



## Research report

# To what extent is sleep rebound effective in reversing the effects of paradoxical sleep deprivation on gene expression in the brain?

Camila Guindalini\*, Monica L. Andersen, Tathiana Alvarenga, Kil Lee, Sergio Tufik

Department of Psychobiology, Universidade Federal de São Paulo (UNIFESP), Rua Napoleão de Barros, 925, Vila Clementino, SP-04024-002, Brazil

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## ABSTRACT

Sleep is essential to maintaining health and well-being. It has been demonstrated that some of the biological alterations caused by paradoxical sleep deprivation (PSD) are not completely reversed after a period of sleep rebound (SR). The purpose of this study was to determine to what extent the specific molecular changes that occur in the rat cerebral cortex after 96 h of PSD can effectively be reversed during 24 h of recovery. Total RNA from the right cerebral cortex of Wistar male rats and GeneChip® Rat Genome 230 2.0 arrays were used to perform comprehensive microarray analysis of gene expression in control, PSD and SR groups. Microarray data were validated by Real Time qPCR. A total of 78 unique transcripts were differently expressed after PSD relative to control levels. These include genes related to metabolic processes, the circadian sleep–wake cycle, response to stimuli, regulation of cell proliferation and signaling pathways. After 24 h of sleep rebound, ~62% of the sleep deprivation transcripts were again detected as differently expressed in the SR relative to the PSD group, although in the opposite direction. On the other hand, the expression of the remaining transcripts showed intermediate values between control and sleep-deprived animals. In summary, our results provide a unique set of transcripts that might be specific related to regulation of paradoxical sleep phase and sleep homeostasis processes, as well as to the biological basis of sleep disorders.

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## 1. Introduction

Sleep is essential to maintaining both mental health and well-being [1,2]. Sleep deprivation has been the strategy of choice to study the functions of sleep due to the number of behavioral, physiological, and cellular alterations it promotes [3–6]. In humans, sleep deprivation and reduced sleep time have become increasingly prevalent in modern society as demands on productivity increase, highlighting the importance of a better understanding of the biological basis of sleep.

In the last 30 years, our groups has used a standardized protocol of 96 h of paradoxical sleep deprivation (PSD) to demonstrate a number of consistent alterations caused by sleep loss on different aspects of sleep physiology. The majority of these are reversed after a recovery period of 24 h [7–10], although some, such as hormonal profile [8] and estrous cycle disruptions in females [11], remain altered for up to 10 days after the termination of the sleep deprivation period. This indicates that sleep loss may promote deleterious and long-lasting effects on certain physiological processes.

Recent data have demonstrated that sleep deprivation either for short periods of time or prolonged over several days can

also influence the expression of distinct categories of genes in the brain, including: immediate-early genes/transcription factors; genes related to metabolic processes, neuronal plasticity and stress response; vesicle- and synapse-related genes; neurotransmitter/hormone receptors and transporters; and enzymes [12–15]. Moreover, it has been shown that this pattern of changes is consistent across species [16] and in different brain regions [12]. However, to date, no study has focused on the reversibility of the molecular changes promoted by extended paradoxical sleep deprivation (PSD) followed by a period of sleep rebound (SR). Thus, the purpose of this study was to determine to what extent the specific molecular changes occurring in the rat cerebral cortex after 96 h of PSD could effectively be reversed during 24 h of SR.

The choice of the cerebral cortex as the region of interest was due to its well known importance in mediating cognitive and other behavioral functions in response to sleep loss [17,18], as well as due to previous finding of widespread changes in gene expression of this particular brain region caused by different periods of sleep deprivation [13,14].

## 2. Methods

### 2.1. Subjects

Naïve male Wistar–Hannover strain rats were bred and raised in the animal facility of the CEDEME of Universidade Federal de São Paulo. The animals were housed

\* Corresponding author. Tel.: +55 11 2149 0189; fax: +55 11 5572 5092.

E-mail address: [camilascg@gmail.com](mailto:camilascg@gmail.com) (C. Guindalini).

in a colony maintained at 22 °C with a 12:12 h light–dark cycle (lights on at 07:00 h) and allowed free access to food and water inside standard polypropylene cages. The rats were three months old at the beginning of the experiment. All rats used in this study were maintained and treated in accordance with the guidelines established by the Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, 1996).

## 2.2. Paradoxical sleep deprivation (PSD)

The animals were subjected to PSD over a period of 96 h using the modified multiple platform method. Nine male rats were placed inside a tilted water tank (143 cm × 41 cm × 30 cm), containing 14 circular platforms, 6.5 cm in diameter, in water up to within 1 cm of their upper surface. The rats could thus move around inside the tank by jumping from one platform to another. When they reached the paradoxical phase of sleep, muscle atonia set in, and they fell into the water and awoke. The cage control group was maintained in the same room as the experimental rats for the duration of the study. We recently noticed that the large platform technique that is known in the literature to be standard control design also reduces PS in approximately 80% in the four days of the experiment [19]. That is the justification that led us to make use of the home-cage control. The narrow platform procedure causes complete and selective loss of PS during all four days. Therefore, our aim in choosing this technique was to evaluate the molecular changes that occur in the rat cerebral cortex that would reflect a predominant suppression of PS over four days. Thus, it seems appropriate to refer to these animals as being paradoxical sleep deprived, rather than being exclusively deprived of sleep. Food and water were provided *ad libitum* by placing chow pellets and water bottles on a grid located on top of the tank. Tank water was changed everyday throughout the PSD period. Sleep-deprived animals were sacrificed in the light period and at the same circadian time as the non-sleep-deprived controls and the rebound group, to assess the effects of behavioral state independently of circadian factors.

## 2.3. Sleep recording

In order to characterize the dynamics of our PSD procedure, we also carried out sleep recordings in a separate experiment. The animals were operated for implantation of the electrodes for electrocorticographic (ECoG) and electromyographic (EMG) activities, thus permitting the assessment of the sleep–wake cycle. The ECoG and EMG electrodes were implanted on the basis of our well-established procedure. Briefly, anesthesia was induced with diazepam (10 mg/kg i.p.) and ketamine (90 mg/kg i.p.). To record cortical ECoG with a minimum of theta activity, one pair of screw electrodes was placed through the skull ipsilaterally: 1 mm posterior to bregma, 3 mm lateral to the central suture; and 1 mm anterior to lambda, 4 mm lateral to the central suture. EMG electrodes were implanted in the neck muscles, soldered to a 6-pin socket, and covered with dental acrylic cement. Body temperature was maintained at 37 °C with a regulated electric heating pad (Harvard Apparatus, USA). After the surgical procedure was performed, rats received an i.m. injection of 0.5 mL/rat of antibiotics and sodium diclofenaco (0.1 mL/rat, vo). The rats were individually placed in rounded transparent plastic cages and allowed a 10 day surgery recovery period, followed by a four day adaptation period with the cable connected. Four controls and four animals that were sleep deprived for 96 h had their sleep recorded for 24 h at baseline and during the 24 and 48 h period of SR. Electrophysiological recordings were collected using a digital polygraph (Neurofax QP 223A<sup>®</sup> Nihon Kohden, Tokyo, Japan) at a sampling rate of 200 Hz. Recordings were displayed every 30 s on a high-resolution PC monitor and visually classified as wakefulness–W (active–AW, >40  $\mu$ V), slow wave sleep–SWS and PS–paradoxical sleep as previously described [20].

## 2.4. Microarray experiment

After sleep deprivation and rebound period, rats were sacrificed by decapitation without anesthesia. Brains were rapidly removed, and the right cerebral cortex was dissected, immediately frozen on dry ice and stored at –80 °C. Total RNA was isolated from the entire right cerebral cortex of each animal using Trizol (Gibco-BRL, Gaithersburg, MD, USA) according to the manufacturer's instructions, followed by Qiagen RNeasy (Qiagen, Chatsworth, CA) cleanup and DNase I (Invitrogen, Carlsbad, CA, USA) treatment to remove any residual genomic DNA. Total RNA concentration was measured by a NanoDrop<sup>®</sup> Spectrophotometer. An equal mass amount of cortical RNA from each animal was used to hybridize nine independent sets of GeneChip<sup>®</sup> Rat Genome 230 2.0 arrays ( $n = 3$  for control, PSD and SR groups). Microarray labeling, hybridization and expression analyses were performed according to the Affymetrix GeneChip Expression Analysis Technical Manual (Affymetrix Inc., Santa Clara, CA, USA). Briefly, an equal mass amount of total cortical RNA from nine animals was converted into double-stranded cDNA using T7-Oligo(dT) promoter primers. cDNA was then converted to biotinylated cRNA by *in vitro* transcription, according to the manufacturer's instructions (One-Cycle Target Labeling kit, Affymetrix, Santa Clara, USA). The cRNA was fragmented and the size range of cRNA before (0.5 kb and longer) and after (35–200 base fragments) fragmentation was checked by agarose electrophoresis. The arrays were subsequently washed, stained with phycoerythrin streptavidin and scanned. Quality control criteria of the cRNA labeling and hybridization were assessed for all scanned arrays using Microarray Suite 5.0

(Affymetrix). Protocols were performed in the Molecular Core-Microarray Facility, Sao Paulo, Brazil. Analysis of the microarray data was performed using DNA chip (dChip) software (<http://biosun1.harvard.edu/complab/dchip/>). Data from all chips were normalized against the array with median overall intensity using an invariant set method. Model-based expression values were computed using the PM/MM difference model, with outlier detection and correction [21]. To minimize the number of false positive results, highly strict criteria were used for condition-to-condition comparisons: genes on the array were filtered for a Present call in all array sets, lower-bound 90% confidence intervals of >1.2-fold change, two-sample *t*-test  $p < 0.05$  and median False Discovery Rate (FDR) <5%, after 500 permutations. Hierarchical clustering analysis was performed using dChip software. In order to identify specific functional Gene Ontology categories overrepresented among the differentially expressed genes, the bioinformatics software Gostat (<http://gostat.wehi.edu.au>) and GoMiner (<http://discover.nci.nih.gov/gominer/>) were used.

## 2.5. Real-time qPCR confirmation

To technically validate the microarray results, qPCR was run on 5 genes (~5% of total differentially expressed transcripts), which will actually be used in 10 independent comparisons, involving the three groups of animals (control, PSD and SR). The Brain Derived Neurotrophic Factor (BDNF), the early growth factor 2, period homolog 2 (*per2*) and VGF nerve growth factor inducible genes were identified by the microarray analysis as up-regulated in PSD group compared to controls, as well as down-regulated in the SR compared to PSD group. The metallothionein 1a gene expression was identified as up-regulated after sleep deprivation, but as not changing after 24 h of sleep rebound, when compared to PSD by the microarray analysis. In brief, 1  $\mu$ g of total RNA was reverse transcribed into cDNA using the using Superscript III reverse transcriptase, according to manufacture protocol (Invitrogen, Carlsbad, CA, USA). Each cDNA was then used as template for real-time PCR amplification, performed by using Platinum<sup>®</sup> SYBR<sup>®</sup> Green qPCR SuperMix (Invitrogen, Carlsbad, CA, USA) and pairs of forward/reverse primers for both selected genes and beta-actin housekeeping gene. Independent reactions were performed in triplicate on Applied Biosystems 7500 Real-Time PCR system, according to the manufacturer's instructions. Threshold cycle (CT) values were exported into spreadsheets, and normalized to the housekeeping gene, beta-actin ( $\Delta$ CT). Relative changes in gene expression were then calculated using the  $2^{-\Delta\Delta CT}$  method, as previously described [22].

## 3. Results

In order to better understand the effects of PSD and SR at the molecular level, we performed global gene expression profiling using Affymetrix microarray assays on the cerebral cortex of rats subjected to 96 h of PSD, PSD followed by 24 h of SR, or simply left on their cage during the experimental period (control animals). Sleep records of an independent set of animals showed that, as described by previous studies [19], the modified multiple platform method effectively abolished paradoxical sleep during the deprivation period, as illustrated by a significant increase of the percentage of time spent by the SR animals in the paradoxical sleep phase ( $p < 0.02$ , Fig. 1).

Among over 30,000 transcripts surveyed, high stringency data analysis identified a set of 78 unique transcripts that were differently expressed in PSD group compared to controls (up-regulated:  $n = 59$  and down-regulated:  $n = 19$ ). Among those, 50 were annotated genes that could be grouped into the following categories identified by Gostat and GoMiner bioinformatic analysis: (a) metabolic processes: glycerol-3-phosphate dehydrogenase 1; insulin receptor substrate 2; hydroxysteroid 11-beta dehydrogenase 1; insulin induced gene 1; (b) circadian sleep/wake cycle: VGF nerve growth factor inducible, period homolog 2; cortistatin, albumin; (c) response to stimulus: heat shock 70 kDa protein 1A; crystallin, alpha B; sulfotransferase family 1A1; fibronectin 1; allograft inflammatory factor 1; (d) regulation of cell proliferation: brain derived neurotrophic factor (*Bdnf*); gap junction membrane channel protein beta 6; prostaglandin-endoperoxide synthase 2; secretogranin 2; (e) signaling pathway: neuronal regeneration related protein; connective tissue growth factor; HtrA serine peptidase 1; (f) other biological processes: metallothionein 1a; glutamate receptor, metabotropic 2; opioid binding protein/cell adhesion molecule-like; vasoactive intestinal polypeptide, among others. The complete list of known genes can be found in the Table 1, together with the respective levels of changes. All Gene

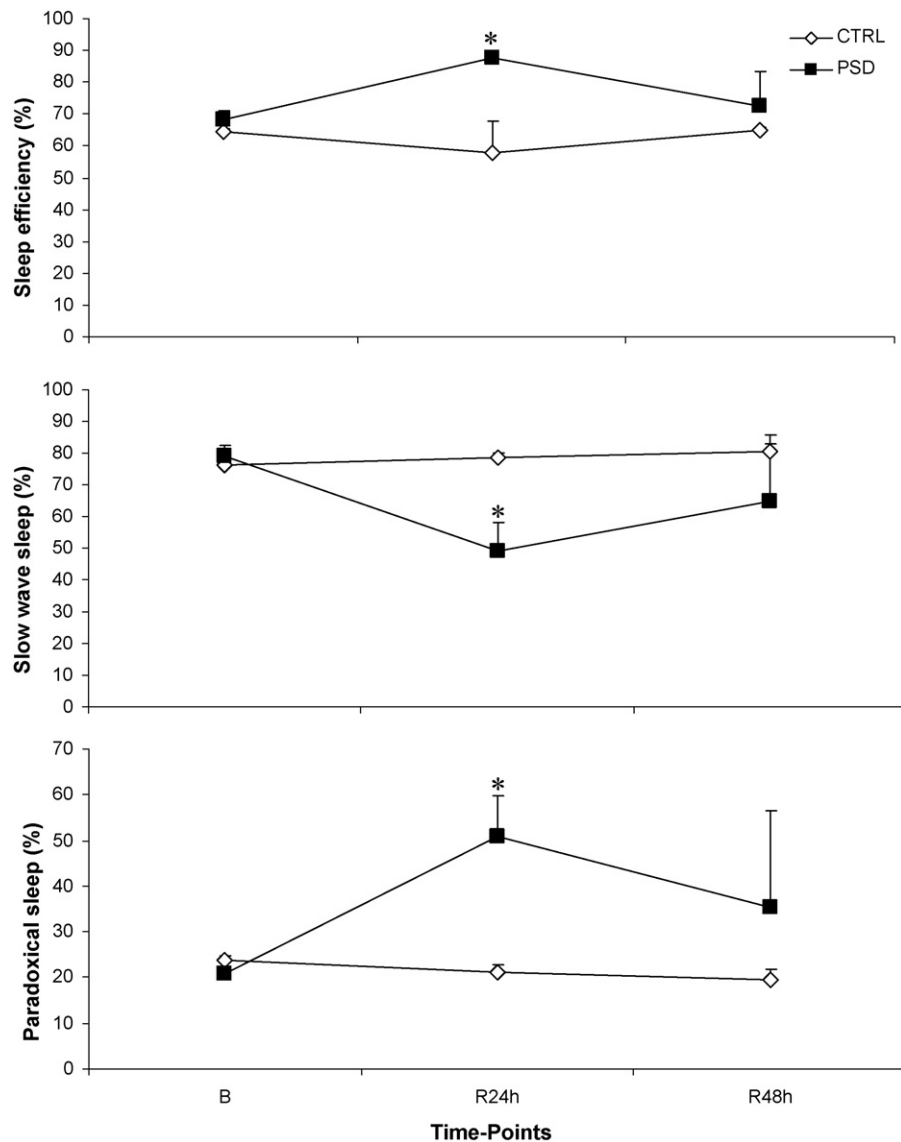
**Table 1**

List of genes found to be differently expressed after 96 h of paradoxical sleep deprivation relative to control levels.

Symbol	Name	GenBank	Fold change
<b>Response to stress</b>			
Ptgs2	Prostaglandin-endoperoxide synthase 2	U03389	1.6
Nrep	Neuronal regeneration related protein	BE107450	–1.40
Insig1	Insulin induced gene 1	NM_022392	–1.31
Hspa1a	Heat shock 70 kDa protein 1A	NM_031971	3.27
Plip*	Plasma membrane proteolipid	NM_022533	–1.53
Sult1a1	Sulfotransferase family 1A, phenol-preferring, member 1	AF394783	3.35
Fn1	Fibronectin 1	AA893484	2.31
Aif1	Allograft inflammatory factor 1	NM_017196	–1.53
Cryab	Crystallin, alpha B	NM_012935	1.74
Ctgf	Connective tissue growth factor	NM_022266	1.99
Bcl6	B-cell leukemia/lymphoma 6	AI237606	1.39
Scg2	Secretogranin 2	NM_022669	1.35
<b>Metabolic processes</b>			
Gpd1	Glycerol-3-phosphate dehydrogenase 1	BI277042	1.87
Irs2	Insulin receptor substrate 2	BE118080	1.30
Hsd11b1*	Hydroxysteroid 11-beta dehydrogenase 1	NM_017080	1.47
<b>Circadian rhythm</b>			
Cort*	Cortistatin	NM_012835	1.45
Alb*	Albumin	NM_134326	2.03
Per2	Period homolog 2	NM_031678	1.72
<b>Receptor binding</b>			
Ifrd1	Interferon-related developmental regulator 1	NM_019242	1.41
Vgf	VGF nerve growth factor inducible	NM_030997	1.50
Vip	Vasoactive intestinal polypeptide	AI412212	1.43
Nov*	Nephroblastoma over expressed gene	NM_030868	1.54
<b>Oxidoreductase activity</b>			
Phyh1	Phytanoyl-CoA dioxygenase domain containing 1	BI275763	1.69
Fdft1	Farnesyl diphosphate farnesyl transferase 1	NM_019238	–1.30
Smox*	Spermine oxidase	BI289700	1.45
<b>Biological regulation</b>			
Cml3	Camello-like 3	AF187814	–1.70
Bdnf	Brain derived neurotrophic factor	NM_012513	1.59
Tnnc2	Troponin C type 2	BG663128	–1.64
Mt1a*	Metallothionein 1a	AF411318	2.01
Dusp6	Dual specificity phosphatase 6	AI602811	1.46
Cited2	Cbp/p300-interacting transactivator	AI013390	1.39
Cables1*	Cdk5 and Abl enzyme substrate 1	BI296696	1.50
Htra1*	Htra serine peptidase 1	NM_031721	1.67
Gjb6	Gap junction membrane channel protein beta 6	NM_053388	1.68
Grm2	Glutamate receptor, metabotropic 2	M92075	–1.60
<b>Others</b>			
Arhgef3	Rho guanine nucleotide exchange factor 3	AI454536	1.31
Camk1g	Calcium/calmodulin-dependent protein kinase I gamma	BG381458	1.53
Cbs*	Cystathionine beta synthase	NM_012522	1.36
CPG2	CPG2 protein	NM_019355	1.44
Cr16	SH3 domain binding protein CR16	U25281	1.82
Dact2	Dapper homolog 2, antagonist of beta-catenin	BI288833	–1.45
Fabp7*	Fatty acid binding protein 7, brain	NM_030832	–1.42
Fkbp5	FK506 binding protein 5	AW534837	1.60
G10*	Maternal G10 transcript	NM_053556	1.31
Gpatch4	Gpatch4 G patch domain containing 4	BI288424	2.25
Gpr37*	G protein-coupled receptor 37	NM_057201	–1.43
Igfbp5	Insulin-like growth factor binding protein 5	BE104060	–2.08
LOC499941	SCF apoptosis response protein 1	BI294932	–1.33
LOC682565*	Similar to Proline oxidase, mitochondrial precursor	AI411345	1.49
Lyz*	Lysozyme	L12458	1.35
Mt2	Metallothionein-2	BM383531	1.62
Opclm1*	Opioid binding protein/cell adhesion molecule-like	M88709	1.51
Rbm34	RNA binding motif protein 34	BM392304	1.40
RGD1310773*	Similar to hypothetical protein FLJ31810	BE097267	–1.30
RGD1359529*	Similar to chromosome 1 open reading frame 63	BE096504	1.68
RGD1559896*	Similar to RIKEN cDNA2310022B05	AI1 03552	–1.41
Rprml	Reprimo-like	BM387395	1.39
S100a13*	S100 calcium binding protein A13	AW141940	–1.38
Tmem100	Tmem100 transmembrane protein 100	BM391248	2.00
Usp54	Ubiquitin specific protease 54	BI285307	1.39
Vof16*	Ischemia related factor vof-16	BE107282	1.82

List of genes differently expressed in the cerebral cortex of rats subjected to 96 h of paradoxical sleep deprivation relative to control animals, simply left on their cage during the experimental period, as indicated by high-density microarray analysis (GeneChip® Rat Genome 230 2.0, Affymetrix Inc., Santa Clara, CA, USA). Statistical analysis based on present call in all array sets, >1.2-fold change, *t*-test *p* < 0.05 and median False Discovery Rate (FDR) < 5% (Dchip Software-(<http://biosun1.harvard.edu/complab/dchip/>)).

\* Genes with expression levels not fully restored after 24 h of Sleep Rebound.



**Fig. 1.** Effect of paradoxical sleep deprivation on the sleep parameters (panel A: sleep efficiency, panel B: slow wave sleep, and panel C: paradoxical sleep) in different time-points (baseline and recovery period). \* Different from control group ( $p < 0.05$ ). Values are expressed as mean  $\pm$  s.e.m.

Ontology terms were overrepresented at a false discovery rate of  $p < 0.05$ .

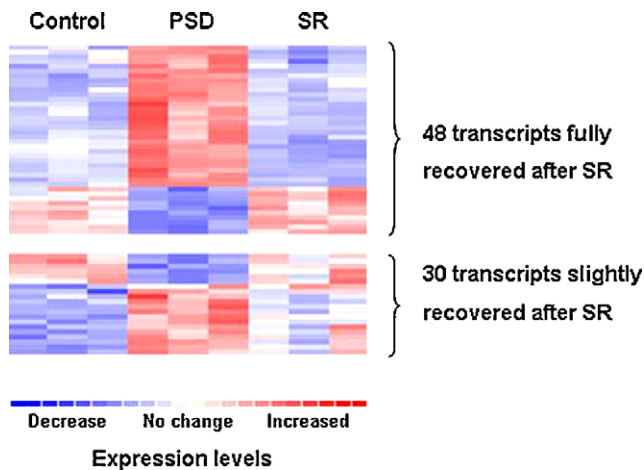
After 24 h of SR, independent analysis showed that  $\sim 61.5\%$  (40 known genes, 8 EST) of the transcripts previously affected by PSD were again detected as differently expressed in the SR group compared to the PSD group, but in the opposite direction. The known genes are presented in Fig. 2, where the red scale reflects up-regulated transcripts, and the blue scale denotes down-regulation of gene expression. For the remaining 30 transcripts (21 known genes, 9 EST) initially induced by PSD, the mean expression values were not significantly different between PSD and SR groups, indicating, a partial recovery, as demonstrated by the intermediate levels of expression (data not shown).

Since the aim of this study was to provide a global view of the paradoxical sleep deprivation and sleep recovery influence on the expression patterns in the brain, we decided to technically validate the microarray results, running real-time RT-PCR on five randomly selected genes. The results confirmed the up-regulation (1.8; 1.9; 2.2; and 1.8-fold changes) in the PSD group for the period homolog 2, *Bdnf*, metallothionein and VGF nerve growth factor inducible gene expression, respectively; the subsequent reversion of *Bdnf* and

VGF nerve growth factor inducible levels in the SR group, and the partial reestablishment of the metallothionein expression after 24 h of SR ( $-1.7$  in the SR group, compared to 2.1-fold increase in the PSD group). In the microarray experiment, the *EGR2* gene expression was shown to be up-regulated (1.4-fold change) in the PSD compared to control group and down-regulated ( $-4.5$ ) in the SR group compared to sleep deprived animals, although the former was not statistically significant after Bonferroni correction. Both results were confirmed in the qPCR experiments with a 1.5-fold-change up-regulation in the PSD group and 5-fold change down-regulation in the SR, compared to control animals. The only exception was the transcript for *per2* gene in the comparison PSD versus SR groups, which according to the array analysis was decreased ( $-1.5$  fold-change) in SR rats, whereas the qPCR showed a slighter reduction of 1.1-fold-change.

#### 4. Discussion

The aim of this study was to assess whether the molecular changes caused by 96 h of PSD could be restored after 24 h of SR. We demonstrated here that the sleep recovery period reversed



**Fig. 2.** Results of gene expression analysis on the cerebral cortex of rats subjected to 96 h of paradoxical sleep deprivation (PSD), PSD followed by 24 h of sleep rebound (SR) or simply left on their cage during the experimental period (CONTROL), using high-density microarray assays (GeneChip® Rat Genome 230 2.0, Affymetrix Inc. (Santa Clara, CA, USA). Statistical analysis based on present call in all array sets, >1.2-fold change,  $t$ -test  $p < 0.05$  and median False Discovery Rate (FDR) <5% (Dchip Software-(<http://biosun1.harvard.edu/complab/dchip/>)). Higher expression levels are shaded in red, and lower levels are shaded in blue. For gene names please see Table 1 (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

the expression of the majority of transcripts modulated by PSD. This is in accordance with behavioral and biological modifications reversion [23], such as impairment of visual attention [9], corticosterone hormone levels and genital reflexes [7,8], as well as the gene expression and protein levels of BDNF [24] and Bip/GRP78 [25], respectively. On the other hand, the expression of a subset of transcripts initially modulated by PSD was only partially restored in the SR group, showing intermediate values between those observed for control and PSD groups. Conceivably, a longer recovery period might be necessary. This partial reversion nicely reflects the sleep rebound-induced partial restoration of other physiological effects of PSD measured by our group in earlier studies, such as thermal pain threshold [26], corticosterone concentrations [8], body composition [10], estrous cycles disruption [11], learning abilities [27], endocrinological molecules, catecholaminergic compounds [8] and homocysteine levels [28]. In these studies it was shown that the effects of PSD were still detectable after a recovery period of 24 h.

Similar to other type of SD paradigms [12–14], our PSD protocol primarily affected genes related to metabolic processes, response to stimulus (including stress and inflammation), circadian/sleep cycles, regulation of cell proliferation and signaling pathways. Moreover, several genes identified in this study had been previously reported by others, highlighting the importance of those in the sleep homeostasis mechanisms occurring after a period of wakefulness [12–14]. These included transcripts encoding BDNF, a well-known key protein in neural plasticity and long-term potentiation that has repeatedly been associated with different sleep deprivation conditions [29,30]; the heat shock 70 kD protein 1A and crystalline- $\alpha$ B, two proteins associated with stress response [31]; metallothionein, a highly conserved low-molecular-weight cysteine-rich protein with antioxidative roles [32]; glycerol-3-phosphate dehydrogenase and farnesyl diphosphate farnesyl transferase1, enzymes involved in carbohydrate metabolic processes and cholesterol biosynthesis [33]; and cortistatin, a neuropeptide synthesized by cortical GABAergic interneurons, which has been found to depress neuronal electrical activity [34]. Notably, sulfotransferase family 1A1—the primary enzyme responsible for the catabolism of norepinephrine, dopamine and to a lesser extent serotonin [35] was the most highly

up-regulated transcript in the PSD compared to control conditions. Interestingly, this gene was also identified in an extreme sleep loss condition [13] and after short periods of spontaneous or forced wakefulness [12,29].

On the other hand, our PSD protocol induced the expression of several genes not described in other types of sleep deprivation, suggesting that those might play a more specific role in the paradoxical stage of sleep. Of particular interest, we found up-regulation of the gene encoding the Insulin receptor substrate 2 (IRS2), a protein thought to have an important role in modulating the actions of insulin in the brain [36]. Recently, Lin et al. [37] demonstrated that a conditional knockout of the insulin receptor substrate 2 (*Irs2*) in mouse pancreas  $\beta$  cells and parts of the brain resulted in increased appetite, lean and fat body mass, linear growth, as well as elevated insulin resistance progressing to diabetes. Thus, up-regulation of the *Irs2* gene in PSD and subsequent recovery during the rebound period is consistent with our previously published data showing that PSD for 96 h promoted a significant decrease in body weight and fat content. This occurred as a result of reduction in food intake [10,38,39] and quickly returned to baseline levels during the sleep recovery period. In humans, on the other hand, short sleep times and/or sleep fragmentation, as occurs in patients with Obstructive Apnea Syndrome (OSA), have been consistently associated with adverse effects on glucose metabolism, obesity and increased risk for development of type 2 diabetes (for review, see Tasali et al. [40]). Taken together, these results suggest that the *Irs2* gene might represent a new candidate for further studies on the complex interactions between sleep loss, obesity and diabetes.

Both circadian and homeostatic processes are known to regulate the cycling between the states of sleep and wakefulness. The circadian process controls the timing of sleep and intensity across the 24-h day, whereas the homeostatic mechanism is a reflection of the duration of prior wakefulness and it is thought to influence sleep under baseline conditions and after periods of sleep deprivation, during recovery [17]. Therefore, the genes modulated by PSD, in particular the ones with expression levels fully recovered during sleep rebound appear to be indicative of a homeostatic response on the molecular level and suggest novel mechanistic insights for underlying the regulatory processes involved in initiating and maintaining the normal sleep.

One limitation of the current study may be the small number of samples used in the microarray analysis which could potentially compromise the reliability of the results. However, we believe this problem is partially overcome by three important factors: (1) the great deal of overlap of genes identified by this and previously published studies, regarding the effect of different sleep deprivation paradigms; (2) the genes identified in opposite direction in both independent comparisons, e.g., control versus PSD and PSD versus SR groups in opposite directions. This can be interpreted as a biological replication of the role of these genes in sleep homeostasis; (3) the technical confirmation by the real time PCR of the size and direction of the effects in nine of ten analysis.

In conclusion, our results suggest that PSD promotes a pattern of gene expression modulation that shares a number of common features with other types of sleep deprivation paradigms, but also has specific characteristics possibly related to the regulation of paradoxical sleep phase. In addition, we demonstrated that 24 h of sleep recovery was not sufficient to completely reverse all effects caused by prolonged periods of sleep deprivation. This suggests that, as it occurs with a number of previously examined behavioral and physiological parameters, some of the molecular consequences of sleep loss are long-lasting and might have a greater impact on physical and mental health. Finally, several transcripts, whose expression was affected in opposite directions by PSD and SR, could be potential candidate genes for understanding sleep homeostasis and the biological basis of sleep-wake cycles and sleep disorders.

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