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Microarray analysis of liver and brain tissues after REM sleep deprivation in rats

Atul Pandey[1, 2], Santosh Kar[1, 3]*

[1]School of Biotechnology, Jawaharlal Nehru University, New Delhi, India

Present Addresses:

[2] Department of Ecology, Evolution, and Behavior, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, 91904, Israel

[3] School of Biotechnology, KIIT, Bhubaneswar, Odisha, India

***Corresponding author**

Prof. Santosh K Kar, Email: santoshkariis@rediffmail.com, Phone: +91-9937085111

Short title:

REM sleep deprivation differentially affects the genes of liver and brain in rats.

Key words:

Microarray; Sleep; Rapid eye movement sleep; Circadian rhythms, Hepatocytes; Gene expression; Reactive oxygen species; Acute phase response; Inflammation and cell death.

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Highlights of the study:

- Microarray analysis compared the expression pattern of genes related to many biological, molecular and physiological processes in brain and liver.
- Analysis revealed about the basic genetic tool-kit involved with REM sleep deprivation and its loss in brain and liver.
- Many of the genes involved in critical physiological processes like apoptosis and circadian rhythms are found differentially expresses in brain and liver.

Abstract

Fundamental questions about sleep and its universal existence remain elusive. The common presence of sleep across phyla suggests that it must serve some indispensable cellular and/or molecular function needed for survival. Microarray studies, performed in several model systems, have identified classes of genes that are believed to regulate "sleep-state" or vice-versa. This has led to the following concepts: first, a function of sleep is to maintain synaptic homeostasis; second, sleep is a stage of macromolecule biosynthesis which is needed during the waking period; third, extending wakefulness leads to the downregulation of several important metabolic pathways which needs to be balanced for extended survival; and, fourth, extending wakefulness leads to endoplasmic reticulum stress. In human sleep studies, microarrays are being pragmatic to the identification of biomarkers for sleepiness and for the common sleep disorders. This study tries to find out the correlative processes which happen across different tissue system in the maintenance of sleep. We compared the gene expression profile in brain and liver due to REM sleep deprivation. Our result suggests that sleep deprivation affects a different set of genes in the brain and liver.

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1. Introduction:

Sleep loss has fatal effects on individuals (Rechtschaffen et al., 1983; Webb and Agnew, 1962). The degree of fatality or behavioral or physiological change depends on the extent of sleep loss as such in the form of acute or chronic loss. Recent advancement in sleep research has thrown a light on a functional aspect of sleep in general as well as on REM sleep. Many theories explain the evolutionary significance and functions of sleep starting from “null” to “synaptic plasticity” hypothesis. To summarize all, sleep must be having some core common function for all individuals who evolved to have sleep and the simplest hypothesis (after the null hypothesis) is that there may be a single core function that requires sleep and related functions that take advantage of sleep. The synaptic plasticity theory of sleep explains the requirement of sleep as a need to discharge the synaptic load. Many sleep-related studies correlate its negative effect along with physiology, behavior, immunity and hormonal regulations. Sleep also affects cellular functions in different individuals reported. All the above indicates that sleep is essential and can’t be replenished. Studies ranging from cellular to behavioral scale promised an ideal platform to discuss the realm of information on sleep.

A microarray is a very useful technology to understand the dynamics of the gene expression in a tissue related to specific physiological or behavioral conditions. Typically, DNA microarray technology follows naturally from the basic structure and replication dynamics of the DNA molecule and the genetic code. During cell replication, the double helix formed due to the base pairing of nucleotides comes unzipped briefly until new bonds are formed. Microarray usually arranges the thousands of small pieces of the unzipped DNA in a known order on a small chip. Each small piece of DNA (or oligonucleotide) can act as a molecular "probe." Such a probe is "sticky" and just waiting to attach or zipper itself to a corresponding piece of DNA that makes up a "target" gene in any solution that is incubated or "hybridized" with the probes on the chip. Thus microarray can offer an advantage of measuring out the

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differences between the physiological stages across different body tissues, which promise to answer the facilitating questions in sleep biology research (Cirelli and Bushey, 2008; Cirelli and Tononi, 2008; Mignot, 2008). Overall, these questions are quite relevant to the modern problems of sleep deprivation in the current world and also relevant to the many pathophysiological conditions of sleep disorders (Colten et al., 2006). Most of the studies involving microarrays addressing the sleep functions or related questions basically focused on the brain region only in various species studied (Cirelli, 2004; Cirelli et al., 2004; Cirelli et al., 2005; Jones et al., 2008; Mackiewicz et al., 2007; Maret et al., 2007; Zimmerman et al., 2006), although a few recent studies focused on the other organs (Maret et al., 2007). Studies of the transcriptome in model organisms have demonstrated 10% changes in the level of transcripts of many genes between sleep and wakefulness (Mackiewicz et al., 2007). Many of the array studies were performed to date have used different platform and design, while some evaluated the difference using fold change (Cirelli et al., 2004), while other used false-discovery rate strategies (Mackiewicz et al., 2007). Despite these differences, there is a resemblance in the results with respect to pathways in the brain that are affected by sleep and wakefulness (Mackiewicz et al., 2009).

Recently, more important advances in sleep research has been reported in non-mammalian model systems including, the fruit fly (Drosophila melanogaster) (Hendricks et al., 2000; Tononi, 2000; Zimmerman et al., 2008), the zebrafish (*Danio rerio*) (Prober et al., 2006; Yokogawa et al., 2007; Zhdanova et al., 2001), the nematode (*Caenorhabditis elegans*) (Raizen et al., 2008) and bees (*Apis mellifera*) (Eban-Rothschild and Bloch, 2008; Eban-Rothschild and Bloch, 2012; Klein and Seeley, 2011). A crucial point here is that for the identification of sleep like stage in these model systems, behavioral criteria was used rather than electrophysiological parameters. Authors used the different behavioral criteria, in addition to the quiescence like elevated arousal threshold, homeostasis and rhythmic

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expression of the clock genes. Many aspects of the sleep regulation are conserved among different model systems unlike the behavioral aspect, where both neurotransmitter systems and molecular signaling pathways that regulate sleep were also ecologically conserved (Zimmerman et al., 2008). For example, signaling mechanisms involving cyclic AMP, which promotes wakefulness (Graves et al., 2003; Hendricks et al., 2001), and epidermal growth factor, which promotes sleep (Foltenyi et al., 2007; Van Buskirk and Sternberg, 2007), have the same role in different model systems [for review, see (Zimmerman et al., 2008)]. Microarray provides a great advantage to investigate sleep and sleep-like processes in these non-model systems. Numerous genes were identified which were involved in the acquisition and potentiation of synaptic plasticity as these genes were predominantly expressed during wakefulness compared to genes involved in synaptic consolidation or depression, which were found more expressed during sleep (Cirelli et al., 2004). These findings further support the 'synaptic homeostasis theory' of sleep–wake control (Tononi and Cirelli, 2003). This theory postulates that wakefulness is accompanied by synaptic potentiation of cortical networks through brain-derived neurotrophic factor (BDNF)-dependent and other signaling mechanisms. Activities and learning are postulated to achieve such synaptic potentiation during wakefulness in the neuronal circuits (Vyazovskiy et al., 2000; Vyazovskiy et al., 2004). This observation supports the idea that there is a potentiation of synaptic strength in the barrel cortex that receives and processes tactile information derived from the contralateral face of the animal. It is postulated that an increase in overall synaptic potentiation during wakefulness will require more resources (energy, space) to maintain this level of potentiation. The slow-wave sleep further was assumed to downscale the synaptic strength, crucial for neuronal functions in the imminent days.

REM sleep deprivation was found affecting the 652 brain genes along with 426 liver genes, which oscillated with REM sleep loss like conditions (Turek and Bringmann, 2014). We do

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moved ahead with a lot of information from previous studies having a few more basic questions in our mind like why do brain largely gets affected by sleep loss? Do other major organs also have physiological consequences? How the body does balance the stress created by sleep loss? In the present study we compared the gene expression pattern from brain and liver in respect to REM sleep loss. We report here, that REM sleep loss affected a major number of genes in brain and liver along with physiological processes. A number of pathways got affected by REM sleep loss in the brain and liver in-common were very limited suggests that each organ in the body deals the stress at different degree and extent. In common, a number of genes getting upregulated compared to downregulated was less suggesting sleep loss affects the living system in a negative way and tries to shut-down the system rather than enforce it. We observed here that a large number of genes got affected in the brain (652) compared to liver (426). Several of these genes were not reported with previous studies and thus taken together; we provide a dataset of genes that oscillate with the REM sleep loss, and thus a useful base for future research and identification of candidate gene for sleep like behavior.

2. Material and Methods:

Male wistar rats weighing between 220-260 gm were used in this study. Animals were housed in the institutional animal house facility with a 12:12 hr L: D cycle (lights on at 7.00 am). Food and water were provided *ad libtium.* All experiments were conducted as per the protocol approved by the University's Institutional Animal Ethics Committee.

2.1 REM sleep deprivation procedure

Rats were REM sleep deprived for 9 days by exposing the rats to flower-pot methods (Hicks et al., 1977; van Hulzen and Coenen, 1981). In this method, animals were kept on a small raised platform (6.5 cm diameter) surrounded by water compared to cage control (CC) rats, which were maintained in rectangular plastic cages at laboratory condition. Although the

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animals could sit, crouch and have non-REM sleep on this island, but due to the postural muscle atonia during REM sleep, they were unable to maintain their extended relaxed body posture on the small platform and tended to fall into the surrounding water. As a result they wake up at the onset or prior to the appearance of REM sleep and thus deprived of it. The rats were collected and sacrificed on day 9 and total brain and liver were collected for further analysis.

2.2 RNA extraction and quality analysis:

Total RNA was isolated from whole brain and liver samples using Qiagen kit. Briefly, rats were briefly anesthetized with isoflurane and quickly head was decapitated and flash frozen in to the liquid nitrogen. Total RNA was isolated from the entire brain and liver of every animal by using Trizol (Gibco-BRL, Gaithersburg,MD, USA) according to the manufacturer's instructions.The concentration of the total RNA was measured using Nanodrop and quality were analyzed using Bioanalyzer. The array was performed of the good quality samples.

2.3 Microarray: labelling, hybridization and data analysis

For microarray, we took equal amount of total RNA from brain and liver. We outsourced the genomic facility of Ocimum Biosolutions (https://ocimumbio.com) for microarray analysis. In brief, we used Affymetrix Rat Gene 1.0 ST Arrays containing more than 7000 annotated sequences and 18000 expressed sequence tags (ESTs). Microarray labelling, hybridization and expression analyses were performed according to the Affymetrix Gene Chip Expression Analysis Technical Manual (Affymetrix Inc., Santa Clara, CA, USA) and essentially as previously reported (Cirelli, 2004). Briefly, an equal mass amount of total RNA from brain and liver were converted into cDNA using Superscript II - reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA). The cDNA prepared was then converted to biotinylated cRNA using the ENZO Bio Array High Yield In-vitro Transcription kit (Enzo

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Life Sciences, Farmingdale, NY, USA) according to the manufacturer's instructions. The cRNA was fragmented at 0.5 g/mL final concentration in a fragmentation buffer (40mM Tris-acetate, pH 8.1, 100mM potassium acetate, 30mM magnesium acetate). Analysis was done using Affymetrix Expression Console and Programming language-R (Computing, 2013; R Foundation for Statistical Computing, 2016).

Validation of Focal Transcripts with Quantitative Real-Time q-PCR

Six transcripts for which our microarray analyses predicted significant alternations were further analyzed using RT-PCR. The controls were maintained respectively along with experimental conditions to rule out other possibilities of influence of external factors in our experimental set up. Respective mRNA levels were measured with real-time RT-PCR. Briefly, samples were collected from total liver and total brain tissues, freeze dried and were stored individually at −80°C until mRNA quantification. Total RNA was isolated using Trizol methods, and reverse-transcribed using ABI reverse transcription kit (Applied Biosystems, Catalog number: 4368814). TaqMan gene expression Master Mix (Applied Biosystems, Catalog number: 4369016) and probes (Applied Biosystems, Supplementary figure, Table S2) were used for quantitative analysis of mRNA. Each cDNA sample was analyzed in triplicate. RT-PCR reactions for all focal genes and *GAPDH* were measured from the same cDNA sample and were loaded on the same 96-well analysis plate. Gene levels were quantified with the $2^{-\Delta\Delta Ct}$ method and *GAPDH* was used as a reference control gene for expression level normalization.

3. Results and Discussion

3.1 General Results

Affymetrix Rat Gene 1.0 ST Array was used in the current study. The primary data analysis was performed by using Affymetrix Expression Console and R. We observed that total 209 genes upregulated compared to 217 genes which were found downregulated in liver in

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response to REM sleep deprivation. Similarly, REM sleep deprivation upregulated the total 311 genes in brain, while 341 genes in total were found downregulated in brains (Supplementary figure, Table: S1). Interestingly, none of the genes were found in common in brain and liver, which got shared. There were number of genes found affected in common for brain and liver. For example, total of 4 genes were found downregulated in brain, while same 4 genes where found upregulated in liver. Similarly, there were 6 genes which got upregulated in brain but same time got downregulated in liver. We observed only 2 and 11 genes which were either upregulated or downregulated in brain and liver tissues simultaneously (Fig.2 and Supplementary figure, Table: S3). Based on this primary observations, we proceeded with gene ontology analysis and pathway analysis.

3.2 Gene Ontology Analysis

Functional annotation of the differentially expressed genes was obtained from the Gene Ontology Consortium database, based on their respective molecular function, biological process, or cellular component (Ashburner et al., 2001). Functional categories enriched within genes that were differentially expressed between control and REMSD at each time point were determined (Fig. 3, 4 & 5). A variant of the one-tailed Fisher exact probability test based on the hypergeometric distribution was used to calculate *P* values.

3.3 Pathway Analysis:

We performed pathway analysis of microarray data using KEGG analysis. Most of the biochemical pathways are stored in KEGG database describing physiological processes. Though, most of the biological processes are species–specific and there is always inadequate annotation of genes. These considerations makes it difficult to have good analysis of the microarray results for the pathways. Therefore we tried to search for general pathways and species specific pathways. Whenever, data from rat genome was not available, we searched other species database and compared with reference pathways used in analysis.

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3.4 Validation of array data:

Following to microarray data analysis a set of genes were chosen for validation by qPCR based on their degree of expression change. Correlation analysis was done between the microarray and qPCR results and statistical significance was determined. For the microarray, the data input into the correlation analysis was the Log2 ratio value of the weighted average for each gene on the composite array representing all replicate animals. For qPCR, we used the mean Log2 ratio value reported by qPCR from all replicate animals. Prior to performing correlation analyses, the data were tested for normality using the Shapiro-Wilk test. Because the data was not normally distributed, Spearman's Rho was used. Spearman's Rho is the rank-based non-parametric equivalent of the more commonly used Pearson's correlation calculation. The effects of Ct, array spot p value, degree of change, direction of change, and array spot intensity on correlation were investigated by binning subsets of genes according to these criteria. Oneway ANOVAs were then used to determine the relationship between the observed correlations. All statistical analyses considered a p value of 0.05 significant and were performed using SPSS version 12.0.

4. Conclusions:

Our microarray analysis revealed that many of the fundamental and physiological processes and genes are regulated differentially in brain and liver tissues in response to REM sleep loss. This implies the general idea of body responding to the stress created by REM sleep loss, where liver seems like counter attacking the stress response of the body. In general, brain cells were found more responsive and affecting processes like apoptosis, learning and memory, oxidative stress and circadian rhythms in response to REM sleep loss, while hepatocytes were found more affected with processes like protein synthesis, stress balance and detoxification processes. This study provides an excellent platform to visualize the

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effects of the REM sleep loss in system and can be further extended as a basic platform for further studies.

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Conflict of Interest:

The authors have no conflict of interest among themselves and all the funding and scientific contributions are fully acknowledged.

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Figures:

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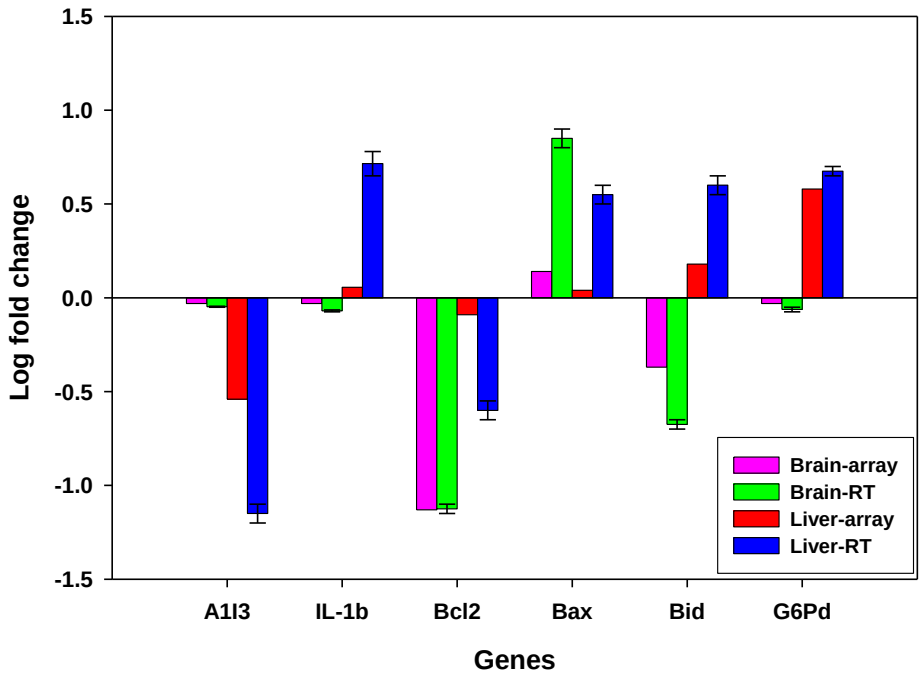

Fig.1: Comparative expression of candidate genes from brain and liver using real-time PCR and microarray.

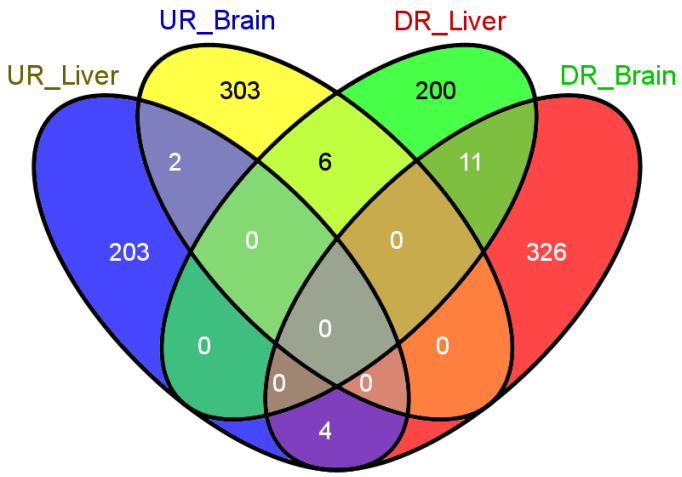

Fig.2: Schematic representation of genes regulated after rapid eye moment sleep deprivation in liver and brain. UR_Liver: upregulated liver; UR_Brain: upregulated brain; DR_Liver: downregulated liver; DR_Brain: downregulated brain. Number in different shaded panel represent the genes commonly affected in both the tissues either in similar direction or opposite.

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Fig.3: Comparison of biological processes getting differentially up-regulated (A) and down-regulated (B) in brain and liver after rapid eye movement sleep deprivation in rats.

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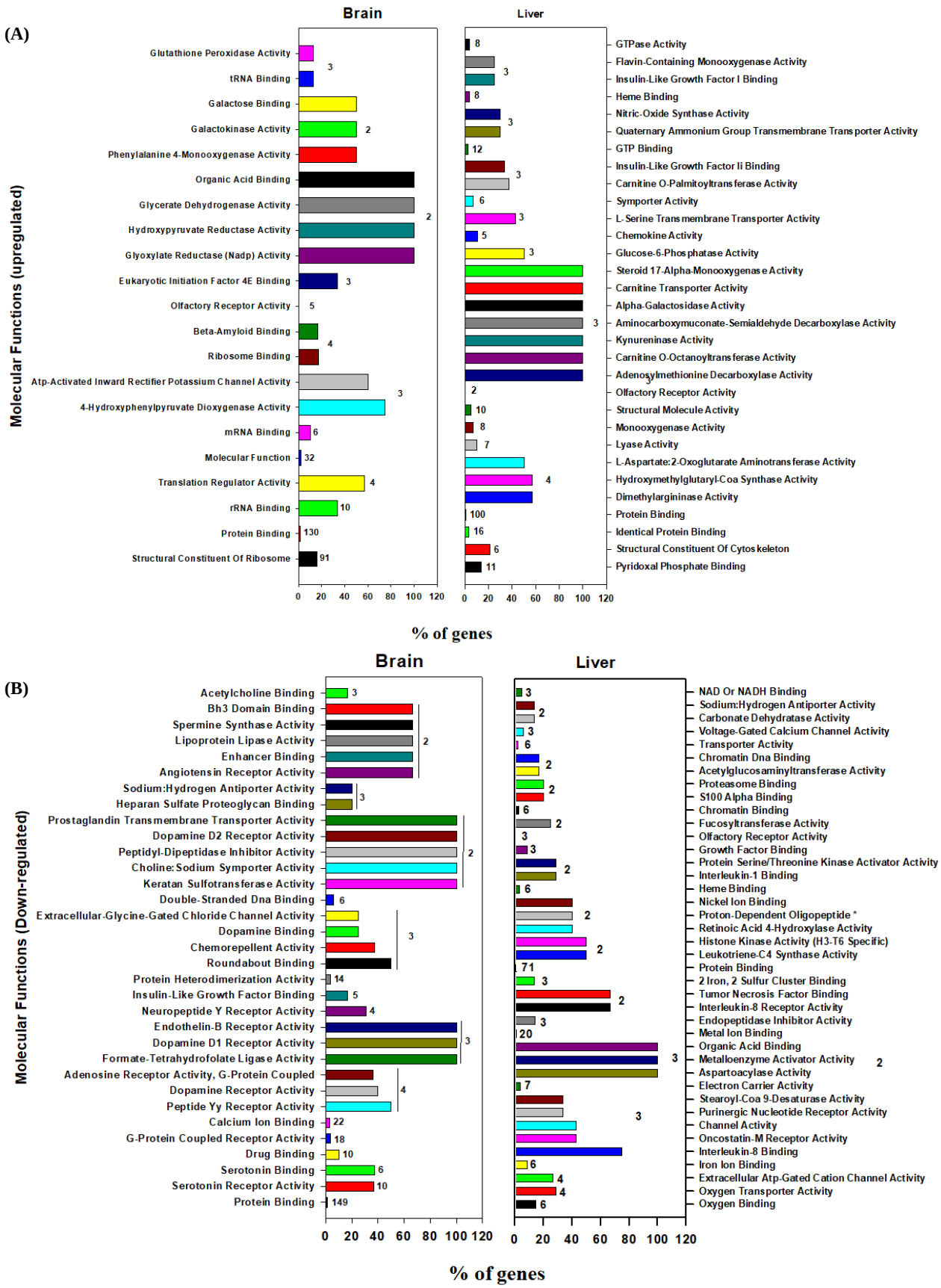

Fig.4: Comparison of molecular functions getting differentially up-regulated (A) and down-regulated (B) in brain and liver after rapid eye movement sleep deprivation in rats.

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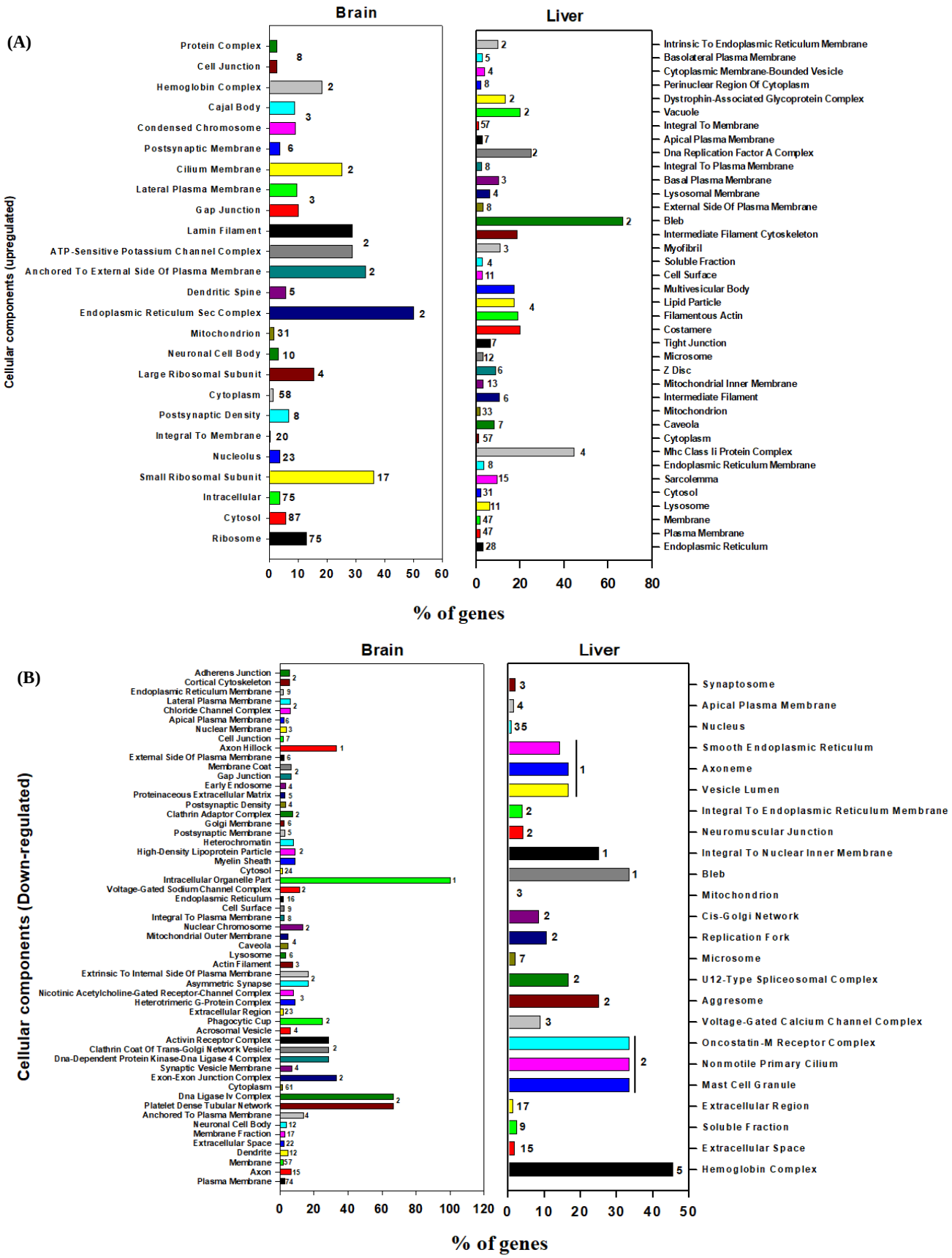

Fig.5: Comparison of cellular components getting differentially up-regulated (A) and down-regulated (B) in brain and liver after rapid eye movement sleep deprivation in rats.

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Supplementary Figure:

Table S1: Number of genes getting either up-regulated or down-regulated more than 1.8 fold compared to control.

S. No	Comparison	Fold Change	# Up-regulated Genes	# Down-regulated Genes
1	Control vs Sleep Deprived Liver	1.8	209	217
2	Control vs Sleep Deprived Brain	1.8	311	341

Table S2: List of real time PCR gene primers used in microarray validation experiments.

S.N	**Primer/probe**	**Primer/probe sequence ID's**
1	Alpha 1 inhibitor 3	Rn00440636-m1
2	Interleukin-1b	Rn00580432_m1
3	TP53	Rn00755717_m1
4	Bcl2	Rn99999125_m1
5	Bax	Rn02532082_g1
6	Bid	Rn01459517_m1
7	G6pD	Rn01529640_g1
8	GAPDH	Rn01749022_g1
9	Master mix	Cat. 4304437

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Table S3: List of common genes affected in brain and liver

(A) Common genes "down-regulated in brain" and "down-regulated in liver":

Gene Symbol	Accession No.	GO term molecular/biological functions
Wee1	NM_001012742	Protein tyrosine kinase activity
Slc2a12	NM_001107451	Carbohydrate transmembrane transporter activity
Hrk	NM_057130	Apoptosis regulation, Bleb assembly
Fam110b	NM_001024341.1	---N.A.---

(B) Common genes "up-regulated in brain" and " down-regulated in liver":

Gene Symbol	Accession	GO term molecular/biological functions
Hba-a2	NM_013096	Heme binding, Oxidoreductase activity, Drug transport
Hba-a2	NM_013096	Heme binding, Oxidoreductase activity, Drug transport
Mup5	NM_203325.1	Fatty acid biosynthetic process, Catalytic activity

(C) Common genes "down-regulated in brain" and " up-regulated in liver":

Gene Symbol	Accession	GO term molecular/biological functions
RT1-Da	NM_001008847	Response to stimulus
Zbtb6	NM_001108953	--N.A.--
Tmem106b	NM_001004267	--N.A.--