BEHAVIORAL AND BIOCHEMICAL CONSEQUENCES OF EARLY LIFE SLEEP DEPRIVATION IN RATS

A Dissertation Presented to

The Faculty of the Pharmacological and Pharmaceutical Sciences Department

University of Houston, College of Pharmacy

In Partial Fulfillment

Of the Requirements for the Degree

Doctor of Philosophy

By

Fatin Atrooz

August 2018

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Fatin.

ABSTRACT

Adequate sleep is essential for normal brain function, especially during the critical period of early developmental stages (childhood and adolescence). Many epidemiological and clinical studies have linked early life sleep deprivation (SD) with the occurrence of later life behavioral impairment. However, the mechanism by which early life SD causes behavioral impairment is not fully understood. Animal studies have provided useful insights. Previous reports from our laboratory have suggested a potential role for oxidative stress in induction of behavioral impairments in a rat model of acute sleep deprivation. Others have reported that extended wakefulness increases cellular metabolism and induces formation of reactive oxygen species leading to oxidative stress. Moreover, synaptic pruning and neuronal myelination occurs during early life. These processes are essential for neural circuitry maturation which underlies the behavioral changes. Sleep enhances synaptic pruning and neuronal myelination, strengthening neuronal connections. Therefore, we suggested that sleep deprivation during early life, by engaging stress pathway(s), adversely affects neuronal development and function leading to long-lasting behavioral impairment. We examined the effect of early life SD in rats. The rats at postnatal day (PND) 19 were exposed to SD 6-8h/day for 14 days. The behavioral consequences of SD were examined at different developmental stages; early adolescent: PND33, late adolescent: PND60, and adulthood: PND90. The biochemical markers of stress and synaptic density/

plasticity were measured in different brain regions at PND33 and PND90. We found that anxiety-like behavior was induced in SD rats at early life (PND33 and 60). However, at later life (PND90), anxiety-like behavior disappeared and depression-like behavior developed. Interestingly, we found a reduction in synaptic density and plasticity in prefrontal cortex (PFC) of SD rats at PND33 and PND90 as compared to control rats. The protein level of the stress response phosphatase (MKP1), was upregulated while the protein level of the neurotrophic factor (BDNF) was down-regulated in PFC of SD rats. The results suggest that PND 19-32 in rats (2-11 years in humans), is a sensitive period to SD. Sleep deprivation during this developmental stage alters cortical maturation most likely by reducing synaptic density and plasticity contributing to the behavioral deficits.

ABBREVIATIONS

Ach: Acetylcholine

AMPARs: α-amono-3-hydroxy-methyl-4isoxazole propionic acid receptors

ARAS: Ascending reticular activating system

BBB: Blood-brain barrier

BDNF: Brain-derived neurotrophic factor

JNK: Jun c-terminal kinase

CaMKII: Ca^{2+}/calmodulin-dependent protein kinase II

cAMP: Cyclic- adenosine mono phosphate

CREB: Cyclic AMP response element binding protein

DA: Dopamine

dlPFC: Dorsolateral prefrontal cortex

4EBP2: 4E binding protein

EEG: Electroencephalogram

EMG: Electromyogram

EOG: Electrooculogram

EPM: Elevated plus maze

ERK: Extracellular signaling-regulated kinase

FST: Forced swim test

GABA: Gamma-aminobutyric acid

GLO-1: Glyoxalase

Glu: Glutamate

GPx: Glutathione peroxidase

GSH: Glutathione

GSR: Glutathione reductase

GSSG: Glutathione disulfite

HO-1: Heme oxygenase-1

5-HT: 5-hydroxytryptamine

HPA: Hypothalamic- pituitary- adrenal

IL-1β: Interleukin-1β

IL6: Interleukin 6

LD: Light/Dark

LTP: Long-term potentiation

LTD: Long-term depression

MAPK: Mitogen-activated protein kinases

MEK: MAP/ERK kinase

Mn-SOD: Manganese superoxide

MRI: Magnetic resonance imaging

mTORC1: The mechanistic target of rapamycin complex 1

MKP1: Mitogen-activated protein kinase phosphatase

NE; Noradrenaline

NMDARs: N-methyl-D aspartate receptors

NO: Nitric oxide

NPY: Neuropeptide Y

NREM: None REM

OFT: Open field test

PFC: Prefrontal cortex

PKA: Protein kinase A

PLC: Phospholipase C

PND: Postnatal day

PTSD: Posttraumatic stress disorder

PUFAs: Polyunsaturated fatty acids

RAWM: Radial water maze

REM: Rapid eye movement

ROS: Reactive oxygen species

RNS: Reactive nitrogen species

SCN: Suprachiasmatic nucleus

SOD: Superoxide dismutase

SSRI: Selective serotonin reuptake inhibitors

S/W: Sleep/wakefulness

SWA: Slow wave activity

SD: Sleep deprivation

SWA: Slow wave activity

TrkB: Tropomyosin-related kinase B

TNF-α: Tumor necrosis factor-α

PTSD: Posttraumatic stress disorder

vmPFC: Ventro medial prefrontal cortex

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1. INTRODUCTION

It is well known that sleep, especially during early life, is essential for proper brain development and function (Orzel-Gryglewska, 2010). Consequently, sleep deprivation (SD) in children and adolescents has been associated with affective and behavioral difficulties (Astill, Van der Heijden, Van Ijzendoorn, & Van Someren, 2012; Dewald, Meijer, Oort, Kerkhof, & Bogels, 2010). Relevant to this, in a longitudinal study, SD in children, 2.5-6 years old, was found to be associated with hyperactivity and behavioral abnormalities (Touchette et al., 2009). Later onset of anxiety and depression has also been associated with early life sleep problems (Gregory et al., 2005). In fact, some longitudinal studies suggest that early life sleep problems are highly predictive of future affective and behavioral problems (Gessa, Pani, Fadda, & Fratta, 1995).

Although the negative consequences of early life sleep deprivation are well recognized, yet, many aspects remain unknown. For example, what is the most vulnerable age when early life SD exerts most serious and long-lasting effects? Whether early life SD causes one psychiatric condition such as anxiety or depression and whether one or both persist over time or does one disorder transform into another? Whether early life SD causes learning and memory impairments? Are these deficits permanent? What is the biochemical basis of the behavioral impairment induced by early life SD? Answers to these questions

cannot be easily sought, as conducting these studies in humans is quite challenging and often impossible. Although animal models cannot accurately reveal the impact of early life SD on psychiatric symptoms occurring in later life, however, rodent models are excellent tools that can provide useful insights.

Previous reports from our laboratory have suggested a potential role for oxidative stress in the induction of behavioral impairments in a rat model of acute sleep deprivation. Others have reported that extended wakefulness increases cellular metabolism and induces reactive oxygen species (ROS) formation leading to oxidative stress. Induction of oxidative stress as a result of extended wakefulness is important, as the brain is considered highly susceptible to oxidative stress because of its high oxygen consumption. The connection between oxidative stress, sleep deprivation, and behavior deficits seems further strengthened from the knowledge that, oxidative stress enhances inflammation and expression of mitogen-activated protein kinase (MAPKs) such as P38, leading to augmentation of inflammation and stress. Enhanced expression of MAPKs reportedly activates a phosphatase enzyme that is involved in stress response mechanisms, mitogen-activated protein kinase phosphatase (MKP1). MKP1 dephosphorylates MAPKs and reverse its harmful effects. On the other hand, MKP1 negatively regulates the brain-derived neurotrophic factor (BDNF)-mediated downstream signaling by causing de-phosphorylation of extracellular signaling-regulated kinase (ERK) and MAP/ERK kinase (MEK). Hence, overexpression of MKP-1 reduces BDNF-MAPKs

downstream signaling pathway. Therefore, BDNF depletion and overexpression of MKP-1 impairs neuronal development and function of synapses which are considered critical for maintenance of behavioral and cognitive functions. Moreover, synaptic pruning and neuronal myelination occurs during early life. These processes are associated with the maturation of neural circuitry which underlies behavioral changes. Sleep enhances synaptic pruning and neuronal myelination, strengthening neuronal connections. Therefore, we suggest that SD during early life, by engaging stress pathway(s), adversely affects neuronal development and function leading to long-lasting behavioral impairment. We examined the effect of early life SD in Sprague-Dawley rats starting at postnatal day (PND) 19. The behavioral effects of early life SD were studied at different developmental stages, and the mechanistic basis of these effects was also examined. The first specific aim of this study is to examine the effects of early life SD on the behavior of the rats using a gentle method tailored to cause SD in pups. The second aim is to study the role of oxidative stress in early life SD-induced behavioral impairment. The third aim is to study the effect of SD on synaptic density/plasticity markers in different brain regions. Understanding the molecular and structural effects of early life SD on the brain is significant in revealing novel molecular targets and developing new approaches to counteract the detrimental effects of SD.

To induce sleep deprivation in the rat pups, we used a customized SD apparatus. Rats at postnatal day (PND) 19 were used for inducing SD. The SD protocol lasted for 14 days (6-8 hours per day), until PND32. This developmental stage in rodents mimics late childhood and early adolescence in humans (Semple, Blomgren, Gimlin, Ferriero, & Noble-Haeusslein, 2013). The **rationale** for choosing this developmental stage is the following. Postnatal brain development continues until late adolescent stage. Synaptogenesis, the formation of new contacts between neurons, increases rapidly during ages 2-3 years in humans (PND 20-21 in rodents) (Semple et al., 2013). Moreover, synaptic pruning and neuronal myelination, processes that are critical for maturation of neuronal circuits, occur until late adolescent period and early adult stage (18-20 years in humans, PND45-60 in rodents) (Semple et al., 2013). Interestingly, in adolescent mice, cortical spine turnover was reported to be associated with sleep, while spine gain was associated with wakefulness, suggesting that adequate sleep during this critical period of development is essential for spine pruning and refinement (Eagle, Fitzpatrick, & Perrine, 2013). Furthermore, analysis of the whole genome transcripts in mice oligodendrocytes showed that; genes involved in neuronal myelination and oligodendrocytes proliferation are preferentially transcribed during sleep (Toth & Neumann, 2013). Thus, sleep seems to play an essential role in synaptic remodeling and neuronal circuit maturation, during early life stages. Therefore, it seems reasonable to target this developmental stage for disrupting

sleep and to follow the impact of SD on behavioral and cognitive functions later in life.

Another consideration was to achieve a model that solely induces SD stress without the concern of adding non-relevant stress. Current models of SD do not seem to be the best for inducing SD in rat pups due to the concerns of confounding factors such as physical stress, isolation stress or fatigue (Zhao, Zhao, & Veasey, 2017). Therefore, we used the pinnacle automated SD system. This system utilizes an automated rotating bar which gently pushes the pups to move in a timed manner. This system is not only gently and continuously disrupts sleep of pups but it also eliminates personnel involvement. Furthermore, in this model, we can place two littermate pups in the SD apparatus together. Having the two littermate pups togother during SD hours is a major procedural advantage as this further eliminates the concern of adding isolation stress during SD protocol. Finally, the software analysis enabled us to quantitatively measure sleep patterns and sleep behavior. Using this method of SD, the rats at PND19 were exposed to SD 6-8h/day for 14 days, following which, the behavioral and cognitive consequences of SD at different developmental stages (early adolescent: PND33, late adolescent: PND60, and adulthood: PND90) were examined. The biochemical markers of stress and synaptic density/ plasticity were measured in different brain regions including the prefrontal cortex (PFC), hippocampus, and the amygdala at PND33 and PND90.

We found that anxiety-like behavior was induced as the first response to SD protocol (PND33 and 60). However, at later life (PND90) anxiety-like behavior transformed into depression-like behavior. Interestingly, we found a significant reduction in synaptic density as well as synaptic plasticity in SD rats at PND33 and PND90. The protein level of the stress response phosphatase (MKP1), was upregulated while the protein level of the neurotrophic factor BDNF was down-regulated in PFC of SD rats. The results suggest that PND 19-32 in rats, which is correlated with 2-11 years old in humans, is a sensitive period of development highly susceptible to the harmful effects of sleep deprivation. We postulate that sleep deprivation during this developmental stage alters cortical maturation most likely by reducing synaptic density and plasticity contributing to the behavioral deficits.

STATEMENT OF PROBLEM

In otherwise healthy children and adolescents, behavioral problems, affective symptoms, and reduced school performance have been associated with sleep deprivation (SD). However, the link between early life SD and the time-line of the appearance of affective, behavioral, and cognitive problems is not fully understood. Sleep deprivation is a huge problem in modern societies, especially in young adults. Hence understanding the link between early life SD and cognitive deficits and psychiatric symptoms across the developmental course is critical. These studies are difficult to perform in children. Therefore, establishing an animal model to study the short and long-term effects of early life SD on behavior and cognition will be very useful. Furthermore, establishing an animal model of early life SD, the underlying mechanisms regulating early life SD-induced behavioral impairments can also be revealed, paving the way for identification of new therapeutic approaches to tackle early life SD-related effects.

2. LITERATURE REVIEW

2.1. Sleep Physiology

Sleep is a physiological state characterized by a lack of response to environmental stimuli. In the vertebrates, sleep is defined as a specific pattern of synchronized electrical activity of the cortical area of the brain. Sleep is typically evaluated by polysomnography (PSG), which involves the simultaneous recording of brain's cortical activity using electroencephalogram (EEG), eye movement using electrooculogram (EOG), and skeletal muscles' activity using electromyogram (EMG). In humans and other mammals, sleep consists of two main phases: rapid eye movement (REM) sleep and non REM (NREM) sleep. NREM sleep is characterized by high amplitude low frequency EEG waves known as slow wave decreased muscle tone, and slow eye movement. While REM sleep is characterized by low amplitude high-frequency waves known as active sleep, muscle atonia, and rapid eye movement. In humans, NREM sleep is further divided into three stages; N1, N2, and N3, each stage has a defined EEG wave. During sleep, these phases alternate in a highly regulated pattern forming sleep cycles (Stenberg & Porkka-Heiskanen, 1990). Sleep/wakefulness state is regulated by the interaction of two processes; the circadian system (process C) and the sleep homeostasis (process S) (Borbely, 1982a, b). Process C is controlled by the suprachiasmatic nucleus (SCN) within the hypothalamus. The SCN serves as the neural pacemaker of the circadian timing cycle. It is mainly

synchronized to environmental light/dark cycle and projects signals that control the proper timing of physiological functions including sleep/ wakefulness cycle. The daily circadian rhythm is 24 hours under normal light/ dark cycles, with sleep onset and wakefulness onset occurring at the same time every day. Sleep homeostasis or process S, is regulated by the sleep pressure generated after a period of wakefulness, hence, the level of process S increases with extended wakefulness and decreases after sleep onset. There are two hypothesis for sleep pressure generation; one is an accumulation of sleep promoting substances such as adenosine and nitric oxide, second is the need for restoration of energy resources (Abrahamson & Moore, 2006). The interaction of the sleep homeostasis and the circadian rhythm regulates the timing, depth and duration of sleep. In fact, the alignment between the two processes is essential for body metabolism and brain function. Furthermore, disruption in sleep/circadian rhythm is associated with many metabolic, psychiatric, and neurodegenerative diseases (Wulff, Gatti, Wettstein, & Foster, 2010).

The wakefulness state is coordinated by several defined nuclei in the pons and midbrain areas known as ascending reticular activating system (ARAS). These wakefulness promoting cells are; noradrenergic (NE) cells in locus coeruleus, serotonergic (5-HT) cells in raphe nuclei, cholinergic (Ach) cells in pedunculopontine tegmentum and laterodorsal tegmentum, glutamatergic cells

(Glu) in the midbrain, and dopaminergic (DA) cells in the substantia nigra and ventral tegmental area. Projections of these waking promoting cells activate thalamocortical, hypothalamo-cortical and cortico-basal systems. In addition to ARAS, there are five other groups of cells that promote wakefulness these groups include; histaminergic cells of posterior hypothalamic area, the hypocretin/orexin cells in lateral hypothalamus, cholinergic cells in basal forebrain, neuropeptide Y(NPY) containing cells in suprachiasmatic nucleus, and glutamatergic neurons in ventro medial prefrontal cortex (vmPFC). Activation of these systems maintains wakefulness by activation of cortex. However, lesion of one specific wakefulness promoting group may not induce changes in amount of wakefulness, suggesting that all these groups of brain regions together contribute in the promotion and maintenance of wakefulness but none of them is absolutely necessary for generation of wakefulness. Understanding sleep initiation and maintenance has been a challenge for decades. Initiation of sleep is suggested to be a result of successive passive and active physiological processes which leads to blockage of cortical sensory gates (Datta, 2010). The passive process that contributes to sleep initiation results from accumulation of neuronal activity-dependent metabolites. During wakefulness, metabolic products such as adenosine, nitric oxide, cytokines, and prostaglandins accumulate leading to slowing down of wakefulness-prompting neuronal activity, which ultimately slows down the production of metabolites. Reducing the activity of wake-promoting neurons

represents the active process of sleep initiation. Metabolite-dependent neuronal activity is known as metabolite homeostasis (Datta, 2010). Lately, electrophysiological studies have suggested that the thalamic reticular nucleus is the pacemaker of sleep spindles generation (Steriade, McCormick, & Sejnowski, 1993). The thalamic reticular nucleus consists exclusively of GABAergic neurons, it also serves as the sensory and internal signal at the gateway to the cerebral cortex. The thalamic reticular nucleus contains two different types of neurons; the thalamocortical relay neurons which relay incoming sensory signals to the cerebral cortex, and thalamo-reticular neurons which prevents the formal one from sending the sensory signals to the cerebral cortex. During wakefulness, the activity of the thalamo- cortical relay neurons is proportional to the activity of wake-promoting noradrenergic, serotonergic, and cholinergic cells, while the thalamic-reticular cells remain inactive. During the passive step of sleep initiation, reduction in wake-promoting cells leads to reduction in activity of thalamocortical relay and enhancement of the thalamic-reticular cell. Excitation of thalamic-reticular relay further inhibits thalamocortical relay by activation of the inhibitory postsynaptic GABA-B receptors. Therefore, the gate for cerebral cortex sensory signals is closed and the cerebral cortex is deprived of external information, which is reflected by high voltage and slow brain waves in NREM sleep, an indication of cortical inactivity (Datta, 2010; Kalia, 2006; Stenberg & Porkka-Heiskanen, 1990).

Sleep occurs in all organisms with the neuronal/glial network, suggesting that sleep is a property of every neuronal network. While many theories have been presented to explain the function of sleep such as; energy restoration, waste elimination, thermoregulation, and neuronal connectivity and plasticity augmentation, sleep is still a challenging enigma for scientists (Krueger, Frank, Wisor, & Roy, 2016; Krueger & Obal, 2003).

2.2. Sleep Function and Mental Health

Sleep is an essential physiological state that has been conserved with evolution. Many functions of sleep have been proposed. One of the leading theories of sleep is the synaptic homeostasis hypothesis. The synaptic homeostasis hypothesis proposes that; extensive strengthening of synapse during wakefulness increases the need for energy resources and hence saturates learning processes (Ringli & Huber, 2011; Tononi & Cirelli, 2014). During sleep, spontaneous neuronal activity normalizes the synaptic strength and restores the cellular homeostasis. The synchronized neuronal activity forms high amplitude slow oscillation reflected by increased slow wave activity (SWA) in EEG. Slow wave oscillation results from repeated sequences of depolarization and hyperpolarization which leads to a reduction in synaptic strength. Ultimately, this leads to a reduction in synaptic strength which reduces the amplitude and the synchronization of the slow oscillation reflected by the decrease of the SWA with

time after sleep. Hence, by recalibrating synaptic strength to the baseline level, sleep promotes synaptic homeostasis (Ringli & Huber, 2011; Tononi & Cirelli, 2014). Accordingly, sleep plays an essential role in energy restoration and neuronal plasticity and connectivity. Moreover, neuronal plasticity underlies sleep-dependent memory consolidation. In fact, many studies had linked alterations of sleep dependent-plasticity and memory functions to neurobehavioral and psychiatric disorders especially when SD occurred during the developmental stages of early life.

Another proposed function of sleep is the regulation of affective brain function. Neuroimaging studies have shown that the activity of the emotion-related brain regions including the amygdala, striatum, hippocampus, and the prefrontal cortex increases during REM sleep. Enhanced activity of emotional regulation regions is paralleled by a reduction in the level of adrenergic locus coeruleus tone during REM sleep (Dang-Vu et al., 2010; Kametani & Kawamura, 1990; Ouyang, Hellman, Abel, & Thomas, 2004). Reduction of adrenergic tone during REM sleep is important because affective experiences are associated with a burst of adrenergic tone during the time of traumatic and stressful experiences. Elevation of adrenergic tone and autonomic reactions tag these affective experiences with emotion forming salient emotional memory (Goldstein & Walker, 2014). During REM sleep, this emotional memory is recalled with reduced adrenergic tone leading to decoupling of emotions from the information of the experience. Removal

of the affective emotions that initially tagged the emotional experiences is a preventive mechanism from developing anxiety disorders that result from persistence of affective charges within memory networks. Hence, the emotional regulation function of sleep is considered as a mechanism to forget the emotional tone of affective experiences, while retaining the memory of those experiences (Goldstein & Walker, 2014). Additionally, serval studies have suggested that reduction of the locus coeruleus activity during REM sleep recalibrates next day noradrenergic response to emotional stimuli. Recalibration of noradrenergic response during REM sleep allows a balance between emotional sensitivity and specificity to salient stimuli governed by top-down prefrontal cortex control of the amygdala (Goldstein & Walker, 2014; Hermans et al., 2011; Mallick & Singh, 2011; van Marle, Hermans, Qin, & Fernandez, 2009). The emotional regulation function of sleep might explain the complicated relationship between sleep, sleep deprivation, and psychiatric disorders.

2.3. Consequences of Sleep Deprivation

While sleep has an essential role in the maintenance of body and brain functions, sleep deprivation (SD) has been associated with many physiological and psychological disorders. For example, in healthy individuals, SD has been linked to metabolic disorders including diabetes 2 and obesity (Knutson, Spiegel, Penev,

& Van Cauter, 2007), and increased the risk of cardiovascular diseases (Cappuccio, Cooper, D'Elia, Strazzullo, & Miller, 2011; Dettoni et al., 2012). The association between SD and mental health has been suggested in many clinical and epidemiological studies. The detrimental effects of SD on memory encoding and learning have been extensively reported in human and animal studies (Drummond et al., 2000; Guan, Peng, & Fang, 2004; Harrison & Horne, 2000). Moreover, in both human and animal models of fear conditioning, SD negatively affects fear extinction following exposure to traumatic event contributing to the development of posttraumatic stress disorder (PTSD) (Pace-Schott, Germain, & Milad, 2015). Additionally, affective disorders including anxiety and depression are highly correlated with sleep loss (Babson, Trainor, Feldner, & Blumenthal, 2010; Kahn-Greene, Killgore, Kamimori, Balkin, & Killgore, 2007). While the detrimental effects of sleep loss on mental health are extensively studied, the mechanisms by which sleep loss affects brain function are unclear. However, human studies, supported by animal models of sleep deprivation have provided important mechanistic explanations.

2.3.1. Sleep Deprivation and Depression

Several epidemiological studies have indicated the remarkable link between sleep and psychiatric disorders. For instance, 75% of depressed subjects have insomnia symptoms, and 20% of patients with insomnia experienced depressive

symptoms, (Nutt, Wilson, & Paterson, 2008; Tsuno, Besset, & Ritchie, 2005). Furthermore, sleep EEG in depressed patients showed alterations in sleep architecture including; a decrease in REM latency (the time from sleep start and first period of REM), an increase in total REM sleep time and density, and reduction in slow wave activity (SWA). Additionally, high-risk studies including relatives of depressed patients indicated that REM sleep alterations might precede the onset of depression and hence, might be useful in identifying subjects at high risk for depression (Palagini, Baglioni, Ciapparelli, Gemignani, & Riemann, 2013). In fact, one hypothesis has suggested the involvement of REM sleep dysregulation in the etiology and pathophysiology of depression (Palagini et al., 2013). Indeed, prospective studies have suggested that sleep deprivation in adolescents increases the risk for major depression which in turn increases the risk of sleep reduction (Roberts & Duong, 2014). Data from animal studies suggest that chronic sleep restriction may gradually induce changes in neuroendocrine stress systems, serotonergic neurotransmission, neurogenesis and neuronal plasticity (Meerlo, Havekes, & Steiger, 2015). Alteration of synaptic plasticity and strength by chronic SD might lead to alteration in connectivity within the different brain regions involved in the regulation of emotion and mood contributing to the pathophysiology of depression (Meerlo et al., 2015; Meerlo, Sgoifo, & Suchecki, 2008)

2.3.2. Sleep Deprivation and Anxiety Disorders

In healthy adults, acute sleep deprivation is reported to increase self-reported symptoms of affective psychopathology (Babson et al., 2010; Kahn-Greene et al., 2007). Some studies have suggested that anxiety disorders and sleep problems are prevalent and comorbid. In a German survey, examination of the relationships between anxiety disorders and sleep quality revealed that most anxiety disorders are moderately associated with poor sleep quality. Moreover, individuals with anxiety disorders and poor sleep have compromised mental health and increased disability relative to those with anxiety disorders alone (Ramsawh, Stein, Belik, Jacobi, & Sareen, 2009). Other studies have suggested that sleep disturbance might contribute to anxiety disorders and it might exacerbate the severity of the symptoms of anxiety and related disorders. Sleep disturbance may also diminish treatment efficacy in these disorders suggesting that addressing sleep problems in the treatment of anxiety disorders may enhance treatment efficacy (Cox & Olatunji, 2016).

Animal models of SD are crucial for investigating the link between sleep deprivation and anxiety. However, the effect of SD on anxiety produced inconsistent results in rodents. Some studies have reported that SD induces anxiety-like behavior, while others have reported reduced anxiety-like behavior following SD which makes it hard to reproduce sleep deprivation-induced anxiety

in humans (Pires, Bezerra, Tufik, & Andersen, 2016). The lack of consistency in animal models might be attributed to the variation of SD methods and protocols used (Alkadhi et al., 2013), or to the lack of the sensitivity of methodologies used for the evaluation of the anxiety-like behavior (Pires et al., 2016). Concerning the methodology, some studies have suggested the analysis of self-grooming for evaluation of anxiety-like behavior following SD (Pires et al., 2012; Pires, Tufik, & Andersen, 2013).

2.3.3. Sleep Deprivation and Cognition

Impairment of memory processing by sleep loss has been addressed in human and animal models of SD (Havekes, Vecsey, & Abel, 2012). In humans, functional magnetic resonance imaging (fMRI) studies involved sleep-deprived and well-rested brains demonstrated the importance of sleep for optimal cognitive function and learning (Chee & Chuah, 2008). Chronic sleep-restriction experiments which mimic sleep loss experienced by many individuals in modern societies and by individuals with sleep disorders demonstrate that over time, cognitive deficits accumulate to severe levels. Functional neuroimaging has revealed that sleep deprivation associated cognitive deficits involve scattered changes in the brain regions including frontal and parietal control areas, secondary sensory processing areas, and thalamic areas. However, the degree of cognitive vulnerability to sleep loss varies among individuals, an effect that may be attributed to the basis of genes

involved in sleep homeostasis and circadian rhythms (Goel, Rao, Durmer, & Dinges, 2009). Animal models of SD provided useful insights for the molecular processes that might play a role in the interaction between sleep and memory. SD studies indicate that hippocampus-dependent memory formation and working memory are mainly sensitive for SD and that there is a specific period following training during which SD impacts memory consolidation. The most sensitive period for SD was found to be 5-6 hours following training. However, the sensitive period varied depending on the nature of the training (Havekes et al., 2012). Electrophysiological studies were conducted both in vivo and in vitro to determine the effect of SD on hippocampal synaptic plasticity. SD was reported to inhibit synaptic potentiation in the hippocampus but facilitating specific forms of synaptic depression (Aleisa et al., 2011; Alhaider, Aleisa, Tran, Alzoubi, & Alkadhi, 2010; Hagewoud et al., 2010; Kopp, Longordo, Nicholson, & Luthi, 2006). Moreover, the brief period of SD (5-6 hours) impairs the maintenance of long-term potentiation (LTP). LTP is a form of synaptic plasticity, which depends on cyclic AMP (cAMP) and protein kinase A (PKA) pathway which is critical for memory consolidation (Abel et al., 1997). The cAMP-PKA pathway, by phosphorylation of GluA1 subunit of α-amono-3-hydroxy-methyl-4-isoxazole propionic acid receptors (AMPAR), controls AMPAR function and hence, synaptic plasticity. Long periods of total SD impaired spatial working memory and reduced phosphorylation of GluA1 in mice hippocampus (Hagewoud et al., 2010). Additionally, the cAMP-PKA pathway and

the MAP kinase (MAPK) pathway represented by the extracellular signal-regulated kinase (ERK), together, those pathways regulate changes in synaptic efficacy which are essential for memory formation. Seventy-five hours of REM sleep deprivation using platform over water method, decreased synaptic plasticity and transmission which were associated with reduced ERK phosphorylation and GluA1 expression in mice hippocampus (Ravassard et al., 2009). The role of protein synthesis in sleep-dependent cortical plasticity during memory consolidation has been suggested by several studies (Sweatt & Hawkins, 2016). Translational initiation is a critical step in protein synthesis which involves the translation initiation factors 4E and 4G in eukaryotes (eIF4E and eIF4G). In the brain, eIF4E is sequestered by 4E binding protein2 (4EBP2) which prevents its interaction with eIF4G. The mechanistic target of rapamycin complex 1 (mTORC1) is essential for release of eIF4E by phosphorylation of 4EBP2 allowing eIF4E- eIF4G binding and subsequent protein synthesis initiation (Sweatt & Hawkins, 2016). A series of experiments have demonstrated that protein synthesis regulated by mTORC1-dependent de-phosphorylation of 4EBP2 is a critical signaling mechanism that underlies memory deficits induced by sleep loss (Graves, Heller, Pack, & Abel, 2003; Hernandez & Abel, 2008; Tudor et al., 2016).

2.4. Sleep Deprivation and Allostatic Load

Allostatic load is a measure of cumulative physiological dysregulation across biological systems. Allostasis is the process of maintaining homeostasis; it is an active process that involves metabolic mediators such as those of the autonomic nervous system, the neuroendocrine system and the immune system (McEwen, 2006). Chronic stress or deficits of homeostasis regulating systems enhance allostatic load/overload promoting brain and body dysfunction. Consequences of SD and circadian disruption involve elevation of allostatic load mediators such as pro-inflammatory cytokines, cortisol, oxidative stress, glycogen, and insulin (Hirotsu, Tufik, & Andersen, 2015). Also, SD is reported to induce hypertension, reduce parasympathetic tone, increase the risk for obesity, and increase the risk of cardiovascular diseases and diabetes (Hirotsu et al., 2015). The consequences of sleep deprivation suggested that SD is a stressor that contributes to allostatic load leading to brain dysfunction and precipitation of physiological diseases (McEwen, 2006; McEwen & Karatsoreos, 2015).

2.5. Sleep Deprivation and Stress Systems

During sleep, the activity of stress systems declines, consequently, sleep deprivation or extended wakefulness maintains the activity of these systems. Autonomic sympathoadrenal system and hypothalamic-pituitary-adrenal (HPA)

axis are the major neuroendocrine stress response systems; their activation is indicated by elevation of catecholamines and glucocorticoids, respectively. Catecholamines levels show a clear daily rhythm. The plasma levels of adrenaline and noradrenaline are higher during the circadian waking cycle in both humans and rodents (Akerstedt & Froberg, 1979; De Boer & Van der Gugten, 1987). Sleep deprivation is associated with an increase of sympathetic activity indicated by an increase in plasma levels of catecholamine and elevation of blood pressure and heart rate (Lusardi et al., 1996; Tiemeier, Pelzer, Jonck, Moller, & Rao, 2002). Although these changes in sympathetic changes are brief and not significant, chronic or repeated sleep deprivation might, with time, cause permanent changes in the basal level of sympathetic activity increasing the risk of hypertension and cardiovascular diseases (Meerlo et al., 2008). Similarly, the activity of HPA axis showed a pronounced daily pattern regulated by the circadian rhythm; in humans glucocorticoids level is low during the night then peaks in the morning, just before the end of the sleeping phase (Weitzman et al., 1971). In rodents, glucocorticoids level is low during the light phase (resting phase), then rises just before the dark phase (active phase) (De Boer & Van der Gugten, 1987). It has also been suggested that sleep has a suppressive effect on glucocorticoids (Weitzman et al., 1971). However, the effect of SD on cortisol levels in humans is not clear, with some studies reporting mild elevation of cortisol following SD (Leproult, Copinschi, Buxton, & Van Cauter, 1997), others reporting no changes or slight decrease

(Kant, Genser, Thorne, Pfalser, & Mougey, 1984; Leproult et al., 1997). Similar variability in the effect of SD on the HPA axis also was reported in animal studies; with some reporting mild elevation of HPA axis activity following SD (Tobler, Murison, Ursin, Ursin, & Borbely, 1983), others suggesting no effect of SD on glucocorticoids levels (Rechtschaffen, Gilliland, Bergmann, & Winter, 1983). The mixed effects of SD on HPA axis activation might be related to the method, duration, and conditions of SD paradigm used. Additionally, in humans, the mild activation of the HPA axis might not be related to sleep loss per se, but rather due to the physical and mental activities during wakefulness. Although the activation of HPA axis might not be due to the sleep loss per se, it reflects the consequences of daily life stress-related SD, which is usually associated with mental and physical activities of dealing with daily life challenges (Meerlo et al., 2008). In cases of repeated sleep deprivation or chronic insomnia symptoms, the recurrent mild increase of cortisol might be additive leading to cortisol overload. As data suggested that elevation of cortisol might reduce neuronal plasticity and neurogenesis (Sapolsky, 2000), activation of the HPA axis might be the link between sleep loss and mood disorders.

2.6. Sleep Deprivation and Oxidative Stress

Oxidative stress occurs when the production of reactive oxygen species (ROS) or reactive nitrogen species (RNS) overwhelms the antioxidant capacity of the cells (Betteridge, 2000). ROS and RNS comprise of free radical species and non-radical species/oxidants (Pham-Huy, He, & Pham-Huy, 2008). Free radicals are molecules or atoms with unpaired electrons in their outer shell such as superoxide ($O_2^{\cdot}$), hydroxyl ($OH^{\cdot}$), peroxyl ($ROO^{\cdot}$), nitric oxide ($NO^{\cdot}$) and nitrogen dioxide ($NO_2^{\cdot}$). Free radicals are highly unstable and readily participate in further reactions forming non-radical species that are also called oxidants. Examples of non-radical species are peroxynitrite (ONOO-), hydrogen peroxide (H_2O_2), ozone (O_3), hypochlorous acid (HOCl), nitrous acid (HNO_2), and others. These non-radicals, in turn, can efficiently produce free radicals by losing one or more electrons (Halliwell, 2007).

Usually, ROS and RNS are produced in the body as a consequence of redox reactions that accompany different metabolic processes such as oxidative phosphorylation, prostaglandin synthesis or various enzymatic reactions that involve cytochrome P450 enzyme complex (Pacher, Beckman, & Liaudet, 2007). Activation of pathways associated with inflammation, stress, and aging also result in the production of ROS and RNS. At physiologically low to moderate levels, ROS and RNS play significant roles in performing different cellular functions and are

critical for optimum cellular health. For instance, ROS/ RNS participate in various signaling process and cascades in cardiac, epithelial, vascular and neuronal cells. Nitric oxide is an example of oxidant which serves as an intercellular signaling molecule that regulates dilation of arteries, clotting of blood, gastric motility and transmission between neurons (Pacher et al., 2007). Also, ROS plays an essential role in long-term potentiation (LTP) which is a form of synaptic plasticity considered essential for learning and memory (Serrano & Klann, 2004). ROS causes activation of mitogen-activated protein kinases (MAPKs), protein kinases that regulate cellular signal transduction (Son et al., 2011). Moreover, ROS are also a critical part of cellular immune response as they lead to activation of proinflammatory cytokines such as IL-1β and TNF-α (Arsenijevic et al., 2000; Wang, Malo, & Hekimi, 2010). Oxidants such as H_2O_2 oxidize proteins thereby altering their structure and function which then leads to transcription of specific genes or activates specific signaling cascades that regulate normal cellular proliferation, maturation or controlled degradation (Reczek & Chandel, 2015; Schieber & Chandel, 2014).

Generally, antioxidant defense systems in the body ensure that ROS and RNS production in the body is just enough to achieve physiologically beneficial effects. The antioxidant defense mechanisms include enzymatic and non-enzymatic systems. Enzymatic antioxidant defenses include superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT). Non-enzymatic antioxidants

include ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), glutathione (GSH), carotenoids, flavonoids, and others. SOD exists in two forms: cytosolic Cu/Zn-SOD, and mitochondrial Mn-SOD. SOD catalyzes the dismutation of the highly reactive superoxide anion $O_2^{\cdot}$ to the less reactive species; H_2O_2 and O_2. Hydrogen peroxide can be destroyed by CAT or GPX reactions. CAT reacts very efficiently with H_2O_2 to form water and molecular oxygen. Glutathione peroxidase (GPx) catalyzes the reduction of hydro peroxides using GSH (Kleinova et al., 2007). Glutathione system is one of the essential anti-oxidative defense mechanisms. Glutathione (GSH) is a tripeptide that contains L-cysteine, L-glutamic acid and glycine. Glutathione exists in reduced (GSH) and oxidized (GSSG) states. Reduced glutathione (GSH) is a major antioxidant molecule in cells. It provides reducing equivalents for the GPx which results in the formation of a disulfide bond between two GSH molecules generating oxidized glutathione (GSSG). The enzyme glutathione reductase (GSR) recycles GSSG to GSH (Masella, Di Benedetto, Vari, Filesi, & Giovannini, 2005).

In normal conditions, there is a balance between pro-oxidant production and the intracellular levels of antioxidants. This balance is essential for the survival of organisms and their health (Valko et al., 2007). However, various factors lead to excessive production of these reactive species setting off the balance maintained by the antioxidants, thus resulting in oxidative stress **(Figure 1)**. Once the levels of ROS and RNS increase beyond an optimum level, the resultant oxidative stress

leads to cellular damage by oxidizing lipids, proteins, and DNA. Oxidative stress-associated cellular damage and alteration of cellular components initiates a cascade of underlying mechanisms that eventually lead to different diseases. Increase in oxidative stress induces an inflammatory response via the release of proinflammatory cytokines such as TNF-α and IL-6. Other mediators of inflammation such as nuclear factor-kappa B (NF-κB), vascular cell adhesion molecule-1 (VCAM-1) are also activated by ROS and RNS. This inflammatory response is an underlying causal mechanism for various diseases such as diabetes, chronic renal failure, hypertension, chronic obstructive pulmonary diseases (COPD) and rheumatoid arthritis as well as neurodegenerative disorders like Parkinson's disease and multiple sclerosis (Aruoma, Grootveld, & Bahorun, 2006; Butterfield, Castegna, Drake, Scapagnini, & Calabrese, 2002; Hoshino & Mishima, 2008; MacNee, 2001; Prabhakar, 2013).

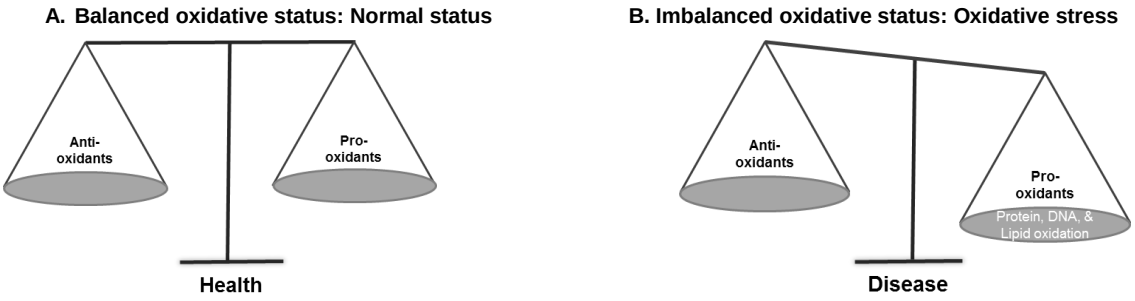


Figure 1. Oxidative status in health and disease: A. Balanced oxidative status, the production of pro-oxidants is equal to anti-oxidant concentration, maintaining optimum health. **B.** Imbalanced oxidative status, the production of pro-oxidants outweigh the concentration of antioxidants, causing oxidative stress and related diseases.

The central nervous system (CNS) is especially vulnerable to the effects of oxidative stress owing to the relatively low level of antioxidants in the brain and high level of transition metals such as Fe^{2+} that form reactive, unstable complexes and generate more free radicals (Rahal et al., 2014). Also, levels of polyunsaturated fatty acids (PUFAs) and lipids are very high in the CNS. These lipids and PUFAs are well-known targets of oxidative stress and undergo free-radical induced lipid peroxidation and oxidation (Cherubini, Ruggiero, Polidori, & Mecocci, 2005). Moreover, various redox reactions that result in the production of ROS and RNS occur in the brain to meet the high oxygen and energy requirement of the (Butterfield et al., 2002). Therefore, it seems likely that excessive levels of ROS and RNS in the brain might alter the structure and function of different brain regions resulting in neurological and psychological conditions. However, neuronal response to oxidative stress is not uniform, which makes some regions of the brain more vulnerable to oxidative stress than others. For example, it has been reported that the cortex and the hippocampus are the most sensitive brain regions to oxidative stress (Wang & Michaelis, 2010).

Both clinical and preclinical studies have indicated that behavioral and memory deficits are associated with oxidative stress elevation. For example, in an animal model of stress, elevation in markers of oxidative stress in the hippocampus of socially defeated rats was associated with induction of anxiety, depression-like behavior and memory impairment (Patki, Solanki, Atrooz, Allam, & Salim, 2013).

Depressed patients showed an increase in oxidative stress markers in serum indicated by a decrease in antioxidant enzymes and an increase in the lipid peroxidation marker, malondialdehyde (MDA), as compared to age-matched control group (Stefanescu & Ciobica, 2012). Anxiety disorders in humans were also associated with elevation of lipid peroxidation products and alteration of antioxidant enzymes (Ng, Berk, Dean, & Bush, 2008). Additionally, children and adolescents with anxiety disorders showed plasma elevation of oxidative stress markers (Guney et al., 2014).

One of the proposed functions of sleep is promoting anti-oxidative mechanisms. This perhaps is an adaptive response to sleep loss/deprivation as SD or sleep loss might induce oxidative stress. For instance, it has been suggested that sleep promotes the removal of free radicals accumulated during wakefulness (Reimund, 1994). Peroxide infusion into preoptic/anterior hypothalamus region of brain induced sleep in rats by promoting the release of neuromodulators, nitric oxide, and adenosine (Ikeda et al., 2005). Furthermore, intraventricular infusion of the oxidized glutathione (GSSG) enhanced the SWA sleep and REM sleep (Honda, Komoda, & Inoue, 1994). Indeed, some studies have suggested that oxidative stress is the underlying pathophysiological mechanism for the development of cardiovascular and neurobehavioral complications mediated by obstructive sleep apnea (Gozal & Kheirandish-Gozal, 2008). Animal models also have provided valuable insights into the relationship between oxidative stress and

sleep deprivation (Villafuerte et al., 2015). Chronic SD in rats decreased SOD activity in the hippocampus and brainstem (Ramanathan, Gulyani, Nienhuis, & Siegel, 2002), and decreased total glutathione (GSH) levels and catalase activity in liver, but increased glutathione peroxidase (GPx) activity in the heart (Everson, Laatsch, & Hogg, 2005). Short periods of SD (96h) decreased the GSH levels in rat hippocampus (D'Almeida et al., 1998). Twenty-four hours sleep deprivation in rats reduced lipid peroxidation in the hippocampus (Melgarejo-Gutierrez et al., 2013) and increased the level of glyoxalase (GLO-1) and glutathione reductase (GSR-1) in the prefrontal cortex, hippocampus, and amygdala (Vollert et al., 2011). While acute SD (6h) in rats by gentle handling increased the level of the reduced glutathione (GSH) levels in the cortex, brain stem, and forebrain and enhanced the activity of GPx in hippocampus and cerebellum (Ramanathan, Hu, Frautschy, & Siegel, 2010). Collectively, these data suggest that while short periods of SD enhances anti-oxidant response in the brain and other organs, long SD periods reduce the anti-oxidant response suggesting that extended wakefulness induces chronic oxidative stress that leads to failure of antioxidant mechanisms to sustain the accumulation of pro-oxidants.

2.7. Sleep Deprivation and Synaptic Plasticity

Synaptic plasticity represents the activity-dependent modification of strength or efficacy of synaptic transmission at pre-existing synapses. It plays an essential role in the capacity of the brain to incorporate new experiences into persistent memory circuits. Synaptic plasticity is the cellular mechanism underpinning learning and memory; it also involves in fear conditioning and stress reaction. Synaptic plasticity has a vital role in the development of neuronal circuits during early life brain development, in fact, deficits of synaptic plasticity mechanism contribute to the development of psychiatric disorders (Citri & Malenka, 2008). Excitatory synapses express different forms of synaptic plasticity, the most prominent forms are long-term potentiation (LTP) and long-term depression (LTD). Synaptic plasticity is indicated by changes in synapse number/size or by measurement of synaptic strength indicated by LTP and LTD. Two types of glutamate receptors contribute to postsynaptic response and synaptic plasticity include α-amono-3-hydroxy-methyl-4-isoxazole propionic acid receptors (AMPAR) and N-methyl-D aspartate receptors (NMDAR). AMPARs are ionotropic channels that are permeable to K^+ and Na^+; they are composed of four subunits, named GluA1-4. Phosphorylation of GluA1 subunit is fundamental for functional regulation of AMPARs mediated synaptic plasticity. In fact, synaptic trafficking of AMPARs is strongly correlated with synaptic strength (Kessels & Malinow, 2009). AMPARs mediate the majority of fast excitatory synaptic transmission. When the cell is close

to its resting membrane potential, activation of AMPARs generates the excitatory postsynaptic response. NMDARs are assembled from seven types of subunits (GluN1, GluN2A-D, and GluN3A-B). The distinct combination of NMDAR subunits is critical for determination of the polarity of synaptic plasticity, for instance, blockage of GluN2A abolished the induction of LTP, while blockage of GluN2B abolished the induction of LTD in hippocampal synapses (Liu et al., 2004). NMDARs are voltage dependent because of the blockage of its channel with Mg^{2+} at the resting membrane potential when the cell is depolarized, Mg^{2+} dissociates from the channel allowing entrance of Na^{+} and Ca^{2+} into the cell. The influx of Ca^{2+} activates autophosphorylation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). Indeed, NMDA-dependent LTP is associated with activation of CaMKII (Fukunaga, Stoppini, Miyamoto, & Muller, 1993). Activation of CaMKII regulates the recruitment of GluA1subunit to the cell membrane which enhances synaptic potentiation and hence synaptic strength (Appleby et al., 2011). Furthermore, synaptic plasticity is associated with increased expression of many other proteins such as presynaptic synapsin 1 and postsynaptic density protein (PSD95), in addition to dendritic CaMKII and microtubule-associated protein 2 (MAP2) (Kornau, Schenker, Kennedy, & Seeburg, 1995; Roberts et al., 1998; Sato, Morimoto, Suemaru, Sato, & Yamada, 2000). Interestingly, expression of PSD95 and NMDA receptors showed a similar pattern and GluN2B subunits co-localized

with PSD95 in cultured rat hippocampal neurons, suggesting the importance of this interaction on synaptic plasticity at excitatory synapses (Kornau et al., 1995).

Synaptic plasticity is also regulated by many other factors such as the neuronal growth factors and downstream signaling pathways. The brain-derived neurotrophic factor (BDNF), and the cyclic AMP response element binding protein (CREB) are among the factors that play an important role in synaptic plasticity (Figurov, Pozzo-Miller, Olafsson, Wang, & Lu, 1996). These factors and the pathways that control them might be critical for stress response, and disruption of these factors by repeated or severe stress might contribute to the pathophysiology of mood disorders (**Figure 2**) modified from (Duman, 2002b).

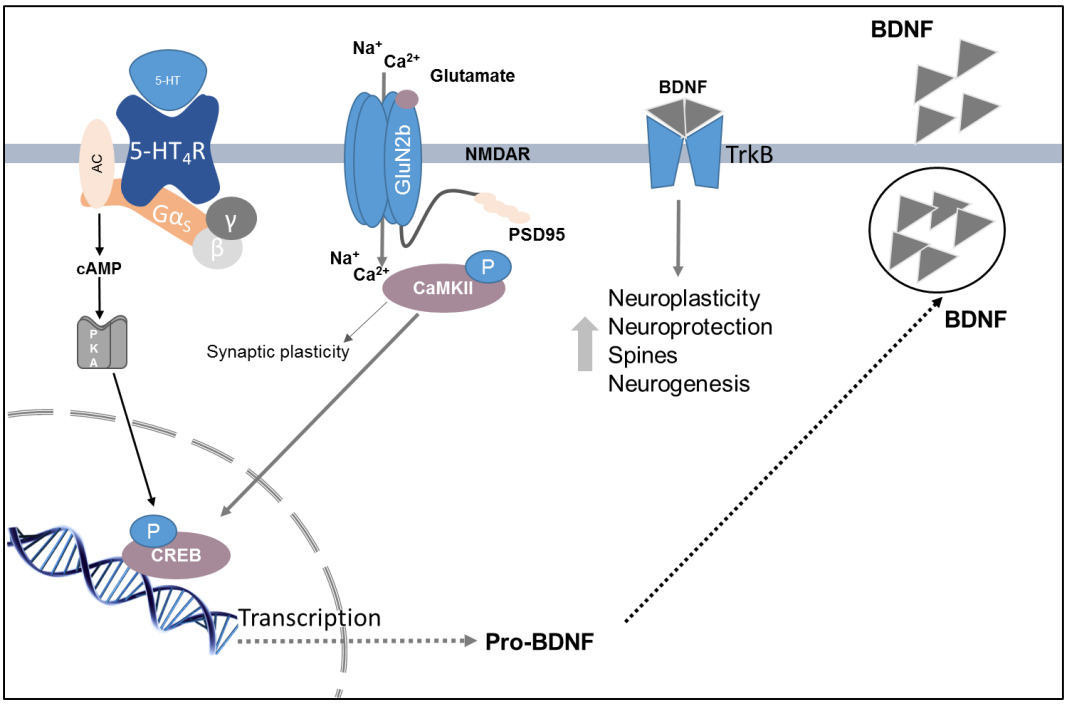


Figure 2. Brain-derived neurotrophic factor (BDNF) and neuronal plasticity. Neuronal activity enhances phosphorylation of cAMP response elements (CREB) leading to induction of the neurotrophic factor BDNF which in turn induces neuronal plasticity, synaptic growth, and neurogenesis. Modified from (Duman, 2002b).

In fact, post-mortem studies have found a reduction in CREB immunoreactivity in the temporal cortex of depressed subjects (Dowlatshahi, MacQueen, Wang, & Young, 1998). Moreover, agents that are used for the treatment of mood disorders such as the selective serotonin reuptake inhibitors (SSRI) have been reported to upregulate CREB and BDNF (Manji, Moore, & Chen, 2000). Studies utilizing

animal models of stress have demonstrated that stress down-regulates BDNF and disrupts CREB function. Disruption of CREB function is proposed to contribute to the pathology of depression-like behavior in rodents (Duman, 2002a; Duman, Malberg, Nakagawa, & D'Sa, 2000). Interestingly, overexpression of CREB or BDNF in the dentate gyrus of rat hippocampus produced an anti-depressant like effect as indicated by a forced swim and learned helplessness tests (Chen, Shirayama, Shin, Neve, & Duman, 2001; Shirayama, Chen, Nakagawa, Russell, & Duman, 2002).

BDNF is the most abundant neurotrophic factor in the brain. BDNF by binding to tropomyosin-related kinase B (TrkB) receptor, activates three major pathways: Phosphatidyl inositol 3-kinase (PI3K-Akt), Ras-mitogen-activated protein kinases (Ras-MAPK), and phospholipase Cγ (PLCγ)-Ca^{2+} pathways. BDNF activated pathways regulate neuronal survival, growth, and plasticity. The Ras-MAPK pathway including extracellular regulated kinase (ERK) and MAP/ERK kinase (MEK) and the (PI3K-Akt) pathway plays a role in depression and treatment response. The activity of ERK and MEK are negatively regulated by the dual specificity phosphatase, MAPK phosphatase (MKP-1) **(Figure 3)**.

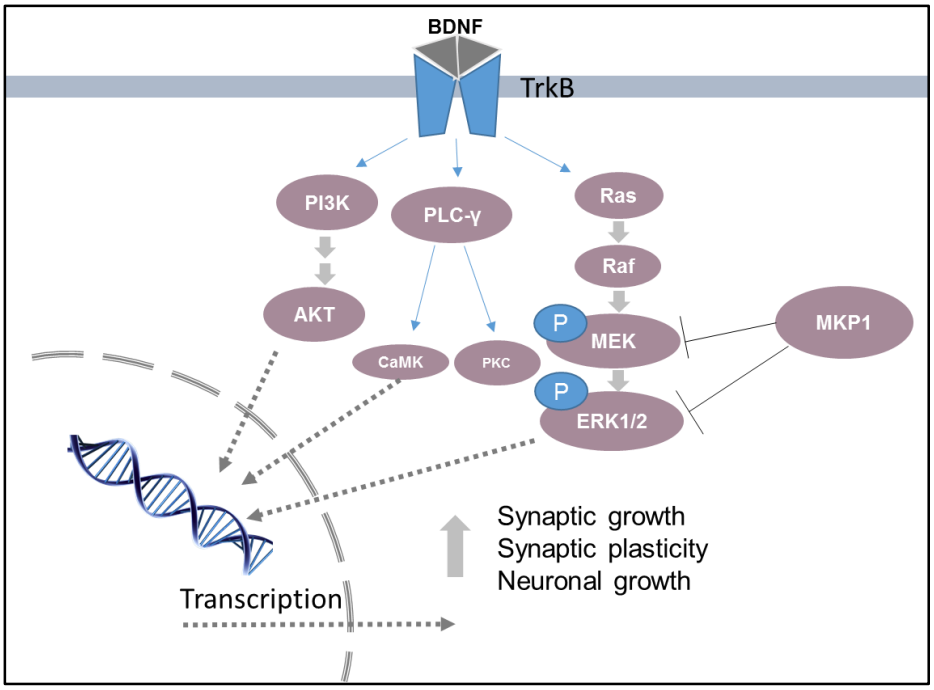


Figure 3. The neurotrophic response in depression. BDNF activated pathways regulate neuronal survival, growth, and plasticity which play a role in depression and treatment response. BDNF by binding to tropomyosin-related kinase B (TrkB) receptor, activates three major pathways: Phosphatidyl inositol 3-kinase (PI3K-Akt), Ras-mitogen-activated protein kinases (Ras-MAPK), and phospholipase Cγ (PLCγ) pathways. The activity of ERK and MEK are negatively regulated by the dual specificity phosphatase, MAPK phosphatase (MKP-1), modified from (Duman, 2004).

BDNF and its downstream signaling pathways are decreased in stress and depression. As a negative regulator of MAPK signaling pathway, MKP-1 expression was found to be significantly increased in hippocampal regions of depressed subjects as well as in rodent models of depression (Goldstein & Walker, 2014). BDNF and MKP-1 levels are altered as a response to stress and elevation

in oxidative stress. Oxidative stress induces HPA axis activation, which reportedly increases glucocorticoids expression, promoting glutamate release and increasing intracellular calcium concentrations. Calcium activates calpain causing calpain-dependent BDNF degradation (Salim et al., 2011). Glucocorticoid receptors also bind to *MKP-1* promoter and induce its expression (Kaushal, Nair, Gozal, & Ramesh, 2012). Furthermore, stress, inflammation, or apoptosis cause activation of p38 and JNK kinases which increases stress response and neuroinflammation leading to neuronal cell death. As a result, *MKP-1* expression increases leading to dephosphorylation of P38 and JNK and hence attenuates their detrimental effects (Collins, Downer, Toulouse, & Nolan, 2015). However, accumulation of MKP-1 negatively affects BDNF-Ras-MAPK signaling cascade. Interestingly, expression of *MKP-1* is regulated by sensory neuronal activity and it is frequently correlated with BDNF (Doi et al., 2007; Genoud, Knott, Sakata, Lu, & Welker, 2004; Horita, Wada, Rivas, Hara, & Jarvis, 2010). Indeed, conditional deletion of BDNF in mice PFC resulted in decreased MKP1 expression (Glorioso et al., 2006), and treatment of cultured neurons with BDNF significantly enhanced the level of MKP1 expression (Jeanneteau, Deinhardt, Miyoshi, Bennett, & Chao, 2010), suggesting that BDNF might regulate *MKP1* expression (Jeanneteau & Deinhardt, 2011). During development, MKP1 was suggested to regulate axonal arborization promoted by BDNF and neuronal activity (Jeanneteau & Deinhardt, 2011). Dephosphorylation of the c-Jun N-terminal kinase (JNK) which contribute to

microtubule stabilization is suggested to be the mechanism by which MKP1 overexpression alters axonal branching (Jeanneteau & Deinhardt, 2011). Knowing that MKP1 is an inducible stress gene, the role of MKP1 in microtubule stabilization and hence axonal and dendritic branching might explain the link between stress exposure during critical periods of brain maturation and developmental of affective disorders. Indeed, in fact, it has been suggested that chronic overexpression of MKP1 might contribute to the gradual development of depression (Jeanneteau & Deinhardt, 2011).

A growing body of evidence suggests that alteration of synaptic density and plasticity is associated with mood disorders. Reduction in volume of PFC and hippocampus is consistently reported in major depressive disorders (Campbell, Marriott, Nahmias, & MacQueen, 2004; MacQueen, Yucel, Taylor, Macdonald, & Joffe, 2008; Savitz & Drevets, 2009). Reduction in volume is correlated with decreased number and size of glial cells, and neuronal shrinkage and loss of synapses (Price & Drevets, 2010). Post-mortem studies on depressed subjects showed a reduction in synapse numbers in PFC (Kang et al., 2012). Reduced expression of synaptic proteins (e.g., synapsin, SNAP25, PSD95), AMPAR subunits (GluA1and 3), NMDAR subunits (GluN1, GluN2A, and GluN2B), and BDNF were reported in PFC and hippocampus of post-mortem samples obtained from subjects with the major depressive disorder (Marsden, 2013). The data provided evidence of the disruption of synaptic plasticity and density within the

hippocampus and PFC in depression. Alteration of synaptic plasticity in depression is further implicated with cognition and emotion disruption (Marsden, 2013).

An emerging body of data suggests a tight association between sleep and neuronal recovery and plasticity (Frank, Issa, & Stryker, 2001; Huber & Born, 2014; Wilhelm et al., 2014). Synaptic plasticity is crucial for learning, memory consolidation, and emotion (Krueger, Obal, & Fang, 1999; Stickgold, 1998; Tononi & Cirelli, 2014). Ultimately, SD results in impairment of neuronal plasticity and hence, cognition deficits and emotion dysregulation (Kreutzmann, Havekes, Abel, & Meerlo, 2015). The synaptic homeostasis of sleep/wakefulness regulation suggests that during sleep spontaneous neuronal activity recalibrates synaptic strength and restores cellular homeostasis (Tononi & Cirelli, 2014). Measurement of synaptic strength in the human cortex using noninvasive EEG recording, (Kuhn et al., 2016) revealed that synaptic strength was increased while LTP was decreased after sleep deprivation. Neuroimaging studies in humans reported a reduction in hippocampus volume after sleep deprivation in consecutive nights as well as in patients with insomnia or other sleep disorders (Dusak et al., 2013; Riemann et al., 2007; Taki et al., 2012). The reduction in hippocampal volume following SD might be due to dendritic atrophy or loss of synapse. As a result downregulation of neurotrophic signaling pathways (Kreutzmann et al., 2015). In animal models of SD, induction of LTP was inhibited in hippocampus slices isolated from rats after 12 hours of SD (Campbell, Guinan, & Horowitz, 2002).

Additionally, 24 and 48 hours SD in rats caused LTP impairment in hippocampus as indicated by in vivo extracellular recording (Alhaider 2011, Alhaider, 2010). Expression of synaptic plasticity markers including; BDNF, synapsin 1, CREB, and CaMKII was significantly reduced in the hippocampus of rats following 8 or 24 hours SD (Guzman-Marin et al., 2006). Moreover, phosphorylation levels of CaMKII were significantly reduced following high field stimulation of CA1 region of hippocampus in rats that had been deprived of sleep for 24 hours as compared to rested rats (Alhaider, 2010). The above data suggest the fundamental role of sleep in synaptic plasticity which is potentially impaired in psychiatric and cognition disorders.

2.8. Sleep Deprivation in Children and Adolescents

According to a national survey conducted in 2014 by the National Sleep Foundation, approximately 55% of children and adolescents do not obtain adequate sleep per night. Further, inadequate sleep is more common at older ages; for example, 31% of children 6-11 years old sleep less than the recommended 11 hours per night, while 56% of adolescents 15-17 years old sleep less than the recommended sleep hours per night **(Figure 4)** (National Sleep Foundation, 2014 sleep in America poll). Comparisons between cross-sectional studies conducted between 2003 and 2012 by the National Survey of Children's

Health on children 6 to 17 years old, (Hawkins & Takeuchi, 2016) found a consistent increase of inadequate sleep among all studied age groups, and the shorter sleep duration increased with age. Sleep deprivation in youth has been associated with adverse physical, mental and behavioral outcomes, therefore, increasing the amount of sleep in youth has become a public health priority in the United States. In fact, members of the American Academy of Sleep Medicine have released a consensus statement of the recommended amount of sleep for youth to promote optimal physical and mental health (Paruthi et al., 2016).

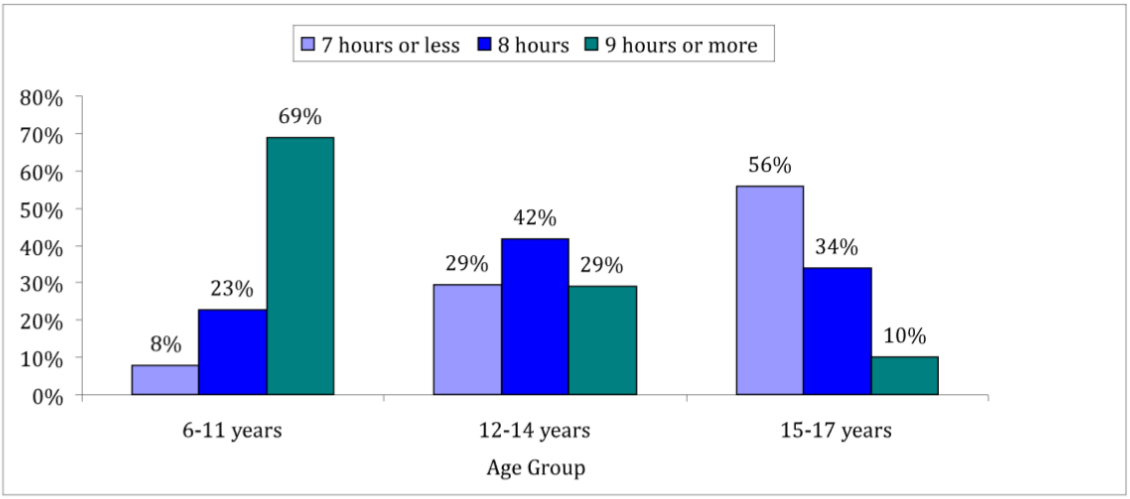


Figure 4. Sleep hours in children and adolescents. Data from the US population-based studies, National sleep foundation, 2014.

Why children and adolescents are getting less sleep? Several studies investigated the causes of a decrease in sleep duration among youth. Bedtime

delay and short sleep duration in school-aged children and adolescents have been consistently related to the extensive exposure of electronic media including television viewing, use of computers, electronic gaming, mobile telephones, and music (Cain & Gradisar, 2010). Early school start times and academic workload are also lead to insufficient sleep in school-aged children and adolescents (Wheaton, Chapman, & Croft, 2016). Furthermore, sleep disturbances are common among youth with psychiatric disorders such as anxiety, depression, autism, or schizophrenia (Alfano, Pina, Zerr, & Villalta, 2010; Gregory & Sadeh, 2016; Hodge, Carollo, Lewin, Hoffman, & Sweeney, 2014; Yilmaz, Sedky, & Bennett, 2013) and in children who are exposed to violent events (Spilsbury, Babineau, Frame, Juhas, & Rork, 2014). The detrimental effects of insufficient sleep in youth on mental and behavioral outcomes have been reported in many epidemiological and clinical studies, some are discussed in the next section.

2.9. Consequences of Sleep Deprivation in Children and Adolescents

In otherwise healthy children, insufficient sleep has been linked to poor school performance (Astill et al., 2012; Dewald et al., 2010), behavioral problems such as attention deficit, and emotional symptoms such as anxiety and depression (Gregory & Sadeh, 2012; Maski & Kothare, 2013). In a longitudinal study, sleep deprivation in children 2.5-6 years old was associated with hyperactivity and poor

behavior at school (Touchette et al., 2009; Touchette et al., 2008). Later onset of anxiety and depression are also associated with early life (childhood and adolescent) sleep disturbances (Gessa et al., 1995; Gregory et al., 2005). The longitudinal studies suggest that early life sleep deprivation are highly predictive of future affective and behavioral problems (Maski & Kothare, 2013). It is not understood how sleep at early life modulates neurobehavioral functioning and cognition. Some investigators have suggested that sleep spindles and slow wave activity, which show significant changes through childhood and adolescents might have a role in neurobehavioral and executive functional development. However, further research is needed to investigate the association between sleep and neurobehavioral development (Kurth, Olini, Huber, & LeBourgeois, 2015; Lopez, Hoffmann, & Armitage, 2010; Maski & Kothare, 2013).

2.10. Sleep and Postnatal Brain Development

Brain development starts prenatally and continues postnatally in all mammals. Postnatal brain development is characterized by cortical gray matter expansion/regression and white matter development. Gray matter maturation is indicated by a massive increase of synapse (synaptogenesis), that peaks at childhood (2-3 years old) followed by rapid elimination and refinement of excess synapses (synaptic pruning) which continues until adolescent stage. White matter

development is indicated by neuronal myelination which occur during early life stages (childhood and adolescence) and continues until early adult stage (**Figure 5)**. These processes are important for optimization of neuronal connectivity and maturation of neurobehavioral functions (Semple et al., 2013).

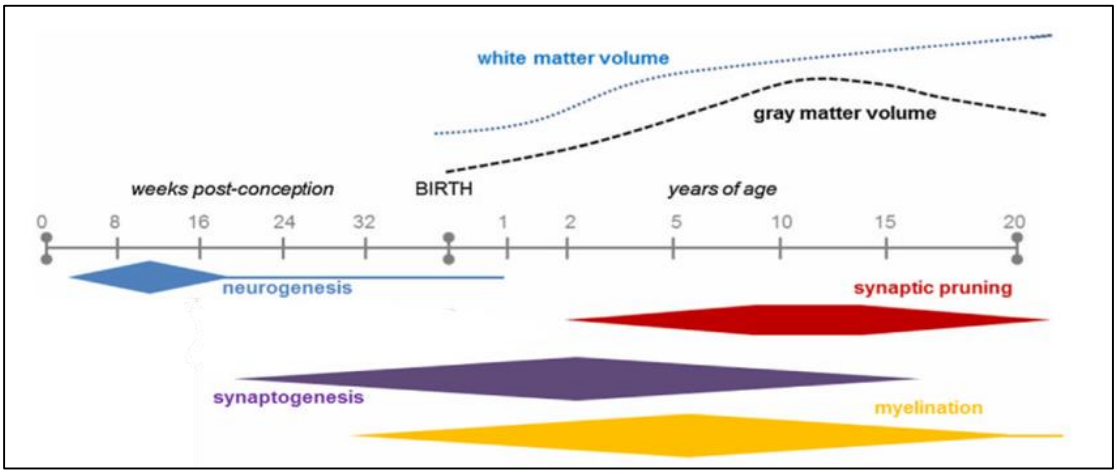


Figure 5. Major developmental processes in human brain during gestation and up to 20 years age. Synaptogenesis (purple color) is associated with the increase of grey matter volume, and synaptic pruning (red color) is associated with its reduction. Neuronal myelination (yellow color) is associated with the increase of white matter volume, modified from (Semple et al., 2013).

Interestingly, sleep architecture shows remarkable changes during development that parallel the time course of postnatal brain maturation, suggesting a relationship between postnatal brain development and sleep (Kurth et al., 2015;

Semple et al., 2013). REM sleep is highly concentrated in newborns consisting around 50% of their total sleep. During the first two years of life, REM sleep diminishes to 20-25% of total sleep time that maintained throughout development (Louis, Cannard, Bastuji, & Challamel, 1997). REM sleep is found to promote cortical plasticity necessary for consolidation of waking experiences in developing brain (Dumoulin Bridi et al., 2015). Furthermore, localization, distribution, and coherence of sleep EEG from early childhood to late adolescence reflect the brain maturational processes. Cortical maturation follows a posterior to anterior trajectory, similarly, SWA shifts from posterior to anterior brain regions with maturation (Kurth et al., 2010b). Also, the coherence of EEG activity, which measures the functional connectivity strengthen by neuronal myelination, increases with maturation in a region-specific manner (Kurth, Achermann, Rusterholz, & Lebourgeois, 2013; Tarokh, Carskadon, & Achermann, 2010). Myelination is important for brain connectivity and functional network maturation. Oligodendrocytes wrap the axons with myelin sheaths forming multiple insulating layers that increase the action potential conduction by 1000 folds. Neuronal myelination is reflected by the increase in the volume of the white matter in the brain. In humans, neuronal myelination started prenatally and continued through the early adult life. In rodents, neuronal myelination continues until at least postnatal day (PND) 30 to 40. Interestingly, oligodendrocytes proliferation and myelin synthesis occur preferentially during sleep (Toth & Neumann, 2013), which

suggests that neuronal activity during sleep, by inducing neuronal myelination, enhances functional connectivity during brain development (Kurth et al., 2013; Kurth et al., 2015). Hence, sleep is essential for neuronal network connectivity and brain maturation during the sensitive window of childhood.

Another important process during postnatal brain development is synaptogenesis. Synaptogenesis is the process of formation of new synapses between neurons. During brain development, there is a period of synapse overproduction followed by synapse pruning and elimination. These processes reflect the refinement of neuronal circuits during childhood and adolescence inducing synaptic plasticity and cortical maturation essential for efficient processing of adult cognition. In humans, the density of synapses increases rapidly after birth to reach 50% over the adult level by the age of 2-3 years (Huttenlocher, 1979, 1984; Huttenlocher & Dabholkar, 1997; Huttenlocher, de Courten, Garey, & Van der Loos, 1982). In rodents, synaptic density peaks during the second week after birth and reaches the adult level by week 3 to 4 of age (Crain, Cotman, Taylor, & Lynch, 1973; Semple et al., 2013). However, the timing of appearance of synapse peak and elimination is region specific. The increase in synaptic density is correlated with the increase of N-Methyl-D-Aspartic Acid Receptors (NMDARs) density in the cortex which peaks at the age of 1-22 years in humans and PND28 in rats (McDonald, Johnston, & Young, 1990; Zhang, 2006; Zhong, Carrozza, Williams, Pritchett, & Molinoff, 1995). Interestingly, Studying the synaptic density

using two-photon microscopy in adolescent mice showed that sleep is associated with a net loss of cortical spine while net gain of the spine was increased during wakefulness, (Maret, Faraguna, Nelson, Cirelli, & Tononi, 2011). Furthermore, in a longitudinal study of sleep EEG across childhood and adolescence, (Campbell & Feinberg, 2009; Feinberg & Campbell, 2010), a very steep decline of NREM delta and theta waves were reported during adolescent stage (11-16.5 years). The study suggested that EEG changes might reflect synaptic pruning indicated by a reduction in cortical thickness as measured by MRI in adolescents (Shaw et al., 2008). In a cross-sectional study including children and adolescents, the topography of SWA was highest over the posterior brain region in children then shifted to the frontal cortex in adolescents, which matches the course of gray matter maturation (Kurth et al., 2010a) (Smith, Wilkins, Mogavero, & Veenema, 2015). The fact that SWA amplitude increases during childhood to reach maximum during puberty then decreases during adolescence, suggests that SWA might be the driving mechanism underlying brain maturation processes (Ringli & Huber, 2011). Indeed, measuring the cortical volume and the synaptic density across development in humans and rats found a clear correlation between the trajectories of SWA coherence and cortical maturation **(Figure 6)** (Kurth et al., 2015). This proposed role of SWA in brain maturation is further strengthened by the emergence of synaptic homeostasis hypothesis mentioned above. Hence, SWA can be an indicator of synaptic density and strength during development (Ringli &

Huber, 2011). Cortical maturation is also correlated with progressive changes in behavioral and cognitive functions from childhood to adulthood, this behavioral and cognitive development is thought to rely on synaptic pruning and neuronal myelination (Luna & Sweeney, 2004), supporting the link between SWA, cortical maturation and behavioral development. Therefore, this window of development is highly sensitive to sleep deprivation. Yet the exact relationship between brain maturation, SWA and behavior needs further investigation, as several neurobehavioral disorders are associated with sleep problems, suggesting a bidirectional relationship between sleep and neurobehavioral disorders.

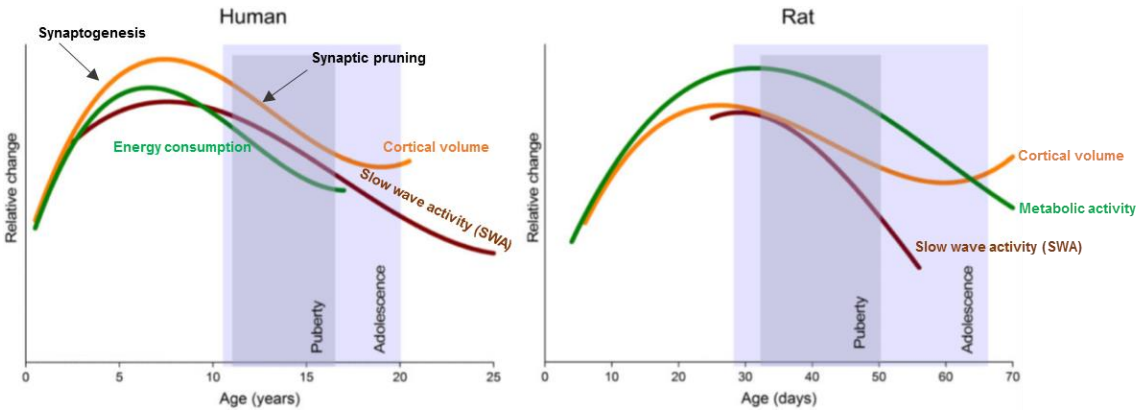


Figure 6. Sleep and cortical maturation in humans and rats. Changes in cortical volume and synaptic density occur in parallel with changes of slow wave activity (SWA) and metabolism, modified from (Kurth et al., 2015).

2.11. Brain Regions of Interest

The association between PFC and the limbic structures including the hippocampus, the amygdala, and the hypothalamus are critical for long-term memory and processing of internal states such as mood and motivation. Disruption of this circuits that originate in PFC results in neuropsychiatric and cognitive disorders (Carlson, Singh, Zarate, Drevets, & Manji, 2006). The structure and function of these brain regions that are part of the limbic circuit are discussed in the following sections.

Prefrontal cortex

The prefrontal cortex (PFC) is a collection of interconnected neocortical areas that sends and receives projections from cortical sensory systems, motor systems, and subcortical structures. The PFC provides a setting where information from wide-ranging brain systems can interact through relatively local circuitry (Miller & Cohen, 2001). The PFC is particularly important for the executive control of behavior. Patients with damaged PFC have normal perceptive and motor behavior and may perform normally in intelligence tests, yet, they are unable to effectively function in their daily life. They lack concentration and hence their behavior is disorganized and they are unable to carry out plans. They are also characterized by abnormal emotional state (Miller & Cohen, 2001).

The prefrontal cortex consists of the dorsolateral-PFC (dlPFC) and the ventromedial-PFC (vmPFC). The dlPFC is critical for cognitive control of behavior. The vmPFC is critical for social emotions and related feelings, patients with damage to this region of PFC results in impaired social emotions characterized by antisocial personalities. During an emotional response, the vmPFC operates in parallel with the amygdala to shape the mental plans conceived as a response to the triggering stimuli by governing the attention accorded to certain stimuli and influence the content retrieved from the memory (Kandel et al., 2013). The critical function of PFC is the regulation of top-down processing. When the mappings between sensory inputs, thoughts and the behavior is weakly established, the PFC is needed to coordinate between the internal representations of goals and the means to achieve them (Miller & Cohen, 2001). Studies on humans and monkeys showed that the PFC exhibit the properties required for top-down cognitive control of behavior. The multimodal convergence and integration of behaviorally relevant information, the sustained activity in the face of interference, the feedback pathways that can influence other brain structures, and the continuous plasticity that allows adaptation to the demands are unique characteristics of the PFC that supports its role in cognitive control of behavior (Miller & Cohen, 2001).

Hippocampus

The hippocampus formation consists of the hippocampus, the dentate gyrus, and the subiculum. The hippocampus receives inputs from cortical regions including mainly the entorhinal cortex in addition to perirhinal cortex and postrhinal cortex. Hippocampus also receives inputs from subcortical regions including; the medial septum, the locus cereolus, the raphe nucleus, and the amygdala. The hippocampus has a major outputs to the lateral septum, the nucleus accumbens, the PFC, and the amygdala (Knierim, 2015).

The major function of the hippocampus and the adjacent brain regions is to support the creation of new declarative memories. Patients with hippocampal damage can retain memories of events that occurred prior to hippocampus damage, but they cannot form new episodic memories to remember events of their daily lives (Knierim, 2015). The hippocampus is also implicated in mood disorders. Hippocampal atrophy has been correlated with depression, however, whether reduced volume of hippocampus is a result of or a risk factor for depression it is not known yet (Kandel et al, 2013).

Amygdala

The amygdala is involved in analyzing the emotional and the motivational significance of the sensory stimuli. The amygdala receives inputs directly from sensory systems, and it projects neuronal relays to the neocortex, basal ganglia,

hippocampus, hypothalamus, and other subcortical structures (Kandel et al., 2013). The amygdala is a critical regulatory region in circuits of emotion, damage to amygdala consistently alters the emotional behavior. Studies on both animals and humans showed that amygdala is implicated in fear responses. Patients with damage to amygdala fail to recognize facial expressions of fear, they also fail to undergo fear conditioning. Amygdala is also involved in positive emotions particularly, the processing of rewards.

Giving the fact that amygdala is involved in processing of negative emotions, it is not surprising to know that amygdala is also implicated in anxiety and mood disorders. The enlargement of the amygdala and the increase in its basal activity have been repeatedly reported in depression, bipolar, and anxiety disorders (Kandel et al., 2013).

2.12. Methods for Sleep Deprivation in Animals

To elucidate the cellular and molecular basis of sleep deprivation associated detrimental effects, several methods have been developed to induce sleep deprivation in animals, mainly in rodents. Some of these methods and the advantages/disadvantages of these methods are discussed below.

2.12.1. Platform over Water

This method selectively deprives rodents of REM sleep. In this method, the rodents are placed in a tank with single or multiple platforms surrounded by water. The size of the platforms allows the animals to sleep on it, but once they lose their muscle tone as they enter REM sleep, they fall in the water and are awakened, hence preventing them from entering REM sleep (Jouvet, Vimont, Delorme, & Jouvet, 1964). The control animals placed in a similar chamber with larger platforms that allow the animals to sleep. However, control animals in this method have been reported to show some alterations in behavioral and neuronal functions, suggesting the involvement of factors other than REM sleep deprivation as being responsible for some of the phenotypes (Marks & Wayner, 2005).

2.12.2. Forced Locomotion

The animals in this method are placed individually in a chamber with a rotating drum or revolving floor which forces the animals to keep moving. This method induces total SD; it can also be modified to selectively target one sleep stage by continuous EEG monitoring (Friedman, Bergmann, & Rechtschaffen, 1979; Roman, Van der Borght, Leemburg, Van der Zee, & Meerlo, 2005). However, the forced activity may cause fatigue for the animals inducing external stress, which has the potential to mask the effect of SD (Alkadhi et al., 2013; Havekes et al., 2012)

2.12.3. Gentle Handling

The animals in this method are disturbed by the gentle shaking of their home cages or continuous introduction of new objects or nesting materials (Alkadhi et al., 2013; Hagewoud et al., 2010). This method is very effective at inducing total SD. However, this method involves constant vigilance by the investigator which makes it suitable only for short periods of SD. Moreover, personnel involvement and the introduction of new objects or nesting materials might present as additional confounding factors (Alkadhi et al., 2013; Havekes et al., 2012)

2.12.4. Pinnacle Automated SD System

This apparatus consists of a cylindrical cage with a rotating bar at the base, while the bar is rotating, it gently touches the animal's feet keeping them awake. This system effectively produces sleep deprivation in rats as validated by polysomnography (Hines, Schmitt, Hines, Moss, & Haydon, 2013; Wooden et al., 2014).

In the present study, we used the pinnacle automated SD system to deprive the rats of sleep. This system utilizes an automated rotating bar which gently pushes the pups to move in a timed manner. The rotating bar is not only gently and constantly disrupts the sleep of pups but also eliminates personnel involvement. Furthermore, in this model, we can place two littermate pups in the SD apparatus together. Having the littermate pups together during SD protocol is a

significant procedural advantage as this further eliminates the concern of adding isolation stress. Finally, the software analysis enabled us to quantitatively measure sleep patterns and duration.

3. THE RATIONALE FOR THE STUDY

In otherwise healthy children and adolescents, behavioral problems, affective symptoms, and reduced school performance have been associated with sleep deprivation (SD). However, the link between early life SD and the time-line of the appearance of affective, behavioral, and cognitive problems are not fully understood. Sleep deprivation is a huge problem in modern societies, especially in young adults. Hence, understanding the link between early life SD and cognitive deficits and psychiatric symptoms across the developmental course is critical. These studies are difficult to perform in children. Therefore, establishing an animal model to study the short and long-term effects of early life SD on behavior and cognition will be very useful. Furthermore, establishing an animal model of early life SD, the underlying mechanisms regulating early life SD-induced behavioral impairments can also be revealed, paving the way for identification of new approaches to tackle early life SD-related effects.

Many animal models are employed to study the effect of SD on behavioral and cognitive functions. Most of the studies so far have been done on adult animals.

However, no study has investigated the behavioral and biochemical consequences following early life SD. The brain development continues during childhood and adolescence. These stages have unique vulnerabilities to neuropsychiatric and cognitive impairment. Therefore, we studied the effect of SD during early life developmental stages in rats, starting at PND 19. The animals' behavior and the molecular changes in the brain were assessed immediately after the sleep deprivation protocol and later when the rats were adult. In this unique experimental design, the short term, as well as the long term effects of early life SD on the rats' brain molecular structure and behavior, were studied. The study design elucidates part of the mechanism underlying the behavioral impairment induced by EL-SD in the developing brain.

4. SPECIFIC AIMS

4.1. Specific Aim 1:

To examine the effects of early life SD on behavioral and cognitive functions in rats.

Sprague-Dawley male rats, (PND) 19 were employed. Two groups were employed in this aim; control (CON) and sleep-deprived (SD). The rats in the SD group were deprived of sleep using pinnacle automated SD system for 6-8 hours per day for 14 days, starting at lights on. Behavior tests were conducted at different developmental stages (early adolescent: PND33, late adolescent: PND60, and

adulthood: PND90). First, we measured the exploration behavior using open field test, then we measured anxiety-like behavior in rats using light/dark and elevated plus maze tests. We also measured the sociability in rats using the three compartment paradigm. For depression-like behavior, we used forced swim test. And for cognitive function, we measured short-term and long-term learning and memory functions using radial water maze (RAWM) test.

4.2. Specific Aim 2:

To examine the role of oxidative stress in early life SD–mediated behavioral and cognitive impairment.

We suggest that oxidative stress is involved in the impairments of behavioral and cognitive functions associated with sleep deprivation. Therefore, we measured oxidative markers in plasma and brain regions that are susceptible to oxidative stress including the PFC, hippocampus and the amygdala. In plasma, we measured the levels of 8-isoprostane as an indicator of oxidative stress. We measured the oxidative status in brain by measuring the oxidation and nitrosylation levels of proteins in brain regions. We also measured the total anti-oxidant capacity and the protein levels of anti-oxidant enzymes in the brain regions. We followed the sleep deprivation protocols as in aim 1. Plasma and brain tissues were collected at two developmental stages; at the early adolescent stage: PND33, and adulthood: PND90. The brain regions we collected are; PFC, hippocampus and

the amygdala. These brain regions are susceptible to oxidative stress and they are part of the limbic circuit that regulates behavior and memory.

4.3. Specific Aim 3:

To examine the effects of early life SD on synaptic density and plasticity in specific brain regions.

The developmental stages targeted by SD in our model are late childhood and early adolescence (PND19-32). This period is characterized by cortical maturation and neuronal circuit formation. Synaptogenesis followed by synaptic pruning are critical processes in cortical maturation during these developmental stages, and sleep has been suggested to promote these processes. Therefore, we measured the effect of SD at these stages on synaptic density and plasticity. We measured the expression level of synaptic density and synaptic plasticity markers including; AMPAR subunits, NMDAR subunits PSD95 and BDNF. WE also measured the phosphorylation level of the synaptic plasticity marker CaMKII, in addition to the neurotrophic factor downstream signaling markers including ERK1/2 and CREB. We followed the sleep deprivation protocols as in aim 1. Protein levels of synaptic density and synaptic plasticity markers were examined in the hippocampus, amygdala, and prefrontal cortex immediately after the SD protocol (PND33) and when the rats are adult (PND90).

5. METHODS AND MATERIALS

5.1. Animals and Housing Conditions

Consolidated litters of lactating Sprague-Dawley male pups with mothers were purchased from Envigo, USA. The pups arrived at our animal facility at PND11. The rats were acclimatized in the animal facility for seven days with 12 h light/12 h dark cycle. At PND18, the pups were separated from their mother, and housed as two rats per cage to eliminate isolation stress and provided with water gel and food ad libitum.

5.2. Sleep Deprivation Apparatus

Pinnacle automated sleep deprivation system was used to induce sleep deprivation (Pinnacle Technology, Lawrence, KS, USA), (**Figure 7)**. This system effectively produces sleep deprivation in rats as validated by polysomnography in previous studies (Hines et al., 2013; Wooden et al., 2014). The apparatus is a Plexiglas cylindrical cage with a rotating bar at the base. The bar is controlled by a software (Sirenia Acquisition) which enables controlled rotation to maintain desired speed and direction. Random bar rotation was selected to prevent adaptation to bar rotation, and moderate rotation speed at 10-40 rotations per minutes was selected, so the rotating bars gently touch the rat feet and disturb their sleep. The cages were layered with corn cob bedding and equipped with water bottles and food.

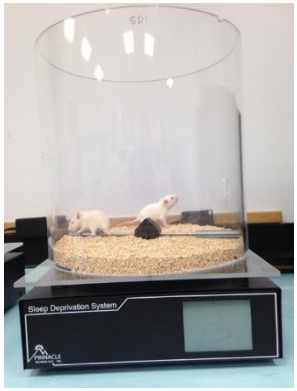


Figure 7. Pinnacle automated sleep deprivation (SD) apparatus. The SD apparatus is a Plexiglas cylindrical cage with a rotating bar at the base controlled by a software system (Sirenia Acquisition), the software enables controlled bar rotation to maintain desired speed and direction

5.3. Sleep Deprivation Protocol

All experiments were conducted in accordance with NIH guidelines using approved protocols from the University of Houston Animal Care Committee. The rats were randomly assigned into two groups; sleep deprivation (SD) and control (CON) groups, 12 rats per group. At PND18, the rats were acclimatized in the SD apparatus for 2 hours. At PND19, the SD rats were subjected to sleep deprivation 6 hours per day starting at ZT1 for seven days; two rats were placed in each sleep deprivation apparatus to exclude social isolation stress. After seven days, the SD duration increased for 8 hours per day for seven more days as the percentage of wakefulness during light cycle increases with development in rats (Alfoldi, Tobler, & Borbely, 1990). The control rats were placed in similar cages (two rats per cage) and stayed undisturbed in the same room. At the end of the sleep deprivation protocol; at PND33, behavior tests were conducted as described in the following

sections. The behavior tests were repeated at PND60 and 90. A schematic representation of the sleep deprivation protocol is provided in **Figure 8**.

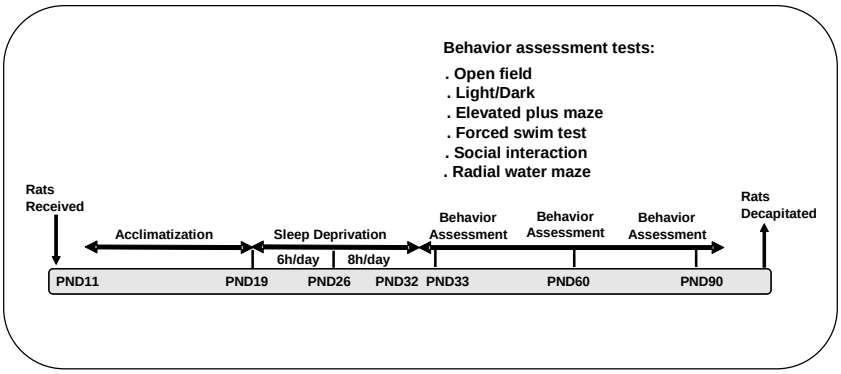


Figure 8: Schematic presentation of the study design. The pups arrived at postnatal day (PND) 11, and were acclimatized in the animal facility for seven days. At PND19, the rats were randomly assigned into sleep deprivation (SD) or control (CON) groups. SD group was subjected to SD 6h/day for seven days. At PND26, the SD rats were sleep deprived for 8h/day for 7days. CON rats were placed in a similar apparatus and allowed to sleep in the same room. Behavior and cognitive function tests were performed, at PND33, 60, and 90. Rats were decapitated at PND92.

5.4. Justification and Feasibility of the Model

Sleep has a significant impact on behavioral development during early life developmental stages. Behavioral functions following SD were investigated in adolescent and adult animal models (Wooden et al., 2014). However, no study has been conducted in earlier life stages, which justified the need to study the effect of SD in rats at early life stages. Rats will be deprived of sleep 6-8h/day for 14 days

beginning at PND19. The effect of SD will be studied right after SD protocol (PND33), when the rats are late adolescents (PND60), and adults (PND90). The studies at later life stages will elucidate if SD is detrimental and permanently impacts the rats' behavior later in their life. This is also justified by the finding that sleep disturbance during childhood was correlated with the development of anxiety-like behavior during adulthood (Gregory et al., 2005).

5.5. Assessment of Sleep and Wakefulness

We used a noninvasive method to estimate the sleep/wakefulness (S/W) pattern in rats. This method is based on analyzing the activity of the rats in their home-cages through continuous video acquisition. Estimation of a sleeping phenotype depends on the continuous recording of rat's activity/ inactivity, the longer the rat stays inactive, the more likely the rat is asleep (Pack et al., 2007). Pack and his colleaques also have reported that there is 92% agreement between sleep bouts determined by activity/inactivity assessment and EEG/EMG assessment. While this method lacks detection of micro-sleep bouts and rebound sleep, it allows high throughput phenotyping of sleep in rats without the need for surgery and chronic electrode implantation. For digital analysis of videos, we used AnyMaze software (Stoelting Co, USA) to track the activity of the rats throughout the SD protocol. The following activities were detected and analyzed by the

software; rearing time, a number of rears, freezing time, number of freezing episodes, immobility time, immobility episodes, and mobility time. The software detects freezing and immobility of the animals at minimal threshold. We estimated the amount of sleep and wakefulness in rats according to their activity versus inactivity (the mobility versus immobility). If the rat stays immobile for a long bout, it represents a sleep phenotype. We estimated the minimum time of inactivity (immobility) as 45s according to investigator observations and previous studies (Knutson et al., 2007). Tracking was conducted continuously for 24h/day for 15 days. We started tracking the rats one day before starting SD protocol to determine baseline S/W pattern. Also, we continued the tracking for one night after SD protocol, to determine if the rats' S/W pattern changed after the conclusion of SD protocol. We analyzed the immobility time over one-hour time segment every 24 hours. Furthermore, we analyzed the total immobility time over the 12 hours dark cycle. We also analyzed the total rearing time during the dark cycle throughout the whole experiment days (15 days).

5.6. Behavioral and Cognitive Assessments

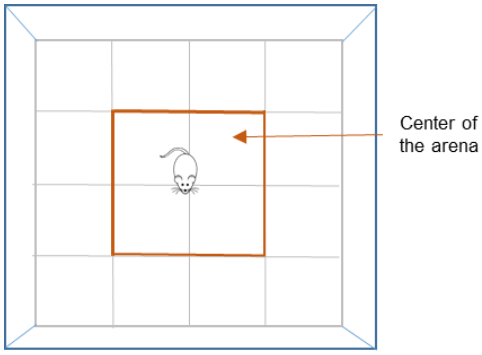
After the conclusion of SD protocol, behavioral tests were performed at PND33, 60, and 90. All behavior tests were conducted during the daytime starting at ZT2. Behavior tests were carried out in the order of least to most stressful test. We performed open field (OFT) followed by light/dark (LD) test, then an elevated

plus maze (EPM) test at PND33. Social interaction (SI), and forced swim test (FST) at PND34. Similarly, we performed OF, LD test, followed by EPM test at PND60. SI followed by FST at PND61, and short-term and long-term memory function tests at PND62 and PND63, respectively. At PND90, we performed OF, LD test, followed by the EPM test. At PND91, SI, and FST. At PND92, and PND93 short-term and long-term memory function tests respectively.

5.6.1. Measurement Activity and Exploration Behavior

Open Field Test (OFT) was conducted first to measure the rats' activity and exploration behavior, the test was performed as previously published by our lab (Patki et al., 2015; Solanki, Alkadhi, Atrooz, Patki, & Salim, 2015; Vollert et al., 2011). The OFT apparatus consists of an open arena (17.5" x 17.5") surrounded by transparent Plexiglas walls **(Figure 9).** Each rat was placed in the arena for 15 min in a room with a dim light. The rat was allowed to move freely throughout the arena. Infrared light sensors in the apparatus recorded the rat's movement which was quantified by Opto-Varimex Micro Activity Meter v2.00 software (Optomax, Columbus Instruments; OH). Total rat activity, ambulatory activity and the time spent in the center of the arena were analyzed. The OFT is suitable for assessing the rats' locomotion and normal exploration behavior when they are exposed to a novel environment (Archer, 1973; Roth & Katz, 1979).

Figure 9: Open field apparatus

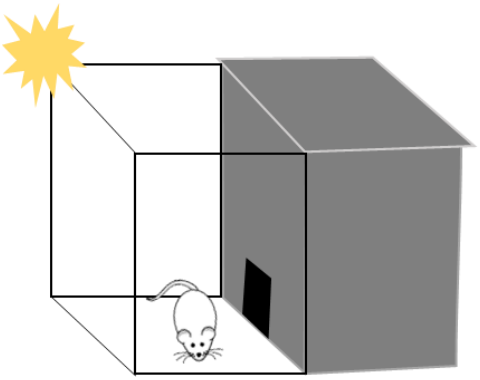


5.6.2. Measurement of Anxiety-like Behavior

A light/dark test was conducted, followed by elevated-plus maze test, as previously published by our lab (Patki et al., 2015; Solanki et al., 2015; Vollert et al., 2011).

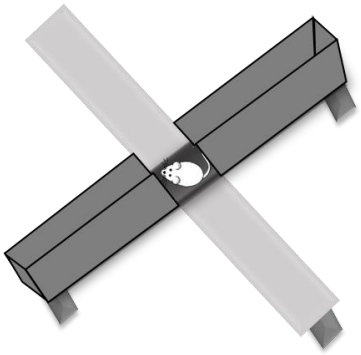
Light/Dark (LD) Test: The LD test apparatus is a box that consists of two compartments: a lit compartment (27 x 27 x 27 cm) and a dark compartment (27 x 18 x 27 cm), the rats have free access to both compartments through a single opening (7 x 7 cm) between the two compartments (**Figure 10**). Each rat was placed in the lit compartment and was given 5 min to explore both compartments. Time spent in the lit area was recorded as previously published (Allam et al., 2013; Patki et al., 2015). Less time spent in the lit compartment is an indication of anxiety-like behavior.

Figure 10: Light/dark apparatus



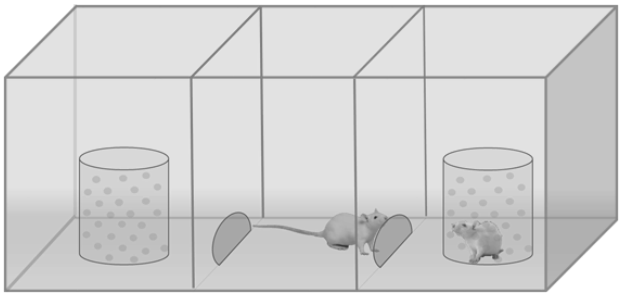
Elevated Plus Maze (EPM) Test: The EPM apparatus consists of two open and two closed arms (10 cm x 50 cm) that intersected to create a plus shape at an elevation of about 60 cm from the ground (**Figure 11**). The apparatus was obtained from Med Associates Inc., (St. Albans, VT). Each rat was placed in the intersection area facing the open arms of the maze and allowed to explore the maze. Movement of rat between the arms was recorded for 5 min as previously published (Salim et al., 2011; Vollert et al., 2011). Reduced time spent by a rat in the open arms is an indication of anxiety-like behavior.

Figure 11: Elevated plus maze apparatus



Social Interaction Test: The social interaction apparatus consists of three-compartments connected via a sliding partition. The middle compartment is 25cm wide X 35 cm long X 35 cm high, served as habituation compartment, each of the two end compartments is 25 cm wide X 50 cm long X 35 cm high and contained a wire cup **(Figure 12)**. A stranger Sprague Dawley male rat, from a different litter, was placed under one of the wire cups and the second cup left empty. The test consists of a habituation session and a sociability session. During the habituation session, the test rat was placed in the middle empty compartment for 5 minutes for habituation. In the second "sociability" session, the rat was allowed to encounter a stranger rat in one compartment and an empty cup in the other compartment. The sociability session lasted for 10 minutes. The time spent sniffing and interacting with each cup, the time spent in each compartment, and the number of entries into each compartment were recorded. Normal sociability in rats is indicated by their preference to spend more time interacting with another rat (stranger) rather than the empty cup. (Eagle et al., 2013; Smith et al., 2015; Toth & Neumann, 2013).

Figure 12: Social interaction apparatus



5.6.3. Measurement of Depression-like Behavior

Forced Swim Test (FST): Depression-like behavior was assessed using FST. The apparatus of FST consists of a tank (24 cm in diameter and 30 cm high) filled with water (25° C) (**Figure 13**). Each rat was placed the tank, and their mobility was recorded for 5 min. Rats exhibiting an immobile posture and motionless floating is an indication of depression-like behavior (Hogan, Kovalycsik, Sun, Rajagopalan, & Nelson, 2015). The total time spent immobile by the rat was recorded as previously published (Solanki, Salvi, Patki, & Salim, 2017). More time the rat spent immobile, is an indication of depression-like behavior.

Figure 13: Forced swim test apparatus

5.6.4. Measurement of Short and Long Term Memory Functions

Radial Arm Water Maze (RAWM) Test: RAWM apparatus consists of a circular water pool that had six swimming arms and an open central area. The test was performed in a dimly lit room **(Figure 14)**. Each rat was randomly assigned a goal arm with a hidden platform at the end of the arm that was submerged 1 cm

under water. The test began with 12 practice trials in which the rat was released from one of the arms (except the goal arm) and was given 1 min to swim through the pool and find the hidden platform. The room had visual cues on the walls to help the rats in locating the platform. If the rat could not find the platform in 1 min, they were manually guided to the platform. Once on the platform, the rat was given a rest period of 15 sec before the next learning trial began. The purpose of the practice trials was to help the rat learn the location of the platform. 30 min following practice trials, short-term memory (STM) test was conducted wherein the rat was again released from a randomly selected arm and given 1 min to find the hidden platform. Cognitive function of the rat was quantified based on a number of errors the rat made in locating the platform. Every time the rat entered halfway into any arm or entered the goal arm but failed to reach the platform was counted as an error. If the rat failed to find the platform in 1 min, it was given a score of 7. The same procedure was repeated 24 hours after the STM test and was denoted as long-term memory (LTM) test.

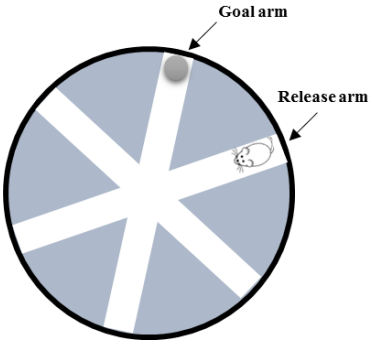


Figure 14: Radial water maze apparatus

5.7. Urine Samples Collection

To measure the direct effect of SD protocol on stress response, we measured corticosterone in urine during sleep deprivation protocol. Urine samples were collected on Day 1 of SD protocol at ZT7 (PND19), and on day 14 of SD protocol at ZT9 (PND32), right after the conclusion of SD hours. Urine samples were collected by placing the rats individually in a clean Plexiglas cages lined with cling wrap and allowing them to urinate spontaneously without any compulsion. Once the rats urinate, they were taken back to their home cages and the voided urine samples were aspirated into a micro centrifuge tubes (Kurien, Everds, & Scofield, 2004; Kurien & Scofield, 1999). Then, the samples were centrifuged at 800 X g for 10 minutes using a refrigerated centrifuge (4◦C). The urine samples were carefully aspirated and transferred into a new tube and immediately stored at -80◦C.

5.8. Brains Dissection and Collection of Plasma

Twenty four hours after termination of behavioral and cognitive tests, at PND35 and PND92, different set of rats were decapitated during the light phase between ZT2-4. Rats were anesthetized using isoflurane (cat. #57319-479-06, Phoenix Pharmaceuticals). Blood was collected from the left ventricle of rats' heart in ethylene diamine tetra acetic acid (EDTA) tubes. Plasma was separated by centrifugation at 2000 X g for 20 minutes at 4° C, plasma then collected in a new

tube and stored at -80° C. The rats were quickly decapitated, and brains were removed. Three brain regions, namely, pre-frontal cortex (PFC), hippocampus and amygdala were dissected out according to (Heffner, Hartman, & Seiden, 1980). The regions were immediately flash frozen in liquid nitrogen then stored at -80° C.

5.9. Tissue Homogenization and Protein Estimation

The brain tissues were homogenized using lysis buffer containing 20 mM Tris-HCl, 4 mM ethylene-diamine-tetra-acetic acid (EDTA), protease inhibitors, 100 µg/ml phenyl-methyl-sulfonyl fluoride (PMSF), 1 µg/ml leupeptin, 1 µg/ml aprotinin and 1 µg/ml pepstatin (Salim & Dessauer, 2004). The protein concentration of the lysates was estimated using micro BCA assay kit (Pierce, Rockford, IL).

5.10. Measurement of Corticosterone Levels

Corticosterone levels in urine were measured on day 1 and day 14 of SD protocol. Corticosterone concentration was measured using an EIA kit (#K014, Arbor Assays, Ann Arbor, MI) as per manufacturer's instructions. The corticosterone levels were normalized with creatinine concentration in urine samples. Creatinine was measured using a colorimetric assay kit (#ab 65340, Abcam, Cambridge, MA) as per manufacturer's instructions. Plasma levels of

corticosterone were measured using an EIA based kit (#500651, Cayman Chem. Co. Ann Arbor, MI) as per manufacturer's instructions.

5.11. Measurement of Indices of Oxidative Stress

5.11.1. Measurement of Plasma 8-Isoprostane Levels

8-Isoprostane is an eicosanoid generated as a result of phospholipid oxidation by free radicals. Therefore, 8-isoprostane is considered as a marker of oxidative stress. The plasma level of 8-isoprostane was measured using an ELISA kit (cat # 516351, Cayman Chemical, MI) as per manufacturer's instructions. 8-Isoprostane kit is a competitive ELISA kit that based on the competition between 8-isoprostane in the sample with 8-isoprostane-acetylcholine esterase conjugate for a limited number of 8-isoprostane molecules.

5.11.2. Measurement of Protein Oxidation in the Brain

Protein oxidation refers to the addition of carbonyl group into protein side chains by free radicals. The oxidative modification of proteins alters proteins biochemical characteristics and hence impairs protein functions. The levels of protein oxidation were measured in brain homogenates using OxyBlot protein oxidation detection kit (Cat# S7250, Millipore, MA) following the manufacturer's

instructions. In this kit, the carbonyl groups in the protein side chains are derivatized to 2,4-dinitrophenylhydrazone (DNP-hydrazone) by reaction with 2,4-dinitrophenylhydrazine (DNPH). After blotting the proteins onto PVDF membrane, the DNP-moiety of the protein is detected by specific antibody followed by an HRP conjugated secondary antibody.

5.11.3. Measurement of Protein Nitrosylation in the Brain

Protein nitrosylation refers to the addition of NO by reactive nitrogen species (RNS) to thiol (SH) group of tyrosine residues in protein leading to the formation of S-NO moiety (Mannick & Schonhoff, 2002). Protein nitrosylation is a post-translational modification which regulates physiological activities of target proteins (Jaffrey, Erdjument-Bromage, Ferris, Tempst, & Snyder, 2001). However, the increased level of nitrosylation in proteins is an indication of oxidative stress. Protein nitrosylation was measured in brain region homogenates using OxiSelect Nitrotyrosine ELISA detection kit (Cat# STA-305, Cell Biolabs, Inc, CA) following the manufacturer's instructions. The nitrotyrosine quantitation kit is a competitive ELISA. The unknown protein sample or nitrated BSA standards are first added to a nitrated BSA pre-absorbed EIA plate. Nitrosylation is detected by addition of anti-nitrotyrosine antibody, followed by an HRP conjugated secondary antibody. The protein nitrotyrosine content in the unknown sample is determined by comparing with a standard curve that is prepared from predetermined nitrated BSA standards.

5.12. Measurement of Antioxidant markers

5.12.1. Measurement of Total Antioxidant Capacity (TAC)

Total antioxidant capacity in brain region homogenates was measured using the Total Antioxidant Capacity Assay kit (Cat# MAK187, Sigma-Aldrich, MO) per manufacturer's instructions. This kit estimates the capacity of the total antioxidants in the sample to convert Cu^{2+} to its reduced form, Cu^{+} which chelates with a colorimetric probe, giving a broad absorbance peak at ~ 570 nm. Antioxidant capacity is measured regarding Trolox equivalents which serves as an antioxidant standard.

5.12.2. Measurement of Glutathione (GSH) / (GSSG) Ratio in the Brain

Glutathione existed in reduced (GSH) and oxidized (GSSG) states. Reduced glutathione (GSH) is a primary antioxidant molecule in cells. It provides reducing equivalents for the glutathione peroxidase (GPx) which results in the formation of a disulfide bond between two GSH molecules generating oxidized glutathione (GSSG). The enzyme glutathione reductase (GSR) recycles GSSG to GSH. In regular status, more than 90% of the total glutathione pool is in the reduced form (GSH). When cells are exposed to increased levels of oxidative stress, GSSG accumulates, and the ratio of GSH/GSSG decreases. A decreased ratio of GSH/GSSG is an indication of oxidative stress. The ratio of (GSH) / (GSSG) in

brain region homogenates was measured using an assay kit (Cat# ab138881, Abcam, MA) as per manufacturer's instructions. The kit uses a proprietary non-fluorescent dye that becomes strongly fluorescent upon reacting with GSH. Fluorescence is measured at Ex/Em = 490/520 nm.

5.12.3. Measurement of Protein Levels in the Brain Using Western Blotting

Western blotting was used to measure protein level of antioxidant enzymes, namely catalase (CAT), glutathione-S-reductase (GSR), Heme oxygenase (HO), and Manganese superoxide dismutase (Mn-SOD), in the brain region homogenates. Samples for western blotting were prepared by diluting the respective brain region homogenates in 2x Laemmli buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS and 0.1 mg/ml bromophenol blue). Samples (approximately 20 µg of total protein per sample) were resolved on standard 15-well, 8-16% SDS-PAGE gels. The proteins were transferred to PVDF membrane (Amersham Pharmacia Biotech, Buckinghamshire, England) and then detected as immunoreactive bands using specific primary antibodies and horseradish peroxidase-conjugated secondary antibody. β-actin was used as a loading control. Antibodies dilutions used have been listed in **Table 1**. Chemiluminescence reagent (Cat# 1705060, Biorad, CA) was used for the development of the protein blot. Chemiluminescence was detected using Gene Sys imaging system (Gene Sys

V1.4.1.0, GeneSys, USA) and densitometric analysis was performed using Fluorochem FC8800 software.

Protein	Primary Antibody	Secondary Antibody
CAT	Ab16731 (Abcam, MA) 1:1000	7074S (Cell signaling, MA) 1:2000, Goat anti-rabbit
GSR	Ab124995 (Abcam, MA) 1:1000	7074S (Cell signaling, MA) 1:2000, Goat anti-rabbit
Mn-SOD	06-984 (Millipore, MA) 1:1000	7074S (Cell signaling, MA) 1:2000, Goat anti-rabbit
Cu/Zn-SOD	07-403 (Millipore, MA) 1:1000	7074S (Cell signaling, MA) 1:2000, Goat anti-rabbit
HO	Ab13243 (Abcam, MA) 1:1000	7074S (Cell signaling, MA) 1:2000, Goat anti-rabbit
β-actin	3700S (Cell signaling, MA) 1:1000	7076S Cell signaling, MA) 1:2000, Goat anti-mouse

Table 1. **Antibodies for antioxidant enzymes, dilutions, and sources**. Details of primary and secondary antibodies used in detecting the levels of specific proteins

Western blotting was also used to measure protein levels of stress and proinflammatory markers including; P38, JNK, TNF-α, IL-6. Primary and secondary antibody dilutions used have been listed in **Table 2.**

Protein	Primary Antibody	Secondary Antibody
P-P38	Ab47363 (Abcam, MA) 1:1000	7074S (Cell Signaling, MA) 1:2000, Goat anti-rabbit
P38	Ab31828 (Abcam, MA) 1:1000	7076S Cell Signaling, MA) 1:2000, Goat anti-mouse
P-JNK1,2,3	Ab124956 (Abcam, MA) 1:1000	7074S (Cell Signaling, MA, MA) 1:2000, Goat anti-rabbit
JNK1,2,3	Ab208035 (Abcam, MA) 1:1000	7074S (Cell Signaling, MA, MA) 1:2000, Goat anti-rabbit
TNF-α	3707 (Cell Signaling, MA) 1:1000	7074S (Cell Signaling, MA) 1:2000, Goat anti-rabbit
IL-6	ARC0062 (Thermo Fisher Scientific, USA), 1:1000	7076S (Cell Signaling, MA) 1:2000, Goat anti-mouse
β-actin	3700S (Cell Signaling, MA) 1:1000	7076S (Cell Signaling, MA) 1:2000, Goat anti-mouse

Table 2. Antibodies for stress and proinflammatory markers, dilutions, and sources. Details of primary and secondary antibodies used in detecting the protein levels of inflammatory markers

Western blotting was used to measure protein levels of synaptic density markers including; post synaptic density protein (PSD) 95, AMPAR subunit A1 (GluA1), and NMDAR subunit 2b (GluN2b). The protein levels of synaptic plasticity markers were also measured using the western blot technique including; CaMKII phosphorylation level (p-CaMKII), and CREB phosphorylation level (p-CREB). Primary and secondary antibodies dilutions used have been listed in **Table 3.**

Protein	Primary Antibody	Secondary Antibody
PSD95	MA1-045 (Thermo fisher Scientific, USA), 1:1000	7076S Cell Signaling, MA) 1:2000, Goat anti-mouse
GluA1	D4N9V (Cell Signaling, MA) 1:1000	7074S (Cell Signaling, MA) 1:2000, Goat anti-rabbit
GluN2b	Ab65783 (Abcam, MA) 1:1000	7074S (Cell Signaling, MA) 1:2000, Goat anti-rabbit
P-CaMKII	SC-32289 (Santa Cruz, TX) 1:1000	7076S Cell Signaling, MA) 1:2000, Goat anti-mouse
CaMKII	SC-9035 (Santa Cruz, TX) 1:1000	7074S (Cell Signaling, MA) 1:2000, Goat anti-rabbit
P-CREB	06-519 (Cell Signaling, MA) 1:250	7074S (Cell Signaling, MA) 1:2000, Goat anti-rabbit
CREB	SC-58 (Santa Cruz, TX) 1:1000	7074S (Cell Signaling, MA) 1:2000, Goat anti-rabbit
β-actin	3700S (Cell Signaling, MA) 1:1000	7076S Cell Signaling, MA) 1:2000, Goat anti-mouse

Table 3. Antibodies for synaptic density/ plasticity markers, dilutions and sources. Details of primary and secondary antibodies used in detecting the protein levels of synaptic density markers

Western blotting was also used to measure protein levels of markers of neurotrophic signaling pathway including; BDNF, MKP1, ERK1/2, Primary and secondary antibodies dilutions used have been listed in **Table 4.**

Protein	Primary Antibody	Secondary Antibody
BDNF	SC-546 (Santa Cruz, TX) 1:500	7074S (Cell Signaling, MA) 1:2000, Goat anti-rabbit
MKP1	PA5-17973 (Thermo fisher Scientific, USA), 1:1000	81-1620 (Thermo fisher Scientific, USA), 1:2000 Rabbit Anti-goat
P-ERK$_{1/2}$	9106S (Cell Signaling, MA) 1:1000	7076S Cell Signaling, MA) 1:2000, Goat anti-mouse
ERK$_{1/2}$	9107S (Cell Signaling, MA) 1:1000	7074S (Cell Signaling, MA) 1:2000, Goat anti-rabbit
β-actin	3700S (Cell Signaling, MA) 1:1000	7076S Cell Signaling, MA) 1:2000, Goat anti-mouse

Table 4. Antibodies for neurotrophic signaling pathway markers, dilutions and sources. Details of primary and secondary antibodies used in detecting the protein levels of neurotrophic pathway markers

5.13. Statistical Analysis

All values are reported as mean ± SEM. Comparison between groups was made either by Student's t-test (behavioral and biochemical analysis), or two-way ANOVA (Social interaction test, total rearing time, and sleep/wakefulness pattern). Total rearing time and sleep/wakefulness pattern were further analyzed using repeated measures ANOVA test. Graph Pad prism was used for the statistical analysis (Graph Pad 5 Software, Inc., San Diego, CA). $P < 0.05$ was used to denote statistically significant groups.

6. RESULTS

In all studies, body weight did not vary between the different groups of rats throughout the study, suggesting the absence of any behavior associated with illness (**Figure 1, appendix**).

6.1. Sleep/Wakefulness (S/W) Pattern

To validate our SD model, we measured the S/W pattern in rats before, during, and after SD hours throughout the whole experiment. We estimated the immobility for ≥ 45s as a sleeping phenotype. First, the baseline S/W pattern was measured in both SD and CON groups. At day 0, before starting the sleep deprivation protocol, the CON and SD rats showed similar patterns of immobility within 20 hours measured at the one-hour interval **(Figure 15A**). At day 2, the rats in SD group were awake during the 6 hours SD period (light cycle), while the rats in CON group showed high immobility scores; suggestive of sleeping behavior of CON rats and wakefulness behavior of SD rats. Right after the conclusion of SD protocol, the SD rats showed an increase in immobility score for the rest of the light cycle as compared to CON group, though, the increase was insignificant. During the dark cycle, both groups exhibited comparable S/W pattern **(Figure 15B)**. At day 11, after 8 hours of sleep deprivation, the SD and CON rats showed no difference in S/W pattern for the rest of the day **(Figure 15C)**. During the dark cycle of day

14, following the last day of sleep deprivation, SD and CON rats showed no difference in S/W pattern **(Figure 15D)**. Analysis of the immobility bouts over 24 hours at one hour time interval for the rest of the experiment days showed no changes in S/W pattern between SD and CON rats following sleep deprivation hours. However, analysis of the total immobility time over the 12 hours dark cycles throughout the15 days showed that SD rats and CON rats spent comparable time being immobile in most of the days except for 3 days when SD rats displayed significantly longer time being immobile as compared to CON rats; nights of day 2, 12 and 13 of SD protocol, see **table 5**. The results suggest that SD rats stayed awake during sleep deprivation hours while CON rats showed the sleeping phenotype. Interestingly, the SD rats did not show changes in S/W pattern compared to CON rats throughout the experiment. However, SD rats showed an increase in total immobility time during the dark cycle of three days, which suggests that SD rats most likely slept extra time during the dark cycles of few days while in total still got fewer sleep hours as compared to CON rats.

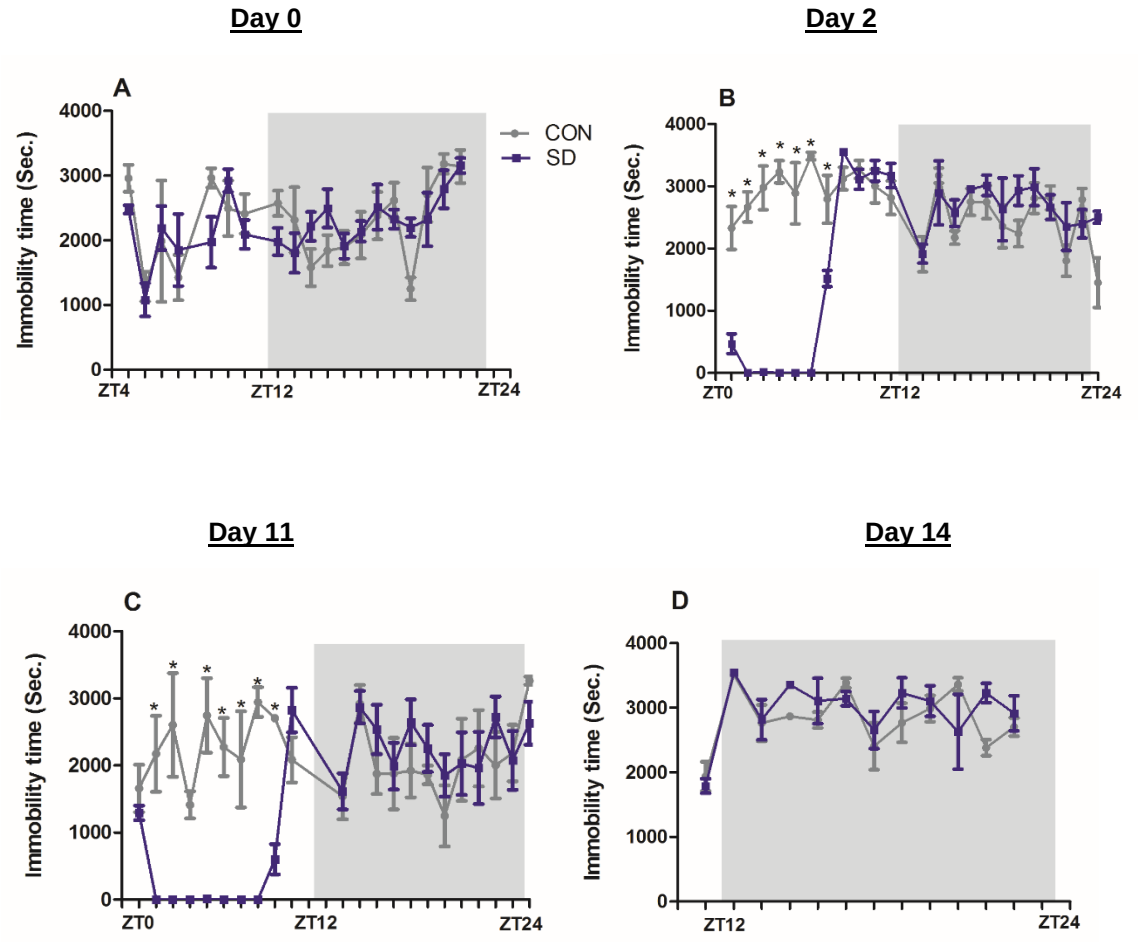


Figure 15. Sleep/ wakefulness (S/W) pattern during light/dark cycles of sleep deprivation protocol. Sleep/ wakefulness (S/W) pattern was analyzed by AnyMaze software. Baseline S/W pattern in rats exhibited by immobility time, measured in seconds (sec) over one-hour time segment, represented at day 0 before the commencement of the sleep deprivation protocol **(A).** Immobility patterns of rats are represented at day two during both light and dark cycle **(B),** at day 11 during both light and dark cycle **(C)**, and day 14 during the dark hours **(D)**. Lines are means of immobility time in seconds ±SEM, n=4 rats/group. Data were analyzed using a two-way ANOVA test. (*) Significantly different at $p<0.05$. Group designations: control (CON), sleep deprivation (SD).The light areas represent the light cycle (ZT0-ZT12), while the shaded areas represent the dark cycle (ZT12-ZT24).

Total Immobility time (seconds)				Immobility Episodes (seconds)		
Day	**CON (mean±SEM)**	**SD (mean±SEM)**	**P**	**CON (mean±SEM)**	**SD (mean±SEM)**	**P**
Day 0	39492 ± 1736	38810 ± 2073	0.82	237.7 ± 10.40	270.3 ± 25.89	0.35
Day 1	35646 ± 808.0	33690 ± 3904	0.64	117.0 ± 25.31	92.25 ± 8.250	0.39
Day 2	28689 ± 988.7	32183 ± 797.1	0.048*	151.0 ± 14.39	123.3 ± 14.08	0.24
Day 3	17251 ± 4762	28897 ± 82.02	0.09	86.25 ± 25.72	129.0 ± 3.000	0.22
Day 4	27037 ± 854.4	21837 ± 4492	0.299	146.0 ± 11.75	85.0 ± 12.51	0.012*
Day 5	27193 ± 1205	28705 ± 602.4	0.30	126.0 ± 11.63	112.8 ± 6.981	0.37
Day 6	24898 ± 1381	28140 ± 1168	0.12	117.3 ± 8.645	123.8 ± 8.340	0.61
Day 7	21739 ± 3326	26230 ± 511.7	0.23	75.00 ± 19.81	102.0 ± 12.87	0.297
Day 8	25190 ± 3381	26611 ± 3897	0.79	113.3 ± 11.27	99.0 ± 14.34	0.46
Day 9	20009 ± 4970	26267 ± 2794	0.31	102.5 ± 8.005	80.25 ± 17.80	0.298
Day 10	20326 ± 2646	26914 ± 409.1	0.049	80.75 ± 9.953	116.3 ± 2.323	0.013*
Day 11	23708 ± 1939	27180 ± 861.9	0.153	99.00 ± 3.189	116.0 ± 7.927	0.094
Day 12	22080 ± 1899	27595 ± 437.6	0.03*	96.00 ± 7.348	113.3 ± 0.7500	0.058
Day 13	22302 ± 1893	27531 ± 584.0	0.039*	103.8 ± 8.654	116.8 ± 12.11	0.416
Day 14	33601 ± 888.7	35144 ± 1629	0.44	94.25 ± 7.952	89.75 ± 23.42	0.86

Table 5. Total immobility time and immobility episodes during dark cycles of sleep deprivation protocol, as measured by AnyMaze. The total time the rats spent immobile (sec.) and number of immobility episodes measured during the 12 hours dark cycles of the 15 days experimental course, starting at dark cycle of day 0, one day before commencement of the sleep deprivation protocol, and ending at dark cycle of day 14, the last day of sleep deprivation protocol. N= 4rats/group. Data were analyzed using two-tailed unpaired t-test. (*) significantly different at $p<0.05$. Group designations: control (CON), sleep deprivation (SD).

6.2. Total Rearing Time

We measured the time the rats spent rearing during the dark cycles that follow the SD hours to investigate the effect of SD on the rats' behavior. Analysis of the total rearing time over the 12 hours of dark cycle throughout the experiment **(Figure 16)**, showed that; during the first night before starting SD protocol (day 0) and at the first day of SD protocol (day 1), SD and CON rats spent comparable time rearing during the dark cycles. At day 0, total time rearing was comparable between the two groups (CON =:876.8 ± 204.8 sec; SD =876.3±101.2 sec, p=0.998). Similarly, at day 2, SD and CON rats spent comparable time rearing (CON=237.2±53.51 sec, SD=223.7±57.70 sec, p=0.26). However, in the successive days, SD rats showed a consistent reduction in total time rearing as compared to CON rats. This reduction was significant on the last three days of the experiment; Day 12, day 13, and day 14 **(Figure 16)**. Total rearing time at day 12 (CON=1046±173.3 sec; SD=434.5±80.84 sec, p= 0.0187), day 13 (CON= 599.2±71.15 sec; SD=304.3±81.66 sec, p=0.048), and day 14 (CON=687.9±113.6 sec; SD=335.8±29.87 sec, p=0.024) was significantly different between the two groups. The results suggest that SD over time impacted rearing behavior in rats although it did not affect their mobility/immobility pattern.

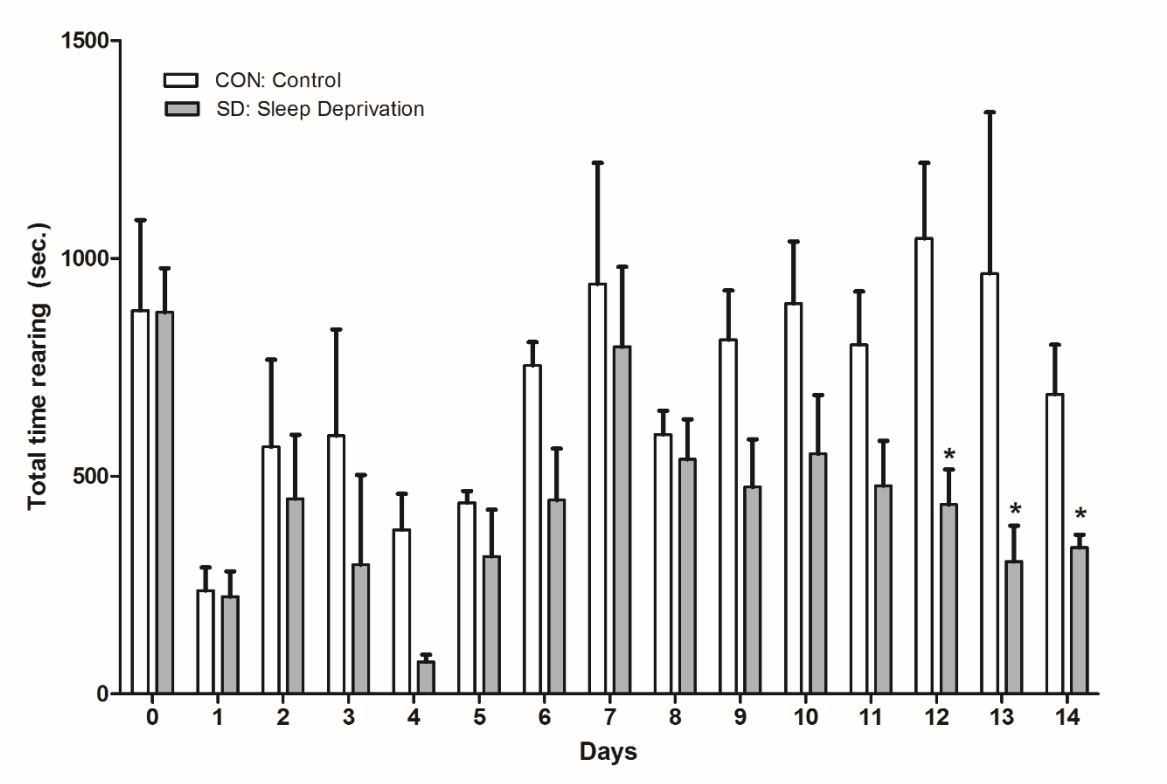


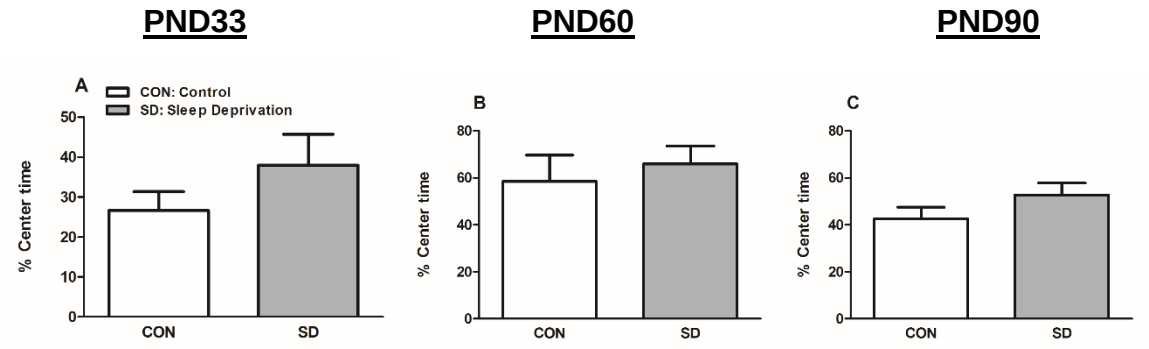
Figure 16. Total rearing time during the dark cycles of sleep deprivation protocol. The rearing time was measured by AnyMaze software. The total time the rats spent rearing was measured during the 12 hours dark cycle of the 15 days experimental course, starting at the dark cycle of day 0, one day before the commencement of the sleep deprivation protocol, and ending at the dark cycle of day 14, the last day of sleep deprivation protocol. Bars are means of rearing time in seconds ±SEM, n=4 rats/group. Data were analyzed using two-way ANOVA test. (*) significantly different at $p<0.05$. Group designations: control (CON), sleep deprivation (SD)

6.3. Behavioral and Cognitive Assessments

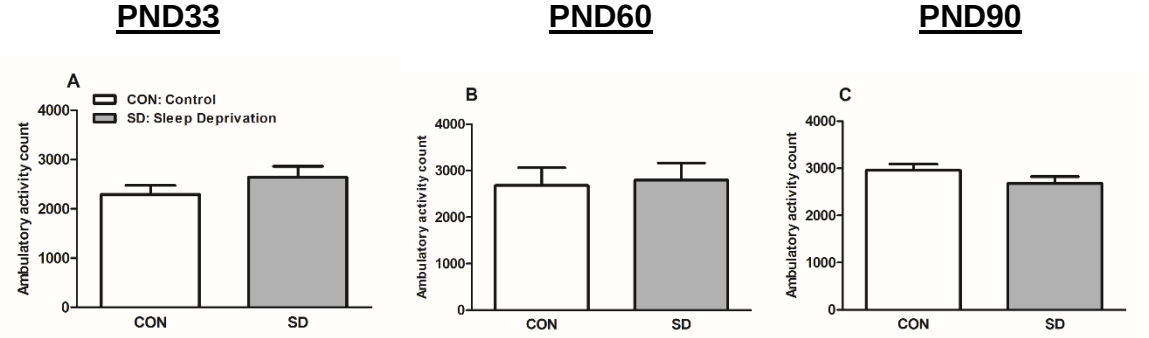
6.3.1. Examination of Anxiety-Like Behavior in the Open Field Test (OFT)

Open field test is commonly utilized to study anxiety-like behavior in rodents. Reduced time spent in the center of the arena of the OFT apparatus is an indication of increased anxiety-like behavior (Gould, Dao, & Kovacsics, 2009; Salim et al., 2011). In the open field test, SD rats did not exhibit any significant difference in terms of percentage of time spent in central part of the open field arena as compared to CON rats at PND33, 60 or 90 (The mean of percentage of center time CON and SD rats ±SEM. PND33; CON=26.67±4.7; SD=37.98±7.74, p=0.23; PND60; CON=58.52±11.20; SD=56.94±7.57, p=0.59; PND90; CON=42.58±4.96; SD=52.62±5.24, p=0.19) (Figure 15; IA. PND33 IB. PND60 IC. PND90). No significant difference was noted in total activity or ambulatory activity between SD and CON rats at PND33, 60 or 90 **(Figure 17).**

I. Center time



II. Ambulatory activity



III. Total activity

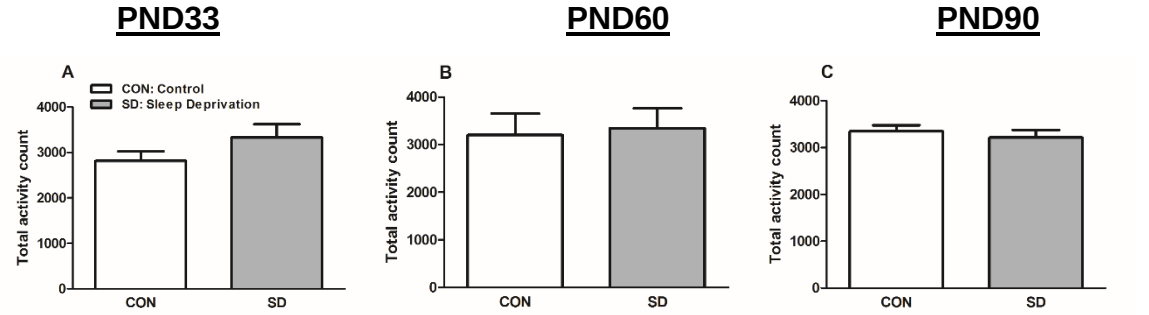


Figure 17. Examination of anxiety-like behavior using an open field test. (I) Percent time spent in the center of the open field arena was analyzed at PND33 **(A)**, PND60 **(B)**, and PND90 **(C)** using **the** Opto-Varimex software**.** (**II)** Ambulatory activity was measured

at PND33 **(A)**, PND60 **(B)**, and PND90 **(C). (II)** Total activity was measured at PND33 **(A)**, PND60 **(B)**, and PND90 **(D)**. Bars are means ± SEM, n=8 rats/group. Data were analyzed using two-tailed unpaired t-test. PND: postnatal day. Group designations: control (CON), sleep deprivation (SD).

6.3.2. Examination of Anxiety-Like Behavior in the Light/Dark (LD) Test

The LD test measures anxiety-like behavior based on rodents' aversion for lit areas. Less time spent in the lit compartment of the LD box suggests an increased anxiety-like behavior. In light/dark test, the SD rats exhibited anxiety-like behavior at PND33 indicated by SD rats spending significantly less time in the lit area as compared to CON rats (PND33; CON: 89.13 ± 12.77 sec, SD: 56.92 ± 7.150 sec; 32.2 %, p=0.024) **(Figure 18 A)**. However, at PND60 and 90, the SD rats did not show anxiety-like behavior as SD rats spent comparable time as CON rats in the lit area (PND60; CON: 70.13 ± 14.94 sec, SD: 74.75 ± 11.68 sec, p=0.81; PND90; CON: 43.50 ± 11.19 sec, SD: 58.38 ± 12.16 sec, p=0.38) **(Figure 18 B, C)**.

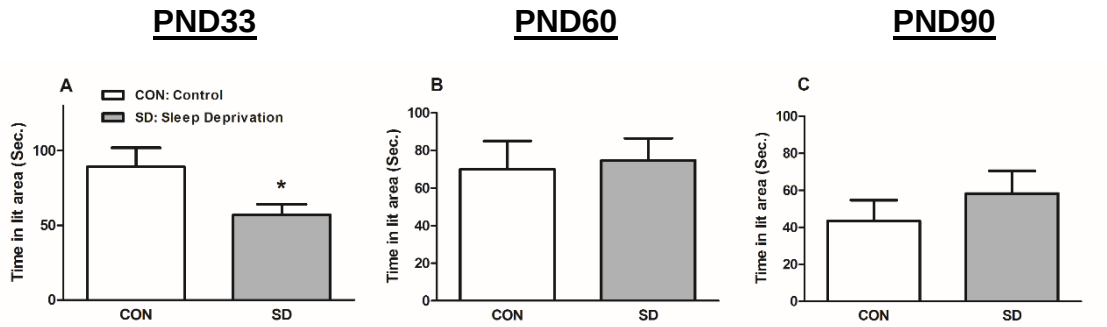


Figure 18. Examination of anxiety-like behavior using Light/Dark test. Bars represent the time spent in the lit area measured in seconds (sec) at PND33 **(A),** PND60 **(B),** and PND90 **(C**). Bars are means of time spent in open arms in seconds± SEM, n=8 rats/group. Data were analyzed using two-tailed unpaired t-test. (*) significantly different at $p<0.05$. PND: Postnatal day. Group designations: control (CON), sleep deprivation (SD).

6.3.3. Examination of Anxiety-Like Behavior in the Elevated Plus Maze (EPM) Test

The EPM test measures anxiety-like behavior based on rodents' aversion for elevated and open areas (Patki et al., 2015). Less time spent in the open arms of the EPM test apparatus is an indication of increased anxiety-like behavior. In elevated plus maze test, the SD rats spent significantly less time in open arms as compared to CON rats at PND33 and 60 **(Figure 19 A, B)**, an indication of anxiety-like behavior (PND33; CON: 68.43 ± 10.90 sec, SD: 41.56 ± 6.468 sec; 26.87%, p=0.019; PND60; CON: 69.38 ± 7.066 sec, SD: 43.25 ± 9.292 sec; 26.13%,

p=0.04). While at PND90, the SD and CON rats spent comparable time in open arms (CON: 62.00 ± 17.42 sec, SD: 90.71 ± 5.375 sec, p=0.14) **(Figure 19 C),** which suggested that the SD rats not exhibit anxiety-like behavior at the adult stage.

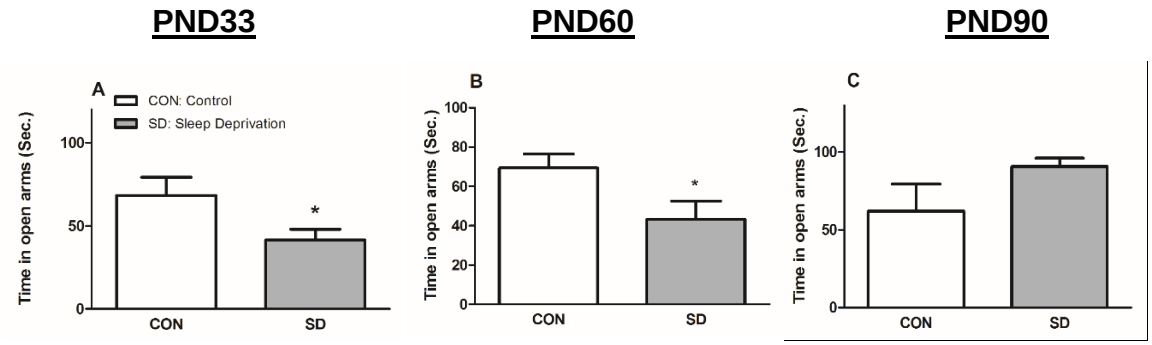


Figure 19. Examination of anxiety-like behavior using elevated plus maze test. Bars represent the time spent in open arms measured in seconds (sec). At PND33 **(A),** PND60 **(B),** and PND90 **(D).** Bars are means of time spent in open arms in seconds± SEM, n=8 rats/group. Data were analyzed using two-tailed unpaired t-test. (*) significantly different at $p<0.05$. PND: Postnatal day. Group designations: control (CON), sleep deprivation (SD).

6.3.4. Examination of Anxiety-Like Behavior in the Social Interaction Test

The sociability in rats was not affected by sleep deprivation. In the sociability test, rats in SD and CON groups showed equal preference to interact with stranger rat rather than the empty cup when this test was conducted at PND33, 60 and 90,

(Figure 20 A, B, C). The results suggest that sleep deprivation did not affect the high sociability behavior in rats.

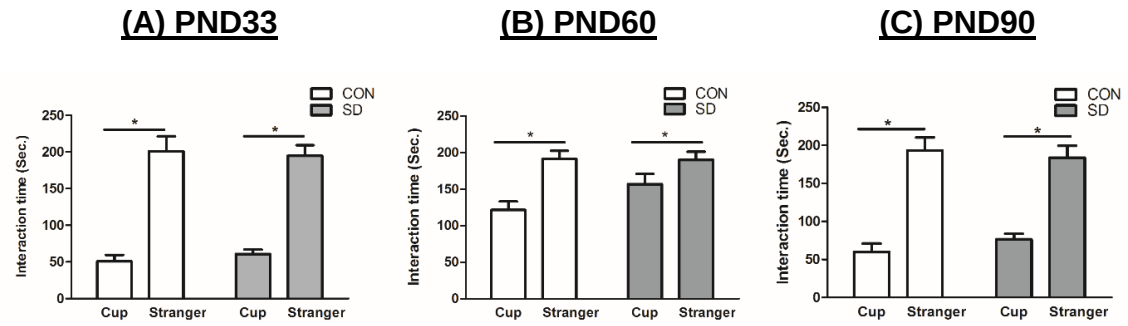


Figure 20. Examination of social interaction behavior using the three-compartment paradigm test. Bars represent the time the rats spent interacting with stranger rat or with the empty cup, measured in seconds (sec) at PND33 **(A**), PND60 **(B),** and PND90 **(C).** Bars are means of time spent interacting with a cup or stranger rat in seconds ± SEM, n=8 rats/group. Data were analyzed using two way ANOVA test. (*) significantly different at $p<0.05$. PND: Postnatal day. Group designations: control (C), sleep deprivation (SD).

6.3.5. Examination of Depression-Like Behavior in the Forced Swim Test (FST)

The FST is widely used to study depression-like behavior in rodents (Hogan et al., 2015). More time spent by the rat in an immobile position is an indication of increased depression-like behavior. FST was conducted at PND33, 60 and 90. Interestingly, rats at PND33 were too active when placed in the FST water tank and showed zero immobility. Hence, reliable data was not achieved. FST was successfully conducted at PND60. SD and CON rats spent comparable

time immobile at PND60 (CON: 7.750 ± 2.266 sec, SD: 6.375 ± 2.275 sec, p=0.67) **(Figure 21A**). However, at PND90, SD rats spent longer time immobile as compared to control rats (CON: 44.13 ± 4.121 sec, SD: 75.75 ± 13.73 sec, 31.63%, p=0.045) **(Figure 21B),** an indication of depression-like behavior. The results suggest the later onset of depression-like behavior.

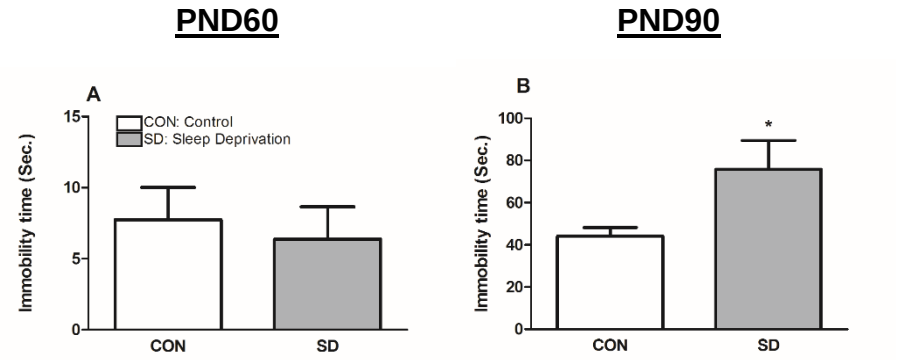
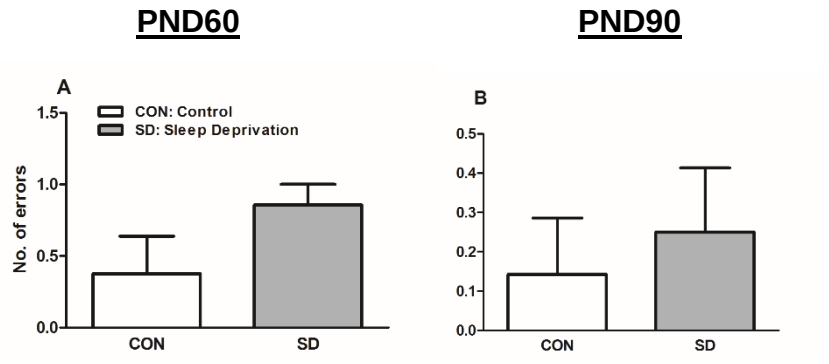


Figure 21. Examination of depression-like behavior using forced swim test. Bars represent the time the rats stayed immobile in water within 5 minutes test duration, measured in seconds (sec) at PND60 **(A**), and PND90 **(B)**. Bars are means of immobility time in seconds± SEM, n=8 rats/group. Data were analyzed using two-tailed unpaired t-test. (*) significantly different at p<0.05. PND: Postnatal day. Group designations: control (CON), sleep deprivation (SD).

6.3.6. Examination of Learning-Memory Function in the Radial Arm Water Maze (RAWM) Test

The radial water maze test is used to measure learning and memory function in rodents. More errors made by the rat in locating the hidden platform is an indication of memory impairment. RAWM test was conducted at PND33, 60 and 90. As observed in the FST paradigm, rats at PND33 were too panicked when placed in the RAWM water tank and did not swim towards the arms to find the platform. Hence reliable data was not achieved. RAWM was successfully conducted at PND60 and PND90. SD and control rats showed no impairment in the short term or long term memory functions. At PND60, SD and CON rats made comparable number of errors in short-term memory test (mean number of errors for CON and SD groups ±SEM, CON=0.38±0.26 errors; SD=0.86±0.14 errors, $t(14)=1.2$, $p=0.15$) **(Figure 22 IA),** as well as in long-term memory test (mean number of errors for CON and SD groups ±SEM, CON=1.78± 0.81 errors, SD=3.50±0.94 errors, $t(14)=1.30$, $p=0.32$) **(Figure 22 IIA)**. At PND90, SD and CON rats made comparable number of errors in finding the platform in short-term memory test (mean number of errors for CON and SD groups ±SEM, CON=0.12±0.12 errors; SD: 0.25±0.16 errors, $t(14)=0.61$, $p=0.63$) **(Figure 22 IB)**, and in long-term memory test (mean number of errors for CON and SD groups ±SEM, CON=0.38±0.26 errors; SD=0.38±0.18 errors, $t(14)=0.0$, $p=1.00$) (**Figure 22 IIB).**

I. Short-term memory



II. Long-term memory

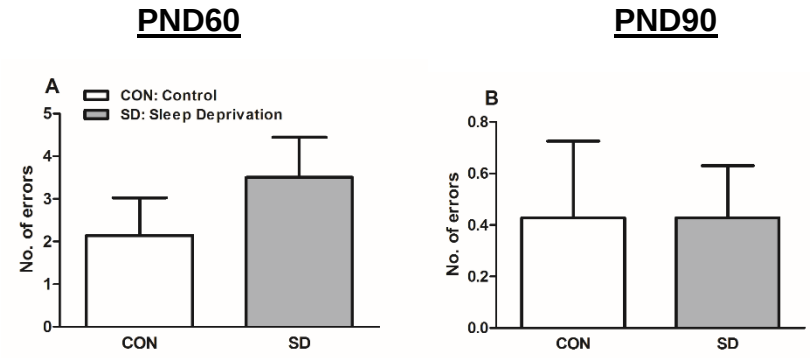


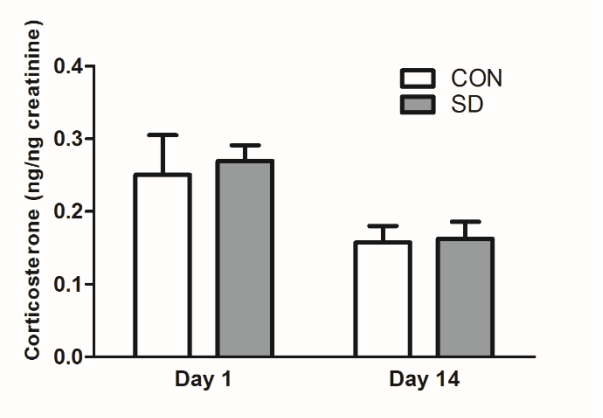
Figure 22. Examination of learning and memory using radial arm water maze test, bars represent the number of errors the rats made before finding the platform in radial arm water maze. Short term memory test **(I)**, conducted 30 minutes following learning trials at PND60 **(A)**, and PND90 **(B)**. Long-term memory test **(II)**, conducted 24 hours following learning trials at PND60 **(A)**, PND90 **(B)**. Bars are means of a number of errors ± SEM, n=8 rats/group. Data were analyzed using two-tailed unpaired t-test. (*) significantly different at $p<0.05$. PND: Postnatal day, Group designations: control (CON), sleep deprivation (SD).

6.4. Biochemical Parameters

6.4.1. Effect of Early Life (EL)-SD on Corticosterone Levels

The increase in systemic stress leads to the release of corticosterone from the adrenal cortex of rodents (Smith and Vale 2006). Therefore an increase in the levels of corticosterone is an indication of systemic stress. To investigate the effect of SD protocol on stress response, we measured corticosterone levels in urine collected during SD protocol right after the conclusion of SD hours on day 1 and day 14. We found that SD rats did not show an increase in corticosterone level in urine as compared to CON rats at day 1 or day 14 (mean concentration of corticosterone in CON and SD groups ±SEM, CON=1.315±0.1689; SD= 1.834±0.25, p=0.832) **(Figure 23 I)**. The results suggest that SD protocol was not stressful for rats. Additionally, we measured corticosterone level in plasma of rats at PND33 and 90. Corticosterone levels showed a mild increase in SD rats at PND33 as compared to CON rats, though the increase was insignificant (mean concentration of corticosterone in CON and SD groups ±SEM, Day 1: CON=0.251±0.054; SD= 0.270±0.021; Day 14: CON= 0.157±0.023; SD= 0.162±0.024, p= 0.83) **(Figure 23 IIA)**. At PND90, Corticosterone levels were comparable in CON and SD groups (mean concentration of corticosterone in CON and SD groups ±SEM, CON= 1.250±0.36; SD= 1.278±0.37, p=0.96) **(Figure 23 IIB)**.

I. Corticosterone levels in urine



II. Plasma corticosterone levels

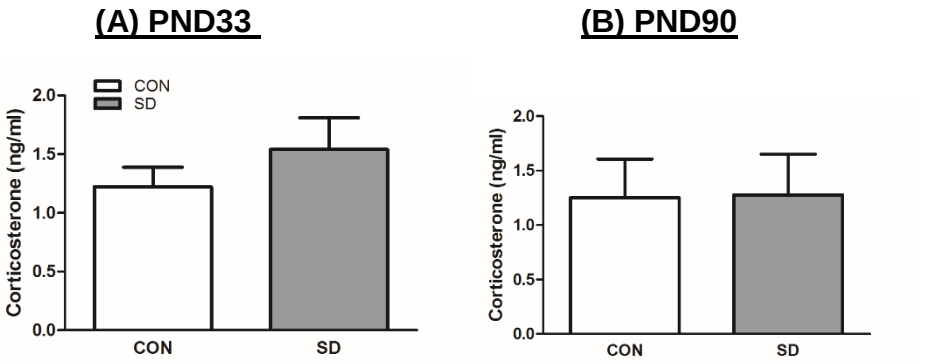


Figure 23. Corticosterone levels in urine and plasma. Corticosterone level in urine **(I).** Corticosterone was measured in urine collected at Day1 and Day 14 of SD protocol, right after SD hours. Bars are means of corticosterone concentration (ng/ng creatinine) ±SEM. Data were analyzed using a two-way ANOVA test. n=8 rats/group. Plasma corticosterone level **(II).** Corticosterone concentration was measured in plasma of rats at PND33 **(A)** and PND90 **(B)**. Bars are means of corticosterone concentration (ng/ml) ±SEM, n=8 rats/group. Data were analyzed using two-tailed unpaired t-test. Group designations: control (CON), sleep deprivation (SD).

6.4.2. Effect of EL-SD on Plasma 8-Isoprostane Levels

Increased oxidative stress and subsequent buildup of free radicals in the body leads to oxidation of free fatty acids which results in the production of isoprostanes (Betteridge, 2000). Therefore, the increase in 8-isoprostane levels is an indication of increased oxidative stress. Plasma 8-Isoprostane levels were significantly elevated in SD rats as compared to CON rats at PND33, suggesting a systematic elevation of oxidative stress, (mean concentration of 8-isoprostane in CON and SD groups ±SEM, CON=73.23 ± 6.394; SD= 104.8 ± 10.06, p=0.033) **(Figure 24A)**. Plasma 8-Isoprostane levels were comparable in SD and CON rats at PND90, (mean concentration of 8-isoprostane in CON and SD groups ±SEM, CON=17.81 ± 2.520; SD= 18.26 ± 2.035, p=0.8901), suggesting mitigation of systemic oxidative stress over time **(Figure 24B)**.

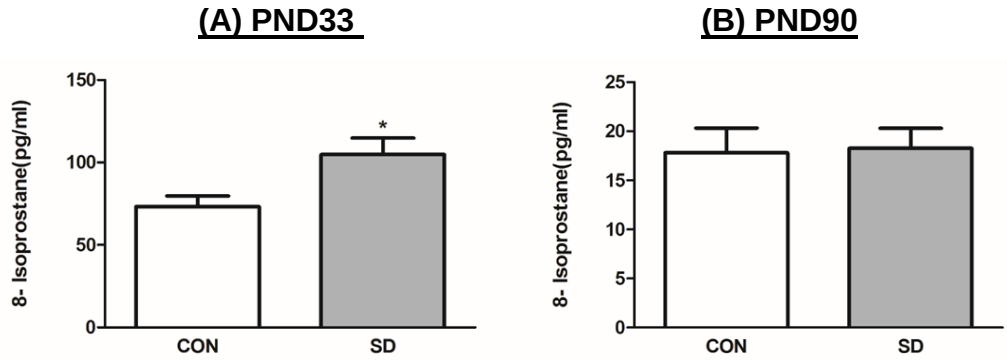


Figure 24. 8-Isoprostane levels in plasma. 8-Isoprostane concentration was measured in plasma of rats at PND33 **(A)** and PND90 **(B)**. Bars are means of 8-isoprostane concentration (pg/ml) ±SEM, n=8 rats/group Data were analyzed using two-tailed unpaired t-test. Group designations: control (CON), sleep deprivation (SD).

6.4.3. Effect of EL-SD on Protein Oxidation Status in the Brain

Oxidation modification in proteins is induced by free radicals; it is an indication of oxidation stress. We measured the level of protein oxidation in the brain regions at PND33. We found a significant elevation of protein oxidation in the hippocampus of SD rats as compared to CON rats (mean oxidation amount in CON and SD groups ± SEM; CON=258900 ± 1285; SD=350900 ± 1678, p=0.0023) **(Figure 25B)**. While SD rats did not show changes in protein oxidation level in PFC and amygdala compared to corresponding regions in CON rats (mean oxidation amount in CON and SD groups ± SEM; PFC: CON= 230500 ± 32130; SD= 248100 ± 4557, p= 0.7738; amygdala: CON= 379500 ± 28; SD= 332100 ± 330, p= 0.306) **(Figure 25 A,C)**.

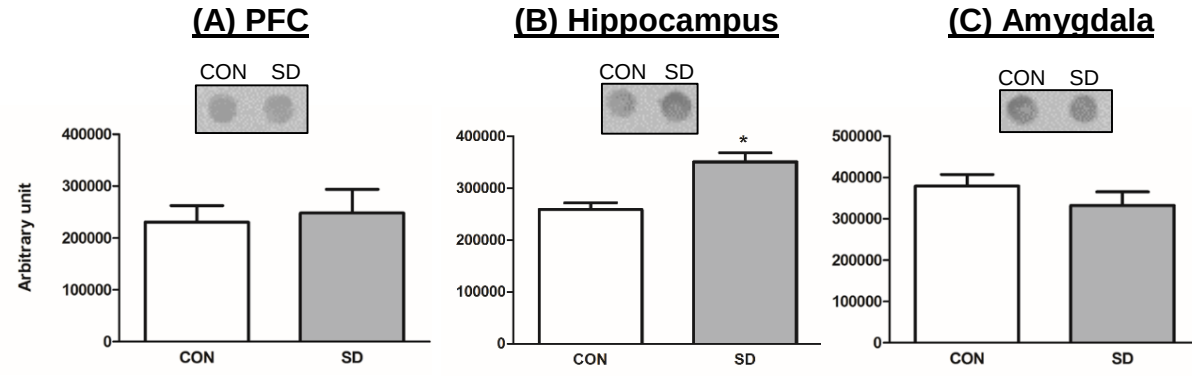


Figure 25. Protein oxidation status in the brain. Protein oxidation status was measured at PND33 in PFC **(A)**, hippocampus **(B)**, and amygdala **(C)**. Bars are means of protein oxidation amount ±SEM, n=6 rats/group Data was analyzed using two-tailed unpaired t-test. (*) significantly different at $p<0.05$. Group designations: control (CON), sleep deprivation (SD). Top panels are representative images of dot blots.

6.4.4. Effect of EL-SD on Protein Nitrosylation Status in the Brain

Protein nitrosylation by reactive nitrogen species (RNS) is an indication of oxidative/ nitrosatvie stress. We measured the nitrosylation level in brain homogenate of PFC, hippocampus and amygdala at PND33. We did not find any change in nitrosylation level between SD and CON rats in the three tested brain regions **(Figure 26)**. (Mean nitrotyrosine concentration in CON and SD groups ± SEM; PFC; CON= 0.114 ± 0.061; SD= 0.0779 ± 0.041, p= 0.646; Hippocampus; CON= 0.202 ± 0.0433; SD= 0.229 ± 0.087, p= 0.790; amygdala; CON= 0.184 ± 0.046; SD= 0.1991 ± 0.035, p= 0.808). The data suggest that protein nitrosylation did not affect by EL-SD.

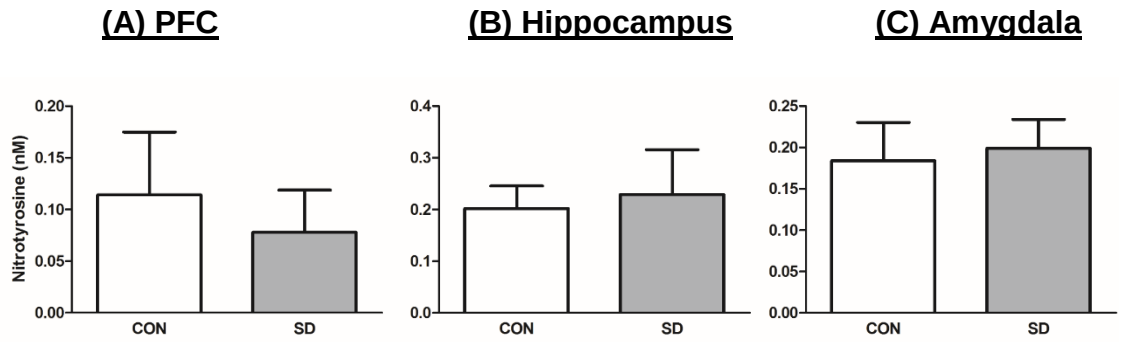


Figure 26. Protein nitrosylation levels in the brain. Protein nitrosylation level was measured at PND33 in PFC **(A)**, hippocampus **(B)**, and amygdala **(C)**. Bars are means of nitrotyrosine concentration ± SEM, n=6 rats/group Data was analyzed using two-tailed unpaired t-test. Group designations: control (CON), sleep deprivation (SD).

6.4.5. Effect of EL-SD on Total Antioxidant Capacity in the Brain

As a response to the increase of pro-oxidants production, the antioxidant defense system increases. In this study, the total antioxidant capacity in PFC, hippocampus, and amygdala was studied at PND33. We did not find a significant change in antioxidant capacity between SD and CON rats in the three brain regions **(Figure 27).** (Mean antioxidant capacity for CON and SD groups ± SEM; PFC; CON=0.0086 ± 0.003; SD=0.013 ± 0.0023, p=0.32; hippocampus; CON=0.032 ± 0.012; SD=0.023 ± 0.005, p=0.489; Amygdala; CON=0.0257 ± 0.0072; SD=0.035 ± 0.0076, p=0.428).

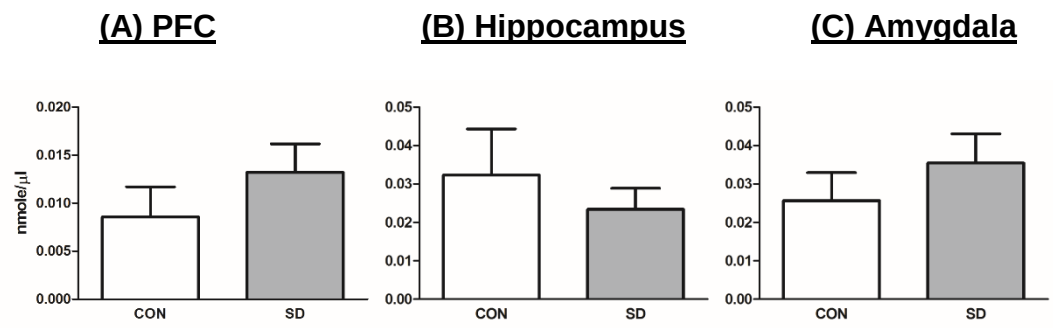


Figure 27. Total antioxidant capacity in the brain. Total antioxidant capacity was measured at PND33 in PFC **(A)**, hippocampus **(B)**, and amygdala **(C)**. Bars are means of antioxidant capacity ±SEM, n=4 rats/group. Data were analyzed using two-tailed unpaired t-test. Group designations: control (CON), sleep deprivation (SD).

6.4.6. Effect of EL-SD on Glutathione GSH/GSSG Ratio in the Brain

When the production of pro-oxidants increase, GSSG accumulates, and the ratio of GSH/GSSG decreases. A decreased ratio of GSH/GSSG is an indication of oxidative stress. We measured the GSH/GSSG ratio in brain regions including; PFC, hippocampus, and amygdala at PND33. We found a significant increase in GSH/GSSG ratio in PFC of SD rats as compared to CON rats (mean of GSH/GSSG ratio ± SEM in CON and SD groups; CON= 2.88 ± 0.162; SD= 3.766 ± 0.378, p= 0.037) **(Figure 28A)**. While no changes in GSH/GSSG ratio in hippocampus and amygdala of SD rats compared to CON rats (mean of GSH/GSSG ratio ± SEM in CON and SD groups; Hippocampus; CON= 3.679 ± 0.439; SD= 3.322 ± 0.351, p= 0.548; Amygdala; CON= 3.142 ± 0.812; SD= 2.571 ± 1.168, p= 0.689) **(Figure 28 B, C)**. The data suggest that sleep deprivation induced pro-oxidant production in PFC leading to the elevation of the antioxidant response.

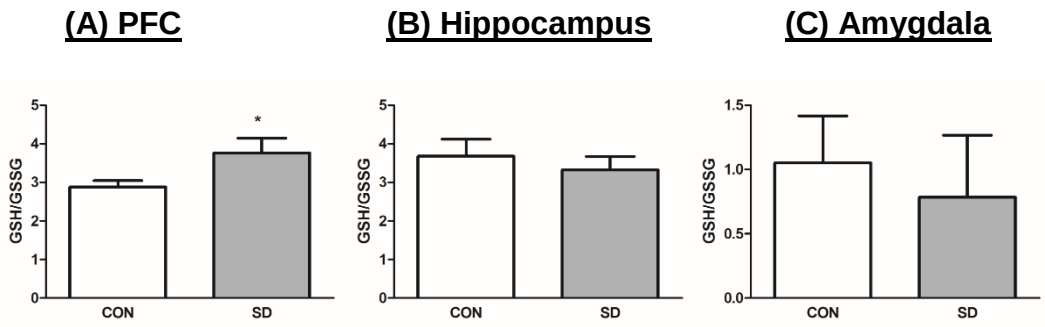


Figure 28. Glutathione GSH/GSSG ratio in the brain. The ratio of the reduced form of glutathione (GSH) to the oxidized form (GSSG) was measured at PND33 in PFC **(A)**, hippocampus **(B)**, and amygdala **(C)**. Bars are means of GSH/GSSG ratio ±SEM, n=6 rats/group. Data were analyzed using the two-tailed unpaired t-test. (*) significantly different at $p<0.05$. Group designations: control (CON), sleep deprivation (SD).

6.4.7. Effect of EL-SD on Protein Level of Antioxidant Enzymes in the Brain

As a result of the elevation of pro-oxidants, the level of antioxidant enzymes increases to counteract the detrimental effects of pro-oxidants. We measured the protein levels of the antioxidant enzymes including; CAT, HO, GSR, and Mn-SOD in brain regions at PND33 and Cu/Zn-SOD and GSR at PND90.

6.4.7.1. Effect of EL-SD on Protein Levels of Catalase (CAT)

The protein levels of the antioxidant enzyme CAT did not change in the tested brain regions of SD when compared to CON rats **(Figure 29)** at PND33. In PFC, CAT level was comparable in CON and SD rats (mean of normalized CAT level ± SEM in CON and SD groups; CON= 0.2149 ± 0.037; SD=0.2603 ± 0.029,

p=0.349). Similarly, SD and CON rats had the same level of CAT in hippocampus (Mean of normalized CAT level ± SEM in CON and SD groups; CON=0.1077 ± 0.007; SD=0.1004 ± 0.010, p=0.576), and amygdala (mean of normalized CAT level ± SEM in CON and SD groups; CON=0.003873 ± 0.0004; SD=0.003353 ± 0.0003, p=0.341).

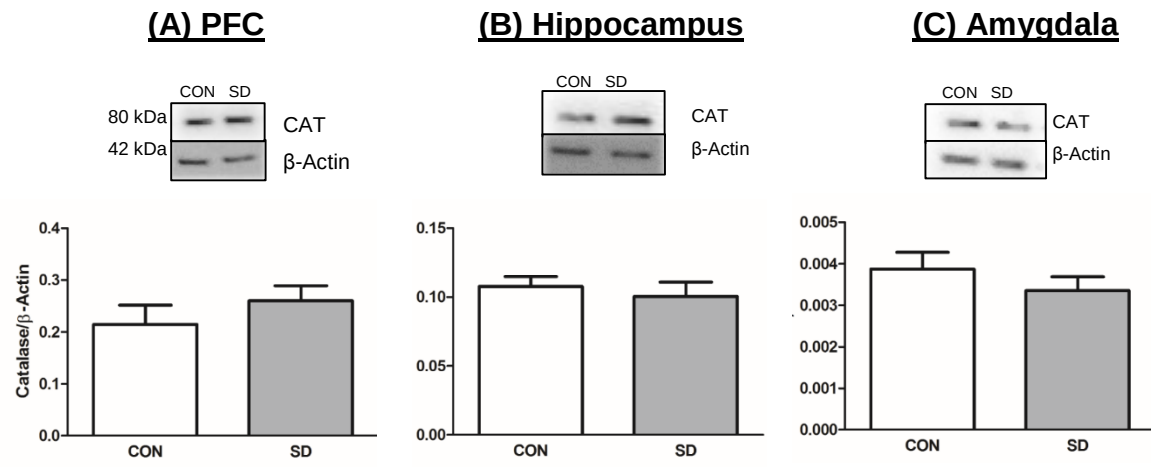


Figure 29. Protein levels of catalase (CAT) in the brain at PND33. The protein levels of CAT were measured in PFC **(A)**, hippocampus **(B)**, and amygdala **(C)** using the western blot technique. The protein levels were normalized with the loading control β-actin. Bars are means of normalized CAT level ± SEM, n=7 rats/group. Data were analyzed using the two-tailed unpaired t-test. (*) significantly different at $p<0.05$. PND: Postnatal day. Group designations: control (CON), sleep deprivation (SD). Top panels are representative western blot images.

6.4.7.2. Effect of EL-SD on Protein Levels of Glutathione Reductase (GSR)

The protein levels of GSR were significantly increased in PFC of SD rats when compared to CON rats **(Figure 30 A)** at PND33 (mean of normalized GSR level ± SEM in CON and SD groups; CON= 0.1022 ± 0.0059; SD= 0.1275 ± 0.0097, p= 0.045). While no change in GSR levels in hippocampus and amygdala of SD rats compared to CON rats **(Figure 30 B, C)** (mean of normalized GSR level ± SEM in CON and SD groups; hippocampus; CON= 0.1380 ± 0.0077; SD= 0.1373 ± 0.006, p= 0.9469; amygdala; CON= 0.1605 ± 0.002; SD= 0.1493 ± 0.010, p= 0.317). The data suggest that SD induced the antioxidant response in PFC.

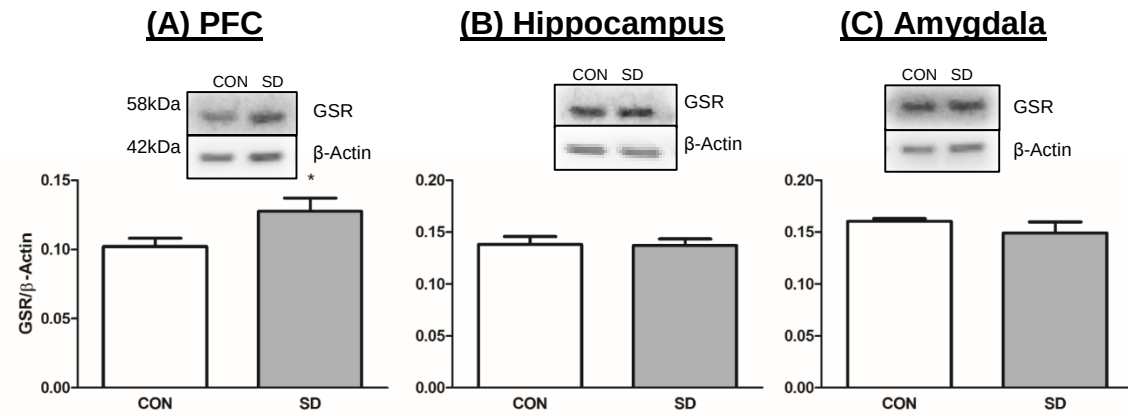


Figure 30. Protein levels of glutathione reductase (GSR) in the brain at PND33. The protein level of GSR was measured in PFC **(A)**, hippocampus **(B)**, and amygdala **(C)**, using the western blot technique. The protein levels were normalized with the loading control β-actin. Bars are means of normalized GSR level ± SEM, n=7 rats/group. Data were analyzed using the two-tailed unpaired t-test. (*) significantly different at $p<0.05$. PND: Postnatal day. Group designations: control (CON), sleep deprivation (SD). Top panels are representative western blot images.

6.4.7.3. Effect of EL-SD on Protein Levels of Manganese superoxide dismutase (Mn-SOD)

The protein levels of Mn-SOD did not change in the tested brain regions of SD rats as compared to CON rats **(Figure 31)** at PND33. In PFC, Mn-SOD level was comparable in CON and SD rats (mean of normalized Mn-SOD level ± SEM in CON and SD groups; CON=13.39 ± 1.343; SD=12.75 ± 1.062, p=0.713). Similarly, SD and CON rats had same level of Mn/SOD in hippocampus (mean of normalized Mn/SOD level ± SEM in CON and SD groups; CON= 2.073 ± 0.342; SD= 1.775 ± 0.310, p= 0.534), and amygdala (mean of normalized Mn-SOD level ± SEM in CON and SD groups; CON= 6.621 ± 0.809; SD= 4.703 ± 0.322, p= 0.059).

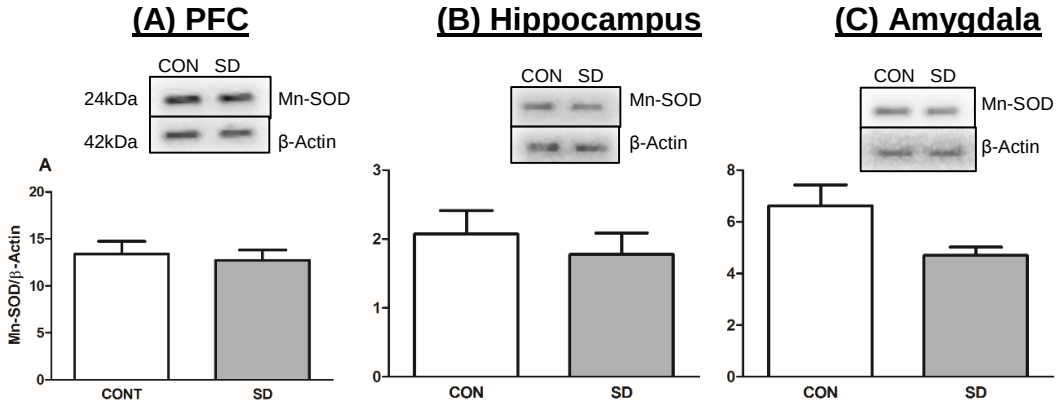


Figure 31. Protein levels of manganese superoxide dismutase (Mn-SOD) in the brain at PND33. The protein level of Mn-SOD was measured in PFC **(A)**, hippocampus **(B)**, and amygdala **(C)**, using the western blot technique. The protein levels were normalized with the loading control β-actin. Bars are means of normalized Mn-SOD level ± SEM, n=7 rats/group. Data were analyzed using the two-tailed unpaired t-test. (*) significantly different at p<0.05. PND: Postnatal day. Group designations: control (CON), sleep deprivation (SD). Top panels are representative western blot images.

6.4.7.4. Effect of EL-SD on Proteins Level of Heme oxygenase (HO)

Protein level of HO did not change in tested brain regions of SD rats when compared to CON rats **(Figure 32)** at PND33. In PFC (mean of normalized HO level ± SEM in CON and SD groups; CON= 0.5647 ± 0.0176; SD= 0.5552 ± 0.053, p= 0.867). In hippocampus (Means of normalized HO level ± SEM in CON and SD groups; CON=1.494 ± 0.142; SD= 1.566 ± 0.115, p= 0.703). In amygdala, (mean of normalized HO level ± SEM in CON and SD groups; CON=0.7020 ± 0.084; SD=0.5611 ± 0.116, p=0.349).

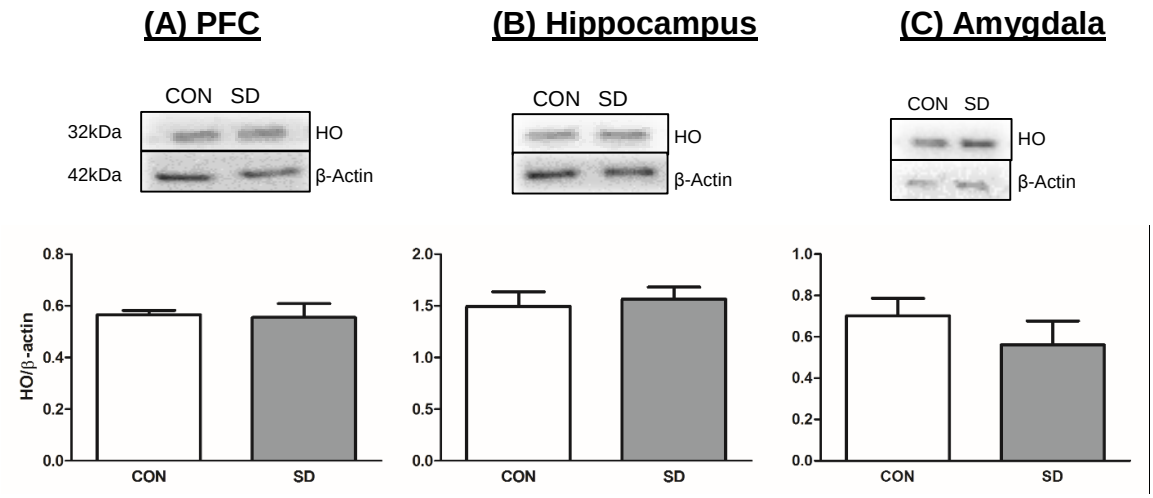


Figure 32. Protein levels of heme oxygenase (HO) in the brain at PND33. The protein level of HO was measured in PFC **(A)**, hippocampus **(B)**, and amygdala **(C)**, using the western blot technique. The protein levels were normalized with the loading control β-actin. Bars are means of normalized HO level ± SEM, n=7 rats/group. Data were analyzed using the two-tailed unpaired t-test. PND: Postnatal day. Group designations: control (CON), sleep deprivation (SD). Top panels are representative western blot images.

We also measured the protein levels of antioxidant enzymes in brain regions at PND90. The protein levels of Cu/Zn-SOD and GSR did not change in SD rat's brain at PND90 **(Figure 2, 3, appendix)**, suggesting mitigation of oxidative stress over time.

6.4.8. Effect of EL-SD on Protein Levels of Stress and Inflammatory Markers in the Brain

Extended wakefulness enhances cellular metabolism leading to an increase in pro-oxidants and stress pathways which in turn induce an inflammatory response (Villafuerte et al., 2015). Elevation of the antioxidant defense mechanisms was evident only in PFC at PND33 where it showed the elevation of glutathione system. Therefore, we measured the protein levels of stress and proinflammatory markers including P38, JNK, TNFα, and lL-6 in PFC at PND33 and 90.

6.4.8.1. Effect of EL-SD on Phosphorylation of MAPKs Stress Markers in PFC

Stress, inflammation or apoptosis leads to activation of p38 and JNK MAP kinases which contribute to alteration of neuronal growth and induction of stress response and inflammation(Collins et al., 2015). We measured the phosphorylation level of p38 and JNK in brain regions at PND33 and 90.

At PND90, the phosphorylation levels of p38 were significantly increased in PFC of SD rats **(Figure 33A)** (mean of normalized phosphorylated p38 levels ± SEM in CON and SD groups: CON= 0.4137 ± 0.078; SD= 0.6896 ± 0.072, p= 0.036). However, we did not find changes in phosphorylation level of p38 in the other brain regions **(Figure 33B, C)** (mean of normalized phosphorylated p38 levels ± SEM in CON and SD groups: Hippocampus; CON= 1.480 ± 0.384; SD= 1.361 ± 0.366, p= 0.829; amygdala: CON= 0.2490 ± 0.0444; SD= 0.3767 ± 0.049, p= 0.076).

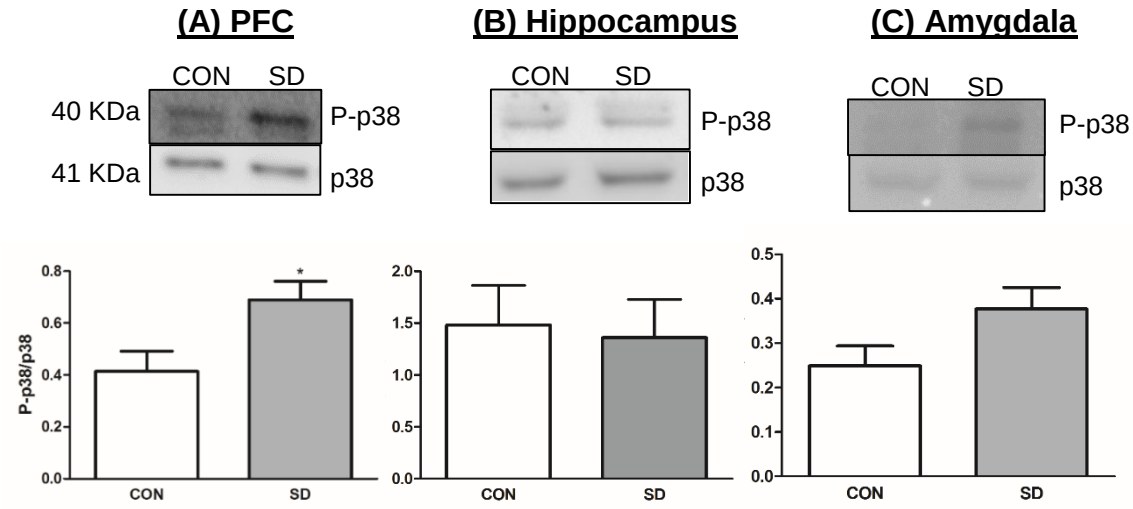


Figure 33. Phosphorylation levels of p38 MAPK in the brain at PND90. The Phosphorylation levels of p38 were measured at PND90 in PFC **(A)**, Hippocampus **(B)**, and amygdala **(C)**, using the western blot technique. The protein phosphorylation levels were normalized with total p38. Bars are means of normalized p38 level ± SEM, n=7 rats/group. Data were analyzed using two-tailed unpaired t-test. (*) significantly different at $p<0.05$. PND: Postnatal day. Group designations: control (CON), sleep deprivation (SD). Top panels are a representative western blot images.

However, the phosphorylation level of JNK did not change in PFC neither in hippocampus at PND90 **(Figure 34A, B)** (means of normalized phosphorylated JNK levels ± SEM in CON and SD groups: PFC; CON= 1.297 ± 0.413; SD= 1.185 ± 0.219, p= 0.806; hippocampus: CON= 0.4617 ± 0.060; SD= 0.5552 ± 0.02764, p= 0.2106).

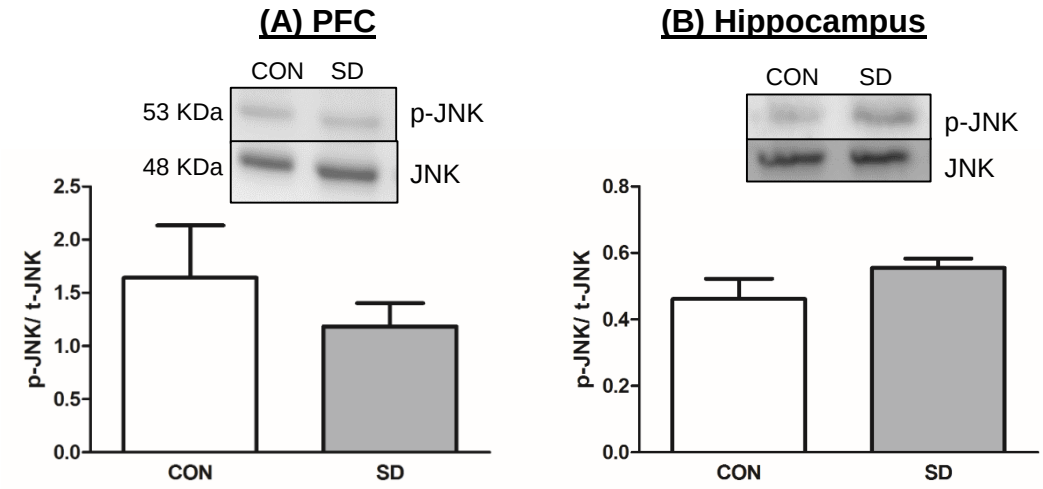


Figure 34. Phosphorylation levels of JNK MAPK in the brain at PND90. The Phosphorylation levels of JNK were measured at PND90 in PFC **(A)**, and hippocampus **(B)**, using the western blot technique. The protein phosphorylation levels were normalized with total JNK. Bars are means of normalized JNK level ± SEM, n=7 rats/group. Data were analyzed using two-tailed unpaired t-test. PND: Postnatal day. Group designations: control (CON), sleep deprivation (SD). Top panels are representative western blot images.

At PND33, we measured the phosphorylation level of p38 and JNK MAPKs in PFC because we found the changes in the phosphorylation level of p38 only in PFC at PND90. However, we did not find any changes in the phosphorylation level

of neither p38 nor JNK in PFC of SD rats as compared to CON rats. For p38 **(Figure 35 A)** (mean of normalized phosphorylated p38 levels ± SEM in CON and SD groups: CON=0.1687 ± 0.0243; SD=0.1223 ± 0.0194, p=0.174) For JNK **(Figure 35B)** (mean of normalized phosphorylated JNK levels ± SEM in CON and SD groups: CON=0.1709 ± 0.0126; SD=0.1456 ± 0.0287, p=0.434).

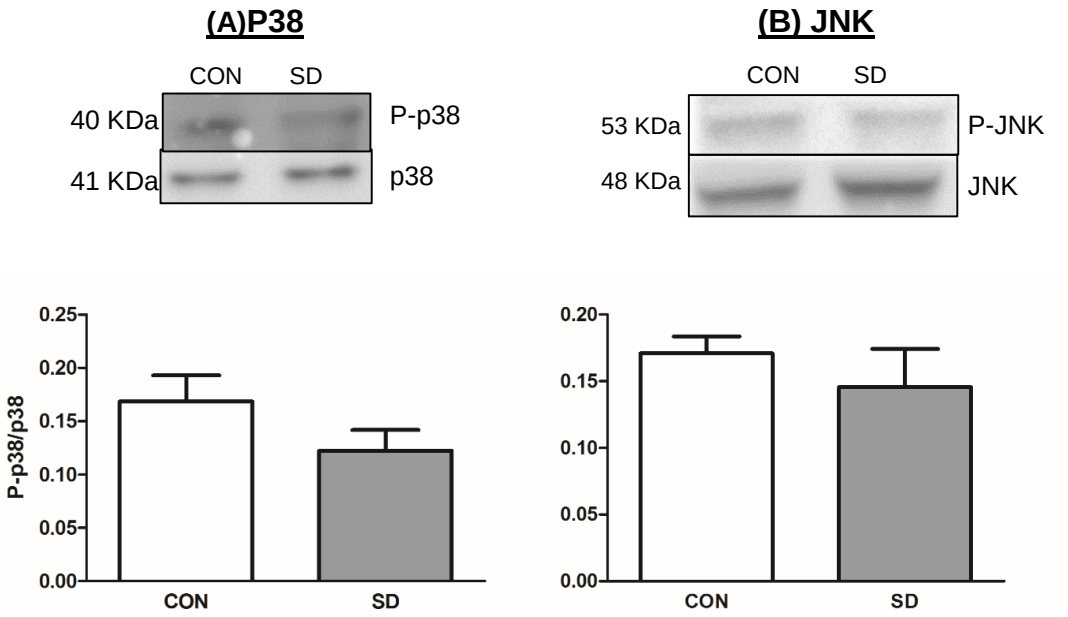


Figure 35. Phosphorylation levels of the MAPKs; p38 and JNK in PFC at PND33. The Phosphorylation levels of p38 were measured at PND33 in PFC **(A)** using the western blot technique. The protein phosphorylation levels were normalized with total p38. Bars are means of normalized p38 level ± SEM, n=7 rats/group. The phosphorylation levels of JNK were measured at PND33 in PFC **(B)**, using the western blot technique. The protein phosphorylation levels were normalized with total JNK. Bars are means of normalized JNK level ± SEM, n=7 rats/group. Data were analyzed using two-tailed unpaired t-test. PND: Postnatal day. Group designations: control (CON), sleep deprivation (SD). Top panels are representative western blot images.

6.4.8.2. Effect of EL-SD on Protein Levels of Proinflammatory Markers in PFC

We measured the protein levels of proinflammatory markers; interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) in PFC at PND33 and 90. The protein levels of IL-6 were significantly increased in PFC of SD rats at PND33 **(Figure 36A)** (means of normalized IL-6 level ± SEM in CON and SD groups; CON= 0.4689 ± 0.056; SD= 0.6710 ± 0.057, p= 0.034). Similarly, the protein levels of IL6 were significantly increased in PFC of SD rats at PND90 **(Figure 36B)** (mean of normalized IL-6 level ± SEM in CON and SD groups; CON= 0.7355 ± 0.127; SD= 1.530 ± 0.243, p= 0.0159). The data suggest that EL-SD induced biosynthesis of proinflammatory markers in PFC which persist until adult stage.

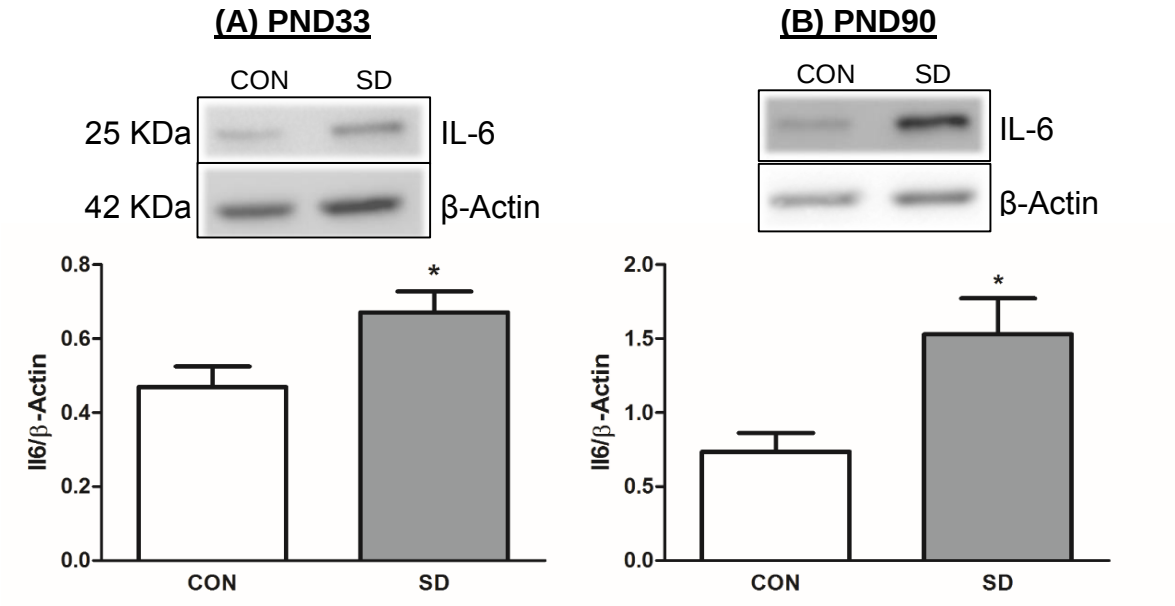


Figure 36. Protein levels of interleukin-6 (IL-6) in PFC at PND33 and PND90. The protein level of IL-6 was measured in PFC at PND33 **(A)**, and PND90 **(B)**, using the western blot technique. The protein levels were normalized with the loading control β-actin. Bars are means of normalized IL6 level ± SEM, n=7 rats/group. Data were analyzed using two-tailed unpaired t-test. (*) significantly different at $p<0.05$. PND: Postnatal day. Group designations: control (CON), sleep deprivation (SD). Top panels are representative western blot images.

However, the protein level of TNF-α did not change in PFC of SD rats **(Figure 37 A, B)** (means of normalized TNF-α level ± SEM in CON and SD groups; PND33: CON= 0.6541 ± 0.066; SD= 0.6341 ± 0.079; p= 0.853; PND90: CON= 1.512 ± 0.285; SD= 1.784 ± 0.182, p= 0.441)

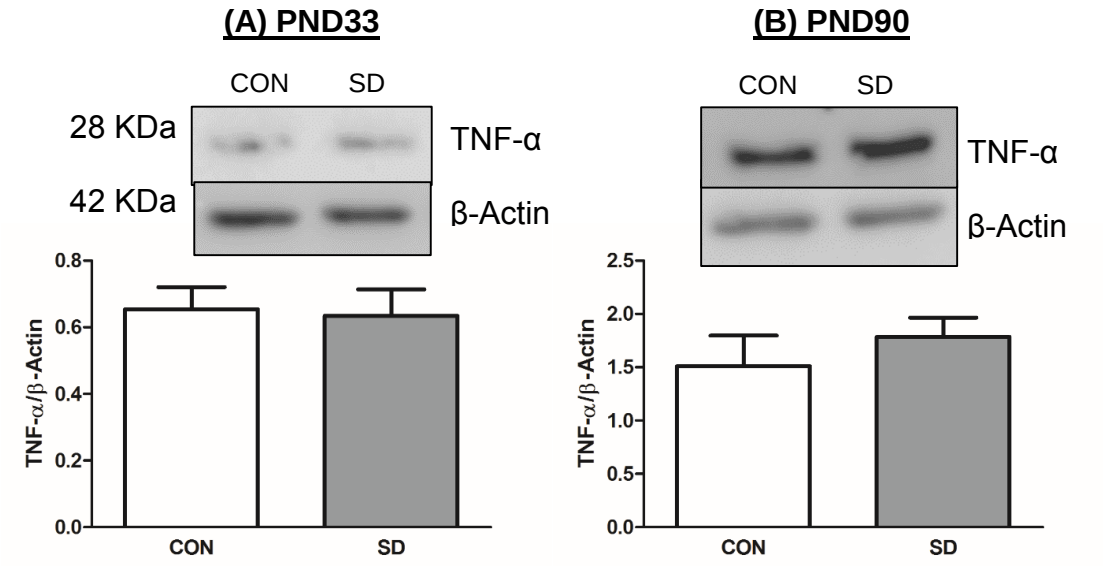


Figure 37. Protein levels of the tumor necrosis factor-α (TNF-α) in PFC at PND33 and 90. The protein level of TNF-α was measured in PFC at PND33 **(A)**, and PND90 **(B)**, using the western blot technique. The protein levels were normalized with the loading control β-actin. Bars are means of normalized TNF-α level ± SEM, n=7 rats/group. Data were analyzed using two-tailed unpaired t-test. PND: Postnatal day. Group designations: control (CON), sleep deprivation (SD). Top panels are representative western blot images.

6.4.9. Effect of EL-SD on Protein Level of Neurotrophic Signaling Pathway Markers in the Brain

The brain-derived neurotrophic factor (BDNF) is critical for neuronal survival, growth and synaptic plasticity (Duman, 2002b). We measured the effect of EL-SD on protein levels of BDNF and the downstream signaling pathway markers including; extracellular regulated kinase ($ERK_{1/2}$) and MAPK phosphatase (MKP1) in PFC of rats at PND33 and 90.

The protein levels of BDNF did not change in PFC of SD rats at PND33 **(Figure 38A)** (mean of normalized BDNF level ± SEM in CON and SD groups; CON=0.9578 ± 0.0519; SD=1.045 ± 0.0345, p=0.215), whereas the protein levels of BDNF were significantly decreased in PFC of SD rats at PND90 **(Figure 38B)** (mean of normalized BDNF level ± SEM in CON and SD groups; CON=1.114 ± 0.01362; SD=0.8040 ± 0.111, p=0.0134).

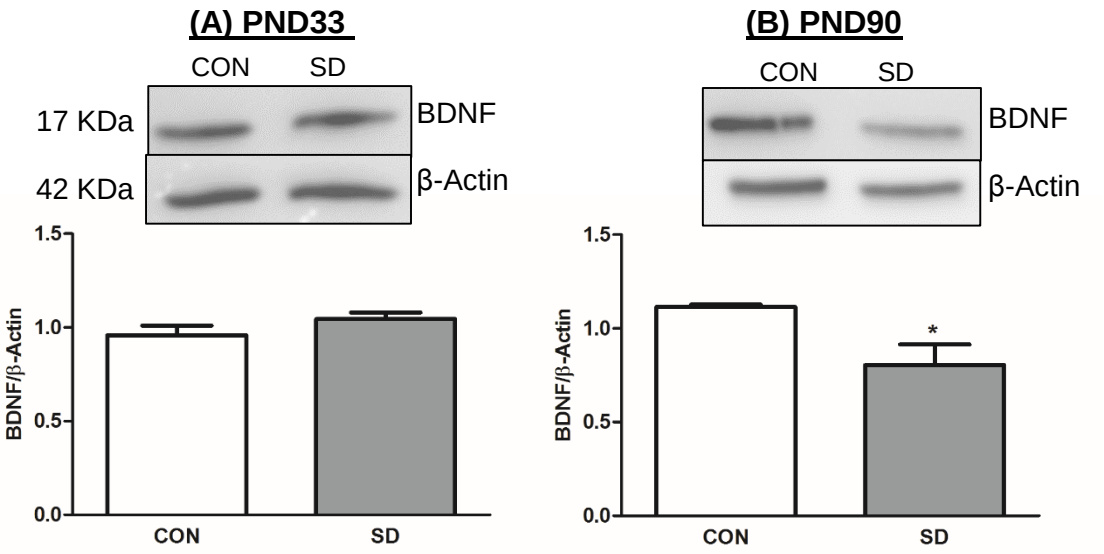


Figure 38. Protein levels of the brain-derived neurotrophic factor (BDNF) in PFC at PND33 and 90. The protein level of BDNF was measured in PFC at PND33 **(A)**, and PND90 **(B)**, using the western blot technique. The protein levels were normalized with the loading control β-actin. Bars are means of normalized BDNF level ± SEM, n=7 rats/group. Data were analyzed using two-tailed unpaired t-test. (*) significantly different at $p<0.05$. PND: Postnatal day. Group designations: control (CON), sleep deprivation (SD). Top panels are representative western blot images.

However, we did not find the change in BDNF levels in the hippocampus and amygdala of SD rats at PND90 **(Figure 4, appendix)**. The data indicated that BDNF protein level was decreased only in PFC of SD rats at PND90.

The phosphorylation levels of $ERK_{1/2}$ were significantly decreased in PFC of SD rats at PND33 and PND90 as compared to CON rats **(Figure 39A, B)** (mean of normalized phosphorylated $ERK_{1/2}$ levels ± SEM in CON and SD groups; PND33: CON= 0.1549 ± 0.010; SD= 0.08802 ± 0.0146, p= 0.003; PND90: CON= 0.4681 ± 0.110; SD= 0.1776 ± 0.0125, p= 0.035). However, $ERK_{1/2}$ phosphorylation level did not change in the hippocampus and amygdala at PND 90 **(Figure 5, appendix)**. The findings indicate that as a result of EL-SD, $ERK_{1/2}$ activation decreased in PFC.

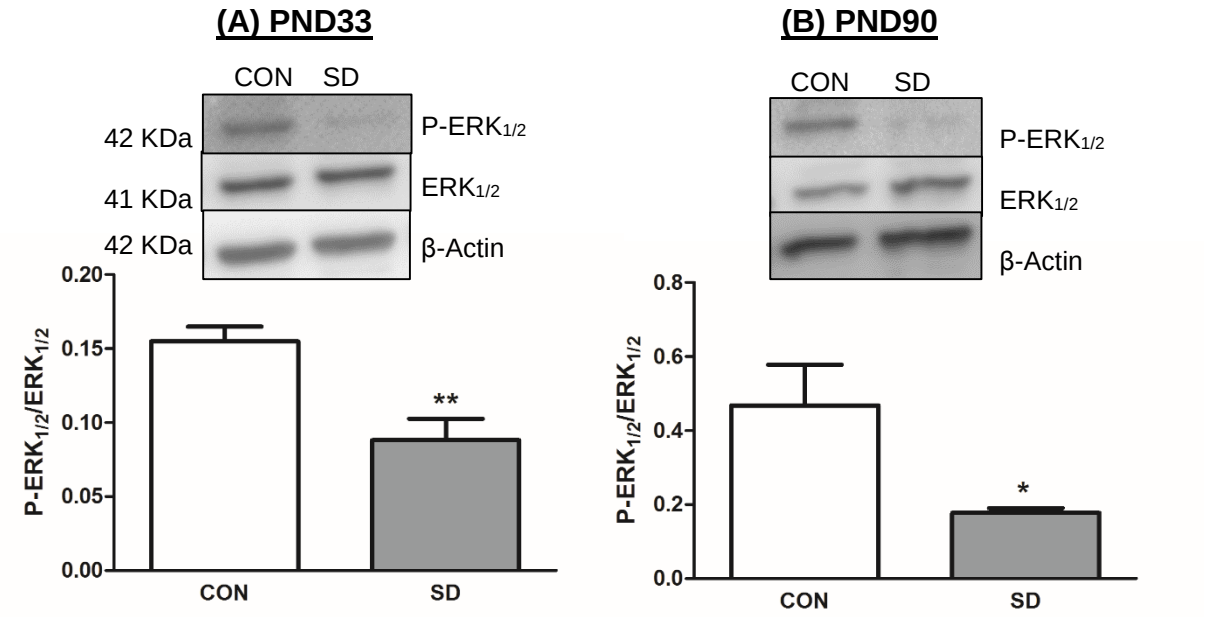


Figure 39. Phosphorylation levels of the extracellular signal-regulated kinase ($ERK_{1/2}$) in PFC at PND33 and 90. The Phosphorylation levels of $ERK_{1/2}$ were measured in PFC at PND33 **(A)**, and PND90 **(B)**, using the western blot technique. The protein phosphorylation levels were normalized with total $ERK_{1/2}$. Bars are means of normalized $ERK_{1/2}$ level ± SEM, n=7 rats/group. Data were analyzed using two-tailed unpaired t-test. (**) significantly different at $p<0.01$. (*) significantly different at $p<0.05$. PND: Postnatal day. Group designations: control (CON), sleep deprivation (SD). Top panels are representative western blot images.

The protein levels of MKP1 did not change in PFC of SD rats at PND33 when compared with CON rats **(Figure 40A)** (mean of normalized MKP1 levels ± SEM in CON and SD groups: CON= 0.1964 ± 0.022; SD= 0.2372 ± 0.026, p= 0.252), whereas at PND90, MKP1 protein levels were significantly increased in PFC of SD rats as compared to CON rats **(Figure 40B)** (mean of normalized MKP1 levels ± SEM in CON and SD groups: CON= 0.2439 ± 0.077; SD= 0.4484 ± 0.038, p= 0.039).

However, the protein level of MKP1 did not alter by EL-SD in other brain regions including the hippocampus and amygdala at PND90 **(Figure 6, appendix).**

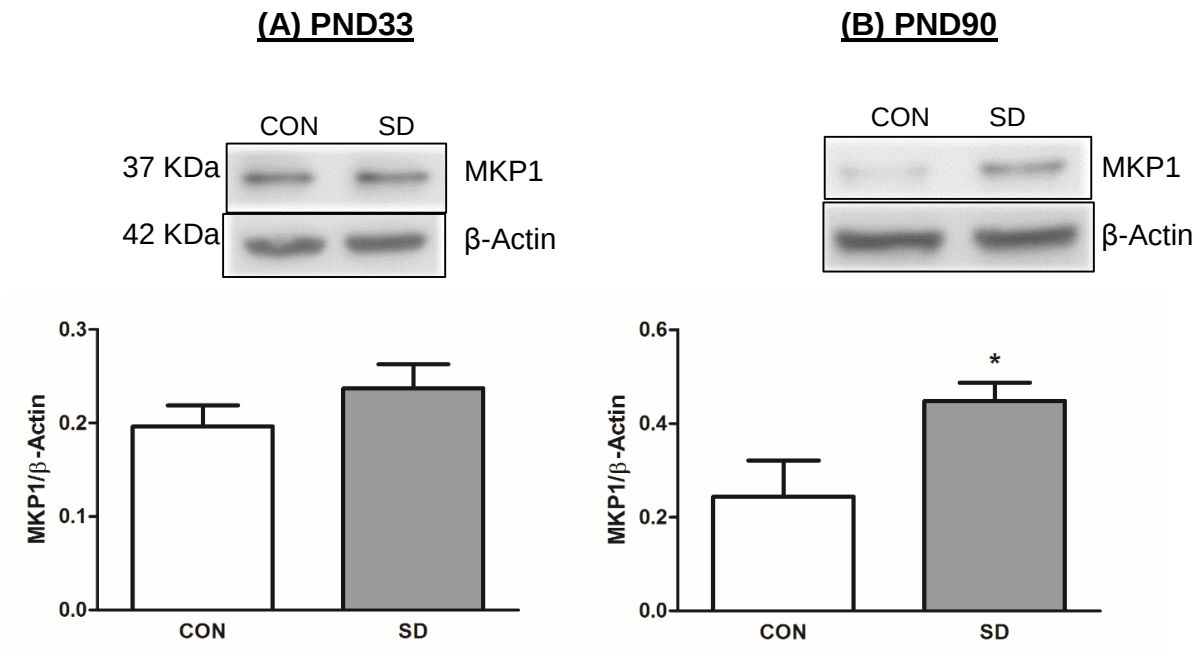


Figure 40. Protein levels of the MAPK phosphatase 1(MKP1) in PFC at PND33 and 90. The protein level of MKP1was measured in PFC at PND33 **(A)**, and PND90 **(B)**, using the western blot technique. The protein levels were normalized with the loading control β-actin. Bars are means of normalized MKP1 level ± SEM, n=7 rats/group. Data were analyzed using two-tailed unpaired t-test. (*) significantly different at $p<0.05$. PND: Postnatal day. Group designations: control (CON), sleep deprivation (SD). Top panels are representative western blot images.

6.4.10. Effect of EL-SD on Protein Level of Synaptic Density Markers in PFC

A growing body of evidence suggests that alteration of synaptic density and plasticity is associated with mood disorders. Expression of AMPA and NMDA receptors is positively correlated with synaptic density and plasticity (Marsden, 2013). Here, we measured the protein levels of GluA1 (AMPA receptor subunit), GluN2b (NMDA receptor subunit), and postsynaptic density protein (PSD95) in PFC of rats at PND33 and 90.

The protein levels of GluA1 were significantly decreased in PFC of SD rats at PND33 and PND90 as compared to CON rats **(Figure 41 A, B)** (mean of normalized GluA1 levels ± SEM in CON and SD groups; PND33: CON= 0.4308 ± 0.1298; SD= 0.1202 ± 0.039, p= 0.041; PND90: CON= 18.90 ± 5.20; SD= 5.962 ± 1.488, p= 0.021). The data suggest that synaptic density and plasticity were decreased in PFC of SD rats as compared to CON rats.

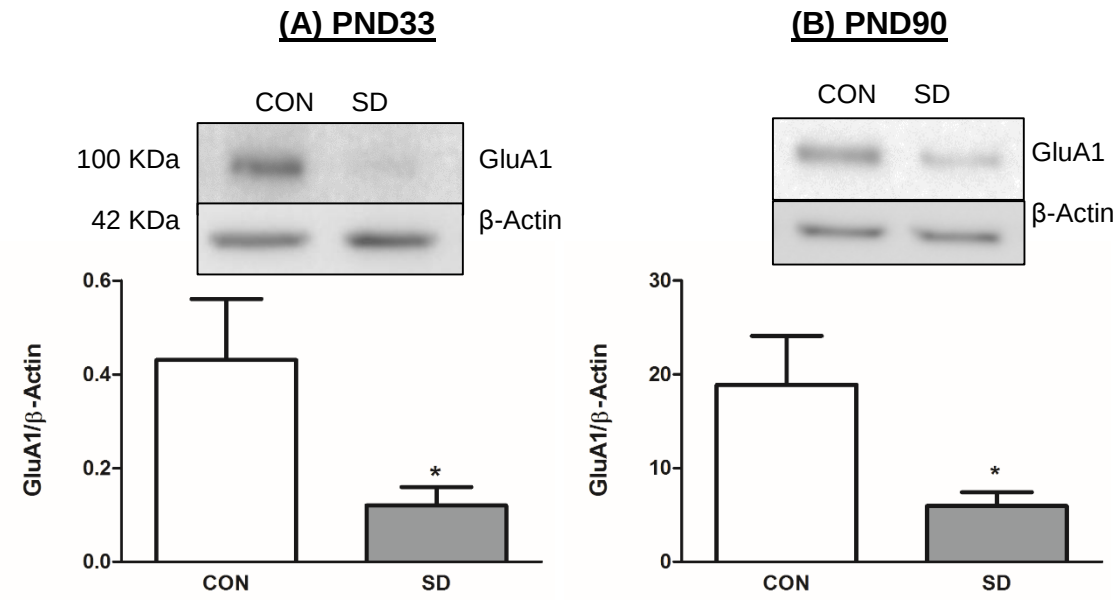


Figure 41. Protein levels of the AMPA receptor subunit (GluA1) in PFC at PND33 and 90. The protein level of GluA11was measured in PFC at PND33 **(A)**, and PND90 **(B)**, using the western blot technique. The protein levels were normalized with the loading control β-actin. Bars are means of normalized GluA1 level ± SEM, n=7 rats/group. Data were analyzed using two-tailed unpaired t-test. (*) significantly different at $p<0.05$. PND: Postnatal day. Group designations: control (CON), sleep deprivation (SD). Top panels are representative western blot images.

The protein levels of GluN2b were significantly decreased in PFC of SD rats at PND33 and 90 as compared to CON rats **(Figure 42 A, B)** (means of normalized GluN2b levels ± SEM in CON and SD groups; PND33: CON= 0.4286 ± 0.0439; SD= 0.1239 ± 0.051, p= 0.0007; PND90: CON= 0.5974 ± 0.107; SD= 0.1121 ± 0.0219, p= 0.0008). The data suggest that synaptic density and plasticity were decreased in PFC of SD rats as compared to CON rats.

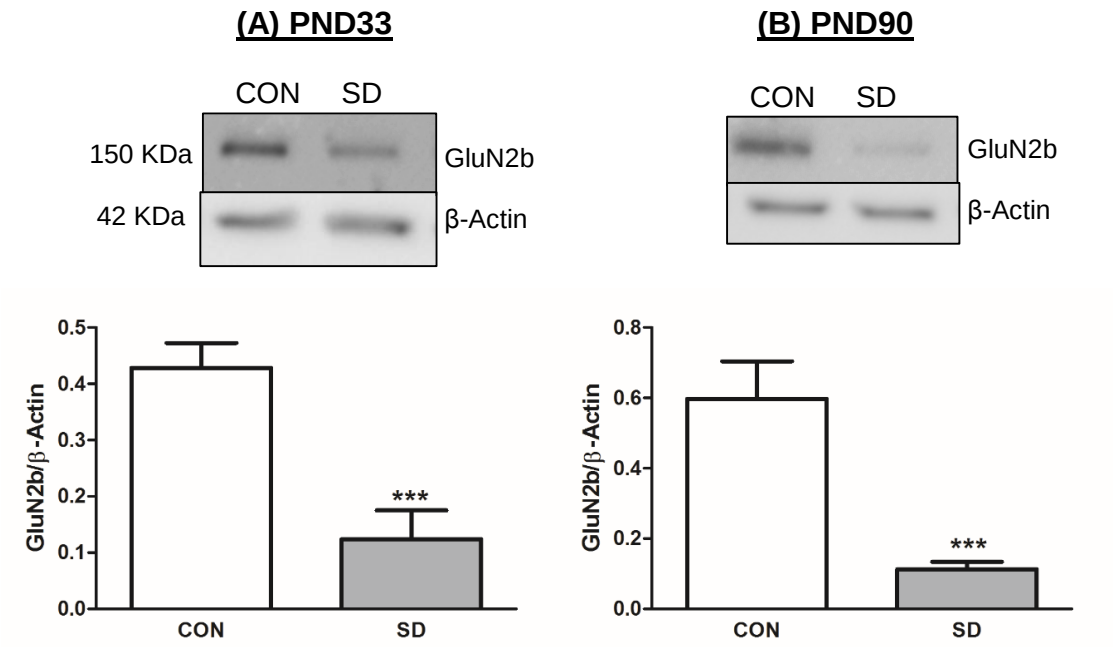


Figure 42. Protein levels of the NMDA receptor subunit (GluN2b) in PFC at PND33 and 90. The protein level of GluA11was measured in PFC at PND33 **(A)**, and PND90 **(B)**, using the western blot technique. The protein levels were normalized with the loading control β-actin. Bars are means of normalized GluN2b level ± SEM, n=7 rats/group. Data were analyzed using two-tailed unpaired t-test. (*) significantly different at p<0.05. (***) significantly different at p<0.005. PND: Postnatal day. Group designations: control (CON), sleep deprivation (SD). Top panels are representative western blot images.

The protein levels of PSD95 were significantly decreased in PFC of SD rats at PND32 and 90 as compared to CON rats **(Figure 43 A, B)** (mean of normalized PSD95 levels ± SEM in CON and SD groups; PND33: CON= 0.3018 ± 0.0369; SD= 0.1702 ± 0.0265, p= 0.017; PND90: CON= 0.3070 ± 0.0696; SD= 0.06972 ± 0.0083, p= 0.0054). The data suggest that synaptic density and plasticity were decreased in PFC of SD rats as compared to CON rats.

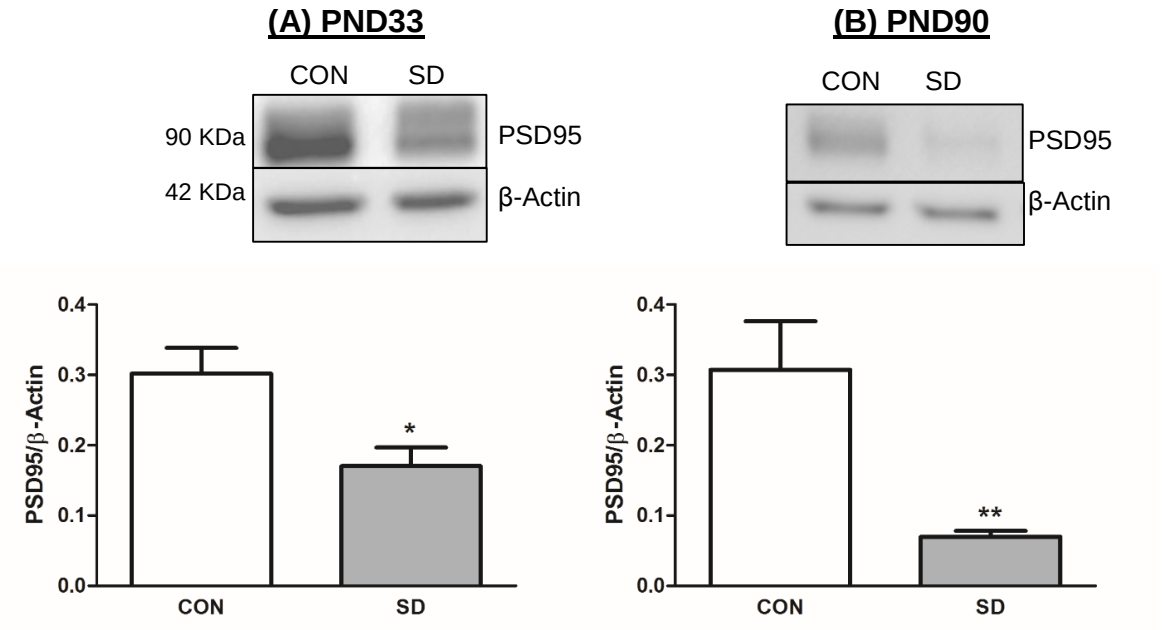


Figure 43. Protein levels of the postsynaptic density protein (PSD95) in PFC at PND33 and 90. The protein level of GluA11was measured in PFC at PND33 **(A)**, and PND90 **(B)**, using the western blot technique. The protein levels were normalized with the loading control β-actin. Bars are means of normalized PSD95 level ± SEM, n=7 rats/group. Data were analyzed using two-tailed unpaired t-test. (*) significantly different at $p<0.05$. (**) significantly different at $p<0.001$. PND: Postnatal day. Group designations: control (CON), sleep deprivation (SD). Top panels are representative western blot images.

6.4.11. Effect of EL-SD on Protein Level of Synaptic Plasticity Markers in PFC

Sleep homeostasis theory suggests that sleep plays a critical role in synaptic plasticity. Moreover, synaptic plasticity has been implicated in mood disorders (Tononi & Cirelli, 2014). Therefore, we measured the activation and protein levels of synaptic plasticity markers including; CaMKII and CREB.

The phosphorylation levels of CaMKII were significantly decreased in PFC of SD rats at PND32 and PND90 as compared to CON rats **(Figure 44 A, B)** (mean of normalized CaMKII levels ± SEM in CON and SD groups; PND33: CON= 0.9310 ± 0.156; SD= 0.3493 ± 0.108, p= 0.012; PND90: CON= 0.387 ± 0.097; SD= 0.129 ± 0.036, p= 0.025). The data suggest that synaptic plasticity was decreased in PFC of SD rats as compared to CON rats.

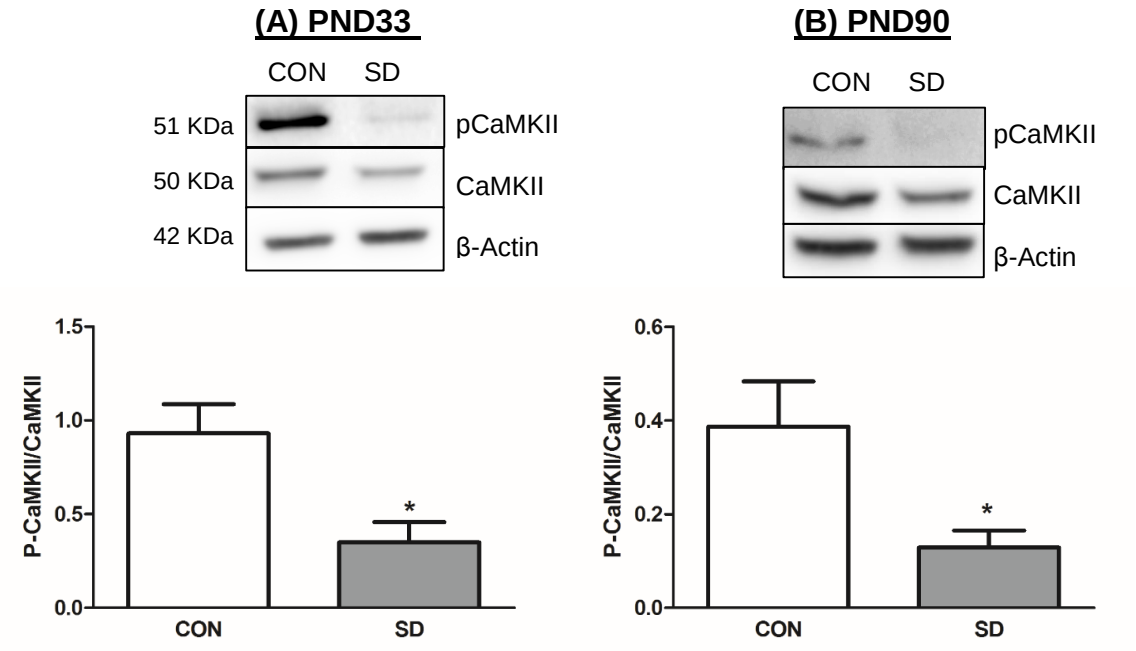


Figure 44. Phosphorylation levels of the calcium/calmodulin-dependent kinase II (CaMKII) in PFC at PND33 and 90. The Phosphorylation levels of CaMKII were measured in PFC at PND33 **(A)**, and PND90 **(B)**, using the western blot technique. The protein phosphorylation levels were normalized with total CaMKII. Bars are means of normalized CaMKII level ± SEM, n=7 rats/group. Data were analyzed using two-tailed unpaired t-test. (*) significantly different at $p<0.05$. PND: Postnatal day. Group designations: control (CON), sleep deprivation (SD). Insets are a representative western blot images.

The phosphorylation levels of CREB in PFC did not change in SD rats at PND33 as compared to CON rats **(Figure 45A)** (mean of normalized CREB levels ± SEM in CON and SD groups; CON=3.093 ± 0.304; SD=3.473 ± 0.375, p=0.446; PND90: CON=0.6105 ± 0.061; SD=0.6266 ± 0.0785, p=0.878), While at PND90, the phosphorylation levels of CREB in PFC of SD rats were significantly reduced when compared to CON rats **(Figure 45B)** (means of normalized CREB levels ± SEM in CON and SD groups; CON=0.7899 ± 0.1069; SD=0.5041 ± 0.0481, p=0.042).

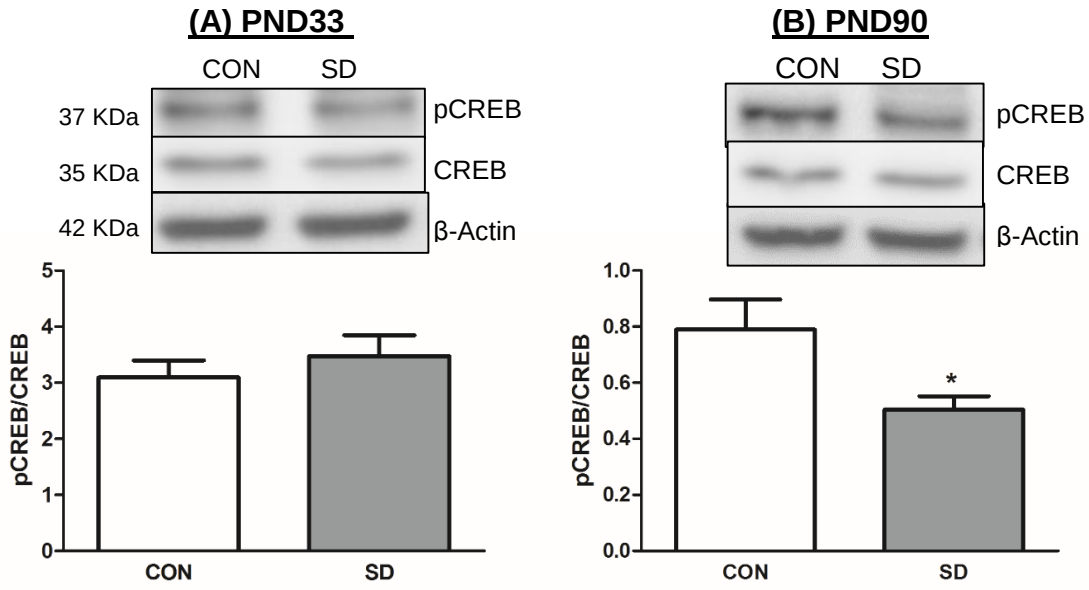


Figure 45. Phosphorylation levels of the cAMP response element binding protein (CREB) in PFC at PND33 and 90. The Phosphorylation levels of CREB were measured in PFC at PND33 **(A)**, and PND90 **(B)**, using the western blot technique. The protein phosphorylation levels were normalized with total CREB. Bars are means of normalized CREB level ± SEM, n=7 rats/group. Data were analyzed using two-tailed unpaired t-test. (*) significantly different at p<0.05. PND: Postnatal day. Group designations: control (CON), sleep deprivation (SD). Top panels are representative western blot images.

7. DISCUSSION

7.1. Behavioral Alterations in Rats Following Early Life Sleep Deprivation

Sleep by promoting neuronal myelination and synaptic remodeling processes (Bellesi et al., 2013; Maret et al., 2011), not only contributes to brain development and maturation (Kurth et al., 2010a; Kurth et al., 2015), but also maintains normal behavior and cognition (Maski & Kothare, 2013). Considering that postnatal brain development occurs early in life, sleep deprivation (SD) during early life can elicit negative consequences in children and adolescents. In fact, children are believed to be highly vulnerable to SD associated behavioral impairment (Astill et al., 2012; Dewald et al., 2010; Maski & Kothare, 2013). Conducting SD studies in children is challenging. Therefore, animal studies are useful in studying the impact of SD on behavior and cognition, especially across different developmental stages. Relevant to this, the present animal study of early life SD offers exciting new insights. Our study has multiple innovative features. *First*, our sleep deprivation system has several procedural advantages as compared to the existing models. While most models of sleep deprivation have issues of confounding factors of external stress, isolation stress, and physical fatigue (Zhao et al., 2017), our model has no such caveats. The method we used gently disturb the pups without forcing them to do an extra physical activity, additionally, having two rats together in the same cage during sleep deprivation eliminates the isolation stress. Further, lack of stress was evident as plasma and

urine levels of corticosterone in SD rats were comparable to CON rats. *Second*, successful sleep deprivation was quantitatively examined via measurement of immobility bouts using a software-based assessment. In our settings, the system detects immobility at ≥45 seconds. Therefore, short bouts of sleep which are ≤45 seconds were possible during sleep deprivation hours. Furthermore, using video recording, we could not measure rebound in sleep as a response to SD, since this requires surgery for probe implanting and EEG acquisition. Hence, we estimated the total hours of sleep and wakefulness with simple noninvasive video recording method. We observed that SD rats showed an insignificant increase in immobility time during the rest of the light cycle right following sleep deprivation hours as compared to CON rats **(Figure 15)**. This increase likely represents compensation for sleep deprivation hours. However, Immobility time and bouts were comparable in SD and CON rats during the following dark cycles throughout the sleep deprivation protocol except for few days **(Table 5)**, which suggests that SD rats in most days did not sleep more than CON rats during the dark active cycles. In general, the data suggest that SD rats slept fewer hours than CON rats throughout the sleep deprivation protocol. Therefore, our model of sleep deprivation efficiently restricted sleep hours in SD rats. *Third*, our observations that early life SD induces an early occurrence of anxiety-like behavior and delayed onset of depression-like behavior in rats, without changes in learning-memory and social interaction behaviors, are translationally relevant. In our study, the anxiety-like behavior was

observed right after induction of SD protocol (PND33) in light/dark (LD) and elevated plus maze (EPM) anxiety tests, but the anxiety-like phenotype seemed to fade by the end of the adolescent stage (PND60). At PND60, SD rats showed anxiety-like behavior in EPM test but not in LD test. Test sensitivity seems to play a role in these results. LD test detects the anxiety indicated by avoidance of lit areas, but EPM test detects anxiety-like behavior that results from avoidance of open, lit, and elevated areas. Thus, while rats at PND60 did not exhibit anxiety-like behavior in a mild LD test (Salim et al., 2010), but the anxiety-like phenotype was evident in a more stringent and robust EPM test (Kochi et al., 2017). Interestingly, in open field test (OFT), SD rats did not show any change in their total activity or time spent in the center area at PND33, 60 or 90, suggesting that exploration and locomotion of rats not be impacted by sleep deprivation. OFT is a movement-based test, and SD rats seemed to be hyperactive. Hence their overall exploration was, in fact, higher than control groups. We have, therefore, focused on SD-related anxiety behavior using LD and EPM data. Additionally, rearing time during the last three days of SD protocol was significantly decreased in SD rats as compared to CON rats **(Figure 16)**. It has been suggested that rearing time is inversely related to the onset of anxiety-like behavior in rats (Urakawa et al., 2013). Interestingly, in our results, SD rats showed reduced rearing time followed by anxiety-like behavior onset.

The issue of panic, hypervigilance, and hyperactivity was also evident in FST and RAWM tests conducted at PND33, as rats swam excessively in the water tanks. Interestingly, hyperactivity diminished at PND60 and PND90, suggesting that perhaps SD induced hyperactivity and hypervigilance, which lasted for some time but diminished later. SD-induced hyperactivity is already reported in humans (Touchette et al., 2009) and animals (Gessa et al., 1995). Early induction of anxiety-like behavior and late onset of depression-like behavior indicate that anxiety potentially transforms into depression during development (Liu, Atrooz, Salvi, & Salim, 2017). The potential transformation of anxiety into depression also raises the intriguing possibility that anxiety is the pathway to depression and that anxiety induces depression (Fairhurst, 1985). Early occurrence of anxiety following SD (PND33 and 60) and later development of depression at PND90 also suggest that perhaps behavior switching mechanisms are marked during the PND33-60 period. Synaptic remodeling and neuronal myelination, which are important for the development of neuronal circuits and synaptic plasticity, occur during childhood and early adolescence in human and rodents (Kurth et al., 2015; Semple et al., 2013). (Lopez et al., 2010) found that REM sleep deprivation hindered hippocampal neuronal circuit indicated by reduced expression of mature glutamatergic synaptic components and instability of long term potentiation (LTP) in the hippocampus of young rats, PND16 (Lopez et al., 2008). Therefore, it is highly likely that early life SD by engaging stress mechanisms, alters synaptic

remodeling and neuronal myelination. Alteration of synaptic remodeling and neuronal myelination weakens neuronal connectivity specifically, the pre-frontal cortex and amygdala circuit, causing anxiety-like behaviors at PND33 and PND60. We postulate that the biochemical sequelae initiated by early life SD continue to compromise the pre-frontal cortex-amygdala circuit (Goldstein & Walker, 2014; Maski & Kothare, 2013) while involving additional brain areas, and causing more permanent changes in synaptic integrity and function, which consequently leads to depression-like behavior at PND90.

7.2. Biochemical Alterations in Rats Following Early Life Sleep Deprivation

Sleep deprivation induces short-term oxidative stress indicated by systemic elevation of plasma 8-isoprostane at PND 33 but not PND90 **(Figure 24)**, and by the enhanced protein oxidation level in the hippocampus at PND33 **(Figure 25)**. Elevation of oxidative stress markers has repeatedly been reported in patients with sleep problems as well as in animal models of SD (Chen et al., 2011; Villafuerte et al., 2015; Vollert et al., 2011; Zhang et al., 2013). Although, other studies did not find evidence of oxidative stress following SD in animal models (Gopalakrishnan, Ji, & Cirelli, 2004). The acute elevation of oxidative stress is further associated with elevation of the glutathione system in PFC of SD rats only following SD protocol (PND33), **(Figures 26A and 28A)**. The data suggest that SD in our model induced

acute oxidative stress, which was mitigated with time as plasma 8-isoprostane levels were comparable in CON and SD rats at PND90 **(Figure 24B)** and no changes in antioxidant enzymes at this time point in brain regions **(Figure 2, 3, appendix)**. The finding that the hippocampus was the only brain region that showed an increase in protein oxidation suggested that the hippocampus is highly vulnerable to oxidative stress. The variability in neuronal vulnerability to oxidative stress was discussed earlier (Wang & Michaelis, 2010). While acute oxidative stress induced during SD period might be mitigated with time, its effect on other signaling pathways might last longer. Relevant to this, pro-inflammatory markers which have been correlated with oxidative stress induction (Salim et al., 2011) were induced in PFC of SD rats at PND33 and PND90 **(Figure 36)**. Our finding that SD caused an elevation in IL-6 in PFC of SD rats is important. Elevation of the proinflammatory marker IL-6 as a response to SD was reported by several studies in human and animal models (Chennaoui et al., 2015; Irwin, Olmstead, & Carroll, 2016; Ritter, Kretschmer, Pfennig, & Soltmann, 2013). In fact, the correlation between sleep architecture and IL-6 suggests that alteration of SD can have a direct effect on the IL-6 level (Hong, Mills, Loredo, Adler, & Dimsdale, 2005). The elevation of IL-6 in SD rats is an interesting finding which might be the link between stress and induction of stress response molecules such as the MAP kinases (p38, JNK) and the MAPK phosphatase (MKP1). In response to stress and inflammation, activity, and expression of p38 MAPK are induced. Activation of p38 MAPK

induces a stress response, inflammatory response and eventually leads to cell death (Collins et al., 2015; Nolan, Vereker, Lynch, & Lynch, 2003). Interestingly, we found the significant elevation of p38 MAPK phosphorylation at PND90 **(Figure 33)**, but not JNK **(Figure 34)**. Enhanced activation of p38 MAPK was accompanied with elevation of IL-6 at PND90 in PFC **(Figure 36)**. The complex and the bidirectional relationship between p38 MAPK and proinflammatory markers might explain our findings. In vitro studies showed that as a response to oxidative stress, the activity of p38 MAPK increased which in turn mediated the production of pro-inflammatory cytokines including IL-6. Additionally, activation of the p38 MAPK pathway was also essential for regulating the expression of the antioxidant enzymes including HO and Mn-SOD (Nahirnyj, Livne-Bar, Guo, & Sivak, 2013). Prolonged activation of p38 and JNK MAPKs is detrimental to cells due to their role in mediating biosynthesis of proinflammatory cytokines, hence, activation of MAPKs, induces the expression of MAPK phosphatase (MKP1) which in turn dephosphorylates p38 and JNK MAPKs and therefore restraints the MAPKs-mediated inflammatory response (Collins et al., 2015). This mechanism of negative feedback regulation is critical for cell survival. Hence, MKP1 is considered as a key factor in regulating the stress response, and its expression is highly regulated transcriptionally, post-transcriptionally, and post-translationally by numerous factors (Kuwano & Gorospe, 2008). In our model, MKP1 levels were induced in PFC of SD rats in parallel with enhanced p38 activation, suggesting its role in cell

survival and growth. On the other hand, MKP1 is also a negative regulator of the neurotrophin signaling pathway mediated by BDNF **(Figure 3)**. MKP1, by dephosphorylation of $ERK_{1/2}$ and MEK, alters the neurotrophic downstream signaling pathway which is essential for neuronal growth, plasticity and cell survival (Duman, 2004; Valjent, Caboche, & Vanhoutte, 2001). In fact, the role of MKP1 in the nervous system is quiet challenging; while its role in the protection of neuronal viability has been suggested by many studies, recently, its contribution to stress-induced depressive disorders has emerged (Collins et al., 2015). It is well known that MKP1 regulates the magnitude and duration of MAPKs signaling, and as the strength of MAPKs signaling determines the duration of biological responses and many physiological outcomes, therefore, the spatial and temporal titration of MKP1 has a fundamental regulatory role. In connection with this, it has been reported that overexpression of MKP1 in the developing cortex contributed to aberrant axon branching, while its knockout resulted in an inability of BDNF and neuronal activity to produce new axonal branches (Jeanneteau et al., 2010). Additionally, chronic overexpression of MKP1 in the hippocampus and the cingulate cortex has been implicated in depressive behavior in clinical and preclinical studies (Barthas et al., 2017; Duric et al., 2010). Therefore, in this study, enhanced MKP1 level in PFC of SD rats at PND90 might be responsible for the development of depression-like behavior evident at this stage. In addition to MKP1 overexpression in PFC of SD rats, we found a significant reduction in the phosphorylation level of $ERK_{1/2}$ at

PND33 and 90 **(Figure 39)**, which suggests that MKP1, in addition to other mechanisms, might contribute to reduction in $ERK_{1/2}$ activity.

The role of ERK/MAPK signal transduction cascade in mechanisms that are components of dendritic spines stabilization, synaptic plasticity, and neuronal growth is well established (Impey, Obrietan, & Storm, 1999). Phosphorylation of $ERK_{1/2}$ by the MAP kinase (MEK) which is initially activated by the MAP kinase kinase (Raf) is regulated by multiple upstream regulators such as activation of adenylate cyclase-coupled receptors, NMDA receptors, or receptor of tyrosine kinases. Once activated, $ERK_{1/2}$ exerts many downstream effects including regulation of transcription factors and eventually gene expression, protein synthesis, and regulation of cellular excitability necessary for synaptic transmission and plasticity.

In the hippocampus, activation of ERK/MAPK signaling is well known as an essential step for neuronal plasticity during memory formation. $ERK_{1/2}$ activation indirectly targets CREB phosphorylation which in turn regulates gene expression necessary for LTP (Impey et al., 1999). In addition to phosphorylation by ERK/MAPK cascade, CREB phosphorylation is targeted by several signal transduction mechanisms including; Ca^{2+}-calmodulin kinase, protein kinase C (PKC) and cAMP-dependent protein kinase A (cAMP-PKA). One of the genes regulated by CREB is BDNF, whose expression is critical for neuronal growth and

plasticity. It is important to mention here that many studies demonstrated that stress down-regulates BDNF and disrupts CREB function which might underlie the molecular mechanism that leads to the pathophysiology of mood disorders (Duman, 2002b). In this study, reduced activity of $ERK_{1/2}$ and reduction of synaptic density/plasticity were evident at PND33 and 90 in PFC of SD rats **(Figure 38)**. Additionally, CREB phosphorylation **(Figure 39B)** and BDNF expression **(Figure 45B)** were significantly reduced in the PFC of SD rats at PND90. Therefore, it is possible that in PFC of SD rats, the reduced activity ERK/MAPK and disruption of CREB activity caused an alteration in gene expression and hence disrupted the synthesis of proteins responsible for neuronal growth and synaptic plasticity, such as the BDNF and glutamate receptor subunits.

The reduction in synaptic density/plasticity in PFC of SD rats was indicated by the reduction in glutamate receptor markers including GluA1 subunit of AMPARs and a GluN2b subunit of NMDARs, as well as the reduction of the postsynaptic density protein, PSD95. AMPARs expression is positively correlated with the size of the spine head as well as synaptic strength at the excitatory synapse (Marsden, 2013). Moreover, phosphorylation of GluA1 is essential for synaptic potentiation mediated by AMPARs (Marsden, 2013). Furthermore, reduction in GluN2b subunits of NMDA receptors in PFC of SD rats might also contribute to a reduction in synaptic plasticity. Subsequent activation of CaMKII following Ca^{2+} entry through NMDARs is critical in synaptic plasticity (Marsden,

2013). Interestingly, CaMKII recruitment to NMDARs is facilitated by GluN2b subunit which has a high affinity to CaMKII (Barria & Malinow, 2005). Interestingly, phosphorylation levels of CaMKII in PFC of SD rats were significantly reduced in association with the reduction in glutamate receptor markers mentioned above. Our data suggest a reduction in synaptic density/plasticity in PFC as a result of EL-SD in rats. Reduction in synaptic density/plasticity in PFC is highly related to the pathophysiology of mood disorders (Duman, 2002b; Marsden, 2013). In this study, the reduction in synaptic density/plasticity at an early life, in association with enhanced inflammatory markers in PFC might be part of the molecular changes that underlie the pathophysiology of anxiety-like behavior evident at early life.

It is well known that synaptic plasticity/density is essential for neuronal circuit formation and maturation during development (Picchioni, Reith, Nadel, & Smith, 2014). The neuronal circuits that connect the limbic regions including PFC, hippocampus, and amygdala are essential for emotion regulation and behavioral development that requires proper synaptic activity and connectivity within and between PFC and other limbic regions. Impairment of synaptic density and plasticity in PFC of SD rats during critical periods of cortical maturation might permanently alter neuronal circuit formation and maturation leading to the development of depression-like behavior later in life.

It has been proposed that SWA is highly correlated with synaptic plasticity/density during cortical maturation (Buchmann et al., 2011; Kurth et al., 2015). Consequently, SD and hence reduction in SWA alters synaptic density and synapse formation during critical periods of development. The molecular basis for the connection between SWA and synaptic plasticity is not fully addressed, but the alteration of protein synthesis was proposed as one of the mechanisms. This study demonstrated that EL-SD reduced synaptic density in PFC as indicated by the decreased expression of glutamate receptors which are essential for mediating synaptic plasticity. Reduction in synaptic density was also evident by significant reduction in CaMKII activity in PFC of SD rats. Disruption of the ERK/MAPK pathway by oxidative stress might be the molecular mechanism that alters synapse formation and plasticity in PFC during critical periods of cortical development leading to behavioral impairment.

8. SUMMARY AND CONCLUSION

1. Early life sleep deprivation (EL-SD) in rats (during childhood and adolescence) caused anxiety-like behavior in early life which disappeared later in life and transformed into depression-like behavior at the adult stage.

2. EL-SD induced oxidative stress in blood and brain indicated by elevation of plasma 8-isoprostane, induction of protein oxidation, and elevation of antioxidant systems in the brain.

3. EL-SD increased proinflammatory markers in PFC of rats, which might be related to the elevation of oxidative stress.

4. Oxidative stress and proinflammatory markers induced the activity of (P38 MAPK) which in turn enhanced biosynthesis of in proinflammatory cytokines; IL-6 in PFC of SD rats.

5. Parallel to enhanced activity of P38 MAPK, expression of MKP1 was increased in PFC of SD rats, suggesting an essential role for the p38 MAP in the induction of MKP1 expression. MKP1 dephosphorylates p38 MAPK and prevents an inflammation-mediated response.

6. Accumulation of MKP1 with other signaling mechanisms, negatively regulated neurotrophic pathway by dephosphorylation of $ERK_{1/2}$ which is downstream of BDNF signaling pathway

7. BDNF protein level was significantly reduced in PFC of SD in association with reduced $ERK_{1/2}$ activity.

8. The phosphorylation level of CREB which is downstream of $ERK_{1/2}$ was significantly reduced in PFC of SD rats.

9. The synaptic density in PFC of SD rats was reduced at PND33 and 90 indicated by a reduction in AMPARs, NMDARs and PSD95 density.

10. The synaptic plasticity in PFC was reduced in SD rats at PND33 and 90 indicated by the significant reduction of CaMKII phosphorylation.

11. Depression of neurotrophic pathways by SD during critical periods of cortical development altered synapse formation and neural plasticity in PFC indicated by a reduction in glutamate receptors and disruption of CaMKII activity.

12. Alteration of the neurotrophic pathway and synaptic plasticity/density in PFC during critical periods of development, characterized by cortical maturation, might contribute to anxiety-like behavior. Disruption of synaptic density/plasticity and reduction of neuronal growth pathways during critical periods of neuronal circuit formation and maturation might permanently alter the connection within and between limbic brain regions that regulate behavior leading to the onset of depression-like behavior at adult life.

We can conclude that EL-SD by engaging oxidative stress pathways, alters cortical maturation and development causing disruption in synaptic density and plasticity which underlies the pathophysiology of behavioral impairment **(Figure 46)**.

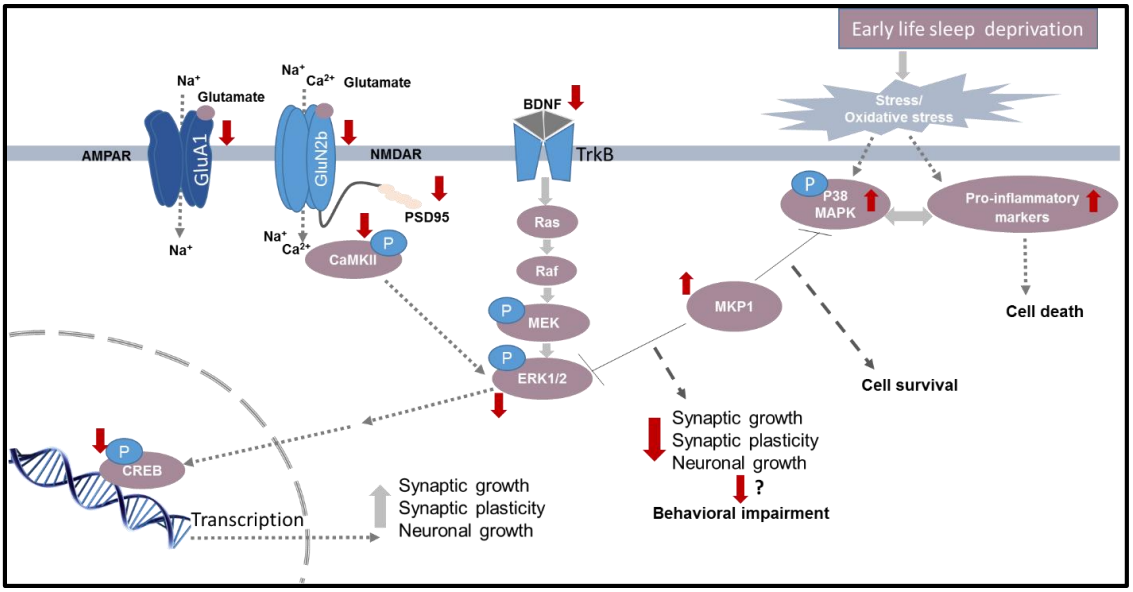


Figure 46. Schematic representation of the events potentially responsible for the behavioral impairment induced by early life sleep deprivation (EL-SD). Red arrows indicate the changes induced by EL-SD

9. PERSPECTIVE

In this study, we found that early life SD in male rats induced anxiety-like behavior at early life (PND33 and 60), later in life (PND90), anxiety-like behavior disappeared and depression-like behavior developed. Analysis of the biochemical changes in plasma and brain at different developmental stages revealed some mechanisms that are potentially responsible for the behavioral impairment induced by early life SD. The findings suggested that EL-SD by engaging oxidative stress pathways, potentially altered cortical maturation and development causing reduction in synaptic density and plasticity which might underlie the pathophysiology of behavioral impairment.

Our findings also indicate that oxidative stress and the stress inducible phosphatase, MKP1, might be a candidate targets for development of new strategies to counteract the detrimental behavioral consequences of EL-SD that is consistently increasing in our modern society.

Further analysis of dendritic spine morphology and number in PFC of rats will reveal further evidence and deeper understanding of the structural changes in PFC following EL-SD. Additionally, further studies that include treatment the rats with anti-oxidant agents such as tempol during SD protocol might provide useful insights. If administration of tempol during SD protocol successfully prevents the detrimental behavioral and biochemical consequences of EL-SD, the causative

relationship between oxidative stress and the detrimental consequences of SD would be suggested.

It is important to point out that this study has done on male Sprague Dawely rats, studying the behavioral and biochemical consequences of EL-SD on female rats should be part of future studies to investigate the effect of sex in vulnerability to EL-SD.

Following sleep/wakefulness pattern of rats during sleep deprivation protocol by video monitoring the rat's immobility versus immobility was an advantageous noninvasive way for validation of our SD model. However, this tracking has a limitation concerning the number of rats (four per group). We could only have four rats in each group because we couldn't place two rats in the same apparatus during SD because the software (AnyMaze) can't track more one rat in one cage. Future experiments with larger number of rats for tracking the rats' sleep/wakefulness pattern are suggested.

10. APPENDIX

It is known that sickness in rodents is generally associated with alteration in general body parameters such as body weight (Ellacott, Morton, Woods, Tso, & Schwartz, 2010; Morton, Meek, & Schwartz, 2014). Therefore, CON and SD rats were monitored throughout sleep deprivation protocol and behavioral tests, starting at PND18 until PND35 to determine if our SD protocol leads to sickness behavior. We found that body weight remained unchanged in SD rats as compared to CON rats throughout the whole experiment (**Figure 1**). Normal weight gain in SD rats suggests that SD protocol does not induce sickness behavior in rats.

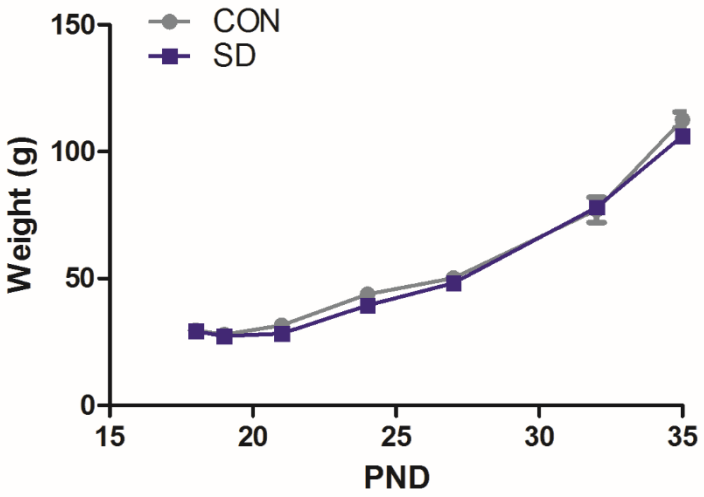


Figure 1. Body weight in grams (g). The rats' weights were measured daily throughout SD protocol starting at PND18 until PND35. Lines are means of body weight ± SEM. Data were analyzed using a two-way ANOVA test, n=16 rats/ group. PND: Postnatal day. Group designations: control (CON), sleep deprivation (SD).

We measured the antioxidant protein level in brain regions at PND 90. The protein level of Cu/Zn- superoxide dismutase (Cu/Zn-SOD) did not alter by EL-SD in PFC, hippocampus, or amygdala **(Figure 2)**.

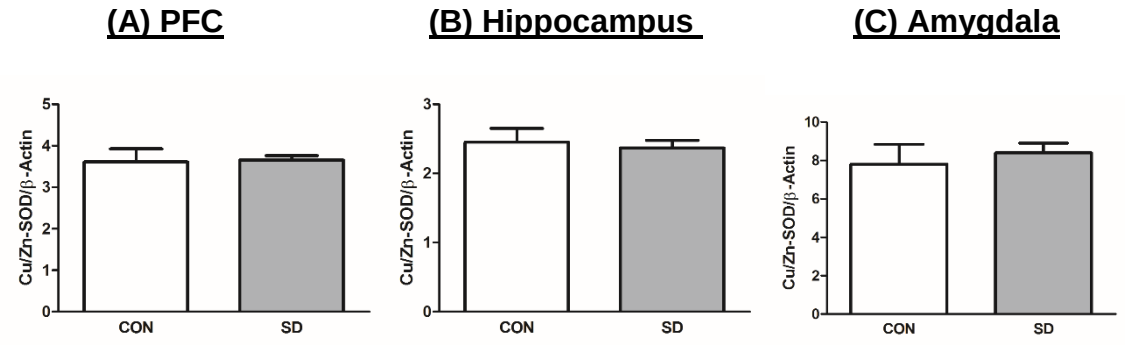


Figure 2. The protein level of copper/zinc superoxide dismutase (Cu/Zn-SOD) in the brain at PND90. The protein level of Cu/Zn-SOD was measured in PFC **(A)**, hippocampus **(B)**, and amygdala **(C)**, using the western blot technique. The protein levels were normalized with the loading control β-actin. Bars are means of normalized Cu/Zn-SOD level ± SEM, n=7 rats/group. Data were analyzed using two-tailed unpaired t-test. PND: Postnatal day. Group designations: control (CON), sleep deprivation (SD).

The protein levels of glutathione reductase also did not change in PFC of SD rats at PND90 as compared to CON rats **(Figure 3).**

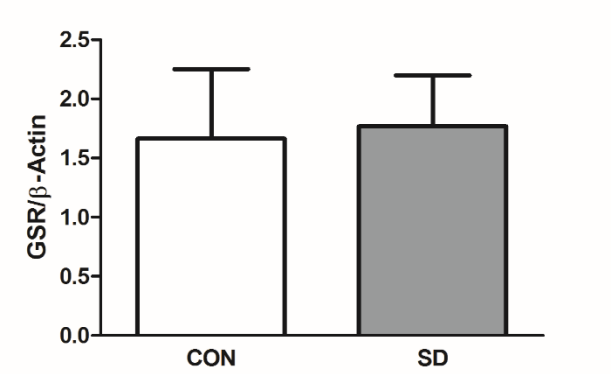


Figure 3. The protein level of glutathione reductase (GSR) in the brain at PND90. The protein level of GSR was measured in PFC **(**using the western blot technique. The protein levels were normalized with the loading control β-actin. Bars are means of normalized GSR level ± SEM, n=7 rats/group. Data were analyzed using two-tailed unpaired t-test. PND: Postnatal day. Group designations: control (CON), sleep deprivation (SD).

We also measured the protein level of the brain-derived neurotrophic factor (BDNF) and the phosphorylation level of its downstream signaling pathway marker (ERK1/2) in hippocampus and amygdala at PND90. The protein levels of MAPK phosphatase (MKP1) were also measured in the hippocampus and amygdala at PND90. EL-SD did not alter the protein level of BDNF neither in the hippocampus nor in the amygdala at PND90 **(Figure 4)**. Also, the phosphorylation level of ERK1/2 did not alter by EL-SD in hippocampus or in the amygdala at PND90

(Figure 5). Moreover, EL-SD did not alter the protein level of MKP1 in hippocampus or in the amygdala at PND90 **(Figure 6)**.

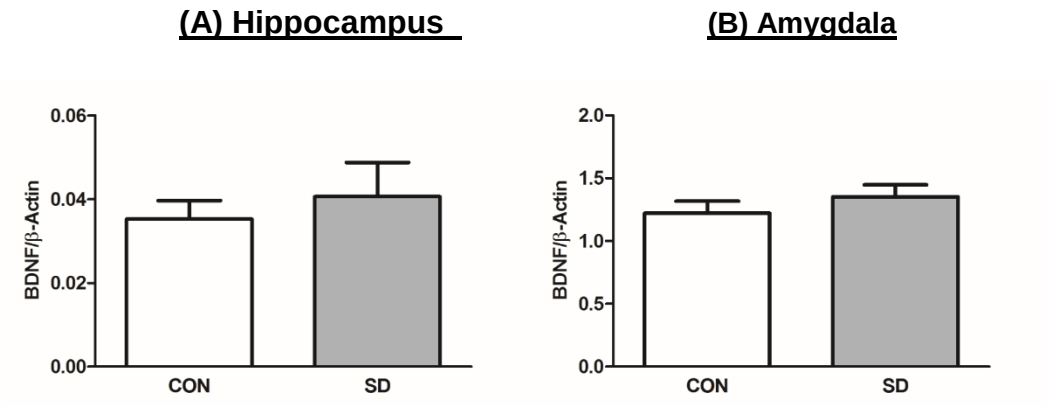


Figure 4. The protein level of the brain-derived neurotrophic factor (BDNF) in the hippocampus and amygdala at PND90. The protein level of BDNF was measured in the hippocampus **(A)**, and amygdala **(B)** at PND90 using the western blot technique. The protein levels were normalized with the loading control β-actin. Bars are means of normalized BDNF level ± SEM, n=7 rats/group. Data were analyzed using two-tailed unpaired t-test. PND: Postnatal day. Group designations: control (CON), sleep deprivation (SD).

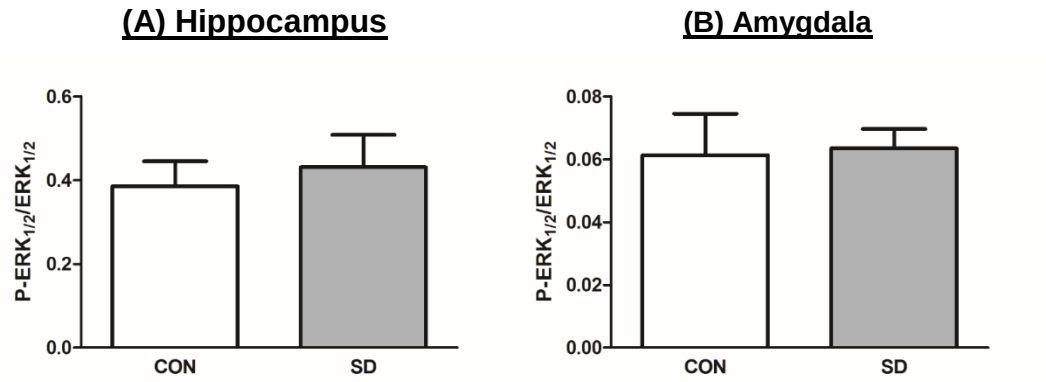


Figure 5. The phosphorylation level of the extracellular regulated kinase ($ERK_{1/2}$) in the brain regions at PND90. The Phosphorylation levels of $ERK_{1/2}$ were measured in the hippocampus **(A)**, and amygdala **(B)** at PND90 using the western blot technique. The protein phosphorylation levels were normalized with total $ERK_{1/2}$. Bars are means of normalized $ERK_{1/2}$ level ± SEM, n=7 rats/group. Data were analyzed using two-tailed unpaired t-test. PND: Postnatal day. Group designations: control (CON), sleep deprivation (SD).

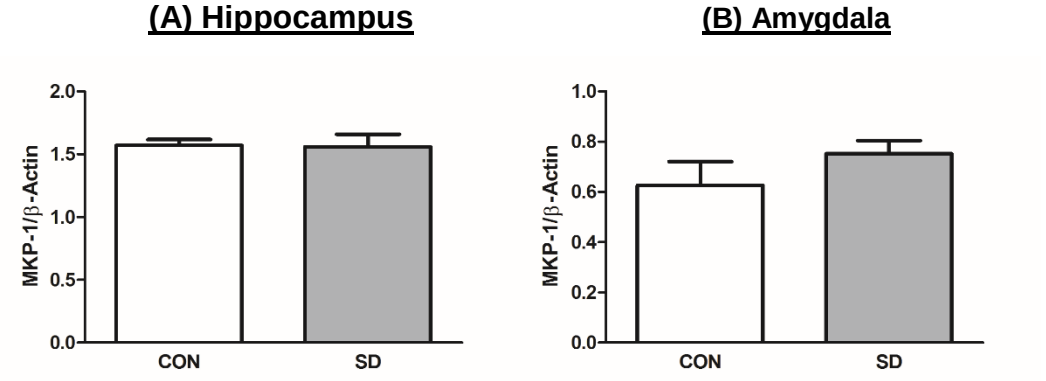


Figure 6. The protein level of the MAPK phosphatase 1 (MKP1) in hippocampus and amygdala at PND90. The protein level of MKP1was measured in the hippocampus **(A)**, and amygdala **(B)** at PND90 using the western blot technique. The protein levels were normalized with the loading control β-actin. Bars are means of normalized MKP1level ± SEM, n=7 rats/group. Data were analyzed using two-tailed unpaired t-test. PND: Postnatal day. Group designations: control (CON), sleep deprivation (SD).

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