EVALUATION OF SLEEP-WAKEFULNESS PROFILE AND THE BEHAVIORAL PHENOTYPE OF MALE RATS DURING MIDDLE AGE THAT ARE BORN TO THE REM SLEEP DEPRIVED RATS DURING PREGNANCY

A THESIS SUBMITTED

BY

S. L. JOHNSY MARY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF PHILOSOPHY

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY
THIRUVANANTHAPURAM – 695011

DECLARATION

I, **S.L. Johnsy Mary**, hereby declare that I had personally carried out the work depicted in the thesis entitled **"Evaluation of Sleep-Wakefulness profile and the behavioral phenotype of male rats during middle age that are born to the REM sleep deprived rats during pregnancy"** under the direct supervision of **Dr. Kamalesh K. Gulia,** Scientist E, Division of Sleep Research, Biomedical technology wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India. External help sought are acknowledged.

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(An institute of national importance under Govt. of India)

CERTIFICATE

This is to certify that the thesis entitled **"Evaluation of Sleep-Wakefulness profile and the behavioral phenotype of male rats during middle age that are born to the REM sleep deprived rats during pregnancy"** submitted by **S.L. Johnsy Mary** (2016/MPhil/09) in partial fulfillment for the **Degree of Master of Philosophy** in Biomedical Technology to be awarded by this institute. The entire work was done by her under my supervision and guidance at Division of Sleep Research, Department of Applied Biology, Biomedical technology wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India.

Place: Thiruvananthapuram
Date:

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The Thesis

Entitled

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For

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Of

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Evaluated and approved

By

Signature Signature

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(S.L. Johnsy Mary)

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LIST OF ABBREVIATIONS

S.NO	EXPANDED FORM	ABBREVIATED FORM
1.	Sleep wakefulness	S-W
2.	Electrooculogram	EOG
3.	Electroencephalogram	EEG
4.	Electromyogram	EMG
5.	Non Rapid Eye Movement	NREM
6.	Rapid Eye Movement	REM
7.	Rapid Eye Movement Sleep Deprivation	REMSD
8.	Suprachaismatic Nuclei	SCN
9.	Active wakefulness	W1
10.	Quite wakefulness	W2
11.	Light slow wave sleep	S1
12.	Deep slow wave sleep	S2
13.	Anterio-Posterior	A/P
14.	Medio-Lateral	M/L
15.	Dorso-Ventral	D/V
16.	Biopac Student Label	BSL
17.	Elevated Plus Maze	EPM
18.	Forced Swim Test	FST

LIST OF NOTATIONS

1.	Hour	h
2.	Hertz	Hz
3.	Second	S
4.	Minute	min
5.	Microvolt	µV
6.	Milligram	mg
7.	Millimetre	mm
8.	Weight	Wt
9.	Degree Celsius	ºC
10.	Percentage	%
11.	Gram	G
12.	Intramuscular	i.m
13.	Kilogram	Kg
14.	Centimetre	cm
15.	Plus or minus	±
16.	Less than or equal to	≤

SYNOPSIS

Sleep quality deteriorates during normal aging. Sleep consists of rapid eye movement (REM) and non-REM sleep. It is noted that sleep disturbances and reduction in REM sleep are common among pregnant women during last trimester which might affect the development of the network in growing newborns. REM sleep deprivation during pregnancy is shown to impair the newborn's sleep. However, it is not known if REM sleep deprivation during pregnancy has any extended effect on sleep-wake pattern of offspring when they grow older. The present study aimed to evaluate the effects of prenatal REM sleep deprivation on the sleep-wakefulness (S-W) profile of the offspring when they reached middle age, which is also referred as middle adulthood, in rat model to assess onset of changes in S-W. In addition, behavior profile of animals was assessed for anxiety using elevated plus maze (EPM) test and depression traits through forced swim test (FST) during the middle age. The study was conducted on 10 male rats that were born to either control (n=5) or REM sleep deprived dams during pregnancy (n=5). These rats maintained in standard laboratory conditions of 12:12 h light: dark schedule at ambient temperature of 26±1 °C with *ad libitum* food and water. After they reached middle age (13-16 months), rats were stereotaxically implanted with electrooculogram, electroencephalogram and electromyogram electrodes under anaesthesia for objective assessment of S-W. After post-surgical recovery, individual rat was recorded for S-W of 24 h for three consecutive days. The S-W signals were acquired using data acquisition system MP-150 and were scored manually (offline) taking epochs of 30 sec duration into active wake (W1), quiet wake (W2), NREM (S1 and S2) and REM sleep. For each stage of S-W, the percentage time, frequency and average bout durations and circadian alterations, latency to REM sleep, Delta power during NREM sleep were calculated. Behavior parameters in the EPM and FST were recorded after completing their S-W

recordings. The results showed that there was no change in the total percentage of S-W between two groups during the middle age. However, a significant increase in the bout frequencies of W1 and S1 were evident especially during the day time in the REM sleep deprived group in comparison to the control group. The bout duration of W1 was also shorter during the day time compared to control. Latency to REM sleep was slightly lower in the rats born to REM sleep challenged dams. Even though not much changes were observed in the circadian pattern in the NREM sleep but delta power of NREM sleep was reduced in the REM sleep deprived group in comparison to control. This probably suggested decrease in the homeostatic drive in the prenatally sleep deprived group. No changes were observed in any of the parameters between the control and the REM sleep deprived rats in the EPM test. In the FST test, the time spent in immobility was higher but the time spent in swimming was lower in the REM sleep deprived dams compared to the control rats. These findings indicate that the REM sleep deprived groups have an inability to restore their sleep patterns during their middle age.

1. INTRODUCTION

1.1. Background

Sleep is a dynamic process that undergoes changes in quality and quantity from the time of birth to old age. Sleep is a behavioural state distinguishable from wakefulness based on characteristics features including reduced responsiveness to stimulation, altered level of consciousness level, and most importantly reversibility. In 1953, rapid eye movement (REM) sleep was discovered by Aserinsky and Kleitman (Aserinsky and Kleitman, 1953). Consequently, sleep was categorized into two distinct states in mammals, i.e. rapid eye movement (REM) and non-REM sleep. REM sleep is also observed in the avian species.

1.2. REVIEW OF LITERATURE

1.2.1 How to assess sleep?

Sleep can be assessed objectively based on **t**hree primary physiological measures i.e. electroencephalogram (EEG), electromyogram (EMG) and electrooculogram (EOG). The EEG was first recorded in human by Hans Berger in 1929. The EEG is recorded by placing electrodes on the skull, EOG records eye movement and EMG records the electrical activity of the active muscles. These three parameters are simultaneously recorded to assess the S-W.

1.2.2. Differences in sleep of human and rats

The sleep in human is slightly different from the most commonly used animal model rats. In human, sleep consists of 3 stages in the Non-REM sleep (N1, N2, N3) as per the new classification (American Sleep Medicine Association, 2007). In the N1 stage of sleep (S1 stage as per previous classification), alpha activity decreases, EEG mostly contains low voltage activity with mixed frequency and slow rolling eye movements are observed. The

EMG becomes moderately low. In the N2 stage (S2 as per previous classification) bursts of “sleep spindle” (12 - 14 Hz sinusoidal waves) appears in the EEG against the background of low voltage mixed frequency activity. In N3 stage (S3 and S4 stages of the previous classification) high voltage (more than 75 μV), slow waves called delta waves appear in the EEG. This is followed by the REM sleep in which the EEG reverts to a low voltage mixed frequency pattern similar to stage 1. Bursts of rapid eye movement in the EOG while the EMG nearly becomes absent (muscle atonia), many small muscle twitches may occur background.

Sleep pattern in the human is different from the rats as humans sleep during the night whereas rats being “Nocturnal” sleep more during the day. Sleep in the rat is polycyclic in nature i.e. they undergo several cycles of sleep during 24 h whereas sleep in human is monophasic. The NREM sleep in rats consists of only two stages S1 and S2. Nevertheless, the brain mechanisms to regulate S-W are nearly similar in these two species. Rat is a good model to study various mechanism involved in regulation of S-W.

1.2.3. Sleep during ageing

Sleep quality deteriorates during normal ageing. It is a recognized fact that sleep undergoes changes during development in altricial species including human and rat species. At the time of birth, human babies spent about 80 % of the time in REM sleep, out of which half the time is spent in active sleep. Moreover sleep is also polycyclic i.e. there are several cycles of S-W of shorter durations. With further growth, sleep consolidation is attained and circadian rhythm continues to develop. Sleep gradually becomes biphasic in children and monophasic in adolescents with slightly longer duration and reach to an adult profile thereafter.

Sleep in the old age is of poor quality in human due to deficit in total sleep and sleep efficiency, increased sleep latency and increased awakening during the night (Mendelson, 1987; Feinberg, 1968; Miner and Kryger, 2017; Roepke and Sonia-Ancoli, 2010). It is also reported that reduction in the total sleep time is small in magnitude after the middle age which precedes the old age (Williams et al., 1974). Since sleep disturbance in the older adults is associated with increased morbidity and mortality, it is a crucial health issue as not many studies are done on the aging population. Similarly, studies in the rats reported that sleep declines in the light phase but not in the dark phase under normal condition (Van Gool and Mirmiran, 1983, 1986; Rosenberg et al., 1979).

1.2.4. Increase in ageing population and related issues

It is also acknowledged that the overall average life expectancy (the average number of years a person born in a given country) has increased in recent years in comparison to last 5-6 decades due to better healthcare conditions, reasonable control on life threatening diseases and improved lifestyle facilities. Japan tops the list with a life expectancy at 83.7 years while in life expectancy India is 68.3 as per the United Nations World Population Prospects (2015). Worldwide, the average life expectancy at birth is 71.5 years (68 years and 4 months for males and 72 years and 8 months for females) over the period 2010-2015. As a result, there is growth in the aged population all over the world in 21st century. As per the estimate of the United Nation, between 2015 and 2030, the number of people in the world aged 60 years or over is projected to grow by 56 %, from 901 million to 1.4 billion, and by 2050, the global population of older persons is projected to more than double its size in 2015, reaching nearly 2.1 billion. Globally, the number of people aged 80 years or over, the “oldest-old” persons, is growing even faster than the number of older

persons overall. Japan is home to the world's most aged population 33% cent were aged 60 years or over in 2015. In India, the proportion of elderly persons in the total population is expected to increase from 8.3 percent in 2011 to 12.4 percent in 2025 (IIM-B report). The need for health care increases with age as people above 65 years spend on average 1.5 times on healthcare compared to those in the 60-64 age category (Mahal and Berman, 2002). The expenditure on health care is expected to be high for the elderly because of chronic diseases. The elderly in India suffer from cardio-vascular illnesses, circulatory diseases, cancer, arthritis, hyper tension, osteoporosis, communicable diseases, high blood pressure, kidney problems, vision problems, diabetes, rheumatism and digestive disorders (Kumari, 2001; Jha et al., 2006). As a consequence, there are several health and socioeconomic issues in association to increasing span in old age. There is global concern associated with health in ageing population.

Sleep is one such issue which has received due attention in recent years. As per the National Sleep Foundation (USA), several older adult's report of being less satisfied with their sleep and feel more tired during the day. Studies on the sleep habits of older Americans show an increase in the time to fall asleep (sleep latency), an overall decline in REM sleep, and an increase in sleep fragmentation (waking up during the night) with age. The prevalence of sleep disorders also tends to increase with age (Roepke and Ancoli-Israel, 2010; Wolkove et al., 2007; Neikrug and Ancoli-Israel, 2010; da Silva et al., 2016; Miner and Kryger, 2017). Some of the sleep disturbances among the elderly are attributed to physical and psychiatric illnesses and the medications used to treat them.

The reports regarding of prevalence of insomnia among middle aged adults (age group 35-54 years) from some countries including India, France, Brazil (Gupta, 2015; Panda et

al., 2012; Ohayon and Lemoine, 2004; Hirotsu et al., 2014) show high percentage (18 to 21 %) while this percentage remain low (5 to 10%) in some of the countries like Germany, Australia, China (Xiang et al., 2008; Schlack et al., 2013; Hillman and Lack, 2013). The variations in prevalence could be due to difference in socioeconomic and cultural characteristics of the populations.

1.2.5. Sleep during pregnancy and effects of sleep deprivation during pregnancy

In addition to normal ageing, the sleep is also altered during normal pregnancy due to several associated changes including anatomical, physiological and hormonal leading to increased arousals from sleep (Izci-Balserak and Pien, 2010; Pein and Schwab, 2004; Driver and Shapiro, 1992; Sahota et al., 2003; Wilson et al., 2011; Mindell et al., 2015; Reichner, 2015). These changes can alter sleep patterns especially during third trimester of pregnancy (eg, increased frequency of snoring, poor sleep quality and quantity, daytime sleepiness, and decreased daytime alertness) are observed (Facco et al., 2010; Pien et al., 2005). The sleep quality usually disrupts with the increasing gestational week (Kizilirmak et al., 2012).

Sleep deprivation during gestational period of women are at higher risk (Pien and Schwab, 2004). It is already shown that REM sleep deprivation during third trimester of pregnancy gives rise to newborn with immature sleep in rats (Aswathy et al., 2017). These newborns continue to show higher amount of active sleep (analogue of adult REM sleep), and reduced quiet sleep (analogue of adult NREM sleep), reduced latency to REM sleep even till postnatal day 21 (Aswathy et al., 2017). It is not known if these parameters of S-W ever resume normal levels after weaning during later development. Moreover, the REM sleep deprivation during 3rd term of pregnancy also impair the early development of

behavior and give rise to depression like symptoms during adult age (Gulia and Kumar, 2018). The prolonged sleep disturbances during pregnancy have impact on normal development in the growing offspring (Chang et al., 2010; Gulia and Kumar, 2018; Gulia et al., 2014, 2015; Micheli et al., 2011; Radhakrishnan et al., 2015). Some reports quoted that sleep deprivation during pregnancy impairs brain development and memory processes by affecting neurogenesis in the hippocampus and functioning of the glial cell (Peng et al., 2016; Zhao et al., 2014, 2015).

1.2.6. Regulation of sleep-wakefulness

Regulation of sleep-wake cycle involves interaction of two distinct biological mechanisms, the homeostatic process (Process S) and the circadian rhythm (Process C) referred as Borbely's "Two Process Model" proposed in 1980 (Borbely et al., 2016). According to this model, Process C governs the regulation of the body's internal processes and alertness levels which is governed by the internal biological or circadian clock. The Process S controls sleep-wake homeostasis based on the accumulation of sleep-inducing substances in the brain that generates a homeostatic sleep drive. Both of these processes are affected by various external factors like food, drugs, ambient temperature, meal times, naps, stress, exercise, daily schedules, alarm clocks, etc can also have a direct or indirect effect on an individual's S-W cycle. We feel sleepy at night since we have been awake so long during the day time which actually increases the "sleep drive" homeostatically controlled by the time of prior wakefulness. Likewise homeostatic sleep drive dissipates which promote us to wake up in the morning. When homeostatic sleep drive increases throughout the day, effectively makes a person more and more sleepy and it is countered and moderated by "circadian drive for arousal" which removes the alerting system starts to produce sleep-inducing "melatonin" substance in the brain. This opens the "sleep gate"

exactly by which mechanism occurs is not fully understood. But “neuronal group theory of sleep” suggests that individual group of neurons enters into a state of sleep when certain threshold of activity reached. Once group of neurons come in this state, whole organism falls to asleep. Circadian rhythm regulates the body internal process and alertness level drive for arousal which is governed by circadian clock. Importantly, these two processes consolidate sleep and wake when individuals are attained at a normal phase angle, means that we sleep during the night when melatonin levels are high and core body temperature is less, and are awake during the day.

Circadian rhythms in aged people are impaired (Costa et al., 2013). Aging disrupts amplitude in circadian level, melatonin level, sleep-wake disruptions, lowered locomotor activity (Hofman and Swab, 2006: Yamazaki et al., 2002; Duffy and Czeisler, 2002; Valentinuzzi et al., 1997; Weinert, 2000; Yoon et al., 2003). Further, consequence of phase shifts and re-entrainment difficulty are seen in aging population (Gibson et al., 2009; Scarbrough et al., 1997; Valentinuzzi et al., 1997). Though many factors account for these physical changes, the central clock suprachiasmatic nuclei (SCN) in brain act as a key element responsible for this age-related decline. The activities of human SCN both for the diurnal and the seasonal rhythms altered during aging.

Evaluation of sleep during middle-age can help in understanding the onset of changes from the young adult to the aged population on as would be contain an expression of transition of various changes during this period. However, not much is known about the sleep in middle age population.

2. LACUNAE

There are no reports on the effects of prenatal maternal sleep deprivation on the sleep-wakefulness profiles of the offspring during aging. It is important to understand the effects of pregnancy associated sleep deprivation on the sleep patterns of offspring during middle age as sleep quality is deteriorated in the aged population. Not much is known regarding deterioration in sleep architecture during normal aging.

It is also not known if the sleep deprivation in dams during pregnancy can affect the anxiety behavioral states like anxiety and depression in the first generation offspring.

3. OBJECTIVES OF THE STUDY

1. **To evaluate the effects of prenatal REM sleep deprivation on sleep-wakefulness profiles and the homeostatic sleep drives of the offspring during middle age in the rat model.**

2. **To assess the expression of anxiety and depression in the middle aged rats that were born the REM sleep deprived rats during pregnancy.**

4. MATERIALS AND METHODS

4.1. Experimental animals and maintenance

The study was carried out in the male rats born to the pregnant Wistar rats which had undergone REM sleep deprivation of 22 h each day during day 15 to 20 of pregnancy (Gulia et al., 2014). These male rats were allowed to grow and their sleep-wakefulness (S-W) was studied when they reached age of 13-16 months. Age matched control rats (n=5) was used to compare the S-W of the experimental sleep deprived group (n=5). Body weights of all the rats ranged between 400 to 550 g.

These animals were bred and reared in the central animal facility of Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum, India. These were housed individually in transparent polypropylene cages (44.5 X 29.5 X 18.5 cm) and were maintained at the controlled temperature (26° C) and 12 h light (illumination above 200 lux) and 12 h dark (illumination below 5 lux) cycle, with light on from 06:00 h (controlled by timer) and had *ad libitum* access to food and water. The study was approved and performed in accordance with the guidelines laid down by the Institutional Animal Ethics Committee of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum, Kerala.

This study was conducted in rat model for the following reasons:

a. Rats are comparatively less distracted by the surroundings; therefore they are more suitable for sleep studies.
b. Many of the classical studies on S-W are performed on rats including our laboratory.
c. Rats are less prone to infection and suitable for chronic experiment.
d. The research laboratory, ours, is well equipped and standardized for experiment on rat.

e. Rats are easily available and economical to maintain.

f. Handling is easier during chronic recordings.

4.2. Chemicals Used

I. Ketamine was obtained from Miracalus Pharma Pvt Ltd, india. It was used at a dose of 50 mg/kg body weight (i.m.) for anaesthetizing the animals for surgery.

II. Xylazine (Indian Immunologicals limited, Telengana, India) used at a dose of 5mg/kg.

III. Betadine (7.5% Povidine- Iodine solution, JEPS pharmaceuticals, Mumbai) was used as antiseptic.

IV. Neomycin-bacitracin sulphacetamide powder (Pfizer ltd, Thane, India), betadine (JEPS pharmaceuticals, India), Ampicillin sodium (Ranbaxy, Mumbai, India) were used as antibiotics.

V. Melaxicam (Intas pharmaceuticals ltd, Ahmedabad, India) were used as Analgesic.

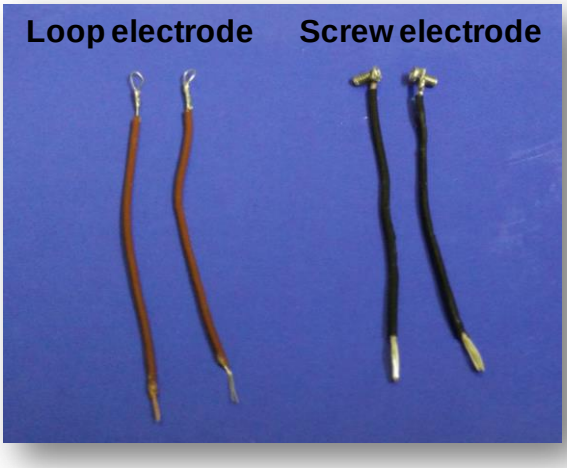
VI. Dental cement and fluid (Bombay Burmah Trading corporation Ltd, Mumbai, India).

4.3. Electrodes (EEG, EOG and EMG) for recording sleep-wakefulness

a) Electroencephalogram (EEG) electrodes: Stainless steel screw (1mm) was used for making EEG electrodes (**Fig. 1**). By soldering lightweight flexible radio wire to this screw, EEG electrodes were made. The screw electrodes were chronically fixed on the skull just above the dura for recording EEG.

b) Electromyogram (EMG) and electrooculogram (EOG) electrodes: Small loop were made from 40G stainless steel wires. The ends of the loops were soldered with small flexible radio wire (**Fig. 1**).

Figure 1. Picture of the different types of electrodes



4.4. Stereotaxic instrument

In this study, small animal stereotaxic instrument (Model KOPF 957, California), was used for implantation of electrodes for electrophysiological recording S-W in free moving animals. The stereotaxic instrument consists of two lateral bars that are graduated horizontally and are connected to a horizontal plate to form 'H' shaped frame (**Fig. 2 A**). There are two calibrated ear bars at right angles to the lateral bars. The centre of meeting point of two ear bar is considered as instrumental zero. At the centre of the body of the 'H' shaped structure, a detachable head holder is mounted which has a longitudinal groove. The purpose of this groove is to allow its movement in the anteroposterior (A/P) direction to the desired position. The head holder also has a horizontal incision bar on which rested the incisors of the animal. A nasal bar presses down the head of the animal. The electrode carrier is attached to one of the lateral bars, which has calibration. The carrier is adapted to fit on the lateral bars of the 'H' shaped frame with the help of a sliding clamp. The movement of the carrier on the lateral bar achieved the rostro-caudal movement of the IC socket (for soldering electrodes to it) fixed on the carrier. The carrier has also the provision for medio-lateral (M/L) and dorso-ventral (D/V) movements.

4.5. Procedure for implantation of electrodes for S-W

The surgery for implantation of electrodes for recording S-W was carried out in middle aged rats under aseptic condition, under ketamine (50mg/kg body weight, i.m.) and xylazine (5mg/kg body weight, i.m.) (Gulia et al., 2005; Gulia et al., 2008; Sivadas et al., 2017). After the rat was anaesthetized, the head was shaved using electric shaver and the skin overlying the head was cleaned with betadine solution. Animals were fixed on the stereotaxic instrument with help of ear bar and incision bar (**Fig. 2B**). The incision bar was set 3.3 mm above the interaural line in accordance with Paxinos and Watson atlas (Paxinos and Watson, 1997).

A midline longitudinal incision was made on the skin over the skull, with a sterilized scalpel blade. The periosteal muscle was retracted with a blunt spatula, to expose a small portion of the frontal bone, the bregma, the coronal and the sagittal sutures, the lamba and small portion of the occipital bone. The skin was retracted on both the sides. Two small burr holes were drilled using micromotor drill for implanting bilateral screws electrodes on the skull, 2 mm posterior to the bregma and 3 mm lateral to the mid-sagittal suture, for recording EEG (**Fig. 2 C**).

The EMG electrodes were placed bilaterally in the dorsal nuchal muscle and sutured with a sterilized silk thread. Similarly, two EOG electrodes were sutured to the external canthus of the eye on either side. Ground electrodes (2 in number) were implanted approximately 5 mm away from the EEG electrodes, rostrally at the naso-frontal portion of the skull. To fix the anchoring screws, two separate burr holes were drilled, caudal to the lambda, bilaterally on the either sides. Anchoring screws are used to anchor the self-curing acrylic cement to the skull along with the electrodes in place. A film of the acrylic

cement was applied on the sides to fix the recording electrodes. All distal ends of the recording electrodes were trimmed and soldered on to an eight-pin IC socket, which was then fixed on the skull. A thin layer on the dental acrylic cement was applied on the surface of the skull before soldering with the socket. After soldering the dental cement was applied all around the IC socket. The whole assembly of electrodes on the IC socket was fixed on the skull with the help of dental cement (**Fig. 2 D**). The animal was placed in a clean labeled cage under dim light till it recovered from anesthesia. Analgesics were given for 3 days and antibiotics for 5 days to these animals. Their food intake, body weight etc were monitored for the post operative recovery of 8-10 days. After post operative recovery of ten days, the rats were individually habituated to the sleep recording chamber with recording cables for 24 h.

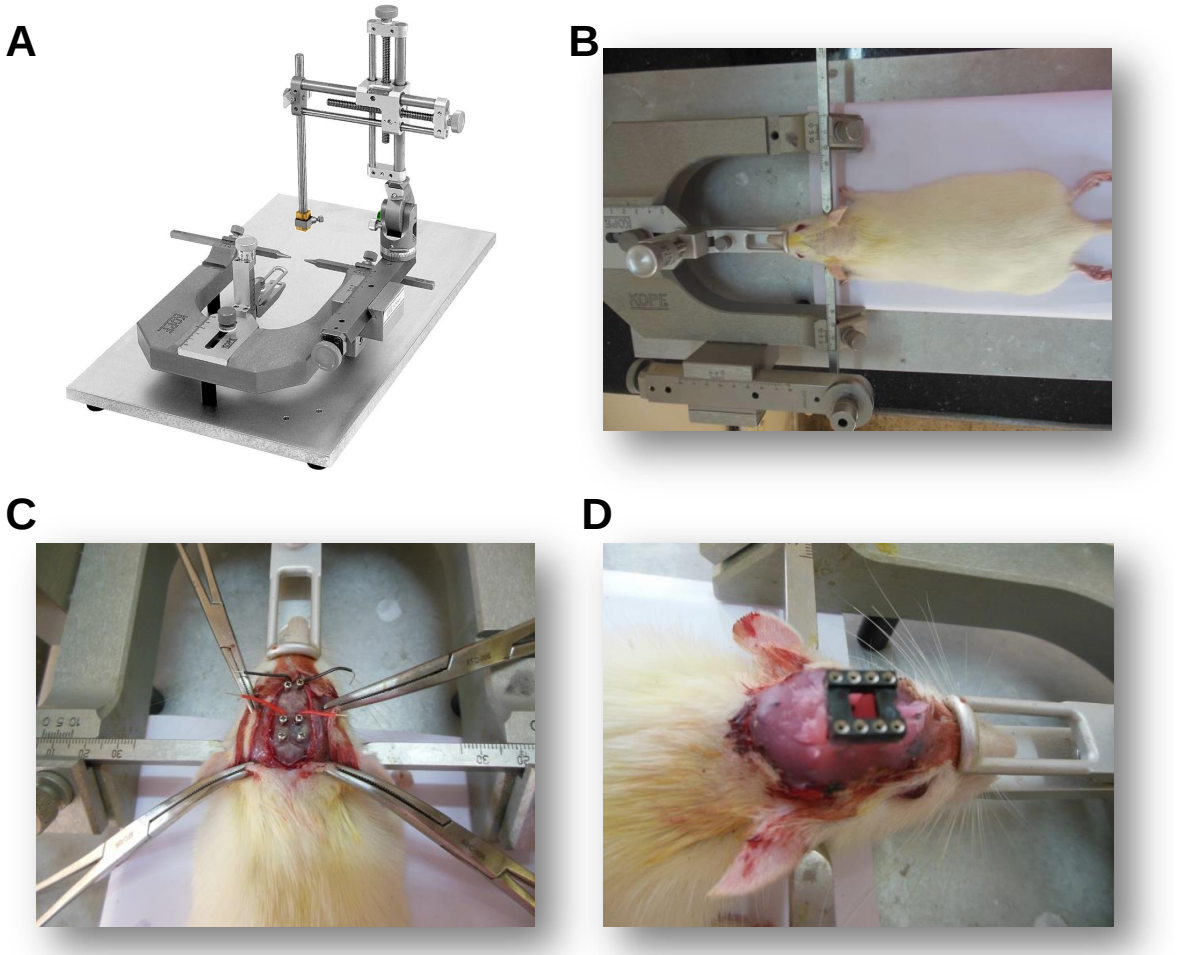
Three recordings of 24 h (6 am to 6 am next day) on consecutive days were taken for all animals in control and the REM sleep deprived group under normal conditions.

4.6. Procedure for recording S-W (Digital polysomnography)

The Biopac Student Lab (BSL) system was used for recording EEG, EMG and EOG (**Fig. 3**). It is a complete system that consists of software and hardware components. The BSL hardware included the MP150 acquisition unit, one amplifier module for each signal with MEC 100 C kit, and electrode cables with pinch clips. The Acknowledge program software version 4.2 was used in the computer (Windows 7). The software reads the electrical signals acquired in the MP150 acquisition system and displayed them in waveforms on the computer screen. In addition, the software was used for off-line analysis of the acquired signals. The unwanted electrical noise or interfering signals, was filtered out and the amplified signal was digitized by the MP150 system. The EEG, EOG

and EMG signals from rats were fed to the MP150 acquisition unit that digitized and displayed it on the monitor. The signals were stored in the hard disc of the computer and analyzed offline by the Acknowledge software program.

Figure 2. The stereotaxic instrument (A) and the steps of the surgical procedure of electrode implantation in the head immobilized anesthetized rat (B-D)

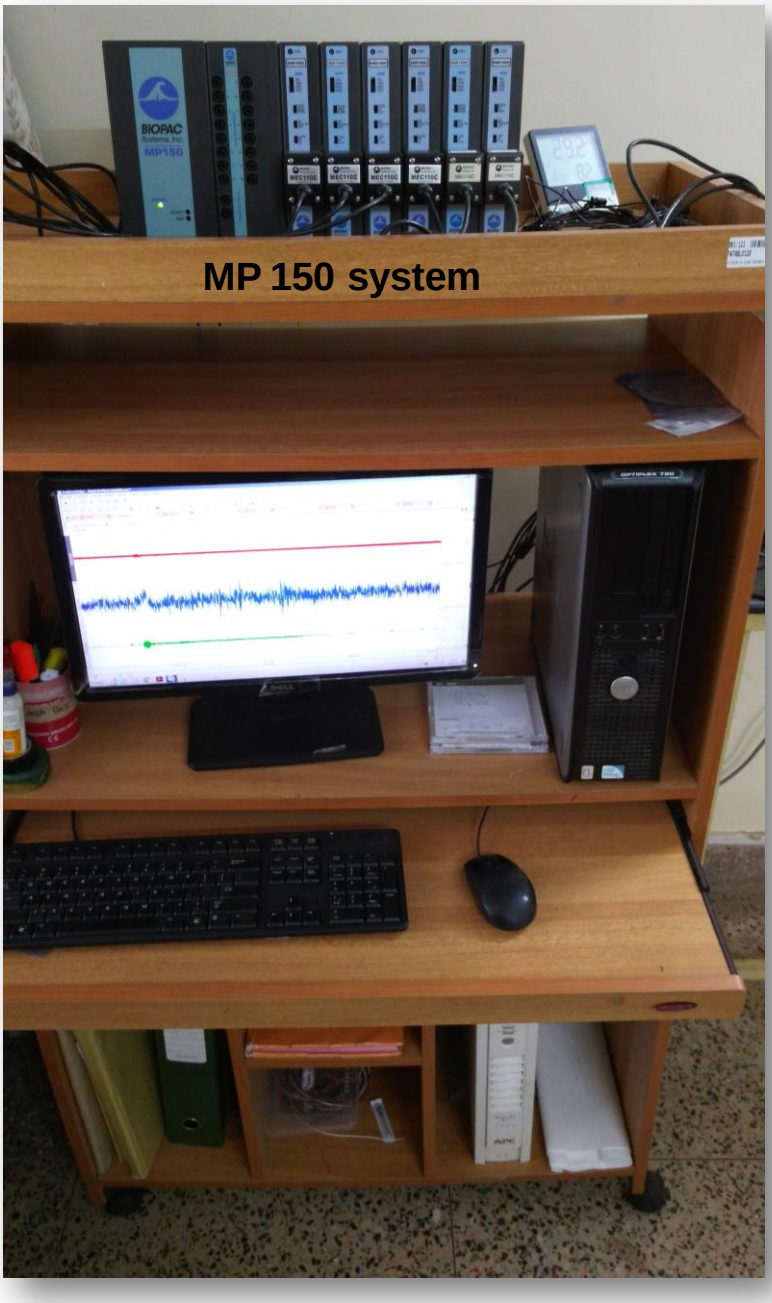


4.7. Analysis of S-W in rats

The S-W signals acquired using data acquisition system MP-150 (Biopac system) were amplified (x5000), filtered (EOG: 1-10 Hz ; EEG: 0.1-35 Hz; EMG: 1-500Hz), and digitized at 1kHz. The S-W recordings of 24 h were visually scored in offline analysis

taking epochs of 30 sec duration. Each epoch was marked for a particular stage of S-W depending on the stage to which exceeded more than 15 sec in each epoch of the record. The S-W was classified into the five different stages of S-W (Kumar et al., 1993, Sivadas et al., 2017).

Figure 3. Recording system for the sleep-wakefulness in rats



4.8. Classification of different S-W stages

Wake consists of two stages, namely active wakefulness (W1), and quiet wakefulness (W2), and sleep was classified into three different stages, namely light slow wave sleep (S1), deep slow wave sleep (S2), and rapid eye movement (REM) sleep (**Fig. 4**). EEG, EOG and EMG signals were visually analyzed offline for assessing different S-W stages.

i) Active wakefulness (W1)

During active wakefulness, EEG waves are desynchronized with higher frequency (25-40 Hz) with lower amplitude (20-40 µV). EMG shows gross body movement artifacts, and EOG shown as high amplitude spiky waves with eyeball movements. Animals showed grooming, scratching, eating and orienting activities during this period.

ii) Quite wakefulness (W2)

Quiet wakefulness identified by the presence of desynchronized EEG waves, though there was a slight decrease in the frequency and increase in the amplitude of the waves. Though the EMG activity remained high, but it did not show any movement artifacts. Animals were found sitting quietly during this period. Spiky movements produced by the eyeballs were also practically absent.

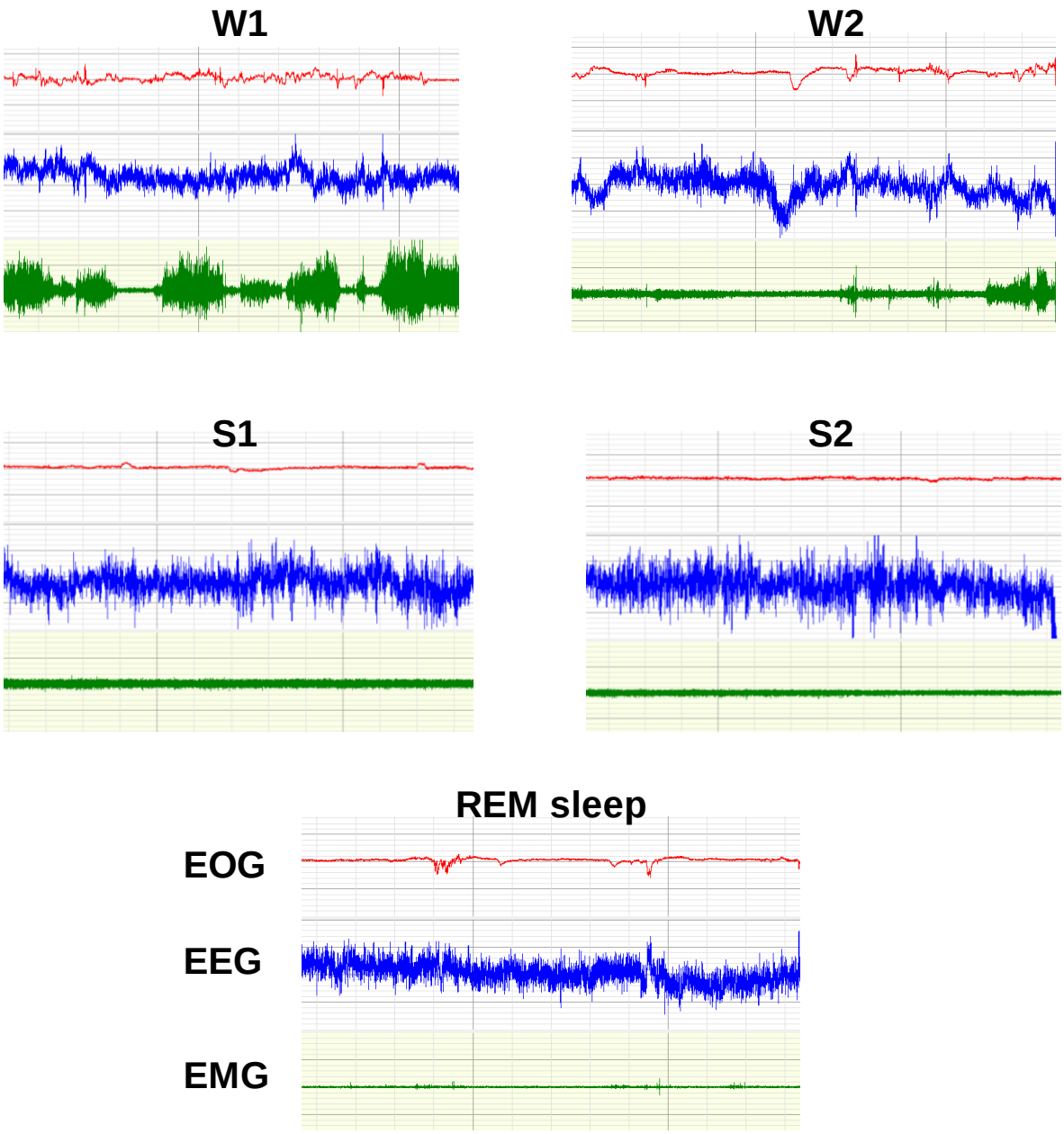
iii) Light slow wave sleep (S1)

EEG waves characterized by the presence of synchronized low frequencies (8-14 Hz) with higher amplitude (150-200 µV) appeared in the form of spindles 1.5-2.5 s duration. The spindles appeared at the rate of 5-12/ min on the background of desynchronized waves. There was considerable reduction in EMG and EOG activity. The rats assumed a sleeping posture during this period.

iv) Deep slow wave sleep (S2)

EEG waves characterized by the presence of low frequency (4-12 Hz) with high amplitude (200-300 Hz), giving a gross appearance of continuous slow wave activity. There was further reduction in EMG as compared to that during S1.

Figure 4. Polysomnographic traces of the W1, W2, S1, S2 and REM sleep of rat



v) Rapid eye movement (REM) sleep

EEG waves are characterized by the desynchronized mean amplitude (25-45 µV). It usually occurs after a period of deep SWS. EMG activity was reduced compared with light SWS (muscle atonia). EOG characterized by the presence of spiky waves due to the rapid rolling of eye movements. Some jerks of head, limbs and vibrissae are observed during the REM sleep.

4.9. Different parameters of the S-W

For each stage of S-W, the percentage time, frequency and average bout durations were calculated. Day and night values (%) of NREM (S1 and S2) and REM sleep, in three hour bins, were compared to find out the circadian alterations. Latency to REM sleep calculated for all the rats. The mean delta power was calculated from artifact-free NREM sleep for every 30 s epoch in 24 h EEG and was averaged in 3 h bin for light phase (06.00 h till 18.00 h) and dark phase (18.00 h till 06.00 the next day). The delta power obtained from NREM sleep was normalized by dividing it with the delta power obtained in the REM sleep of the same bin of 3 h (Naylor et al., 1998; Sivadas et al., 2017)

4.10. Procedure for assessment of anxiety using elevated plus maze (EPM) in rats

Equipment set up:

To find the state-dependent alterations in emotional behavior in rats, anxiety levels were tested in elevated plus maze (EPM) using ANY- maze video-tracking system (version 4.82) from stoelting Co (USA). The EPM consists of a plexiglass maze having two open arms (50 x 10 cm) and two closed arms (50 x 10 x 50 cm) arranged in plus shape. It was kept at a height of 45 cm from the floor. The closed arms were covered with black chart paper from outside. The arms of same types faced each other and were connected through

an open centre zone (10 x 10 cm). The video tracking system with computer interface and video camera is used to automatically collect behavioral data (Gulia et al., 2015). The program installed on a Dell PC computer with a digital video camera mounted overhead on the ceiling. It should be kept close to the centre of the room, and has similar levels of illumination on both open and close arms. The legs of the maze are adjusted so that the maze is perpendicular to the ground and each arm was in level.

EPM Protocol

1. The EPM maze is cleaned with 30% alcohol and dried before use. In the video-tracking system, the data sheets with subject number of animal, date, coded condition is filled and initialed by the experimenter before testing.
2. Bring each animal in an individual temporary transport cage into the behavioral testing room, 5 min prior to the beginning the first trial to habituate the condition of the behavioral testing room.
3. Take animal out of the cage placing at the centre zone of the maze facing the open arms and the test was conducted for 5 min test session. Start the video tracking system and with the timer set for 5 mins. The video-tracking system automatically detects and record when rat enter the open arms, closed arms and the central zone of the maze and the time spent in each arms.
4. The other parameters include total distance travelled in the maze, distance travelled in different arms, percentage of time spent in mobility, percentage entries into various arms were noted.
5. Ethologically derived measures like grooming, rearing, head dipping and stretch attend posture were also counted and manually entered in the system.

6. At the end of the 5-min test, remove the rat from the plus maze and place into a transport cage and then place back inside its home cage.
7. Clean the elevated plus maze with spirit and dry with paper towels before testing with another rat.

Anxiety test is indicated by a decrease in the proportion of time spent in the open arms, and a decrease in the proportion of entries into the open arms.

4.11. Procedure for recording behavioral phenotype of depression like trait using Forced swim test (FST)

Equipment Setup

Forced swim test (FST) is used to assess the antidepressant like behavior in rats. In the FST, a swim cylinder with 65-cm height by 25-cm-diameter filled with water at temperature of 25-26°C to a level (48-50 cm for middle aged rats which is more than length of animal), so that tail does not touch floor for support during the test). After completion of test, absorbable paper towels are used to dry animals. Stopwatch is used to start the time of swim trial. Video camera mounted on a stand and connected to a recording device installed with iball face to face webcam C12.0 for recording that will be used for subsequent analysis

FST protocol

1. The FST is a 2-day procedure (one day of training and one for testing) in which rats swim under particular conditions (swim cylinders) in which escape is not possible.
2. Rats can be singly housed in a separate transport cage before taken into the test room.
3. Fill the swim cylinders with water at temperature of 25-26 º C.
4. On first day, rats were placed in swim cylinders for training for 15 min.

5. After that the rats were removed from the water, dried with towels, and placed in a home cage at room temperature.
6. The cylinders were emptied and cleaned between each recording.
7. Second day, at 24 h after the forced swim test, rats were retested for 5 min (300 s) under identical swim conditions.
8. Retest sessions were videotaped from the side of the cylinders and scored using a behavioral sampling method (Detke et al., 1995; Carlezon et al., 2002).
9. Rats were rated at 1 s interval throughout the duration of the retest session; at each 1s interval, the predominant behavior was assigned to one of four categories: immobility, swimming, climbing, or diving.
10. A rat was judged to be immobile if it was making only movements necessary to keep its head above water; climbing if it was making forceful thrashing movements with its forelimbs directed against the walls of the cylinder; swimming if it was actively making swimming movements that caused it to move within the center of the cylinder; and diving if it swam below the water, toward the bottom of the cylinder.

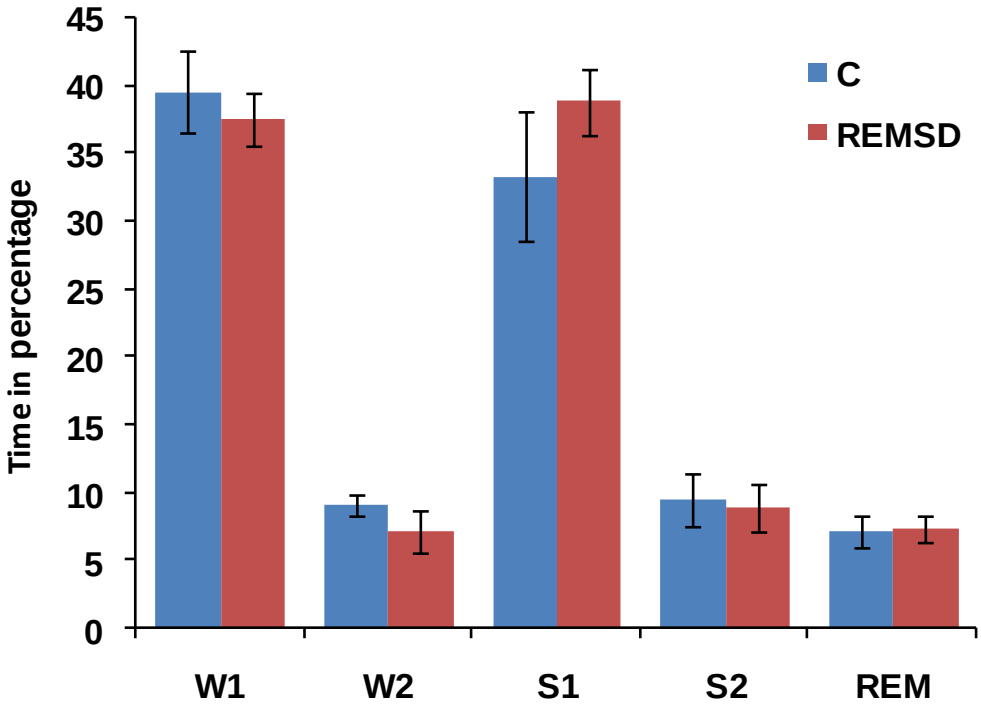
4.12. STATISTICAL ANALYSIS

Comparisons was made between the C and the REMSD group for all the parameters of S-W (the percentage time, frequency and average bout durations, day and night values (%) of S1, S2, and REM sleep, latency to REM sleep) using t-test. Recordings taken on 3 days were compared to see any inter-day variation in S-W. An average of 3 days was taken for comparison with sleep deprived group. Similarly, the parameters obtained in the EPM test and the forced swim test were statistically compared using ‘t’-test.

5. RESULTS

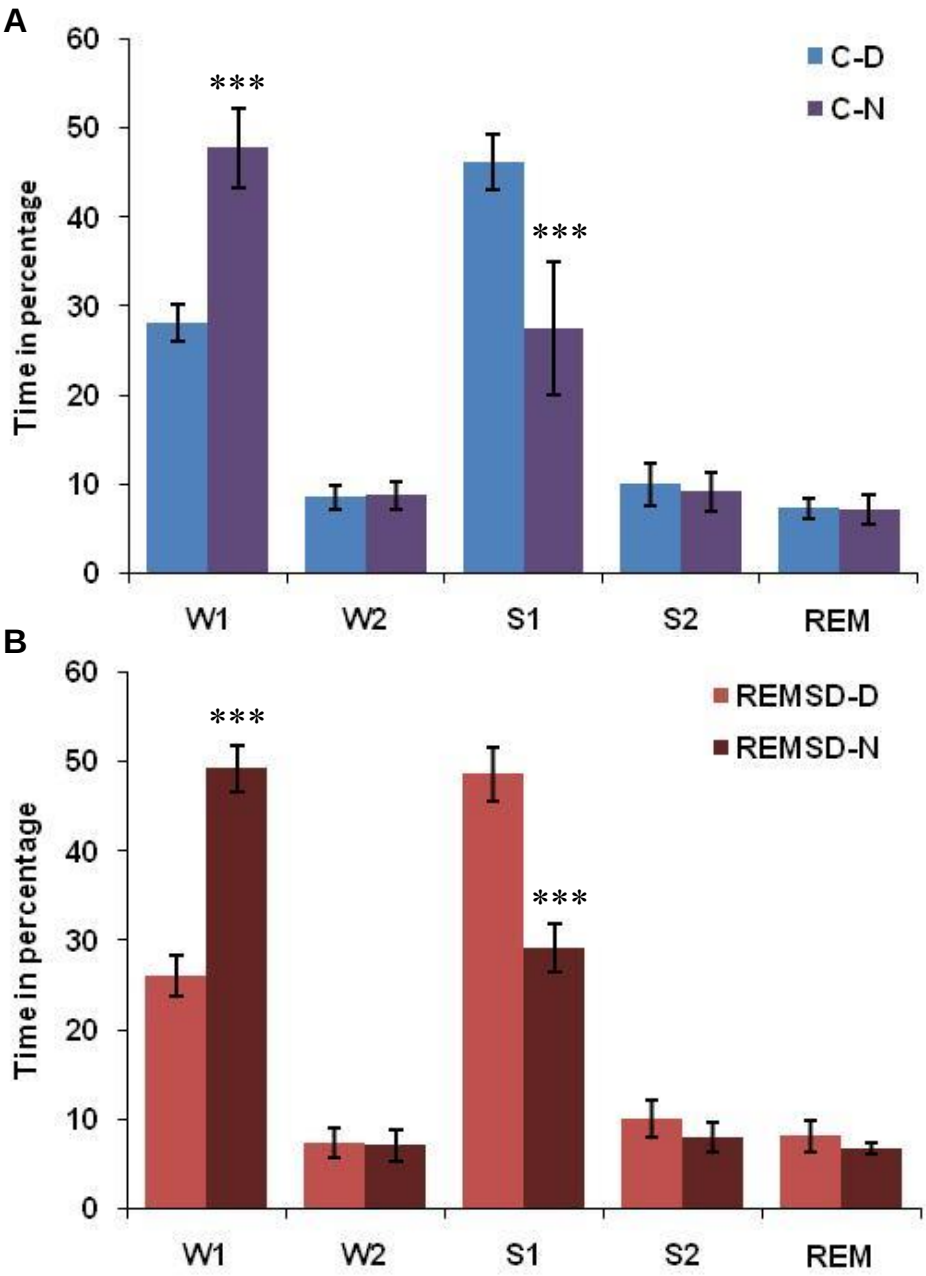
No changes were observed in any of the stages of S-W during 24 h between control and the REM sleep deprived group rats (**Fig. 5**). In the control group, the percentage of W1 during night was significantly higher than the day while the percentage of S1 was higher during the day as compared to night (**Fig. 6A**). Similar trends were observed in the rats from the REM sleep deprived groups (**Fig. 6B**). However, no significant differences were observed between the control and REM sleep deprived groups (**Fig. 7**).

Figure 5. Percent time in various stages of S-W of the middle age rats in the control and the REMSD group in total 24 h.



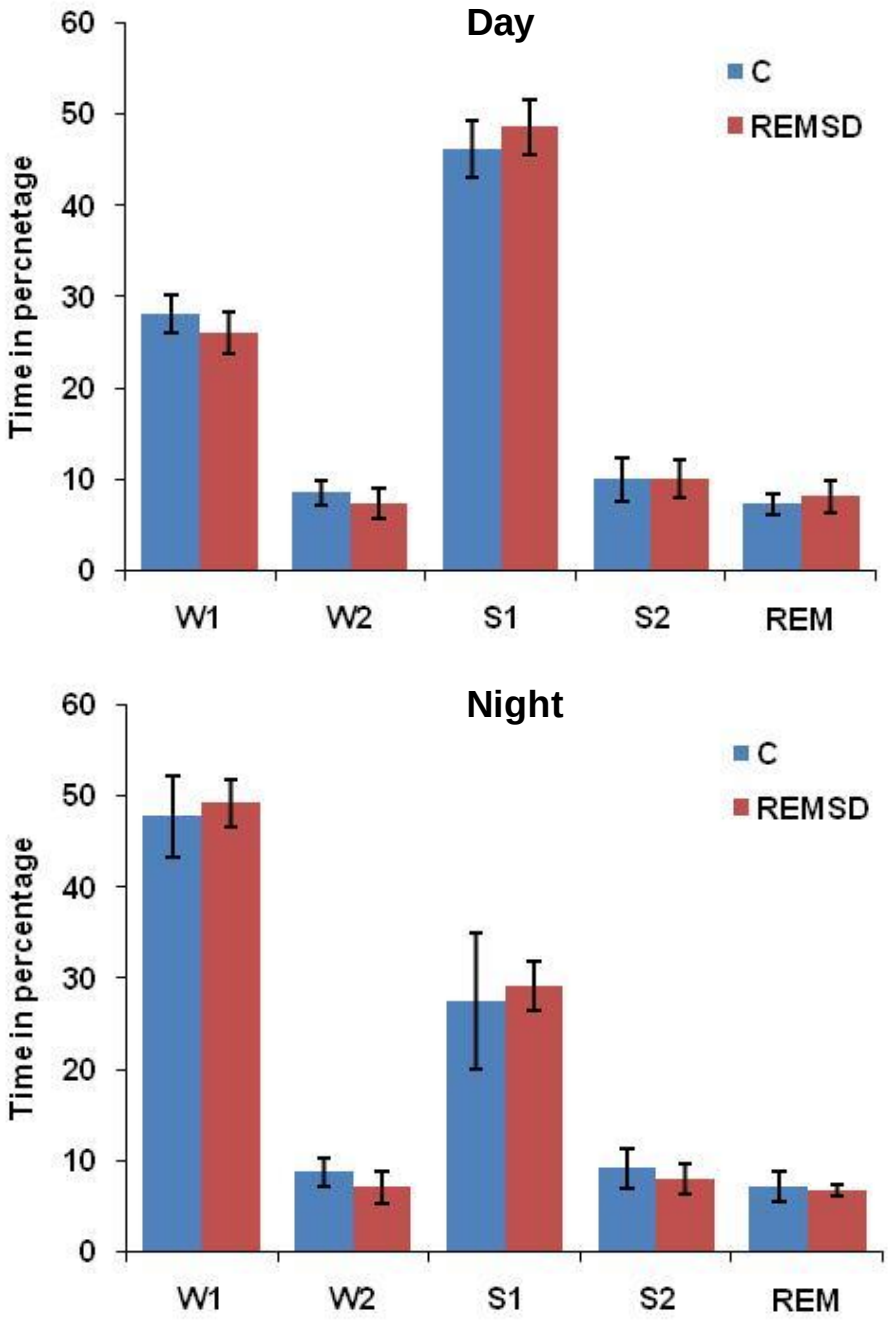
The y-axis represents the time in percentage of active wakefulness (W1)), quiet wakefulness (W2); light slow wave sleep (S1), deep slow wave sleep (S2), and rapid eye movement (REM) sleep, C refers to control and the REMSD stands for REM sleep deprivation group. The values are given as Mean ± SD.

Figure 6. Percent time in various stages of S-W of the middle age rats in the control (A) and the REMSD group (B) during light phase (12 h) and dark phase (12 h).



The y-axis represents the time in percentage of active wakefulness (W1)), quiet wakefulness (W2); light slow wave sleep (S1), deep slow wave sleep (S2), and rapid eye movement (REM) sleep during light phase (D, light color) and dark phase (N, dark color), C refers to control and the REMSD stands for REM sleep deprivation group. The values are given as Mean ± SD. *** $p \leq 0.001$

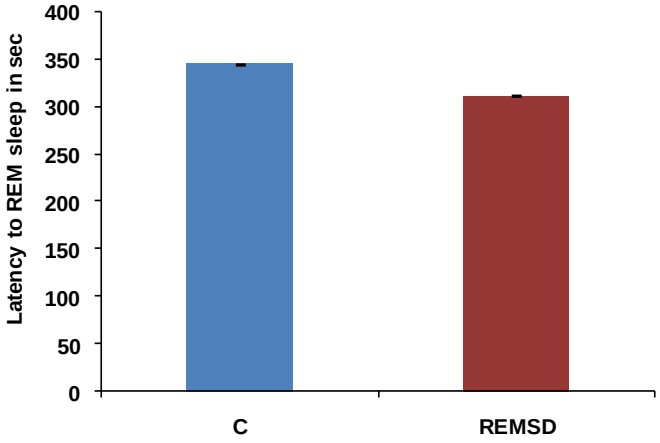
Figure 7. Day-Night changes in the stages of S-W in middle aged rats born to C & REMSD dams during pregnancy



The y-axis represents the time in percentage of active wakefulness (W1)), quiet wakefulness (W2); light slow wave sleep (S1), deep slow wave sleep (S2), and rapid eye movement (REM) sleep during light phase (D) and dark phase (N), C refers to control and the REMSD stands for REM sleep deprivation group. The values are given as Mean ± SD.

Latency to REM sleep in control was 345.0 ± 46.5 s and in REM sleep deprived group was 312.0 ± 39.2 s (**Fig. 8**).

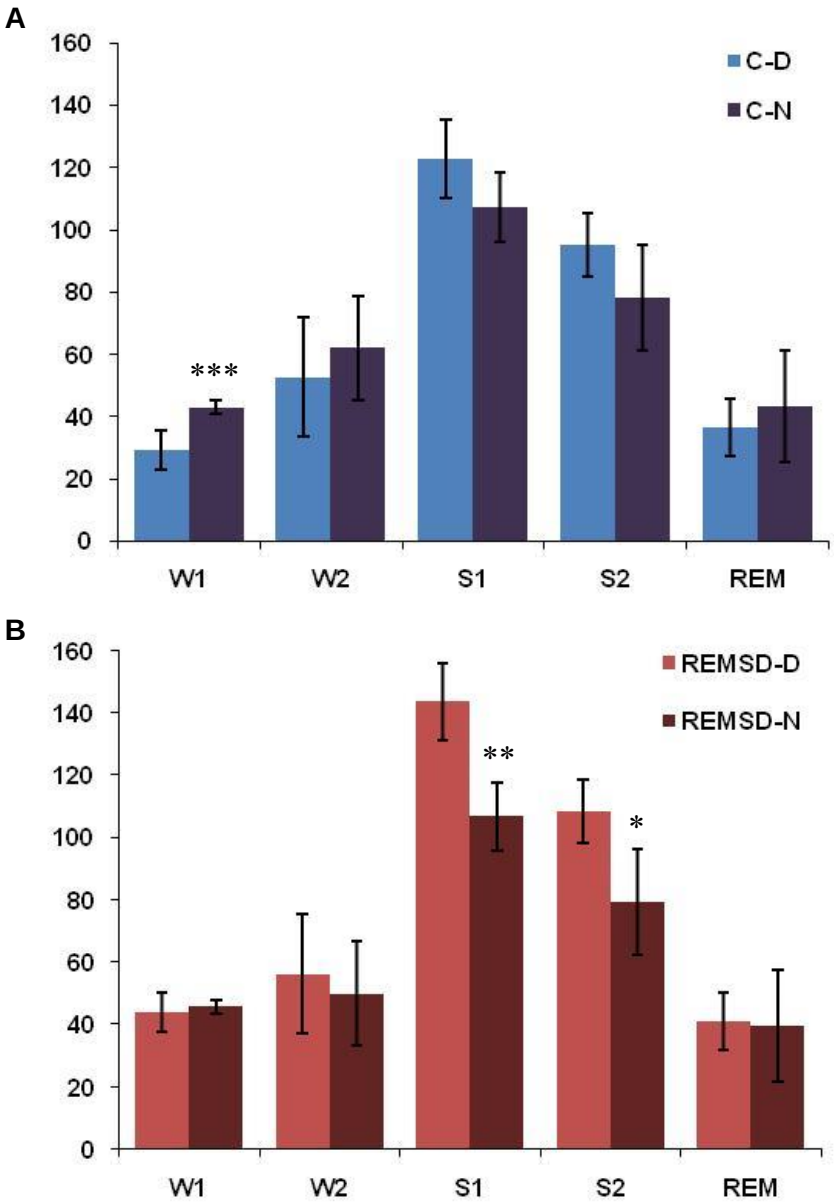
Figure 8. Latency to REM sleep in middle aged rats born to C & REMSD dams group



The y-axis represents the latency to REM sleep in sec, C refers to control and the REMSD stands for REM sleep deprivation group. The values are given as Mean ± SD.

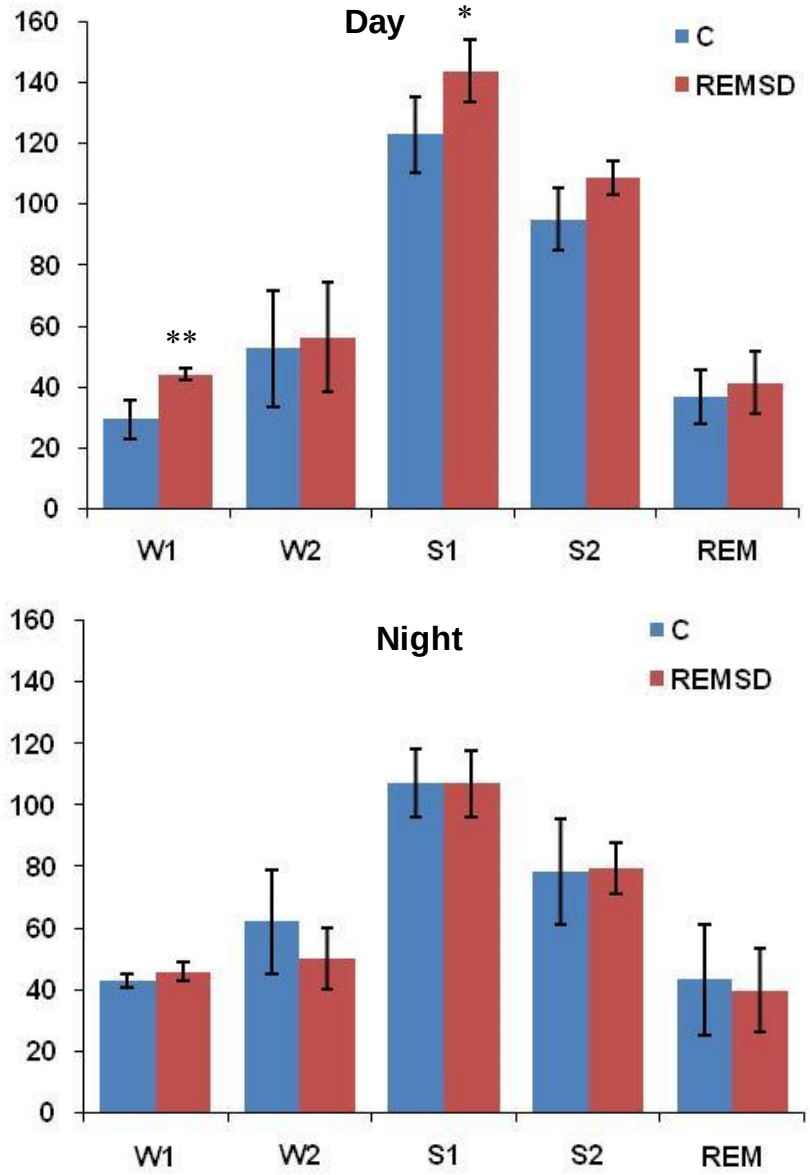
Within control group, the frequency of W1 during the night (43 ± 2.1) was significantly higher than the day (29.2 ± 6.2) whereas the frequency of S1 during the day (122.8 ± 12.5) was significantly higher than the night (107.2 ± 11.1) (**Fig. 9 A**). In comparison to control, the W1 and S1 were significantly higher in the REM sleep deprived during the day (**Fig. 9 B; Fig. 10**). No changes were found in any of the parameters during night. Within control group, the bout duration of S1 during the night was significantly lower than the day whereas the W1 was significantly higher during the dark phase (**Fig. 11 A**). The bout duration of the W1 was significantly lower in the REM sleep deprived group in comparison to control during day (**Fig. 11 B, Fig. 12**). No changes were observed during the night.

Figure 9. Frequencies of the various stages of S-W of the middle age rats in the control (A) and the REMSD group (B) during light phase (12 h) and dark phase (12 h).



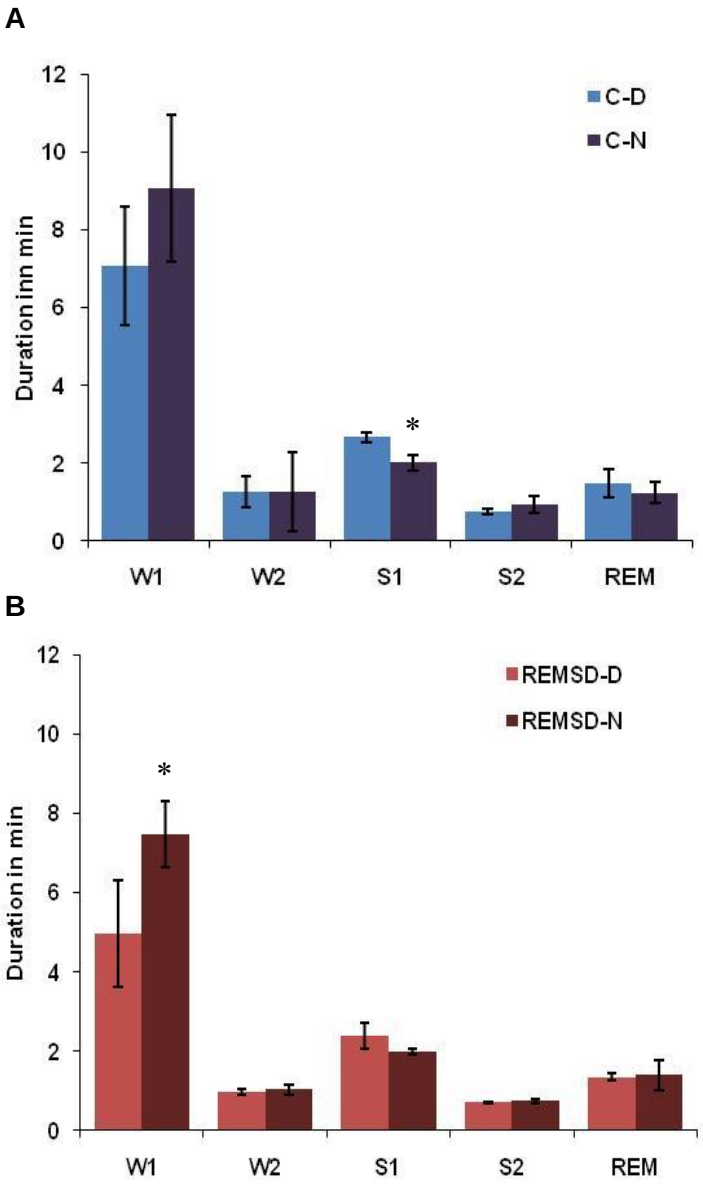
The y-axis represents the frequencies of active wakefulness (W1)), quiet wakefulness (W2); light slow wave sleep (S1), deep slow wave sleep (S2), and rapid eye movement (REM) sleep during light phase (D, light color) and dark phase (N, dark color), C refers to control and the REMSD stands for REM sleep deprivation group. The values are given as Mean ± SD. The level of significance is *** $p \leq 0.001$; ** $p \leq 0.01$; and * $p \leq 0.05$.

Figure 10. Day-Night changes in the frequencies of various stages of S-W in middle aged rats born to C & REMSD dams during pregnancy



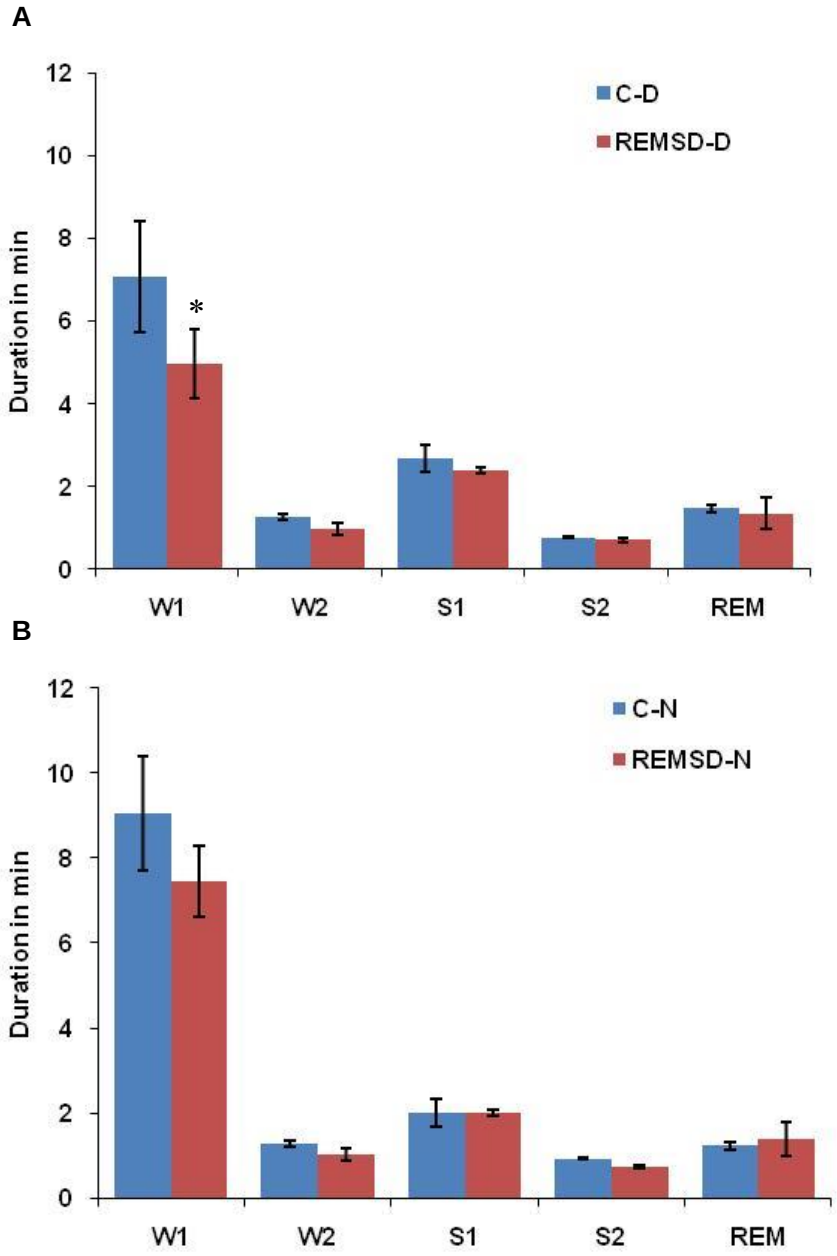
The y-axis represents the frequencies of active wakefulness (W1)), quiet wakefulness (W2); light slow wave sleep (S1), deep slow wave sleep (S2), and rapid eye movement (REM) sleep during light phase (D) and dark phase (N), C refers to control and the REMSD stands for REM sleep deprivation group. The values are given as Mean ± SD. The level of significance is * $p \leq 0.05$ and ** $p \leq 0.01$.

Figure 11. Bout durations of the various stages of S-W of the middle age rats in the control (A) and the REMSD group (B) during light (12 h) and dark phase (12 h).



The y-axis represents the bout duration (sec) of active wakefulness (W1)), quiet wakefulness (W2); light slow wave sleep (S1), deep slow wave sleep (S2), and rapid eye movement (REM) sleep during light phase (D, light color) and dark phase (N, dark color), C refers to control and the REMSD stands for REM sleep deprivation group. The values are given as Mean ± SD. The level of significance is * $p \leq 0.05$

Figure 12. Day-Night changes in the duration of various stages of S-W in middle aged rats born to C & REMSD dams during pregnancy



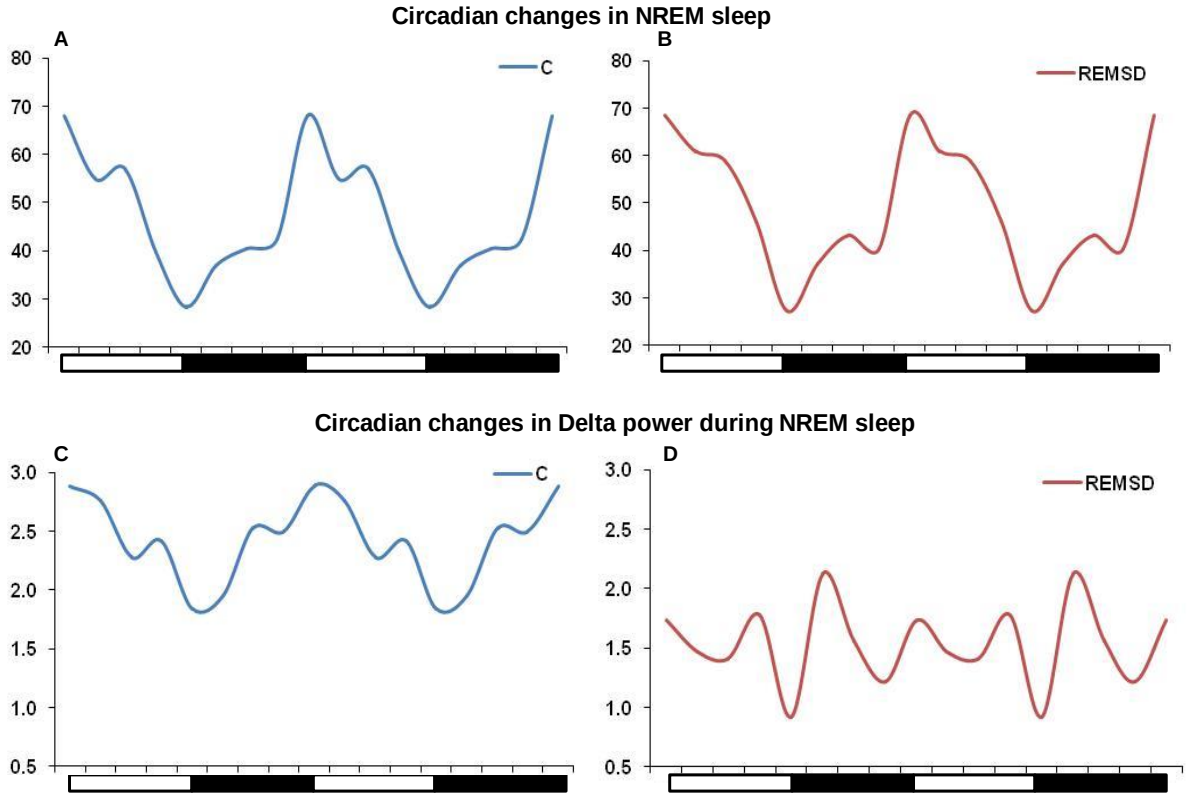
The y-axis represents the bout durations (sec) of active wakefulness (W1)), quiet wakefulness (W2); light slow wave sleep (S1), deep slow wave sleep (S2), and rapid eye movement (REM) sleep during light phase (D) and dark phase (N), C refers to control and the REMSD stands for REM sleep deprivation group. The values are given as Mean ± SD. The level of significance is * $p \leq 0.05$.

The circadian variation in the NREM sleep over day and night in the control and REM sleep deprived groups are shown in 3 h bins (**Fig. 13 A, B**) and delta power during NREM sleep are shown in **Fig. 13 C,D.** In control rats, the NREM sleep peaked at 06.00 h in the beginning of the light period and then gradually decreased and reached the nadir at the beginning of the dark period (18.00 h) (**Fig. 13 A**). There was not much difference in circadian pattern for averaged NREM sleep between two groups. The mean delta power in the control rats also showed day-night changes in such way that it gradually increased across the dark period and decreased during the light period (**Fig. 13 C**). However, overall delta power was lower in the REM sleep deprived group in comparison to control rats. Since this trend is obtained from data of one animal only, inclusion of data from more rats would be providing conclusive trends.

Various parameters obtained in the EPM test in control and REM sleep deprived group are shown in **Table 1**. There was no significant change between two groups in any of the studied parameters. The movement tracking of rats in the EPM test from both groups is shown in **Fig. 14.** Rats spent more than 75% of the time in the closed arm in both the groups.

In the FST, time spent in the immobility was higher but the time spent in swimming was lower in the REM sleep deprived groups in comparison to the control group (**Fig. 15**).

Figure 13. Circadian variation in the NREM sleep and delta power in the middle aged rats in C & REMSD group



The y-axis represents the time in percentage of NREM sleep during light phase and ☐ during dark phase ■ in control (A) and REM sleep deprived group (B); Delta power in control (C) and REM sleep deprived group (D). NREM sleep/ Delta power are shown in 3 hourly bins across night and day on the horizontal axis. This is representative data from one rat each and values are given as Mean ± SD.

Figure 14. Representative diagram of the movement tracking of rats during testing in the EPM (video tracking system) in the middle aged rats in C & REMSD group

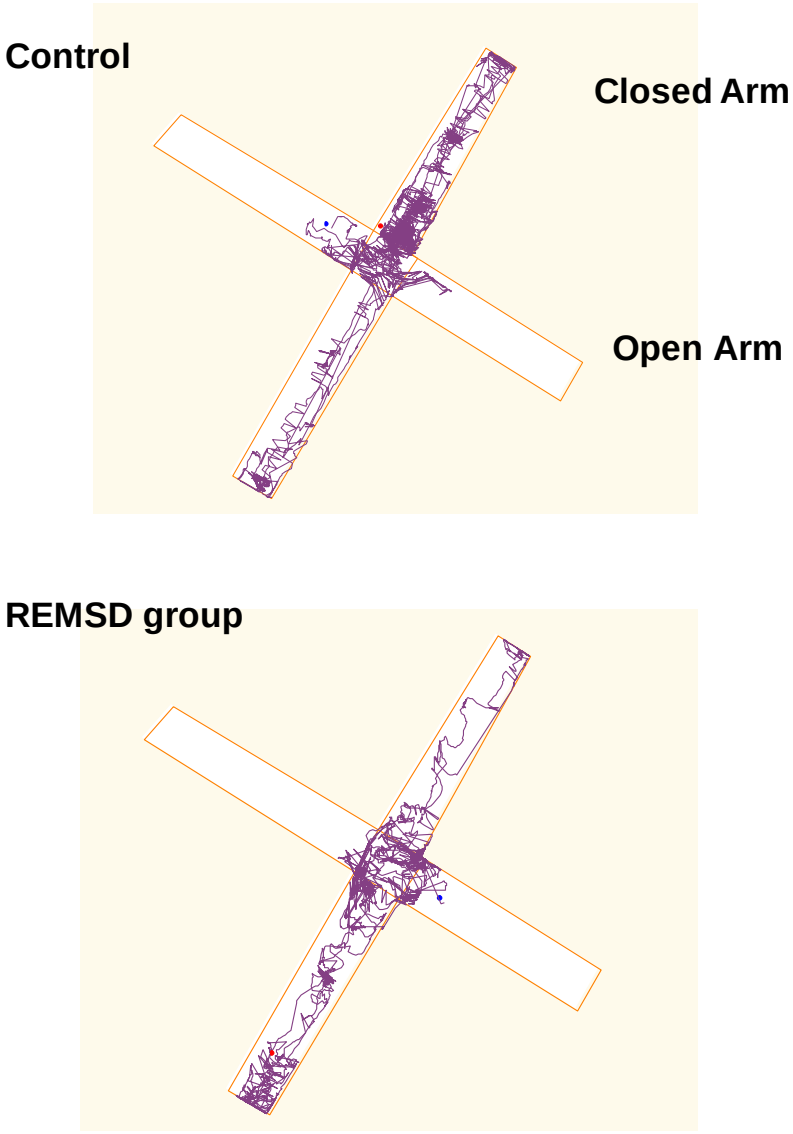
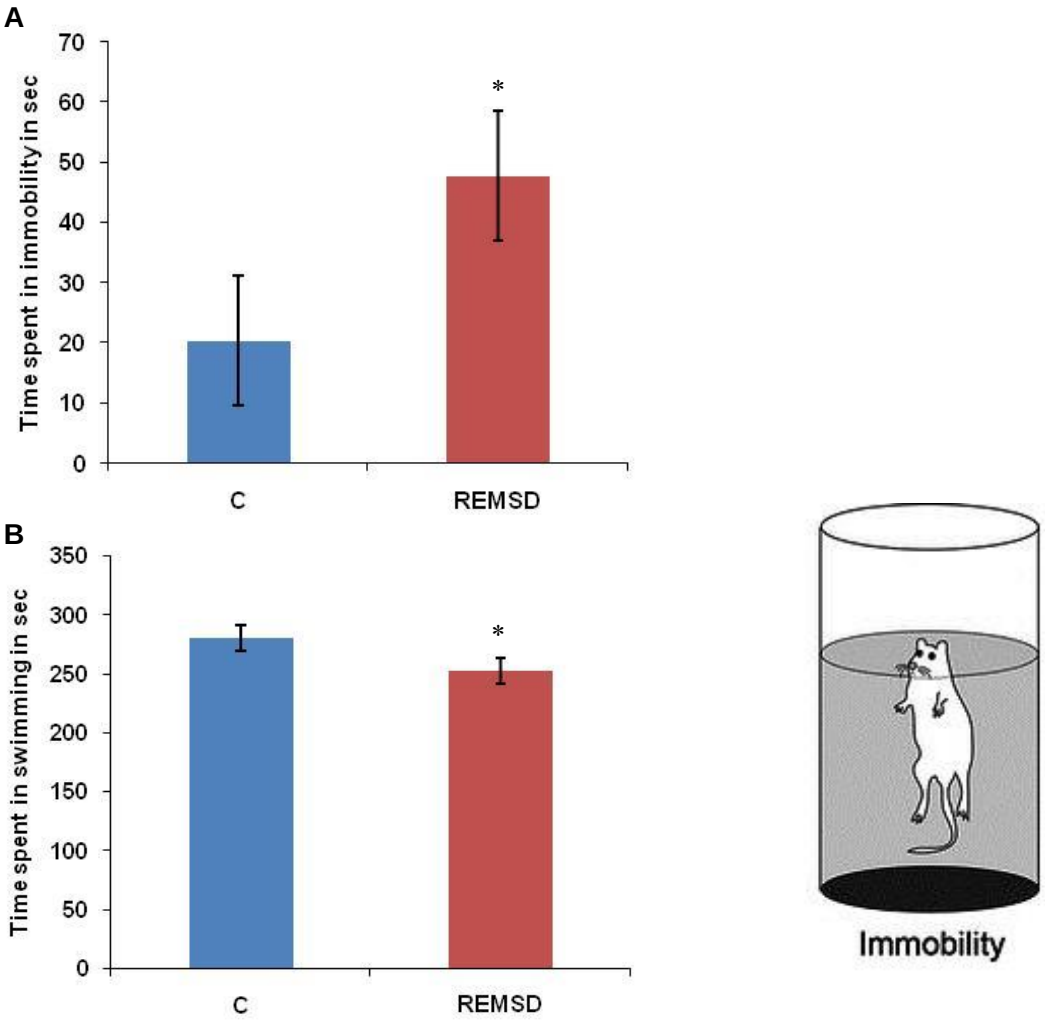


Figure 15. Parameters of the Forced swim test in the middle aged rats in C & REMSD group



The y-axis represents the time in immobility (A) and time spent in swimming (B) in sec in the control (C) and the REM sleep deprivation group. The values are given as Mean ± SD.

* $p \leq 0.05$

Table 1. Different parameters obtained in the control and REM sleep deprived group rats in the elevated plus maze (EPM) test

S. NO	PARAMETERS	CONTROL	REM-SD
		MEAN± SD	MEAN ± SD
1	Total distance travelled (m)	5.5 ± 1.7	3.4 ± 1.9
2	Total time mobile (%)	16.2 ± 6.7	9.78 ± 6.1
3	Number of line crossings	39.8 ± 28.2	21.5 ± 13.0
4	Number of entries to the open arm zone	3.3 ± 3.5	2.2 ± 2.3
5	Number of entries to the closed arm zone	17.3 ± 13.4	9.2 ± 6.1
6	Number of entries to the center zone	20.2 ± 14.0	11.2 ± 6.6
7	Time in the open arm zone (%)	7.3 ± 14.1	1.9 ± 2.5
8	Time in the closed arm zone (%)	76.9 ± 24.9	90.2 ± 8.0
9	Time in the center zone (%)	47.2 ± 40.0	23.6 ± 18.2
10	Distance travelled in the open arm zone (m)	0.6 ± 1.3	0.1 ± 0.2
11	Distance travelled in the open arm zone (m)	4.3 ± 1.5	3.02 ± 1.6
12	Distance travelled in the open arm zone (m)	0.6 ± 0.3	0.29 ± 0.2

Values are presented as Mean ± Standard Deviation (SD) in both the control and the REM sleep deprived group rats.

6. DISCUSSION

The results from the study show no overall changes in the difference in percentage stages in S-W between control and the REM sleep deprived rats during the middle age. The day and night variations in the S-W parameters were evident in both the groups. However, significantly higher frequencies of W1 and significantly lower bout duration of S1 in the REM sleep deprived group during the day in comparison to the age matched control indicated deterioration of sleep due to fragmented sleep during the light phase. Latency to the REM sleep was also slightly lower in the REM sleep deprived group. These animals spent lesser time in swimming and were immobile for a longer time in comparison to the control rats in the FST suggesting expression of the depression like traits. No changes were observed in their anxiety profile based on the EPM test.

The present study was conducted in the rats during middle age (13-16 months), an age that precedes age-bar of aged rats (18 months onwards). In this study, even though there were no changes in quantity of sleep during middle age between the control and the REM sleep deprived group rats as it may be just the beginning of aging. However, significant changes in W1 and S1 in the REM sleep deprived group rats indicated fragmented S-W especially during day time. It is noted that the rats are nocturnal animals that sleep more during the day (Light phase) in comparison to night (Dark phase), and they are more active during night. Thus, observation of more fragmentation of sleep during the light phase indicate prominent and timely emergence of effects during day. Evidently, the persistence of distinct day and night variation in the study may point to a slow emergence of sleep deterioration during this middle age. Since the purpose of study was to observe differences in the S-W pattern in the middle age rats that were born to the mothers who had REM sleep deprivation during third term of pregnancy, there will not be a direct

discussion on the changes from young adult to middle age in the S-W profile in control rats. In comparison to the young adults from another study in rats, probably slightly increased W2 and reduced S1 and S2, and increased frequency of W2 in controlled middle aged rats in the current study probably indicate slow onset of changes in the S-W which is indirectly an expression of reduced functioning of various networks involved in regulation of S-W (Srividya et al., 2006).

In current study, prominent reduction in the delta power during NREM sleep in the REM sleep deprived group in comparison to control indicate reduction in their homeostatic drive. There are studies to show that the delta power decrease in middle age and old age in comparison to young age in control rats (Mendelson and Bergmann, 1999). However, from the current study, it is difficult to compare the changes from the young rats but definitely the middle aged rat in the REM sleep deprived had lower delta power. It is also asserted that the age specific changes in the sleep quality in aged human (60 yrs and above) points more to change in sleep architecture then the macro-changes (Miner and Kryger, 2017; Schwraz et al., 2017). The macrostructure refers to the changes in total percentage of the stages in S-W, while microstructure refers to the changes in delta power etc. In the current study similar trends were observed in the middle age rats.

Old age related atrophy and thinning in the cortical region is also linked with the reduction in slow wave activity (Mander et al., 2013; Dube et al., 2015). Reduction in the delta spectral power and the increased beta power, as an indicator of cortical arousal, are common in older population (Carrier et al., 2001). The lower slow wave activity in older human compared to the younger age is attributed to decrease homeostatic drive (Carrier et al., 2001; Mander et al., 2013). In middle age subjects, the slow wave rebound is also

reduced after sleep deprivation (Lafortune et al., 2012). Difficulty in initiating the sleep is also one of the probable reasons for poor sleep (Klerman and Dijk, 2008).

Reduced latency to REM sleep, and reduced time in swimming and enhanced time in immobility by the middle age rats from the REM sleep deprived groups indicated expression of depression traits without altering their anxiety levels. REM sleep deprivation during pregnancy probably affects the networks for the depression than the anxiety (Gulia et al., 2014; Gulia and Kumar, 2018). Prenatal stress is shown to elicit depression in offspring (Rao et al., 1999).

7. CONCLUSIONS

The S-W in the middle aged rats from the REM sleep deprived group showed fragmented sleep in comparison to the age matched control. These rats also showed traits of depression but did not show any signs of anxiety. These results clearly indicate that the sleep deprivation during pregnancy has sustained effects on the sleep profiles and behavioral phenotypes in offspring.

FUTURE DIRECTIONS

The life expectancy in women is higher than men, so future study can be conducted in female rats also.

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