Nakamura, S., 2006. Selective rapid eye movement sleep deprivation impairs the maintenance of long-term potentiation in the rat hippocampus. Eur. J. Neurosci. 24, 243–248.
Jones, S., Pfister-Genskow, M., Benca, R.M., Cirelli, C., 2008. Molecular correlates of sleep and wakefulness in the brain of the white-crowned sparrow. J. Neurochem. 105, 46–62.
Kasai, H., Fukuda, M., Watanabe, S., Hayashi-Takagi, A., Noguchi, J., 2010. Structural dynamics of dendritic spines in memory and cognition. Trends Neurosci. 33, 121–129.
Klann, E., Sweatt, J.D., 2008. Altered protein synthesis is a trigger for long-term memory formation. Neurobiol. Learn. Mem. 89, 247–259.
Knapp, L.T., Klann, E., 2002. Potentiation of hippocampal synaptic transmission by superoxide requires the oxidative activation of protein kinase C. J. Neurosci. 22, 674–683.
Ledoux, L., Sastre, J.P., Buda, C., Luppi, P.H., Jouvet, M., 1996. Alterations in c-fos expression after different experimental procedures of sleep deprivation in the cat. Brain Res. 735, 108–118.
Lei, Y., Shao, Y., Wang, L., Zhai, T., Zou, F., Ye, E., Jin, X., Li, W., Qi, J., Yang, Z., 2015. Large-scale brain network coupling predicts total sleep deprivation effects on cognitive capacity. PLoS One 10, e0133959.
Mackiewicz, M., Shockley, K.R., Romer, M.A., Galante, R.J., Zimmerman, J.E., Naidoo, N., Baldwin, D.A., Jensen, S.T., Churchill, G.A., Pack, A.I., 2007. Macromolecule biosynthesis: a key function of sleep. Physiol. Genomics 31, 441–457.
Madsen, P.L., Schmidt, J.F., Wildschiødtz, G., Friberg, L., Holm, S., Vorstrup, S., Lassen, N.A., 1991. Cerebral O_2 metabolism and cerebral blood flow in humans during deep and rapid-eye-movement sleep. J. Appl. Physiol. 70, 2597–2601.
Maquet, P., 1995. Sleep function(s) and cerebral metabolism. Behav. Brain Res. 69, 75–83.
Maret, S., Faraguna, U., Nelson, A.B., Cirelli, C., Tononi, G., 2011. Sleep and waking modulate spine turnover in the adolescent mouse cortex. Nat. Neurosci. 14, 1418–1420.
Martin, K.C., Barad, M., Kandel, E.R., 2000. Local protein synthesis and its role in synapse-specific plasticity. Curr. Opin. Neurobiol. 10, 587–592.
Matsuzaki, M., Honkura, N., Ellis-Davies, G.C., Kasai, H., 2004. Structural basis of long-term potentiation in single dendritic spines. Nature 429, 761–766.
McDermott, C.M., LaHoste, G.J., Chen, C., Musto, A., Bazan, N.G., Magee, J.C., 2003. Sleep deprivation causes behavioral, synaptic, and membrane excitability alterations in hippocampal neurons. J. Neurosci. 23, 9687–9695.
Murphy, M.J., Peterson, M.J., 2015. Sleep disturbances in depression. Sleep Med. Clin. 10, 17–23.
Nakanishi, H., Sun, Y., Nakamura, R.K., Mori, K., Ito, M., Suda, S., Namba, H., Storch, F.I., Dang, T.P., Mendelson, W., Mishkin, M., Kennedy, C., Gillin, J.C., Smith, C.B., Sokoloff, L., 1997. Positive correlations between cerebral protein synthesis rates and deep sleep in *Macaca mulatta*. Eur. J. Neurosci. 9, 271–279.
Pawlyk, A.C., Ferber, M., Shah, A., Pack, A.I., Naidoo, N., 2007. Proteomic analysis of the effects and interactions of sleep deprivation and aging in mouse cerebral cortex. J. Neurochem. 103, 2301–2313.
Picchioni, D., Duyn, J.H., Horovitz, S.G., 2013. Sleep and the functional connectome. NeuroImage 80, 387–396.
Pielot, R., Smalla, K.H., Müller, A., Landgraf, P., Lehmann, A.C., Eisenschmidt, E., Haus, U.U., Weismantel, R., Gundelfinger, E.D., Dieterich, D.C., 2012. SynProt: a database for proteins of detergent-resistant synaptic protein preparations. Front Synaptic Neurosci. 4, 1.
Poirrier, J.E., Guillonneau, F., Renaut, J., Sergeant, K., Luxen, A., Maquet, P., Leprince, P., 2008. Proteomic changes in rat hippocampus and adrenals following short-term sleep deprivation. Proteome Sci. 6, 14.
Ramanathan, L., Siegel, J.M., 2011. Sleep deprivation under sustained hypoxia protects against oxidative stress. Free Radic. Biol. Med. 51, 1842–1848.
Ramanathan, L., Hu, S., Frautschy, S.A., Siegel, J.M., 2010. Short-term total sleep deprivation in the rat increases antioxidant responses in multiple brain regions without impairing spontaneous alternation behavior. Behav. Brain Res. 207, 305–309.
Ramm, P., Smith, C.T., 1990. Rates of cerebral protein synthesis are linked to slow wave sleep in the rat. Physiol. Behav. 48, 749–753.
Rechtschaffen, A., Bergmann, B.M., Gilliland, M.A., Bauer, K., 1999. Effects of method, duration, and sleep stage on rebounds from sleep deprivation in the rat. Sleep 22, 11–31.
Reimund, E., 1994. The free radical flux theory of sleep. Med. Hypotheses 43, 231–233.
Reynolds 3rd, C.F., Hoch, C.C., Stack, J., Campbell, D., 1988. The nature and management of sleep/wake disturbance in Alzheimer's dementia. Psychopharmacol. Bull. 24, 43–48.
Rumpel, S., LeDoux, J., Zador, A., Malinow, R., 2005. Postsynaptic receptor trafficking underlying a form of associative learning. Science 308, 83–88.
Short, M.A., Louca, M., 2015. Sleep deprivation leads to mood deficits in healthy adolescents. Sleep Med. 16, 987–993.
Starkstein, S.E., Preziosi, T.J., Robinson, R.G., 1991. Sleep disorders, pain, and depression in Parkinson's disease. Eur. Neurol. 31, 352–355.
Steriade, M., 2006. Grouping of brain rhythms in corticothalamic systems. Neuroscience 137, 1087–1106.
Steriade, M., Llinás, R.R., 1988. The functional states of the thalamus and the associated neuronal interplay. Physiol. Rev. 68, 649–742.
Stickgold, R., 2005. Sleep-dependent memory consolidation. Nature 437, 1272–1278.
Sweatt, J.D., 1999. Toward a molecular explanation for long-term potentiation. Learn. Mem. 6, 399–416.
Szabó, Z., Szeline Szomor, J., Földi, I., Janáky, T., 2012. Mass spectrometry-based label free quantification of gel separated proteins. J. Proteome 75, 5544–5553.
Szegő, E.M., Janáky, T., Szabó, Z., Csorba, A., Kompagne, H., Müller, G., Lévay, G., Simor, A., Juhász, G., Kékesi, K.A., 2010. A mouse model of anxiety molecularly characterized by altered protein networks in the brain proteome. Eur. Neuropsychopharmacol. 20, 96–111.
Terao, A., Greco, M.A., Davis, R.W., Heller, H.C., Kilduff, T.S., 2003a. Region-specific changes in immediate early gene expression in response to sleep deprivation and recovery sleep in the mouse brain. Neuroscience 120, 1115–1124.
Terao, A., Steininger, T.L., Hyder, K., Apte-Deshpande, A., Ding, J., Rishipathak, D., Davis, R.W., Heller, H.C., Kilduff, T.S., 2003b. Differential increase in the expression of heat shock protein family members during sleep deprivation and during sleep. Neuroscience 116, 187–200.
Tomasi, D., Wang, R.L., Telang, F., Boronikolas, V., Jayne, M.C., Wang, G.J., Fowler, J.S., Volkow, N.D., 2009. Impairment of attentional networks after 1 night of sleep deprivation. Cereb. Cortex 19, 233–240.
Tominaga-Yoshino, K., Urakubo, T., Okada, M., Matsuda, H., Ogura, A., 2008. Repetitive induction of late-phase LTP produces long-lasting synaptic enhancement accompanied by synaptogenesis in cultured hippocampal slices. Hippocampus 18, 281–293.
Tononi, G., Cirelli, C., 2003. Sleep and synaptic homeostasis: a hypothesis. Brain Res. Bull. 620, 143–150.
Tononi, G., Cirelli, C., 2006. Sleep function and synaptic homeostasis. Sleep Med. Rev. 100, 49–62.
Tononi, G., Cirelli, C., 2014. Sleep and the price of plasticity: from synaptic and cellular homeostasis to memory consolidation and integration. Neuron 81, 12–34.
Trachtenberg, J.T., Chen, B.E., Knott, G.W., Feng, G., Sanes, J.R., Welker, E., Svoboda, K., 2002. Long-term in vivo imaging of experience-dependent synaptic plasticity in adult cortex. Nature 420, 788–794.
Vallee, R.B., Bloom, G.S., 1991. Mechanisms of fast and slow axonal transport. Annu. Rev. Neurosci. 14, 59–92.
Vecsey, C.G., Peixoto, L., Choi, J.H., Wimmer, M., Jaganath, D., Hernandez, P.J., Blackwell, J., Meda, K., Park, A.J., Hannenhalli, S., Abel, T., 2012. Genomic analysis of sleep deprivation reveals translational regulation in the hippocampus. Physiol. Genomics 44, 981–991.
Vitiello, M.V., Prinz, P.N., Williams, D.E., Frommlet, M.S., Ries, R.K., 1990. Sleep disturbances in patients with mild-stage Alzheimer's disease. J. Gerontol. 45, M131–M138.

Vitiello, M.V., Poceta, J.S., Prinz, P.N., 1991. Sleep in Alzheimer's disease and other dementing disorders. Can. J. Psychol. 45, 221–239.
Vyazovskiy, V.V., Cirelli, C., Tononi, G., Tobler, I., 2008. Cortical metabolic rates as measured by 2-deoxyglucose-uptake are increased after waking and decreased after sleep in mice. Brain Res. Bull. 75, 591–597.
Watson, B.O., Buzsáki, G., 2015. Sleep, memory & brain rhythms. Daedalus 144, 67–82.
Wiegand, M., Möller, A.A., Schreiber, W., Lauer, C., Krieg, J.C., 1991. Brain morphology and sleep EEG in patients with Huntington's disease. Eur. Arch. Psychiatry Clin. Neurosci. 240, 148–152.
Yang, G., Pan, F., Gan, W.B., 2009. Stably maintained dendritic spines are associated with lifelong memories. Nature 462, 920–924.
Yang, G., Lai, C.S., Cichon, J., Ma, L., Li, W., Gan, W.B., 2014. Sleep promotes branch-specific formation of dendritic spines after learning. Science 344, 1173–1178.
Youngblood, B.D., Zhou, J., Smagin, G.N., Ryan, D.H., Harris, R.B., 1997. Sleep deprivation by the "flower pot" technique and spatial reference memory. Physiol. Behav. 61, 249–256.