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Heat Shock Protein Expression in Sleep Deprivation and Recovery Sleep

A Thesis

Presented to

The Faculty of The Department of Biological Sciences

San Jose State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Sciences

By

Deepali Rishipathak

May 2003

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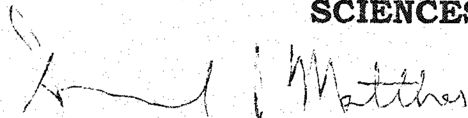
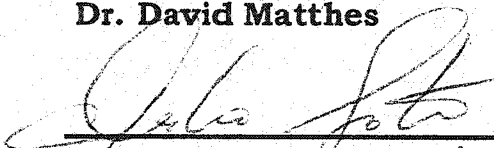


Dr. David Matthes



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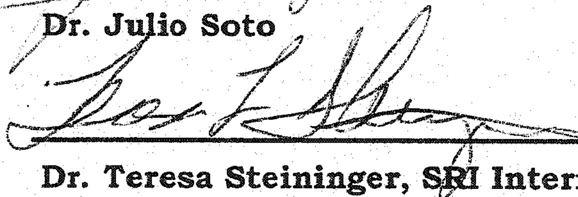

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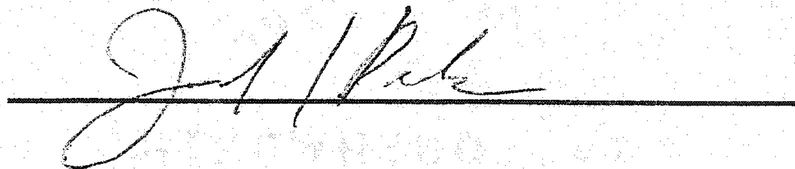

ABSTRACT

Sleep is a dynamic brain process. Several studies have found that the expression of genes changes during sleep deprivation. Few studies have examined the expression of genes during recovery sleep. Studies in our laboratory have identified candidate genes, which change during sleep deprivation and recovery sleep. The candidates for this study are glucose regulated proteins GRp94, GRp78 and endoplasmic reticulum protein ERp72. mRNA analysis shows that these genes are up regulated in the cerebral cortex and medulla. Immunohistochemical techniques were employed to study their protein expression. Two different conditions were studied: sleep deprivation, recovery sleep and controls at the equivalent time points. It was found that all three proteins were up-regulated in the cortex and only GRp94 proteins were up-regulated in the medulla. Functional differences between the cortical and medullary areas may suggest that different proteins are required for sleep and waking.

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TABLE OF CONTENTS

Section	Topic		Page
1	**Introduction**		1
		Sleep Physiology	1
		Sleep In Rodents	5
		Sleep Deprivation and Recovery Sleep	6
		Effects of Sleep Deprivation in Rodents	10
		Gene Expression Changes During Sleep Deprivation and Recovery Sleep	12
		Heat Shock Proteins	14
2	**Purpose**		20
3	**Research design and Methods**		22
		Mouse Sleep studies	22
		Immunohistochemistry	23
		Quantitative Analysis	25
		Cerebral Cortex Analysis	25
		Medulla Analysis	26
		Statistical Analysis	27
4	**Results**		28
		Cortex	28
		Dorsal Cortex	30
		Lateral Cortex	30
		Medulla	31
5	**Discussion**		35
		Long term studies	39

LIST OF FIGURES

1	The various stages of sleep to REM sleep, represented from the EEG traces	4
2	A typical Sleep Pattern in Rodents	5
3	Delta power Changes During Recovery Sleep after sleep deprivation	8
4	Effect of 6h sleep deprivation in mice	11
5	HSp Family Gene Expression Changes Across Mouse Brains, panel A, sleep deprivation and panel B, recovery sleep	19
6	The diagrams depict coronal sections at the three Levels of cerebral cortex chosen for this study, rostral, mid-rostral and caudal	26
7	GRp78 Immunoreactive Neurons in the Lateral Cortex	29
8	GRp94 Immunoreactive Neurons in the Dorsal Medulla	32
9	Graphical representation of cell counts in Cortex and Medulla, for all 4 conditions	34

INTRODUCTION

Sleep physiology

In humans, sleep is divided into two main stages, non rapid eye movement sleep (NREM) and rapid eye movement sleep (REM). These phases progress in a cycle from NREM to REM sleep and this cycle is repeated with a cycle length of ~90-120 minutes (Herman et al., 1984).

Sleep stages can be determined by polysomnography (Fontaine, 1989; Lavie, 1981). The main polysomnographic variables include, the electroencephalogram (EEG), which measures brain activity in the cortex, the electromyogram (EMG), which measures muscle tone and the electrooculogram (EOG), which measures the eye movements. A polygraph records voltages generated in the various tissues, which are in the micro-volt range. These are amplified and are displayed on a polygraph.

Wakefulness is characterized by desynchronized EEG, low voltage of about 10-30 micro-volts and high frequency (16-25 Hz) activity in the EEG, high tonic EMG activity, and extensive, coordinated eye movements. Drowsiness also shows alpha activity, the sinusoidal pattern with about 20-40 micro-volts and 8-12 Hz frequency. The transition from wakefulness to Stage 1 sleep results in slowing of the frequencies recorded in the EEG.

The four stages of NREM sleep are primarily characterized by the amplitude and frequency of EEG and EMG activity (Kryger et al., 1989). Sleep begins by descending from wakefulness to Stage one, a light sleep. In this stage, the muscles relax and the EEG shows high amplitude and low frequency brain waves at about 3-7 Hz. Rapid eye movements are absent, but slow rolling eye movements appear. The EMG is moderate to low. This stage comprises about 2 to 5 percent of sleep. In Stage 2, the brain waves become larger, with bursts of electrical activity. It is characterized by continuous low voltage and mixed frequency activity. Bursts of distinctive, 12-14 Hz sinusoidal waves called sleep spindles appear in the EEG (Hofle et al., 1997). Eye movements are rare, and the EMG is low to moderate. Stage 2 sleep comprises about 45 to 55 per cent of sleep. The cycle then progresses into deep sleep, Stages 3 and 4, where the EEG shows high amplitude, >75 mV and low frequency waves in the delta frequency (0.5-2 Hz). Stage 3 comprises about 3 to 8 per cent of sleep. During stage 4, there is a quantitative increase in delta waves. This stage comprises about 10 to 15 percent of sleep. This stage is very responsive to the amount of prior wakefulness (Kandel and Schwartz, 1985).

After about 80-90 minutes, sleep progresses into a highly active stage characterized by rapid eye movements, hence the name REM sleep. During REM sleep, the EEG shows low amplitude and high frequency

waves, similar to that seen during wakefulness. The EMG is virtually absent, but many small muscle twitches along with the other indicators of autonomic activity, may occur against this low background. The mental activity of human REM sleep is associated with dreaming, which depends on the internal sleep-waking rhythm (Islas-Marrooquin and Delgado-Brambila, 1998). About 75 percent of the night is spent in NREM sleep and about 25 percent is spent in REM sleep. The first third of the night is generally characterized by NREM sleep, which is usually 75 to 80 percent of sleep, whereas the latter third of the night is predominantly REM sleep, which is about 20 to 25 per cent of sleep. The various stages of sleep are represented in Figure 1.

A central circadian pacemaker, located in the suprachiasmatic nucleus in the brain is the regulator of physiology and behavior. This pacemaker is well adapted to the environmental settings and it synchronizes to the environmental time. This is an important factor of the biological clock for the proper timing of species-specific behaviors. Exposure to light during day and darkness at night is critical for the proper functioning of the circadian clock and maintaining the circadian rhythm during the 24-h time period. An exposure to even an equivalent of candlelight during wakefulness is sufficient to maintain the circadian entrainment to 24h day (Wright et al., 2001).

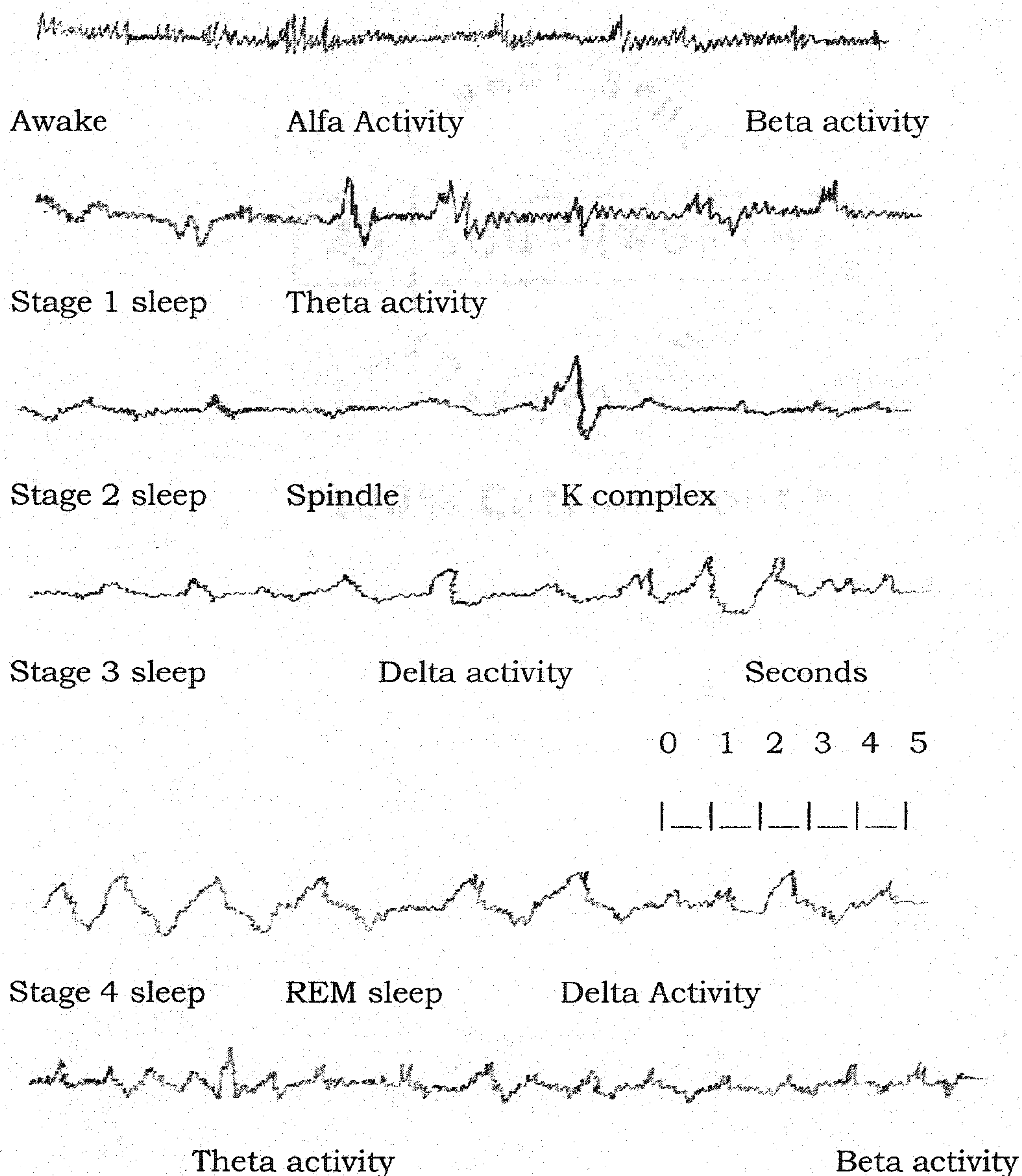

Figure-1. The various stages of sleep to REM sleep, represented from the EEG traces (Antrobus, 1993). The various EEG frequencies such as alpha, beta, theta, delta, etc. are a characteristic of the individual stages and they are presented at that particular stage.

Sleep in rodents

Rodents are nocturnal animals, which sleep during the day and are awake at night. The total daily sleep time of laboratory animals is 13 hours and the sleep cycle length is 11 minutes (Kryger et al., 1989). The typical sleep pattern in rodents is depicted in Figure 2, where the differences in measurements of the EEG and EMG during sleep and wakefulness are shown.

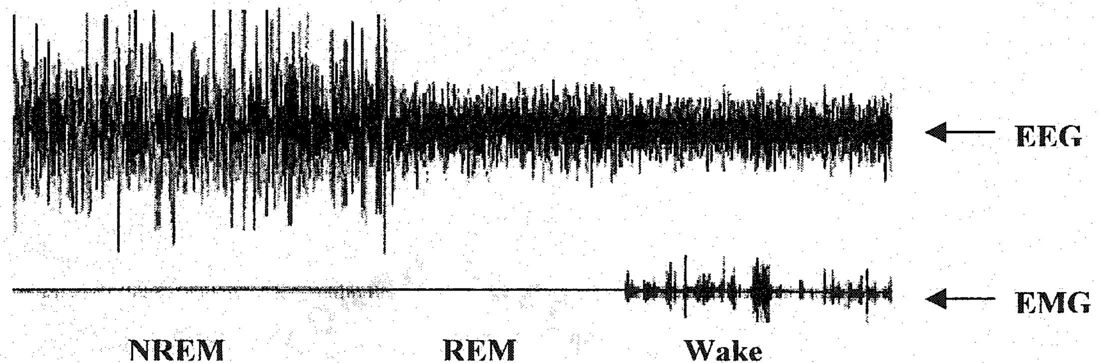

Figure-2. A typical sleep pattern in rodents. The EEG during NREM sleep shows high amplitude and low frequency waves, during REM and wakefulness the EEG shows low amplitude and high frequency waves. The EMG during sleep shows high amplitude whereas during Wakefulness it shows low amplitude.

The typical sleep pattern of rodents, like humans, consists of cycles of wakefulness, NREM and REM. During wakefulness the EEG waves are of low amplitude and high frequency waves. When sleep progresses into NREM sleep, EEG waves become high amplitude and low frequency. During REM sleep, EEG patterns are similar to those during wakefulness comprising of high frequency and low amplitude waves. The EMG patterns during wakefulness are of high amplitude. During NREM sleep, the EMG is of low amplitude, and during REM sleep, the EMG is typically flat, due to the active motor inhibition occurring during this state. Each cycle of sleep is approximately 11 minutes long.

Sleep deprivation and recovery sleep

The maintenance of optimal performance is dependent on the neurobiological mechanism that facilitates sustained alertness and attention. A major factor in the maintenance of sustained alertness is optimal sleep on the preceding night.

Sleep is a regulated physiological response. Short-term sleep deprivation in humans have resulted into decreased glucose tolerance and increased blood pressure (Ayas et al., 2003). A previous finding suggests that sleep periods reduced by as little as 1.3 to 1.5 hours for 1 night result in reduction of daytime alertness by as much as 32% as measured by the Multiple Sleep Latency Test (Bonnet and Arand, 1995; Carskadon and dement, 1979). A long wakefulness period or sleep

deprivation impairs cognition, and prolonged sleep deprivation results in impaired physiological function. Research on cognitive performance after sleep deprivation has reported that total sleep deprivation may cause monotonic decrease in performance of a broad range of variables including vigilance, reaction time, short-term and long-term memory, arithmetic computations, psychomotor tasks, and logical reasoning task (Jeong et al., 2002). Failure to perceive and respond to critical visual and auditory information correctly has also been reported from this study. REM sleep deprivation increases cortical excitability, alters food consumption and may increase stimulus-evoked aggressive behavior. The study has also reported to impair memory of past events and acquisition of new data (Mendelson, 1987) suggesting that REM sleep deprivation has serious psychological consequences. It is hypothesized that sleep protects against fatigue and enhances memory. Sleep is not caused due to a global effect of fatigue on the brain, but is a specific effect on the brain regions involved in a particular task the body performs (Maquet et al., 2002). More time spent in NREM sleep helps in the recovery process, also suggesting the importance of NREM sleep. It is also known that deprivation of a stage in sleep leads to an excessive amount of that stage during recovery sleep (Mendelson, 1987).

One of the most important functional parameters of EEG, known as the slow wave activity or Delta power, refers to the quantity of EEG

waves in the delta frequency range of approximately 0.5 to 4.5 Hz (Borbely and Achermann, 1983) . Delta power has a quantitative and predictive relationship with prior wakefulness. Sleep loss induces an increase in Delta power during subsequent sleep and excess sleep induces a decrease (Franken et al., 2002). Delta power is thought to reflect the need for slow wave sleep and its underlying homeostatically regulated recovery process. Delta power declines exponentially in the course of daily rest period and is enhanced after prolonged wakefulness. These changes are thought to reflect the homeostatic process underlying slow wave sleep regulation (Franken et al., 1999). Figure 3 represents the Delta power changes during sleep and recovery sleep.

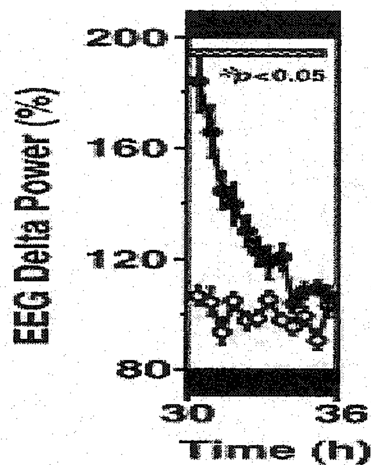

Figure-3. Delta power changes during recovery sleep after sleep deprivation. Increased EEG delta power in male C57BL/6 mice (n=10) is seen during the first 6h of recovery sleep. During recovery sleep following sleep deprivation, delta power increases to a range of 160% to 200%, which gradually decreases.

While short duration (12 h or less) of sleep deprivation results in a rebound of NREM sleep, a longer sleep deprivation (24 h) results in a rebound of REM sleep, and also increased the rate of entry to REM sleep. This study suggests that the amount of REM sleep lost during sleep deprivation predicts the subsequent REM sleep rebound during recovery sleep (Franken, 2002).

The NREM sleep rebound is seen following sleep deprivation in humans, and in many species of animals including cats, rabbits, rats, hamsters, mice, chipmunks, ground squirrels, dogs and dolphins (Huber et al., 2000) . The large proportion of our lifespan is spent in the sleep state and the ubiquity of sleep in mammalian species suggest that some essential physiological function occurs during sleep that cannot be fulfilled during wakefulness. These various considerations have led to a search for parameters of sleep that vary systematically with time spent awake.

Neurons in many different regions of the brain exhibit changes in activity related to the states of sleep and wakefulness. During NREM sleep, many neurons in the brain exhibit a reduced mean discharge rate. During tonic REM sleep, the mean firing rate in many regions increases compared with NREM or wakefulness. The overall metabolism of brain tissue is greatly decreased in most brain areas during NREM sleep. Several approaches have been taken to study the molecular basis for

sleep. One hypothesis that is being investigated is that sleep serves a restorative function that is that cellular constituents become depleted during wakefulness (Pompeiano et al., 1994) . Such constituents might include cellular energy stores such as glycogen or adenosine (Alanko et al., 2003), or that membrane ion gradients must be restored, such as the sodium-potassium gradient, also that macromolecules such as lipids, co-factors, proteins, etc. may be restored.

The focus of the present study is to examine the compensatory changes in gene expression in the brain that occur as a consequence of perturbation of sleep homeostasis. The hypothesis to be examined is that the processes that occur during sleep most likely involve a change in macromolecular synthesis that facilitates neuronal recovery or restoration from prior waking activities. Several genes are known to be differentially expressed in the various brain regions during sleep deprivation and during recovery sleep.

To study the biochemical nature of sleep homeostasis and to further understand the molecular basis of sleep, it is important to study those genes that are up regulated during sleep deprivation and during recovery sleep.

Effects of sleep deprivation in rodents

Previous studies have examined the effects of sleep deprivation using rodent models. Rats and mice, when deprived of sleep for 6 h and

then allowed to sleep for a few hours are known to sleep intensely and for longer period of time. These animals sleep for 90% of time as opposed to the normal 70 to 80 % of time. They would then resume their normal sleep routine. Total sleep loss of few hours elicits a homeostatic increase in sleep duration and intensity in rats and also in humans (Cirelli et al., 1995) . The effect of sleep deprivation and recovery sleep on mice is depicted in Figure 4.

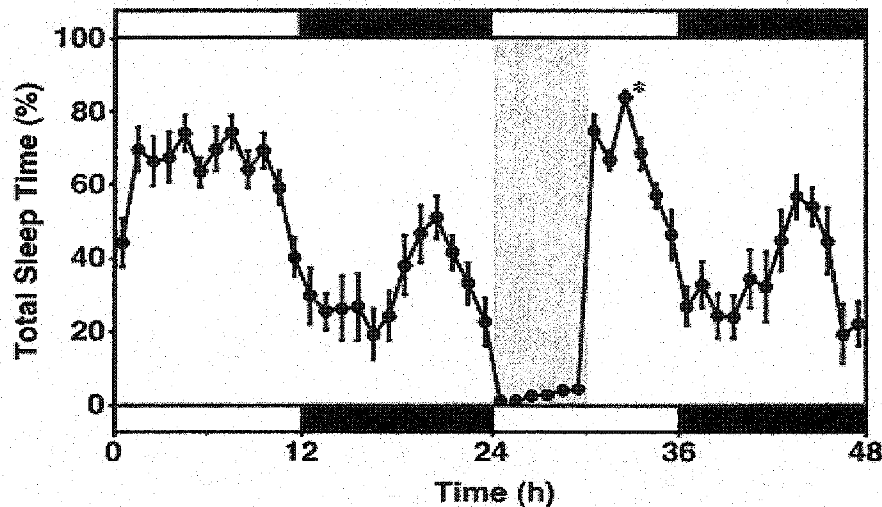

Figure-4. Effect of 6h sleep deprivation in mice. The total sleep time in male C57BL/6 mice (n=8) over day/night, with a comparative effect between 6h sleep deprivation and subsequent recovery sleep following sleep deprivation, suggesting the increase in intensity of sleep, hence called recovery sleep.

This figure shows the total sleep time over a 24h day. These mice under study sleep for approximately 12-13h during daytime and the total sleep time is reduced considerably during night. When they are deprived of sleep for 6h, the effect of sleep deprivation is enhanced and the total

sleep time is considerably increased. This is thus the recovery sleep, where some additional time is spent in sleep to recover from the previous sleep deprivation.

Gene expression changes during sleep deprivation and recovery sleep

Although molecular studies of sleep are in their infancy, there are four experimental approaches that have been used to study gene expression and the sleep/wake cycle: 1) gene expression associated with sleep deprivation, 2) gene expression associated with recovery sleep after sleep deprivation, 3) gene expression in association with spontaneous variation in arousal state, and 4) gene expression associated with drug-induced sleep. The proposed research will focus mainly on the first two parameters.

A study shows that about 10,000 genes are expressed in the cerebral cortex of rats after 8 hours of sleep, spontaneous waking, or sleep deprivation (Cirelli and Tononi, 2000). This study provides a comprehensive evaluation of changes in gene expression as a function of behavioral state, but only in the cortex and only in rats. Two approaches were taken for this study, mRNA differential display to screen random mRNA samples and cDNA microarray technology to screen 1176 known mRNAs. In a similar study, the expression pattern of about 7000 genes was analyzed in the cerebral cortex of rats after 3 hours of spontaneous

sleep, spontaneous waking and sleep deprivation respectively (Cirelli and Tononi, 1999) . Studies have focused cerebral cortex, which appears to be most significantly affected by sleep deprivation in humans. It is the target of the restorative effects of sleep according to several studies about the functions and the local mechanism of sleep. Among several parts of the brain, only cortex is the site of cognition, memory and perception. Sleep deprivation can modify gene transcription and translation in the brain and induce the production of several substances that have been considered as potential sleep factors (Cirelli et al., 2000).

The interest of the laboratory and this research is to study the molecular basis of sleep. The experimental approach taken include, identifying the genes involved in sleep. For this, some candidate genes that increase expression with sleep deprivation and recovery sleep were identified using DNA arrays. Animals were sleep deprived for 6 h and control animals were sleeping normally. After sleep depriving animals they were allowed to sleep for 4 h, called recovery sleep and control animals were sleeping normally. Brain samples for these conditions related to sleep were collected. cDNA from the different brain regions like basal forebrain, cerebral cortex, thalamus, hypothalamus, pons, cerebellum and medulla was hybridized to DNA arrays. Candidate genes chosen from the arrays were confirmed for their expression using Taqman real time PCR. These genes were then studied for protein

expression using immunohistochemistry techniques. Whole brain samples were used to identify the specific localization of the candidate proteins chosen.

Heat-shock proteins

Heat shock proteins are a family of molecular chaperones that facilitate the folding of most newly synthesized proteins in the cell (Cirelli and Tononi, 2000). Heat shock proteins are induced by denatured proteins produced during heat shock and also during ischemia and other stresses induce these heat-shock proteins [HSps) (Sharp et al., 1999)· HSps belong to the family of ubiquitous polypeptides that have been highly conserved from yeast through humans. They have been found in plant, yeast, bacterial and mammalian cells. HSps bind to exposed peptide sequences of unfolded proteins and facilitate a renaturation or repair process. In addition, HSps may bind to abnormal proteins to disrupt or prevent their aggregation, which could lead to protein precipitation. HSps may also target abnormal proteins for breakdown by proteolysis. A second major function of HSps, particularly the glucose-regulated (GRp) and endoplasmic reticulum-associated (ERp) proteins is in the genesis of new peptides. These HSps bind to newly formed proteins where they may assist in the precise folding and assembly of newly synthesized proteins (Hamos et al., 1991).

HSps have been relatively little studied in the field of neuroscience of sleep. In one study, it was shown that following a neurotoxic stimulus like hyperthermia, ischemia, etc. HSp70 were expressed, suggesting a neuroprotective role in the brain (Rajdev and Sharp, 2000). Another study on HSp70 was done on Spermophilus lateralis, a diurnal ground squirrel (Bitting et al., 1999). Spermophilus lateralis is a seasonal hibernator. It was found that HSp70 expression was low during daytime and also during hibernation and it remained low during hibernation. This result suggests that the expression of a highly conserved HSp70 gene expression involved in protection from cellular stress, changes with the arousal state of animals. Recent studies have shown that heat shock and viral overproduction of HSps protects the brain cells against injuries that produce necrosis and apoptosis. The heat-shock proteins and the glucose-regulated proteins (GRps) provide molecular markers of specific types of cell stress. GRps belong to the highly conserved family of stress proteins, which act as molecular chaperones (Barnes and Smoak, 1997). The GRps are induced by a variety of stimuli, including glucose deprivation, glycosylation inhibitors and depletion of endoplasmic reticulum (ER) Ca^{++}. The major GRps include, GRp78 and GRp94, which are proteins of molecular weights 78,000 and 94,000 and they share an amino acid sequence identity to the HSp70 and HSp90 proteins respectively (Little et al., 1996). The GRps are localized to the ER, a

major store of the intracellular Ca^{2+} ions. Both the GRp78 and the GRp94 proteins are calcium-binding proteins and GRp94 has been shown to bind Ca^{++} ions in a high-capacity but low-affinity manner. In a study it was shown that Ca^{2+} in the presence of ATP stimulates the release of GRp98 and ERp72 (Nigam et al., 1994) . Some particular types of HSps and GRps could protect against specific types of injury or against a variety of pathological processes. It has been reported that GRp78 and GRp94 genes are up regulated following kainate treatment, cerebral ischemia or head trauma in adult rat brains. In another study molecular chaperones like GRp78, GRp94 and ERp72 were identified and characterized to assist in the folding of a secretory protein (Linnik and Herscovitz, 1998) . These proteins have been found to associate intracellularly with nascent polypeptides such as thyroglobulin, thrombospondin, major histocompatibility complex class II molecules and human chorionic gonadotropin subunit. It was found from this study that GRp94 and ERp72 interacts with both early and more advanced folding intermediates of the secretory protein and they mediate its folding into a secretion-competent form. Another study on GRp78, GRp94 and ERp72 has detected these proteins in complexes with denatured or incorrectly folded proteins and other chaperones. This study indicates that GRp78 and GRp94 associate in the endoplasmic reticulum and that ERp72 functions in association with other

chaperones like GRp78 and GRp94, to mediate correct protein folding in the endoplasmic reticulum (Dhahbi et al., 1997) . Although they have distinct cytoplasmic localizations, ERps and GRps have been proposed to serve similar proteins and translocation of both normal and damaged proteins.

Studies in our laboratory

Previous findings have identified some candidate heat shock proteins for future studies, GRp94, GRp78 and ERp72 (Terao et al., 2001). According to the Taqman real time PCR results, GRp94 is up-regulated only in the basal forebrain during sleep deprivation, but is expressed in the cortex and the medulla regions during recovery sleep. The GRp78 gene is up regulated in the cortex, basal forebrain, the pons and the medulla regions during sleep deprivation and remains up-regulated during recovery sleep in the cortex and the medulla. The ERp72 gene shows up regulation in the cortex and the basal forebrain regions, but during recovery it is expressed only in the cortex region of the brain. Table 1 shows the summary of results from Taqman studies done in our laboratory.

A pictorial representation of mouse brain, to summarize the HSp family gene expression changes is represented in figure 5. In this figure, A represents the sleep deprived mouse brain and B represents the sleep recovered mouse brain.

NAME OF GENE	CONDITION	CTX	BF	THAL	CB	HYP	PONS	MD
ERp72	SD	↑↑	↑↑	-	-	-	-	-
	RS	↑	-	-	-	-	-	-
GRp78	SD	↑↑	↑↑↑	-	↑↑	-	↑↑	↑↑
	RS	↑	-	-	-	-	-	↑↑
GRp94	SD	-	↑↑	-	-	-	-	-
	RS	↑↑	-	-	-	-	-	↑↑↑

TABLE 1: Results of the Taqman real time PCR studies done in our laboratory. The three genes studied include the ERp72, GRp78 and the GRp94. A comparison between the sleep deprived animals (for 6 hours) and the recovered animals (allowed to sleep for 4 hours after sleep deprivation) for the different regions of the brain have been presented here. The arrows show the up regulation of the genes in that particular brain area and the number of arrows represent the magnitude of gene expression changes.

KEY: -, No significant change; ↑, 1-1.5 fold increase; ↑↑, 1.5-2.5 fold increase; ↑↑↑, 2.5-3.5 fold increase; ↑↑↑↑, 3.5-4.5 fold increase; ↑↑↑↑↑, 4.5-5.5 fold increase; ↑↑↑↑↑↑, 5.5-6.5 fold increase; ↑↑↑↑↑↑↑, 6.5-7.5 fold increase; ↑↑↑↑↑↑↑↑, 7.5-8.5 fold increase.
CTX, Cortex; BF, Basal forebrain; THAL, Thalamus; CB, Cerebellum; HYP, Hypothalamus; MD, Medulla; SD, Sleep deprived; RS, Recovery sleep.

The seven major brain regions at their tentative locations are shown. The number of arrows shows the mRNAs for the GRps and ERps expressed in the specific areas of the brain.

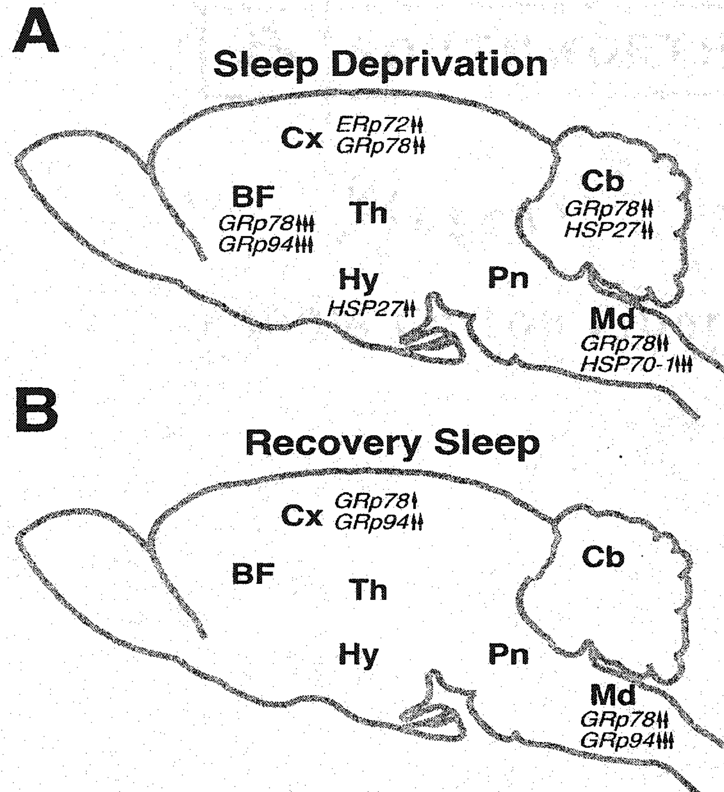

Figure-5: HSp family gene expression changes across mouse brains, panel A, sleep deprivation and panel B, recovery sleep. The seven different areas are shown and the number of arrows indicates x-fold changes as observed from Taqman real-time PCR analysis.
Key: ↑, gene is significantly upregulated but magnitude is 1.5 fold or less; ↑↑, indicates gene is significantly upregulated and magnitude is between 1.5-2.5 fold; ↑↑↑, indicates gene is significantly upregulated and magnitude is between 2.5-3.5 fold.

PURPOSE

The objective of the proposed research is to examine the role of heat shock protein expression associated with normal sleep, sleep deprivation, and the homeostatic response to sleep deprivation. The research will be done to test the following hypothesis.

Certain heat shock proteins are localized to the specific regions of the cortex and medulla that play a significant role in sleep and waking. This facilitates neuronal recovery or restoration of sleep.

The specific aims of this research proposal are as follows:

1. Localize the protein products of candidate sleep-regulated genes – members of the heat shock protein family.

- Perform single-label immunohistochemistry on brain sections from mice that were sleep deprived or have undergone recovery sleep, plus the appropriate circadian time-matched controls, to localize the expression of GRp78, GRp94, and ERp72 under these conditions.

2. To determine whether these proteins are upregulated in cortex and medulla following sleep deprivation and recovery sleep in concert with mRNA levels.

- Perform quantitative analysis of GRp78, GRp94, and ERp72 immunoreactive neurons in the cerebral cortex and medulla in the four experimental conditions.

Since the candidate genes show up regulation in cortex and medulla, protein expression pattern will be studied in these areas. GRp78, GRp94 and ERp72 are specifically chosen for the present study, as they are upregulated during recovery sleep. This is a novel finding since no earlier studies have looked at recovery sleep.

RESEARCH DESIGN AND METHODS

Mouse sleep studies

Male mice inbred strain C57BL/6 were used for this research. All research was performed in accordance with US Public Health Service guidelines and approved by the Stanford University and SRI International institutional Animal Care and Use Committee. Animals were maintained under an LD12:12 photoperiod with food and water. At 10 weeks of age these mice were surgically implanted with electrodes for electroencephalogram (EEG) and electromyogram (EMG) for sleep analysis. Each mouse was allowed three weeks recovery after surgery and one week of adaptation to the recording cable and chamber.. EEG and EMG recordings were made continuously for 24 h baseline sleep, during 6 h sleep deprivation and during 18 h recovery sleep. Sleep deprivation was initiated at light onset (Zeitgeber Time 0 or ZT0) by disturbing cage bedding around mice, stroking the vibrissae using an artist's brush and, toward the end of sleep deprivation period, stroking fur with the brush only when slow waves were evident on the EEG. After 48 h recording period, mice were kept in recording chambers for 6 days until the experimental day. Arousal states (Wake, NREM, REM) were determined in 10 sec epochs using a computerized algorithm and EEG was digitized for offline spectral analysis. Twenty-four h EEG and EMG were continuously recorded on the experimental day and the animals

were then sacrificed according to experimental protocols. (1) 6 h sleep deprivation at ZT0 followed by sacrificed at ZT6 (n=8); (2) Controls that experienced undisturbed sleep and were sacrificed at ZT6 (n=8); (3) 6 h sleep deprivation initiated at ZT0 followed by 4 h recovery sleep from ZT6 to ZT10 and then sacrificed at ZT10 (n=8); (4) Controls that experienced undisturbed sleep and were sacrificed at ZT10 (n=8).

Mice were sacrificed by transcardial perfusion of 4% paraformaldehyde. The brains were removed were stored in sucrose solution at 4°C for histology studies. Akira Terao; PhD conducted the mouse sleep studies at Stanford University sleep research laboratory.

Immunohistochemistry

Mice were prepared and recorded in a similar manner as they were prepared for sleep studies. At the end of the recording period, mice (n=4 per group) were anesthetized with sodium pentobarbital interperitonially (50 mg/kg) and perfused transcardinally with 14 ml saline, followed by 40-50 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The brains were removed and equilibrated in sucrose (30% in PB), and stored at 4°C until processed.

Brains from 16 mice (n-4 per group) were cut into 30 μm sections on a freezing microtome. A 1:6 series of sections through the entire brain was treated with H2O2 to quench endogenous peroxidase activity. The sections were incubated in primary antisera (rabbit-anti-GRp78

1:2000, Stressgen Biotechnologies Corp, Victoria, BC), (rabbit-anti-GRp94 1:5000, Stressgen Biotechnologies Corp, Victoria, BC) (and rabbit-anti-ERp72 1:5000, Stressgen Biotechnologies Corp, Victoria, BC) in diluent (0.1% Triton X-100, 0.1% casein, and 0.1% sodium azide in 0.01M phosphate buffered saline (PBS) for 66 hours at 4°C with agitation. Sections were rinsed in PBS + 0.2% Triton X-100 (PBST) and incubated in secondary antibody (biotinylated goat-anti-rabbit, 1:200, Vector Laboratories, Burlingame, CA) in 3% normal goat serum (Pel Freeze Biologicals, Rogers, AR) in PBST for 1.5h at RT. Sections were rinsed in PBST and incubated in avidin-biotin-peroxidase complex (ABC, Vector Elite kit, 1:100, Vector Laboratories) diluted in PBST for 45 min at RT. Sections were again rinsed in PBST and then 0.05 M Tris buffer pH 7.6, then reacted for 5 min in 0.05% 3,3' diaminobenzidine (DAB) and 0.01% H_2O_2 in 0.05 M Tris. As a negative control, additional sections were treated similarly, but the primary antibody was omitted. The reaction was stopped with generous rinses of PBS. The sections were mounted onto glass slides (Super Frost Plus, Fischer Scientific), air-dried, dehydrated in ethanol, cleared in xylene and cover slipped. Photomicrographs were prepared with a Spot 2 digital camera and software (Diagnostic Instruments, Inc, Sterling Heights, MI).

Quantitative analysis

Analysis includes quantifying immunoreactive cells in cerebral cortex and medulla regions of brain. Based on the results of the gene expression studies, two cortical regions and three regions of the medulla were chosen for analysis. A comparative study was done from these results and the data from the earlier cDNA microarray and Taqman real time PCR experiments.

Cerebral cortex analysis

Three different rostrocaudal levels of cerebral cortex formation were chosen for the analysis. They were called rostral, mid-rostral and caudal levels (Fig. 6). Immunoreactive cells from dorsal and lateral sides of all three-cortex levels were quantified manually using a 7X7 cm grid and a cell-counter. The grid was placed on grayscale photomicrographs (134X magnification) containing layers II through V of dorsal and lateral cortex. Dorsal cortex was sampled at the midline and lateral cortex was sampled dorsal to the rhinal sulcus. The dorsal and lateral sides were numbered 1 through 6 from rostral through caudal respectively. Immuno reactive neurons for the four conditions (n=4 per group) were counted within the circumscribed region, and counts were average across the 3 rostrocaudal levels. The cell counts were then statistically analyzed.

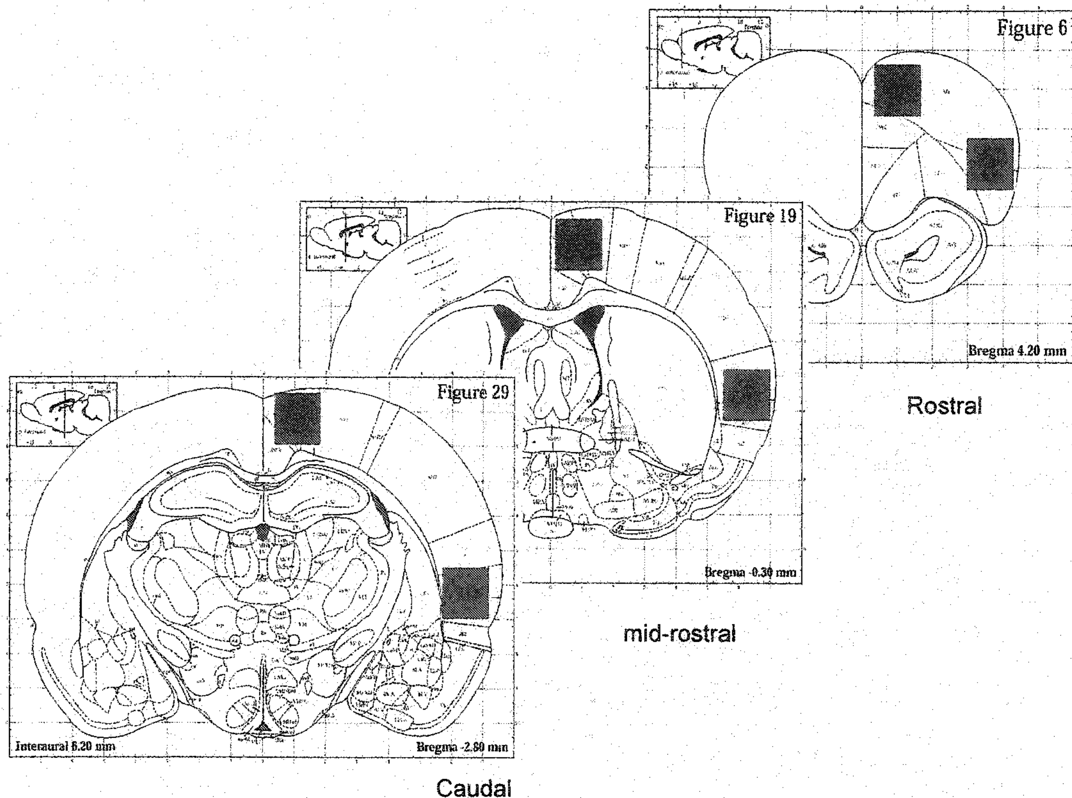

Figure-6. The diagrams depict coronal sections at the three levels of cerebral cortex chosen for this study, rostral, mid-rostral and caudal. The red boxes depict the region of cell counts in the dorsal cortex, and the blue boxes indicate the region of cell counts in the lateral cortex

Medulla analysis

For the medullary analysis, counts of immunoreactive neurons were made within the boundaries of the caudal nucleus tractus solitarius (NTS), dorsal motor vagal nucleus (DMX) and rostroventrolateral medulla (RVL), which were identified by anatomical landmarks on grayscale photomicrographs (189X magnification).

Statistical analysis

The neuronal measurements obtained from the three proteins under study were analyzed using SASW in 8.2 (SAS Institute Inc., Cary, NC). A logarithmic transformation was applied to the data to stabilize the variances (Sokal and Rohlf, 1995). Prior to the transformation, the standard deviations of the measurements were proportional to the mean values of the measurements; after the transformation, the standard deviations were no longer related to the means. The transformed data were initially analyzed by Mixed Model analysis of variance (ANOVA) to determine differences between treatment groups. Since the expression of five genes was measured, alpha was set at 0.01 to control for Type II errors. Where significant differences were indicated between treatment groups, Mixed Model ANOVA by brain region was employed to determine in which brain region(s) significant variation occurred. Finally, for those brain regions in which the mixed procedure indicated significant effects, p values were calculated for the appropriate contrasts (sleep deprivation vs. ZT 6 controls; recovery sleep vs. ZT 10 controls) with alpha corrected for multiple comparisons (Hochberg, 1998).

RESULTS

Results of this research have been published (Terao et al., 2003).

Immunoreactive cells for GRp94, GRp78 and ERp72 proteins were evident throughout the brain in both ZT6 and ZT10 control mice. All the three proteins are located in the cytoplasm hence antibodies used to stain the proteins in the neurons showed cytoplasmic staining. These stained neurons were manually counted and the quantitative data was analyzed. Results of the analysis for the 2 brain regions, cortex and medulla, are described below.

Cortex

A typical immuno-reactive staining for GRp78 neurons in the lateral cortex is represented in figure 7, with a box indicating the grid, which was used to count the immunoreactive cells. In this figure, A and C are the controls brains for sleep deprivation and recovery sleep respectively. B and C are the sleep deprived and sleep recovered brains.

Neurons were observed in all cortical layers in sleep deprivation, recovery sleep and control brains. All three proteins had an increase in number of immuno-reactive neurons in dorsal and lateral layers of the sleep-deprived group. The immunoreactive cells were counted for the three layers, rostral, mid-rostral and caudal cortical levels.

Mixed model ANOVA indicates significant effects of condition for the number of GRp78, GRp94, and ERp72 immunoreactive cells. The

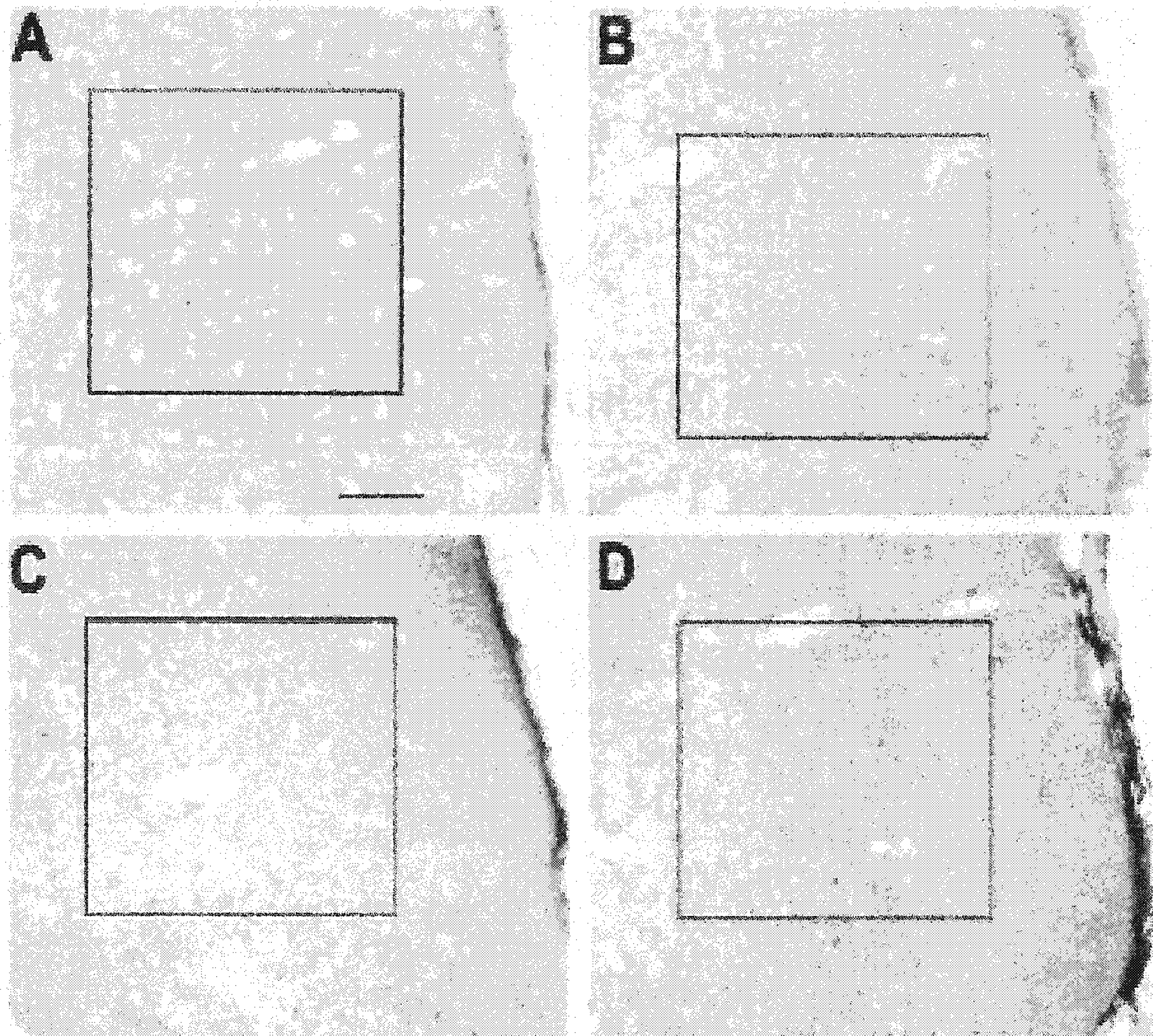

Figure-7: GRp78 immunoreactive neurons in the lateral cortex. A, control mouse sacrificed at ZT6; B, mouse sacrificed at Zt6 after sleep deprivation; C, control mouse sacrificed at Zt10; D, mouse sacrificed at Zt10 after 6h sleep deprivation followed 4h period of recovery sleep. Box indicates the outline of grid used for manually counting the immunoreactive neurons. Scale bar = 100μm.

observed probability value was less than 0.0001 for each gene. The effect of condition was significant for both dorsal and lateral cortex for each of these genes and the probability value was less than 0.02. The results for the dorsal and lateral cortical areas are described below with the significant probability values.

Dorsal cortex

In the dorsal cortex, compared to the time matched controls, the number of GRp78 stained cells in the sleep-deprived group was significantly increased by 35-45% (p=0.002). The number of GRp94 stained cells was also significantly increased [p=0.007]. Similarly, the number of ERp72 cells was significantly increased by 35-45% [p=0.008]. Interestingly, although all proteins were significantly increased in sleep deprived group of animals, compared to the time matched controls, the number of stained cells for any of the three proteins were significantly changed in the recovery sleep group, compared to the time matched controls. The graphical representation of the results is presented in figure 8

Lateral cortex

In the sleep-deprived group, the number of immuno-reactive cells was significantly increased by 21-66% compared to the time matched controls in the lateral cortex. GRp78 [p=0.001], GRp94 [p=0.021] and ERp72 [p=0.004]. In recovery sleep, the number of immunoreactive

neurons for all the three proteins was significantly increased by 31-39%. GRp78 [p=0.05], GRp94 [p=0.001] and ERp72 [p=0.04]. It was observed that increase in the immunoreactive cells in the recovery sleep groups was mainly due to an increased number of cells in cortical layers III-V and not in layer II.

Medulla

A typical immunoreactive staining for GRp94 neurons in the dorsal medulla is represented in figure 8. In this figure, A and C represent the control brains for sleep deprivation and recovery sleep. B and C represent the sleep deprived and sleep recovered brains. The doted lines indicate the regions within the medulla where the immuno-reactive neurons were counted.

As with the cortex, immunoreactive cells were evident in all the medullary regions, for all the four conditions under study. However, significant changes were evident only for the GRp94 immunoreactive cells and not for GRp78 and ERp72 proteins. Mixed model ANOVA results indicate significant effect of condition only for the number of GRp94 immuno-reactive cells; the probability value was less than 0.0001. Mixed model ANOVA also indicates significant effects of condition for the number of GRp94 cells in all three medullary regions, namely NTS, DMX and RVL, [p=0.01]. This result was different than the results observed for the cortex, where significant effects of condition were

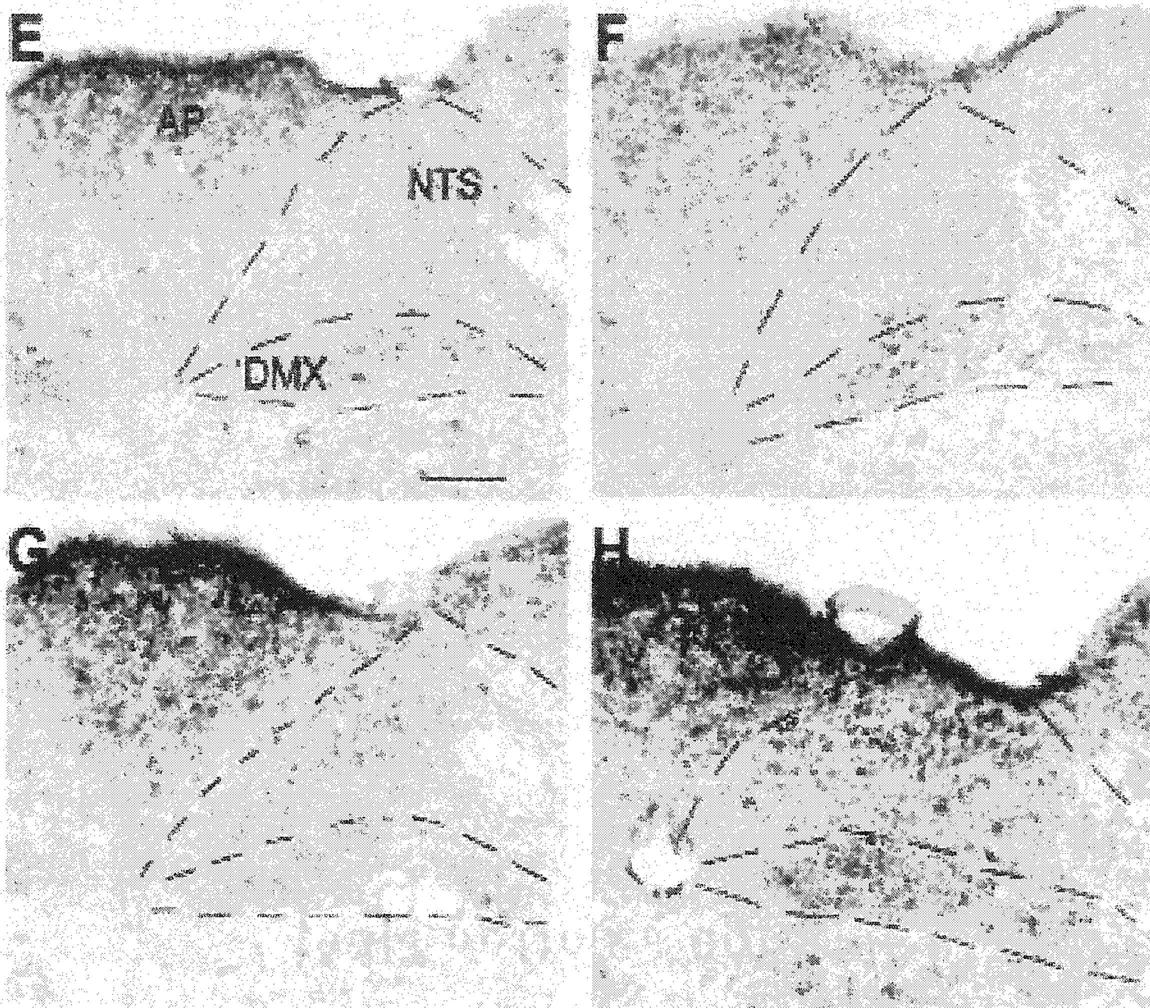

Figure-8: GRp94 immunoreactive neurons in the dorsal medulla. A, mouse sacrificed at ZT6; B, mouse sacrificed at ZT6 after sleep deprivation; C, mouse sacrificed at ZT10; D, mouse sacrificed at ZT10 after 6h sleep deprivation followed 4h period of recovery sleep. Box indicates the outline of grid used for manually counting the immunoreactive neurons. Scale bar = 100μm, where, A, area postrema; CC, central canal; DMX, dorsal motor vagal nucleus; NTS, nucleus tractus solitarius.

observed for all three proteins. The number of GRp94 immuno-reactive cells also showed a significant increase in the recovery sleep group as compared to the control ZT 10 groups. This increase was evident for all three medullary areas. For the DMX region, there was a significant 60.4% increase, [p=0.0003]. For the NTS region, there was a significant increase of 45.6 %, [p=0.0001]. For the RVL region there was a significant increase of 45.9 %, [p=0.0037]. Interestingly this result was also different compared to the results observed in the cortex, where significant changes were observed mostly in the sleep-deprived group.

A graphical representation of the results for all the three proteins in dorsal and lateral cortex and NTS, DMX and RVL layers of medulla has been represented in Figure 9.

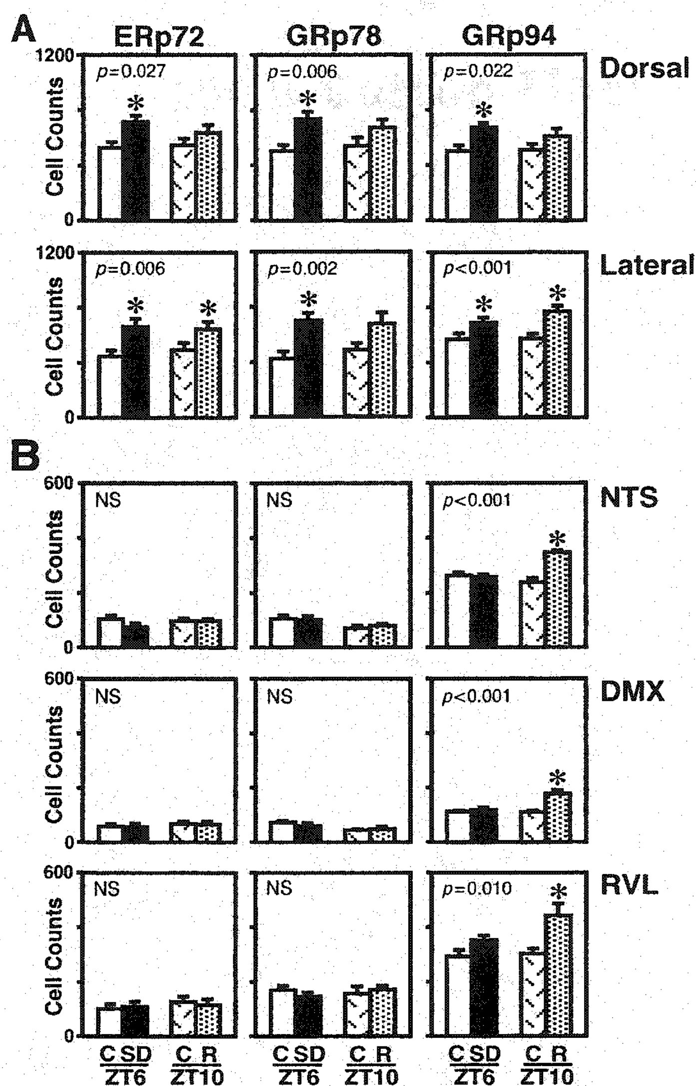

Figure-9: Graphical representation of cell counts in cortex and medulla, for all 4 conditions. A comparison between sleep deprivation and recovery sleep groups for dorsal and lateral cortex, panel A and between NTS, DMX and RVL medulla, panel B is presented. Where significant cell counts were observed, it has been shown with an asterix for that condition and the probability values are shown for each.

Key: NS, Not significant; C, Control for sleep deprivation or recovery sleep ; SD, sleep deprivation; ZT6, Zeitgeber time 6; RS, recovery sleep; ZT10, Zeitgeber time 10; NTS, nucleus tractus solitarius; DMX, dorsal motor vagal nucleus; RVL, rostroventrolateral medulla.

DISCUSSION

Although immunohistochemistry was performed on the entire brain from all 4 groups under study, focus was on the cortex and medulla regions of the brain. All three proteins show significant increase in the cortex during sleep deprivation, both in the dorsal and lateral cortex. GRp94 proteins, which did not show significant increase in mRNA levels in cortex during sleep deprivation, showed an increase in the protein levels. In the medulla region, none of the three proteins showed any significant changes during sleep deprivation in the protein expression, but GRp94 mRNA levels were significantly increased in the medulla.

During recovery sleep, all three proteins showed significant increase only in the lateral cortex, but not in the dorsal cortex. GRp78 and GRp94 mRNA levels were also significantly increased in the cortex. In the medulla region, only GRp94 cells showed a significant increase. This is consistent with the mRNA studies, where GRp94 was significantly increased, along with GRp78 protein levels. Interestingly, GRp94 proteins showed significant increase in mRNA and protein expression levels only during recovery sleep in both, cortex and medulla regions. GRp94 proteins were not affected during sleep deprivation.

The results of this research suggest that neuronal cells are differentially expressed in the various regions of the brain. With a focus on the heat-shock proteins, which are expressed during situations of stress may imply that sleep deprivation may also be causing some stressful situations. This effect of sleep-deprived stress may be pronounced in the brain. As mentioned earlier, heat shock proteins are generally induced due to denatured proteins produced during many pathological conditions. Heat shock proteins and glucose related proteins provide molecular markers of specific types of cell stress. They are generally induced to protect against specific types of injury or other pathological processes. Some of these conditions include ischemia, seizures, etc. The expression of genes encoding HSps and GRps is induced in specific cells or in specific organelles. These stress proteins can be produced either by non-specific stimuli, such as low glucose levels or heme (heme oxygenase-I). The cellular and organelle specificity demonstrates specific functions of these heat-shock or glucose-regulated proteins that could be unique for each stress, cell or organelle. These specific responses provide molecular markers of the type and location of injury in cells. This suggests that some stress proteins could protect against only certain types of stress, but will not protect against any other unrelated types of stress. Other than in response to stress due to pathological conditions, we have now studied that some GRps are

induced in response to sleep deprivation. This is interesting and implies that sleep deprivation acts as a stress on neurons. These specific GRps are induced in the specific sleep deprived neurons and their function as expected may be in re-naturing the proteins. The expression of the specific GRps for protection of neurons may also compel the brain to sleep more intensely, and hence the EEG parameter, the delta power is high (Figure-4). Recovery sleep after sleep deprivation causes induction of some other specific GRps like GRp94, which may play a role in neuronal recovery.

Another important feature of these GRps, which were found in specific locations in the brain, may suggest that they are anatomically restricted. Hence, they were specifically expressed in the cortex and medulla and more so, in the specific regions of the cortex and medulla. There are functionally differences between the regions of the cortex. The dorsal cortex is limbic cortex, associated with processing of emotional and other internal stimuli, whereas the lateral cortex is more related to sensory processing. These functional differences between the two cortical areas may have different proteins or other needs during sleep and waking. In case of medulla regions, the area surrounding the nucleus tractus solitarius (NTS) has been implicated in EEG synchronization and slow wave sleep. Increased expression of HSps and

GRps in the NTS and other medullary regions may be related to EEG synchronization and in turn, also with restorative sleep.

Thus, this research has helped confirm the results obtained from the preliminary studies and has shown that heat shock proteins are expressed during sleep deprivation and recovery sleep. Immunohistochemistry approach helped reveal and understand several parameters of sleep; some of them are listed below.

1. Sleep has a neuronal function.
2. Certain heat shock proteins are expressed in particular areas of the brain, which may be the vital regions involved in sleep.
3. The exact localization of the heat shock proteins like GRps and ERps in the specific areas of the brain.
4. The protein expression levels through the different rostro-caudal regions of the brain by performing immunohistochemistry on the entire brain.
5. The protein expression pattern in the specific regions within the cortex and medulla.
6. The quantitative data for the expressed proteins was generated by manually counting the immuno-reactive neurons.

The specific proteins expressed during sleep deprived and recovery sleep and thus helped us better understand the function of sleep and the restorative effect of sleep.

Long-term studies

After studying the localization of specific GRps and ERps induced in specific areas of the brain there may be some future studies that could be conducted. The initial list of potential genes was generated from the Atlas™ Mouse cDNA Expression Array. Lately, cDNA microarrays and high-density oligonucleotide arrays are available and thousands of genes can be analyzed at a time. There are a variety of mice strains available, hence mice were chosen as experimental organisms for this study. Since the mouse genome project, large sequence repository and database are available, which aid in finding genes easily. After a more complete representation of mouse genome becomes available, combination of cDNA microarrays, Taqman analysis and immunohistochemistry to study the specific localization of the expressed proteins across mouse strains will likely be a robust and complete approach in finding the genes involved in sleep homeostasis. Finally, after confirming specific genes involved in sleep, knock out experiments may be conducted to further strengthen the current data.

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