EFFECT OF CHRONIC SLEEP DEPRIVATION INDUCED OXIDATIVE STRESS AND SLEEP RECOVERY ON THE STRUCTURAL INTEGRITY OF HIPPOCAMPUS, COGNITION, AND BEHAVIOR OF RATS

Thesis submitted to
SRI DEVARAJ URS ACADEMY OF HIGHER EDUCATION AND RESEARCH (Deemed to be University)
for the degree of Doctor of Philosophy (Ph.D.)

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DECLARATION BY THE CANDIDATE

I, **Konakanchi Suresh** hereby declare that this thesis entitled **"Effect of chronic sleep deprivation induced oxidative stress and sleep recovery on the structural integrity of hippocampus, cognition, and behavior of rats"** is an original research work carried out by me during the period from August - 2017 to August - 2022 for the award of **Doctor of Philosophy** in the subject of Physiology (Faculty of Medicine) under the supervision of **Dr. Vinutha Shankar MS,** Professor and Head, Department of Physiology, and Co-supervision of **Dr. Harendra Kumar ML,** Former Professor of Pathology, Sri Devaraj Urs Medical College, Sri Devaraj Urs Academy of Higher Education and Research (Deemed to be University).

No part of this thesis has formed the basis for the award of any degree or fellowship previously elsewhere.

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LIST OF ABBREVATIONS

µg	Micrograms
µl	Microliters
ml	Milli liters
mg	Milligrams
µM	Micro molar
ANOVA	Analysis of variance
BDNF	Brain derived neurotrophic factor
BSA	Bovine serum albumin
℃	Celsius
CAT	Catalase
CA1	Cornu ammonis1
CA3	Cornu ammonis2
CC	Concentric circles
cDNA	Complementary-Deoxyribonucleic acid
CNPase	2', 3’-cyclic nucleotide 3'-phosphodiesterase
CNS	Central nervous system
CPCSEA	Committee for the Purpose of Control and Supervision of Experiments on Animals
CSD	Chronic sleep deprivation
CSD+21D SR	Chronic sleep deprivation + 21 days sleep recovery
CSD+5D SR	Chronic sleep deprivation + 5 days sleep recovery
CT	Cycle threshold
CZ	Concentric zone
DBP	Dendritic branching point
DG	Dentate gyrus
DIS	Dendritic intersection
DNA	Deoxyribonucleic acid
DPX	Distrene polystyrene xylene
EC	Environmental control
ECG	Electroencephalogram

ELISA	Enzyme-linked immunosorbent assay
EPM	Elevated plus maze
GABA	Gamma-aminobutyric acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSH	Glutathione
GSSG	Oxidized glutathione
g	Grams
H_3PO_4	Phosphoric acid
HCl	Hydrochloric acid
$MgCl_2$	Magnesium chloride
h	Hours
HWM	Hebb Williams maze
HPA	Hypothalamic-pituitary-adrenal axis
IAEC	Institute animal ethics committee
$K_2Cr_2O_7$	Potassium dichromate
K_2CrO_4	Potassium chromate
LC	Locus ceruleus
LTP	Long-term potentiation
MBP	Myelin basic protein
MDA	Malondialdehyde
MMPI	Modified multiple platform instrument
MMPM	Modified multiple platform method
mRNA	Messenger RNA
MWM	Morris water maze
n	Number of rats
Na_2CO_3	Sodium carbonate
Na_2HPO_4	Di-sodium hydrogen phosphate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NIMHANS	National Institute of Mental Health and Neurosciences
REM	Rapid eye movement
NREM	Non-rapid eye movement rapid eye moment
OA	Open arm

OCD	Obsessive-compulsive disorder
OD	Optical density
OFM	Open field maze
PBS	Phosphate buffer saline
PLP	Proteolipid protein
PTSD	Post-traumatic stress disorder
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
REM	Rapid eye movement
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPM	Revelations per minute
RT	Room temperature
S	Soma
SDUAHER	Sri Devaraj Urs Academy of Higher Education and Research
SDUMC	Sri Devaraj Urs Medical College
Sec	Seconds
SEM	Standard error of the mean
SOD	Superoxide dismutase
SPSS	Statistical package for social sciences
SR	Sleep recovery
TAC	Total antioxidant capacity
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TBS	Tris-buffered solution
TBS-T	Triton X in TBS
TCA	Trichloroacetic acid
TMP	Tetra methoxy propane
USA	United States America
VLPO	Ventrolateral preoptic nucleus
ZT	Zeitgeber time

CONTENTS

Abstract

Sleep is an important biological phenomenon that is essential for cognition, memory, health, and the overall quality of life of human beings. The recommended sleep for adult human beings by the Sleep Research Society and American Academy of Sleep Medicine is more than seven hours. Sleep and its quality depend on factors such as the duration of sleep, appropriate time of sleep, absence of sleep disturbances, lifestyle, and sleep disorders. Sleep loss is a principal factor that disturbs the quality of life by degrading the health and productivity of human beings in the modern-day world. Sleep loss can be categorized as acute sleep deprivation and chronic sleep deprivation (CSD). Both kinds of sleep deprivation were stated to alter the functions of the brain such as attention, alertness, vigilance, decision making, learning, memory, and behavior in both animal and human studies. Long-term sleep loss is also reported to enhance the risk of obesity, diabetes, hypertension, heart diseases, stroke, depression, and neurological disorders such as multiple sclerosis, Alzheimer's disease, and Parkinson's disease.

Key aspects of neurobehavioral functions such as learning, memory, and behavior are regulated in the hippocampus and depend on the quality of sleep. Several experiments have proved that acute or chronic sleep deprivation increases the signs of anxiety-like behavior and impairs memory in both humans and animals. The causes of sleep deprivation induced behavioral deficits including memory and efficacy of sleep recovery have not been fully understood at the cellular and molecular levels. The accumulation of free radicals after prolonged waking and the inability of antioxidant defense mechanisms to scavenge progressively accumulated free radicals results in oxidative stress which may damage the neurons and neuroglial cells leading to neurobehavioral deficits. Understanding the cellular or molecular level changes after sleep deprivation and sleep recovery might help in better management of individuals

suffering from sleep loss/disorders. Therefore, the present study aims to find the extent of the effect of 21-days of CSD, and 5- and 21-days of sleep recovery after CSD on spatial learning and memory, anxiety-like behavior, oxidative stress, dendritic arborization of hippocampal CA3 neurons, and the structural integrity of oligodendrocytes in the hippocampus of Sprague Dawley rats.

Sprague Dawley rats (male; n=60) were grouped (five groups: n=12/group) as control, environmental control (EC), CSD, 5-days sleep recovery (CSD+5D SR), and 21-days sleep recovery (CSD+21D SR). CSD, CSD+5D SR, and CSD+21D SR group rats were sleep deprived for 21-days (18 hours/day) using a modified multiple platform instrument. After CSD, the CSD+5D SR and CSD+21D SR rats were sleep recovered for 5- and 21-days respectively. At the end of sleep deprivation and sleep recovery, spatial learning and memory, anxiety-like behavior, hippocampal oxidative stress, CA3 neuronal dendritic arborization, and changes in oligodendrocytes were assessed.

Spatial learning and memory and anxiety-like behavior were assessed using different behavioral tasks in all five groups. The spatial learning performance was improved in all five groups when the number of trails increased during the acquisition phase in both Hebb-Williams Maze and Morris water maze tasks. However, during the testing phase, the performance of spatial memory significantly decreased in the CSD compared to the control group ($p<0.05$). The rats showed an improvement in their spatial memory performance in CSD+5D SR and CSD+21D SR groups compared to the CSD group, however, it did not reach control levels. Further, the rats were exposed to an elevated plus maze and open field maze tasks to assess the levels of anxiety and we observed a significant increase in anxiety-like behavior in the CSD group compared to the control group. The anxiety-like behavior was reduced in the CSD+5D SR and

CSD+21D SR groups compared to the CSD group, however, it did not reach control levels. The results suggest that the CSD led to impairment of spatial memory, and increased anxiety-like behavior in rats. The 21-days of sleep recovery showed an improvement in spatial memory and decreased anxiety-like behavior compared to the CSD; however, these changes did not reach the values observed in the control group. Further, the study was extended to find the extent of 21-days of CSD-induced damage and sleep recovery on oxidative stress in the hippocampus.

The oxidative markers such as malondialdehyde (MDA), total glutathione, oxidized and reduced glutathione (GSSG/GSH) ratio, and total antioxidant capacity (TAC) were measured in hippocampal tissue, and levels of corticosterone were measured in serum samples. The levels of MDA and ratio of GSSG/GSH increased significantly, whereas the total glutathione and TAC levels decreased significantly in the CSD group compared to the control ($p<0.001$). The levels of MDA and the ratio of GSSG/GSH decreased significantly in CSD+21D SR when compared to the CSD group ($p<0.05$). A decrease in the levels of these markers was observed in CSD+5D SR as compared to CSD, but it was not significant. The total glutathione and TAC levels increased significantly in the CSD+21D SR group compared to the CSD group ($p<0.05$). An increase in the levels of these markers was observed in CSD+5D SR as compared to CSD, but it was not significant. However, the MDA, total glutathione ($p<0.01$), the ratio of GSSG/GSH ($p<0.05$), and TAC ($p<0.001$) levels obtained from the CSD+21D SR group showed significant alteration, but did not reach to control level. The results indicate that CSD increased the oxidative stress in the hippocampus and 21-days of sleep recovery decreased the oxidative stress which did not completely reach the control level. Though the extent of CSD-induced spatial memory impairment, behavioral alterations, and oxidative damage has been well studied, the impact of CSD induced

oxidative damage and efficacy of sleep recovery at the cellular and molecular level has not been fully understood. Therefore, the study was further extended to find the impact of CSD and sleep recovery on the dendritic arborization of CA3 neurons and the structural integrity of oligodendrocytes in the hippocampus.

The mean basal/apical branching points and intersections of CA3 neurons were significantly decreased in the CSD compared to the control group ($p<0.05$). The mean basal/apical branching points and intersections of CA3 neurons increased non significantly in CSD+5D SR, and CSD+21D SR groups compared to the CSD group. Furthermore, the increased basal/apical branching points and intersections obtained from CSD+5D SR and CSD+21D SR did not reach to control level. The CNPase+ve oligodendrocytes in CA1 ($p<0.01$) and dentate gyrus (DG) regions ($p<0.001$) decreased significantly, whereas, the same was not observed in CA3 region in CSD compared to the control group. The number of CNPase+ve oligodendrocytes in CA1, CA3, and DG regions increased in the CSD+5D SR and CSD+21D SR groups when compared to the CSD group, but the increase was not statistically significant ($p<0.05$). Further, the increase in the number of CNPase+ve oligodendrocytes did not reach control levels in both CA1 and DG regions ($p<0.05$).

The intensity of CNPase protein (an indirect measure of myelin content) in CA1, CA3, and DG regions was significantly reduced ($p<0.001$) in CSD when compared to the control group. The intensity of CNPase in CA1, CA3, and DG regions showed an increase, though not significant in CSD+5D SR ($p>0.05$) and significant in CSD+21D SR group rats ($p<0.05$) when compared to the CSD group. Though the intensity of CNPase in CA1, CA3, and DG regions improved in the CSD+21D SR group, it did not reach control levels ($p<0.001$). The expression of the *CNPase* gene was 12.56-fold

down-regulated after 21-days of CSD, which was significant as compared to the control group. After 21-days of sleep recovery, the expression of the *CNPase* gene increased, which was still 5.5-fold down-regulated as compared to control.

Overall, the study results highlight that the 21-days of CSD impaired spatial memory and increased anxiety-like behavior and oxidative stress in the hippocampus of rats. These changes are associated with reduced dendritic arborization of CA3 neurons and decreased oligodendrocytes in the hippocampus after 21-days of CSD. The spatial memory and anxiety-like behavior significantly improved though the dendritic arborization of hippocampal CA3 neurons and CNPase+ve oligodendrocytes did not improve significantly after 21-days of sleep recovery, which might be due to the reduced oxidative stress in the hippocampus. Together these results suggest that the 21-days of sleep recovery is not sufficient to reverse the 21-days of CSD-induced damage in the hippocampus of rats.

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Introduction

1.1. Sleep

Sleep is a naturally occurring state of mind and body that is characterized by altered consciousness, reduced responsiveness to external stimuli, and suppressed motor activity in both animals and humans. Sleep plays a major role in the proper functioning of central neuronal systems (CNS), which regulate behavior, cognition, autonomic functions, arousal, movements, etc. (Krueger et al., 2016).

1.1.1. The architecture of sleep-in humans

The basic structural organization of normal sleep is referred to as sleep architecture. Electroencephalographic (EEG) recordings, which trace the electrical patterns of brain activity were used to uncover the sleep cycles and their stages. Sleep is majorly divided into two states: non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep. NREM sleep is further divided into stages 1, 2, 3, and 4. Both REM and NREM sleep has distinct characteristics such as differences in eye movements, muscle tone, brain wave patterns, etc. (Loomis et al., 1937, Dement and Kleitman, 1957).

Sleep cycles

The episode of sleep begins with a brief period of NREM stage 1, followed by stages 3 and 4, and finally REM. NREM sleep accounts for roughly 75-80% of total sleep time, with REM sleep accounting for 20-25%. The NREM-REM sleep cycle lasts 70-100 min on average. In healthy adults, REM sleep increases throughout the night and lasts the longest in the last one third of the sleep episode (Carskadon and Dement, 2005).

Stages in NREM sleep

The sleep episode begins with NREM stage 1. It typically lasts for 1-7 minutes in the first cycle, accounting for 2-5% of the total duration of sleep. In stage 1, the brain pattern of waves on the EEG shifts from wakefulness (represented by rhythmic alpha waves) to low voltage, mixed-frequency waves. Alpha waves have a frequency of 8-13 cycles/sec and are associated with a wakeful relaxation state (Carskadon and Dement, 2005).

Stage 2 of NREM sleep lasts about 10-25 min in the first cycle and gets longer with each subsequent cycle, eventually accounting for 45-55% of the total sleep episode. EEG shows a relatively mixed frequency activity and low voltage, which is characterized by the presence of K complexes and sleep spindles. Sleep spindles are thought to be important for memory consolidation. Individuals who learn a new task have significantly more sleep spindles than those in the control group (Gais et al., 2002).

Stages 3 and 4 of NREM sleep are collectively considered slow wave sleep. Stage 3 lasts for a few minutes and accounts for 3-8% of total sleep time. The EEG reveals an increase in high voltage and slow wave activity. The final stage of NREM is stage 4, which lasts for about 20-40 min in the first cycle and accounts for about 10-15% of total sleep time. The arousal threshold is highest in stage 4 for all NREM stages. This stage is distinguished by increased high voltage, and slow wave activity on the EEG recordings (Carskadon and Dement, 2005).

REM sleep

REM sleep is evident by the presence of desynchronized (mixed-frequency, low-voltage) brain wave activity, bursts of rapid eye movements, and muscle atonia (Carskadon and Dement, 2005). REM sleep is also characterized by "sawtooth"

waveforms, theta activity (3-7 counts/sec), and slow alpha activity. The REM sleep period may last for only 1-5 min in the initial cycle, which becomes progressively prolong as sleep episode progresses. Table 1.1 shows the physiological differences between NREM and REM sleep in humans (Carskadon and Dement, 2005).

Table 1.1: The physiological changes in NREM and REM sleep in humans

Physiological characteristics	NREM sleep	REM sleep
		(Related to wakefulness)
Brain activity	↓	↑ sensory and motor area while other areas are similar to NREM
Heart rate	↓	↑
Blood pressure	↓	↑
Respiration	↓	↑
Airway resistance	↑	↑
Sympathetic activity	↓	↑
Muscle tone	↓	↓↓
Blood flow to the brain	↓	↑ depends on the brain region
Body temperature	↓	Not regulated

1.1.2. Regulation of sleep wake cycle in the brain

Wakefulness is promoted by an ascending arousal system that originates in the brainstem and keeps the forebrain awake. Ascending arousal pathway begins in the rostral pons and runs through the midbrain reticular formation. This path is divided into two major branches. The first pathway has a significant input to the thalamic relay and reticular nuclei (Figure 1.1: yellow pathway) which comes from cholinergic (ACh) cell groups in the upper pons, pedunculopontine (PPT), and laterodorsally tegmental nuclei (LDT) that aid thalamocortical transmission. A second pathway (Figure 1.1: red pathway) activates the cerebral cortex to aid in the processing of thalamic inputs. This is caused by neurons in the monoaminergic cell groups, such as the tuberomammillary nucleus (TMN) which contains histamine (His), the A10 cell group, which contains

dopamine (DA), the dorsal and median raphe nuclei, which contain serotonin (5-HT), and the locus coeruleus (LC), which contains noradrenaline (NA). This pathway is also supported by peptidergic neurons in the lateral hypothalamus (LHA) that contain orexin (ORX) or melanin-concentrating hormone (MCH), as well as neurons in the basal forebrain (BF) that contain gamma-aminobutyric acid or Ach (Saper et al., 2005). Sleep is promoted by the ventrolateral preoptic neurons: the VLPO was found to send outputs to all of the major cell groups in the hypothalamus and brainstem that participate in arousal (Figure 1.1) (Saper et al., 2005).

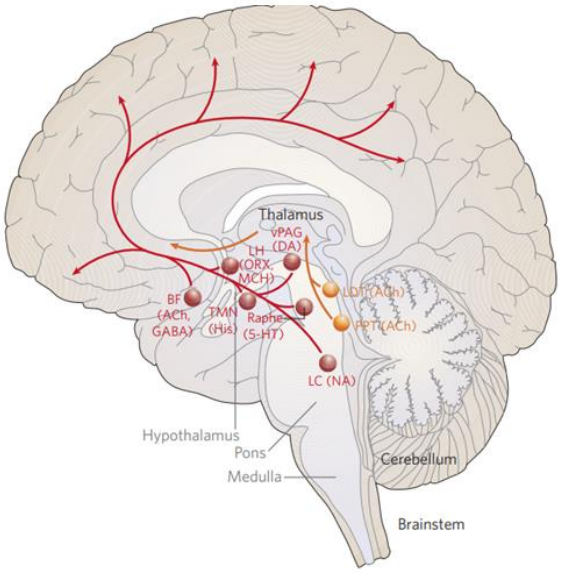


Figure 1.1: Representative image of ascending arousal system in the brain (Source: Saper et al., 2005)

The flip-flop switches

During wakefulness, monoaminergic nuclei (red) inhibit VLPO (purple), thereby relieving the inhibition of the monoaminergic cells and that of the orexin (ORX) neurons (green), and the cholinergic pedunculopontine (PPT) and laterodorsally tegmental nuclei (LDT; yellow) (Figure 1.2A). The firing of the VLPO neurons inhibits the monoaminergic cell groups thereby relieving their inhibition during sleep. This enables it to block orexin neurons, reducing monoaminergic activity even more. Direct mutual

inhibition between the monoaminergic and VLPO cell groups creates a characteristic flip-flop switch that causes abrupt state changes but is also comparatively unstable. The addition of the inputs from orexin neurons stabilizes the switch (Figure 1.2B) (Saper et al., 2005, Saper et al., 2010).

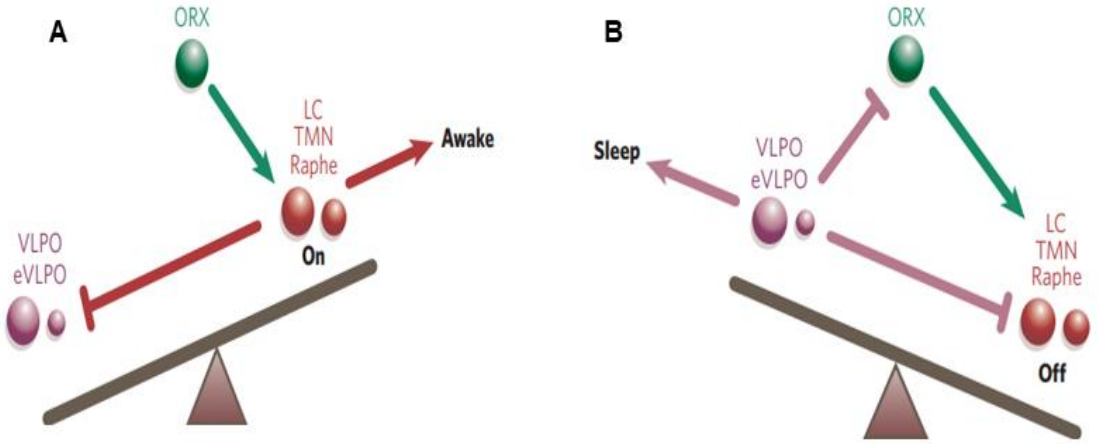


Figure 1.2: Representative image of flip-flop switch model of wake and sleep cycle (Source: Saper et al., 2005).

1.1.3. Sleep and sleep stages in rodents

The sleep wake cycles differ between humans and rodents. In general, the animals used in the laboratory are nocturnal and spend the majority of the daytime (light phase) resting and nighttime as active phase (dark phase). The rodents are typically polyphasic as they sleep repeatedly across the 12:12 hour light/dark cycle. However, they spend 70-80% of their sleep in the light phase and around 20-30% in the dark phase asleep. Similar to humans, rodents have two states of sleep: NREM, and REM sleep and the duration of each NREM and REM sleep state sleep cycle lasts about 12 min for rats (Colavito et al., 2013).

1.2. Sleep loss

Sleep loss affects the quality of life by degrading the health and productivity of human beings in the modern-day world (Pilcher and Morris, 2020). Sleep loss is the

main factor that has led to some of the biggest disasters in recent history. The consequences of errors in judgment contribute to the disasters such as the space shuttle challenger, three-mile island nuclear plant, Chornobyl nuclear plant, and Air India express flight 812 in 2010 (Mitler et al., 1988, State of Alaska 1990, Walker et al., 2010). Discreet consequences of sleep loss are more prevalent and they have an impact on nearly every key indicator of public health such as mortality, morbidity, accidents and injuries, performance, family well-being, quality of life, and health care utilization.

Depending on the duration of sleeplessness, sleep loss can be categorized as Acute or chronic sleep deprivation (CSD). Continuous sleep deprivation for several hours to a week can be defined as acute sleep deprivation; whereas, sleep deprivation for a few hours (typically 3-8 hours/day) daily in 24 hours sleep wake cycle for several weeks or months can be defined as CSD (Alzoubi et al., 2016). A recent study over 9 years (2010-2018) in the working adult American population found that one third of the adult population experienced short sleep duration (<7 hours) (Khubchandani and Price, 2020). In South Korea, the average reported sleep time for teenagers was 4.9 hours/night (Yang et al., 2005). A recent study on sleep time and its quality in Indian adults found that mean sleep time was 6.9 hours/night and poor sleep quality was widely prevalent (Banthiya et al., 2021).

1.2.1. Etiology and risk factors of sleep loss

The causes for sleep loss are multifactorial in humans which can fall majorly on lifestyle, occupational, and sleep disorders. These include lifestyle and occupational factors such as shift work, prolonged working hours, jet lag, irregular sleep schedules, and sleep disorders such as insomnia, obstructive sleep apnoea, restless leg syndrome, and circadian rhythm disorders. Sleep loss is being driven primarily by broad societal

changes, such as a greater reliance on long work hours, shift work, and increased access to mobile, television, and computers (Ong and Crawford, 2013).

1.2.2. Effect of sleep loss on human health

Excessive daytime sleepiness is the most common symptom of sleep deprivation, but other symptoms include fatigue, depressed mood, and poor memory or concentration (Kryger et al., 2010). Chronic sleep loss has serious consequences on health, performance, and safety. Prospective cohorts studies on sleep suggested that <7 hours of sleep/night for a long time may have a wide range of effects on the cardiovascular, endocrine, immune, and nervous systems and is also associated with obesity, impaired glucose tolerance, hypertension, anxiety, depression, etc. (Medic et al., 2017). Insufficient sleep for the long term is associated with detrimental effects on the physiology of the nervous system such as cognition, vigilance, attention, learning, and memory as well as performing complex real world tasks and alterations in behavior (Banks and Dinges, 2007). Long term chronic sleep loss or impaired neural functions leads to depression, and neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, etc., (Abbott and Videnovic, 2016). In addition to neurological disorders, health complications such as diabetes, obesity, hypertension, cardiovascular diseases, etc. have also been reported to be increased due to sleep loss (Medic et al., 2017). A recent meta-analysis on the impact of sleep loss on the risk of diabetes mellitus highlights that one-hour sleep loss compared to 7 hours/day of sleep results in an increase in 9% risk of type 2 diabetes mellitus (Shan et al., 2015). A similar meta-analysis of published articles on sleep loss and the risk of cardiovascular disease showed that <5 hours sleep/day increased the risk of coronary heart disease (relative risk of 1.48) and stroke (relative risk of 1.15) compared to 7-8 hours of sleep (Cappuccio et al., 2011).

Recent studies highlight the negative impact of sleep loss on human health. Hence, understanding the extent of sleep loss and its impact at molecular, biochemical, and cellular levels (neurons and neuroglial cells) in the brain, and learning and memory and behavioral changes might help for better management of individuals suffering from sleep loss/sleep disorders. Proper management of individuals suffering from sleep loss can improve their health and behavior and reduce the public health burden.

Chapter II

Review of Literature

2.1. Sleep and sleep deprivation

Sleep is required to replenish the energy of our body and mind. Sleep loss is an important factor that negatively affects the cognition, behavior, health, and overall quality of life of human beings. The recommended sleep for adult human beings by the Sleep Research Society and American Academy of Sleep Medicine is 7-9 hours (Panel: et al., 2015). Sleep loss is a major problem, approximately 70 million Americans and 45 million Europeans are experiencing chronic sleep loss, which is affecting their daily performance and health status (Medic et al., 2017). Sleep and its quality depend on factors such as the duration of sleep, appropriate time of sleep, absence of sleep disturbances, lifestyle, sleep disorders, etc., (Altevogt and Colten, 2006). Sleep loss can be categorized as acute and chronic sleep deprivation (CSD), both kinds of sleep deprivation were reported to alter the functions of the central nervous system such as attention, alertness, vigilance, decision making, learning, memory, and behavior in both animal and human studies (Abel et al., 2013, Havekes et al., 2016). In addition to impaired neural functions, CSD may also lead to neurodegenerative diseases (Abbott and Videnovic, 2016).

Professionals such as nurses, doctors, security guards, and call center workers have altered their lifestyles, or sleep disorders resulting in chronic partial sleep deprivation (Carey et al., 2011). CSD is predisposed to poor health and diseases such as cardiovascular complications, metabolic syndrome, gastrointestinal dysfunction, an enhanced risk of cancer, and hormone imbalances. In addition, it may lead to neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (Abbott and Videnovic, 2016). CSD has an impact not only on individuals but also on the government's healthcare burden. In comparison to the growing number of people

affected by CSD, relatively few clinical and preclinical studies have been conducted to determine its neurological impact. Thus, more studies at cellular, molecular, physiological, and community levels are necessary for a better understanding of how CSD affects human health and leads to several disease conditions.

2.2. Sleep deprivation techniques used in rodents

To study the long-term sleep loss effect on health, we need a model of sleep loss in both humans and animals. The sleep deprivation protocols used in humans are laborious and expensive to carry out due to sampling size, length of sleep deprivation, etc. The sleep deprivation protocol in rodents can provide insights into CSD-induced cognitive or behavioral abnormalities at the cellular and molecular levels in the brain for the better management of sleep-associated disorders. Revel et al., (2009) reviewed different sleep deprivation models used for insomnia in rodents i.e.; stress models: psychological stress, water tank, grid over water paradigms, foot electroshock, fear and fear-conditioned stress, sensory stimulation, chronic mild stress, and perinatal stress; Pharmacological: administration of caffeine, psychostimulants, and modafinil; genetic models: DBA/2J mice, clock gene mutants; Modification of the light-dark cycle and circadian perturbation, etc., (Revel et al., 2009). Total sleep deprivation is distinct as preventing the sleep for the desired amount of time and independent of the sleep state, in contrast, partial sleep deprivation, a specific sleep state is selectively targeted for deprivation. It is critical to distinguish between total and partial or state-dependent sleep deprivation. In the majority of cases, partial sleep deprivation is restricted to REM sleep although NREM can be accomplished. The most commonly used experimental sleep deprivation techniques in rodents have been discussed in the following section.

Gentle hand method: The gentle hand approach, which relies on the experimenter actively keeping the rodents awake, is the most often used technique for completely depriving animals of their ability to sleep. The operator's task is to keep the animals awake by providing just enough stimulation when they become sleepy or seek to assume a sleeping position. Sleep deprivation by gentle hand effectively suppresses nearly all sleep activity in rodents. Sleep deprivation by gentle handle can reduce NREM sleep by 92% and REM sleep by 100% in rats (Franken et al., 1991).

Automated total sleep deprivation: Total sleep deprivation can be administered by inducing specific patterns of locomotor activity either continuously or whenever the animal exhibits behavioral and/or electrophysiological signs of impending sleep. Rechtschaffen and co-workers introduced a “disk over water” apparatus by using this technique sleep time were reduced to 91% in experimental animals (Rechtschaffen, 1983).

REM sleep deprivation models: A simple flowerpot technique can be used to induce REM sleep deprivation. The technique was first intended to make cats lose their sleep, but it has since been modified to prevent mice and rats from getting enough REM sleep (Jouvet et al., 1964). This method has been widely used due to its simplicity, contributing to a better understanding of the roles and mechanisms of REM sleep. However, it should be noted that the procedure primarily targets REM sleep, significant loss of NREM sleep has also been reported (Grahnstedt and Ursin, 1985, Machado, 2004). Furthermore, the procedure is associated with stress, which may confound the interpretation of the results (Revel et al., 2009). To reduce stress, the protocol has been modified by placing multiple platforms in a larger tank and sleep-deprived several animals at the same time. As a result, the animals are free to move around the cage,

jumping from platform to platform, and interact with their cohorts, by which immobility and social isolation could be avoided. The flowerpot method can also be performed without the use of an electrophysiological recording. The "head-lifting method" is another protocol for REM sleep deprivation that has been proposed to alleviate some of the drawbacks associated with the majority of REM sleep deprivation methods (Colavito et al., 2013).

2.3. Sleep deprivation induced changes in brain and behavior

Several studies demonstrated that sleep deprivation impairs memory in a variety of behavioral tests. The cause of memory deficits after sleep deprivation is not fully understood, but some of the effects have been hypothesized that the accumulation of free radicals during prolonged wakefulness due to increased metabolic activity and the inability of antioxidant defense mechanisms to scavenge progressively accumulated free radicals, which intensify oxidative stress, causing biochemical distress in the brain specifically the hippocampus. The CSD induced oxidative stress in CNS might lead to potential damage to neurons and neuroglial cells, which in turn may result in neurodegeneration. The following sections summarise the recent study results on both chronic and acute sleep loss-induced changes in spatial learning and memory, anxiety-like behavior, oxidative stress, and the structural integrity of the neurons and oligodendrocytes in both animals and humans.

2.3.1. Impact of sleep deprivation on spatial learning and memory

Spatial learning and memory is a brain function involved in a variety of behaviors, such as planning a route to a certain destination or remembering an object's location. It is commonly defined as the capacity to learn, retain, and retrieve spatial information. Spatial memory has a high correlation between the hippocampus and related networks

in both animal and human studies (Bird and Burgess, 2008). The identification of so-called "place cells" in the rat hippocampus served as a major catalyst for the initial scientific interest in spatial memory. The neuroscientist John O'Keefe proposed that these "place cells" were the basis for the formation of a spatial map (O'Keefe and Dostrovsky, 1971).

The homeostasis of many biological systems, including sound sleep is necessary for the development of spatial memory. Sleep is thought to encourage the stabilization of initially labile spatial memory traces and their integration into neocortical networks for long-term storage (Björn and Born, 2013). Sleep deprivation studies from both human and animal experiments revealed the importance of sleep in learning and memory. Sleep likely modulates the synaptic connections in the hippocampus, which plays an important role in learning and memory (Yang et al., 2014). Hippocampal-dependent memory processes are especially susceptible to disruptions of sleep. Prominently, limited evidence has also suggested that sleep deprivation in humans impaired hippocampal-dependent spatial memory. For example, insomnia is a sleep disorder characterized by difficulty sleeping, interrupted, non-restorative sleep, and impairment of cognitive functioning has been reported compared to healthy controls (Li et al., 2016, He et al., 2021). Similarly, subjects with poor "insomnia-like" sleep quality perform worse in a spatial task than those with good quality sleep (Valera et al., 2016). Neuroimaging studies also reported that reduction in hippocampal volume in insomnia patients compared to healthy controls (Joo et al., 2014, Emamian et al., 2021). The converging evidence also suggests that other cognitive domains such as verbal memory, executive functioning, attention, and mood regulation, emotional processing have shown to be vulnerable to sleep deprivation (McCoy and Strecker, 2011).

Learning and memory can be assessed in rodents using the Hebb Williams Maze (HWM), Morris Water Maze (MWM), Radial Arm Maze, and T Maze (Colavito et al., 2013). The rats were given continuous 72 hours of REM sleep deprivation with the flowerpot method, which was demonstrated to impair the spatial learning in the MWM and the capacity to memorize the position of the platform in the following probing test (Zhao et al., 2014). Rats were shown to be impaired in spatial learning and even more significantly impaired spatial memory performance in MWM after being subjected to 5-days of total sleep deprivation. The mice showed a marked reduction of spatial learning, and memory retention in the WMW test when they were chronically sleep-deprived for 30-days (3 hours/day). The spatial memory impairment in mice was observed after 24- and 48 hours of sleep deprivation (Cao et al., 2019). Both short- and long-term spatial memory of rats were impaired in radial arm water maze after 6 weeks of CSD (8 hours/day), and it was associated with increased oxidative stress (Alzoubi et al., 2012, Alzoubi et al., 2016). It has been demonstrated that the CSD induced oxidative stress in the hippocampus might result in the damage of neurons and impairment of cognition (Alkadhi et al., 2013, Salim, 2017). Twenty-one days of CSD (18 hours/day) leads to low-grade neuroinflammation (increased inflammatory cytokines) in the hippocampus, heightened anxiety-like behavior, and impaired memory in rats (Manchanda et al., 2018). Eight weeks of CSD (8 hours/day) leads to increased oxidative damage in the hippocampus and impaired short-term and long-term memory in rats (Alzoubi et al., 2019). Seven days of intermittent and paradoxical sleep deprivation also increased the anxiety-like behavior and impaired memory of mice when compared to 3-days (Yin et al., 2017). Overall, the existing literature suggests both acute or chronic sleep deprivation leads to spatial learning and memory impairment in rodents as well as in humans. Indeed, the converging evidence of animal experiments specified

that sleep deprivation impaired spatial learning and memory was correlated with oxidative stress in the hippocampus (Table: 2.1).

2.3.2. Impact of sleep deprivation on anxiety-like behavior

Excessive dread or worry caused by anxiety increases the risk of heart disease and diabetes as well as disrupts daily life. General anxiety disorder, social anxiety disorder, panic disorder, particular phobias, obsessive-compulsive disorder (OCD), and post-traumatic stress disorder are several types of anxiety disorders (PTSD). The limbic system is a part of the brain that controls emotion. The amygdala, prefrontal cortex, and hypothalamus, which are all involved in emotions, are closely connected to the ventral hippocampus in rodents. More recently, “anxiety cells” have been discovered in ventral hippocampal CA1 in mice (Jimenez et al., 2018). The pioneering experiment with sleep deprivation in humans reported the anxiety-like behavior effect for the first time (Dement and Kleitman, 1957). Anxiety-like behavior is one of the most serious side effects of sleep loss. People who were not getting enough sleep had a triad of neurobehavioral comorbidities, including increased anxiety, attention deficit, and aggression. Animal experiments demonstrated that different durations of sleep deprivation are shown to increase anxiety-like behavior. Anxiety-like behavior in experimental animals can be assessed using a variety of models such as Elevated Plus Maze (EPM), Open Field Maze (OFM), and light-dark box tests (Pires et al., 2016) discriminative avoidance task (Silva et al., 2004) and the T Maze are capable of assessing anxious-type behavior to varying degrees in rodents. Indeed, these models are useful experimental instruments for the study of anxiety, and they yield data that is both relevant and clinically useful. The anxiety-like behavior of rats assessed using an OFM showed higher anxiety levels after 24 hours of REM sleep deprivation compared to

control (Xie et al., 2018). The 21-days of CSD (18 hours/day) increase the anxiety-like behavior in rats (da Silva Rocha-Lopes et al., 2018). Twenty-one days of CSD (18 hours/day) leads to low-grade neuroinflammation (increased inflammatory cytokines) in the hippocampus, heightened anxiety-like behavior, and impaired memory in rats (Manchanda et al., 2018). Yin et al., (2017) observed that the anxiety-like behavior or memory impairment was more prominent when there was an increased duration (from 3-7-days) of sleep deprivation in mice (Yin et al., 2017). The sleep loss-induced changes in behavior alterations and memory impairment are often correlated with oxidative stress hippocampus (Yin et al., 2017, Salim, 2017). Reciprocal interactions between sleep deprivation, oxidative stress, structural/functional changes in the hippocampus as well as their associations with memory impairment and behavioral abnormality explicate that oxidative stress might mediate the link between sleep deprivation, spatial memory impairment, and behavioral abnormalities (Table 2.1).

2.3.3. Role of the hippocampus in cognitive and behavioral functions

Hippocampus is known to play a role in cognition as well as spatial navigation, emotional behavior, and regulation of hypothalamic functions. Production of new neurons and preservation of dendritic structures are essential for the hippocampal-dependent cognitive processes because they offer the synaptic plasticity required for learning and memory formation. Structurally, the hippocampus can be divided into hippocampus proper (CA1, CA2, and CA3), dentate gyrus (DG), subiculum, and entorhinal region (Schultz and Engelhardt, 2014). The hippocampus receives afferent connections from the parietal, temporal, and occipital areas via the entorhinal cortex through the polysynaptic pathway (Rao et al., 2022) (Figure 2.1). The internal connections such as the entorhinal cortex have been shown input to the dentate gyrus

(layer II of the perforant route). Layer II and IV axons of the entorhinal cortex project to CA3 pyramidal cells, while layer III and IV of the entorhinal cortex axons project to CA1 pyramidal cells and subiculum pyramidal cells. CA1 pyramidal cells deliver their axons to the entorhinal cortex, subiculum, and deep layers. Shaffer's collaterals are axons that project from CA3 cells to the CA1 region. Especially, the hippocampal CA3 neuronal dendrites receive the input signals from DG (mossy fibers), the entorhinal cortex (perforant path), recurrent fibers of CA3, contralateral CA3, and the septal area (Figure 2.1). The dendritic intersections and branching points of CA3 neurons play a major role in synaptic connectivity, which is associated with the formation of memory. To date, learning-induced modifications have mostly been studied at the synaptic level and at the time of encoding, with changes in synaptic strength in the hippocampus (Takeuchi et al., 2014, Anand and Dhikav, 2012).

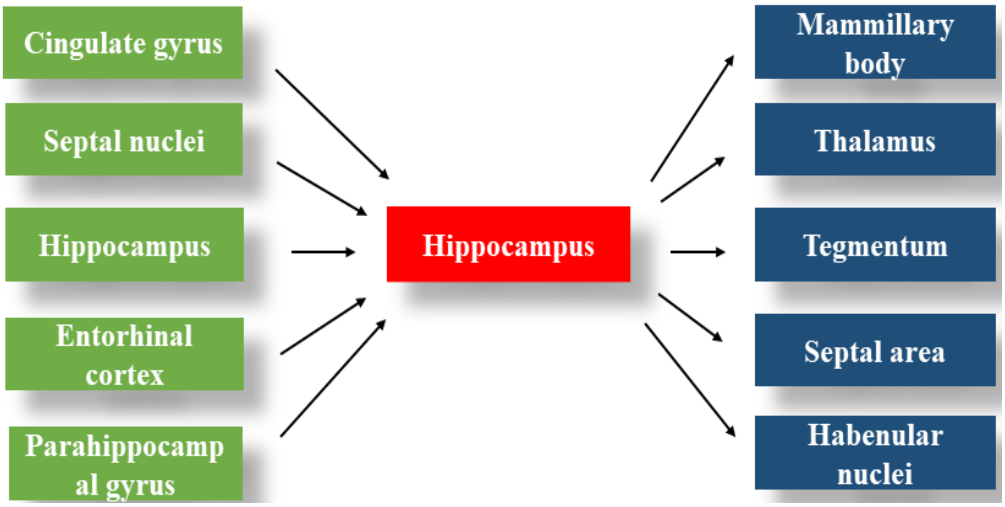


Figure 2.1: Afferent and efferent connections of the hippocampus (Rao YL et al., 2022).

The speed of conduction along axons may also affect neural circuit plasticity. Neuronal conduction speed along the axon depends on the axon diameter together with the thickness and spacing of myelin. Myelin-forming oligodendrocytes are regulated by neuronal activity (Hill et al., 2018). Myelin patterning may dynamically remodel

activated circuits in an experience-dependent manner (Hughes et al., 2018). This type of plasticity may also be significant during "offline" periods following the learning of new information because proper consolidation of spatial memory is assumed to require coupling of rhythmic oscillations in hippocampal-cortical networks (Buzsáki, 1996, Diekelmann and Born, 2010). Reduced antioxidant capacity and exposure to any form of stress/sleep deprivation can significantly impede hippocampal-dependent functions of learning and memory by decreasing the formation of new neurons, changing dendritic structures, altering neuronal connectivity the neuroglial activity in the hippocampus. Hippocampus is the most extensively studied and the atrophy of this region has clinical consequences. It is the earliest and most severely affected structure in several neuropsychiatric diseases such as Alzheimer's and epilepsy (Anand and Dhikav, 2012).

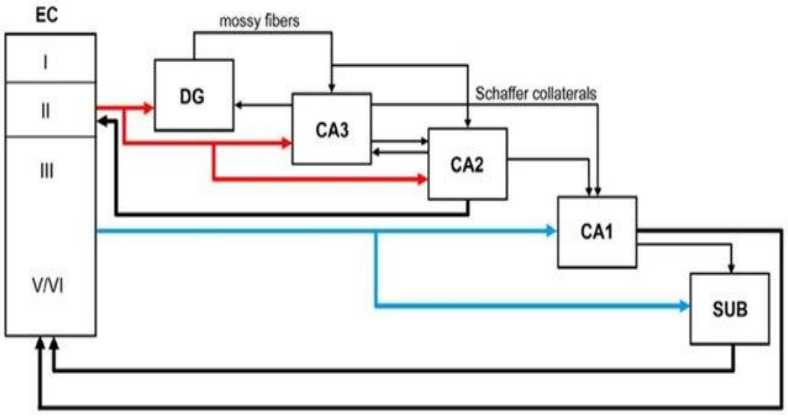


Figure 2.2: Internal connections of the hippocampus (Source: Anand and Dhikav, 2012).

2.3.4. Impact of sleep deprivation on oxidative stress in the brain

The mitochondrial oxidative phosphorylation is a primary source of ATP. This process generates free radicals, also called reactive nitrogen species (RNS), reactive oxygen species (ROS), and carbon- and sulfur radicals as a by-product (Pero et al., 1990). ROS are required for brain development and function in moderate concentrations, while excessive levels are harmful. Long-term potentiation (LTP) is

promoted by ROS generated nitrous oxide (NO), and carbon monoxide (CO) via glutamate-dependent processes (Stevens and Wang, 1993). Under normal circumstances, the antioxidant system neutralizes the negative effects of ROS formation during aerobic metabolism, allowing the brain to properly manage its oxygen consumption and cellular redox capacity. The ROS causes oxidative damage, cellular deterioration, and eventually a reduction in functional ability if they are more powerful than the antioxidant response system's ability to scavenge them. This is referred to as an oxidative stress condition, and it is very hazardous to the brain's physiological functioning. Excessive ROS triggers cellular damage, the damaged molecules themselves may act as and/or become ROS, which is why oxidative stress is often regarded as a self-propagating process. The cells in the CNS are more susceptible to the damage caused by free radicals due to the presence of unsaturated bonds in lipids and greater consumption of oxygen, and low antioxidant capacity (Cobley et al., 2018). In addition, it also contains free radical generating substances such as ascorbate, iron, and glutamate which are involved in redox reactions (Walton et al., 2012). Oxidative stress becomes more severe when the brain is exposed to unfavourable conditions, like stress, sleep deprivation, aging, etc. Several health conditions, including aging, traumatic brain injury, and Alzheimer's disease have been linked to cognitive impairments caused by oxidative stress (Salim, 2017). Indeed, the majority of studies on cognitive impairment in Alzheimer's disease attribute it to oxidative stress via decreased levels of antioxidant enzymes.

The low-molecular-weight antioxidant and antioxidant enzyme systems and two types of protective systems that work in the brain to combat the harm posed by ROS (Kohen and Nyska, 2002, Kohen et al., 1999). Superoxide dismutase (SOD), glutathione reductase, glyoxalase, catalase (CAT), and glutathione peroxidase are all antioxidant

enzymes (Griendling et al., 2000). Mn-SOD and Cu-Zn SOD are SOD enzymes that aid in the dismutation of superoxide radicals to produce H_2O_2, which is then eradicated by glutathione peroxidase enzymes and CAT (Saso and Firuzi, 2014). Glutathione, ascorbic acid, melatonin, and uric acid, are low-molecular-weight antioxidants that act as neutralizers by generating transition metal chelation (Chance et al., 1979). Glutathione is foremost the nonenzymatic endogenous antioxidant. The reduced (GSH) and oxidized glutathione (GSSG) are the two forms of glutathione. It is regenerated by glutathione reductase with the consumption of NADPH (Gul et al., 2000). The endogenous ratio of oxidized and reduced glutathione is used to determine redox equilibrium in a cell. Glutathione also serves as a cofactor for a variety of enzymes, including peroxidase, and glyoxalase (Kohen and Nyska, 2002).

Wakefulness requires a high level of neuronal metabolism to maintain neuronal electrical potentials, which usually require a large amount of oxygen, resulting in substantial production of oxidants. Thus, sleep is a state with increased antioxidant activity, which promotes brain protection against free radicals by decreasing oxidant production (Reimund, 1994). ROS and other oxidative stress markers may accumulate in brain tissue during wakefulness, and after reaching a threshold, may act as sleep promoters (Inoué et al., 1995). However, prolonged wakefulness produced ROS which dominates the antioxidant capacity and eventually leads to oxidative stress. There is concrete evidence that CSD can increase oxidative stress in the CNS (Villafuerte et al., 2015). Therefore, the CSD-induced oxidative stress in CNS leads to the potential damage to neurons and neuron-supporting cells, which in turn results in neurodegeneration (Salim, 2017, Owen and Veasey, 2020). Indeed, numerous animal studies have demonstrated that various forms of experimental sleep deprivation, including sleep fragmentation as well as partial and total sleep deprivation, consistently

cause significant deficits in hippocampus-dependent spatial memory performance and increase oxidative stress in the hippocampus (Colavito et al., 2013). For example, sleep deprivation for 72 hours results in a passive avoidance retention deficit and is correlated with the extent of oxidative stress in the hippocampus of mice (Silva et al., 2004). Twenty-one days of CSD (18 hours/day) leads to low-grade neuroinflammation (increased inflammatory cytokines) in the hippocampus, heightened anxiety-like behavior, and impaired memory in rats (Manchanda et al., 2018). Eight weeks of CSD (8 hours/day) leads to increased oxidative damage in the hippocampus and impaired short-term and long-term memory in rats (Alzoubi et al., 2019). While sleep disruption has been linked to a decline in a variety of cognitive domains (spatial learning and memory), experimental data show that the hippocampus and spatial memory are especially vulnerable to the effects of sleep loss (Havekes et al., 2016). The converging evidence suggests that acute or chronic sleep deprivation predominantly increases the oxidative stress in the hippocampus/brain which may contribute the neuron and neuroglial damage, specifically in areas of the brain that are responsible for memory encoding and consolidation. The consolidated information on CSD induced changes in behavior, cognition, and oxidative stress damage was provided in table 2.1.

Table 2.1: Published reports on the effect of sleep deprivation on behavior, cognition, and oxidative stress damage.

Author and year	Intervention	SD model	Behavioral changes	Biochemical changes
Manchanda et al., 2018	21-days (18 h/day) of rats	MMPM	Impaired working memory and increased anxiety levels	Elevated levels of TNFα, IL-1β, NFκB, AP1, and transcription factors in the hippocampus

Zielinski et al., 2014	5-days of TSD rats	Rotating wheel	-	Elevated levels of IL-1β, TNF-α and reduced BDNF mRNA levels in the hippocampus
Kaur et al., 2017	12 h of TSD rats	GH	Increased anxiety	-
Alzoubi et al., 2019	6 weeks (8 h/day) rats	MMPM	Impaired short- and long-term memory	Decreased GPx, catalase, SOD, GSH/GSSG ratio, and increased GSSG in the hippocampus
Alzoubi et al., 2012	8 h/day for 6 weeks in rats	MMPM	Impaired short- and long-term memory	Increased GSSG, decreased catalase, GPx, and SOD
Yin et al., 2017	48 h in mice	MMPM	Impaired the spatial memory	Excessive autophagy and apoptosis of hippocampal neurons
McCoy et al., 2013	18 h/day for 5-days in rats	Wheel movement	Impaired the spatial memory	-
Zhu et al., 2012	24 h in mice	Moving rockers	Impaired hippocampal depended on learning and memory	Increased pro-inflammatory cytokine IL-6 levels and induced microglia activation in the mouse hippocampus
Süer et al., 2011	21-days (18 h/day) in rats	MMPM	Not shown anxiety-like behavior	Increased the MDA, decreased SOD, GPx and impaired the LTP
da Silva Rocha-Lopes et al., 2018	18 h/day for 21-days in rats	MMPM	Increased anxiety-like behavior in juvenile rats	BDNF levels were elevated in the dorsal hippocampus
Xue et al., 2019	20 h/day for 7-days in mice	MMPM	-	Increased the TNF-α, IL-1β, and IL-6 and decreased the Nrf-2, and HO-1

	antioxidant enzymes in the hippocampus

TSD: Total sleep deprivation, **GH:** Gentle handle, **MMPM:** Modified multiple platform method, **CA:** Cornus amminus 1, **DG:** Dentate gyrus and **h**: hours, **SD:** Sleep deprivation, **IL-1β:** Interleukin-1β, **IL-6:** Interleukin-6 **TNFα:** Tumour necrosis factor α, **BDNF:** Brain-derived neurotrophic factor, **NFκB:** Nuclear factor kappa B, **GPx:** Glutathione peroxidase, **LTP:** Long term potentials, **Nrf-2:** Nuclear factor erythroid 2–related factor, **HO-1:** Hemeoxygenase-1, **MDA:** Malondialdehyde, **SOD:** Superoxide dismutase, **AP-1:** Activator protein-1, **GSH/GSSG ratio:** Reduced and oxidized glutathione ratio.

2.3.5. Effect of sleep deprivation on neuronal loss in brain

Brain volume changes have been reported after CSD using the multiple platform method (Kamali et al., 2017, Noorafshan et al., 2017a, Noorafshan et al., 2017b). CSD in rats for 3-4 weeks reduced the volume of the medial prefrontal cortex and the hippocampus (Noorafshan et al., 2017a). The hippocampal regions showed signs of volume decrease in CA1 (29%), and dentate gyrus (31%) after 21-days of CSD (Noorafshan et al., 2017a). After a 3-4 week recovery period, volume reductions in the medial prefrontal cortex were investigated, demonstrating that the effect of sleep deprivation in rats does not fully reverse in this region (Noorafshan et al., 2017b). Sleep disruption has also been linked to cell loss in several regions of the brain. Three days of sleep restriction resulted in a 30% reduction in the number of loci coeruleus (LC) neurons in rodents (Zhang et al., 2014, Zhu et al., 2016). All neuronal populations are not vulnerable to sleep deprivation: For example, in response to chronic short sleep, a group of sleep-active neurons, melanin-concentrating hormone neurons are not destroyed. The same paradigm was used for a longer period (12 weeks followed by a 6-month recovery), but no more LC neurons were lost implying that susceptible neurons are only lost early in sleep loss (Zhu et al., 2018). After CSD in rats (18 hours/day for 21-days), the total number of neurons in the parabrachial nuclei and the nucleus tractus

solitarius decreased by around 10% (Kamali et al., 2017). CSD (18 hours/day) for 21-days in adult rats resulted showed a 48% decline in CA1 pyramidal neurons and a 25% loss of dentate gyrus neurons when compared to controls (Noorafshan et al., 2017a). Furthermore, mice with 2-4-days of REM sleep deprivation have considerably fewer viable pyramidal neurons in the CA1 region of the hippocampus in the rats (Hou et al., 2019, Olonode et al., 2019). However, one major issue is that neither of this research used stereology to estimate cell numbers; consequently, future investigations should confirm these findings to be convinced that sleep deprivation can result in neuronal loss. Overall, these recent study results demonstrate that sleep loss causes significant volumetric and neuronal loss in several brain regions, which does not rapidly reverse with recovery sleep. Alterations in the vasculature, changes in the extracellular space, increased cell death, decreased neurogenesis, changes to dendrites and/or myelination, and so on could all be contributing to neuronal and volume loss. In addition to the neuronal loss, structural changes in the neurons, loss of neuroglial cells, and myelination may also contribute to the volumetric loss in several brain regions.

2.3.6. Effect of sleep deprivation on dendritic arborization of pyramidal neurons in the hippocampus

As a collection of neural networks, the brain's functionality is largely dependent on the integration of signals of neurons. The integration is influenced by the neuron level of excitability, as well as the type and quantity of neurotransmitters produced. Additionally, at the microstructural level, the type and quantity of synapses (spines) on neurons would affect the integration of inputs. Axonal and dendritic branches, as well as their arborization, would all affect the number of synaptic transmissions. All these factors have been suggested to be impacted by sleep deprivation. Structurally, the CA1

region is made up of homogenous pyramidal neurons and has 1-5 primary basal dendrites in addition to 1-2 primary apical dendrites. The furthest region is CA3, which is distinguished by heterogeneous pyramidal cells of variable organization and dendritic length. The CA3 pyramidal neurons have 1-3 primary apical dendrites that branch off from the soma to produce four or more thick secondary apical dendrites. The factors which decrease the dendritic arborization reflects in reduce the synaptic connectivity (Villanueva Espino et al., 2020, Giri et al., 2021).

Factors other than neuronal loss may account for cognitive and behavioral alterations observed after sleep loss. These include a decrease in the dendrites and myelination of axons. Studies have demonstrated that oxidative stress in the hippocampus can negatively affect learning and memory by changing the structure of dendrites (Huang et al., 2015). Sleep deprivation reduces synaptic plasticity and impairs memory consolidation (Long term potentiation) in the hippocampus, which is a common cellular correlate of learning and memory functions (Prince and Abel, 2013). Several studies demonstrated the impact of sleep deprivation on the dendritic arborization of hippocampal neurons. Acosta et al., (2015) observed that sleep deprivation for 24 hours found no change in the dendritic length and branching whereas dendritic spine density was decreased in the CA1 region of the hippocampus (Acosta-peña et al., 2015). A decreased dendritic spine number was observed in the CA1 and DG subregions of the hippocampus after 5-hours of sleep deprivation in mice (Havekes et al., 2016, Raven et al., 2019). In contrast, a recent study demonstrated that 5-hours of sleep deprivation increased spine density in the apical or basal dendrites of the CA1 pyramidal neurons of the hippocampus in mice (Gisabella et al., 2020). The variance in dendritic spine density following sleep deprivation in mice is probably caused by the use of different dendritic spine imaging techniques, quantification techniques, or statistical methodologies.

The acute sleep deprivation for 21-days of CSD (18 hours/day) decreases the spine density and dendritic length in the hippocampal CA1 region of rats (Noorafshan et al., 2018). Chen et al., (2009) observed that both basal and apical dendrites spines of both CA1 and CA3 neurons were reduced due to fatigue induced by 5-days of sleep deprivation in rats (Chen et al., 2009). The length of dendrites, branching points, and spine density of hippocampal CA1 and CA3 neurons were decreased after continuous REM sleep deprivation of rats for 6-days (Giri et al., 2021). Together these studies suggest that reduced spine density and dendritic arborization could impact synaptic plasticity/connectivity after both acute or chronic sleep deprivation. Interestingly, the extent of damage caused by sleep deprivation on the loss of the spine in the hippocampus pyramidal neurons is region specific. Acute sleep deprivation (5 hours) was shown a significant decrease in dendritic spines in the CA1 and DG regions but not in the CA3 pyramidal neurons (Havekes et al., 2016, Raven et al., 2019). Whether the CSD reduces the dendritic arborization in the hippocampus is region specific and these effects are reversible with sleep recovery should be explored. Which could reveal the underlying mechanisms of CSD-induced spatial memory impairment (Table 2.2).

Table 2.2: Published reports on the effect of sleep deprivation on structural changes in the hippocampus.

Author and year	Intervention	SD model	Findings
Havekes et al., 2016	5 h of TSD in mice	GH	Reduced CA1 neuronal spines, but not in the CA3
Raven et al., 2019	5 h of TSD in mice	GH	Decrease in spine density in the dentate gyrus
Brodin et al., 2022	5 h of TSD in mice	GH	No changes in the dendritic length and spine density in the CA1 region.
Gisabella et al., 2020	5 h of TSD in mice	GH	Increased the density of spines in CA1
Acosta-peña et al., 2015	24 h of TSD in rats	GH	Spine density was reduced in the CA1 and no differences in total

			dendritic length and branching length
Giri et al., 2021	6-days of REM SD in rats	MMPM	Reduced CA1 and CA3 neuronal dendritic length, branching, arborization, and spine density
Chen et al., 2009	5-days of TSD in rats	MMPM	Dendritic spines were reduced in both CA1 and CA3 pyramidal neurons.
Noorafshan et al., 2017a	21-days (18h/day) of CSD in rats	MMPM	CSD decreases the dendritic length and spine density in CA1
Noorafshan et al., 2017b	21-days (18 h/day) in rats	MMPM	Reduced the CA1 (48%) and DG (25%) pyramidal neurons
Aleisa et al., 2011	48 h of REM SD	MMPM	Significant impairment of long-term potentials in CA1 and DG.
Tang et al., 2019	4 weeks of chronic stress	-	Reduced the CNPase+ve oligodendrocytes in the DG and CA3 region of the hippocampus
Bellesi et al., 2018	4 ½ CSD	GH	Reduced thickness of myelination

TSD: Total sleep deprivation, **REM SD:** Rapid eye movement sleep deprivation, **SD:** Sleep deprivation, **CSD:** Chronic sleep deprivation, **GH:** Gentle handle, **MMPM:** Modified multiple platform method, **CA1:** Cornus-amminus 1, **CA3:** Cornus-amminus 3, **DG:** Dentate gyrus and **h:** hours, **CNPase+ve:** 2′,3′-cyclic nucleotide-3′-phosphodiesterase.

2.3.7. Impact of sleep deprivation on neuroglial cells

Astrocytes, microglia, and oligodendrocytes are the neuronal supporting cells that contribute to 80-90 % of brain cells, even the ratio of neurons and neuroglia was shown as 1:10 in humans. Neuroglia cells provide vital metabolic support to maintain their physiological functions. Glia has emerged as key regulators of the brain structure and function, but their role in sleep deprivation responses is considerably less known. More studies are required to highlight the importance of neuroglial cells such as oligodendrocytes and the generation of myelin in the brain circuits related to cognition and behavior in sleep and sleep deprivation.

2.3.7.1. Oligodendrocytes biology and functions

Oligodendrocytes are the non-neuronal cells that form the myelin in the CNS, they play a role in the ionic balance of the CNS and provide metabolic and tropic supply for axonal integrity and functionality (Ettle et al., 2016, Simons). The oligodendrocytes are derived from the oligodendrocyte precursor cells in the CNS (Barres and Raff, 1993). Each mature oligodendrocyte can enwrap the 20 axonal segments on average with multiple lamellae of the membrane in each segment (Bellesi, 2015). Overall, each oligodendrocyte can make 100 myelin sheaths (Xin and Chan, 2020). Myelin is rich in lipids and a variety of proteins, which include proteolipid protein (PLP), myelin basic protein (MBP), 2′,3′-cyclic nucleotide-3′-phosphodiesterase (CNP) around the axon (Aggarwal et al., 2011, Jahn et al., 2009). Myelin can protect and insulate the axons which enhances the salutatory conduction of electrical impulses through the axon (Xin and Chan, 2020). The optimal speed of neural transmission is crucial for the successful integration of information across distinct brain regions (Xin and Chan, 2020). Oligodendrogenesis play a role in memory consolidation most likely through the fine-tuning of myelin patterns in hippocampus circuits. Increased myelination and oligodendrogenesis were observed in rats subjected to learning tasks, however, the disruption of oligodendrogenesis reduced the myelination, impaired memory consolidation, and contextual fear (Steadman et al., 2020). Myelin and oligodendrocytes abnormalities were determined to be changes associated with cognitive impairment in neurodegenerative diseases (Chao et al., 2021). Further, the four weeks of chronic unpredictable stress resulted in a decrease of oligodendrocytes in the rat hippocampus resulting in depression (Tang et al., 2019). Four weeks of chronic stress showed a decrease in oligodendrocytes gene expression in the medial prefrontal cortex of the mouse (Liu et al., 2018). Collectively, explaining that oligodendrocytes and the optimal

myelin sheath are crucial for the neurophysiological functioning of the brain including cognition, behavior, motor activity, and sensory perception. The condition which may cause the reduction in the mature oligodendrocytes and decreased thickness of myelination could detrimentally affect the functioning of CNS.

Myelin proteolipid protein, MBP, galactocerebroside, and 2':3'-cyclic nucleotide-3'-phosphodiesterase (CNPase) are the markers expressed either in oligodendroglia or myelin (Kanno et al., 2010). The CNPase is expressed in oligodendrocytes and Schwann cells and consider for marker myelin-forming cells, although it is found in other cells including lymphocytes and photoreceptors (Yuan et al., 2002). CNPase catalyzes the nucleoside 2',3'-cyclic phosphate+H_2O the nucleoside 2'-phosphate, however, specific physiological functions are still ambiguous (Kursula, 2008). The CNPase expression becomes higher when myelination is most active which indicates that CNPase plays an important role in myelin genesis (Kanfer et al., 1989). CNPase is distributed in the cell membrane region of the oligodendrocytes and peri-axonal regions of the myelin sheath and it is the third most abundant myelin protein in the CNS, representing 4% of CNS myelin proteins (Trapp et al., 1988). Sleep is associated with the transcription of *MBP* and *CNP* genes.

2.3.7.2. Impact of sleep and sleep deprivation on the oligodendrocytes and myelination in the brain

Oligodendrocytes produce myelination that wraps the axons to increase conducting velocity of electrical signals. This oligodendrocytes functioning is influenced by sleep-wake cycles. Bellasi et al., (2015) observed that the number of oligodendrocytes precursor cells proliferating doubles during sleep relative to spontaneous wake and sleep deprivation (Bellesi, 2015). Sleep may replenish the energy

for oligodendrocytes to re-establish cellular homeostasis and promotes myelin growth. Consequently, sleep may be crucial to the process that results in enhanced myelination after learning or increased neural stimulation. In contrast, oligodendrocytes may show a specific vulnerability to sleep loss because of their higher metabolism, and high susceptibility to oxidative stress. Indeed, acute/chronic sleep deprivation increases oxidative stress in the brain. Therefore, sleep deprivation-induced oxidative stress may lead to oligodendrocytes dysfunction. The emerging evidence reported that myelin structure was not affected in acute sleep deprivation (6-8 hours) though the molecular studies showed that the expression of several genes involved in myelin formation is decreased (Bellesi et al., 2013). Chronic sleep loss for 4 ½ days results in the decreased thickness of myelin in the corpus callosum of adolescent mice (Bellesi et al., 2018). Similarly, human studies also have reported that loss of white matter and a decrease in myelination were observed in obstructive sleep apnoea and restless leg syndrome patients a disease often associated with insufficient sleep (Connor et al., 2011, Kumar et al., 2014). These investigations imply that sleep deprivation may be detrimental to oligodendrocytes and more specifically to myelin, Although, a direct demonstration that CSD can cause oligodendrocyte dysfunction and myelin change is still lacking. Along with these cellular changes sleep deprivation might also influence at the molecular level.

Wake-related and sleep-related mRNAs fall under different functional categories, and there are considerable variations in brain gene expression between sleep and wake (Cirelli et al., 2009). In comparison to baseline sleep, many genes are involved in apoptosis and cellular stress (*Acin1, Bcat1, Otud7b, Nr4a1, Hip1, Irf8, Traf6,* and *HSP90aa1*) were elevated during spontaneous wakefulness and brief (4 hours) sleep deprivation. In contrast, several genes are involved in lipid metabolism, specifically glycerophospholipid biosynthesis *Dgat2, Cds1, Elovl7,* and *Chka* as well as in

myelination *Opalin, Pllp,* and *Qk* were preferentially transcribed during sleep (Cirelli et al., 2006). The CSD (5-days) significantly enhanced the expression of *IL-1β* and *TNF-α* mRNA whereas significantly decreased the *BDNF* mRNA levels in the hippocampus of rats. Sleep is required for the transcription of several genes coding for myelin structural protein (*MOBP, MAG, Plasmolipnin,* and *CD9*), myelin-related receptors (insulin-like growth factor binding protein 2), and enzymes (CNP, carbonic anhydrase II, and methionine adenosyl-transferase) (Bellesi et al., 2013). The rats subjected to CSD for one week showed the down-regulated expression of genes involved in the synthesis of myelin and associated proteins (Cirelli et al., 2006). In view of previous studies, sleep and sleep deprivation may detrimentally affect oligodendrocytes at cellular and molecular levels in CNS. However, there is a lack of evidences on the extent of CSD induced damage to oligodendrocytes and the duration of sleep recovery required to reverse the CSD induced changes.

2.3.8. Effect of sleep recovery on sleep deprivation-induced behavioral, biochemical, and structural changes in the brain

Sleep deprivation is considered a risk factor for disease. The biochemical, cellular, and molecular changes produced by sleep and sleep deprivation lead to health consequences, however, are largely unknown. Following sleep deprivation, a period of recovery sleep may beneficial to cognitive performance. However, important issues about the nature of such benefits remain unanswered. Most sleep deprivation studies especially chronic sleep loss experiments neither include nor report the efficacy of sleep recovery and that the damage is reversible or long-lasting. The rebound/recover sleep pattern is characterized as latency of sleep shorter, the efficiency of sleep higher, amount of REM sleep and deep sleep duration would be more in the condition of acute sleep

deprivation (Machado et al., 2004). However, the studies have not demonstrated a pattern of sleep recovery, which was after CSD. Sleep architecture was not recovered to baseline with three nights of sleep after multiple nights of sleep restriction in adolescence (Ong et al., 2016). Overall, it is indicating that recovery sleep architecture is not similar to normal sleep. The free radical flux hypothesis proposed that the core function of sleep is to serve as an antioxidant system for the brain. Therefore, sleep can reduce the free radicals produced during a wakeful state (Reimund, 1994). There is limited evidence on the effect of sleep recovery on cognition, behavior, neurochemical and neurodegenerative changes induced by sleep deprivation and reported as follows.

Sleep deprivation leads to biochemical, molecular, and structural changes in the brain/hippocampus. Earlier studies showed sleep recovery has a beneficial effect on acute sleep deprivation-induced damage. Sleep recovery led to an ameliorative effect on cognition especially on learning and memory in 3-days sleep deprived rats (Shehata and Rizk, 2015). Studies also demonstrated that acute sleep deprivation-induced oxidative stress in the brain can be reversible with sleep recovery (Mathangi et al., 2012). In our previous study we also observed that 72 hour total REM sleep deprivation elevated serum neuron-specific enolase, creatine kinase brain fraction, and lactate dehydrogenase brain fraction, however, these circulatory neuroinflammatory markers were restored after 24 hours of sleep recovery (Suresh et al., 2021). He et al., (2021) also reported that the permeability of the blood-brain barrier was attenuated within 24 hours of sleep recovery (He et al., 2021). A brief period of sleep deprivation (5 hours) resulted in a dendritic spine loss in CA1 neurons of the hippocampus was reversible with 3 hours of sleep recovery in mice (Havekes et al., 2016). In contrast, sleep deprivation (48 hours) induced spatial reference memory impairment was not restored with 48 hours sleep recovery in rats (Li et al., 2009). Seventy-two hours of sleep deprivation-induced

impairment of spatial memory and hippocampus neuronal apoptosis was not reversed even after 3 weeks of sleep recovery in mice (Soto-Rodriguez et al., 2016). The converging evidence suggests that sleep recovery has a beneficial effect on acute sleep deprivation-induced damage.

CSD is reported to be behavioral abnormalities and cognitive impairment. The harmful cellular consequences of prolonged sleep deprivation may result from oxidative mechanisms and excitotoxicity which sensitize the brain cells to neurodegeneration and subsequent changes in behavioral abnormality and cognitive impairment. The CSD-induced behavioral abnormality and cellular damage were not readily reversible with recovery sleep (Owen and Veasey, 2020). Ninety days of repeated and intermittent paradoxical sleep deprivation-induced impaired memory and hippocampal inflammation also persisted even after 3 weeks of sleep recovery in mice (Yin et al., 2017). In contrast, CSD (4 ½ days) mice showed a decrease in myelin thickness and it was not reversible with 32 hours of recovery sleep (Bellesi et al., 2018). Similar to the animal models chronically sleep restricted (4 hours/night for 5-days) human subjects also showed impairment in the neurobehavioral functions, which were not reversed with one night of sleep recovery (Banks et al., 2010). Several studies have recently demonstrated that a week of shorter sleep in healthy adults causes cumulative vigilance impairments with inadequate recovery following three full nights of recovery sleep (Belenky et al., 2003). In contrast, Everson et al., (2005) investigated the impact of two days of sleep recovery following 10-days of total sleep deprivation on DNA damage, apoptotic cell signaling and death, cell proliferation, and concentrations of glutathione peroxidase and catalase activity in the rat plasma, liver, lung, intestine, heart, and spleen. They observed that total sleep deprivation in rats causes a deleterious impact on these organs, however, two days of recovery sleep restored the balance between DNA damage

and repair and restored the metabolic burdens and oxidative damage (Everson et al., 2005). Additionally, 6-days of continuous REM sleep deprivation reduced hippocampal dendritic arborization was reversible with 3-days of rebound sleep (Giri et al., 2021). The converging literature suggests that unlike acute sleep deprivation the CSD induced behavioral abnormality and cellular damage were not readily reversible with recovery sleep. However, the CSD induced damage is either reversible or long-lasting also depending on the duration and type of sleep deprivation, and the subsequent sleep recovery period. In contrast to acute sleep deprivation, the CSD induced behavioral and morphological alterations and the duration of sleep recovery required to reverse the effects caused by CSD have not been thoroughly investigated.

2.4. Lacunae in knowledge

The imbalance between free radicals (increase) and antioxidants (decrease) results in oxidative stress. There is concrete evidence that CSD can increase oxidative stress in the CNS. The key aspects of learning and memory functions are regulated in the hippocampus and are dependent on sleep. It has been reported that CSD induced oxidative stress in the hippocampus might result in impairment of spatial memory and anxiety-like behavior. However, the cellular and molecular mechanisms by which CSD brings about its effects remain unclear, and also the duration of sleep recovery required to improve spatial memory and anxiety-like behavior has not been fully evaluated.

Studies have demonstrated oxidative stress in the hippocampus can negatively affect learning and memory by changing the structure of dendrites. A decreased dendritic spine number was observed in the CA1 and DG regions of the hippocampus after 5 hours of sleep deprivation in mice. In addition, 21-days of CSD decreases the dendritic length and spine density in the hippocampal CA1 region of rats. However,

little is known about the extent of 21-days of CSD-induced changes in dendritic arborization of hippocampal CA3 neurons, and the duration of sleep recovery required to reverse the changes has not been fully explored. In addition to neuronal damage, sleep deprivation might affect neuronal supporting cells, especially oligodendrocytes (cells are responsible for the synthesis of myelin). The evidence suggests that CSD for 4 ½ days results in a reduction of myelin thickness in the corpus callosum of adolescent mice. In addition, the expression of genes codes myelin structural proteins (*MBP and MOBP*), enzymes (*CNPase* and *carbonic anhydrase*), and myelination (*Opalin*, *Pllp*, and *Qk*) were altered by sleep and wake states in mice. The review of the studies highlights that very limited information is available on CSD induced damage to oligodendrocytes and the duration of sleep recovery required to reverse the damage caused by sleep deprivation.

The free radical flux hypothesis proposed that the core function of sleep is to serve as an antioxidant system for the brain. Therefore, sleep can reduce the free radicals produced during a wakeful state. Studies have also demonstrated that acute sleep deprivation induced oxidative stress in the brain can be reversible with sleep recovery. In contrast to studies on acute sleep deprivation, the CSD induced behavioral and morphological alterations and the duration of sleep recovery required to reverse the biochemical, cellular, and molecular changes and behavioral effects caused by CSD have not been thoroughly investigated. Hence, the present study aimed to investigate the effect of 21-days (18 hours/day) of CSD and 5- and 21-days of sleep recovery on spatial learning and memory, anxiety-like behavior, oxidative stress markers, dendritic arborization of hippocampal CA3 neurons, number of CNPase+ve oligodendrocytes, and CNPase protein and gene expression changes in the hippocampus of rats.

Aim and Objectives

Aim

To study the effect of oxidative stress induced by chronic sleep deprivation and the efficacy of sleep recovery on the structural integrity of dendritic arborization of CA3 neurons and oligodendrocytes in the hippocampus as well as cognition and behavior of rats.

Objectives

- To evaluate the effect of oxidative stress induced by CSD on cognitive functions and anxiety-like behavior of rats.
- To evaluate the effect of CSD on dendritic arborization of CA3 neurons and oligodendrocytes in the hippocampus of rats.
- To investigate the efficacy of sleep recovery in reversing the CSD induced oxidative stress, structural integrity of CA3 neurons and oligodendrocytes, and cognition and anxiety-like behavior of rats.

Chapter III

Materials and Methods

3.1. Materials

3.1.1. Chemicals and solvents

Trichloroacetic acid (TCA)

2-Vinylpyridine

Anti-mouse IgG antibodies (Vector, #Cat No pk-4002, Vector Laboratories, USA)

Avidin biotin complex (Vector, #Cat No pk-4002, Vector Laboratories, USA)

Bovine serum albumin

Coomassie brilliant blue G-250

Diaminobenzidine

Disodium hydrogen phosphate (Na_2HPO_4)

Distilled water

Distrene Polystyrene Xylene (DPX)

Ethanol

Ethyl alcohol

Food pellets

Glacial acetic acid

Hydrochloric acid (HCl)

Hydrogen peroxide

Imidazole

Ketamine

Liquid nitrogen

Magnesium chloride ($MgCl_2$)

Methanol

Mouse Anti-CNPase monoclonal antibodies (Abcam # Cat No: ab6319, USA)

N-Butanol

Paraformaldehyde

Phosphoric acid (H_3PO_4)

Potassium chloride (KCL)

Potassium chromate (K_2CrO_4)

Potassium dichromate ($K_2Cr_2O_7$)

Potassium dihydrogen phosphate (KH_2PO_4)

Sodium acetate anhydrate ($C_2H_3NaO_2$)

sodium carbonate (Na_2CO_3)

Sodium Chloride (NaCl)

Sodium Hydroxide (NaOH)

Sucrose ($C_{12}H_{22}O_{11}$)

Tetra methoxy propane ($C_7H_{16}O_4$)

Thiobarbituric acid reagent (TBA)

Tris

Triton-X 100

Try sodium citrate ($Na_3C_6H_5O_7$)

Tween 20

Xylazine

Xylene

TRIzol reagent

High-capacity cDNA conversion kit (Thermo Scientific, USA)

Chloroform

SYBR green (VNIR, India)

Nuclease free water

3.1.2. Instruments

Modified multiple platform instrument

Elevated plus maze

Open field maze

Hebb-William maze

Morris water maze

Spectrophotometer (Lambda, Perkin Elmer, USA)

ELISA reader (Rayto, China)

Microscope (Olympus, Japan)

Digital microscope (Carl Zeiss, Germany)

Camara Lucida

Vibratome microtome (Leica VT 1000S)

Magnetic stirrer with hotplate

pH meter (Sartorius)

Water bath

Hot air oven

Autoclave

Stopwatch

Timer

Deep freezers: (0-4, -20, and -80 ℃): Bio-Equip (Model-BPS-345S), Cryo Scientific (Model- URC-V-700-4)

Vortex mixer (REMI; CM-101)

Rotary shaker

Pipettes (1, 5, 20, 200, and 1000 µl)

Microtome

Real-time PCR machine (Bio-Rad, Model-781BR16930, USA)

PCR machine (Bio-Rad, USA)

Nanodrop (Thermo Fisher Scientific, USA)

Microcentrifuge **(**Tarsons; Model-1000)

Cooling centrifuge (Thermo Fisher Scientific, Model-Sarvall legend XTR)

Thermometer

3.1.3. Glassware and plastic wares

Flasks (Borosil)

Beakers (Borosil)

Graduated cylinders (Borosil)

Test tubes (Borosil)

Funnels (Borosil)

Measuring cylinders

Falcon tubes (15 ml) (Abdos)

Cryo Box (1.8 ml) (Tarsons)

PCR tubes (0.2 ml) (Tarsons)

Microtips (0.2-10 µl) (Abdos)

Microtips (2-20 µl) (Abdos)

Microtips (100-1000 µl) (Abdos)

Cuvette

Eppendorf tubes

EDTA coated blood collection tubes

Syringes

Bottles and jars

Parafilm

96-well plates

24-well plates

Reagent bottles **(**Borosil)

Gelatin coated slides

3.1.4. Materials used for animal handling

Polypropylene rat cages (Vishnu traders, India)

Cage water bottles (200 ml)

Paddy husk (Ramu rice industries, Kolar, India)

Food pellets (Champaka feeds and foods for laboratory animals, Bangalore, India)

Rat perfusion and dissection kit

3.1.5. Reagent preparation

Phosphate buffer saline (PBS): 1 L

Sodium Chloride	- 8 g
Potassium chloride	- 200 mg
Disodium hydrogen phosphate	- 1.44 g
Potassium dihydrogen phosphate	- 240 mg

All the ingredients were added to 800 ml of distilled water and then adjusted the pH=7.4 (Hydrochloric acid and Sodium Hydroxide were used to adjust the pH) with a final volume of 1 L. PBS was stored at 4 ℃ temperature until further use (Dulbecco and Vogt, 1954).

Tris-buffered solution (TBS): 1 L

Tris	- 6.05 g
NaCl	- 8.76 g
Distilled water	- 1000 ml
Adjusted the pH=7.4	

Bradford reagent

Coomassie brilliant blue G-250	- 25 mg
Methanol	- 25 ml
85% Phosphoric acid (H_3PO_4)	- 50 ml
Distilled water	- 425 ml

The final volume of 500 ml Bradford reagent was stored in the dark brown bottle at 4 ℃ and filtered before use.

0.08% of Thiobarbituric acid reagent

8 mg of TBA were dissolved in 1 ml of distilled water.

24% Trichloroacetic acid:

24 g of TCA were dissolved in 100 ml of distilled water.

0.1% Triton X in TBS (TBS-T)

0.1 ml of Triton X 100 in 100 ml of TBS.

Sodium citrate buffer

10 mM trisodium citrate, 0.05% tween 20 with pH=6.0.

0.3% H_2O_2

0.3 ml H_2O_2 in 100 ml of 70% methanol.

3% bovine serum albumin

3 ml bovine serum albumin in 100 ml of TBS.

Diaminobenzidine in acetate imidazole buffer

0.1 M Imidazole buffer: 0.68 g of imidazole in 100 ml of distilled water.

0.1 M sodium acetate anhydrate: 0.82 g of sodium acetate in 100 ml of distilled water.

Mixed both imidazole and sodium acetate buffers in equal volume and adjusted the pH=7.4.

Primary antibodies: Anti-CNPase monoclonal antibodies

Dilution: 1:1000 (1 µl of anti-CNPase antibodies diluted in 999 µl of TBS having 1% Bovine serum albumin (BSA).

Secondary antibodies: Anti-mouse IgG antibodies, Avidin biotin complex kit

Anti-mouse IgG antibodies: 1:200 dilutions: 50 µl of anti-mouse IgG antibodies in 10 ml of TBS.

Avidin biotin complex: 1:100 dilution: 100 µl of avidin-biotin complex in 10 ml of TBS.

Golgi cox stain: 500 ml

Solution A: 5% potassium dichromate ($K_2Cr_2O_7$) - 5 g/100 ml

5% Magnesium chloride (MgCl2) - 5 g/100 ml

Solution B: 5% potassium chromate (K_2CrO_4)

5 g/100 ml (Prepared in a dark beaker)

To solution B added 200 ml of distilled water. Solution A was added slowly for about 30-45 min into solution B used a magnetic stirrer for mixing. The precipitate was allowed to settle down to the bottom overnight at room temperature followed by filtered the supernatant.

8% Sodium carbonate (Na_2CO_3)

8 g of Na_2CO_3 was added to 100 ml of distilled water and mixed thoroughly

3.2. Methods

3.2.1. Animals and intervention

3.2.1.1. Experimental setup

The study was approved (Ref No: IAEC/PHARMA/SDUMC/2017-18/05a) by the Institute Animal Ethics Committee, Sri Devaraj Urs Medical College, Kolar, Karnataka, India. Sixty male rats (Sprague Dawley, n=60) aged between 12-16 weeks and with a bodyweight of 200-220 g were procured from Biogen Laboratory Animal Facility, Bangalore, India (CPCSEA Registration No. 971/bc/06). All the rats were acclimatized to the laboratory for 7-days before the start of the study and divided into two sets (n=30/set). The rats were maintained at 12:12 hour light and dark cycle (lights on at 6.00 am and lights off at 6.00 pm) with a room temperature of 22±2 ℃ by providing ad libitum of food and water. The overall experimental design of the present study was shown in (Figure 3.1).

The sample size of the present study was estimated based on a study by Lima et al., (2014). The difference of Malondialdehyde levels (oxidative stress marker) in the control and sleep deprivation group (48 hours), the outcome assessment for an MDA in the hippocampus, the minimum difference of 7.0 μM and standard deviation of 5.0, 90% statistical power, 5% level of significance, the sample size per group 6 is adequate.

Figure 3.1: Representative image of Sprague Dawley rats in a polypropylene rat cage.

3.2.1.2. Sleep deprivation and sleep recovery of rats

The rats were grouped as control, environmental control (EC), chronic sleep deprivation (CSD), CSD+5 days sleep recovery (CSD+5D SR), and CSD+21 days sleep recovery (CSD+21D SR). The rats were sleep deprived using the Modified Multiple Platform Method (MMPM) as described in our previous study (Suresh et al., 2021). Briefly, the Modified Multiple Platform Instrument (MMPI) has 12 platforms (110 x 44 x 45 cm), each platform has a 5.5 cm diameter and 6.0 cm height. All platforms were surrounded by water up to 1 cm beneath the platform. In this setup, whenever the rat tries to sleep, it will lose muscle tone and falls into the water, and climbs back to the platform. The rats were habituated to MMPI for 3-days (one hour/day) before subjecting them to sleep deprivation. The CSD, CSD+5D SR, and CSD+21D SR groups rats were sleep deprived for 21-days (18 hours/day: 2.00 pm-8.00 am in MMPI and 6 hours/day: 8.00 am-2.00 pm in a home cage) at 12:12 hour light and dark cycle (lights on at 6.00 am and lights off at 6.00 pm) by supplying ad libitum of food and water (Figure 3.2A). The water in the instrument was changed daily throughout the CSD period. Furthermore, the CSD+5D SR and CSD+21D SR group rats were sleep recovered for 5- and 21-days in home cages kept at 12:12 hour light and dark cycle (lights on at 6.00 am and lights off at 6.00 pm) by supplying ad libitum of food and water. Control group rats were placed in their home cages without interruption of sleep. The environmental control group rats were kept in MMPI with stainless steel grid (2.3 mm pore size) placed 1 cm above the water levels (18 hours/day: 2.00 pm-8.00 am and 6 hours/day: 8.00 am-2.00 pm in a home cage) (Figure 3.2B). In this setup, the animals can sleep on a stainless-steel grid without falling into the water (Suresh et al., 2021).

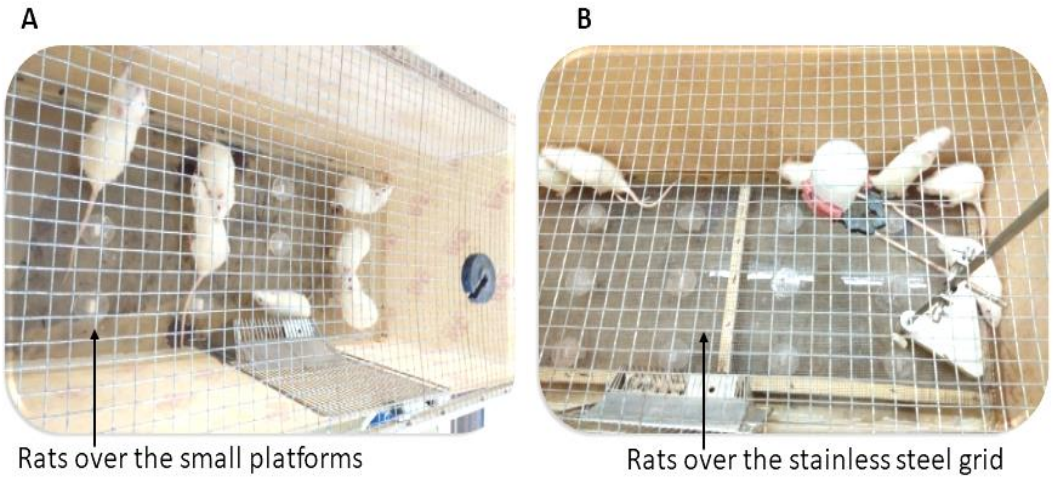


Figure 3.2: Representative image of modified multiple platform instrument. (A) Rats over the small platforms used for CSD. (B) Rats over the stainless-steel grid used for the EC group.

At the end of 21-days of sleep deprivation/5- and 21-days of sleep recovery, the first set of rats (five groups; n=6/group) were used to assess the spatial memory and anxiety-like behavior using the Hebb-Williams maze (HWM) and Elevated plus maze (EPM) respectively. Following the behavioral tasks, the rats were dissected and harvested the hippocampus from each cerebral hemisphere. The right hippocampal tissue was used for the measurement of oxidative markers such as malondialdehyde (MDA), total glutathione, GSSG/GSH ratio, and total antioxidant capacity (TAC). The left hippocampus was used to measure the *CNPase* gene expression using qRT-PCR. The second set of rats (five groups; n=6/group) was used to assess spatial learning and memory using the Morris Water maze (MWM) and anxiety-like behavior was assessed by using the open field maze (OFM). Following the behavioral tasks, the rats were dissected and harvested brain samples. The right cerebral hemisphere from each brain sample was processed to assess the dendritic arborization of hippocampal CA3 neurons using the Golgi cox method and the left cerebral hemisphere was processed for the immunohistochemistry to measure the CNPase+ve oligodendrocytes, additionally, the blood sample was collected and used to measure corticosterone levels (Figure 3.3).

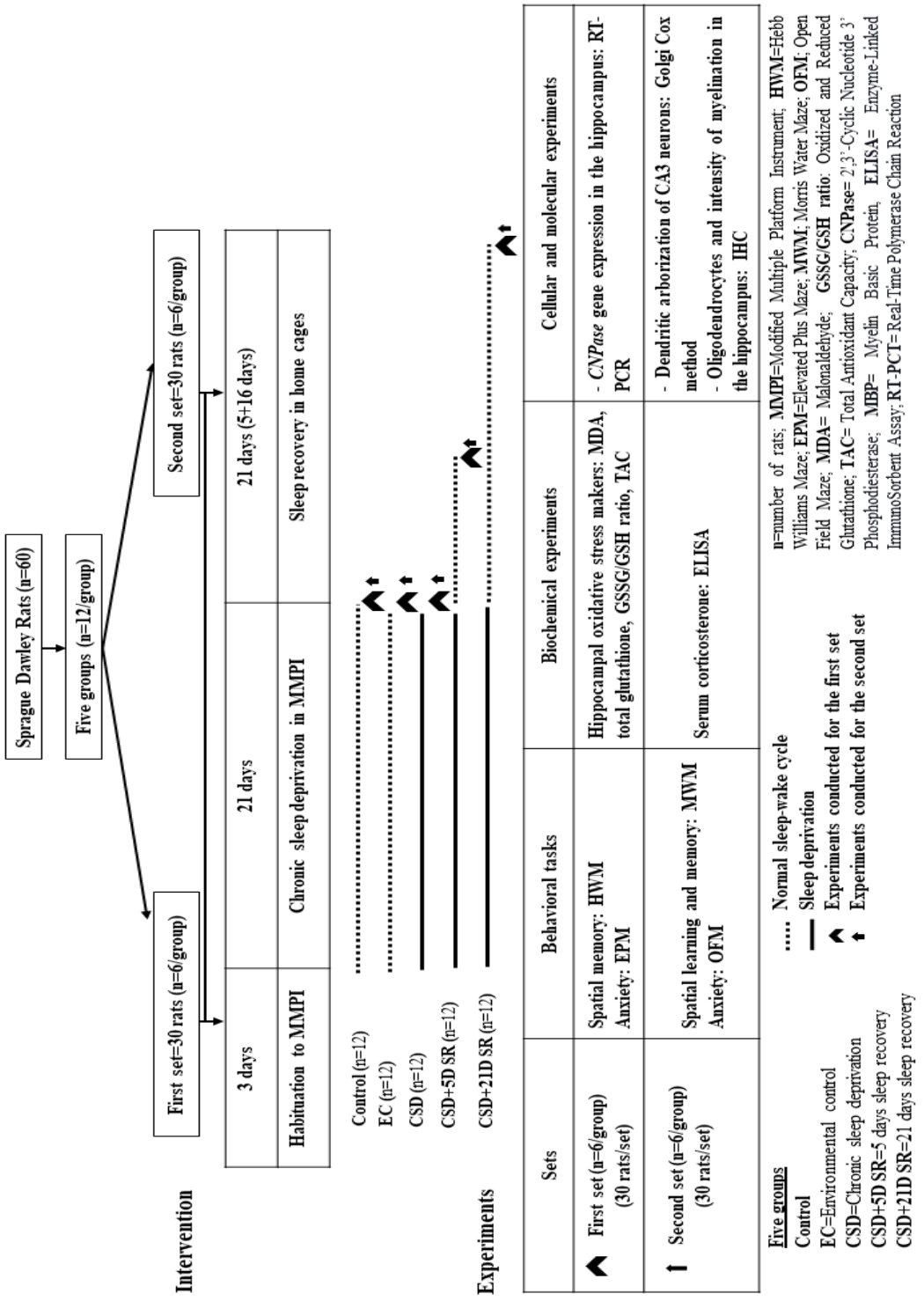


Figure 3.3: Overview of the experimental methodology followed for the current study.

3.2.2. Behavioral experiments

The spatial learning and memory were assessed using the HWM and MWM. Anxiety-like behavior was assessed using the EPM and OFM.

3.2.2.1. Hebb Williams maze task

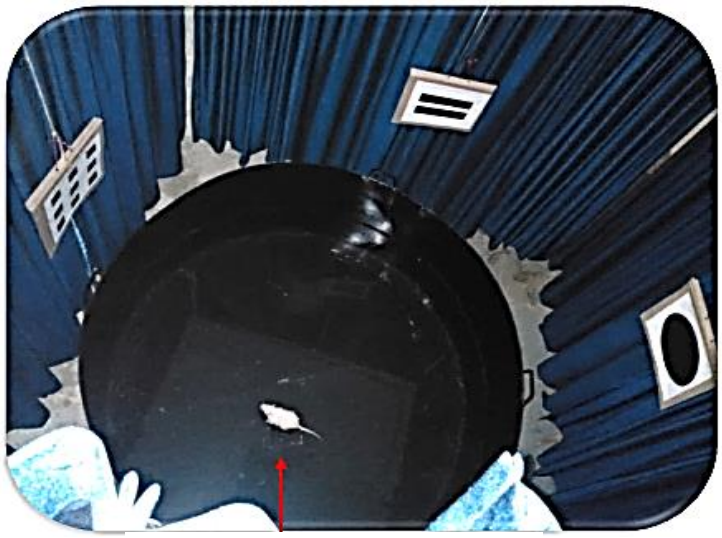
The rats were assessed for spatial memory as described in Hebb et al., (1946) (Hebb and Williams, 1946). The HWM consists of a similar-sized start and goal box, which are located on opposite corners of the maze (Figure 3.4). The time taken by the rats to navigate from the start box to the goal box in a maze is called 'latency time'. The experiment consists of three phases: habituation, acquisition, and testing. The first set of rats (n=6/group) were habituated to HWM for 3-days (10 min/day) without internal walls in the maze. After habituation, the rats were exposed to four simple maze patterns for 7-days (acquisition phase: 4 trials/day) to attain the performance criteria (the rats need to trace any two maze patterns in <25 sec). After obtaining the performance criteria, the rats were subjected to 21-days of sleep deprivation and 5- and 21-days of sleep recovery. At the end of sleep deprivation/sleep recovery, the rats were tested for spatial memory by exposing them to any two maze patterns and the latency time (sec) was recorded from all the groups. The mean latency time of both the acquisition phase and testing phase from all the groups was recorded. All the spatial learning and memory experiments were performed between ZT 4-ZT 5.5.

3.2.2.2. Morris water maze task

It was used to assess the spatial learning and memory of rats (n=6/group) as described in (Bindu et al., 2005). The MWM is iron made circular pool (diameter 178 cm and height 68 cm) painted with black colour and filled with water (temperature 24-26 ℃) depth of 34 cm. The escape platform (diameter 14 cm) submerged 1 cm below the water surface and the position (south quadrant) was remains the same throughout the experiment (Figure 3.5).

Figure 3.4: Representative image of Hebb-William's maze used for the assessment of spatial learning and memory in rats.

Additionally, the reference "cues" which are outside of the maze also remained unchanged throughout the experiment. Rats were allowed to swim for 90 sec without the availability of an escape platform during the habituation trial (2 trials/day for days) in the water maze. Overall the experiment consists of the acquisition phase and the probe trial**.** Acquisition phase: In the last 4-days of EC, CSD, CSD+5D SR, and CSD+21D SR intervention, the second set of rats underwent training in MWM (4 trails/day: one trial from each quadrant i.e south (S), west (W), east (E) and north (N) to reach the escape platform). The maximum trial period was 90 sec to find an escape platform. Initially, if the rat did not find the platform during the trial it was gently guided to reach the platform and allowed to remain there for 30 sec. The given inter-trial interval was 30 min. Probe trial: Next day to the acquisition phase (after CSD and sleep recovery intervention) probe trial was performed, in which the platform was removed from the pool and allowed the rat to swim for 60 sec. The time spent in each quadrant was measured. The video was recorded for all acquisition phase trials and probe trials. All the spatial learning experiments were performed between ZT 1-ZT 2 and spatial memory assessment was performed between the ZT 4-ZT 5.5.



Rat over the escape platform

Figure 3.5: Representative image of Morris water maze used for assessment of spatial learning and memory in rats.

3.2.2.3. Elevated Plus Maze task

It was used to measure the anxiety-like behavior of rats as described in Pellow et al., (1985). EPM is comprised of two closed arms with high walls 50 × 10 × 40 cm (L×W×H) and two open arms 50 × 10 cm (L×W). The arms were connected with a central square (10 × 10 cm) to give the apparatus a plus sign appearance. The maze arms were elevated 60 cm above the floor (Figure 3.6). At the end of sleep deprivation/sleep recovery, the first set of rats (n=6/group) was used to assess the levels of anxiety. The rats were gently placed in the central square facing the open arm. The rats were freely allowed to explore the maze for 5 min and a video was recorded. The time spent in the closed arm, open arm, the number of entries into the open arm, and the total number of crossings were collected from the recorded video. The anxiety index was calculated as described earlier by Subhadeep et al., (2020) (Subhadeep et al., 2020). The anxiety index ranges between 0-100%. The experiments to measure anxiety was conducted between ZT 6-ZT 7.

$$\text{Anxiety Index \%} = 1 - \left[\frac{\left[\left(\frac{\text{Time spent in OA}}{\text{Total time of test}}\right) + \left(\frac{\text{Entries in OAs}}{\text{Total entries}}\right)\right]}{2}\right] \times 100$$

Figure 3.6: Representative image of elevated plus maze used for the assessment of anxiety-like behavior in rats.

3.2.2.4. Open field maze task

It was used to assess the anxiety-like behavior of the rats as described in (Subhadeep et al., 2020). The OFM was made up of a wooden box (90 × 90 × 45 cm) coated with black acrylic polyvinyl paint. The floor had 36 squares (15 × 15 cm) in a grid-like fashion, these were considered as central zone (central 16 squares) and peripheral zone (peripheral 20 squares) (Figure 3.7). At the end of sleep deprivation and sleep recovery, the second set of rats (n=6/group) from each group was subjected to OFM to assess the anxiety-like behavior. The rat gently dropped to the corner of OFM facing the opaque wall. It was freely allowed to expose the maze for 5 min and a video was recorded to assess the initial and acclimatization response to the novel environment. The parameters such as latency time to enter the central zone, time spent in the central or peripheral zone, frequency of zone transmission, resting time, fecal boli count, and total distance traveled were collected from video to assess the behavior of rats. After end intervention (CSD and sleep recovery) the experiment was conducted at the time zone of ZT 6 to ZT 7. OFM was cleaned with 70% ethanol before testing each rat.

Figure 3.7: Representative image of open field maze used for the assessment of anxiety-like behavior in rats.

3.2.3. Biochemical experiments

At the end of sleep deprivation/sleep recovery and behavioral experiments, all the rats (from both sets) were euthanized by injecting a combination of ketamine (92 mg/kg body weight) and xylazine (9.2 mg/kg body weight) intraperitoneally (ZT 7-ZT 9). The rats were perfused with PBS and brain samples (hippocampus) were collected from all the groups. The first set of rat samples (n=6/group) were used to measure oxidative stress markers and gene expression (CNPase) in the hippocampus. The second set of rats was used to measure the serum corticosterone, dendritic arborization of hippocampal CA3 neurons, and CNPase+ve oligodendrocytes in the hippocampus from the collected blood and brain samples (Figure 3.3).

3.2.3.1. Measurement of oxidative stress markers in hippocampal tissue

Hippocampal tissues were homogenized using phosphate buffer saline (PBS) with a ratio of 1:10 (W/V). The homogenate was centrifuged using 10000 rpm for 20 min at 4 ℃, the supernatants were collected, aliquoted, and stored at -20 ℃ until further use. Total protein concentration was measured in the supernatant using the Bradford method. Further, the supernatants were used to measure the levels of malondialdehyde (MDA),

total glutathione, oxidized and reduced glutathione ratio (GSSG/GSH ratio), and Total antioxidant capacity (TAC).

3.2.3.1.1. Estimation of total protein in the hippocampus

Method: Bradford (Bradford, 1976).

Principle: Binding of protein molecules to Coomassie dye under acidic conditions results in a colour change from brown to blue. The colour intensity was measured at 595 nm using a spectrophotometer.

Standard preparation: Bovine serum albumin was used as standard.

Stock solution: 10 mg of BSA was mixed in 5 ml of PBS and the final concentration of the stock solution was 2 mg/ml (2000 µg/ml).

Working standards: Different concentration standards were prepared using the stock solution as shown in table 3.1 and the working procedure in table 3.2.

Table 3.1: Bovine serum albumin standards.

S. No	Standard	PBS (µl)	Stock BSA (µl)	Final Conc. (µg/ml)
1	B	1000	0	0
2	S1	925	75	150
3	S2	850	150	300
4	S3	775	225	450
5	S4	700	300	600
6	S5	625	375	750
7	S6	550	450	900
8	S7	475	525	1050
9	S8	400	600	1200

Sample: Homogenized hippocampus samples were used for total protein analysis. The sample was diluted on a 1:6 ratio by using PBS.

Table 3.2: Working procedure followed for the measurement of total protein levels.

S. No.	Tubes	Bradford Reagent (ml)	PBS (µl)	Standard (µl)	Sample (µl)
1	Blank	1.5	30		
2	Standard	1.5		30	
3	Test	1.5			30

The samples were incubated at 37 ℃ for 5 min and developed colour intensity was measured at 595 nm in a spectrophotometer (Figure 3.8).

Calculation: Y=4296.X-20.01

Y=Concentration of sample; X=OD values of the sample

The final value was multiplied with the dilution factor and the values are expressed in mg/ml.

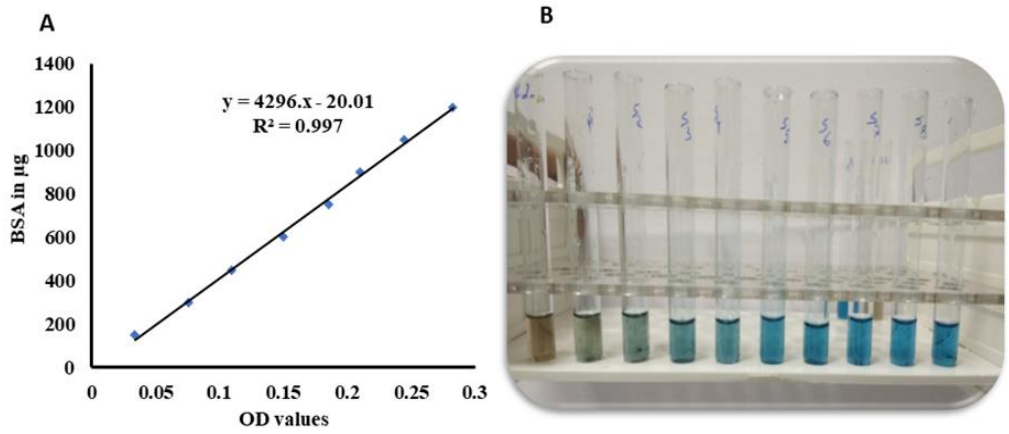


Figure 3.8: Representative images of (A) total protein standard curve and (B) total protein standard tubes.

3.2.3.1.2. Estimation of lipid peroxidation (malondialdehyde) in the hippocampus

Method: Thiobarbituric acid reactive substances (TBARS) (Wills, 1966).

Principle: Free MDA as measured by lipid peroxides derived from polyunsaturated fatty acids is unstable and decomposes to form a complex series of compounds which include reactive carbonyl compounds such as MDA. TBARS assay was used to assess the MDA. To the deproteinated sample, 0.8% thiobarbituric acid was added and incubated at 90 ℃ in a water bath for one hour. The MDA in the sample reacts with thiobarbituric acid

and forms thiobarbituric acid reactive substances and develops the yellow colour. The colour intensity of the solution was measured at 530 nm. The tetra methoxy propane (TMP) was used as a standard solution.

Standard preparation: Stock solution: 16.5 mg of TMP was dissolved in 10 ml of PBS that equal to the 10 M/L of TMP.

1 ml of 10 M mol/L: TMP stock was dissolved in 100 ml of PBS to obtain the 100 µM/L of TMP.

20 µ mol/L of TMP: 1 ml of 100 µM/L of TMP were dissolved in 4 ml of PBS to obtain the 20 µM/L of TMP.

Different concentrations of working standards were prepared using the 20 µM/L of TMP (Tables 3.3 and 3.4).

Table 3.3: Composition of tetra methoxy propane standards.

S. No	Standard	PBS (µl)	Stock TMP (µl)	Final Conc. (µM/L)
1	B	750	0	0
2	S1	0	1500	20
3	S2	750	750 of S1	10
4	S3	750	750 of S2	5
5	S4	750	750 of S3	2.5
6	S5	750	750 of S4	1.25

Table 3.4: Working procedure followed for measurement of malonaldehyde.

S. No	Reagents	Blank (µl)	Standard (µl)	Test (µl)
1	PBS	500		
2	Standard		500	
3	Sample			500
4	24% TCA	1000	1000	1000
Centrifuged at 3000 RPM/30 min and collected the 1000 µl of supernatant				
5	Supernatant	1000	1000	1000
6	0.08% TBA	250	250	250
Tubes were kept in a 90 ℃ boiling water bath for 1 h and cooled the tubes				
7	N-Butanol	500	500	500
All the tubes were vortexed for 1 min				

Sample: Homogenized hippocampus samples were used for MDA measurement. The sample was diluted on a 1:3 ratio by using PBS.

The colour intensity was measured at 532 nm (Figure 3.9).

Calculation: Y= 46.8.X-2.661

Y=Concentration of samples, X=sample OD values

The obtained sample concentration was multiplied with the dilution factor and values were represented in µM/L.

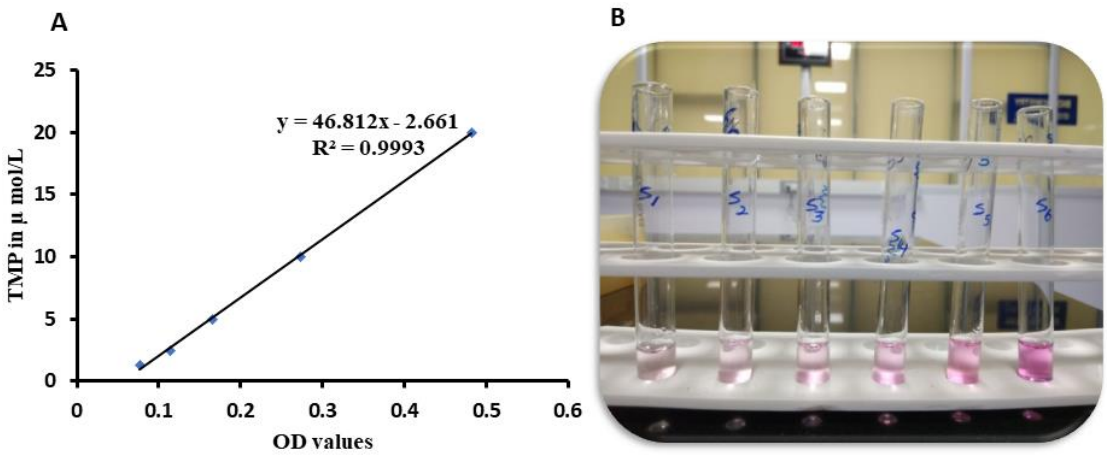


Figure 3.9: Representative images of (A) MDA standard curve and (B) MDA standard tubes image.

3.2.3.1.3. Estimation of total antioxidant capacity in the hippocampus

Total antioxidant capacity was measured using a total antioxidant capacity assay kit from the immune tag (Cat log# ITAK1310). The experiments were carried out according to manufacturer instructions.

Principle: Enzymatic and nonenzymatic antioxidant levels in the sample, under acidic conditions Fe3+ - TPTZ were reduced to Fe2+ - TPTZ and developed a blue color. The rate of reaction reflected the TAC in the sample. The color intensity was measured in a spectrophotometer at 593 nm and the units were expressed as µM/mg of protein.

Items supplied: Reagent I- 35 ml, Reagent II- 20 ml, Reagent III- 5 ml.

Solution mixture: Reagent I+II+III with the ratio of 7:1:1 mixture.

Standard preparation: 10 mg of $FeSO_4$. 7 H_2O: The working solution was prepared with the addition of 1.75 ml of distilled water and 1 drop of concentrated sulphuric acid and forms the final concentration of 20 µM/ml $FeSo_4$ standard solution.

Dilute 20 µM/ml FeSO4 standard solution to 0.2, 0.1, 0.05, 0.025, 0.0125, 0.00625, 0.003125, 0.00156 µM/ml, and added the 500 µl of reagent II then the final concentration $FeSO_4$ was 0.1, 0.05, 0.025, 0.0125, 0.00625, 0.003125. Taken the 500 µl of distilled water mixed with 500 µl of reagent II. Then mixed thoroughly for 10 min and taken reading using a spectrophotometer at 593 nm (Figure 3.10).

For the test samples: added the reagents according to the following procedure.

S. No	Reagents	Blank (µl)	Test (µl)
1	Solution mixture	900	900
2	Sample	0	30
3	Distilled water	120	90
Tubes were mixed for 10 min and the colour intensity was measured at 593 nm			

Calculation: Y=0. 123.X-0.004

Units for mg of protein used the following formula according to manufacturer instructions.

X×Vrv÷(Vs×Cpr)= 34×X÷Cpr, Vrv: Total reaction volume-1.02 ml, Vs: sample volume-0.03 ml, Cpr: Sample protein concentration, mg/ml. X: sample concentration.

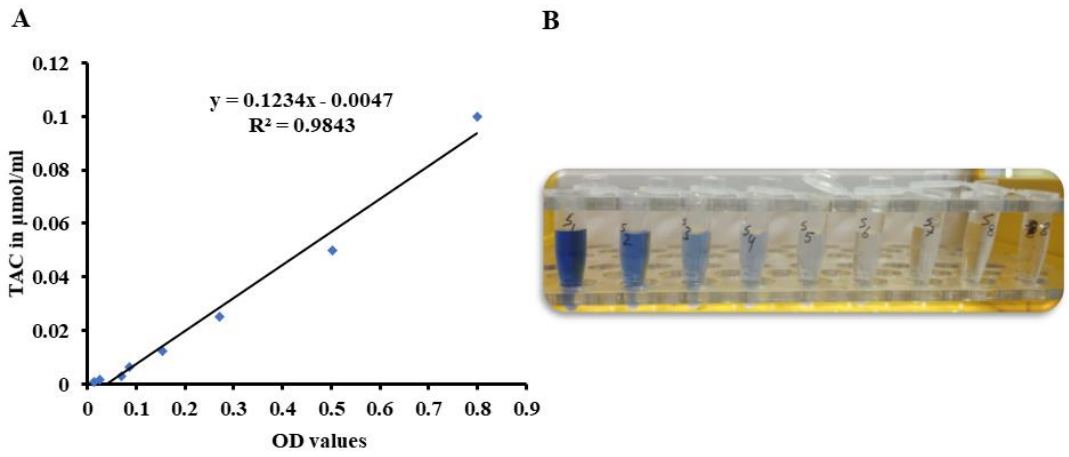


Figure 3.10: Representative images of (A) TAC standard curve and (B) TAC standard tubes.

3.2.3.1.4. Estimation of total glutathione, oxidized and reduced glutathione ratio in the hippocampus

Total glutathione and GSSG/GSH ratio were assessed by using the Glutathione assay kit (Cayman chemicals; cat log 703002). Since glutathione reductase is used in the Cayman GSH assay, both GSH and GSSG are measured, and the assay reflects the total glutathione. The experiments were carried out according to the manufacturer's instructions.

Principle: The sulfhydryl group of GSH (reduced glutathione) reacts with DTNB (5,5'-dithio-bis-2 (nitrobenzoic acid), Ellman's reagent) and produces the yellow-colored 5-thio-2-nitrobenzoic acid (TNB). The oxidized glutathione was also converted to reduced GSH in the presence of glutathione reductase. TNB production was directly proportional to the concentration of GSH in the sample. The colour intensity was measured at 405 nm in a microplate reader. Exclusive measurement of GSSG is accomplished by the exclusion of GSH in the sample by derivatizing GSH with 2 vinyl pyridine.

Materials required: GSH MES buffer, GSSG standard, GSH co-factor mixture, GSH TNB.

Reagents: GSH MES Buffer: It consists of 2-(N-morpholino) ethanesulphuric acid, 0.1 M phosphate, and 2 mM EDTA with pH=6.0.

GSH Co-Factor mixture: The vials contain lyophilized powder of NADP+ and glucose 6 phosphates. Reconstituted the vial with 0.5 ml of distilled water.

GSH Enzyme mixture: 0.2 ml of Glutathione reductase and glucose 6 phosphate dehydrogenase was diluted with MES buffer.

GSH DTNB: 4 vials: Each vial contains a lyophilized powder of DTNB (5,5'-dithio-bis-2 (nitrobenzoic acid), Ellman's reagent). Reconstituted the vial with 0.5 ml water and mixed well. Note: The reconstituted reagent must be used within 10 min.

GSSG standard: 25 µM GSSG in MES buffer (Table 3.5).

Sample preparation for exclusive measurement of GSSG: Quantification of GSSG, exclusive of GSH, is accomplished by first derivatizing GSH with 2-vinyl pyridine.

Prepared 1 M solution of 2-vinyl pyridine in ethanol by mixing 108 µl of 2-vinyl pyridine in 892 µl of ethanol.

Added the 10 µl of 2-vinyl pyridine solution for ml of sample and incubated at room temperature for about 60 min.

Table 3.5: Composition of GSSG standards

Tube	GSSG (µl)	MES buffer (µl)	Final Conc. GSSG (µM)	Equivalent total GSH (µM)
A	0	500	0	0
B	5	495	0.25	0.5
C	10	490	0.5	1.0
D	20	480	1.0	2.0
E	40	460	2.0	4.0
F	80	420	4.0	8.0
G	120	380	6.0	12.0
H	160	340	8.0	16.0

Under assay conditions, GSSG is reduced to produce 2-mole equivalents of GSH. The homogenized hippocampus was diluted 3 times in PBS (1:3).

Procedure: Added 50 µl of sample/standards in the microplate wells. Covered the plate with the plate cover provided. Prepared the assay cocktail by a mixture of the following reagents in a 20 ml vial: MES buffer (11.25 ml) reconstituted cofactor mixture (0.45 ml) reconstituted enzyme mixture (2.1 ml) water (2.3 ml) and reconstituted DTNB (0.45 ml). Removed the plate cover and added 150 µl of freshly prepared assay cocktail to each well-containing standard and sample using a pipette followed by incubating the plate in the dark on an orbital shaker for 25 min.

The colour intensity was measured at 405 nm (Figure 3.11).

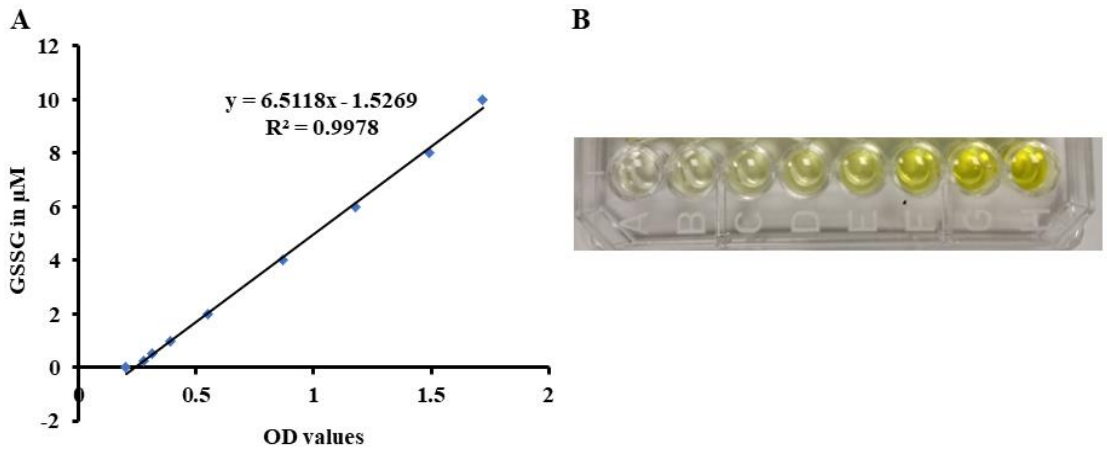


Figure 3.11: Representative image of (A) GSSG standard curve and (B) GSSG standard wells.

Calculation: Total glutathione

$$\frac{\text{Absorbance at 405 nm- Y-intercept} \times \text{sample dilution}}{\text{Slope}}$$

Reduced glutathione= Total glutathione - oxidized glutathione

The ratio of GSSG/GSH was calculated from the obtained oxidized and reduced glutathione values.

3.2.3.2. Measurement of serum corticosterone levels

About 2 ml of blood was collected through cardiac puncture from the anesthetized rats. Blood samples were collected at a fixed time from the second set of rats. The blood samples were allowed to coagulate for 30 min and the serum was collected by centrifuge at 3000 rpm for 20 min at room temperature and stored at -20 °C until use further. The levels of corticosterone were measured using an ELISA kit (Kinesis Dx, USA). Briefly, 40 µl of undiluted samples, 10 µl of biotinylated rat corticosterone antibodies, and 50 µl of streptavidin-horseradish peroxidase were added to anti-rat corticosterone antibodies coated microplate wells and incubated at 37 °C for one hour. After incubation, the solution was discarded, and the wells were washed four times using 1X wash buffer. To the wells, 50 µl of 3, 3', 5,5' tetramethylbenzidine substrate A and B was added and

incubated for 10 min. The reaction was stopped by adding 50 µl of stop solution. The optical density of the yellow colour was measured at 450 nm using an ELISA reader, and the level of serum corticosterone was calculated from the standard curve and expressed as ng/ml (Figure 3.12).

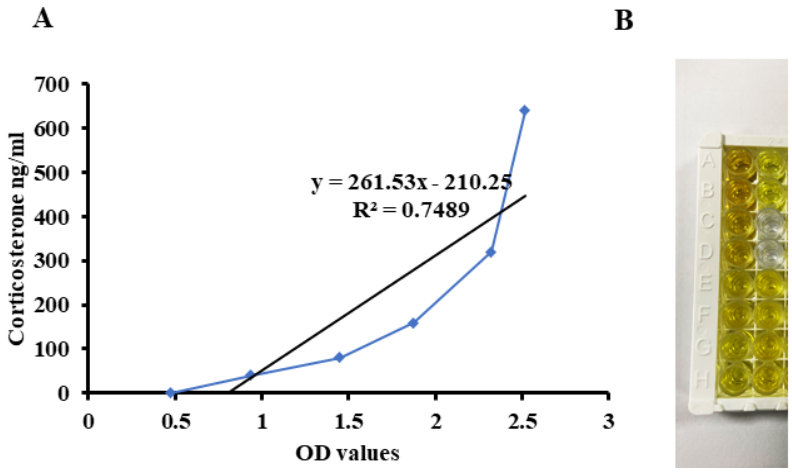


Figure 3.12: Representative images of (A) corticosterone standard curve and (B) Corticosterone standard wells.

3.2.4. Cellular and molecular experiments

At the end of sleep deprivation/sleep recovery intervention and behavioral tasks the second set of rats was euthanized and perfused brain with PBS, before being extracted. The right cerebral hemisphere from each brain sample was processed for the dendritic arborization of hippocampal CA3 neurons using the Golgi-cox method and the left cerebral hemisphere was processed for the immunohistochemistry to measure the CNPase+ve oligodendrocytes.

3.2.4.1. Dendritic arborization of hippocampal CA3 neurons

Method: The method of Golgi Cox staining technique was adopted from Shankaranarayana et al., 2004 (Shankaranarayana Rao and Raju, 2004).

3.2.4.1.1. Collection and processing of the brain sample

The right hemisphere of the brain sample was placed in a Golgi-cox solution and incubated for 9-days in a dark chamber (Figures 3.13A and B). Approximately 160 µm thickness sections were taken from ventral to dorsal hippocampus using a vibratome microtome (Leica VT 1000S, Germany) on charged slides (Figures 3.13C and D).

3.2.4.1.2. Golgi-cox staining procedure

The sections on the charged slides were pressed with blotting paper and kept in a moisture environment for 1 hour. Washed the slides with distilled water (4 times). Further, the slides are treated with the 8% Na_2CO_3 for 2 hours. Washed the slides with distilled water (4 times). Transferred the slides into the 70% ethyl alcohol for 15 min and absolute alcohol for 1 min (4 dips). Thereafter the sections were incubated with cedarwood oil overnight. The following day sections are cleared using xylene and mounted with DPX (Figure 3.14A).

3.2.4.1.3. Tracing of hippocampal CA3 neurons

Tracing of hippocampal CA3 neurons Camera Lucida Tracing method. Briefly, Golgi cox impregnated CA3 neuronal dendritic arborization was traced (8-10 neurons/rat: 50 neurons/group) using light microscopy equipped with camera Lucida in 200X magnification (Olympus, Japan) (Figures 3.14B-D). Dendritic branching points and dendritic intersections were quantified using a concentric circle method as described earlier (Sholl, 1953). Briefly, adjacent concentric circles were drawn on a transparent sheet with a distance of 20 µm each which were calibrated according to the 200X magnification. The dendrite branching points (basal and apical) and intersections were counted in each given concentric circle up to the radius of 100 µm from the center of the cell body of the CA3 neurons (Figure 3.14E).

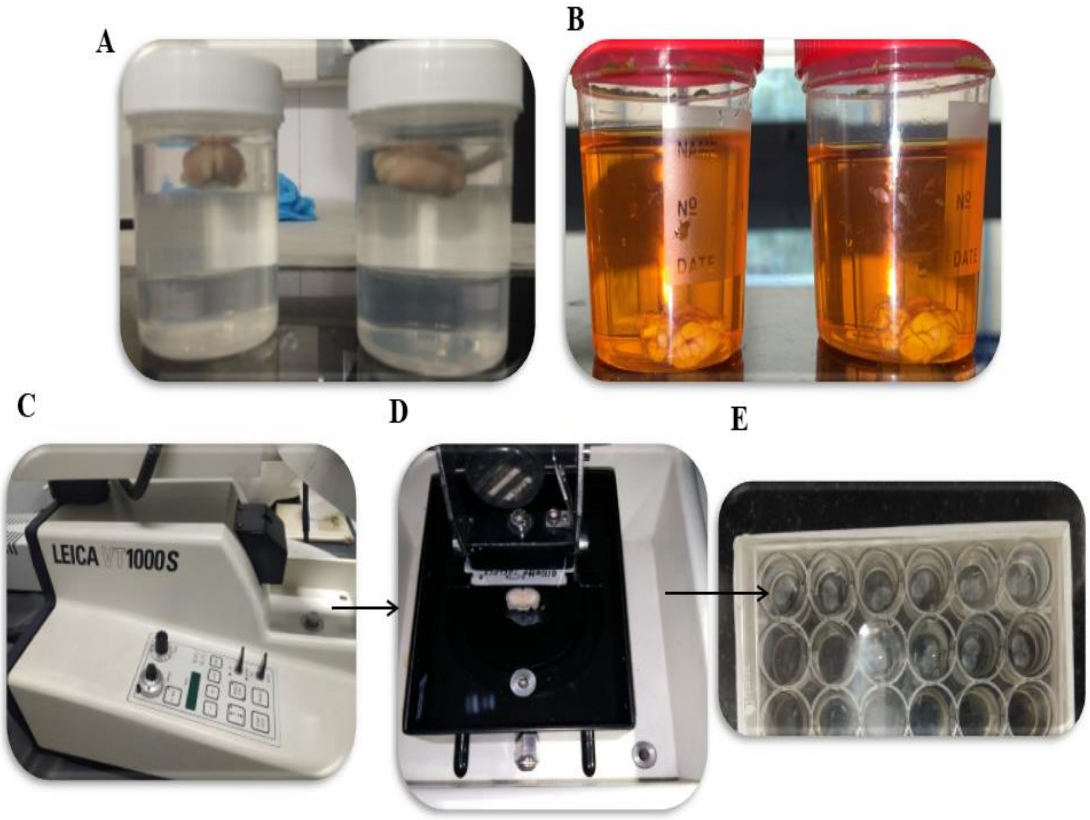


Figure 3.13: Representative images of samples and vibratome microtome sections. (A) Brain fixed in sucrose solution (IHC), (B) Golgi-cox stain impregnated brains, (C and D) Vibratome microtome, (E) Vibratome sections in a well plate.

Criteria adopted for the selection of CA3 neurons for dendritic quantification:

Golgi-impregnated neurons had to satisfy the following criteria:

Presence of untruncated dendrites.

Consistent and dark impregnation along with the entire extent of all of the dendrites.

Relative isolation from neighbouring impregnated neurons.

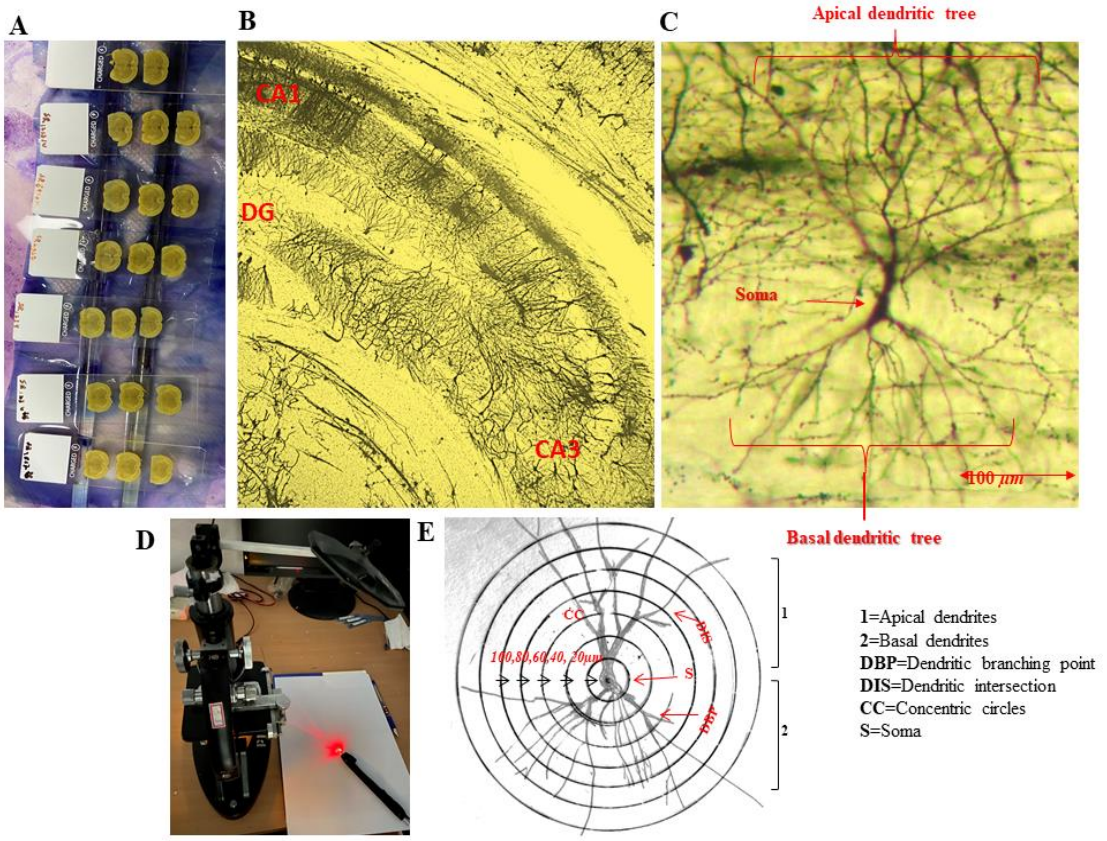


Figure 3.14: Representative images of Golgi Cox impregnated (A) sections (B) hippocampal neurons (4X), (C) apical and basal dendritic arborization of CA3 neurons (10X) and (D) Microscope fixed camera lucida (E) Sholl DA method used to quantify the dendritic arborization of hippocampal CA3 neurons.

3.2.4.2. Measurement of CNPase+ve oligodendrocytes in the hippocampus

3.2.4.2.1. Sample preparation

The brain sample was fixed in 4% paraformaldehyde phosphate buffer overnight at 4 ℃ temperatures. Then the samples were sifted to 15% and 30% sucrose solution for 24 hours each at 4 °C temperature (Figure 3.13A).

Sectioning: 40 µm thick sections were taken using a vibratome microtome (Leica VT1000S) facility available at the department of neurophysiology, NIMHANS, Bangalore (Figures 3.13C and D). Hippocampal sections were collected in a 24-well plate that contain tris buffer solution (Every 6th section has been chosen). The sections were preserved at 4°C till further experiment was carried out (Figure 3.13 E).

3.2.4.2.2. Steps followed for immunohistochemistry

Washed the sections with TBS: 3 × 5 min. Washed the sections with TBS-T: 3 × 5 min. Antigen retrieval: sodium citrate buffer with pH=6, - 20 min at 90 ℃ in a hot air oven. TBS-T washes: 3 × 5 min. Peroxidase block with 0.3% H_2O_2 - 30 min. TBS-T washes: 3 × 5 min. Blocking with 3% BSA, 2 hours. TBS-T washes: 3×5 min. Primary antibodies: 1:1000, Incubated the slides at 22 ℃ for 16 hours. TBS-T washes: 3 × 5 min. Secondary antibodies: 2 hours at room temperature and another 2 hours at 4 ℃ temperature. Washed the sections with TBST. Sections were incubated with ABC for 2 hours at room temperature. Washed the sections with TBS T. Sections were treated with DAB for 1 min. Finally, sections were shifted over the charged slides. Sections were dehydrated in ascending grade ethanol, cleared the slides using xylene, and mounted with DPX (Subhadeep et al., 2021).

3.2.4.2.3. Quantification (counting) of oligodendrocytes in CA1, CA3, and dentate gyrus regions of the hippocampus

CNPase+ve immunostaining hippocampus sections were semi quantified as described earlier by (Yin et al., 2017). Briefly, the micrographs of CNPase+ve immunostaining hippocampal subregions (CA1, CA3, and Dentate gyrus) were captured in 40X magnification by using a digital microscope (Zeiss microscope with ZEN software, Germany) (Figures 3.15A and B). Images in each subregion of the hippocampus were captured and counted the CNPase+ve oligodendrocytes using Image J software. Only oligodendrocytes with complete processes were counted and expressed as a number of CNPase+ve oligodendrocytes per area of 1 mm^2. Four sections/rat (6 rats/group) were processed for immunohistochemistry and from each stained section, eight images were obtained in each (CA1, CA3, and dentate gyrus) subregion hippocampus. Therefore, a total of 200 images/subregion/groups were used to measure

the CNPase+ve oligodendrocytes from the hippocampus to provide a mean value for each group.

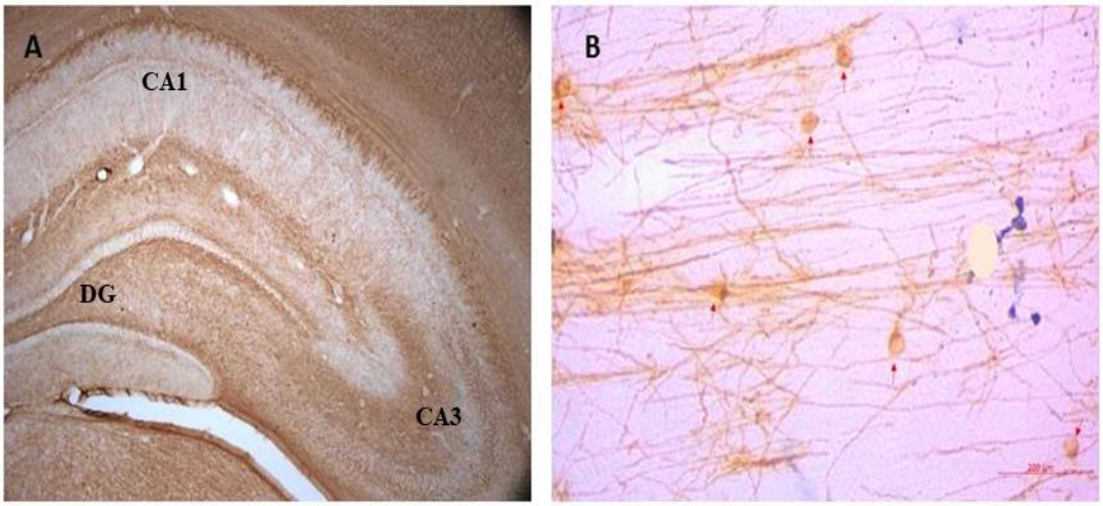


Figure 3.15: Representative image of (A) CNPase+ve reacted hippocampus (4X) and (B) CNPase+ve oligodendrocytes (▲) (40X).

3.2.4.2.4. Quantification of CNPase intensity in CA1, CA3, and dentate gyrus regions of the hippocampus

CNPase intensity was measured by using the Image J software (Image J 1.53a, USA). Briefly, the 8-bit greyscale image has defined areas, and each of its areas measured the mean grey value for the calibration (https://imagej.nih.gov/ij/docs/examples/calibration/) (Figures 3.16A and B). The obtained mean grey value for each selected area is corresponding to a known optical density value under experimental conditions (Razgado-Hernandez et al., 2015). The generated mean grey value and its known optical density calibration curve were used to measure the optical density of CNPase+ve hippocampus images captured in 40X. The selected image was converted to 8-bits in Image J and measured the optical density (intensity). A total of 30 images/subregion/group was used to measure the intensity of CNPase in the hippocampus to provide a mean value for each subregion of the group.

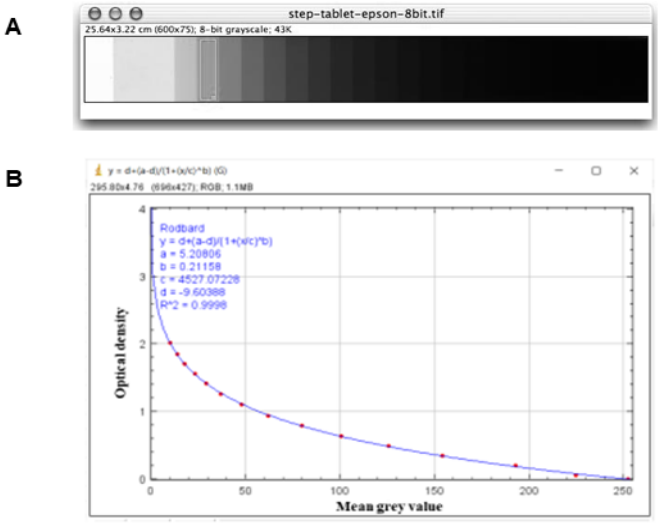


Figure 3.16. Representative images were used to measure the CNPase intensity using Image J software: (A) image of 8-bit greyscale, (B) optical density calibration curve.

3.2.4.3. Expression of *CNPase* gene in the hippocampus

Hippocampal tissue samples stored in RNA later were used to isolate total RNA and performed *CNPase* gene expression. The expression of the *CNPase* gene was measured by using qRT-PCR as described in Hattori *et al*., (2014) and the supernatant was used to isolate total RNA as per the manufacturer's instructions (Hattori et al., 2014).

3.2.4.3.1. Isolation of total RNA from hippocampal tissue

Approximately 50 mg of hippocampal tissue was mixed with 1 ml of Trizol reagent (Thermo Scientific, USA), ground thoroughly using a motor and pestle, and centrifuged at 4 ℃ for 10 min. Added the 200 µl of chloroform to the homogenized sample, vortexed vigorously for 30 sec, and centrifuged for 15 min at 12000 rpm at 4 ℃. The supernatant layer was transferred into a new Eppendorf tube along with 500 µl of 100% isopropanol. After 15 min of incubation at ambient temperature, the tube was centrifuged at 12000 rpm at 4 ℃ for 10 min. Total RNA precipitate formed a white gel like pellet at the bottom of the tube. Discarded the supernatant, the pellet was

resuspended in 1 ml of 75% ethanol and centrifuged tube at 10000 rpm at 4 ℃ for 5 min and repeated the same step. The pellet was allowed to dry at room temperature. The pellet was resuspended in 50 µl of elution solution and preheated at 75 ℃ using a water bath. Finally, the RNA samples were stored at -80 ℃ until further use.

3.2.4.3.2. Measurement of RNA quality and quantity

The concentration of RNA was measured by using nanodrop, the 260/280 and 230/260 values were recorded. RNA having a quality of 1.9-2.1 was used for the conversion of cDNA.

3.2.4.3.3. Conversion of RNA into cDNA

The total RNA sample (1200 ng) was converted to cDNA using a high-capacity cDNA conversion kit (Applied Biosystems, USA) as per the manufacturer's instructions. The composition of the reaction mixture for each 20 µl reaction was as shown in table 3.6. To 10 µl of the reaction mixture, 10 µl of total RNA sample was added and mixed thoroughly, and performed PCR. The reaction conditions used for PCR are as follows (Table 3.7).

Table 3.6: Master mix composition used for cDNA conversion of RNA.

Contents	Volume/reaction (µl)
10X RT buffer	2.0
25 X dNTPs	0.8
10 X RT random primers	2.0
Multiscript reverse transcriptase	1.0
Nuclease free water	4.2

Table 3.7: The PCR reaction conditions for cDNA conversion are as follows.

Steps	Temperature (℃)	Time (min)
Hold	25	10
Hold	37	120
Hold	85	5

3.2.4.3.4. Quantification of *CNPase* gene expression using real-time PCR

Real-time quantification of the *CNPase* gene was performed using SYBR green probe. The primers (Sigma, USA) sets reported by Hattori et al., (2014) were used for the quantification of *CNPase* gene expression (Hattori et al., 2014). The sequence of primers was as follows:

CNPase:5'CAACAGGATGTGGTGAGGA3'/5'CTGTCTTGGGTGTCACAAAG3'

GAPDH:5'GCCTTCTCTTGTGACAAAGTGG3'/5'ATTCTCAGCCTTGACTGTGC C3'. The reaction mixture composition for 20 µl of reaction is as follows. To the reaction mixture, 4 µl of cDNA sample was added and performed real-time quantification (Tables 3.8 and 3.9).

Table 3.8: Composition master mix for qRT-PCR reaction.

Contents	Volume/reaction (µl)
Forward primer	1
Reverse primer	1
SYBR green	10
Nuclease free water	3.5
c-DNA sample	4.5

Table 3.9: The qRT-PCR reaction conditions for each cycle are as follows.

Steps	Temperature (℃)	Time	
Initial denaturation	95	3 min	
Final Denature	95	20 sec	45
Anneal/extent	61.4	45 sec	cycles
Extinction	50		

The cycle threshold (CT) values were collected and used for the analysis of the expression of the *CNPase* gene. The expression of the *CNPase* gene was normalized using the *GAPDH* gene as an internal control. ΔΔCt method was used to calculate the relative expression of the *CNPase* gene (Schmittgen and Livak, 2008).

3.2.5. Statistical analysis

The data was represented as mean±standard error of the mean/percentage. The student 't' test was used to find the significant difference in the latency time obtained from the acquisition and testing phase in the HWM maze. One way analysis of variance with Bonferroni's post hoc analysis was used to find the significant difference in the variable among control, EC, CSD, CSD+5D SR, and CSD+21D SR. Pearson correlation analysis was used to determine the association between MDA vs the number of CNPase+ve oligodendrocytes, MDA vs CNPase intensity, MDA vs *CNPase* gene, and CNPase intensity vs *CNPase* gene expression. All the statistical analysis was performed using SPSS software, version 20 (IBM, USA), and the representative graphs were prepared using GraphPad Prism software (GraphPad Software, USA).

Chapter IV

Results and Discussion

Effect of CSD and sleep recovery on oxidative stress, anxiety-like behavior, spatial learning and memory of rats

4.1. Introduction

Sleep is a physiological phenomenon that is required for the quality of life in human beings. The lack of sleep leads to severe physiological consequences majorly in the brain. Acute/chronic sleep deprivation increases the free radicle formation and decreases the antioxidant capacity, which ultimately leads to oxidative stress in the brain. Acute/chronic sleep deprivation is also reported to impair spatial learning and memory and increased anxiety-like behavior in both humans and rodents. Though the different durations of sleep deprivation-induced oxidative stress on cognitive impairment and behavioral alterations have been extensively studied, the cellular and molecule level mechanisms were not understood clearly. Further, the reversal of CSD induced oxidative stress and behavioral alterations, if any is not fully established. Therefore, in the current study we have investigated the effect of CSD and 5- and 21-days of sleep recovery on the oxidative stress, anxiety-like behavior, spatial learning, and memory of rats.

4.2. Methodology

The spatial learning and memory of rats were assessed using Hebb Williams Maze and Morris water maze after CSD and sleep recovery intervention. The first set of rats (6 rats/group) were exposed to HWM in which the acquisition and testing phase was conducted and the latency time (sec) measured (described in Chapter III; section 3.2.2.1). Whereas the second set of rats (6 rats/group) were subjected to the MWM, in which the acquisition and probe trials were conducted and the latency time and time spent in each quadrant were measured respectively (described in Chapter III; section 3.2.2.2). Further, the elevated plus maze and open field maze test was used to assess the anxiety-like behavior of rats at the end of CSD and sleep recovery. The first set of rats was exposed to EPM and the time spent in the closed arm, open arm (OA), the number

of entries into the open arm, and the total number of crossings were measured, and calculated the anxiety index levels (described in Chapter III; section 3.2.2.3). The second set of rats was subjected to the OFM and the initial and acclimatization response were assessed by measuring the latency time to enter the central zone, time spent in the peripheral zone and central zone, frequency of zone transmission, resting time, fecal boli count, and ambulatory distance (described in Chapter III; section 3.2.2.4). At end of sleep deprivation/sleep recovery intervention and behavioral experiments, the rats were sacrificed and the oxidative stress markers such as MDA, total glutathione, GSSG, and TAC levels were measured in the hippocampus tissue. The MDA, total glutathione, and GSSG levels were expressed as µM/mg of protein and the TAC levels were expressed as units/mg of protein (described in Chapter III; 3.2.3.1).

4.3. Results

The 21-days of CSD and sleep recovery induced changes in the spatial learning and memory, anxiety-like behavior of rats, and oxidative stress markers in the hippocampus were assessed and the obtained results were summarised in the following sections.

4.3.1. Effect of CSD, CSD+5D SR, and CSD+21D SR on spatial learning and memory of rats

The spatial learning and memory of the rats after 21-days of CSD and 5- and 21-days of sleep recovery were assessed using both HWM and MWM tasks.

4.3.1.1. Hebb Williams Maze task

The latency time (sec) in the HWM task was recorded in both the acquisition and testing phases. During the acquisition phase, all the rats from different groups learned to reach the reward box. The mean latency time obtained from all the groups in the

acquisition phase of days 1-7 is shown in (Figure 4.1A) The mean latency time of the acquisition phase is 22±1.2, 22±1.0, 21±0.7, 21±0.8, 21±0.8 sec, and the testing phase is 22±0.8, 23±1.0, 52±3.9, 41±3.4, 30±2.5 sec for control, EC, CSD, CSD+5D SR, and CSD+21D SR groups respectively. The mean latency time during the acquisition phase did not vary among the groups ($p>0.05$). However, the latency time during the testing phase is significantly higher in the CSD group compared to the control and EC group ($p<0.001$). The mean latency time in the testing phase is significantly reduced in both CSD+5D SR ($p<0.0001$) and CSD+21D SR ($p<0.0001$) groups compared to CSD, however, it was not sufficient to reach control and EC groups ($p<0.01$). The mean latency time obtained from the testing and acquisition phase did not differ in both control and EC groups ($p>0.05$). However, the mean latency time in the testing phase is significantly higher in CSD ($p<0.001$), CSD+5D SR ($p<0.001$), and CSD+21D SR ($p<0.01$) groups compared to the acquisition phase (Figure 4.1B).

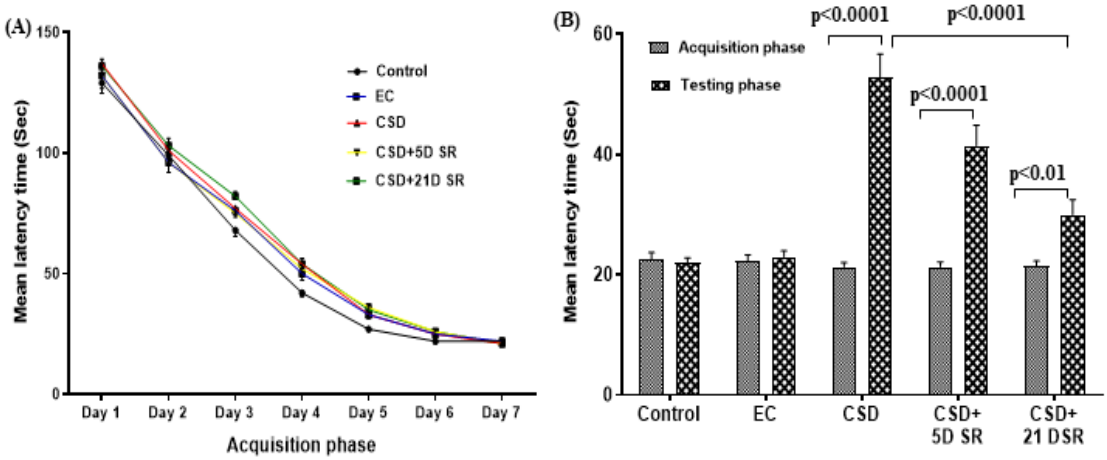


Figure 4.1: Spatial learning and memory performance of rats in HWM (n=6/group): (A) The mean latency time (sec) obtained from control, EC, CSD, CSD+5D SR, and CSD+21D SR groups of rats during days 1-7 of the acquisition phase. (B) The comparison of mean latency time (sec) obtained during acquisition and testing phases in control, EC, CSD, CSD+5D SR, and CSD+21D SR groups of rats.

4.3.1.2. Morris Water Maze task

At the end of 21-days of CSD and sleep recovery, the spatial learning and memory of rats were assessed using the MWM maze. All groups of rats were trained (acquisition) by giving four trials/day for 4-days in MWM. During the acquisition phase, all groups of rats learned to reach the escape platform from S, W, E, and N releasing points in the Morris water maze. The mean latency time decreased gradually from day 1-4 of the acquisition phase in all groups of rats. Further, the mean latency time among the groups on a given day did not show a significant difference ($p>0.05$) (Figure 4.2A). In the probe trial, the time spent in the target quadrant ("S") was significantly higher when compared to the N, E, and W quadrants in both the control and EC groups ($p<0.01$) (Table 4.1). The time spent in the target quadrant did not differ between the control and EC group ($p>0.05$). Whereas, the time (sec) spent in all quadrants did not differ ($p>0.05$) in the CSD group. Further, the CSD rats spent significantly less time in the target quadrant compared to the control and EC groups ($p<0.001$) (Figure 4.2B). The CSD+21D SR group rats spent more time in the target quadrant ("S") compared to the N, E, and W quadrants ($p<0.05$). Similarly, the CSD+21D SR showed a significant improvement in time spent in the target quadrant compared to the CSD group ($p<0.01$), however, the time spent in the target quadrant did not reach the control levels ($p<0.01$) (Figure 4.2B).

4.3.2. Effect of CSD, CSD+5D SR, and CSD+21D SR on anxiety-like behavior of rats

The anxiety-like behavior of rats was assessed using the EPM and OFM after 21-days of CSD, 5- and 21-days of sleep recovery intervention.

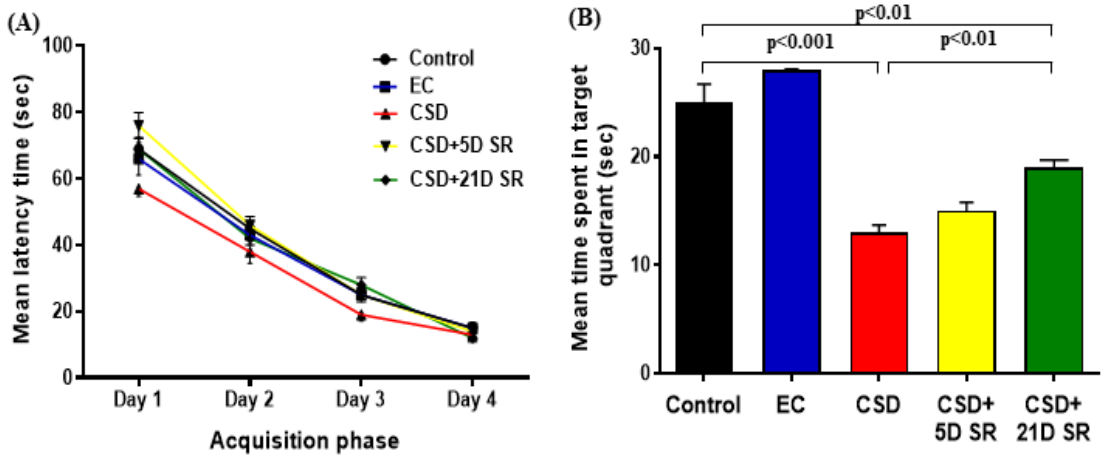


Figure 4.2: Spatial learning and memory performance of rats in MWM (n=6/group): **(A)** The mean latency time (sec) obtained from control, EC, CSD, CSD+5D SR, and CSD+21D SR groups of rats during days 1-4 of the acquisition phase. (B) The comparison of mean time spent in the target quadrant (sec) obtained during probe trial in control, EC, CSD, CSD+5D SR, and CSD+21D SR groups of rats.

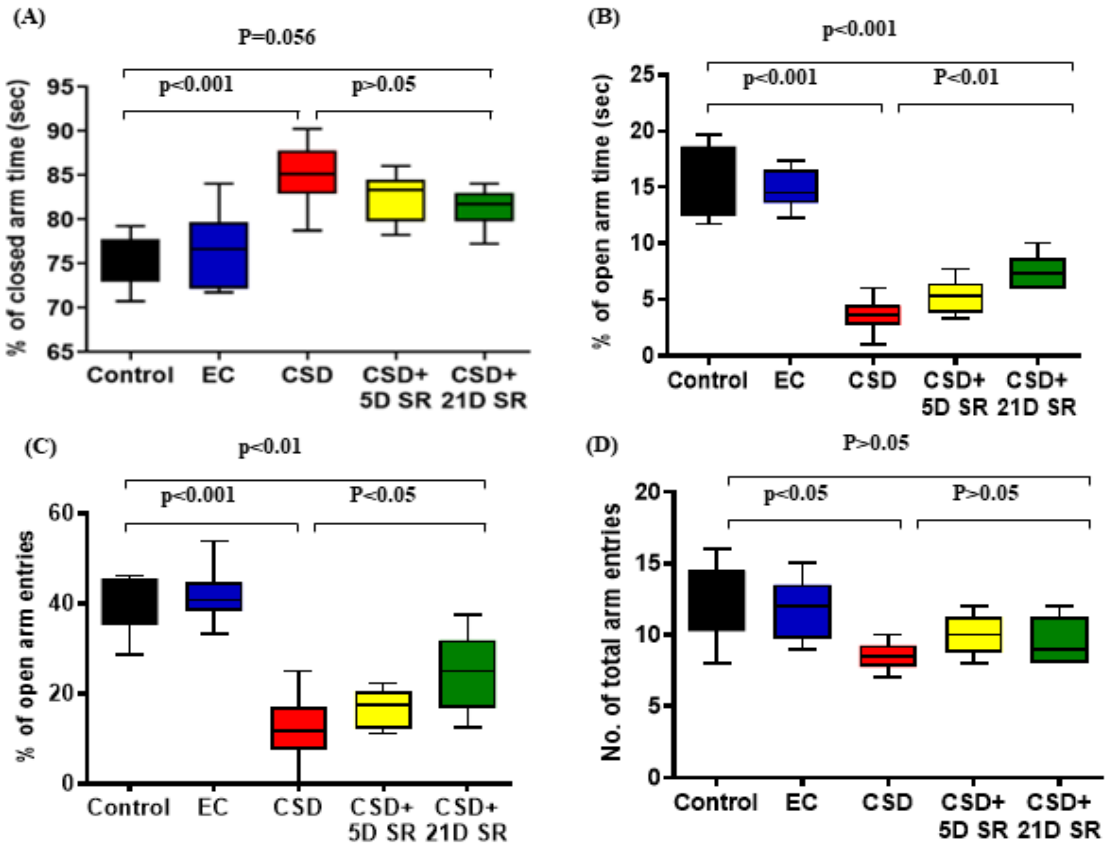
Table 4.1: Time spent (sec) in each quadrant when the platform was removed from MWM during the probe trial.

Groups (n=6)	Mean±SEM time spent (sec) in each quadrant in MWM			
	South	**North**	**East**	**West**
Control	25.6±1.70	12.5±0.70	12.0±1.1	9±0.9
EC	28.2±0.98	10.3±1.20	11.0±1.2	10±1.1
CSD	13.0±0.65	15.8±0.47	17.0±1.2	13±0.6
CSD+5D SR	15.0±0.81	15.17±0.7	17.0±0.7	12.±1.0
CSD+21D SR	19.5±0.70	14.8±1.00	13.5±1.0	12±0.6

4.3.2.1. Elevated Plus Maze task

It is a well-known fact that CSD increases anxiety-like behavior in experimental rats**.** The extent of anxiety-like behavior of rats after 21-days of CSD and 5- and 21-days of sleep recovery was assessed using the EPM task. The percent of time spent in the closed arm, open arm, open arm entries, and the number of total arm entries did not show any significant difference between the control and EC groups ($p>0.05$). The percent of time spent in the closed arm was significantly increased in the CSD group compared to the control and EC groups ($p<0.001$). The time spent in the open arm

($p<0.001$), open arm entries ($p<0.001$), and total arm entries ($p<0.01$) were significantly reduced in the CSD group compared to the control and EC groups. The percent of time spent in the closed arm was reduced in CSD+5D SR and CSD+21D SR groups compared to the CSD group. The time spent on the open arm and open arm entries was increased non significantly ($p>0.05$) in the CSD+5D SR group and significantly in the CSD+21D SR group ($p<0.05$) compared to the CSD group. The total arm entries were increased in CSD+5D SR and CSD+21D SR groups but it is not statistically significant ($p>0.05$) compared to the CSD group. Even though there is a reduction in time spent on the closed arm and increased time spent on the open arm, open arm entries, and total arm entries after CSD+5D SR and CSD+21D SR groups, it did not reach the control group levels (Figures 4.3A-D).



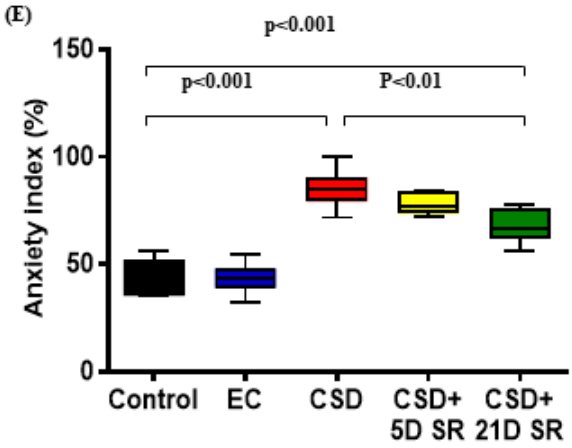


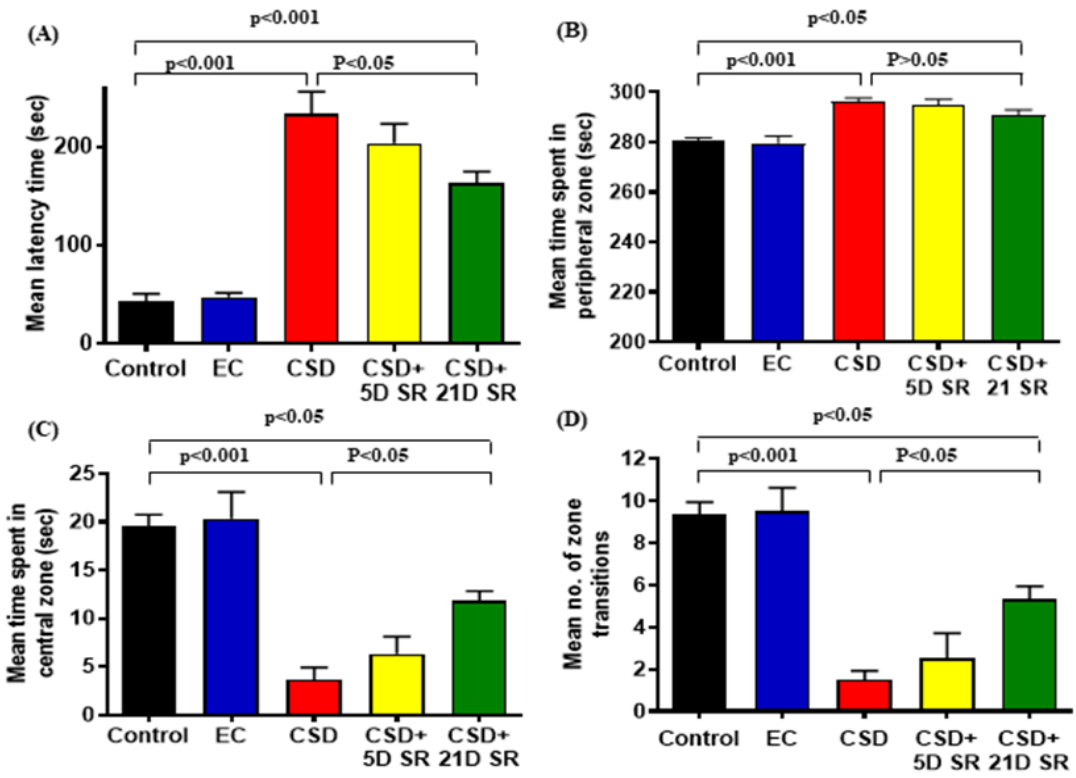
Figure 4.3: Anxiety-like behavior of rats in an elevated plus-maze (n=6/group): (A) Time (sec) spent in the closed arm, (B) Time (sec) spent in the open arm, (C) Open arm entries, (D) Total arm entries, and (E) Anxiety index obtained from control, EC, CSD, CSD+5D SR, and CSD+21D SR groups of rats but was not significant ($p>0.05$).

The anxiety-like behavior of the rats did not show any significant difference between the control and EC groups ($p>0.05$). The anxiety-like behavior was significantly higher in the CSD group compared to the control and EC groups ($p<0.001$). The anxiety-like behavior was reduced in the CSD+5D SR group ($p>0.05$), whereas, the CSD+21D SR group showed a significant ($p<0.01$) reduction compared to the CSD group. Even though there is a reduction in the anxiety-like behavior in both CSD+5D SR and CSD+21D SR groups, it did not reach the control group levels. Figure 4.3E shows the anxiety index obtained from different groups of rats.

4.3.2.2. Open Field Maze task

Anxiety-like behavior of rats after 21-days of sleep deprivation and sleep recovery was assessed using the OFM maze. In the open field test, the mean latency time, time spent in the peripheral zone, resting time, and the number of fecal boli increased significantly ($p<0.001$), whereas, the time spent in the central zone, ambulatory distance, and frequency of zone transmission decreased significantly ($p<0.001$) in CSD group compared to the control and EC groups (Figures 4.4A-G). The mean latency time,

resting time, and the number of fecal boli decreased significantly ($p<0.05$), and the mean time spent in the central zone, ambulatory distance, and frequency of zone transmission increased non-significantly in CSD+5D SR and significantly ($p<0.05$) in the CSD+21D SR group compared to CSD group. The time spent in the peripheral zone decreased in the CSD+5D SR and CSD+21D SR groups compared to the CSD group, but it was not statistically significant ($p>0.05$). Although there was a significant decrease in anxiety-like behavior in the CSD+21D SR group, the levels of anxiety did not reach to control level ($p<0.05$). All the anxiety-like behavior parameters did not show a significant difference between the control and EC groups ($p>0.05$) (Figures 4.4A-G).



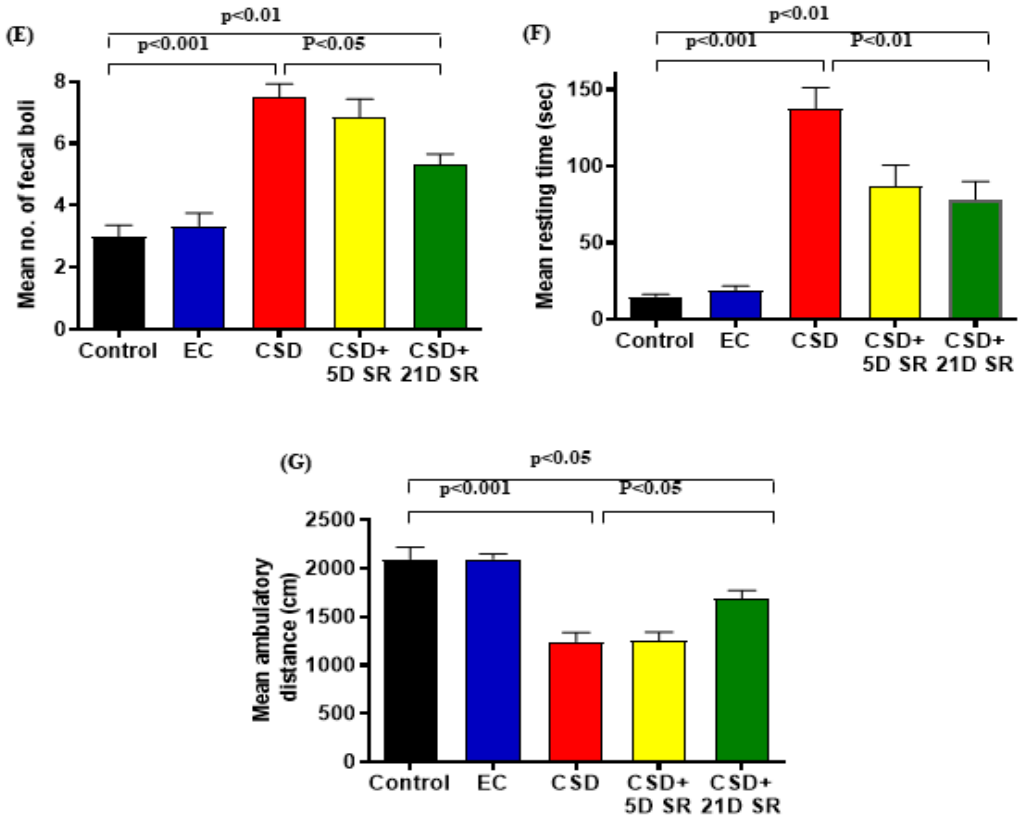


Figure 4.4: Anxiety-like behavior of rats in open field maze: (A) latency time, (B) time spent in the peripheral zone, (C) time spent in the central zone, (D) the number of zone transmission, (E) number of fecal boli, (F) resting time (G) ambulatory distance obtained from control, EC, CSD, CSD+5D SR, and CSD+21D SR groups of rats (n=6/group).

4.3.3. Effect of CSD, CSD+5D SR, and CSD+21D SR on oxidative stress markers in the hippocampus of rats

The extent of oxidative stress after 21-days of CSD, 5- and 21-days of sleep recovery was assessed by measuring the levels of MDA, total glutathione, the ratio of GSSG/GSH, and total antioxidant capacity in the hippocampal tissue of rats.

4.3.3.1. Levels of MDA in CSD, CSD+5D SR, and CSD+21D SR groups of rats

The mean MDA obtained from the control, EC, CSD, CSD+5D SR, and CSD+21D SR groups were 0.84±0.17, 1.1±0.17, 2.25±0.19, 1.7±0.14, and 1.4±0.16 µM/mg of protein respectively (Figure 4.5A). The levels of MDA did not show any significant difference between the control and EC groups ($p>0.05$). The levels of MDA were significantly higher in the CSD group compared to the control and EC groups

(p<0.001). Whereas, the levels of MDA in the CSD+5D SR group reduced when compared to the CSD group but it was not significant (p=0.17). However, the levels of MDA obtained from the CSD+21D SR group showed a significant decrease when compared to the CSD group (p<0.01). The MDA levels obtained from the CSD+21D SR group were comparable with the control group (p=0.14).

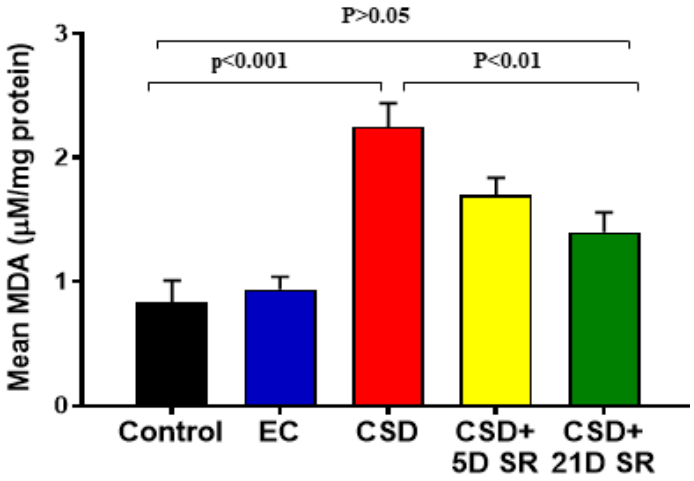


Figure 4.5A: The levels of MDA (µM/mg of protein) in the hippocampus obtained from the control, EC, CSD, CSD+5D SR, and CSD+21D SR group rats (n=6/group).

4.3.3.2. Levels of total glutathione in CSD, CSD+5D SR, and CSD+21D SR groups of rats

The mean total glutathione obtained from control, EC, CSD, CSD+5D SR, and CSD+21D SR groups were 1.24±0.09, 1.22±0.11, 0.51±0.03, 0.66±0.05, and 0.84±0.04 µM/mg of protein respectively (Figure 4.5B). The levels of total glutathione did not show any significant difference between the control and EC groups. Total glutathione levels decreased significantly in the CSD group compared to the control and EC groups (p<0.001). Whereas the levels of total glutathione obtained from the CSD+5D SR group showed a non-significant (p>0.05) increase and the CSD+21D SR groups showed a significant increase (p=0.04) compared to the CSD group. The levels of total glutathione obtained from CSD+5D SR and CSD+21D SR groups did not reach the levels of control and EC groups (p<0.01).

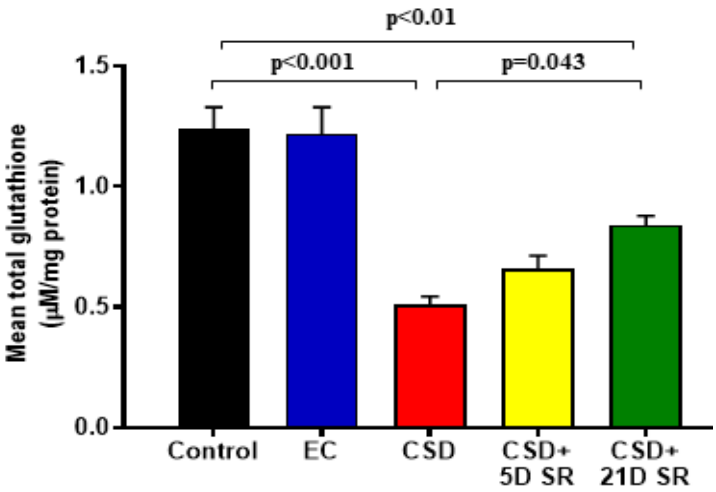


Figure 4.5B: The levels of total glutathione (µM/mg of protein) in the hippocampus obtained from the control, EC, CSD, CSD+5D SR, and CSD+21D SR groups (n=6/group).

4.3.3.3. The ratio of GSSG/GSH in CSD, CSD+5D SR, and CSD+21D SR groups of rats

The mean ratio of GSSG/GSH obtained from the control, EC, CSD, CSD+5D SR, and CSD+21D SR groups were 0.08±0.01, 0.08±0.01, 1.14±0.21, 0.89±0.15, and 0.60±0.04 respectively (Figure 4.5C). The ratio of GSSG/GSH did not show any significant difference between the control and EC groups ($p>0.05$). The ratio of GSSG/GSH was significantly higher in the CSD group compared to the control and EC groups ($p<0.001$). Whereas, the ratio of GSSG/GSH in the CSD+5D SR group was reduced but not significant ($p>0.05$) and CSD+21D SR groups were significantly reduced ($p=0.03$) compared to the CSD group. However, the ratio of GSSG/GSH obtained from CSD+5D SR and CSD+21D SR groups did not reach the levels of control and EC groups ($p<0.01$).

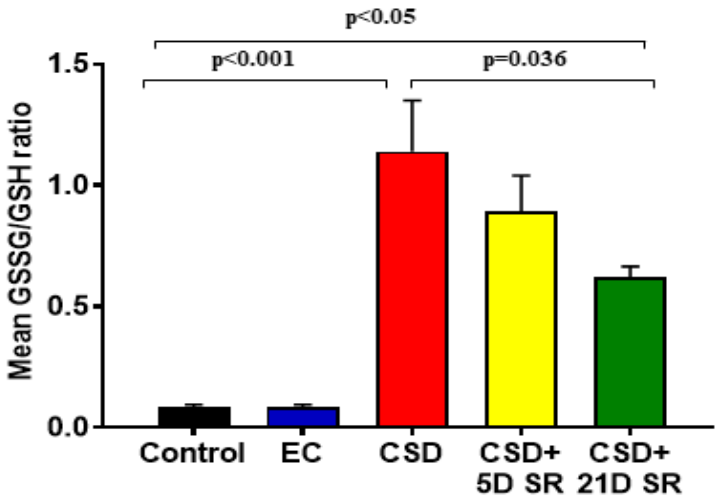


Figure 4.5C: The levels of GSSG/GSH ratio in the hippocampus obtained from control, EC, CSD, CSD+5D SR, and CSD+21D SR groups of rats (n=6/group).

4.3.3.4. Levels of TAC in CSD, CSD+5D SR, and CSD+21D SR groups of rats

The mean TAC levels obtained from control, EC, CSD, CSD+5D SR, and CSD+21D SR groups were 0.13±0.01, 0.138±0.01, 0.019±0.006, 0.04±0.006, and 0.06±0.007 units/mg of protein respectively (Figure 4.5D). The levels of TAC did not show any significant difference between the control and EC groups ($p>0.05$). The levels of TAC were significantly decreased in the CSD group compared to the control and EC groups ($p<0.001$). Whereas, the TAC levels obtained from the CSD+5D SR group showed a non-significant increase ($p>0.05$) and the CSD+21D SR group showed a significant increase ($p<0.02$) compared to the CSD group. However, the levels of TAC obtained from CSD+5D SR and CSD+21D SR groups did not reach the levels of control and EC groups ($p<0.01$).

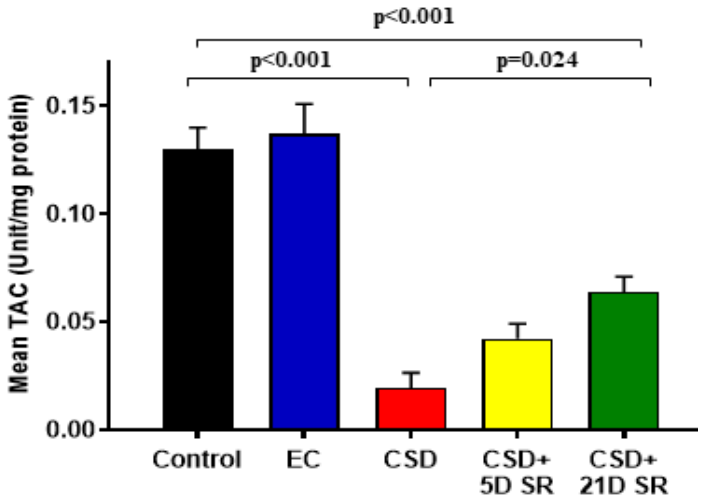


Figure 4.5D: The levels of TAC (units/mg of protein) in the hippocampus obtained from control, EC, CSD, CSD+5D SR, and CSD+21D SR groups of rats (n=6/group).

4.3.4. Effect of CSD, CSD+5D SR, and CSD+21D SR on the levels of serum corticosterone

At the end of 21-days of sleep deprivation and 5- and 21-days of sleep recovery, the serum corticosterone levels were measured. The mean serum corticosterone levels obtained from the control, EC, CSD, CSD+5D SR, and CSD+21D SR groups were 257±20, 236±10, 234±8, 245±27, and 247±15 ng/ml respectively (Figure 4.6). The levels of serum corticosterone did not show any significant difference among the groups ($p>0.05$).

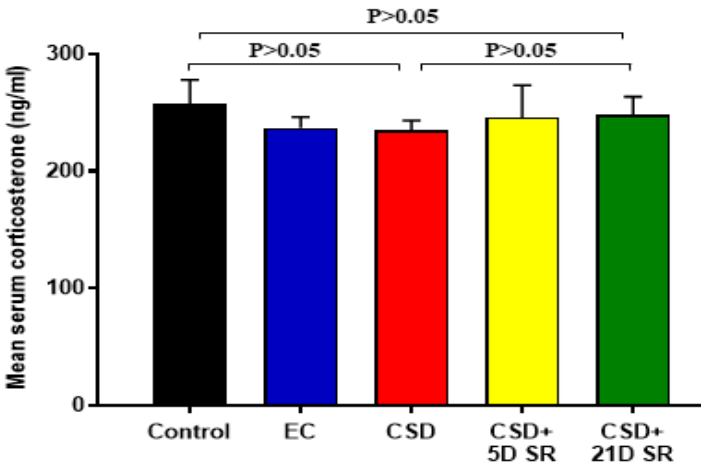


Figure 4.6: The levels of serum corticosterone (ng/ml) obtained from control, EC, CSD, CSD+5D SR, and CSD+21D SR groups of rats (n=6/group).

4.4. Discussion

Sleep is an important physiological function, which is required for the quality of life in modern humans. Lack of required sleep, whether due to acute or chronic sleep deprivation, has been reported to increase the risk of neurological disorders. Even though different durations of CSD induced behavioral alteration and oxidative stress in the brain have been evaluated, the duration of sleep recovery required to reverse the effects of CSD has not been well established. The present study aims to investigate the effect of 21-days of CSD and 5- and 21-days of sleep recovery on spatial memory, anxiety-like behavior, and oxidative stress in rats.

The 21-days (18 hours/day) of CSD rats impaired the spatial memory by decreasing the performance in HWM and MWM tasks and increasing signs of anxiety-like behavior in EPM and OFM tasks. In line with behavioral changes, the oxidative parameters also indicate that CSD induced oxidative stress in the hippocampus. The levels of MDA and ratio GSSG/GSH were increased, whereas the levels of glutathione and TAC levels were decreased in hippocampal tissue after 21-days of CSD, which indicates that the CSD induced oxidative stress in the hippocampus of the rats. Several studies have also shown that both acute and chronic sleep deprivation increased oxidative stress, which in turn leads to the impairment of spatial memory and anxiety-like behavior. Seventy-two hours of sleep deprivation results in increased oxidative stress in the hippocampus, which was correlated with impaired memory in mice (Silva et al., 2004). Twenty-one days of CSD (18 hours/day) leads to low-grade neuroinflammation (increased inflammatory cytokines) in the hippocampus, heightened anxiety-like behavior, and impaired memory in rats (Manchanda et al., 2018). Eight weeks of CSD (8 hours/day) leads to increased oxidative damage in the hippocampus

and impaired short-term and long-term memory in rats (Alzoubi et al., 2019). Seven days of intermittent and paradoxical sleep deprivation also increased the anxiety-like behavior and impaired memory of mice when compared to 3 days (Yin et al., 2017). It is a well-known fact that the metabolism of glucose in the brain during sleep is 30% less compared to wakefulness, which indicates that extended wakefulness increases the metabolism resulting in increased free radicals (Boyle et al., 1994). Sleep deprivation alters the sleep-wake cycle, the altered sleep-wake cycle leads to an imbalance in free radicals and antioxidant levels, which in turn lead to oxidative stress in the CNS specifically the hippocampus (Alkadhi et al., 2013). In line with the reported studies, the current study results have also shown that 21-days (18 hours/day) of CSD induced oxidative stress in the hippocampus is correlated with the impairment of spatial memory and anxiety-like behavior in rats.

In the present study, the rat's performance in MWM (reaching the platform) during the acquisition trials was comparable between the CSD and control. Nonetheless, it does not imply that these CSD rats have better learning than intact controls. The lack of spatial learning impairment might be due the familiarization of CSD rats with the water environment following long-term utilization of MMPI. Similarly, Yin et al., (2017) also observed that the 90-days of intermittent paradoxical sleep-deprivation in mice using an MMPM led to a shorter latency time to reach the escape platform compared to control mice when exposed to the MWM demonstrating intact spatial learning (Yin et al., 2017).

The 21-days of CSD impaired spatial memory increased anxiety-like behavior and raised oxidative stress in the hippocampus of rats. Further, the study was extended to investigate the efficacy of 5- and 21-days of sleep recovery on the damage caused by

21-days of CSD. The 5-days of sleep recovery showed a slight improvement in spatial memory, decreased anxiety-like behavior, and reduced oxidative stress compared to 21-days of CSD, however, it was not statistically significant. In contrast to 5-days of sleep recovery, the 21-days of sleep recovery showed a significant improvement in spatial memory, decreased anxiety-like behavior, and reduced oxidative stress when compared to 21-days of CSD, however, it was not sufficient to completely recover the damage caused by 21-days of CSD. Tung et al., (2005) also demonstrated that the 8 hours of sleep recovery exerted no restorative effect on the suppression of cell proliferation induced by 56 hours of sleep deprivation in rats (Tung et al., 2005). Seventy-two hours of sleep deprivation induced impairment of spatial memory and hippocampus neuronal apoptosis was not reversed even after 3 weeks of sleep recovery in mice (Soto-Rodriguez et al., 2016). Ninety days of repeated and intermittent paradoxical sleep deprivation-induced impaired memory and hippocampal inflammation also persisted even after 3 weeks of sleep recovery in mice (Yin et al., 2017). Furthermore, the levels of cortisol in serum did not affect by sleep recovery after prolonged wakefulness in humans (Brun et al., 1998). In line with the reported studies, the present study results also suggest that the 5- and 21-days of sleep recovery are not sufficient to completely recover the impaired spatial memory, anxiety-like behavior, and oxidative stress caused by 21-days of CSD. However, the 21-days of sleep recovery significantly reduced oxidative stress, increased spatial memory, and reduced anxiety-like behavior due to 21-days of CSD.

4.5. Conclusion

The present study results suggest that 21-days of CSD impaired spatial memory increased anxiety-like behavior, and increased oxidative stress in the hippocampus of

rats. The 21-days of sleep recovery significantly improved spatial memory, reduced anxiety-like behavior, and decreased oxidative stress caused by CSD, however, it did not completely restore to the control levels. Even though different durations of CSD induced behavioral alterations and biochemical changes have been previously evaluated, the effects of CSD and the duration of sleep recovery required to reverse the CSD induced damage have not been well established at the cellular or molecular level. Therefore, this study was further extended to understand the biochemical, cellular, and molecular level changes after 21-days of CSD, and 5- and 21-days of sleep recovery, which might help for better management of individuals suffering from sleep disorders.

Chapter V

Results and Discussion

Effect of CSD and sleep recovery on dendritic arborization of CA3 neurons and structural integrity of oligodendrocytes in the hippocampus of rats

5.1. Introduction

Twenty-one days of CSD impaired spatial memory and increased anxiety-like behavior and oxidative stress in the hippocampus of rats. Several other studies have shown that acute/chronic sleep deprivation increases oxidative stress in the brain, which in turn leads to anxiety-like behavior and impairment of spatial learning and memory. However, the extent of CSD induced oxidative damage at the cellular and molecular level in the brain has not been clearly understood. Therefore, the study was extended to investigate the effect of CSD and sleep recovery on the dendritic arborization of pyramidal CA3 neurons and the structural integrity of oligodendrocytes in the hippocampus of rats.

5.2. Methodology

At end of sleep deprivation/sleep recovery and behavioral experiments, the rats were dissected and the brain/hippocampus harvested from each cerebral hemisphere and processed for the following experiments.

5.2.1. Measurement of dendritic arborization of hippocampal CA3 neurons

The brain samples were processed to measure the dendritic arborization as described in (Shankaranarayana Rao and Raju, 2004). The dendritic arborization of CA3 neurons was analyzed (8-10 neurons/rat: 50 neurons/group) using light microscopy equipped with camera lucida in 20X magnification. Dendritic branching points and dendritic intersections were quantified using a concentric circle method as described earlier (Sholl, 1953) (described in Chapter III; section 3.2.4.1).

5.2.2. Immunostaining of CNPase+ve oligodendrocytes in the hippocampus

The brain samples were processed for immunohistochemistry to measure the CNPase+ve oligodendrocytes using Anti-CNPase primary antibodies (1:1000 dilutions)

(Abcam, USA) (Subhadeep et al., 2021). CNPase+ve oligodendrocytes were quantified as described earlier in (Yin et al., 2017). A total of 200 images/subregion/groups were used to measure the number of CNPase+ve oligodendrocytes. The staining intensity of CNPase in CA1, CA3, and DG subregions of the hippocampus was measured in the captured images using Image J software (Razgado-Hernandez et al., 2015). A total of 30 images/subregion/groups were used to measure the CNPase intensity in the hippocampus (described in Chapter III; section 3.2.4.2).

5.2.3. Expression of *CNPase* gene in the hippocampal tissue

The hippocampal tissue samples were used to isolate total RNA and performed the *CNPase* gene expression. The expression of the *CNPase* gene was measured using qRT-PCR (Hattori et al., 2014). The sequence of the primers: *CNPase*: 5'CAACAGGATGTGGTGAGGA3'/5'CTGTCTTGGGTGTCACAAAG3'&*GAPDH* :5'GCCTTCTCTTGTGACAAAGTGG3'/5'ATTCTCAGCCTTGACTGTGCC3' (described in Chapter III; section 3.2.4.3).

5.3. Results

5.3.1. Effect of CSD, CSD+5D SR, and CSD+21D SR on dendritic arborization of hippocampal CA3 neurons

Hippocampal CA3 neuronal dendrites receive the input signals from DG (mossy fibers), the Entorhinal cortex (perforant path), recurrent fibers of CA3, contralateral CA3, and the septal area. The dendritic branching points and intersections of CA3 neurons play an important role in synaptic connectivity, which is associated with the formation of memory. To find the effect of 21-days of CSD and 5- and 21-days of sleep recovery on dendritic arborization, we measured the basal and apical dendrite branching points and intersections of hippocampal CA3 neurons (8-10 neurons/rat; 50 neurons/group).

5.3.1.1. Basal dendritic branching points of hippocampal CA3 neurons

The representative images of CA3 neurons obtained from hippocampal tissue stained with Golgi-Cox stain and the method used to quantify the dendritic branching points and intersections are shown in figure 5.1. The mean basal branching points of hippocampal CA3 neurons obtained from 0-20, 20-40, 40-60, 60-80, and 80-100 µm concentric zones of control and EC groups did not differ significantly ($p>0.05$). The mean basal branching points of hippocampal CA3 neurons obtained from the CSD showed a significant decrease at different concentric zones of 0-20, 20-40, 60-80, 80-100 ($p<0.001$), and 40-60 µm ($p<0.01$) when compared to control and EC group.

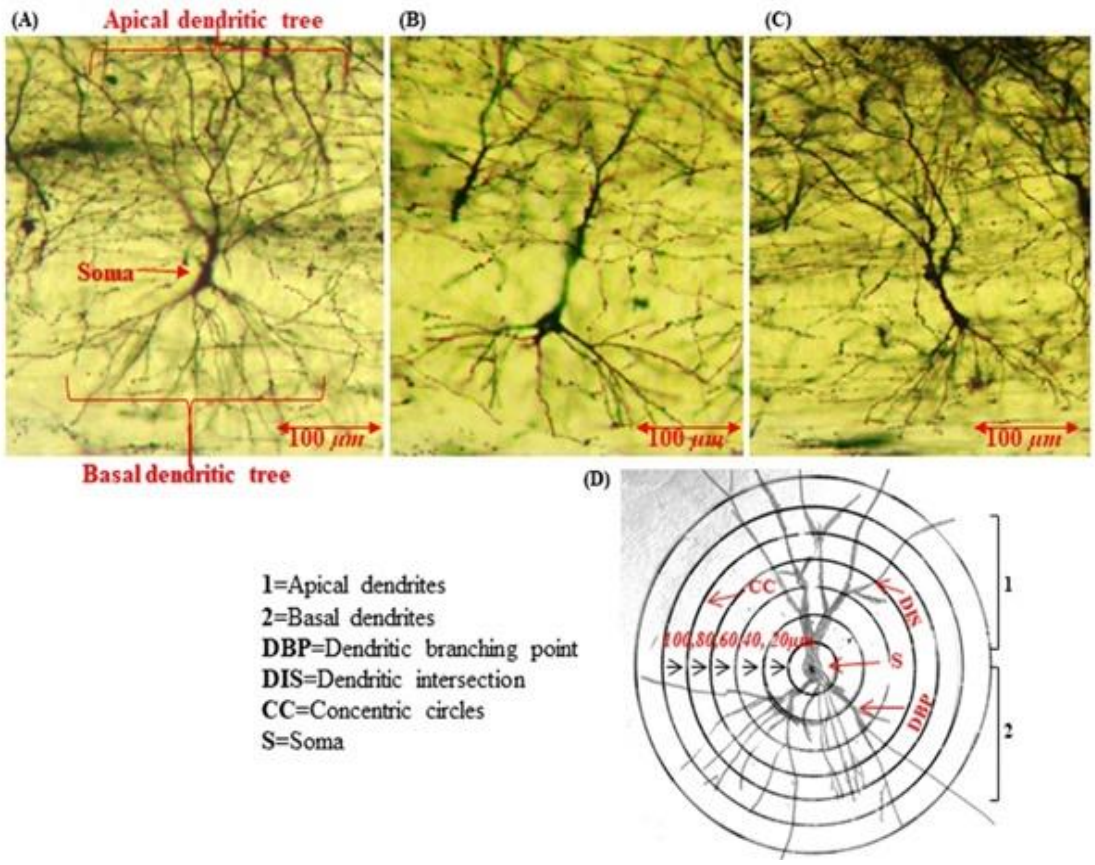


Figure 5.1: Representative images of hippocampal CA3 neurons (basal and apical dendritic tree) obtained after Golgi cox stain (A) Control, (B) CSD, (C) CSD+21D SR groups of rats, and (D) The method used to quantify the dendritic arborization of CA3 Neurons.

The basal dendritic branching points of hippocampal CA3 neurons were increased in CSD+5D SR and CSD+21D SR groups compared to the CSD group, but the increase was not statistically significant in all concentric zones ($p>0.05$). Furthermore, the increased basal branching points obtained from all concentric zones of CSD+5D SR and CSD+21D SR did not reach the control or EC groups ($p<0.05$). Figure 5.2A shows the basal dendritic branching points of hippocampal CA3 neurons obtained from different groups of rats.

5.3.1.2. Apical dendritic branching points of hippocampal CA3 neurons

The mean apical branching points of hippocampal CA3 neurons obtained from 0-20, 20-40, 40-60, 60-80, and 80-100 µm concentric zones of control and EC groups did not differ significantly ($p>0.05$). The mean apical branching points of hippocampal CA3 neurons obtained from the CSD group showed a significant decrease at concentric zones 60-80, 80-100 ($p<0.01$), but non-significant at 0-20, 20-40, and 40-60 µm zones ($p>0.05$) when compared to the control and EC groups. However, the apical dendritic branching points of hippocampal CA3 neurons obtained from CSD+5D SR and CSD+21D SR groups showed a slight increase but non-significant compared to the CSD group at all concentric zones ($p>0.05$). The apical branching points obtained from concentric zones 60-80, 80-100 µm ($p<0.05$) of CSD+5D SR and CSD+21D SR groups did not reach the control and EC groups. Figure 5.2B shows the apical dendritic branching points of hippocampal CA3 neurons obtained from different groups of rats.

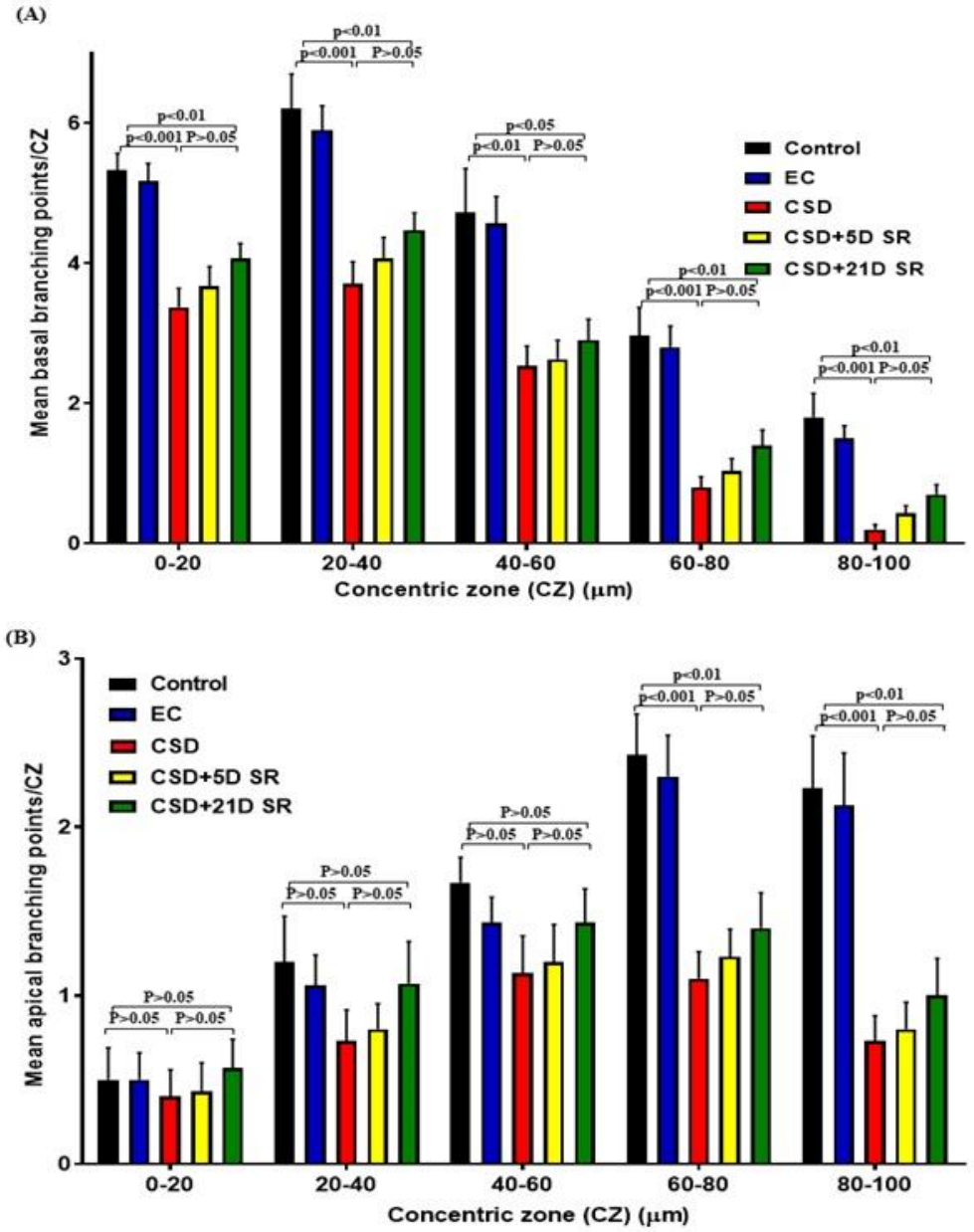


Figure 5.2: Dendritic arborization of hippocampal CA3 neurons (8-10 neurons/rat; 50 neurons/group) obtained from control, EC, CSD, CSD+5D SR, and, CSD+21D SR groups of rats (n=6/group): (A) Mean basal branching points of hippocampal CA3 neurons at different concentric zones (CZ) (0-20, 20-40, 40-60, 60-80, and 80-100 µm distance from the soma). (B) Mean apical branching points of hippocampal CA3 neurons at different concentric zones (CZ) (0-20, 20-40, 40-60, 60-80, and 80-100 µm distance from the soma).

5.3.1.3. Basal dendritic intersections of hippocampal CA3 neurons

The mean basal dendritic intersections of CA3 neurons obtained from 0-20, 20-40, 40-60, 60-80, and 80-100 µm concentric zones of control and EC groups did not differ significantly ($p>0.05$). The mean basal dendritic intersections of hippocampal

CA3 neurons obtained from the CSD group showed a significant decrease at concentric zones of 20-40 ($p<0.001$), 40-60, 60-80, 80-100, ($p<0.01$), but non-significant at 0-20 µm zone ($p>0.05$) when compared to the control and EC groups. However, the basal dendritic intersections of hippocampal CA3 neurons obtained from CSD+5D SR and CSD+21D SR groups showed a slight increase but non-significant compared to the CSD group at all concentric zones ($p>0.05$). The basal dendritic intersections obtained from 20-40, 40-60, 60-80, and 80-100 µm concentric zones ($p<0.05$) of CSD+5D SR and CSD+21D SR groups did not reach the control group. Figure 5.3A shows the basal dendritic intersection of hippocampal CA3 neurons obtained from different groups of rats.

5.3.1.4. Apical dendritic intersections of hippocampal CA3 neurons

The mean apical dendritic intersections of hippocampal CA3 neurons obtained from 0-20, 20-40, 40-60, 60-80, and 80-100 µm concentric zones of control and EC groups did not differ significantly ($p>0.05$). The mean apical dendritic intersections of hippocampal CA3 neurons obtained from the CSD group showed a significant decrease at concentric zones 60-80, 80-100 ($p<0.001$) but non-significant at 0-20, 20-40, and 40-60 µm zones ($p>0.05$) when compared to the control and EC groups. However, the apical dendritic intersections of hippocampal CA3 neurons obtained from concentric zones 60-80, 80-100 µm of CSD+5D SR and CSD+21D SR groups showed a slight increase, but it was non-significant compared to the CSD group ($p>0.05$). The apical dendritic intersections obtained from concentric zones 60-80, 80-100 µm of CSD+5D SR and CSD+21D SR groups did not reach the control level ($p<0.05$). Figure 5.3B shows the apical dendritic intersection of hippocampal CA3 neurons obtained from different groups of rats.

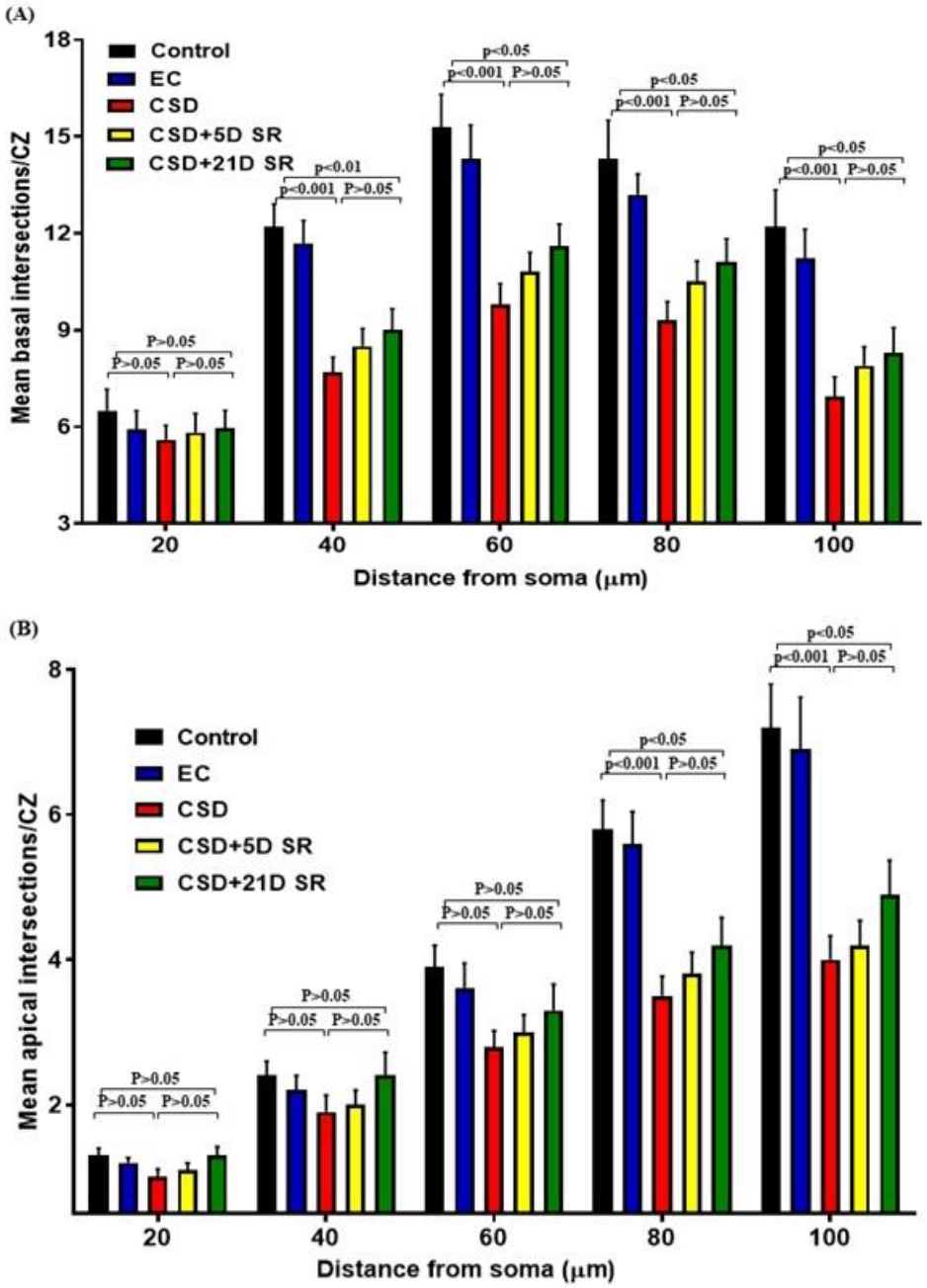


Figure 5.3: Dendritic arborization of hippocampal CA3 neurons (8-10 neurons/rat; 50 neurons/group) obtained from control, EC, CSD, CSD+5D SR, and CSD+21D SR groups of rats (n=6/group): (A) Mean basal dendritic intersections of hippocampal CA3 neurons at different concentric zones (CZ) (0-20, 20-40, 40-60, 60-80 and 80-100 µm distance from the soma). (B) Mean apical dendritic intersections of hippocampal CA3 neurons at different concentric zones (CZ) (0-20, 20-40, 40-60, 60-80, and 80-100 µm distance from the soma).

5.3.2. Effect of CSD, CSD+5D SR, and CSD+21D SR on the number of CNPase+ve oligodendrocytes in the hippocampus of rats

The 21-days of sleep deprivation significantly increased the oxidative stress markers in the hippocampus. To find the extent of the oxidative injury on oligodendrocytes, we measured the number of CNPase+ve oligodendrocytes in the CA1, CA3, and DG regions of the hippocampus. The representative images of CNPase+ve stained hippocampus and CNPase+ve oligodendrocytes were shown in figure 5.4.

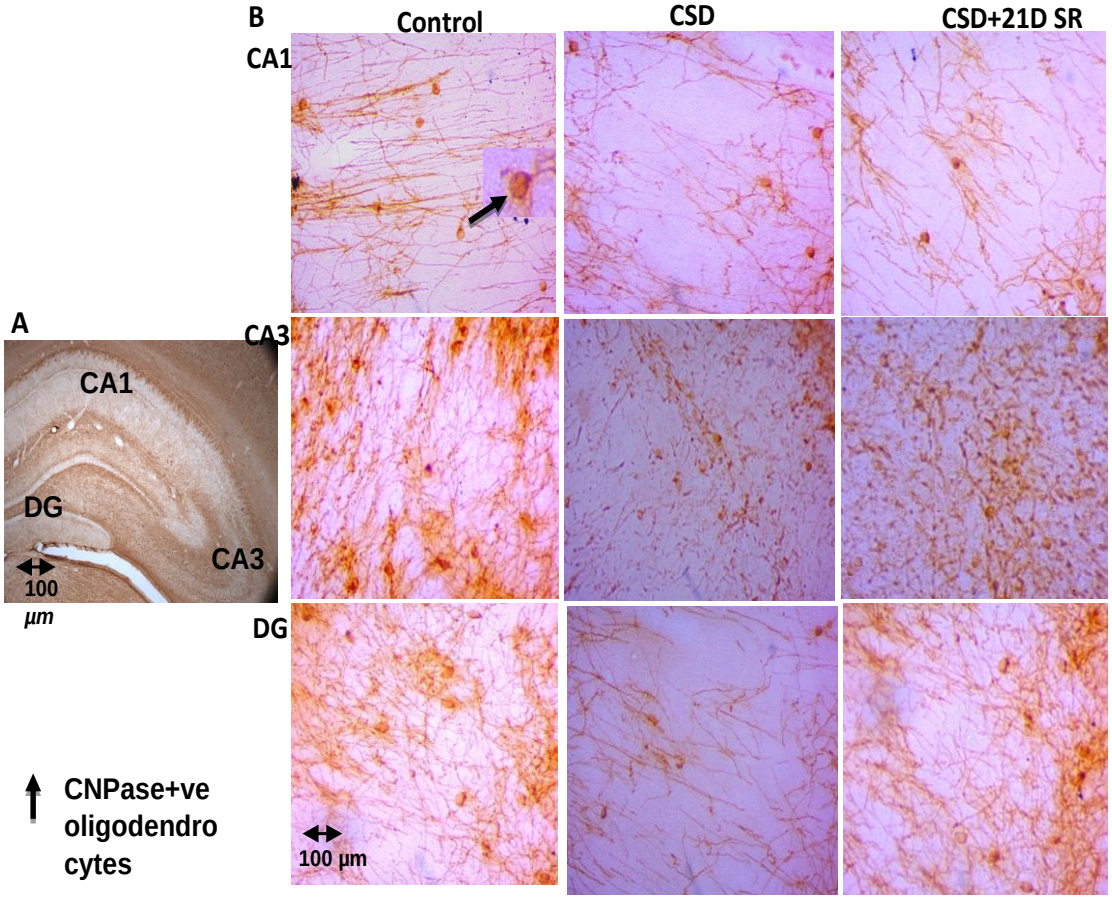


Figure 5.4: Representative images of CNPase stained oligodendrocytes of the hippocampus of control, CSD, and CSD+21D SR groups rats (n=6/group): (A) CNPase stained hippocampus (CA1, CA3, and DG regions), and (B) CNPase+ve oligodendrocytes in CA1, CA3, and DG regions.

5.3.2.1. CNPase+ve oligodendrocytes in the CA1 of the hippocampus

The mean number of CNPase+ve oligodendrocytes in the CA1 subregion of the hippocampus obtained from control, EC, CSD, CSD+5D SR, and CSD+21D SR groups were 8.13±0.25, 8.15±0.23, 6.9±0.2, 7.01±0.23, and 7.2±0.19 cells/mm^2 respectively (Figure 5.5A). The number of CNPase+ve oligodendrocytes in the CA1 region was significantly decreased in the CSD group compared to the control and EC ($p<0.01$) groups. The number of CNPase+ve oligodendrocytes in the CA1 region was increased in the CSD+5D SR and CSD+21D SR groups when compared to the CSD group, but the increase was not statistically significant. However, the increase in the number of CNPase+ve oligodendrocytes in CSD+21D SR has not reached control levels in the CA1 region ($p<0.05$). The CNPase+ve oligodendrocytes obtained from the CA1 region did not show a difference between the control and EC group ($p>0.05$). To find the relation between oxidative markers in the hippocampus and the number of CNPase+ve oligodendrocytes, correlation analysis was performed between MDA and CNPase+ oligodendrocytes of CA1 in the CSD group. A negative correlation was observed between MDA and CNPase+ve oligodendrocytes of the CA1 ($r=-0.87$) region in the hippocampus of the CSD group (Figure 5.5B).

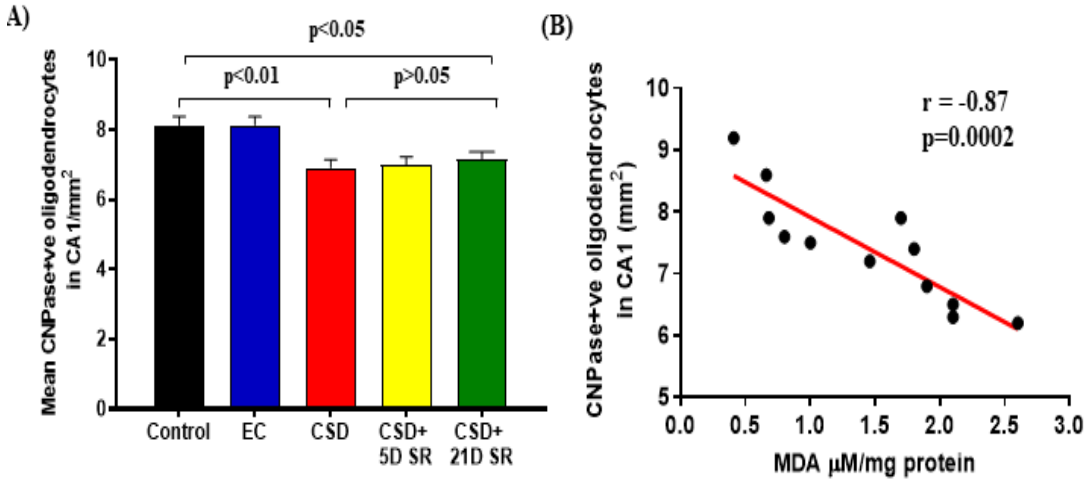


Figure 5.5: Mean number of CNPase+ve oligodendrocytes in the CA1 region of hippocampus obtained from the Control, EC, CSD, CSD+5D SR, and CSD+21D SR (A), Correlation between MDA vs CNPase+ve oligodendrocytes in CA1 (B).

5.3.2.2. CNPase+ve oligodendrocytes in the dentate gyrus of the hippocampus

The mean number of CNPase+ve oligodendrocytes in dentate gyrus obtained from the control, EC, CSD, CSD+5D SR, and CSD+21D SR groups were 9.6±0.23, 9.7±0.24, 7.5±0.22, 7.7±0.21, and 8.0±0.18 cells/mm^2 respectively (Figure 5.6A). The number of CNPase+ve oligodendrocytes in the DG region was significantly decreased in the CSD group compared to the control and EC groups ($p<0.01$). The number of CNPase+ve oligodendrocytes in the DG region was increased in the CSD+5D SR and CSD+21D SR groups when compared to the CSD group, but the increase was not statistically significant. However, the increase in the number of CNPase+ve oligodendrocytes in CSD+21D SR has not reached control levels in the DG region ($p<0.05$). The CNPase+ve oligodendrocytes obtained from the DG region did not show a difference between the control and EC group ($p>0.05$). A negative correlation was observed between MDA and CNPase+ve oligodendrocytes of the DG ($r=-0.9$) region in the hippocampus of the CSD group (Figure 5.6B).

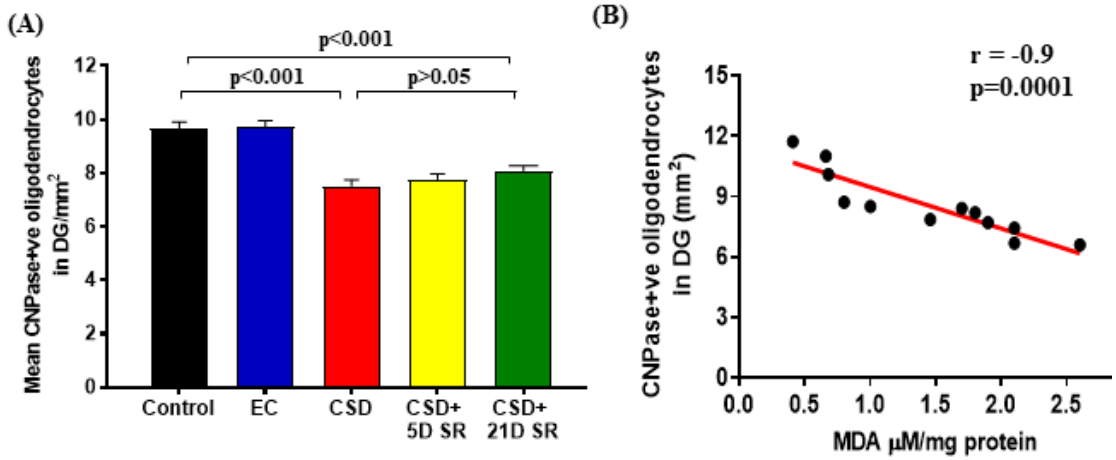


Figure 5.6: Mean number of CNPase+ve oligodendrocytes in the DG region of hippocampus obtained from the Control, EC, CSD, CSD+5D SR, and CSD+21D SR (A), Correlation between MDA vs CNPase+ve oligodendrocytes in DG (B).

5.3.2.3. CNPase+ve oligodendrocytes in CA3 of the hippocampus

The mean number of CNPase+ve oligodendrocytes in the CA3 obtained from the control, EC, CSD CSD+5D SR, and CSD+21D SR groups was 11.8±0.24, 12±0.26, 11±0.26, 11.1±0.26 and 11.9±0.26 cells/mm^2 respectively (Figure 5.7A). The CNPase+ve oligodendrocytes were comparable between the control, EC, CSD, CSD+5D SR, and CSD+21D SR groups. However, a negative correlation was observed between the MDA and CNPase+ve oligodendrocytes of CA3 in the CSD group (r=-0.72). (Figure 5.7B).

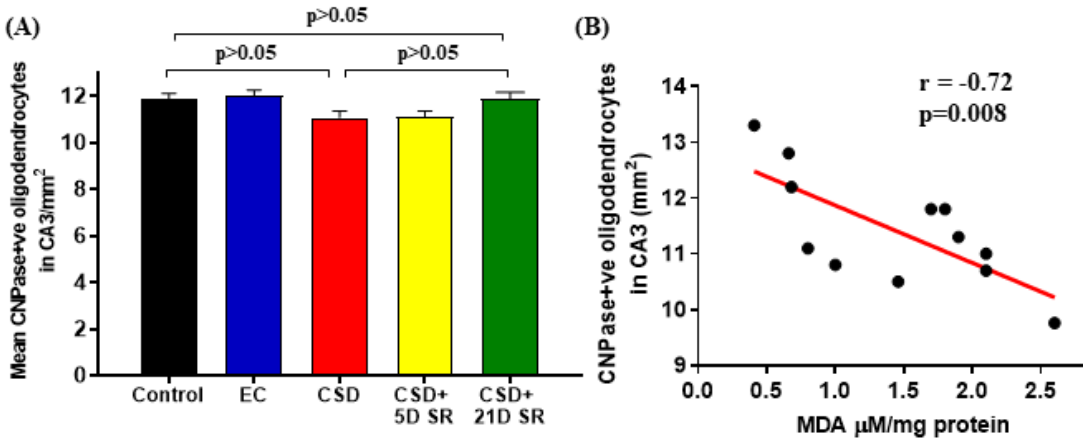


Figure 5.7: Mean number of CNPase+ve oligodendrocytes in the CA3 region of hippocampus obtained from the Control, EC, CSD, CSD+5D SR, and CSD+21D SR (A), Correlation between MDA vs CNPase+ve oligodendrocytes in CA3 (B).

5.3.3. Effect of CSD, CSD+5D SR, and CSD+21D SR on CNPase intensity in the hippocampus of rats

The number of CNPase+ve oligodendrocytes showed a significant decrease after 21-days of sleep deprivation. Further, we have analyzed the optical density (intensity) of CNPase from the images using Image J software (NIH, Bethesda, USA). The intensity of CNPase in CA1, CA3, and DG regions was significantly reduced ($p<0.001$) in the CSD group when compared to the control and EC groups. The intensity of CNPase in CA1, CA3, and DG regions of CSD+21D SR group rats showed a significant increase

($p<0.05$) when compared to the CSD group. Though the intensity of CNPase in CA1, CA3, and DG regions was improved in the CSD+21D SR group, it did not reach control levels ($p<0.001$) (Figures 5.8A-C). Further, a negative correlation was observed between the levels of MDA and CNPase intensity in CA1 (r=-0.97), CA3 (r=-0.98), and DG (r=-0.98) regions in the hippocampus of the CSD group (Figures 5.9A-C).

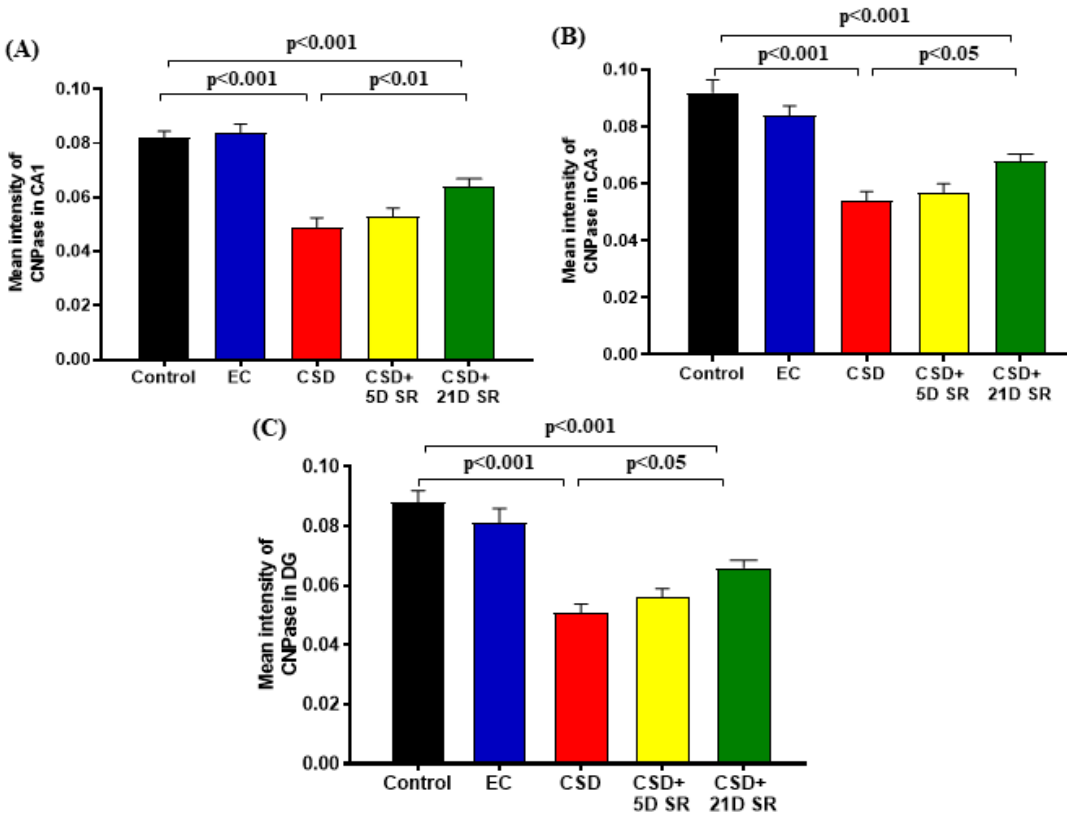


Figure 5.8: Mean intensity of CNPase in the CA1, CA3, and dentate gyrus subregions of hippocampus obtained from the control, EC, CSD, CSD+5D SR, and CSD+21D SR: (A) Intensity of CNPase in CA1, (B) Intensity of CNPase in CA3, (C) Intensity of CNPase in DG region.

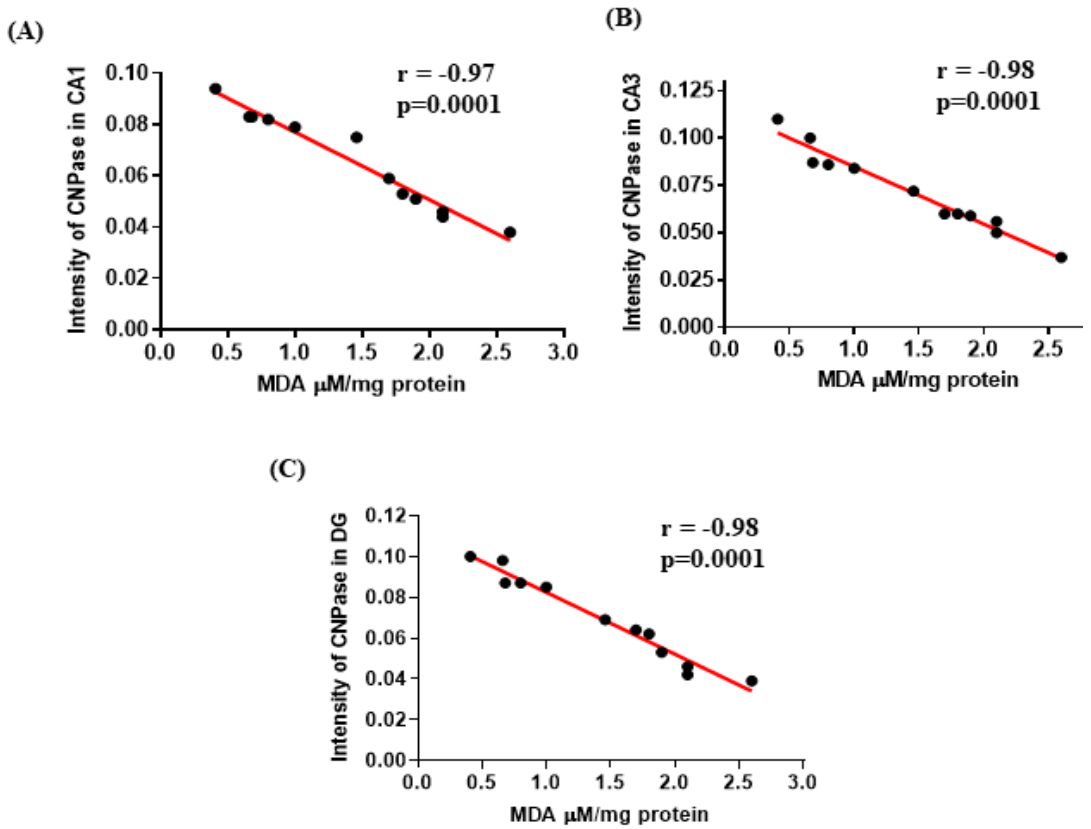


Figure 5.9: Pearson correlation between MDA and intensity of CNPase in CSD rats: (A) Correlation between MDA vs intensity of CNPase in CA1, (B) Correlation between MDA vs intensity of CNPase in CA3, (C) Correlation between MDA vs intensity of CNPase in the DG region.

5.3.4. Effect of CSD, CSD+5D SR, and CSD+21D SR on *CNPase* gene expression in hippocampal tissue

The number of CNPase+ve oligodendrocytes and the intensity of CNPase in hippocampal tissue were reduced after 21-days of CSD. Further, the study was extended to investigate the *CNPase* gene expression change after 21-days of CSD. The expression of the *CNPase* gene was significantly downregulated 12.56-folds after 21-days of CSD compared to control. Even though the 21-days of sleep recovery increased *CNPase* gene expression, it is still 5.5-folds down-regulated compared to control. The fold change expression of the *CNPase* gene after 21-days of CSD and 21-days of sleep recovery was shown in figures 5.10A and B. Correlation analysis was performed: *CNPase* gene expression vs levels of MDA, *CNPase* gene expression vs CNPase intensity. The

normalized Ct (ΔΔCt) of *CNPase* gene expression was positively correlated with oxidative stress (r=0.98) and negatively correlated with the CNPase intensity (r=-0.91) (Figures 5.11A and B).

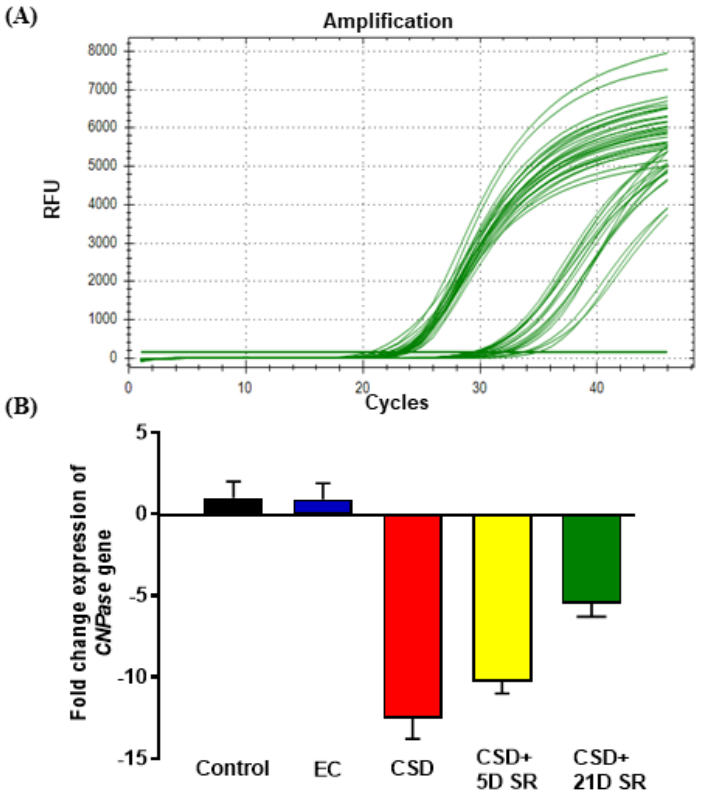


Figure 5.10: *CNPase* gene amplification curve (A), Fold change expression of *CNPase* gene in the hippocampal tissue of control, EC, CSD CSD+5D SR, and CSD+21D SR group rats (n=6/group) (B).

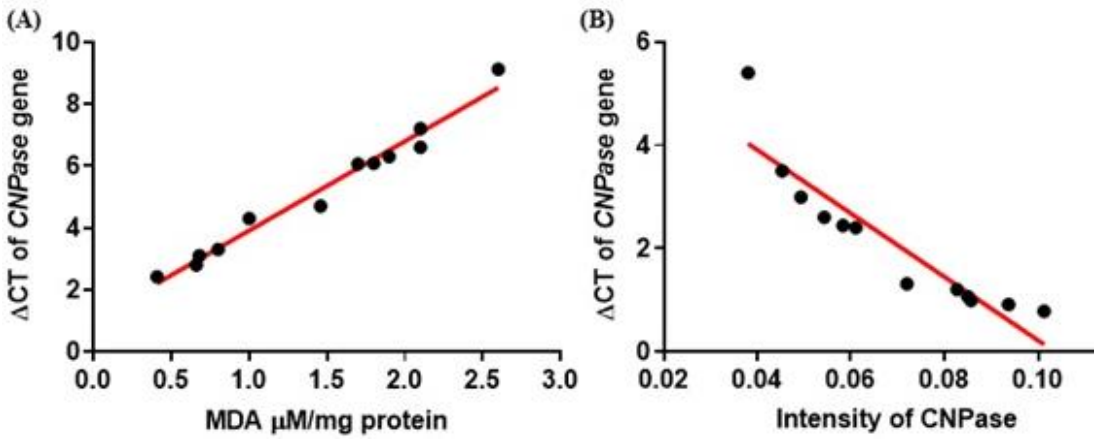


Figure 5.11: Pearson correlation between the ΔCT of *CNPase* gene vs MDA and CNPase intensity in CSD rats: (A) ΔCT of *CNPase* gene vs MDA, (B) ΔCT of *CNPase* gene vs intensity of CNPase.

5.4. Discussion

Sleep plays an important role in learning and memory, and the quality of life of human beings. Loss of sleep has been reported to increase the risk of neurological disorders, type 2 diabetes mellitus, coronary heart diseases, etc. (Shan et al., 2015, Cappuccio et al., 2011). The duration of sleep and the type of sleep deprivation (chronic or acute) can affect the biochemical, cellular, and gene expression functions in the brain and also the behavioral status in both animals and humans (Cirelli, 2002, Kreutzmann et al., 2015). The 21-days of CSD in the present study significantly increased the oxidative stress in the hippocampus, impaired spatial memory, and increased anxiety-like behavior in rats. Further, the study was extended to find the extent of the oxidative injury-induced changes in neurons and neuroglia for a better understanding of the CSD induced damage.

The 21-days (18 hours/day) of CSD in the present study significantly reduced both basal and apical branching points and intersections (at different concentric zones) of hippocampal CA3 neurons. The studies have also shown that the CSD induced atrophy of neurons has reduced the volume of the hippocampus in animal models (Owen and Veasey, 2020). Five hours of sleep deprivation reduced the spine density in CA1 neurons and dentate gyrus regions of the hippocampus in mice (Havekes et al., 2016, Raven et al., 2019). Twenty-one days (18 hours/day) of CSD leads to the shortening and shedding of CA1 dendritic trees in rats (Noorafshan et al., 2018). In addition, 21-days (8 hours/day) of CSD also impaired the maintenance of long-term potentiation in the dentate gyrus in rats (Süer et al., 2011). The hippocampal CA1 neurons are reported to be more susceptible to oxidative injury compared to the CA3 region, however, preliminary studies reported that the CA3 region is also prone to oxidative damage (Salim, 2017). It is a known fact that the dentate gyrus and CA3 region exhibit structural

plasticity with regenerative/remodelling capacity (Sousa et al., 2000). *In vitro* studies showed that both CA1 and CA3 neurons of the hippocampus were susceptible to the damage caused by free radicals (Wilde et al., 1997). One-day sleep deprivation also showed increased levels of interleukin-6 and Ionized calcium-binding adaptor protein-1 positive cells (activated microglia) in the CA3 region of the hippocampus of mice (Zhu et al., 2012). Twenty-eight days (20 hours/day) of sleep deprivation reduced the volume of CA3 cellular sub region in adolescent rats (Novati et al., 2011). In addition, the CA3/dentate gyrus volume was also decreased in patients suffering from insomnia (Neylan et al., 2010). Further, a decreased neuronal density was shown to be more prominent in CA1 and CA3 regions of the hippocampus of Alzheimer's patients compared to controls (Padurariu et al., 2012). In contrast, five hours of sleep deprivation did not alter the dendritic spine density in CA3 neurons in mice (Havekes et al., 2016) and twenty-one days of CSD also did not alter the number of CA3 neurons in rats (Noorafshan et al., 2017). Similar to the reported studies, the present study also showed that CSD can damage the hippocampal CA3 neurons in rats. The elevated levels of corticosterone were known to cause the atrophy of CA3 neurons and dendrites arborization in rats. Recent study reports suggest that 72 or 96 hours of total/partial sleep deprivation increases plasma corticosterone levels in rats (Hipolide et al., 2006, Zager et al., 2007). In contrast to 72 or 96 hours, the 21-days of CSD did not show any changes in plasma corticosterone levels (Süer et al., 2011, Zager et al., 2007). In line with the reported studies, the 21-days of CSD in the present study did not alter the levels of serum corticosterone. Therefore, the reduced apical/basal dendritic branching points/intersections of hippocampal CA3 neurons are not due to corticosterone (a marker of stress).

The 21-days of CSD decreased the dendritic arborization of CA3 neurons in the hippocampus. However, the effect of CSD and duration of sleep recovery on oligodendrocytes and myelination was not fully understood. The present study was extended to find the effect of 21-days of CSD on the number of CNPase+ve oligodendrocytes, CNPase intensity, and *CNPase* gene expression and the efficacy of 21-days of sleep recovery in rats. The 21-days of CSD significantly reduced the number of CNPase+ve oligodendrocytes in CA1 and DG regions of the hippocampus of rats. Further, the increased levels of MDA (oxidative damage marker) were correlated with decreased number of CNPase+ve oligodendrocytes in CA1, CA3, and DG regions of the hippocampus of CSD rats. The results of this study are supported by the published literature on the oxidative stress-induced damage of oligodendrocytes. Oligodendrocytes are prone to generate free radicals in unfavourable conditions, it could be due to the active lipid metabolism and low concentration of antioxidant enzymes (Juurlink, 1997, Thorburne and Juurlink, 1996, Jana and Pahan, 2007). Jana and Pahan et al., (2007) observed that the oxidative stress induced pro-apoptotic signalling cascades result in the loss of cultured human oligodendrocytes (Jana and Pahan, 2007). Oxidative stress can also affect the differentiation of oligodendrocytes by altering the expression of genes responsible for the maturation of cultured oligodendrocytes (French et al., 2009). The reduced number of oligodendrocytes after 21-days of CSD can affect the myelination, memory, and behavior of the rats.

CNPase is an enzyme that is involved in the migration or expansion of membranes during myelination and has distinct roles in subcellular compartments of myelin and axon-glial communication (Chao et al., 2021). To find the 21-days of CSD influence on CNPase protein levels, the CNPase intensity (CNPase protein: an indirect measure of myelin content) was measured using stained images of the hippocampus. The CNPase

intensity was reduced in CA1, CA3, and DG regions of the hippocampus after 21-days of CSD. As there was a decrease in the levels of CNPase protein, the expression of the *CNPase* gene in the hippocampus after 21-days of CSD was analyzed. In line with the number of oligodendrocytes and CNPase intensity, there was a down regulation of *CNPase* gene expression after 21-days of CSD. Both CNPase protein and gene expression levels after 21-days of CSD correlated with increased oxidative stress marker levels. The results of this study support the effect of CSD induced changes on myelin components such as the CNPase protein and *CNPase* gene expression in the hippocampus. Bellesi et al., (2018) reported that CSD for 4 ½ days results in the reduced thickness of myelin in the corpus callosum of adolescent mice (Bellesi et al., 2018). Similarly, decreased myelin thickness and loss of white matter in the brain were observed in obstructive sleep apnea and restless leg syndrome patients (Connor et al., 2011, Kumar et al., 2014). The rats subjected to CSD for one week showed the down-regulated expression of genes involved in the synthesis of myelin and associated proteins (Cirelli et al., 2006). The short (4 hours) sleep deprivation of mice has shown the upregulation of genes (*Acin1, Bcat1, Otud7b, Nr4a1, Hip1, Irf8, Traf6, HSPE1,* and *HSP90aa1)* involved in cellular stress and apoptosis relative to basal levels (Bellesi et al., 2013). Based on the published and the present study results, the increased oxidative stress after 21-days of CSD might be responsible for the decreased number of CNPase+ oligodendrocytes, and reduced expression of both CNPase protein and *CNPase* gene in the hippocampus of rats.

The CSD increased the oxidative stress, and reduced dendritic branching points/intersection in the CA3 neurons and CNPase+ve oligodendrocytes in the hippocampus, which might be the cause of the impairment of spatial memory and increased anxiety-like behavior. Owen et al., (2020) stated that prolonged sleep

deprivation ultimately results in irreversible injury/degeneration of neurons and impairment of memory in animal models (Owen and Veasey, 2020). A recent study also demonstrated that CSD rats (18 hours/day for 21-days) showed anxiety-like behavior and impaired memory was associated with an increase of proinflammatory cytokines in the hippocampus (Manchanda et al., 2018). The demyelinating agent such as lysophosphatidylcholine treated rats also yields evidence of anxiety-like behavior in EPM and OFM when it is injected directly into the hippocampus (Makinodan et al., 2008). The findings from the current study demonstrates that CSD induces oxidative stress, decreases dendritic arborization in the CA3 region, and reduces CNPase+ve oligodendrocytes in the hippocampus which might serve as rationale for the impaired spatial memory and increased anxiety-like behavior observed in these rats. Further studies are in need to enhance our understanding of the impact of CSD and sleep recovery on spatial memory, anxiety-like behavior, and physiological mechanisms.

In the present study we used MMPM to sleep deprive the rats, this method along with the 'flowerpot technique' is being widely used to induce sleep deprivation in rodent models among the laboratories in the world (Machado et al., 2004). It has been proven that the MMPM can cause complete REM sleep deprivation and partial NREM sleep deprivation (Machado et al., 2004). Recent studies also highlighted that continuous sleep deprivation for >24 hours using MMPM results in complete REM sleep deprivation and least effect on non-REM sleep deprivation when compared to environmental control (McDermott et al., 2003, Giri et al., 2021). Therefore, the observed changes (impaired spatial memory, anxiety-like behavior, reduced dendritic arborization of hippocampal CA3 neurons, and increased oxidative stress) in the present study (21-days of CSD) are majorly due to REM sleep deprivation.

Finally, the effect of 5- and 21-days of sleep recovery on the basal/apical dendritic branching points/intersections of hippocampal CA3 neurons, the number of CNPase+ oligodendrocytes, expression of *CNPase* gene, and protein levels were measured in rats. The basal/apical dendritic branching points/intersections of hippocampal CA3 neurons, and the number of CNPase+ve oligodendrocytes improved after 21-days of sleep recovery but it was not significant, whereas, the CNPase intensity and gene expression was significantly increased compared to CSD. Yin et al., (2017) demonstrated a repeated and intermittent paradoxical sleep deprivation results in impairment of memory, inflammation in hippocampal, activation of microglial and neuronal apoptosis, these changes persisted even after 3 weeks of recovery in mice (Yin et al., 2017). In contrast, the rats subjected to 6-days of continuous REM sleep deprivation showed the reduction of hippocampal dendritic arborization (CA1 and CA3) was reversed with 3-days of sleep recovery (Giri et al., 2021). Two months of CSD in mice showed impaired learning and memory, these changes were recovered after three months of sleep recovery (Qiu et al., 2016). Bellesi et al., (2018) reported that 4 ½ days of CSD in adolescent mice results in reduced myelin thickness in the corpus callosum and it was not restored with 36 h of recovery sleep (Bellesi et al., 2018). In line with reported studies, the current study results also highlight that the 21-days of sleep recovery was not sufficient to reverse the damage to CA3 neurons and oligodendrocytes caused by 21-days of CSD to control level.

Even though the dendritic arborization of hippocampal CA3 neurons and CNPase+ve oligodendrocytes was not significantly improved, the spatial memory and anxiety-like behavior were improved significantly after 21-days of sleep recovery related to the CSD, which might be due to the reduced oxidative stress. Together these results suggest that a 21-days of sleep recovery are not enough to restore the CSD

induced alterations. Overall, the present results provided new insights to understand the CSD and sleep recovery induced changes in neurons and neuroglial cells and their impact on spatial learning and memory and behavioral alterations.

5.5. Conclusion

The present study results suggest that there is reduced dendritic arborization of hippocampal CA3 neurons, CNPase+ve oligodendrocytes, and *CNPase* gene expression in the hippocampus after 21-days (18 hours/day) of CSD in rats. The reduced number of oligodendrocytes correlated with the increased oxidative stress in the hippocampus. The decreased dendritic arborization and oligodendrocytes changes were associated with impaired spatial memory and anxiety-like behavior in rats. Even though there was an increased in CA3 neuronal dendritic arborization and number of oligodendrocytes in the hippocampus after 21-days of sleep recovery, it was not sufficient for complete restoration of the damage induced by 21-days of CSD.

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Appendix I

Publications, Presentations, Workshops, and Awards

List of Publications

1. Suresh K, Raavi V, Harendra Kumar ML, Shankar V. Effect of chronic sleep deprivation and sleep recovery on hippocampal CA3 neurons, spatial memory and anxiety-like behavior in rats. ***Neurobiology of Learning and Memory***. 2022 Jan 1; 187:107559. **(Impact Factor: 3.1).**

2. Suresh K, Raavi V, Harendra Kumar ML, Shankar V. Impact of chronic sleep deprivation and sleep recovery on hippocampal oligodendrocytes, anxiety-like behavior, spatial learning and memory of rats. ***Brain Research Bulletin*** **(Under Review).**

3. Suresh K, Shankar V, Dayanand CD. Impact of REM sleep deprivation and sleep recovery on circulatory neuroinflammatory markers. ***Sleep Science***. 2021 Jan;14(1):64.

List of Presentations

1. Poster: Efficiency of sleep recovery on chronic sleep deprivation-induced oxidative stress in hippocampus and spatial memory of rats. ***XXXVIII Indian Academy of Neuroscience.*** *University of Hyderabad and NIPER,* October 4-7th, 2020, Hyderabad, India.

2. Poster: Effect of acute sleep deprivation on circulatory neuroinflammatory markers and efficacy of sleep recovery. ***XXXVIII Indian Academy of Neuroscience.*** *AIIMS,* November 18-21th, 2019, Ansari Nagar, New Delhi, India.

3. Oral: Effect of chronic sleep restriction on behavior and spatial memory in rats and efficacy of Sleep Recovery. ***IBRO/APRC,*** *Neuroscience School, Department of Life Science, Banaras Hindu University*, September 1-14th, 2019, Varanasi, India.

4. Poster: Efficacy of Sleep Recovery on Acute Sleep Deprivation Induced Cognitive and Behavior Alterations **3rd IBRO/APRC,** ***Chandigarh Associated School of Neuroscience***, *Punjab University*, 21-28th, October 2018, Chandigarh, India.

5. Poster: Efficacy of Sleep Recovery on Acute Sleep Deprivation Induced Cognitive and Behavior Alterations. ***India Sleep Conference***, October 2018, Goa, India.

Workshops Attended

1. IBRO/APRC neuroscience school on the molecular basis of neuroinflammation mediated neurodegeneration. Banaras Hindu University, Department of Life Science, Varanasi, September 1-14th, 2019.

2. 3rd IBRO/APRC Chandigarh associated school of Neuroscience, Punjab University, Chandigarh, October 21-28th, 2018.

3. Ad-hoc training course on Laboratory Animal Science and Techniques at National Centre for Laboratory Animal Sciences, NIN, Hyderabad. December 2017.

List of Awards

1. IBRO-international travel grant to participate in 52nd Annual Meeting of American Society for Neurochemistry, Virginia, USA, between April 10-14th, 2022.

2. IBRO-international travel grant, to participate in 40th Annual Scientific Meeting of Australasian Neuroscience Society's, Melbourne, Australia, between December 6-8th, 2021.

List of Events Organized

1. World Sleep Day-2022 in the Department of Physiology, SDUMC, SDUAHER, Tamaka, Kolar.

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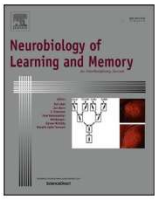

Effect of chronic sleep deprivation and sleep recovery on hippocampal CA3 neurons, spatial memory and anxiety-like behavior in rats

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ABSTRACT

Sleep deprivation-induced degenerative changes in the brain lead to the impairment of memory, anxiety, and quality of life. Several studies have reported the effects of sleep deprivation on CA1 and dentate gyrus regions of the hippocampus; in contrast, there is less known about the impact of chronic sleep deprivation (CSD) and sleep recovery on CA3 neurons and behavior. Hence, the present study aimed to understand the effect of CSD and sleep recovery on hippocampal CA3 neurons and spatial memory, and anxiety-like behavior in rats. Sixty male rats (Sprague Dawley) were grouped as control, environmental control (EC), CSD, 5 days sleep recovery (CSD + 5D SR), and 21 days sleep recovery (CSD + 21D SR). CSD, CSD + 5D SR and, CSD + 21D SR group rats were sleep deprived for 21 days (18 h/day). After CSD, the CSD + 5D SR and CSD + 21D SR rats were sleep recovered for 5- and 21-days respectively. Oxidative stress, dendritic arborization of CA3 neurons, spatial memory, and anxiety-like behavior was assessed. Spatial memory, basal, and apical dendritic branching points/intersections in hippocampal CA3 neurons were reduced, and anxiety-like behavior and oxidative stress increased significantly in the CSD group compared to control ($p < 0.001$). The CSD + 21D SR showed a significant improvement in spatial memory, reduction in anxiety-like behavior, and oxidative stress when compared to the CSD group ($p < 0.05$). The basal and apical dendritic branching points/intersections in hippocampal CA3 neurons were increased after CSD + 21D SR, however, it was not significant ($p > 0.05$). Even though the CSD + 21D SR showed a significant improvement in all the parameters, it did not reach the control level. There was an improvement in all the parameters after CSD + 5D SR but this was not significant compared to the CSD group ($p > 0.05$). Overall results indicate that the CSD-induced impairment of spatial memory and anxiety-like behavior was associated with oxidative stress and reduced dendritic arborization of hippocampal CA3 neurons. The CSD + 21D SR significantly reduced the damage caused by CSD, but it was not sufficient to reach the control level.

1. Introduction

Sleep loss is an important factor that affects the quality of life by degrading the health and productivity of human beings in the modern-day world (Pilcher & Morris, 2020). Depending on the duration of sleeplessness, it can be categorized as Acute or Chronic Sleep Deprivation (CSD). Continuous sleep deprivation for several hours to a week can be defined as acute sleep deprivation; whereas, sleep deprivation for few hours (typically 3–8 h/day) daily in 24 h sleep-wake cycle for several weeks or months can be defined as CSD (Alzoubi et al., 2016)). A recent study over 9 years (2010–2018) in the working adult American population found that one-third of the adult population experienced short sleep duration (<7 h) (Khubchandani & Price, 2020). In addition to work and lifestyle factors, certain health conditions such as chronic

Abbreviations: CSD, Chronic Sleep Deprivation; EC, Environmental Control; CSD+5D SR, 5 Days Sleep Recovery; CSD+21D SR, 21 Days Sleep Recovery; OA, Open Arm; CZ, Concentric Zone; CPCSEA, Committee for the Purpose of Control and Supervision of Experiments on Animals.

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pain, restless leg syndrome, and obstructive sleep apnea also contribute to sleep loss (Ong & Crawford, 2013). Insufficient sleep for the long term is associated with detrimental effects on the physiology of the nervous system such as cognition, vigilance, attention, learning, and memory, as well as performing complex real-world tasks and alterations in behavior (Banks & Dinges, 2007). Long-term chronic sleep loss or impaired neural functions leads to depression, and neurodegenerative diseases such as Parkinson's disease, and Alzheimer's disease, etc., (Abbott & Videnovic, 2016). In addition to neurological disorders, health complications such as diabetes, obesity, hypertension, cardiovascular diseases, etc., have also been reported to be increased due to sleep loss (Medic, Wille, & Hemels, 2017). A recent meta-analysis on the impact of sleep loss on the risk of diabetes highlights that one-hour sleep loss compared to 7 h/day of sleep results in an increase in 9% risk of type 2 diabetes mellitus (Shan et al., 2015). A similar meta-analysis of published articles on sleep loss and the risk of cardiovascular disease showed that <5 h sleep/day increased the risk of coronary heart disease (relative risk of 1.48) and stroke (relative risk of 1.15) compared to 7–8 h of sleep (Cappuccio, Cooper, D'Elia, Strazzullo, & Miller, 2011).

Key aspects of learning and memory functions are regulated in the hippocampus and are dependent on sleep (Hobson & Pace-Schott, 2002). Sleep likely modulates the synaptic connections in the hippocampus, which plays an important role in learning and memory (Yang et al., 2014). It has been described that the CSD-induced oxidative stress in the hippocampus might result in the damage of neurons and impairment of cognition and behavior (Alkadhi, Zagaar, Alhaider, Salim, & Aleisa, 2013; Salim, 2017). The effect of acute or chronic sleep deprivation on the memory of rats can be analyzed using behavioral and cognitive tasks such as a radial arm maze, novel objective recognition, and a morris water maze (Colavito et al., 2013). The impairment of spatial memory in mice was observed after 24- and 48-hours of sleep deprivation (Cao et al., 2019). Both short- and long-term spatial memory of rats were impaired after 6 weeks (8 h/day) of chronic Rapid Eye Movement (REM) sleep-deprivation and it was associated with increased oxidative stress (Alzoubi, Khabour, Albawaana, Alhashimi, & Athamneh, 2016; Alzoubi, Khabour, Rashid, Damaj, & Salah, 2012). Further, studies have also shown that both acute and chronic sleep deprivation increased anxiety levels, assessed using an elevated plus maze and open field test (Pires, Bezerra, Tufik, & Andersen, 2016). The anxiety-like behavior or memory impairment was more prominent when there was an increased duration (from 3 to 7 days) of sleep deprivation (Yin, Chen, Zheng, Pu, Marshall, and Wu et al., 2017). The anxiety-like behavior of rats assessed using an open field maze showed higher anxiety levels after 24 h of paradoxical sleep deprivation when compared to control (Xie et al., 2018). The anxiety-like behavior of rats was also shown to be increased after 21 days (18 h/days) of CSD (da Silva Rocha-Lopes, Machado, & Suchecki, 2018). Similar to animal model experiments, the impairment of working memory was also observed in healthy human beings after 36 h of sleep deprivation (Peng et al., 2020). The sleep loss-induced changes in the hippocampus and the behavior are often correlated with oxidative stress (Salim, 2017; Yin et al., 2017). However, the duration of sleep recovery required to improve spatial memory and anxiety-like behavior was not fully evaluated.

Principally, the CA3 region of the hippocampus is responsible for spatial and episodic memory because it has richer internal connectivity than the other subregions (Cherubini & Miles, 2015). Studies have demonstrated that oxidative stress in the hippocampus can negatively affect learning and memory by changing the structure of dendrites (Huang, Leu, & Zou, 2015). Sleep deprivation reduces synaptic plasticity and impairs memory consolidation (Long term potentiation) in the hippocampus, which is a common cellular correlate of learning and memory functions (Prince & Abel, 2013). Süer et al. (2011) reported that 21 days of CSD impaired long-term potentiation in the hippocampus of rats (Süer et al., 2011). A decreased dendritic spine number was observed in the CA1 and Dentate Gyrus regions (DG) of the hippocampus after 5 h of sleep deprivation in mice (Havekes, Park, Tudor, Luczak, Hansen, and Ferri et al., 2016; Raven, Meerlo, Van der Zee, Abel, & Havekes, 2019). In addition, 21 days of CSD decreases the dendritic length and spine density in the hippocampal CA1 region of rats (Noorafshan, Karimi, Kamali, Karbalay-Doust, & Nami, 2018). Chen et al. (2009) observed that both apical and basal dendritic spines of both CA1 and CA3 neurons were reduced due to fatigue induced by 5 days of sleep deprivation of rats (Chen et al., 2009). The length of dendrites, branching points, and spine density of hippocampal CA1 and CA3 neurons were decreased after continuous REM sleep deprivation of rats for 6 days (Giri, Ranjan, Kumar, Amar, & Mallick, 2021). However, little is known about the extent of 21 days of CSD-induced changes in dendritic arborization of hippocampal CA3 neurons and the duration of sleep recovery was not fully understood.

In a healthy cell, approximately 1% of oxygen will be converted into free radicals (superoxide radical and hydroxyl radical) and non-radical molecules (hydrogen peroxide) in the process of oxidative phosphorylation (Liou & Storz, 2010). The free radicals and non-radical molecules can be eliminated by superoxide dismutase, catalase, glutathione peroxidase enzymes, and antioxidants scavenge systems consisting of vitamin C, vitamin E, carotenoids, and glutathione (Phaniendra, Jestadi, & Periyasamy, 2015). The imbalance between free radicals (increase) and antioxidants (decrease) results in oxidative stress (Hill et al., 2018). There is concrete evidence that CSD can increase oxidative stress in the Central Nervous System (CNS) (Villafuerte et al., 2015). The cells in the CNS are more susceptible to the damage caused by free radicals due to the presence of unsaturated bonds in lipids and greater consumption of oxygen (Cobley, Fiorello, & Bailey, 2018). In addition, it also contains free radical generating substances such as ascorbate, iron, and glutamate which are involved in redox reactions (Walton et al., 2012). Therefore, the CSD-induced oxidative stress in CNS leads to potential damage to neurons and neuron supporting cells, which in turn results in neurodegeneration (Owen & Veasey, 2020; Salim, 2017). However, there is no clear consensus on the duration of the sleep recovery required to reverse the damaging effects caused by CSD.

The free radical flux hypothesis proposed that the core function of sleep is to serve as an antioxidant system for the brain. Therefore, sleep can reduce the free radicals produced during a wakeful state (Reimund, 1994). Studies also demonstrated that acute sleep deprivation-induced oxidative stress in the brain can be reversible with sleep recovery (Mathangi, Shyamala, & Subhashini, 2012). In contrast to acute sleep deprivation, the CSD-induced behavioral and morphological alterations and the duration of sleep recovery required to reverse the effects caused by CSD have not been thoroughly investigated. Hence, the present study was aimed to investigate the effect of 21 days (18 h/day) of CSD and 5- and 21-days of sleep recovery on spatial memory, anxiety-like behavior, dendritic arborization of hippocampal CA3 neurons, and oxidative stress in rats.

2. Materials and methods

2.1. Experimental setup

The study was approved (Ref No: IAEC/PHARMA/SDUMC/2017-18/05a) by the Institute Animal Ethics Committee, Sri Devaraj Urs Medical College, Kolar, Karnataka, India. Sixty male rats (Sprague Dawley, n = 60) aged between 12–16 weeks and a body weight of 170–200 g were procured from Biogen Laboratory Animal Facility, Bangalore, India (CPCSEA Registration No. 971/bc/06). All the rats were acclimatized to the laboratory for 7 days before the start of the study and divided into two sets (n = 30/set). The rats were maintained at 12:12 h light and dark cycle (lights on at 6.00 am and lights off at 6.00 pm) with a room temperature of 22 ± 2 °C by providing ad libitum of food and water. The overall study design of the present study is shown in Fig. 1.

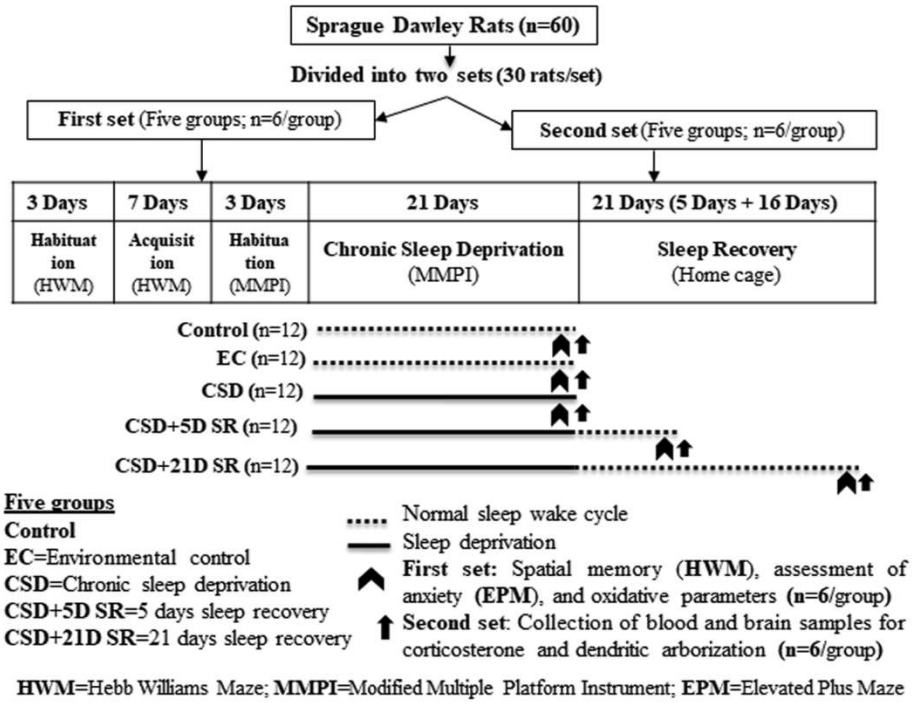

Fig. 1. Overview of the experimental methodology followed in the present study.

2.2. Sleep deprivation and sleep recovery of rats

The rats were grouped as control, environmental control (EC), chronic sleep deprivation (CSD), CSD + 5 days sleep recovery (CSD + 5D SR), and CSD + 21 days sleep recovery (CSD + 21D SR). The rats were sleep deprived using the Modified Multiple Platform Method (MMPM) as described in our previous study (Suresh & Shankar, 2021). Briefly, the Modified Multiple Platform Instrument (MMPI) has 12 platforms (110 × 44 × 45 cm), each platform has a 5.5 cm diameter and 6.0 cm height. All platforms were surrounded by water up to 1 cm beneath the platform. In this setup, whenever the rat tries to sleep, it will lose muscle tone and falls into the water, and climbs back to the platform. The rats were habituated to MMPI for 3 days (one hour/day) before subjecting to sleep deprivation. The CSD, CSD + 5D SR, and CSD + 21D SR groups rats were sleep deprived for 21 days (18 h/day: 2.00 pm-8.00 am in MMPI and 6 h/day: 8.00 am-2.00 pm in a home cage) at 12:12 h light and dark cycle (lights on at 6.00 am and lights off at 6.00 pm) by supplying ad libitum of food and water. The water in the instrument was changed daily throughout the CSD period. Furthermore, the CSD + 5D SR and CSD + 21D SR group rats were sleep recovered for 5- and 21-days in home cages kept at 12:12 h light and dark cycle (lights on at 6.00 am and lights off at 6.00 pm) by supplying ad libitum of food and water.

Control group rats were placed in their home cages without interruption of sleep. The environmental control group rats were kept in MMPI with stainless steel grid (2.3 mm pore size) placed 1 cm above the water levels (18 h/day: 2.00 pm-8.00 am and 6 h/day: 8.00 am-2.00 pm in a home cage). In this setup, the animals can sleep on a stainless-steel grid without falling into the water. The time of lights on was considered as zeitgeber time (ZT) 0. At the end of 21 days of sleep deprivation/5- and 21-days of sleep recovery, the first set of rats (five groups; n = 6/group) were used to assess the behavioral changes and oxidative markers. The second set of rats (five groups; n = 6/group) were used to measure the corticosterone and dendritic arborization (Fig. 1).

2.3. Assessment of spatial memory of rats

The rats were assessed for spatial memory as described in Hebb & Williams, 1946. The Hebb Williams Maze (HWM) consists of a similar-sized start and goal box, which are located on opposite corners of the maze. The time taken by the rats to navigate from the start box to the goal box in a maze is called 'latency time'. The experiment consists of three phases: habituation, acquisition, and testing. The first set of rats were habituated to HWM mage for 3 days (10 min/day) without internal walls in the maze. After habituation, the rats were exposed to four simple maze patterns for 7 days (acquisition phase: 4 trials/day) to attain the performance criteria (the rats need to trace any two maze patterns in < 25 sec). After obtaining the performance criteria, the rats were subjected to 21 days of sleep deprivation and 5- and 21-days of sleep recovery (Fig. 1). At the end of sleep deprivation/sleep recovery, the rats were tested for spatial memory by exposing them to any two maze patterns and the latency time (sec) was recorded from all the groups. The mean latency time of both the acquisition phase and testing phase from all the groups was recorded. All the spatial learning and memory experiments were performed between ZT 4-ZT 5.5.

2.4. Assessment of anxiety-like behavior of rats

Elevated Plus Maze (EPM) was used to measure the anxiety-like behavior of rats as described in Pellow, Chopin, File, & Briley, 1985. EPM comprised of two closed arms with high walls 50 × 10 × 40 cm (L × W × H) and two open arms 50 × 10 cm (L × W). The arms were connected with a central square (10 × 10 cm) to give the apparatus a plus sign appearance. The maze arms were elevated 60 cm above the floor. At the end of sleep deprivation/sleep recovery, the rats were assessed for spatial memory. Further, the same set of rats were used to assess the levels of anxiety. All group rats were gently placed in the central square facing towards the open arm. The rat was freely allowed to explore the maze for 5 min and a video was recorded. The time spent in the closed arm, open arm (OA), the number of entries into the open arm, and the total number of crossings were collected from the recorded video. The anxiety index was calculated as described earlier (Subhadeep, Srikumar, Shankaranarayana Rao, & Kutty, 2020). The anxiety index ranges between 0 and 100%. The experiments to measure anxiety was conducted between ZT 6-ZT 7.

$$\text{Anxiety Index}\,\% = 1 - \left[\frac{\left[\left(\frac{\text{Time spent in OAs}}{\text{Total time of test}}\right) + \left(\frac{\text{Entries in OAs}}{\text{Total entries}}\right)\right]}{2}\right] \times 100$$

2.5. Collection of blood, brain/hippocampal samples

At the end sleep deprivation/sleep recovery and behavioral assessment, all the rats (from both sets) were euthanized by injecting a combination of ketamine (92 mg/kg body weight) and xylazine (9.2 mg/kg body weight) intraperitoneally (ZT 7-ZT 9). Brain samples were collected from the first set of rats and used to measure the levels of oxidative stress markers. Blood and brain samples were collected from the second set of rats to quantifying the serum corticosterone and dendritic arborization of hippocampal CA3 neurons (Fig. 1).

2.6. Measurement of dendritic arborization of hippocampal CA3 neurons

The brain samples were processed to measure the dendritic arborization as described in Shankaranarayana Rao and Raju (2004). Briefly, the brain samples were washed with distilled water and stained with a Golgi-Cox stain (5% $K_2Cr_2O_7$, 5% $HgCl_2$, and 5% K_2CrO_4) in a dark chamber for 9 days. Approximately 160 µm thickness sections were taken from the dorsal and ventral hippocampus using a vibratome microtome (Leica VT 1000S, Germany) on charged slides. The slides were transferred to 8% Na_2CO_3 for one hour and dehydrated in ascending concentrations of ethanol (70, 90, and 100%), and incubated overnight in cedarwood oil. The slides were cleaned using xylene and mounted with DPX. The dendritic arborization of CA3 neurons was analyzed (8–10 neurons/rat: 50 neurons/group) using light microscopy equipped with camera Lucida in 200X magnification (Olympus, Japan). Dendritic branching points and dendritic intersections were quantified using a concentric circle method as described earlier (Sholl, 1953). Briefly, adjacent concentric circles were drawn on a transparent sheet with a distance of 20 µm each which were calibrated according to the 200X magnification. The dendrite branching points (basal and apical) and intersections were counted in each given concentric circle up to the radius of 100 µm from the center of the cell body of the CA3 neurons.

2.7. Measurement of oxidative stress markers in hippocampal tissue

Hippocampal tissues were homogenized using Phosphate Buffer Saline (PBS) with a ratio of 1:10 (W/V). The homogenate was centrifuged using 10,000 rpm for 20 min at 4 °C, the supernatants were collected, aliquoted, and stored at −20 °C until further use. Total protein concentration was measured in the supernatant using the Bradford method (Bradford, 1976). Further, the supernatants were used to measure the levels of Malondialdehyde (MDA), total glutathione, oxidized and reduced glutathione ratio (GSSG/GSH ratio), and Total Antioxidant Capacity (TAC).

2.7.1. Measurement of MDA

Thiobarbituric acid reactive substances assay method was used to assess the levels of MDA as described earlier (Wills, 1966). Briefly, the homogenate samples were deproteinized by adding 24% tri-chloroacetic acid. To the deproteinated sample, 0.8% thiobarbituric acid was added and incubated at 90 °C in a water bath. After one hour, the color intensity was measured at 530 nm using a spectrophotometer (Lambda 35, USA). The tetramethoxypropane was used as standard. The concentration of MDA was calculated from the standard curve and expressed as µmol/mg protein.

2.7.2. Measurement of total glutathione

Total glutathione was measured using a glutathione assay kit (Cayman Chemicals, USA). Briefly, the homogenate was deproteinated with an equal volume of diluted metaphosphoric acid (Sigma Aldrich, USA). Total glutathione was measured by adding about 50 µl of deproteinated sample and 150 µl of assay cocktail (reconstituted GSH MES buffer, GSH Co-factor mixture, GSH enzyme mixture, and GSH DTNB) in a microtiter plate and incubated in an orbital shaker for 25 min in dark (exclusively to measure the oxidized glutathione (GSSG), additionally added 10 µl of 1 M 2 vinylpyridine to 1 ml of homogenized sample). The intensity of the color was measured at 405 nm in a microtiter plate reader (Rayto, China). The total glutathione and concentrations of GSSG were calculated using a standard curve and expressed as µmol/mg protein. Reduced glutathione (GSH) values were obtained by subtracting the GSSG from total glutathione and the ratio of GSSG/GSH was calculated.

2.7.3. Measurement of total antioxidant capacity

Total antioxidant capacity was measured by using a total antioxidant capacity assay kit (ImmunoTag, USA). As per the manufacturer's instructions, 30 µl of homogenate was added to 900 µl of solution mixture and incubated for 10 min. The color intensity was measured at 593 nm using a spectrophotometer (Lambda 35, USA). The levels of TAC were calculated from the standard curve and expressed as units/mg protein.

2.8. Measurement of the levels of corticosterone

About 2 ml of blood was collected through cardiac puncture from the anesthetized rats. Blood samples were collected at a fixed time (ZT 4-ZT 5) from the second set of rats. The blood samples were allowed to coagulate for 30 min and the serum was collected by centrifuge at 3000 rpm for 20 min at room temperature. The levels of corticosterone were measured using an ELISA kit (Kinesis Dx, USA). Briefly, 40 µl of undiluted samples, 10 µl of biotinylated rat corticosterone antibodies, and 50 µl of streptavidin-horseradish peroxidase were added to anti-rat corticosterone antibodies coated microplate wells and incubated at 37 °C for one hour. After incubation, the solution was discarded and the wells were washed four times using 1X wash buffer. To the wells, 50 µl of 3,3′, 5,5′ tetramethylbenzidine substrate A and B was added and incubated for 10 min. The reaction was stopped by adding 50 µl of stop solution. The optical density of yellow color was measured at 450 nm using an ELISA reader (Rayto, China), and the level of serum corticosterone was calculated from the standard curve and expressed as ng/ml.

2.9. Statistical analysis

The experimental data were processed using Microsoft Excel software (Microsoft Corporation, USA). All the data were represented as percentage/mean ± standard error of the mean (SEM). Student 't' test was used to find the significant difference in the latency time obtained from the acquisition and testing phase in the HWM maze. One-way analysis of variance with Bonferroni's posthoc was used to find the significant levels of the variable between control, EC, CSD, CSD + 5D SR, and CSD + 21D SR groups. The p-value < 0.05 was considered statistically significant. All the statistical analysis was performed using SPSS software, version 20 (IBM, USA), and the representative graphs were prepared using GraphPad Prism software (GraphPad Software, USA).

3. Results

3.1. Effect of CSD, CSD + 5D SR, and CSD + 21D SR on spatial memory of rats

The CSD-induced changes in the brain/hippocampus might result in impaired spatial memory. We assessed the impact of 21 days of CSD, 5- and 21-days of sleep recovery on spatial memory of rats using the HWM task. The latency time (sec) in the HWM task was recorded in both acquisition and testing phases. During the acquisition phase, all the rats from different groups learned to reach the reward box. The mean latency time (sec) obtained from all the groups in the acquisition phase of days

1–7 is shown in Fig. 2A. The mean latency time of the acquisition phase is 22 ± 1.2, 22 ± 1.0, 21 ± 0.7, 21 ± 0.8, 21 ± 0.8 sec, and the testing phase is 22 ± 0.8, 23 ± 1.0, 52 ± 3.9, 41 ± 3.4, 30 ± 2.5 sec for control, EC, CSD, CSD + 5D SR, and CSD + 21D SR groups respectively. The mean latency time during the acquisition phase did not vary among the groups ($p > 0.05$). However, the latency time during the testing phase is significantly higher in the CSD group compared to the control and EC group ($p < 0.001$). The mean latency time in the testing phase is significantly reduced in both CSD + 5D SR ($p < 0.0001$) and CSD + 21D SR ($p < 0.0001$) groups compared to CSD, however, it was not sufficient to reach control and EC groups ($p < 0.01$). The mean latency time obtained from the testing and acquisition phase did not differ in both control and EC groups ($p > 0.05$). However, the mean latency time in the testing phase is significantly higher in CSD ($p < 0.001$), CSD + 5D SR ($p < 0.001$), and CSD + 21D SR ($p < 0.01$) groups compared to the acquisition phase (Fig. 2B).

3.2. Effect of CSD, CSD + 5D SR, and CSD + 21D SR on anxiety-like behavior of rats

It is a well-known fact that CSD increases anxiety-like behavior. The extent of anxiety-like behavior after 21 days CSD and 5- and 21-days sleep recovery was assessed using the EPM task. The percent of the time (sec) spent in both closed arm and open arm, open arm entries, and total arm entries were recorded and calculated the anxiety index. The percent of time spent in the closed arm, open arm, open arm entries, and the number of total arm entries did not show any significant difference between control and EC groups ($p > 0.05$). The percent of time spent in the closed arm was significantly increased in the CSD group compared to the control and EC groups ($p < 0.001$). The time spent in open arm ($p < 0.001$), open arm entries ($p < 0.001$), and total arm entries ($p < 0.01$) were significantly reduced in the CSD group compared to control and EC groups. The percent of time spent in the closed arm was reduced in CSD + 5D SR and CSD + 21D SR groups compared to the CSD group but was not significant ($p > 0.05$). The time spent on the open arm and open arm entries were increased non significantly ($p > 0.05$) in the CSD + 5D SR group and significantly in the CSD + 21D SR group ($p < 0.05$) compared to the CSD group. The total arm entries were increased in CSD + 5D SR and CSD + 21D SR groups but it is not statistically significant ($p > 0.05$) compared to the CSD group. Even though there is a reduction in time spent on the closed arm and increased time spent on the open arm, open arm entries, and total arm entries after CSD + 5D SR and CSD + 21D SR groups, it did not reach the control group levels (Fig. 3A, B, C, and D).

The anxiety-like behavior did not show any significant difference between the control and EC groups ($p > 0.05$). The anxiety-like behavior was significantly higher in the CSD group compared to the control and EC groups ($p < 0.001$). The anxiety-like behavior was reduced in the CSD + 5D SR group ($p > 0.05$), whereas, the CSD + 21D SR group showed a significant ($p < 0.01$) reduction compared to the CSD group. Even though there is a reduction in the anxiety-like behavior in both CSD + 5D SR and CSD + 21D SR groups, it did not reach the control group levels. Fig. 3E shows the anxiety index obtained from different groups of rats.

3.3. Effect of CSD, CSD + 5D SR, and CSD + 21D SR on dendritic arborization of hippocampal CA3 neurons

Hippocampal CA3 neuronal dendrites receives the input signals from DG (mossy fibers), the Entorhinal cortex (perforant path), recurrent fibers of CA3, contralateral CA3, and septal area. The dendritic branching points and intersections of CA3 neurons plays an important role in synaptic connectivity, which is associated with the formation of memory. To find the effect of 21 days of CSD and 5- and 21-days of sleep recovery on dendritic arborization, we measured the basal and apical dendrite branching points and intersections of hippocampal CA3 neurons (8–10 neurons/rat; 50 neurons/group).

3.3.1. Basal dendritic branching points of hippocampal CA3 neurons

The representative images of CA3 neurons obtained from hippocampal tissue stained with Golgi-Cox stain and the method used to quantify the dendritic branching points and intersections are shown in Fig. 4. The mean basal branching points of hippocampal CA3 neurons obtained from 0–20, 20–40, 40–60, 60–80, and 80–100 μm concentric zones of control and EC groups did not differ significantly ($p > 0.05$). The mean basal branching points of hippocampal CA3 neurons obtained from the CSD group showed a significant decrease at different concentric zones of 0–20, 20–40, 60–80, 80–100 ($p < 0.001$), and 40–60 μm ($p < 0.01$) when compared to control and EC group. However, the basal dendritic branching points of hippocampal CA3 neurons were increased in CSD + 5D SR and CSD + 21D SR groups compared to the CSD group, but the increase was not statistically significant at all concentric zones ($p > 0.05$). Furthermore, the increased basal branching points obtained from all concentric zones of CSD + 5D SR and CSD + 21D SR did not reach the control or EC groups ($p < 0.05$). Fig. 5A shows the basal dendritic branching points of hippocampal CA3 neurons obtained from different groups of rats.

3.3.2. Apical dendritic branching points of hippocampal CA3 neurons

The mean apical branching points of hippocampal CA3 neurons obtained from 0–20, 20–40, 40–60, 60–80, and 80–100 μm concentric

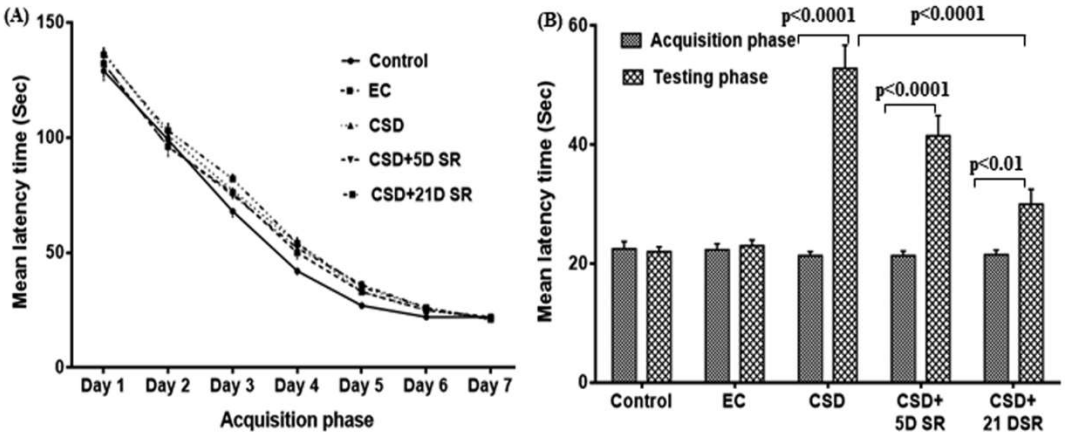

Fig. 2. Spatial learning performance of rats in HWM (n = 6/group): (A) The mean latency time (sec) obtained from control, EC, CSD, CSD + 5D SR, and CSD + 21D SR groups of rats during days 1–7 of the acquisition phase. (B) The comparison of mean latency time (sec) obtained during acquisition and testing phases in control, EC, CSD, CSD + 5D SR, and CSD + 21D SR groups of rats.

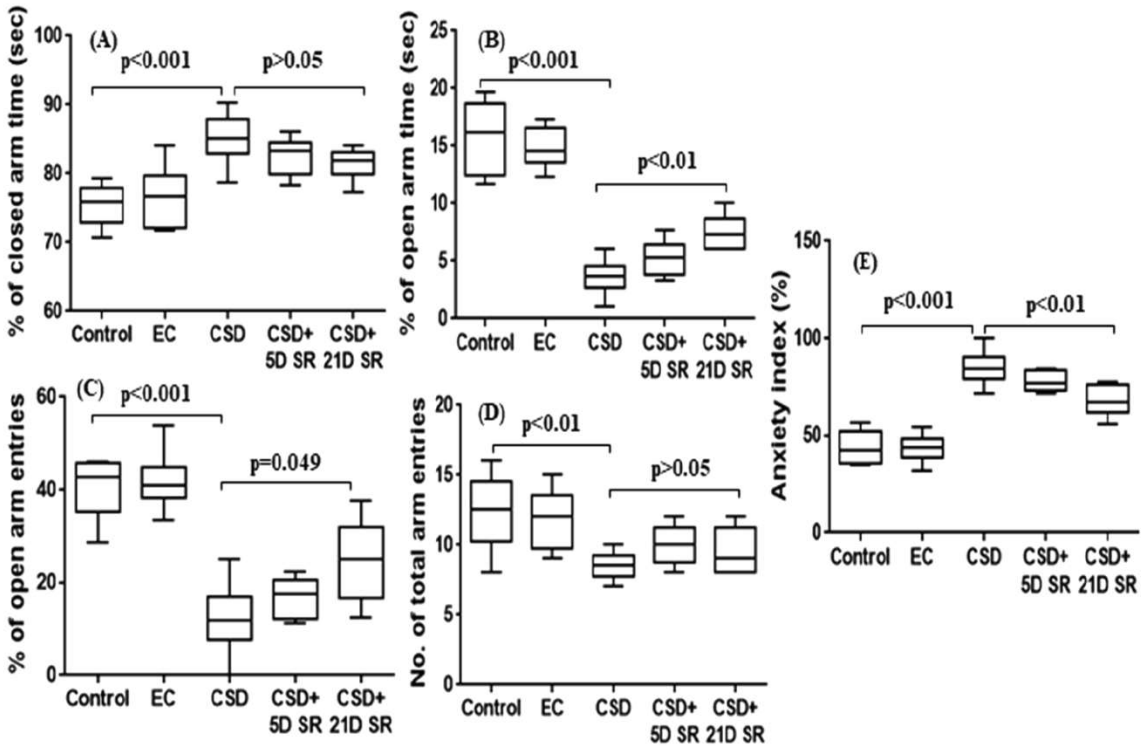

Fig. 3. Anxiety-like behavior of rats in EPM (n = 6/group): (A) Time (sec) spent in the closed arm, (B) Time (sec) spent in open arm, (C) Open arm entries, (D) Total arm entries, and (E) Anxiety index obtained from control, EC, CSD, CSD + 5D SR, and CSD + 21D SR groups of rats.

zones of control and EC groups did not differ significantly ($p > 0.05$). The mean apical branching points of hippocampal CA3 neurons obtained from the CSD group showed a significant decrease at concentric zones 60–80, 80–100 ($p < 0.01$), but non-significant at 0–20, 20–40, and 40–60 μm zones ($p > 0.05$) when compared to the control and EC groups. However, the apical dendritic branching points of hippocampal CA3 neurons obtained from CSD + 5D SR and CSD + 21D SR groups showed a slight increase but non-significant compared to the CSD group at all concentric zones ($p > 0.05$). The apical branching points obtained from concentric zones 60–80, 80–100 μm ($p < 0.05$) of CSD + 5D SR and CSD + 21D SR groups did not reach the control and EC groups. Fig. 5B shows the apical dendritic branching points of hippocampal CA3 neurons obtained from different groups of rats.

3.3.3. Basal dendritic intersections of hippocampal CA3 neurons

The mean basal dendritic intersections of CA3 neurons obtained from 0–20, 20–40, 40–60, 60–80, and 80–100 μm concentric zones of control and EC groups did not differ significantly ($p > 0.05$). The mean basal dendritic intersections of hippocampal CA3 neurons obtained from the CSD group showed a significant decrease at concentric zones of 20–40 ($p < 0.001$), 40–60, 60–80, 80–100, ($p < 0.01$), but non-significant at 0–20 μm zone ($p > 0.05$) when compared to the control and EC groups. However, the basal dendritic intersections of hippocampal CA3 neurons obtained from CSD + 5D SR and CSD + 21D SR groups showed a slight increase but non-significant compared to the CSD group at all concentric zones ($p > 0.05$). The basal dendritic intersections obtained from 20–40, 40–60, 60–80, and 80–100 μm concentric zones ($p < 0.05$) of CSD + 5D SR and CSD + 21D SR groups did not reach the control group. Fig. 6A shows the basal dendritic intersection of hippocampal CA3 neurons obtained from different groups of rats.

3.3.4. Apical dendritic intersections of hippocampal CA3 neurons

The mean apical dendritic intersections of hippocampal CA3 neurons obtained from 0–20, 20–40, 40–60, 60–80, and 80–100 μm concentric zones of control and EC groups did not differ significantly ($p > 0.05$). The mean apical dendritic intersections of hippocampal CA3 neurons obtained from the CSD group showed a significant decrease at concentric zones 60–80, 80–100 ($p < 0.001$) but non-significant at 0–20, 20–40, and 40–60 μm zones ($p > 0.05$) when compared to the control and EC groups. However, the apical dendritic intersections of hippocampal CA3 neurons obtained from concentric zones 60–80, 80–100 μm of CSD + 5D SR and CSD + 21D SR groups showed a slight increase but was non-significant compared to the CSD group ($p > 0.05$). The apical dendritic intersections obtained from concentric zones 60–80, 80–100 μm of CSD + 5D SR and CSD + 21D SR groups did not reach the control group ($p < 0.05$). Fig. 6B shows the apical dendritic intersection of hippocampal CA3 neurons obtained from different groups of rats.

3.4. Effect of CSD, CSD + 5D SR, and CSD + 21D SR on oxidative stress in the hippocampus of rats

It is well known that sleep loss increases the oxidative stress markers in the hippocampus. To find the extent of oxidative stress after 21 days of sleep deprivation, 5- and 21-days of sleep recovery, we have measured the levels of MDA, total glutathione, the ratio of GSSG/GSH, and total antioxidant capacity in the hippocampal tissue of rats.

3.4.1. Levels of MDA in CSD, CSD + 5D SR, and CSD + 21D SR groups of rats

The mean MDA obtained from control, EC, CSD, CSD + 5D SR, and CSD + 21D SR groups were 0.84 ± 0.17, 1.1 ± 0.17, 2.25 ± 0.19, 1.7 ± 0.14, and 1.4 ± 0.16 μmol/mg of protein respectively (Fig. 7A). The levels of MDA did not show any significant difference between the control and EC groups ($p > 0.05$). The levels of MDA were significantly higher in the CSD group compared to the control and EC groups ($p < 0.001$). Whereas, the levels of MDA in the CSD + 5D SR group reduced when compared to the CSD group but it was not significant ($p = 0.17$). However, the levels of MDA obtained from the CSD + 21D SR group showed a significant decrease when compared to the CSD group ($p < 0.01$). The MDA levels obtained from the CSD + 21D SR group were

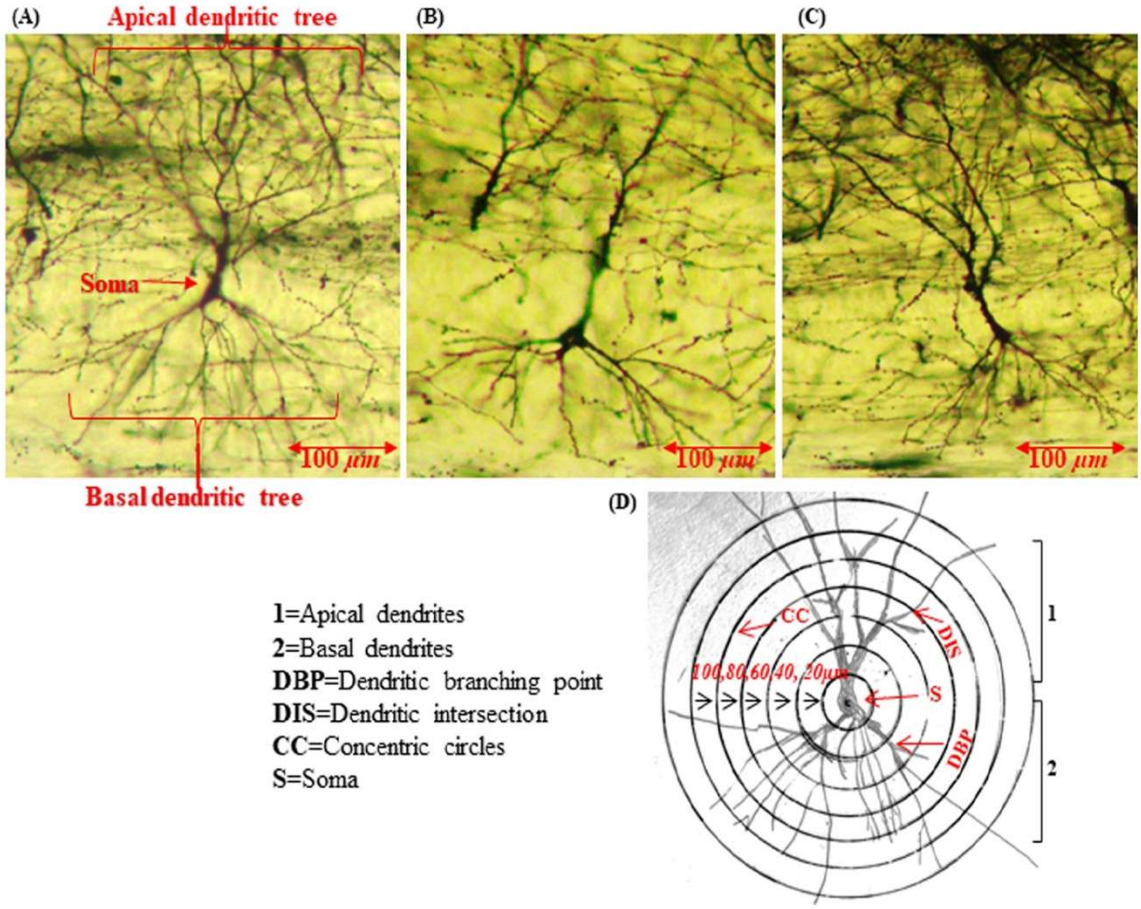

Fig. 4. Representative images of hippocampal CA3 neurons (basal and apical dendritic tree) obtained after Golgi cox stain (A) Control, (B) CSD, (C) CSD + 21D SR groups of rats, and (D) The method used to quantify the dendritic arborization of CA3 Neurons.

comparable with the control group ($p = 0.14$).

3.4.2. Levels of total glutathione in CSD, CSD + 5D SR, and CSD + 21D SR groups of rats

The mean total glutathione obtained from control, EC, CSD, CSD + 5D SR, and CSD + 21D SR groups were 1.24 ± 0.09, 1.22 ± 0.11, 0.51 ± 0.03, 0.66 ± 0.05, and 0.84 ± 0.04 µmol/mg of protein respectively (Fig. 7B). The levels of total glutathione did not show any significant difference between the control and EC groups. Total glutathione levels were decreased significantly in the CSD group compared to the control and EC groups ($p < 0.001$). Whereas the levels of total glutathione obtained from the CSD + 5D SR group showed a non-significant ($p > 0.05$) increase and CSD + 21D SR groups showed a significant increase ($p = 0.04$) compared to the CSD group. The levels of total glutathione obtained from CSD + 5D SR and CSD + 21D SR groups did not reach the levels of control and EC groups ($p < 0.01$).

3.4.3. The ratio of GSSG/GSH in CSD, CSD + 5D SR, and CSD + 21D SR groups of rats

The mean ratio of GSSG/GSH obtained from the control, EC, CSD, CSD + 5D SR, and CSD + 21D SR groups were 0.08 ± 0.01, 0.08 ± 0.01, 1.14 ± 0.21, 0.89 ± 0.15, and 0.60 ± 0.04 respectively (Fig. 7C). The ratio of GSSG/GSH did not show any significant difference between the control and EC groups ($p > 0.05$). The ratio of GSSG/GSH was significantly higher in the CSD group compared to the control and EC groups ($p < 0.001$). Whereas, the ratio of GSSG/GSH in the CSD + 5D SR group was reduced but not significant ($p > 0.05$) and CSD + 21D SR groups were significantly reduced ($p = 0.03$) compared to the CSD group. However, the ratio of GSSG/GSH obtained from CSD + 5D SR and CSD + 21D SR groups did not reach the levels of control and EC groups ($p < 0.01$).

3.4.4. Levels of TAC in CSD, CSD + 5D SR, and CSD + 21D SR groups of rats

The mean TAC levels obtained from control, EC, CSD, CSD + 5D SR, and CSD + 21D SR groups were 0.13 ± 0.01, 0.138 ± 0.01, 0.019 ± 0.006, 0.04 ± 0.006, and 0.06 ± 0.007 units/mg of protein respectively (Fig. 7D). The levels of TAC did not show any significant difference between the control and EC groups ($p > 0.05$). The levels of TAC were significantly decreased in the CSD group compared to the control and EC groups ($p < 0.001$). Whereas, the TAC levels obtained from the CSD + 5D SR group showed a non-significant increase ($p > 0.05$) and the CSD + 21D SR group showed a significant increase ($p = 0.02$) compared to the CSD group. However, the levels of TAC obtained from CSD + 5D SR and CSD + 21D SR groups did not reach the levels of control and EC groups ($p < 0.01$).

3.5. Effect of CSD, CSD + 5D SR, and CSD + 21D SR on the levels of serum corticosterone

At the end of 21 days of sleep deprivation and 5- and 21-days of sleep recovery, the serum cortisone levels were measured. The mean serum corticosterone levels obtained from control, EC, CSD, CSD + 5D SR, and CSD + 21D SR groups were 257 ± 20, 236 ± 10, 234 ± 8, 245 ± 27, and 247 ± 15 ng/ml respectively (Fig. 8). The levels of serum corticosterone did not show any significant difference among the groups ($p > 0.05$).

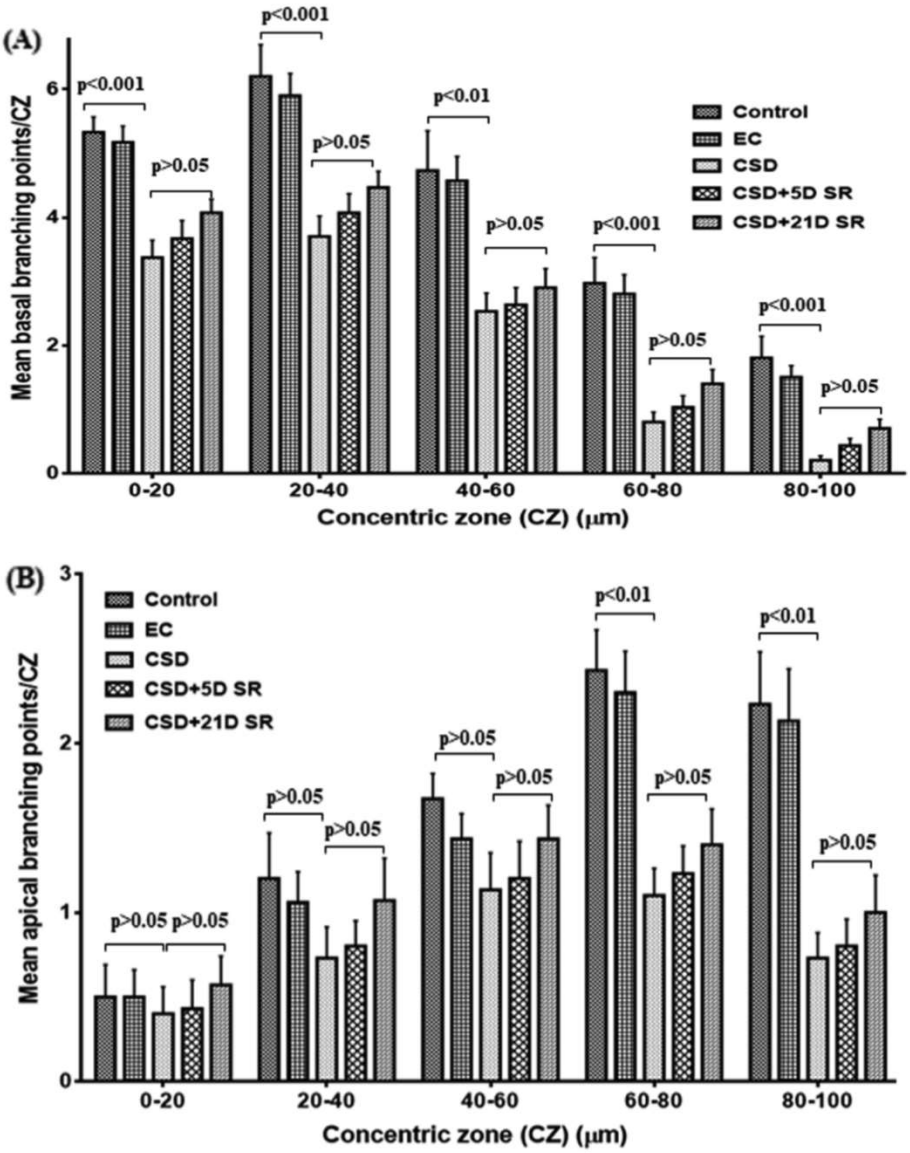

Fig. 5. Dendritic arborization of hippocampal CA3 neurons (8–10 neurons/rat; 50 neurons/group) obtained from control, EC, CSD, CSD + 5D SR, and, CSD + 21D SR groups of rats (n = 6/group): (A) Mean basal branching points of hippocampal CA3 neurons at different concentric zones (CZ) (0–20, 20–40, 40–60, 60–80, and 80–100 μm distance from the soma). (B) Mean apical branching points of hippocampal CA3 neurons at different concentric zones (CZ) (0–20, 20–40, 40–60, 60–80, and 80–100 μm distance from the soma).

4. Discussion

Sleep is an important physiological function, which is required for the quality of life in modern humans. Lack of required sleep, whether due to acute or chronic sleep deprivation, was reported to increase the risk of neurological disorders. Even though different durations of CSD-induced damage were evaluated, the duration of sleep recovery required to reverse the effects of CSD is not well established at the cellular or molecular level. Understanding the cellular or molecular level changes after both sleep deprivation and sleep recovery might help in better management of individuals suffering from sleep disorders. In the present study, we investigated the effect of 21 days of chronic REM sleep deprivation and 5- and 21-days of sleep recovery on spatial memory, anxiety-like behavior, dendritic arborization of hippocampal CA3 neurons, and oxidative stress in rats.

Twenty-one days (18 h/day) of CSD impaired the spatial memory (increased latency time in testing phase compared to acquisition phase) and increased anxiety-like behavior of rats. In line with behavioral changes, the oxidative parameters also indicate that CSD induced oxidative stress. The levels of MDA and ratio GSSG/GSH were increased, whereas the levels of glutathione and TAC levels were decreased in hippocampal tissue after 21 days of CSD, which indicates that the CSD induced oxidative stress in the hippocampus of the rats. Several studies have shown that acute/chronic sleep deprivation increases oxidative stress, which in turn leads to the impairment of spatial memory and anxiety-like behavior. Seventy-two hours of sleep deprivation results in increased oxidative stress in the hippocampus, which correlated with impaired memory in mice (Silva et al., 2004). Twenty-one days of CSD (18 h/day) leads to low-grade neuroinflammation (increased inflammatory cytokines) in the hippocampus, heightened anxiety-like behavior, and impaired memory in rats (Manchanda, Singh, Kaur, & Kaur, 2018). Eight weeks of CSD (8 h/day) leads to increased oxidative damage in the hippocampus and impaired short-term and long-term memory in rats (Alzoubi, Mayyas, & Abu Zamzam, 2019). Seven days of intermittent and paradoxical sleep deprivation also increased the anxiety-like behavior and impaired memory of mice when compared to 3 days (Yin et al., 2017). It is a well-known fact that the metabolism of glucose in the brain during sleep is 30% less compared to wakefulness, which indicates that extended wakefulness increases the metabolism that results in increased free radicals (Boyle et al., 1994). Sleep deprivation alters the sleep-wake cycle, the altered sleep-wake cycle leads to an imbalance in free radicals and antioxidant levels, which in turn lead to oxidative stress of the CNS specifically the hippocampus (Alkadhi et al., 2013). In line with the reported studies, the current study results

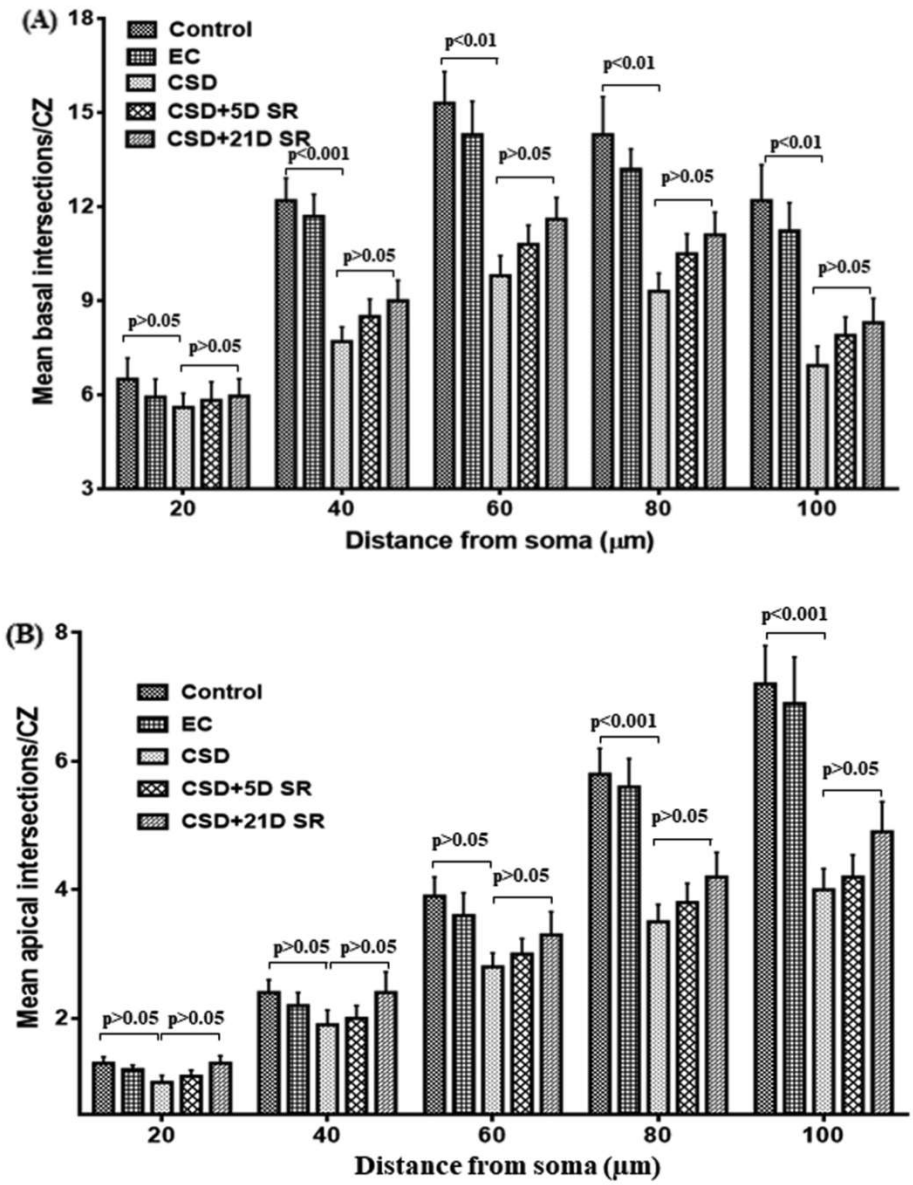

Fig. 6. Dendritic arborization of hippocampal CA3 neurons (8–10 neurons/rat; 50 neurons/group) obtained from control, EC, CSD, CSD + 5D SR, and CSD + 21D SR groups of rats (n = 6/group): (A) Mean basal dendritic intersections of hippocampal CA3 neurons at different concentric zones (CZ) (0–20, 20–40, 40–60, 60–80 and 80–100 μm distance from the soma). (B) Mean apical dendritic intersections of hippocampal CA3 neurons at different concentric zones (CZ) (0–20, 20–40, 40–60, 60–80, and 80–100 μm distance from the soma).

have also shown that 21 days (18 h/day) of CSD-induced oxidative stress in the hippocampus correlated with the impairment of spatial memory and anxiety-like behavior in rats.

Even though the extent of CSD-induced spatial memory and oxidative damage is well studied, the damage at the cellular level is not fully understood. In the present study, we investigated the extent of damage to hippocampal CA3 neurons after 21 days (18 h/day) of CSD. Our results show that the 21 days of CSD significantly reduced both basal and apical branching points and intersections (at different concentric zones) of hippocampal CA3 neurons. Studies have shown that the CSD-induced atrophy of neurons reduced the volume of the hippocampus in animal models (Owen & Veasey, 2020). Five hours of sleep deprivation reduced the spine density in CA1 neurons and dentate gyrus regions of the hippocampus in mice (Havekes et al., 2016; Raven et al., 2019). Twenty-one days (18 h/day) of CSD leads to the shortening and shedding of CA1 dendritic trees in rats (Noorafshan et al., 2018). In addition, 21 days (8 h/day) of CSD also impaired the maintenance of long-term potentiation in the dentate gyrus in rats (Süer et al., 2011). The hippocampal CA1 neurons are reported to be more susceptible to oxidative injury compared to the CA3 region. However, preliminary studies have reported that the CA3 region is also prone to oxidative damage (Salim, 2017). It is a known fact that the dentate gyrus and CA3 region exhibit structural plasticity with regenerative/remodeling capacity (Sousa, Lukoyanov, Madeira, Almeida, & Paula-Barbosa, 2000). *In vitro* studies have shown that both CA1 and CA3 neurons of the hippocampus were susceptible to the damage caused by free radicals (Wilde, Pringle, Wright, & Iannotti, 1997). One-day sleep deprivation also showed increased levels of interleukin-6 and Ionized calcium-binding adaptor protein-1 positive cells (activated microglia) in the CA3 region of the hippocampus of mice (Zhu et al., 2012). Twenty-eight days (20 h/day) of sleep deprivation reduced the volume of CA3 cellular sub-region in adolescent rats (Novati, Hulshof, Koolhaas, Lucassen, & Meerlo, 2011). In addition, the CA3/dentate gyrus volume also decreased in patients suffering from insomnia (Neylan et al., 2010). Further, a decreased neuronal density was shown to be more prominent in CA1 and CA3 regions of the hippocampus of Alzheimer's patients compared to controls (Padurariu, Ciobica, Mavroudis, Fotiou, & Baloyannis, 2012). In contrast, five hours of sleep deprivation did not alter the dendritic spine density in CA3 neurons in mice (Havekes et al., 2016) and twenty-one days of CSD also did not alter the number of CA3 neurons in rats (Noorafshan, Karimi, Kamali, Karbalay-Doust, & Nami, 2017). Similar to the reported studies, our results also indicated that 21 days (18 h/day) of

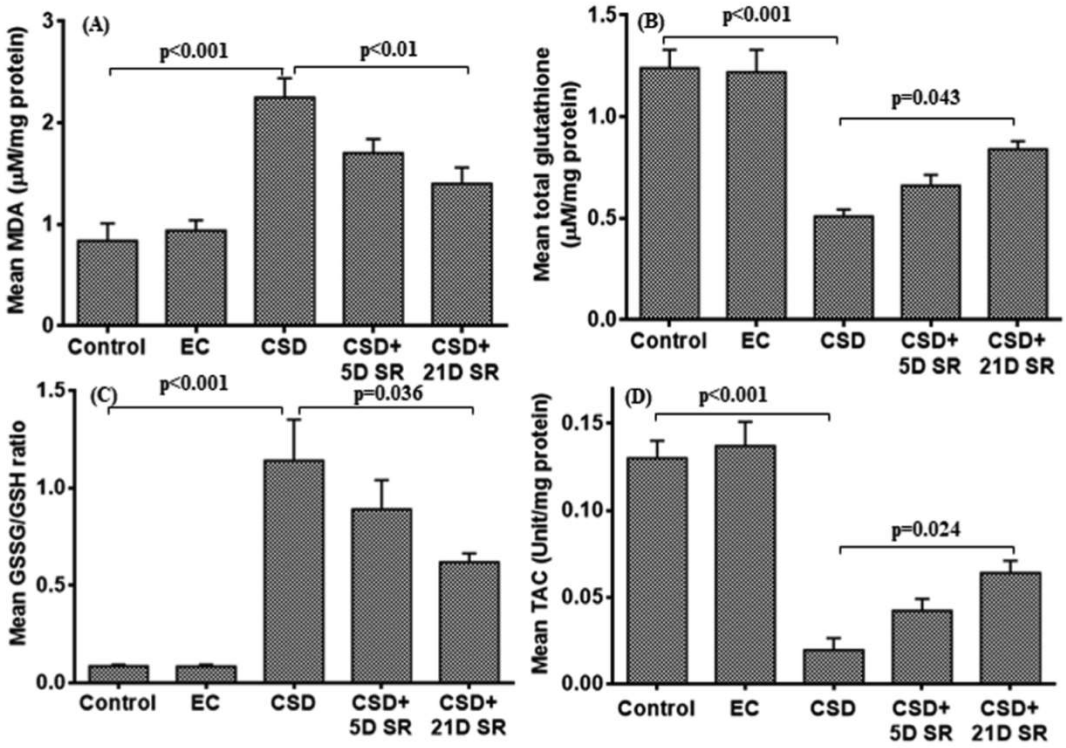

Fig. 7. Oxidative stress markers in the hippocampus of rats (n = 6/group): (A) The levels of MDA (µmol/mg of protein), (B) Total glutathione (µmol/mg of protein), (C) Ratio of GSSG/GSH, and (D) TAC (units/mg of protein) obtained from control, EC, CSD, CSD + 5D SR, and CSD + 21D SR groups of rats.

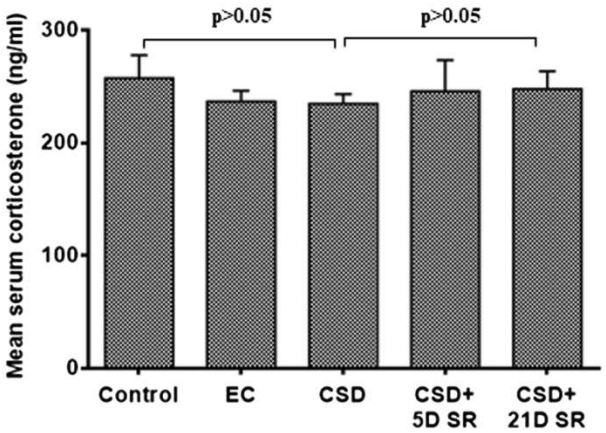

Fig. 8. The levels of serum corticosterone (ng/ml) obtained from control, EC, CSD, CSD + 5D SR, and CSD + 21D SR groups of rats (n = 6/group).

CSD reduced both basal and apical dendritic branching points or intersections in hippocampal CA3 neurons in rats. The reduction in dendritic branching points/intersection in the CA3 region of the hippocampus resulted in the impairment of spatial memory and increased anxiety-like behavior. Owen and Veasey (2020) stated that prolonged sleep deprivation ultimately results in irreversible injury/degeneration of neurons and impairment of memory in animal models (Owen & Veasey, 2020). Elevated corticosterone levels were known to cause the atrophy of CA3 neurons and dendritic arborization in rats. Recent reports suggest that 72 or 96 h of total/partial sleep deprivation increases plasma corticosterone levels in rats (Hipolide et al., 2006; Zager, Andersen, Ruiz, Antunes, & Tufik, 2007). In contrast to 72 or 96 h, 21 days of CSD did not show any changes in plasma corticosterone levels (Süer et al., 2011; Zager et al., 2007). In line with the reported studies, the 21 days of CSD in the present study did not alter the serum corticosterone levels. Therefore, the reduced apical/basal dendritic branching points/intersections of hippocampal CA3 neurons are not due to corticosterone. In the present study we used MMPM to sleep deprive the rats as this method along with the 'flowerpot technique' is being widely used to induce sleep deprivation in rodent models among the laboratories in the world (Machado, Hipólide, Benedito-Silva, & Tufik, 2004). It has been proven that the MMPM can cause complete REM sleep deprivation and partial non-REM sleep deprivation (Machado et al., 2004). Recent studies also highlighted that the continuous sleep deprivation for >24 h using MMPM results in complete REM sleep deprivation and least effect on non-REM sleep deprivation when compared to the environmental control (Giri et al., 2021; McDermott et al., 2003). Therefore, the observed changes (impaired spatial memory, anxiety-like behavior, reduced dendritic arborization of hippocampal CA3 neurons, and increased oxidative stress) in the present study (21 days of CSD) are majorly due to the REM sleep deprivation.

The results clearly indicate that the 21 days of CSD impaired spatial memory, increased anxiety-like behavior, caused dendritic arborization of hippocampal CA3 neurons and raised oxidative stress. Furthermore, we extended the study to investigate the efficacy of 5- and 21-days of sleep recovery on the damage caused by the 21 days of CSD. We observed that the 5 days of sleep recovery showed a slight improvement in spatial memory, decreased anxiety-like behavior, and reduced oxidative stress compared to 21 days of CSD which was not statistically significant. In contrast to 5 days of sleep recovery, 21 days of sleep recovery showed a significant improvement in spatial memory, decreased anxiety-like behavior, and reduced oxidative stress when compared to 21 days of CSD. However, it was not sufficient to completely recover from the damage caused by 21 days of CSD. Tung, Takase, Fornal, and Jacobs (2005) also demonstrated that the 8 h of sleep recovery exerted no restorative effect on the suppression of cell proliferation induced by 56 h sleep deprivation rats (Tung et al., 2005). Seventy-two hours of sleep deprivation-induced impairment of spatial memory and hippocampus neuronal apoptosis was not reversed even after 3 weeks of sleep recovery in mice (Soto-Rodriguez et al., 2016). Ninety days of repeated

and intermittent paradoxical sleep deprivation-induced impaired memory and hippocampal inflammation also persisted even after 3 weeks of sleep recovery in mice (Yin et al., 2017). Furthermore, the cortisol levels were not affected by sleep recovery after prolonged wakefulness in humans (Brun et al., 1998; Moldofsky, Lue, Davidson, & Gorczynski, 1989). In line with the reported studies, our study results also suggest that the 5- and 21-days of sleep recovery are not sufficient to completely recover the impaired spatial memory, anxiety-like behavior, and oxidative stress caused by 21 days of CSD. However, the 21 days of sleep recovery significantly reduced oxidative stress, increased spatial memory, and reduced anxiety-like behavior caused by the 21 days of CSD.

Finally, we also analyzed the effect of the 5- and 21-days of sleep recovery on the basal/apical dendritic branching points/intersections of hippocampal CA3 neurons in rats. We observed that the basal/apical dendritic branching points/intersections of hippocampal CA3 neurons slightly improved after 21 days of sleep recovery compared to CSD but it was not significant. Even though the dendritic arborization of hippocampal CA3 neurons did not significantly improve after 21 days of sleep recovery, the spatial memory and anxiety-like behavior significantly improved, which might be due to the other neuronal cells involved in the spatial memory and anxiety-like behavior. Together these results suggest that a longer duration of sleep recovery might be required to restore the CSD-induced alteration.

5. Conclusion

The present study results suggest that there is increased oxidative stress in the hippocampus and reduced dendritic arborization of hippocampal CA3 neurons after 21 days (18 h/day) of CSD in rats. These morphological changes were associated with impaired spatial memory and anxiety-like behavior of the rats. Even though there was a significant improvement in spatial memory and anxiety-like behavior after 21 days of sleep recovery, it was not sufficient to fully restore the damage induced by CSD. Further increase in the duration of sleep recovery might completely restore the damage induced by 21 days of CSD.

CRediT authorship contribution statement

Suresh Konakanchi: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Resources, Writing - original draft. **Venkateswarlu Raavi:** Methodology, Data curation, Formal analysis, Supervision, Writing - original draft, Writing – review & editing. **Harendra Kumar ML:** Methodology, Supervision, Writing - review & editing. **Vinutha Shankar MS:** Conceptualization, Methodology, Data curation, Project administration, Resources, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Brain Research Bulletin

Impact of chronic sleep deprivation and sleep recovery on hippocampal oligodendrocytes, anxiety-like behavior, spatial learning and memory of rats

--Manuscript Draft--

Manuscript Number:	BRB-D-22-00375
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Abstract:	Sleep and its quality play an important role in memory, cognition, and quality of life. Sleep deprivation-induced changes in hippocampal neurons and behavior were studied widely, in contrast, the extent of damage to oligodendrocytes was not been fully understood. The present study aims to investigate chronic sleep deprivation (CSD) and sleep recovery induced changes in oligodendrocytes of hippocampus, cognition, and behavior of rats. Male Sprague-Dawley rats (n=48) were grouped as control, sham control (SC), CSD, and CSD+sleep recovery (CSD+SR) (n=12/group). CSD and CSD+SR group rats were sleep deprived for 21-days. After CSD, the CSD+SR group rats were sleep recovered for 21-days. Oxidative markers, CNPase+ve oligodendrocytes, CNPase intensity, and CNPase gene expression were measured in the hippocampus, and the anxiety-like behavior, spatial learning and memory were assessed. The 21-days of CSD significantly ($p<0.001$) increased oxidative stress and significantly ($p<0.001$) reduced the number of CNPase+ve oligodendrocytes, CNPase intensity, and CNPase gene expression when compared to controls. The increased oxidative stress was correlated with reduced CNPase+ve oligodendrocytes, CNPase intensity, and CNPase gene expression ($r=-0.9$). In-line with cellular changes, an increased ($p<0.01$) anxiety-like behavior and impaired spatial memory were observed in the CSD group compared to controls. The 21-days of sleep recovery significantly ($p<0.01$) reduced oxidative stress and anxiety-like behavior, improved spatial memory, increased CNPase intensity and CNPase gene expression, and non-significant ($p>0.05$) increase in CNPase+ve oligodendrocytes compared to CSD. Overall, the 21-days of CSD reduced number of CNPase+ve oligodendrocytes in the hippocampus, increased anxiety, and impaired spatial memory in rats. Though the 21-days sleep recovery showed an improvement in all parameters, it was not sufficient to completely reverse the CSD-induced changes to the control level.

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Anita Jagota

Prof. Anita Jagota
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University of Hyderabad

Dr. Shashi Bala Singh
President IAN

Appendix II

Institute Animal Ethics Committee Approval Certificate

Certificate

This is to certify that the project title **Effect of chronic sleep restriction induced oxidative injury on structural integrity of oligodendrocytes in hippocampus and efficacy of sleep recovery. (Project No. IAEC/PHARMA/SDUMC/2017-18/06a)** has been approved by the IAEC.

Approved Sixty Rats

Name of Chairman/ Member Secretary IAEC:
Dr. M.L. Harendra Kumar

Name of CPCSEA nominee:
Dr. P. Krishnamoorthy

P. Krishnamoorthy
28/6/18

Signature with date

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Signature with date

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Summary

- Twenty-one days (18 hours/day) of CSD significantly increased oxidative stress in the hippocampus, anxiety-like behavior, and impaired the spatial memory of rats.
- Twenty-one days (18 hours/day) of CSD significantly reduced the basal/apical dendritic branching points/intersections of hippocampal CA3 neurons.
- Twenty-one days (18 hours/day) of CSD significantly decreased the number of CNPase+ve oligodendrocytes in CA1 and DG subregions of the hippocampus and reduced the expression of CNPase protein and gene expression in the hippocampus of rats.
- The CNPase+ve oligodendrocytes, CNPase protein and gene expression levels were negatively correlated with MDA (oxidative stress marker) in the hippocampus of CSD rats.
- Chronic sleep deprivation followed by 21-days of sleep recovery significantly reduced the oxidative stress in the hippocampus, and anxiety-like behavior, and improved the spatial memory of rats.
- Twenty-one days of sleep recovery increased the dendritic arborization of CA3 neurons, and CNPase+ve oligodendrocytes insignificantly, whereas, the levels of CNPase protein and gene expression levels were significantly increased in the hippocampus of rats.
- Though the 21-days of sleep recovery reversed the CSD induced damage, it was not sufficient to reach control levels.

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