REPUBLIC OF TURKEY
HACETTEPE UNIVERSITY
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THE PROTECTIVE ROLE OF ESCITALOPRAM ON IMPARED HIPPOCAMPAL NEUROPLASTICITY DURING REM DEPRIVATION

Dr. İsmail Mikdat KABAKUŞ

Pharmacology Program
Ph.D. THESIS

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Bu tez Hacettepe Üniversitesi Lisansüstü Eğitim-Öğretim ve Sınav Yönetmeliğinin ilgili maddeleri uyarınca yukandaki jüri tarafından uygun görülmüş ve Sağlık Bilimleri Enstitüsü Yönetim Kurulu kararıyla kabul edilmiştir.

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ÖZET

Kabakuş, İ.M. REM Yoksunluğunda Bozulan Hipokampal Nöroplastisite Üzerine Essitalopramın Koruyucu Etkisi, Hacettepe Üniversitesi Sağlık Bilimleri Enstitüsü, Farmakoloji Doktora Tezi, Ankara, 2013. Uyku bozuklukları depresyon ve anksiyete bozukluğu gibi psikiyatrik rahatsızlıklar ile sıklıkla beraberlik gösterir ve uyku bozukluklarının bu tür hastalarda kognitif fonksiyonlarda bozulmanın altta yatan nedeni olduğu düşünülmektedir. İzole REM (Rapid Eye Movement) yoksunluğunun öğrenme ve nöronal fonksiyonları belirgin derecede zedelediği gösterilmiştir. Öğrenme ilişkili merkezler hipokampüs gibi uyku düzensizliğinden önemli derecede etkilenir. EEG çalışmaları depresif hastalar ile REM yoksunluğu hastalarında benzer patern olduğunu göstermiştir. Serotonin geri alım inhibitörleri depresif hastalarda kognitif fonksiyonları koruyucu etki göstermektedir. Bundan dolayı biz de essitalopram'ın REM mahrumiyeti sonucu oluşan kognitif fonksiyon bozuklukları üzerine koruyucu rolünü araştırdık.

Bu çalışmada, REM yoksunluğu CA1 nöronlarının uyarılabilirliğini azalttı, EPSP ve I/O eğrisini sağa kaydırdı. Çift-dalga uyarımı, kısa dönem nöroplastisitenin bir göstergesi olarak, REM yoksunluğu veya essitalopram ile değişmedi. REM yoksunluğu LTP'yi azalttı. Davranış deneylerinde REM mahrumu sıçanlar obje-tanıma testinde düşük öğrenme kapasitesi, açık-kol labirent testinde azalmış anksiyete ve açık alan testinde artmış lökomotor aktivite gösterdi. Essitalopram tedavisi (2 hafta) almış grupta bu değişimler izlenmedi. REM yoksunluğu veya essitalopram hippokampal, kortikal, beyin sapı nörotransmitter düzeyini değiştirmedi.

Sonuç olarak, 2 hafta essitalopram tedavisi REM yoksunluğu sonucu oluşan öğrenme kapasitesinde azalma, anksiyete ve lökomotor aktivitede değişime karşı sıçanları koruyucu etki gösterdi.

Anahtar kelimeler: REM yoksunluğu, hipokampüs, nöroplatisite, LTP, essitalopram, ssri, serotonin

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ABSTRACT

Kabakus, I.M. The protective role of escitalopram on impaired hippocampal neuroplasticity during REM deprivation , Hacettepe University, Institute of Health, PhD thesis in Pharmacology, Ankara, 2013. Sleep disorders are mostly associated with psychiatric problems like depression, anxiety disorders, etc. and are thought to be the underlying cause of cognitive deficits observed in these patients. It has been shown that isolated REM (Rapid Eye Movement) deprivation markedly impairs learning and neuronal functions. Areas related with learning such as hippocampus significantly affected by sleep disturbances. EEG studies recorded from depressive patients exhibit a similar pattern with that of REM deprivation (REMD). Serotonine reuptake inhibitors was shown to reverse the cognitive functional impairments in depressive patients. Therefore, we investigated the protective effects of escitalopram (ESC) on learning impairment induced by REM deprivation.

In this study, REMD decreased the excitability of CA1 neuron firing and shifted EPSP slope I/O curve to the right. Paired-pulse stimulation as a measure of short-term placticity, was not altered by REMD or ESC. However, REMD significantly reduced LTP (Long Term Potentiation). In behavioral studies, REM-deprivated rats exhibited impaired learning in place recognition test, reduced anxiety in elevated plus arm maze, and increased locomotor activity in open field test. All these electrophysiological and behavioral changes were absent in chronicaly ESC administrated (2 weeks) animals. REMD and/or ESC did not change the neurotransmitter levels in hippocampi, cortex, hindbrain.

In conclusion, 2 weeks of ESC treatment protected the rats from the learning impairment and reversed back the anxiety, locomotor level changes.

Keywords: REM deprivation, hippocampus, neuroplasticity, LTP, escitalopram, ssri, serotonine

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SYMBOLS AND ABBREVIATIONS

5-HT	Hydroxytryptamine
A-1	Adenine-1 receptor
BDNF	Brain derived neurotrophic factor
BZD	Benzodiazepin
CaMK	Calmodulin Dependent Protein Kinase
DG	Dentate gyrus
EEG	Electroencephalography
f-MRI	Functional magnetic resonance imaging
HFS	High-frequency stimulation
LTD	Long-term depression
LTP	Long-term potentiation
NMDA	N-methyl-D-aspartate
NREM	Non-REM
PAG	Periaquaductal gray
REM	Rapid Eye Movement
REMD	Rapid Eye Movement Deprivation
TrkB	Tyrosine-kinase B receptor

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1. Introduction

In our current society, insomnia and other sleep-related disorders are common problems mostly due to irregular sleeping times and reduced duration of sleep. Several genetical, physiological, behavioral, and enviromental reasons were accused in the pathophysiology of insomnia. Occupational night shifts, military service, long distance travels lead the top amoung the enviromental causes. Insomnia is a risk factor for medical and mental disorders, and consequently increased health care costs (1) .

Several animal and human studies revealed the association between sleep loss and mental/psychiatric problems. Sleep loss, especially REM (Rapid Eye Movement) deprivation was shown to cause marked learning impairment and cellular, functional changes in learning related brain regions like hippocampus (2,3) . Besides, sleep deprivation also affects central nervous system areas related to mood. In sleep studies, it was claimed that insomnia has a relation with psychiatric diorders especially depressive disorders. Disinhibited REM sleepmight yield depression (4) . In patients with depression, shortened REM latency and increased REM density as a result of inhibitory aminergic, excitatory cholinergic activity was accused (4) .

Since there is an established reciprocal relationship between insomnia and depression, this brings the question of whether insomnia and depression share the same underlying pathophsiology.

Selective serotonin reuptake inhibitors (SSRIs) are most commonly used and effective drugs for the treatment of depressive disorders. Other anti-depressant drugs such as trazodone, are proven to be effective in insomnia treatment (5) .

The mechanism/s of SSRI action in insomnia should be investigated more throughly to find out if there is recovery effect of SSRIs for insomia related complications like cognitive impairment and mood disturbance.

In experimental studies regarding the mechanism of SSRI action on depression, SSRI's were shown to increase BDNF (Brain Derived Neurotrophic Factor) expression in several regions including hippocampal astrocytes and the authors concluded that increased BDNF prevented neuronal death and promoted neurogenesis in hippocampus, mostly in dentate region (6-8) . Additionally, SSRI actions on 5-HT_{1A} receptors may play a role in insomnia treatment. SSRI treatment

were known to increase serotonin level in central nervous system in acute settings. 5-HT receptors display desensitization when high serotonin levels were attained in CNS. It is proposed that 5-HT_{1A} receptor desensitization in the specific regions of CNS, midbrain raphe, is the key point of mechanism of action in antidepressive actions of SSRIs (9,10) . It was hypothetized that this high serotonin stimulus on the receptors promotes BDNF expression in several regions including hippocampal neurons and astrocytes and increased BDNF levels prevents neuronal death and stimulate neurogenesis in hippocampus, especially in dentate region (6-8) .

We aimed to investigate and correct the alterations of cognition and mood induced by REM sleep deprivation by using a selective serotonin reuptake inhibitor, escitalopram in rats. Secondly, we intended to reveal the role of serotoninergic system in cognitive and mood disorders caused by REM deprivation.

2. Review of Literature

2.1. History

Sleep constitutes more than one third of human life time and this is the period of our lives where we experience an out of reality state which we call it “dreams”. Thus, sleep has always appealed several great minds from the beginning of ancient times and they all tried to understand how and why we sleep. Ancient Egyptians considered that dreams are related to the state of the body and they treated sleep disorders by opium. Hippocrates also mentioned about sleep disturbances in his writings. After the invention of the electroencephalogram (EEG) in 1929, research on sleep and sleep disorders gained a new place. In 1936, Harvey and Loomis by using EEG technique during sleep revealed that the sleep is actually made of several stages, and following studies by Walter and Dovey showed the presence of delta and theta waves. In 1952 REM sleep was discovered by Eugene Aserinsky and Nathaniel Kleitman.

2.2. Why do we sleep and how do we sleep?

In our millennium of struggle to answer the question of why we sleep, finally we begin to get some answers. Modern sleep research showed that sleep have roles on homeostatic restoration of human body, thermoregulation, immune control, growth hormone release and memory processing; Borbely proposed that circadian and homeostatic mechanisms influence sleep (11) . His hypothesis suggests that the circadian rhythm oscillators in the suprachiasmatic nuclei of the hypothalamus control the sleep and awake cycles and the homeostatic process regulates the need for sleep, which increases during the day time and decreases during non-REM (NREM) sleep. It was hypothesized that the accumulation of one or more unknown substances in the brain during the awake hours were responsible for this homeostatic process. Since then, research has shown that prostaglandin D_2, adenosine, nitric oxide, tumor-releasing factor, interleukin-1, growth hormone releasing hormone and other substances are the major candidates for sleep-inducing factors (12-14) . Prostaglandin D_2 and adenosine, a purine nucleoside, accumulate in the brain during wakefulness and they promote sleep. During NREM sleep, adenosine sucked up by

brain cells and serves as a fuel supply in the mitochondria. When prostaglandin D_2 was continuously infused into the third ventricle of rats, it induces NREM and REM sleep in a dose-dependent manner, as became evident by EEG and behavioral studies (15) . In another study, adenosine perfusion into rats basal forebrain resulted in impaired vigilance resembling to the effects of sleep deprivation (16) .

Astrocytes were also shown to contribute an important role by neurotransmission and promote sleep accumulation through adenosine A_1 receptors (17) .

2.3. Sleep and memory

Astrocytes have a role in cognitive decline during sleep loss (18) . When the homeostatic needs of local brain regions are not met, their synaptic efficacy and connectivity are affected, which homeostatic imbalance then adversely influences the related cognitive functions, behaviors and learning. There is mounting evidence attesting to the importance of sleep in cognition (19) and also there is a large body of experiment showing a strong correlation between sleep deprivation and memory impairment in humans and animals (20,21) . Taking all together leads us to that point sleep makes an important contribution to processes of learning, memory and brain plasticity (22) .

The role of sleep in learning and memory consolidation has been around from the first year of 19th century, David Hartley pointed the dream and proposed that it may change the memory links (23) . After that time there have been observations on especially patients with insomnia and experiments, all proving sleep loss decreasing attention, impairing memory consolidation. The first systematic assessment of sleep and memory was conducted over a century later in 1924, when it was shown that, compared with time awake during the day, memory retention was better after a night’s sleep(24) . It was thought that memory consolidation after sleep was due to lack of sensory interference during sleep. They did not take into consideration the physiological processes during sleep might improve memory consolidation. After the discovery of REM and NREM sleep, experiments started going into detail to get more knowledge how sleep or even its specific stages affect neuronal connections, memory consolidation mechanisms.

2.4. Structure of sleep

Sleep has two main states: Rapid eye movement sleep and NREM sleep. NREM sleep is also separated into stages going deeper from 1 to 4.

Different brain regions are activated during stages of sleep. Electrophysiological recordings from brain and muscles showed the difference between stages (Figure 1).

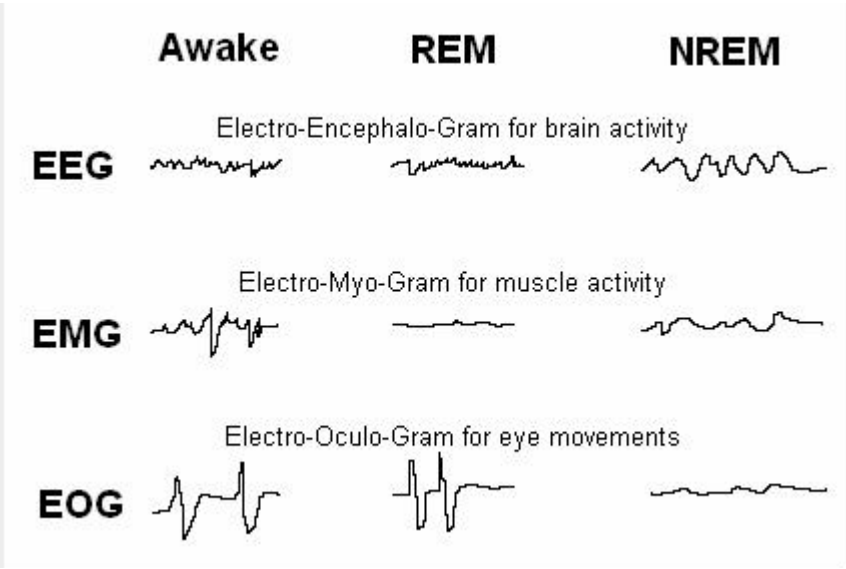

Figure 1.Brain, muscle, ocular activity changes according to awakeness and sleep, its stages.

2.5. Memory types

Memory is not considered a single phenomenon but broadly split into types and memory. Memory types are separated into declarative (consciously accessible memories of fact-based information which includes episodic and semantic memory) and non-declarative (procedural memories of habits, actions or skills, which includes procedural, implicit, non-associative and conditioning memory). Memory stages include several phases: Acquisition/encoding, consolidation, integration, recall and even erasure (25) .

There are still lots of questions in minds about how those different stages of sleep affect memory and particularly which type or stage. Still we don’t know whether the sleep-memory relationship is unidirectional or bidirectional. De Koninck

and colleagues have shown that learning can affect the structure of sleep. Their results indicated that the act of learning can affect sleep and produce changes in the structure of subsequent sleep (26) .

2.6. Hippocampus

The hippocampus is a major component of the brains of human and other vertebrates. It belongs to the limbic system and plays important roles in the consolidation of information from short-term memory to long-term memory and spatial navigation. Humans and other mammals have two hippocampi, one in each side of the hemisphere. The hippocampus is a part of the cerebral cortex, and in primates it is located in the medial temporal lobe, underneath the cortical surface. It contains two main interlocking parts: Ammon's horn and the dentate gyrus.

In rodents, the hippocampus has been extensively studied as part of a brain system responsible for spatial memory and navigation. Many neurons in the rat and mouse hippocampus respond as place cells: That is, they fire bursts of action potentials when the animal passes through a specific part of its environment. Hippocampal place cells interact extensively with head direction cells, whose activity acts as an inertial compass, and conjecturally with grid cells in the neighboring entorhinal cortex.

Since different neuronal cell types are neatly organized into layers in the hippocampus (Figure 2, 3), it has frequently been used as a model system for studying neurophysiology. The form of neural plasticity known as long-term potentiation (LTP) was first discovered to occur in the hippocampus and has often been studied in this structure. LTP is widely accepted to be one of the main neural mechanisms by which memory is stored in the brain (27) .

2.7. Field potentials, short-term and long term potentiation

Field potentials are extracellular potentials recorded from groups of neurons in response to synaptic or to electrical stimulation. In highly laminated organs like hippocampus the synchronous and localized currents generated by synaptic activation of a population of pyramidal cells give rise to easily measured response

called excitatory post-synaptic potentials (EPSP) and after threshold point population action potentials-population spikes (PS). The synaptic response to weak stimulation is below the threshold point for the generation of action potentials. With stronger stimulation, the neurons discharge synchronously giving rise to population spike. Unlike the all or none action potential generated by single neuron the population spike is a graded response. As the stimulus intensity increases, more neurons recruited and population spike amplitude increases (27) .

Population spike amplitude is correlated with the threshold for neuronal firing and excitability.

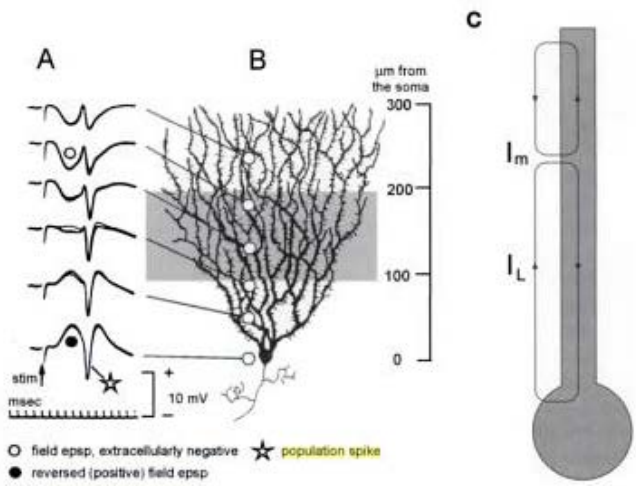

Figure 2. Electrophysiological recordings from different layers hippocampus (27) .

Short-term synaptic plasticity, lasting from a few milliseconds to minutes can be elicited, by paired pulse stimulation.

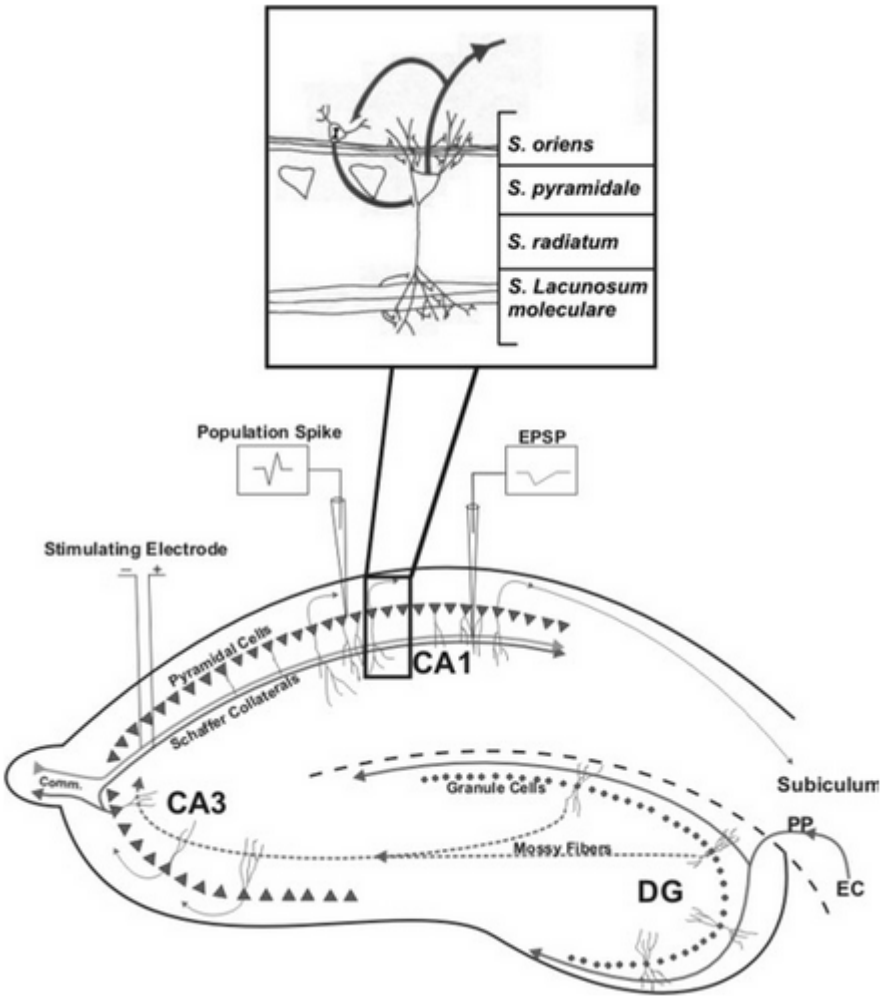

Figure 3. Hippocampal neuronal network, pyramidal neuron organization (27) .

Activation of the Schaffer collaterals by a single pulse of electrical stimulus evokes action potentials in both inhibitory interneurons and the CA1 pyramidal cells. Interneurons activated by the Schaffer collaterals increase their inhibitory tonus on the CA1 neurons, and this is named as feed-forward inhibition. On the other hand, CA1 neuron firings drives further circuits including the inhibitory interneurons within the hippocampus. This interneuron pathway starts a recurrent inhibition on its own synapses and somas of the preceding CA1 neurons. If the first pulse is followed by a second pulse, depending on the difference between time intervals, the subsequent response in the CA1 neuron would be either increased or decreased. This type of pairing of two pulses in rapid succession leads to an inhibition known as paired pulse inhibition (PPI). Changes in the ratio of the amplitudes of the first and

second evoked potentials (in a PPI experiment), can occur through changes in both GABA receptor sensitivity and GABA release. This has been demonstrated experimentally through, an enhancement of PPI with GABA agonists (28) and a decrease in PPI by GABA antagonists (29) .

If the stimuli are further apart (~100 ms) the second stimuli arrives, when the recurrent inhibitory loop has already been inactivated. Therefore, the second response is not inhibited but facilitated due to residual Ca^{2+} after the first stimulus. This is called paired pulse facilitation (PPF). Changes in the ratio of amplitudes of the first and second potentials are generally accepted as a modification in the presynaptic component of the synapse (30,31) .

Long-term potentiation is a long-lasting enhancement in signal transmission between two neurons. It is one of the several phenomena underlying synaptic plasticity, the ability of chemical synapses to change their strength. Since memories are thought to be encoded by the modification of the synaptic strength, LTP is widely considered as the major cellular mechanism that underlies learning and memory (32,33)

LTP was discovered in the rabbit hippocampus by Bliss and Lømo in 1966 and has remained a popular subject of research since (34) . Many modern LTP studies seek to understand better its basic biology, while others aim to draw a causal link between LTP and behavioral learning. Still, scientists are trying to develop methods, pharmacological or otherwise, for enhancing LTP in order to improve learning and memory in disorders characterized with dementia.

The neural networks within the hippocampus can be modulated by the induction of LTP, an electrophysiological correlate of learning and memory (35) . Earlier studies showed that sleep deprivation impairs LTP in the area CA1 of hippocampus (36) . After high-frequency stimulation (HFS), the released glutamate, binds to the glutamatergic receptors, AMPA and N-methyl-D-aspartate (NMDA), on the post-synaptic site and that cause a large influx of Ca^{+2}. Increased Ca^{+2} level activates various kinases including calcium-calmodulin dependent kinase II (CaMKII), which is generally known to be the regulator of short-term memory and LTP (37) . 8 hours sleep deprivation decreases the gene expression of CaMKII (38) .

Stable activity-dependent changes in synaptic efficacy, as seen during long-term potentiation and long-term depression (LTD), depend on new gene expression. Persistent changes in synapses are related to long-term adaptive responses in behavior, including memory formation, motivation, mood, and pain control. LTP induction by high-frequency stimulation of excitatory glutamatergic synapses is typically divided into 2 phases: a transient early phase (hours) and a persistent late phase (days-years) (39) . For late LTP, gene transcription and protein synthesis is needed. Secretory polypeptide brain-derived neurotrophic factor (BDNF) is one of the major regulators of LTP consolidation (40,41) . BDNF is released post-synaptically in response to HFS (High Frequency Stimulation) and signals through TrkB receptor tyrosine kinases located pre- and post-synaptically (42-44) . When applied exogenously BDNF induces a long-term potentiation (BDNF-LTP) that mimics the late phase of LTP (45,46) . LTP induction by BDNF is associated with rapid up-regulation and dendritic transport of mRNA encoded by the immediate early gene, activity-regulated cytoskeleton-associated proteins (Arc, aka Arg3.1) (47,48) . Many human and animal studies suggested that stress-induced depression and the delayed efficacy of antidepressant drugs are related to that gene activity (49-52) . It has been proposed that stress exposure reduces BDNF expression and TrkB signaling in the hippocampal formation and neocortex, while antidepressant treatment increases BDNF expression and TrkB signaling and counteracts the behavioral and mental effects of stress (52-56) .

2.8. Sleep loss causes cellular stress

During the last decade major progress has been made in the understanding of complex molecular changes following sleep deprivation which cause cellular stress (57-59) . Cellular stress is the response of cells to adverse environmental conditions that disturb their homeostasis. During wakefulness, the brain’s energy supplies progressively diminish while during sleep this metabolic energy depletion is refilled (60-62) . When the metabolic needs of the neurons increase various degrees of cellular stress develop depending on the severity and duration of sleep of loss. Cellular stress down-regulates many so-called stress genes and up-regulates many others and the activation of these genes can lead to the production of certain proteins

which are able to protect and repair cells. Interestingly, this process is similar in all living organisms, from bacteria to humans. Under non-stressed conditions the non-productive folding of proteins in cells is prevented. During excessive stress this process may fail, the misfolded proteins begin to accumulate in aggregates and the adaptive cellular functions progressively deteriorate. Excessive cellular stress leads to pathological changes in the mitochondria, macromolecular damage to proteins, DNA, RNA and lipids. In response to significant cellular stress, the unfolded protein response, a quality control system, is initiated that degrades misfolded polypeptides, suppresses the formation of protein aggregates, and ensures the effectiveness of transcription and translation of genes in addition to a number of other complex mechanisms (63,64) . When the excessive stress is prolonged, and the unfolded protein response is unable to compensate, widespread neuronal death and apoptosis may occur. The wear and tear resulting from stress, the “allostatic load”, is cumulative and further influences neurological functions, behavior and health (65-67) .

2.9. Similar neurobiological changes during sleep loss and depression

Interestingly, the pattern of neurobiological changes by sleep deprivation is similar to that seen in depression (68) . This is relevant in that chronic sleep deprivation may be a precursor of depression. As an example, adolescents have high rates of serious sleep disturbances associated with depression and suicidal attempts (69) . Severe restless legs syndrome predisposes to sleep disturbances and depression (70) . Post-partum depressed women are also commonly sleep-deprived (71) .

2.10. REM sleep for memory consolidation

Evidence from experiments have demonstrated that REM sleep plays a crucial role in learning and memory (72,73) . After intense learning activity or exposure to a new environment, REM sleep is augmented (74,75) . Supporting this post-learning REM-deprivation (REMD) impairs learning and memory consolidation, such as shuttle box avoidance and complex maze tasks (76,77) . These

observations suggest that post-learning REM sleep may be a requisite for memory consolidation.

2.11. REM sleep needed for neuroplasticity

Another hypothesis accounting for sleep-dependent memory processing is that synaptic plasticity changes take place during sleep (78) . LTP is thought to be the major cellular model for synaptic plasticity and associated with memory consolidation (79,80) . It is showed that REMD before LTP induction, within the dentate gyrus (DG) or CA1 region of the hippocampus, impairs LTP (36,81) . Knowing that REM sleep is necessary for memory consolidation, it is important and exciting to know what is the exact mechanism underlying.

Sleep duration and quality disruption negatively affects brain sensory processing and cognitive performance (82) . Sleep deprivation associated cellular mechanisms that promote sleepiness and induce physiological and cognitive deficits are largely unknown, although several molecular and biochemical correlates of sleep loss have been identified (38,58,62,83,84) . Cognitive performance impairment during sleep deprivation suggests pre-synaptic, post-synaptic and/or neuronal excitability changes. Hence, the identification of neuronal components that are modified by sleep loss could advance our understanding of the roles for sleep in neuronal functions.

2.12. Waking state/sleep and neurotransmitters

The waking state is controlled by neuro-chemically diverse sets of ascending fibers that release neurotransmitters into diencephalon and telencephalon (85,86) . During prolonged waking, neurotransmitter levels resemble those of the natural waking state. It is known as a common feature of neurotransmitter receptors that if stimulated continuously, their activity alters and attenuates (87,88) . Also chronically active firing in neural circuits provokes some adaptive changes in receptor number and function; that modulate excitability (89,90) . Sleep loss may also cause some circuits activated more and this may change neurotransmitter and their receptor level in central nervous system especially centers related to sleep regulation. Since, sleep

deprivation impairs long-term potentiation which also implicates impaired synaptic plasticity, the question is which neurotransmitter/s or their receptor/s level change have importance in this circumstances and how this impairment can be reversed may be a new treatment option for patients with chronic sleep loss.

The effects of sleep deprivation on the neuronal discharge pattern and neurotransmitter concentration in monoaminergic nuclei has been studied with different techniques. Sleep deprivation increases firing rate in serotonergic system in cats and rats (91,92) ; and this increased serotonin release during sleep deprivation occurs in a stress independent manner (93) . 72 hours REM deprivation led to an increased noradrenaline concentration and turnover in the rat locus ceruleus (94-96) . In a short period of sleep deprivation model, it has been shown that the histamine concentrations in hypothalamic areas of cats were increased (97) . REM deprivation also elevated c-Fos expression in the dopaminergic ventral tegmental area (98) . Taking all into consideration, sleep deprivation seems to increase monoamine levels and monoaminergic neuron firing rate in central nervous system.

Serotonergic system could be comparatively sensitive to insufficient sleep (99) . The serotonergic 5-HT_{1A} receptor is located both pre- and post-synaptically and is implied in the negative-feedback control of serotoninergic neurons (100) . Decreased signaling via 5-HT_{1A} receptors have been linked to mood disorders and anxiety (101) , suggesting that chronic sleep loss could predispose to susceptibility to some mental and neuropsychiatric disorders associated with decreased 5-$_{HT1A}$ receptor function (99) .

2.13. Brain regions related to learning, memory consolidation

Functional magnetic resonance imaging (fMRI) studies suggested that during and after sleep there is differential activation of brain regions and a reorganization of motor memory related regions. And these regions displaying increased activation were detected in the right primary motor cortex, medial prefrontal lobe, hippocampus and left cerebellum (102) . These changes could facilitate faster motor output and more precise mapping of key-press movements. On the contrary, in post-sleep period, reduced activity was recorded in parietal cortices, the left insular cortex, temporal pole and fronto-polar regions that are potentially associated with a

decreased need for conscious spatial monitoring due to automated performance (103) It may be concluded that sleep after learning is required for neural reorganization which is essential for consolidation of memory.

2.14. Human sleep loss and negative remembering bias

In addition to the animal studies, the importance of sleep before learning has also been demonstrated in humans (104) . Sleep deprived human subjects exhibited a striking 40% reduction in the ability to form new memories. Interestingly, in that study it is shown that negative emotional stimuli and its storage in memory appear to be more resistant to the effects of prior sleep loss. Intriguingly, these data may offer novel insights into affective mood disorders that express co-occurring sleep abnormalities (105) . This selective alteration in memory encoding may provide an experimental explanation for the higher incidence of depression in populations expressing impairments in sleep, which, due to these specific deficits, may suffer a negative remembering bias, despite experiencing equally positive and negative reinforcing past events. In addition to performance impairments at a behavioral level, a highly significant and selective deficit in encoding activation was revealed in bilateral regions of the hippocampus in the sleep deprivation. Taken together, these findings indicate the critical need for sleep before learning: without adequate sleep, hippocampal function is markedly disrupted, resulting in a decreased ability to encode new experiences (103) .

2.15. Sleep and depression

Impaired sleep is a common symptom of patients with major depression. Although 80% of patients complain from insomnia, only 15–35% of them suffer from hypersomnia (106,107) . Some characteristical sleep-EEG changes in patients with depression consist of (107,108) :

(i) Impaired sleep continuity (prolonged sleep latency, increased number of intermittent awakenings, early morning awakenings),

(ii) Disruption of REM sleep: shortened REM latency or sleep onset REM periods (SOREMPs, REM latency 0–20 min), prolonged first REM period, elevated

REM density (measure of the frequency of rapid eye movements) particularly during the first REM period,

(iii) Changes of non-REM sleep (decrease of slow wave sleep (SWS), SWA and sleep stage 2, in younger patients, the shift of SWS and SWA from the first to the second non-REM period).

REM-sleep disturbances were reported in depressed patients with insomnia and with hypersomnia as well (109) . The sleep EEG of drug-free depressed patients was investigated during a period of 3 weeks every night. REM latency, sleep continuity and non REM-sleep variables showed permanently changes as characteristic for depression, whereas psychopathology improved (110) . Sleep-EEG variables did not improve between acute depression and stable remission in two studies in adult patients with depression (111,112) . In both studies the patients were drug free at the baseline examination. Then they received treatment with antidepressants and were finally drug free again for several weeks at the retest. In one of these studies sleep stage 4 decreased after remission when compared to the baseline examination (112) . These findings led to the hypothesis that persisting sleep-EEG changes in remitted patients may represent a biological scar.

2.16. Purpose

We aimed to investigate and fix the alterations of cognition and mood induced by REM sleep deprivation by using a serotonin specific reuptake inhibitor, escitalopram, in rats. Secondly, we intended to reveal the role of serotoninergic system in cognitive and mood disorders caused by REM deprivation.

Consequently, we investigated the pathophysiologic changes during REM deprivation, and how these changes affected the memory and the mood; and also the efficacy of escitalopram treatment in these settings.

2.17. Hypothesis

Chronic escitalopram treatment protects from the pathological changes in the cognitive functions and mood caused by REM sleep deprivation.

3. Materials and methods:

3.1. Animals

Adult female Wistar rats, weighing 225–275 g, 12-15 weeks old, housed five per cage with free access to food and water. And all animals were subjected to a 12 h light/dark (lights on at 7:00 am) cycle at 25°C. All procedures involving animals were carried out in accordance with the "National Research Council's Guide for the Care and Use of Laboratory Animals" and on approval of the Hacettepe University Institutional Animal Care and Use Committee. All behavioral and electrophysiological experiments were started at 9:00 am, and were finished no later than 4:00 pm.

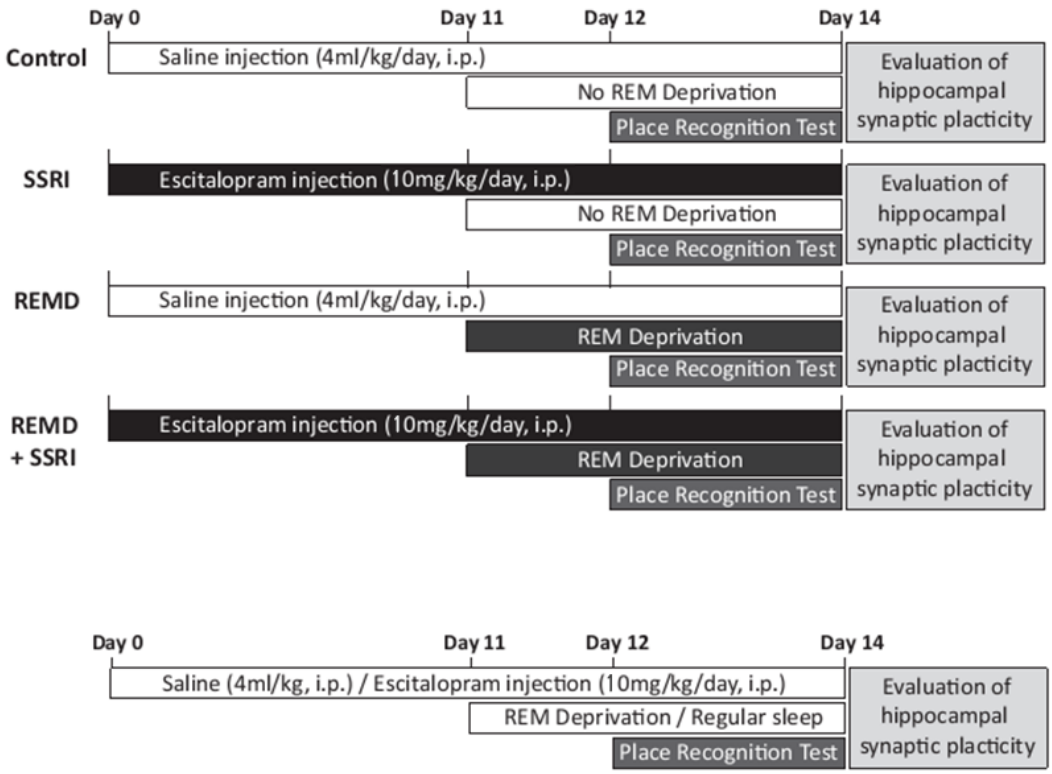

Figure 4. Groups and protocols.

3.2. Treatments

In this study there were mainly four groups: i) Control, ii) REMD, iii) Chronic ESC treatment group, iv) Chronic ESC treatment group plus REM sleep-deprivation (REMD+ESC). Rats treated with ESC received 14 daily injections of escitalopram (10 mg/kg i.p; injection volume was 2 ml/kg, saline); REMD and control group received 14 daily 2 ml/kg saline injection. ESC dose used in our experiments was comparable to that of used in studies of escitalopram' antidepressive effect in rat model of depression (6) .

3.3. REM Sleep Deprivation

REMD and ESC/REMD groups were subjected to 72 hours sleep-deprivation using columns-in-water model as mainly described (113) . In our study, minor changes were made on the model. The animals were placed on platforms (4 platforms; 25 cm high and 6 cm diameter, 7 cm apart edge-to-edge) surrounded by water 22°C in an aquarium where water and food were accessible to animals. The water level in the aquarium was aproximately 1 cm below the edge of the platform. This method has been reported to interfere with total sleep, but it mainly eliminates REM sleep (Grahnstedt and Ursin, 1985). During REM sleep due to muscle tone loss, rats lost the balance on coloumn and rats woke up immediately not to fall into water. In this way, this model spesifically prevents REM sleep but after 72 hours on stage also distrubs total sleep stages of rats.

Figure 5. Rapid Eye Movement sleep deprivation setup.

3.4. Electrophysiological Procedures

In vivo recordings from CA1 hippocampal region of anesthetized rats were performed. The rats were anesthetized with urethane (1.2 g/kg, i.p.) and placed in a stereotaxic frame. Then the skull was exposed and holes were drilled in appropriate places. A concentric bipolar electrode was placed in ventral hippocampal commissure with an angle of 30° to stimulate the area (AP: 3.6 ; L: 1.0; D: 2.1). A glass recording electrode solanoid filled with 2 M NaCl, was placed in the stratum pyramidale or radiatum of CA1 area (AP: 4; L: 2; D: 2.0-2.25 for str. pyramidale and D:3.0 str. Radiatum). Ventral hippocampal commisural fibers were stimulated (square wave pulses, 0.1 ms duration) and population spikes (PS) were recorded from the ipsilateral CA1 region. 30 min was waited for stabilization of the baseline responses and then input–output (I/O) curves, twin-pulses constructed by gradually increasing the stimulus intensities (input) and recording the evoked PS (output). Then, stimulus adjusted to the level of 50% of max response. PS induced by twin-

pulses were recorded by gradually increasing the time interval between twin-pulses. For LTP experiments, baseline excitatory post synaptic potential (fEPSP) slopes were adjusted to evoke 40% of the maximal average slope, hippocampus was stimulated at every 20s. LTP was induced by a train (1 second duration time, 100 Hz) and this train stimuli were repeated 3 times with 2 min intervals. 5-HT and ESC were also administered locally, directly into the hippocampus. For local drug administration recording electrodes (1-5 mOhm) filled with 0.9% saline containing 5-HT (1 mM) or ESC (1mM), and were placed to the CA1 neuronal cell layer using electrophysiological criteria. First, control field potential recordings were obtained by saline filled electrodes and then the drug-loaded electrodes lowered to the CA1 to the regions other than the ones previously used. Signals were amplified and recorded by 1x gain headstage (Batiray, YSEDCo, Turkey) and a DC amplifier (Kaldiray, YSEDCo, Turkey). The slope of fEPSP, which represents the intensity of synaptic activity, and the amplitude of PS, which reflects the number of neurons reaching threshold, were measured. Computer-based recording and analysis were accomplished by the use of scope 3.9.2 software and Powerlab /8SP (AD Instruments, Australia).

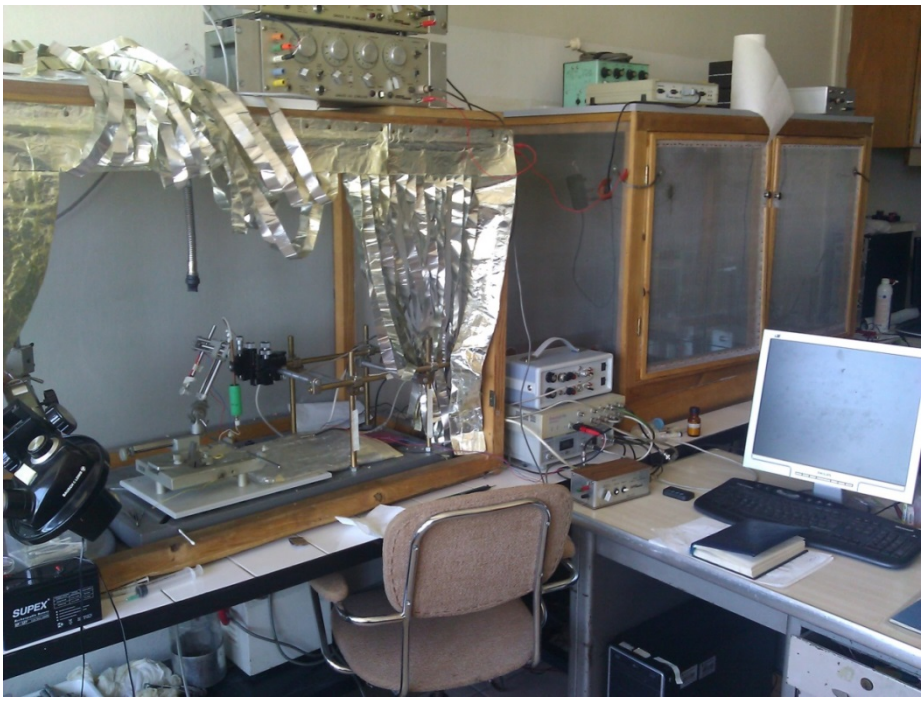

Figure 6. Neuropharmacology Lab. Electrophysiologic Recording setup.

3.5. Place recognition task

The apparatus consisted of an arena (60x60x60 cm) and two identical objects. The arena was surrounded by black curtains to avoid anxiety-provoking situations and cues in the room environment. The apparatus was cleaned between trials to remove olfactory cues. Rat behavior was monitored by webcam (Trust, China) and recorded using an automated tracking system, customized Eyesweb software 5.3.0 (The Eyesweb Project, InfoMus lab, Genoa, Italy) for offline analysis. The task consisted of a sample phase and a test phase delivered 24 h later. In the sample phase, the rats were allowed to explore the arena and the two identical objects for 5 min and then they were removed from the arena. In the test phase, following a 24h delay, one of the objects was moved to a new location; the rats were allowed to explore the arena and objects for 3 min. The time that the rats spent exploring each object was measured. Exploration of an object was defined as directing the nose at a distance <2 cm from the object. The exploration ratio was defined as the ratio of time spent exploring relocated object to total time spent exploring both objects (114) .

Figure 7. Place Recognition Test setup.

3.6. Open Field Test

Animals were placed in a 45x45x45 cm arena with glass walls and a black non-reflective ground for 15 minutes in a homogeneously illuminated room. The activities of the animals were recorded via webcam from above with a sampling rate of 10 frames per second. The recordings were then converted into coordinates using an customized EyesWeb software. For each animal, the total distance travelled, the time spent and distance travelled in the middle 60% of the arena were calculated and compared between groups.

3.7. Elevated Plus Maze

A 75 cm high maze with four 15 x 45 cm arms, two facing arms covered with 45 cm walls, was used for the elevated plus maze experiments. The animals were placed in the center of the maze facing one of the open arms and were recorded on the video for five minutes. The videos were then analyzed for total time spent in open arms and total crossings between arms (115) .

Figure 8. Plus-maze Test setup.

3.8. Biochemical analysis

Animals were decapitated immediately after behavioral tests, and the cortex, hindbrain, hippocampi were dissected and kept frozen at -70 °C. Frozen tissues were homogenized in 0.1 N percloric acid and were sonicated for 30 s at 25 °C. After sonication, the samples were centrifuged at 15,000 rpm for 30 min at 4 °C. The supernatants were removed and filtered through a membrane (Millipore, 0.2Am). Samples were applied to a high-performance liquid chromatograph with electrochemical detection. A stainless-steel CLC-ODS column (25 cm 4.6 mm) was used. The mobile phase consisted of 0.16 M citric acid, 0.69 M octanosulfonic acid,

4% acetonitrile and 17% tetrahidrofurane. The area of each peak was determined and compared with the peak of the corresponding external standard.

Results were expressed as nanomol/g tissue.

3.9. Statistical Analysis

All data were expressed as the mean±SEM. Statistical significance was assessed by analysis of variance (ANOVA) and Students *t* test. A significant ANOVA was followed by post hoc Bonferroni and Tukey test. Differences were considered significant if $p<0.05$.

4. Results

4.1. The effects of REMD and/or chronic escitalopram on excitability of CA1 neurons of rat hippocampus

REM sleep deprivation significantly increased the threshold of the population spike (Figure 9) and shifted the I/O curve to the right. Half maximal PS amplitudes were about 5V and 6.5V for the control and REMD, respectively. Low strength stimulation (5V) yielded 9.0±1.7 mV population spike amplitude in the control group; whereas in the REMD group it was 1.2±1.2 mV. Maximal recruitment of CA1 neurons obtained at about 9V for both control and REMD groups. The PS amplitudes achived at high stimulation strengths (9-15V) were not appreciably different in both groups. Stimulation of input circuitary at 6V resulted in 12.9±0.9 mV population spike in the control group and 5.1±1.9 mV in REMD group (n=6, p<0.01).

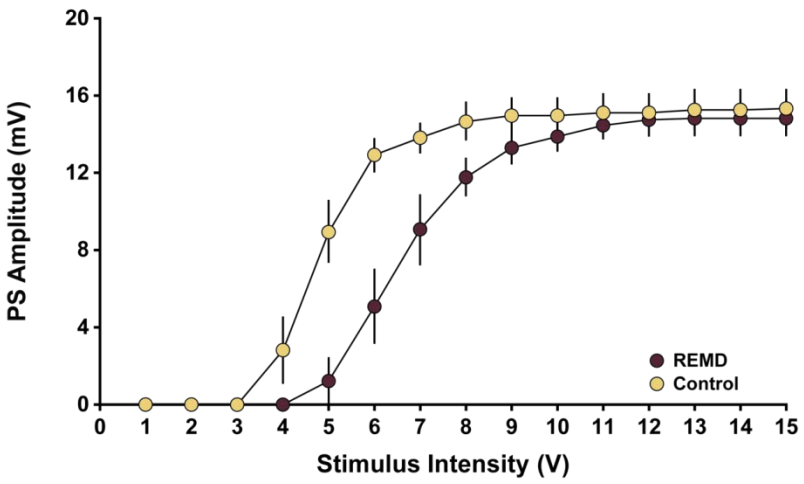

Figure 9. REMD increased the treshold level of population spike amplitude. Population spikes were obtained from hippocampal pyramidal layer of CA1; while anterior hippocampal commissural stimulation was gradually increased. Maximum population spike (PS) amplitudes were not different, but the threshold level for %50 of PS was ~5V in control group and it was ~7V in REMD (Rapid Eye Movement Deprivation) group (n=6, p<0.01). In chronic ESC group, daily ESC (10mg/kg, i.p.) injections were performed for 15 days.

Chronic ESC treatment prevented the increase of population spike treshold in the REMD rats (Figure 9, n=6, p<0.001). The maximum population spike amplitudes in REMD+ESC group were slightly higher than the control group but this was not found to be statistically significant.

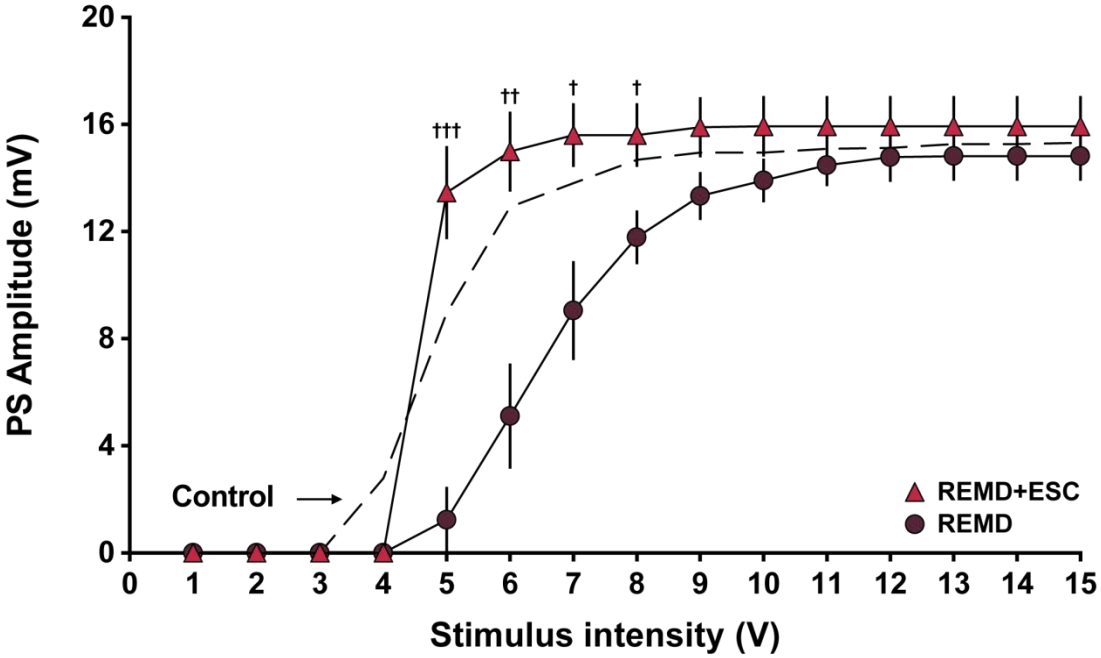

Figure 10. Chronic escitalopram treatment blocked REMD-induced I/O treshold increases. Population spikes were obtained from hippocampal pyramidal layer of CA1; while anterior hippocampal commissural stimulation was gradually increased. The treshold level for 50% of maximum PS amplitude was ~5V in control and treatment (10mg/kg ESC for 14 days) groups (n=6, p<0.001). † p<0.05 REMD+ESC vs. REMD (Rapid Eye Movement Deprivation) (ANOVA, Tukey's test); †† p<0.01 REMD+ESC vs. REMD (ANOVA, Tukey's test); ††† p<0.001 REMD+ESC vs. REMD (ANOVA, Tukey's test).

When chronic, acute ESC treatment and the control groups compared in terms of maximal PS amplitudes, we found that population spike amplitudes were higher in the chronic ESC treatment group and lower in the acute ESC treatment group (Figure 11). The difference betweeen the chronic ESC and acute ESC treatment groups were statically significant, (n=6, p<0.05); but there was no significance with respect to the control group.

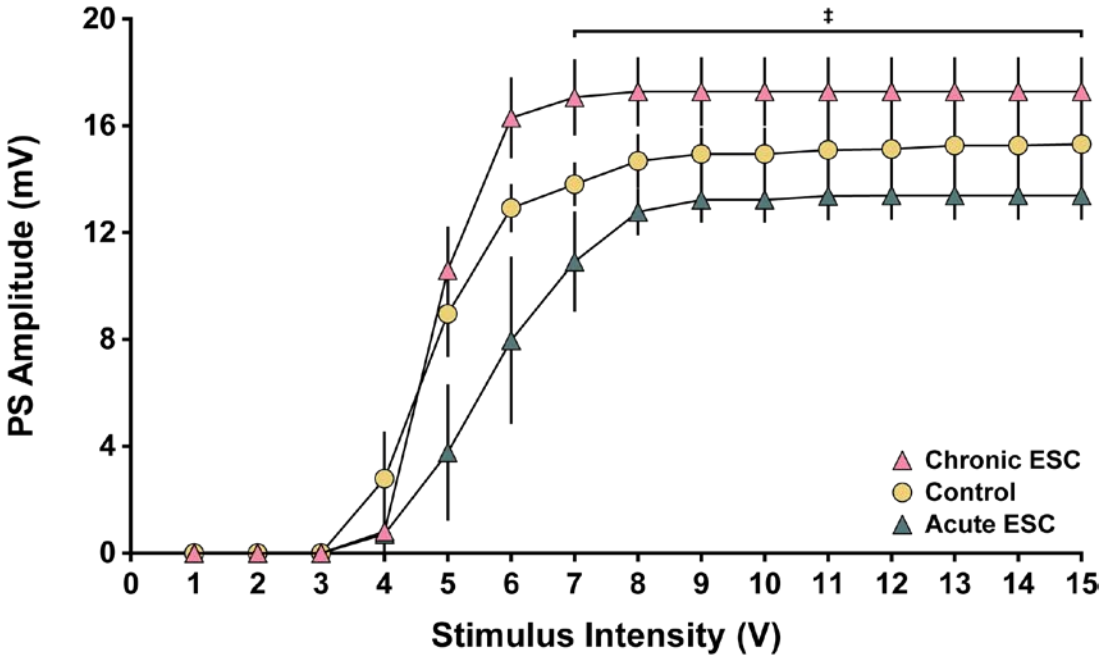

Figure 11. ESC treatments altered the maximum population spike (PS) amplitudes but without statistical significance when compared to that of controls. Population spikes were obtained from hippocampal pyramidal layer of CA1; while anterior hippocampal commissural stimulation was gradually increased. Maximum PS amplitude was around 14 mV, 16 mV and 12mV in control, chronic ESC (10mg/kg ESC for 14 days), and acute ESC (10mg/kg i.p.) groups, respectively. Amplitude changes were statisticaly insignificant when groups were compared to the control values, however, the difference between the acute and chronic ESC treatments was significant (n=6, p<0.05). ‡ p<0.05 Chronic ESC vs. acute ESC (ANOVA, Tukey's test).

4.2. The effects of REMD and/or chronic escitalopram on presynaptic release in CA3-CA1 neurons

In order to evaluate the effects of REMD with or without ESC on CA1 synaptic transmission, we plotted the EPSP slope I/O curves. EPSP average slopes of the REMD group were lower than that of the control stimulation strengths at 3V ($n=6$, $p<0.001$) and 4V ($n=6$, $p<0.05$); but there was no significant difference in the EPSP average slopes at maximum (Figure 12). Maximum EPSP slope of the REMD+ESC (1.8±0.02) group was much higher than the control (1.2±0.07) and the REMD (1.3 ±0.08) groups ($p<0.05$). Although chronic ESC (10mg/kg for 14 days) treated group of animals displayed higher EPSP slopes than the controls this difference was not statistically significant. 50% EPSP average slopes of the control, ESC and ESC treated REMD group were achieved at ~3V and of REMD group at ~4V ($n=6$, $p<0.001$).

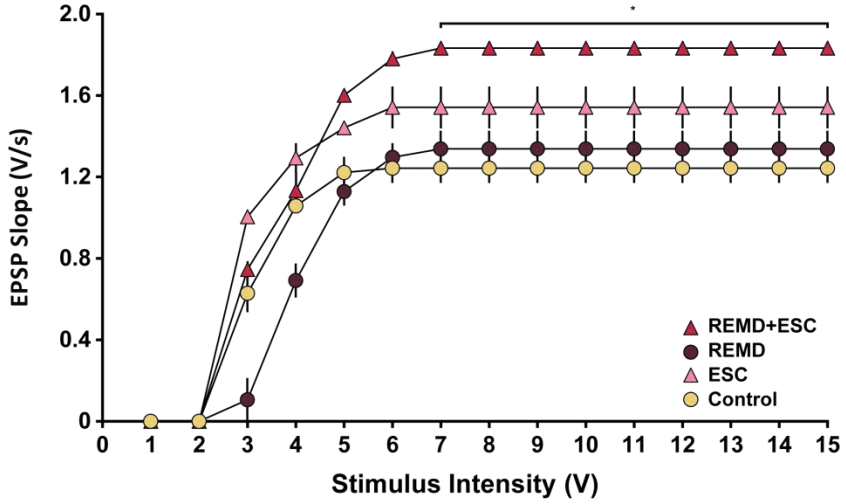

Figure 12. REMD (Rapid Eye Movement Deprivation) and/or ESC altered the EPSP slope I/O curves in stratum radiatum, CA1 synapses of rats. REMD, shifted the I/O curve to the right, implying an increased threshold ($p<0.001$ at 3V and $p<0.05$ at 4V), without altering the maximal responses. ESC slightly shifted the I/O curve to the left and increased the slopes at maximal stimulation strength. Maximum EPSP (Excitatory Post Synaptic Potential) slope amplitude of REMD+ESC were greater than that of the control group. After 2 weeks of ESC treatment (10mg/kg per day), EPSP I/O curves displayed similar I/O pattern at submaximal stimulation strengths, but maximal responses were appreciably higher with respect to the controls (n=6, $p<0.05$). *$p<0.05$ REMD+ESC vs. the control (ANOVA, Tukey's test).

5-HT and ESC were applied via recording electrode to the CA1 str. pyramidale region in vivo. Local ESC application caused a right shift to the PS I/O curve compared to the control group. When the curves are compared statistically, the shift was statistically significant (n=5, $p<0.001$). Local application of 5-HT also shifted PS I/O curve to the right. When compared to the control group, the difference between means of local 5-HT and control groups were statistically significant at 5-9 Volts (Figure 13B, n=5, $p<0.01$ at 6-7V and $p<0.05$ at 5-8-9V). Although local (electrode) or acute (ip) administration of ESC depressed the maximum PS amplitudes, this was not statically significant (Figure 13A, n=5).

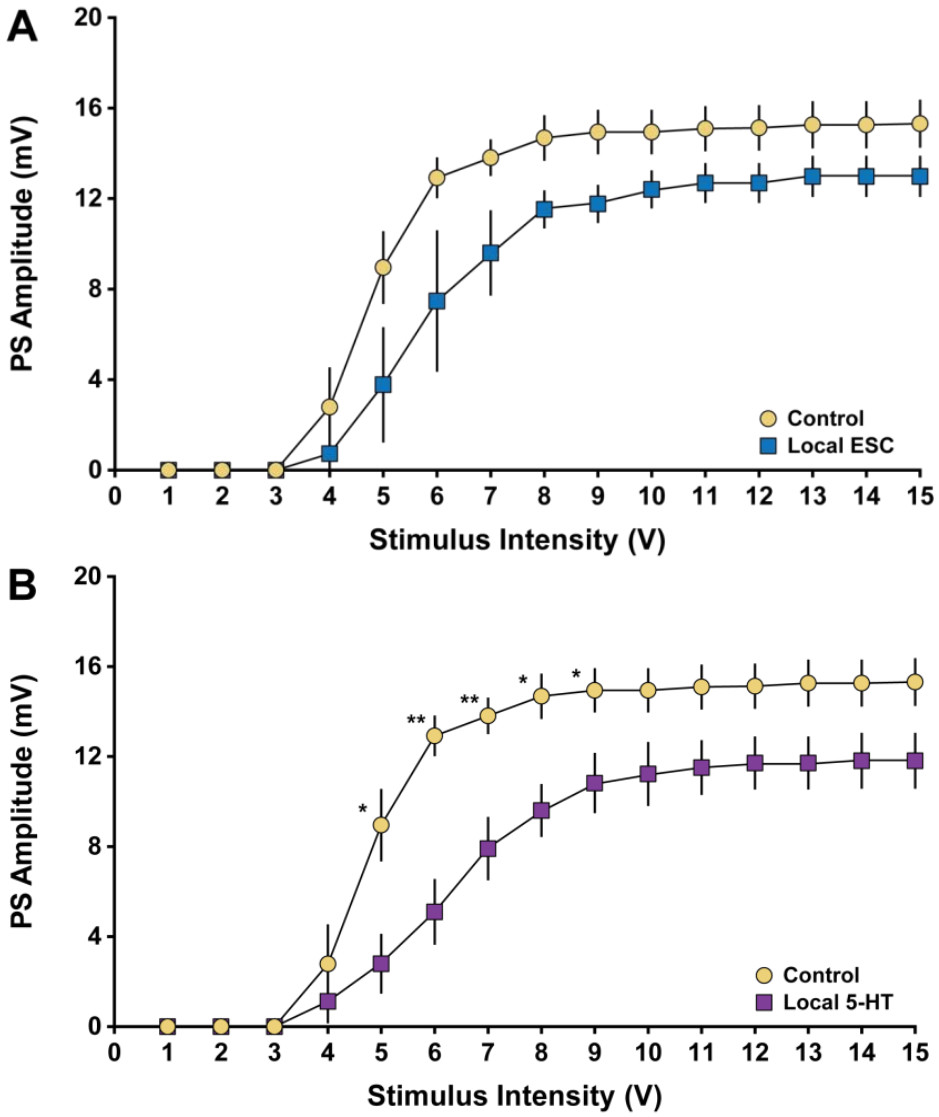

Figure 13A, 13B. Local 5-HT or ESC administration (via recording electrode), shifted I/O curve to the right and depressed the population spike (PS) amplitudes. A) Local ESC (1mM) application via electrode during recording significantly shifted the I/O curve to the right. (n=5, $p<0.001$) B) 5-HT application by electrode in vivo, decreased the excitability in CA1 regions of rats which became evident by the shift of the I/O curve to the right (n=5, $p<0.01$ at 6-7V and $p<0.05$ at 5-8-9V).

4.3. Effects of REMD and chronic escitalopram on short-term synaptic plasticity

Paired-pulse paradigm, as a measure of short-term synaptic plasticity, was compared in all four groups (Figure 14). In control group, interpulse intervals up to 40 ms displayed paired-pulse depression, at greater time intervals depression switched into facilitation and at 1000 ms interval PS1 and PS2 were almost identical. In general, this depression and facilitation pattern did not significantly changed in REMD, ESC (10mg/kg for 14 days), and REMD+ESC groups. Although, at 80 ms and 100 ms intervals, the population spike ratios of the ESC (1.7±0.2 at 80 ms, 1.6±0.3 at 100 ms) group and the REMS+ESC (1.6±0.4 at 80 ms, 1.6±0.3 at 100 ms) were higher than the control group (1.1±0.04 at 80 ms, 1.3±0.07 at 100 ms), when compared to that of controls, these alterations were not statistically significant.

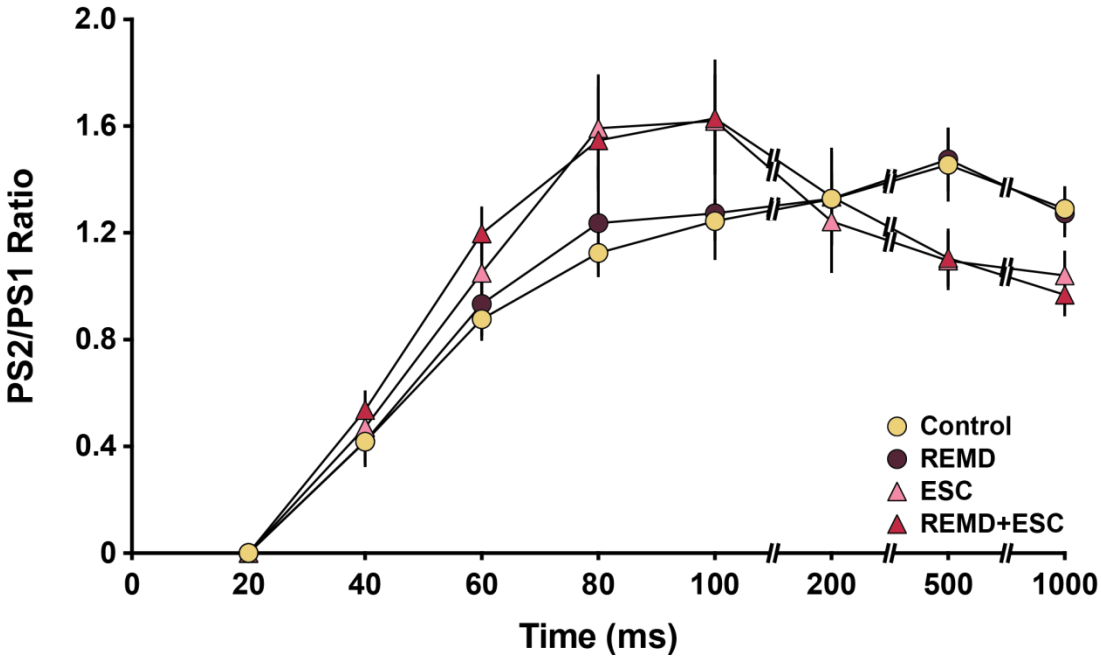

Figure 14. REMD (Rapid Eye Movement Deprivation) and/or ESC did not change the paired pulse paradigm in CA1 neurons of rats. CA3-CA1 pathway were stimulated via paired pulses varying time intervals between 20-1000ms. Although there was no statically significant difference between groups, ESC (10mg/kg for 14 days) and REMD+ESC (10mg/kg for 14 days) groups PS2/PS1 ratios were higher at 60-80 ms interval with respect to the controls.

4.4. Effects of REMD and chronic escitalopram on long-term synaptic plasticity (LTP)

In control group, high frequency stimulation (HFS) of fimbria-commissural patways increased the fEPSP (Field Excitatory PostSynaptic Potential) slope up to 228.9±22.4% of the baseline (Figure 15). Following 72 hours of REM deprivation, animals exhibited response to LTP induction with an increase in fEPSP slope up to 138.9±9.2% of the baseline but this increase was markedly lower than the one obtained from the control group (n=6, p<0.001, Figure 15). 60 minutes after LTP induction, the fEPSP slope of control group was 192.3±14.9% and it was 122.3±9.6% for the REMD group of animals, which was again significantly lower than the control values (n=6, p<0.01, Figure 15).

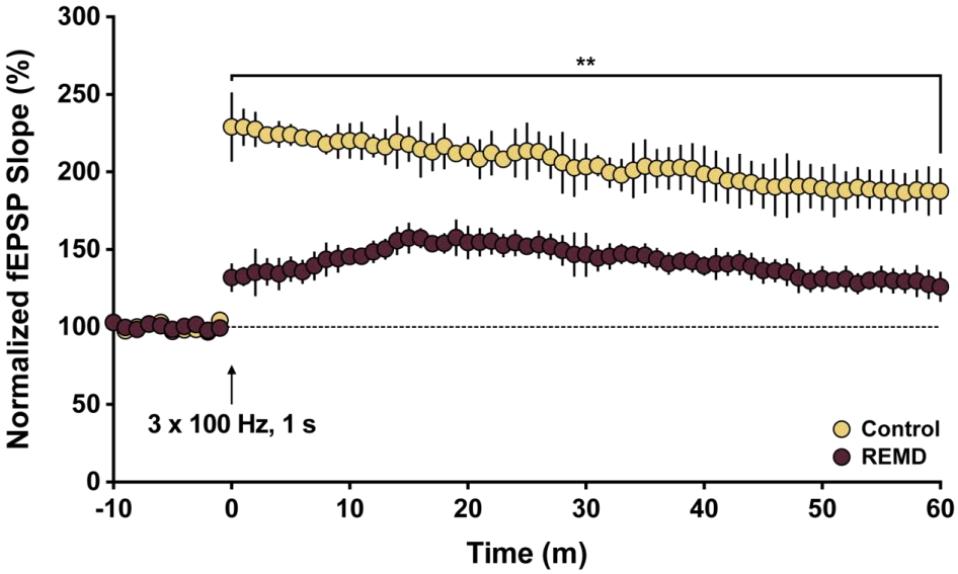

Figure 15. REM (Rapid Eye Movement) sleep deprivation induced significant impairment in LTP of rat CA3-CA1 synapses. Following 30 minutes of baseline recording, LTP was induced by consequent three 100Hz, 1s trains. Recordings were obtained from hippocampal radiatum layer of CA1. LTP induction increased the intial EPSP slope by ~200% in the control group animals (open circles, n=6), while REMD significantly reduced LTP in all post-high frequency stimulation range (60 minutes, closed circles, n=6, p<0.001). **; p<0.01 REMD vs. the control (ANOVA, Tukey's test).

After REM deprivation of chronically ESC (10mg/kg for 14 days) treated rats, LTP induction increased the initial fEPSP slope up to 198.96±8.54%, which was greater than the REMD group of initial LTP (138.9±9.2%, n=6, p<0.01, Figure 16). However, this increase in LTP was found to be statistically significant for only 15 minutes of post-LTP induction period.

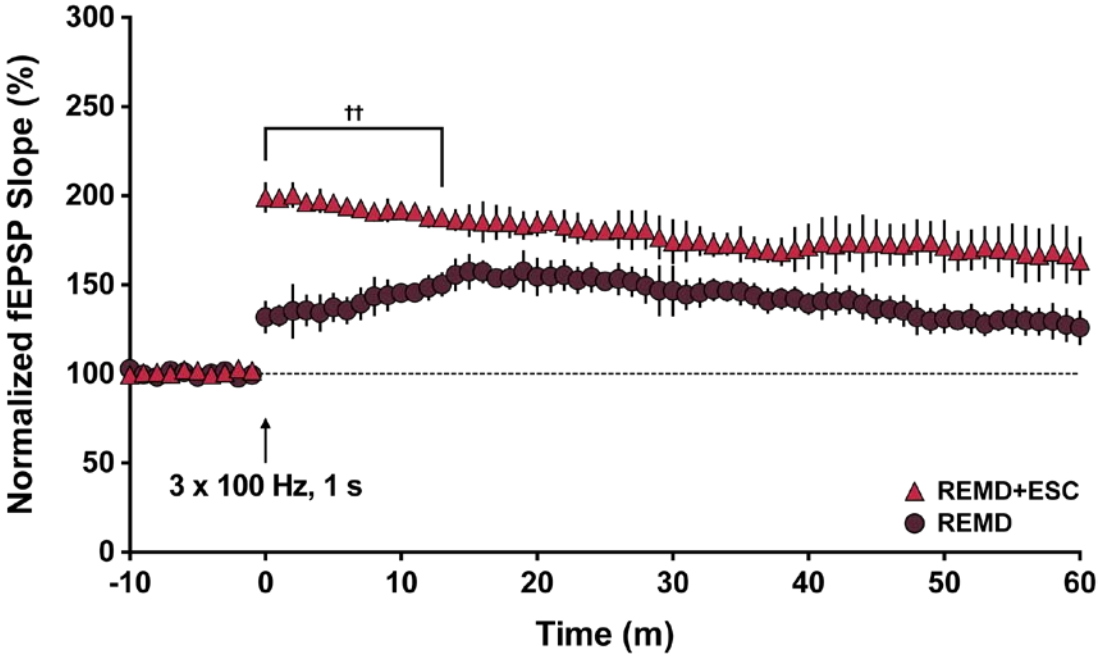

Figure 16. Chronic escitalopram treatment partially prevented REMD (Rapid Eye Movement Deprivation)-induced impairment of LTP. Following 30 minutes of baseline recording, LTP induced by consequent three 100Hz, 1s trains. Recordings were obtained from hippocampal radiatum layer of CA1. HFS stimulation increased EPSP slope up to ~140% of baseline in REMD and up to ~190% of baseline in escitalopram treated REMD group. (n=6). †† p<0.01 REMD+ESC vs. REMD (ANOVA, Tukey's test).

In 2 weeks ESC-treated REMD group, HFS increased the fEPSP slope to 198.9±8.5% of the baseline. When compared to that of the control group, we did not observe any difference in the synaptic strength increases following LTP induction obtained from the control group and the REMD+ESC group (Figure 17). Although

the ESC treatment could not completely prevent the loss of LTP induction in the REMD group, it did significant recovery in the LTP. Unexpectedly, 2 weeks of ESC treatment without REMD weakened the LTP without statistical significance (Figure 17).

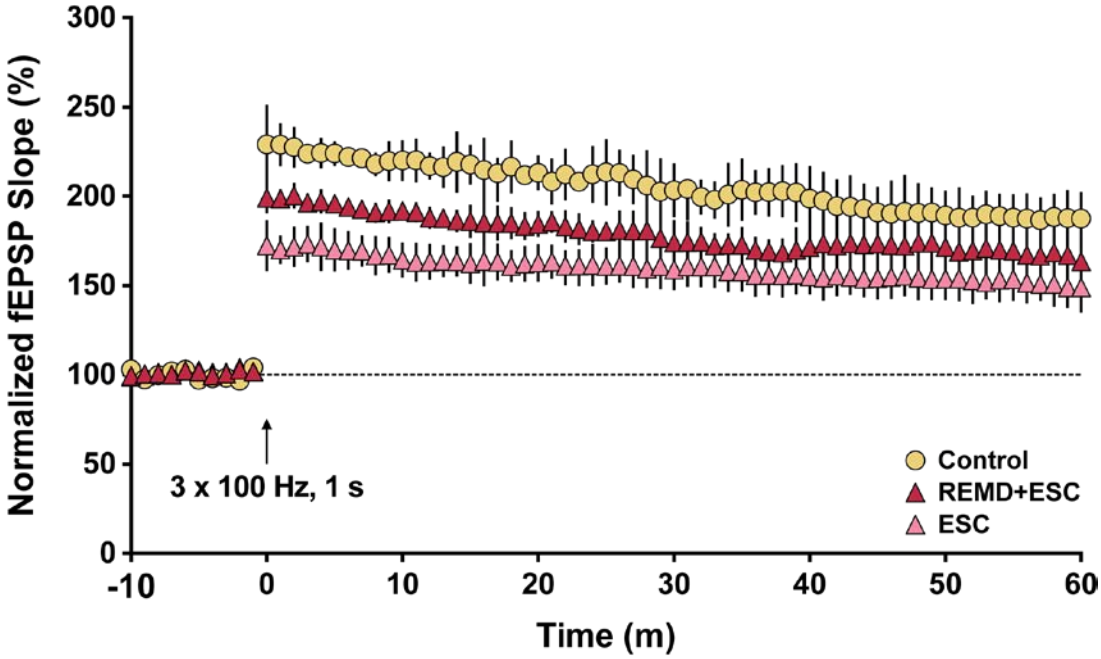

Figure 17. Chronic ESC treatment improved LTP loss following REMD (Rapid Eye Movement Deprivation). Comparison of LTP curves revealed that the synaptic strength reduction observed after REM deprivation was inhibited by chronic ESC (10mg/kg for 14 days) treatment. There was no statistically significant difference between the control and REMD+ESC groups (n=6, $p<0.05$). However, chronic ESC treatment group yielded lower synaptic strenght increase while this was not statistically significant. Following 30 minutes of baseline recording, LTP induced by consequent three 100Hz, 1s trains. Recordings were obtained from hippocampal radiatum layer of CA1.

To assess the acute ESC effects on LTP, ESC (10 mg/kg ip) was injected 1 hour before the LTP induction. Acute ESC treatment depressed the first ~15 minutes of potentiation which corresponds to the early phase of LTP (n=6, $p<0.01$, Figure

18). After 20 minutes of LTP induction the difference between the control group and the acute ESC-treated group disappeared and the curves superimposed (Figure 18).

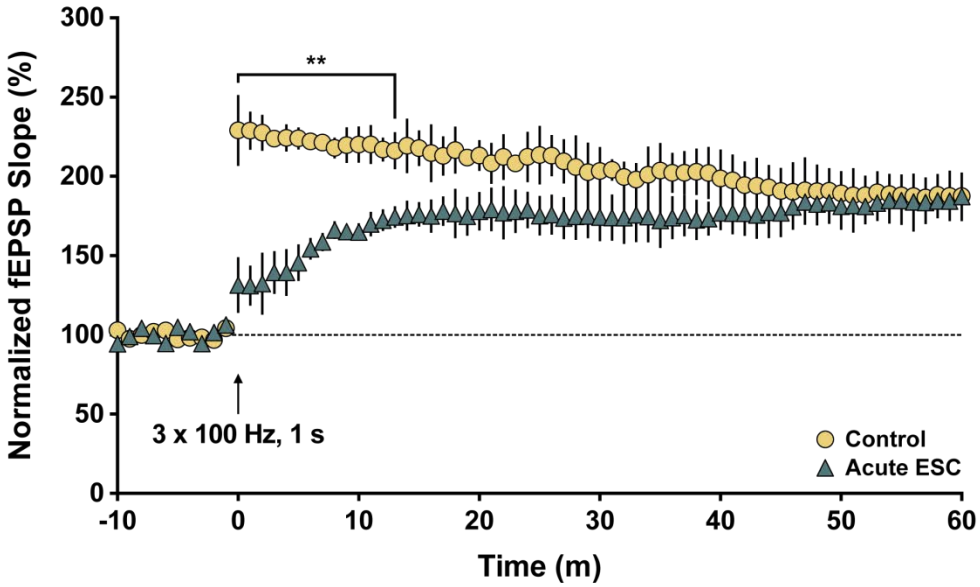

Figure 18. Acute escitalopram treatment inhibited the early phase of LTP (Long Term Potentiation) induction. ESC (10mg/kg ip) was injected 1 hour before the high frequency stimulation. Under this circumstances, LTP induction yielded significantly lower potentiation in the first 15 minutes when compared to the control group. In the post-HFS period, EPSP slopes gradually increased and after 20 minutes they caught up with the control values. Recordings were obtained from hippocampal radiatum layer of CA1.(n=6). ** $p<0.01$ Acute ESC vs. the control (ANOVA, Tukey's test).

4.5. Behavioral Studies

Since our results suggested deficits in LTP after REMD, we also assessed the behavioral alterations induced by REMD and the effects of ESC on these changes.

Place recognition task was used to test the spatial recognition memory (Figure 19). During the sample phase the control group of animals explored the two identical objects with an object-2/(object-1+object-2) ratio of 0.49±0.01; for the REMD, ESC, REMD+ESC groups the ratios were 0.51±0.03; 0.49±0.02; 0.48±0.03,

respectively. Statistical analysis revealed no significance between four groups in the sample phase. In the test phase, control group of animals exhibited increased interest to the object 2 in its new place with a ratio of 0.57±0.01, however, REMD animals failed to explore the replaced object, 0.45±0.02 (n=7, p<0.01). ESC-treated REMD rats recognized the change in the object placement and showed more interest to object-2; the ratio was 0.61±0.02. The difference between the ratios of REMD and REMD+ESC was statistically significant (n=7, p<0.001). ESC treatment alone, increased the ratio in the test phase, but this change was not found to be statistically significant.

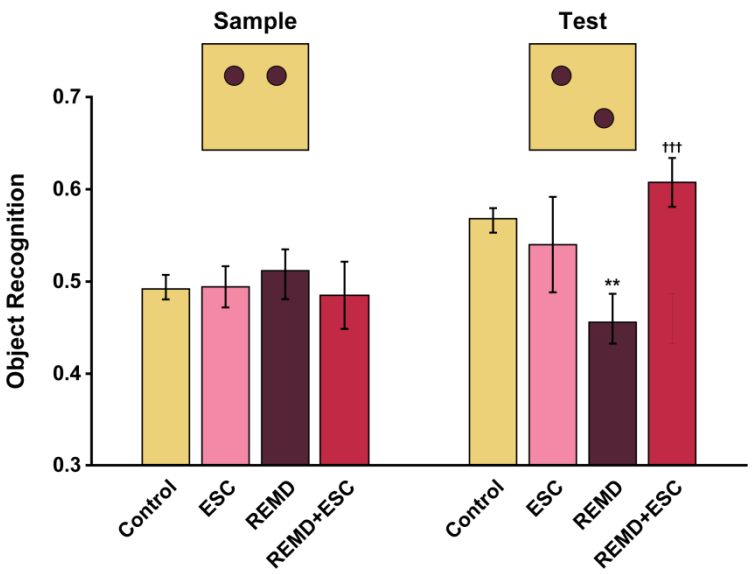

Figure 19. REMD (Rapid Eye Movement Deprivation)+ESC group performed significantly better in object recognition tests than the REMD group. Object recognition test is divided into 2 phases: Sample phase and test phase. In the sample phase, the groups were exposed to 2 object and show approximately same interest to both objects. In the test phase one object is placed to a new location and the ratio of the time spent for the novel object to total exploration time. Comparisons with the control test phase showed REMD group underperformed the test (** p<0.01 REMD vs. The control, n=7) while chronically ESC treated + REM deprived animals performed better than the other groups (††† p<0.001 REMD+ESC vs. REMD, n=7).

Plus-maze is a widely accepted test used to evaluate anxiety levels in animals. Our plus-maze results showed that the REMD group spent much more time in the open arms than the other groups ($n=7$, $p<0.001$ REMD vs. The control, Figure 20A), while the ESC group was the most hesitant to stay in the open arms (Figure 20A). There was no statistical difference between the control and the REMD+ESC groups. As a measure of locomotion we also recorded the number of crosses between the arms. REMD significantly increased the open arm stays ($n=7$, $p<0.001$, Figure 20B) and ESC pretreated REM deprived animals displayed open arm activity identical to that of control group. When compared to REMD group, ESC pretreated alone and REMD groups showed decreased the number of crosses ($n=7$, $p<0.01$ ESC vs. REMD; $p<0.05$ REMD+ESC vs. REMD)

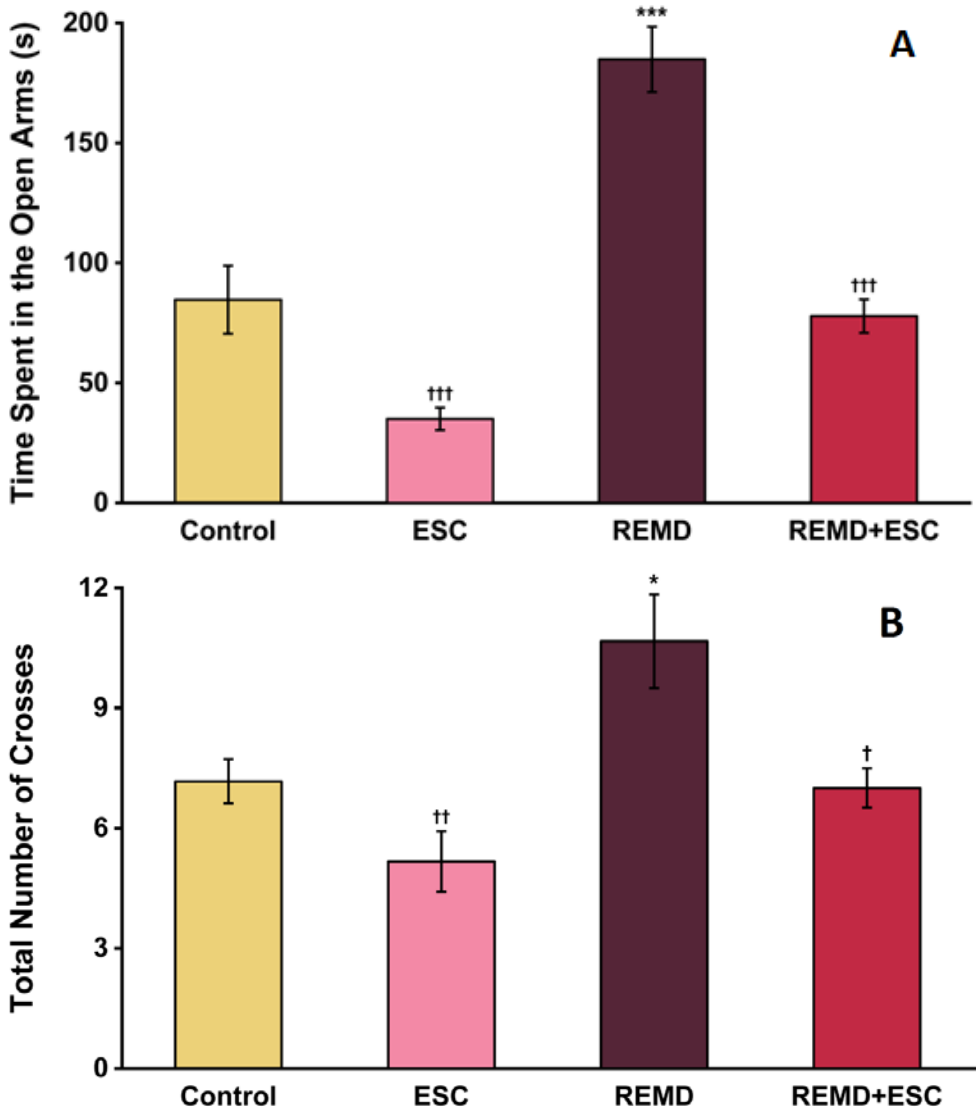

Figure 20A, 20B. REM deprivation (Rapid Eye Movement Deprivation) decreased anxiety and chronic ESC treatment inhibited this effect. Time spent in the open arms and total crosses of arms are related to the anxiety levels and the locomotor activity of the rats. REMD animals spent significantly more time in open arms (175 s, $p<0.001$, F,gure 15A), while the control, ESC, REMD+ESC groups spent nearly 75, 30, 75 seconds, respectively (Figure 20A). Total number of crosses showed the same pattern, it was highest in REMD group. ***; $p<0.001$, with respect to the controls. ††; $p<0.01$, †; $p<0.05$, with respect to the REMD group (n=6).

All animals subjected to open field arena test in order to evaluate their locomotion and anxiety. Control animals travelled 10m in average during their 15 minutes of arena stay (Figure 21). REMD significantly increased the distance travelled up to ~20m (n=6, p<0.01). Chronic ESC treatment, slightly increased the distance travelled without statistical significance. Two weeks of ESC treatment notably blocked REMD-induced hyperactivity (Figure 21). Although, in REMD+ESC group, total distance travelled were somewhat higher than the controls, these values were similar to the ESC alone values and were not statistically different than the controls (Figure 21).

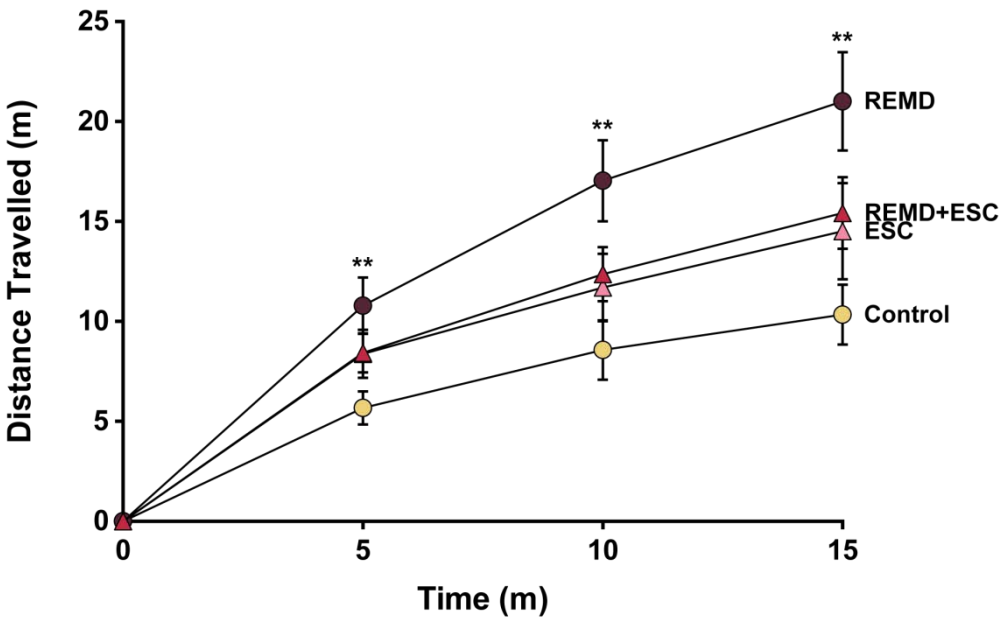

Figure 21. REMD (Rapid Eye Movement Deprivation) drastically increased locomotor activity, chronic ESC treatment blocked the REMD-induced hyperactivity. Rats were placed into a square arena surrounded by glass walls and their activity was recorded for 15 minutes. REMD group travelled significantly greater distance than the control group (n=6, **p<0.01 REMD vs the control group). In the ESC-treated groups with or without REM deprivation the total distance travelled were lower than that of the REMD group. ESC-treated (10 mg/kg for 14 days) groups (ESC, REMD+ESC) did not exhibit statistical difference with respect to the control value.

Since the increase in the time spent and the distance travelled in the center region of the open field was shown to be inversely related to the anxiety levels of the animals, we analyzed center region data for all groups. The control group rats roamed the center region ~2% of their total stay in the arena (Figure 22A) and ~6% of their locomotor activity took place in the center (Figure 22B). On the other hand, REM deprivation increased the time spent in the center to ~6% and locomotion to 12% (Figure 22A). Chronic ESC treatment alone did not alter the center region activity (Figure 22A). In the chronically ESC-treated animals, REM deprivation did not induce increased center region stay and locomotor activity (Figure 22B)

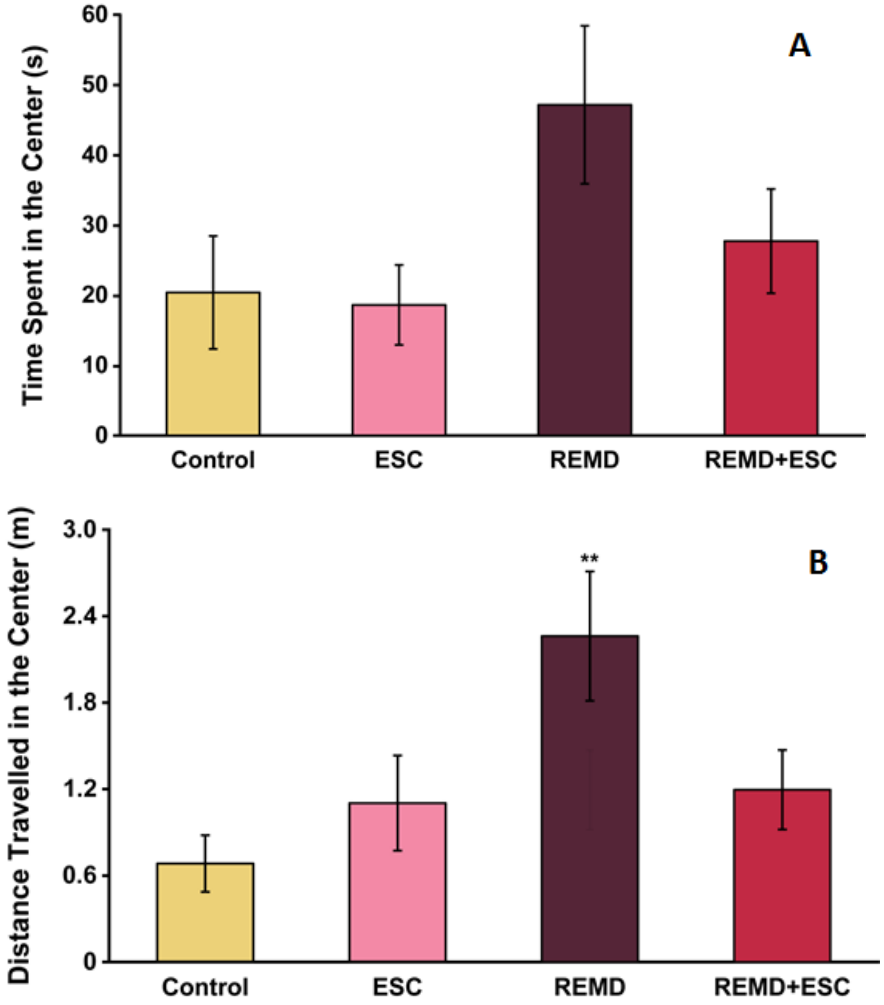

Figure 22 A, 22B. Chronic ESC treatment prevented REM deprivation (Rapid Eye Movement Deprivation)-induced increased locomotor activity and reduced anxiety. Control group animals travelled approximately 0.6 m in the central region of the arena while REMD group of rats travelled more than any other group (~2.4m, **$p<0.01$, REMD vs the control, n=6, Figure 22 B). In the REMD+ESC (10mg/kg for 14 days) and ESC groups, the total distance travelled in the center was found to be comparable to the control values. The time spent in the center region of the arena by the REMD group was more than that of the any other groups (n=6, $p<0.01$, Figure 22 A). ESC-treated animals, did not display increased sojourn in the center regions following REM deprivation. ESC treatment alone did not alter the center sojourn(Figure 22 A).

As ESC effects highly correlated with its inhibition on mainly serotonin reuptaker and to some extend with catecholamine reuptakers, we measured the serotonin, adrenaline, noradrenaline, dopamine concentrations in different brain regions in all four groups of animals.

Table 1. REMD with or without ESC and ESC alone did not alter the neurotransmitter concentrations in rat brain

	5-HT (nM/g)	**NA (nM/g)**	**A (nM/g)**	**DA (nM/g)**
Control				
Hippocampus	83.43±4.87	0.79±0.21	0.13±0.01	0.30±0.04
Cortex	81.11±4.61	0.88±0.33	0.15±0.01	0.52±0.29
Hindbrain	77.53±6.12	0.53±0.09	0.13±0.01	0.29±0.04
REMD				
Hippocampus	81.60±5.76	0.60±0.19	0.14±0.02	0.26±0.02
Cortex	89.62±11.81	0.58±0.19	0.11±0.01	0.21±0.06
Hindbrain	80.19±8.99	1.03±0.47	0.12±0.03	0.26±0.06
ESC				
Hippocampus	71.34±4.68	0.58±0.08	0.15±0.01	0.27±0.01
Cortex	73.71±4.30	0.65±0.09	0.13±0.008	0.21±0.02
Hindbrain	82.15±6.18	0.57±0.13	0.13±0.01	0.22±0.04
REMD+ESC				
Hippocampus	82.04±4.95	1.54±0.66	0.14±0.01	0.24±0.02
Cortex	62.10±4.36	0.52±0.07	0.14±0.01	0.32±0.09
Hindbrain	94.23±11.54	0.50±0.12	0.11±0.02	0.23±0.03

Neurotransmitter concentrations (nM/g wet tissue) from hippocampi, cortex an hindbrain measured by HPLC. Statistical comparisons revealed no significance between the treatments and the brain regions. 5-HT; serotonin, NA; noradrenaline, A; adrenaline, DA; dopamine.

5. Discussion

In this study, we demonstrated that; i) REMD increased the threshold of CA1 neuron firing and decreased the excitability; ESC treatment reversed back the threshold level. ii) REM deprivation shifted EPSP slope I/O curve to the right and chronic ESC treatment inhibited this shift and increased the maximum slope. iii) Paired-pulse stimulation did not differ amoung groups. iv) REMD decreased the LTP. Chronic ESC treatment prevented the reduction in LTP produced by REMD. v) REM deprivation impaired the recognition of the novel place of the object whereas ESC-treatment prevented this impairment in learning vi) Plus maze and arena tests showed REMD animals had lower anxiety and increased locomotor activity, and chronic ESC treatment inhibited the reduced anxiety level and increased locomotor activity caused by REMD. vii) After 14 day of ESC treatment, the 5-HT, A, NA, DA levels in hippocampi, cortex, hindbrain were not found to be different than that of controls.

5.1. REMD increased CA1 neuronal firing treshold but not in ESC-treated rats

In our study, we showed that 3 days of REMD significantly shifted the population spike input/output curve to the right, implicating that the firing threshold of CA1 neurons increased and their excitability decreased. However, after 2 weeks of chronic escitalopram treatment, the I/O curve was resistant to REMD-induced alterations. Population spike amplitude is correlated with number of firing neurons and their excitability. Excitability of the pyramidal neurons in CA1 region is determined by the sum of inhibitory and excitatory activity and as well as intrinsic electrical properties of the neurons. REMD-induced I/O shift could be explained by a reduction in presynaptic glutamatergic output onto the CA1 neurons. Therefore, we analyzed I/O curves plotted by using fEPSP slopes, which were shown to have a correlation with the presynaptic release (116,117) . fEPSP slopes were then analyzed to assess glutamatergic release and in a parallel fashion with the population spike I/O data, fEPSP I/O curves shifted to the right in REMD group. We hypothesized due to changes in presynaptic site, glutamate release is decreased by REMD and recovered by using chronic ESC. It can be also related to postsynaptic site receptor and ligand

interaction. As the impairment caused by REMD is recovered by ESC, serotonin re-uptake inhibitor, neurotransmitter level change and/or receptor change can affect the excitability of presynaptic site. Thus, we measured the 5HT, A, NA, DA levels, and found no change in any of the groups implying that serotonin receptor expression may be changed after REM deprivation. Spindelegger et al. showed chronic escitalopram treatment decreased 5-HT_{1A} binding in limbic system, mainly in hippocampus (118) . 5-HT_{1A} receptors were known to have inhibitory action on CA3-CA1 synapses (119) . That is why we hypothesized chronic ESC treatment plays its role of action through presynaptic 5-HT_{1A} changes, decrease in 5-HT_{1A} receptor level lowers threshold potential and increase excitability. In our studies, direct application of 5HT to the hippocampal pyramidal neurons significantly reduced excitability and PS amplitudes like the effect of acutely administered ESC. The paired-pulse depression phase was reported to be mainly related with GABA-A and GABA-B receptor activity. As in our experiments we did not observe an alteration in this early/inhibitory phase of paired pulse modulation, we suggested that either REMD or ESC treatment did not play a significant role in GABAergic activity.

5.2. REM deprivation does not alter short-term plasticity

Paired-pulse modulation is used to analyze short-term plasticity in Schaffer collateral-CA1 synapses. REMD did not produce any alteration in paired-pulse depression or facilitation when compared to the control group. Similarly, chronic ESC and ESC+REMD groups exhibited no significant changes in their paired-pulse curves implying that REMD, ESC did not affect short-term plasticity. Although chronic treatment with ESC increased facilitation at 80 and 100 ms interval, this was not statistically significant. As the early phase (up to 100ms) mainly related to GABA-A and the late phase (>100 ms) related to GABA-B, these results implied that REMD and/or ESC treatment did not modify GABAergic transmission (31,120,121) .

5.3. Chronic escitalopram treatment inhibited REMD-related LTP impairment

In the present study, we showed that chronic ESC treatment prevented LTP impairment caused by REMD. REMD significantly impaired LTP indicating REMD

severly disturbed learning and/or memory (122) . REMD+ESC groups had stronger potentiation after LTP induction compared to the REMD. These findings were supported by the behavioral experiments showing improved object recognition test performance in REM+ESC animals. Our results favor the idea that chronic ESC treatment is protective for the memorial functions against detrimental effects of REM deprivation. Without REMD, our chronic ESC group of rats did not exhibit stronger synaptic potentiation and better learning performances than the controls. In contrast, in some previous studies, escitalopram and fluoxetine, were shown to increase fEPSP slope, LTP and spatial learning (123-126) . Some other studies support our findings without REMD, ESC treatment did not improve LTP even slightly depressed (127) . This discrapiency can be explained by the indivudual differences in SSRI group of drugs. Additionaly, acute administration (one dose) of ESC significantly impaired the early phase of LTP, which denotes that in early period of SSRI exposure, these drugs may impair learning/memory. This seems to be in accordance with the views that acutely SSRI drugs increase forgetfullnes in patients (128) .

It has been showed that REM sleep deprivation inhibits LTP in rat hippocampus, in vivo (129-131) . Kim et. al. showed that this impairment is independent of REM deprivation related stress (131) . The cytoskeletal remodelling protein cortactin, NMDA receptor subunits 2B, 2A were found to be lower in REM deprivated rat hippocampi (132) . Although we did not assess NMDA and AMPA receptor changes after REMD, our I/O and LTP data could be as a result of NMDA, AMPA receptor downregulation. BDNF(Brain Derived Neurotrophic Factor) is also suggested to play an important role and has been shown that decrease in BDNF level is one of the main reason causing LTP impairment during REM deprivation (133,134) .

Chronic SSRI usage increase neurotrophic factors like BDNF and their commonly accepted mechanism of action in depression treatment is explained by their effects on BDNF and other neurotrophic factors (9,135-137) . That can also explain the protective effect of chronic ESC against REMD.

To evaluate the role of neurotransmitter (5HT, NA, A, DA) level changes on REMD-induced cognition, we analyzed their concentrations in different brain regions. However, our study revealed that after 2 weeks of ESC treatment none of

the neurotransmitters exhibited a level change. We hypothetized that escitalopram, one of the most selcetive serotonin reuptake inhibitor, acutely increased the 5HT levels and increased 5-HT initiated downregulation of 5HT receptor levels. Since, 5-HT_{1A} is the dominant serotonin receptor type in hippocampus, 5-HT_{1A} expression decreased, consequently excitability increased and firing threshold decreased.

5.4. Chronic escitalopram protects from learning impairment caused by REMD

There have been some experiments related to neuroprotective role of SSRI drugs, especially escitalopram and fluoxetine. Escitalopram was shown to enhance the cognitive recovery following stroke and increase cognitive performance compared to control group (138,139) . In some studies related to memory impairment related to chemotherapy, fluoxetine is proven to improve the memory deficit caused by the chemotherapy agent 5-fluorouracil and methotrexate (140,141) . Our results showed that chronic escitalopram treatment recovered the memory impairment caused by REMD. On the other hand, there was no significant difference between the control group and ESC treatment group. BDNF is suggested to play the main role in the mechanism of neuroprotection. Some studies claim that serotonin depletion is related to long-term memory impairment and tryptophan supplementation is associated with improved performance in memory tests (142,143)

5.5. ESC treatment blocked the anxiety reduction by REMD

Exposure to various stressors including restraint stress, tail shock, tail pinch, and high-level (but not low-level) foot shock results in increased 5-HT turnover in the medial prefrontal cortex, nucleus accumbens, amygdala, and lateral hypothalamus in experimental animals (144) . However, exposure to repeated electric shocks sufficient to produce learned helplessness is associated with reduced in vivo release of 5-HT in the frontal cortex (145) , a finding possibly reflecting a state in which 5-HT synthesis is outpaced by the release. Preadministration of benzodiazapine receptor agonists or tricyclic antidepressant drugs prevents stress-induced reductions in 5-HT release and interferes with the acquisition of learned helplessness, whereas infusion of 5-HT into the frontal cortex after stress exposure

reverses learned-helplessness behavior (145,146) . Finally, administration of 5-HT–receptor antagonists produces behavioral deficits resembling those of the learned helplessness seen after inescapable shock during animal stress models that do not ordinarily result in learned helplessness (146) . The effect of stress in activating 5-HT turnover may stimulate both anxiogenic and anxiolytic pathways within the forebrain, depending on the region involved and the 5-HT–receptor subtype that is predominantly stimulated. Microinjection of 5-HT into the amygdala appears to enhance conditioned fear, whereas 5-HT injection into the PAG (periaqueductal gray) inhibits unconditioned fear (147) . Graeff et al. hypothesized that the serotonergic innervation of the amygdala and the hippocampus mediates anxiogenic effects by 5-HT_{2A} receptor stimulation (147) , whereas serotonergic innervation of hippocampal 5-HT_{1A} receptors suppresses formation of new conditioned and unconditioned stimuli associations and provides resilience to aversive events. Potentially compatible with this hypothesis, 5-HT_{1A} receptor knockout mice exhibit behaviors consistent with increased anxiety and fear; long term administration of 5-HT_{1A} receptor partial agonists exerts anxiolytic effects in generalized anxiety disorder (148) . Notably, stress and glucocorticoids exert major effects on the genetic expression of 5-HT_{1A} and 5-HT_{2A} receptors. Postsynaptic 5-HT_{1A} receptor gene expression is under tonic inhibition by adrenal steroids in the hippocampus and possibly other regions where mineralocorticoid receptors are expressed. Thus, 5-HT_{1A} receptor density and mRNA levels decrease in response to chronic stress or CORT administration and increase after adrenalectomy (149) . The stress induced down-regulation of 5-HT_{1A} receptor expression is prevented by adrenalectomy, a finding showing the importance of circulating adrenal steroids in mediating this effect (149) .

Our experiment showed that REMD decreased anxiety. As neurotransmitter levels do not differ between groups, our hypothesis is that REMD probably increases 5-HT_{1A} receptors in fear related regions of central nervous system which is also consistent with 5-HT_{1A} receptor level increases in hippocampus.

Chronic escitalopram treatment normalized the anxiety in REMD. This action can be rationalized by reduced 5-HT_{1A} receptor expression in postsynaptic site by chronical escitalopram treatment.

5.6. The effect of REMD and escitalopram on locomotor activity

In our study, REM sleep deprivation increased the locomotor activity similar to another study (150) . We also showed that chronic escitalopram treatment inhibited the locomotor activity increased by REMD. SSRI drugs, fluoxetine (30 mg/kg) and escitalopram (10 mg/kg) were shown to increase locomotor activity in a dose-dependent manner (151) . Fluoxetine was proven to potentiate cocaine-induced locomotor activity via 5-HT_3 receptors in nucleus accumbens; and receptor blockade by ondansetron diminished the effects of fluoxetine (152) . Taking all into consideration, acute ESC treatment or REMD increased synaptic serotonin levels in brain regions related to locomotor activity like nucleus accumbens. This increased locomotor activity might be related to 5-HT_3 receptors, and chronic escitalopram treatment decreased 5-HT_3 receptors on post-synaptic membranes and normalized locomotor activity.

6. Conclusion

Our study showed that:

- REM deprivation decreased the excitability in hippocampi CA1 region, shifted I/O curves to the right.
- Chronic ESC treatment restored the excitability, whereas acute ESC and also 5-HT (given by in vivo electrode) decreased the excitability.
- Neither ESC treatment nor REMD did not show significant effect on GABAergic system in hippocampal CA1 region neuronal synapses.
- REM deprivation decreased presynaptic neurotransmitter release while chronic ESC treatment reversed back to the control level.
- REMD impaired long term potentiation, chronic ESC recovered the LTP impairment.
- Acute ESC depressed the early phase of LTP.
- REM deprivation significantly disturbed the spatial learning and memory on the other hand chronic ESC treatment showed protective effect over REMD.
- REM deprived rats had more locomotor activity, chronic ESC treatment normalized the increased locomotor activity by REMD. ESC treatment alone did not show significant effect.
- REMD reduced the anxiety. REM deprivation did not change the anxiety level in chronic ESC treated rats.

7. Future Perspective

Our results showed that escitalopram has an important protective effect on REMD. It can be directly related to ESC, the molecule itself or due to a group (antidepressant, SSRI) effect. Other SSRI drugs also should be studied to evaluate whether they will have same effect on REMD. Furthermore, other anti-depressant drugs should also be studied in the future to differentiate if this protective effect of ESC is related to serotoninergic pathways or antidepressant drugs like SNRI, 5-HT and NA reuptake blocker, could have the same effect by acting catecholaminergic pathways.

Although there have been studies in literature about SSRI related to receptor density changes (118) , both presynaptic especially 5-HT_{1A} and also postsynaptic serotoninergic receptor density and pharmacokinetics changes should also be studied. 5-HT_{1A} agonists and antagonists can be used to investigate the importance of 5-HT_{1A} receptors on the protective effect of ESC.

Chronic ESC treatment and its protective effect can be proven by studies on patients with insomnia. Their cognitive performance can be evaluated before and after the treatment.

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APPENDİCES

Appendix 1

(Ethics committee approval)

HACETTEPE ÜNİVERSİTESİ
HAYVAN DENEYLERİ YEREL ETİK KURULU

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03 Eylül 2012

HAYVAN DENEYLERİ YEREL ETİK KURUL KARARI

TOPLANTI TARİHİ	:	03.09.2012 (PAZARTESİ)
TOPLANTI SAYISI	:	2012/5
DOSYA KAYIT NUMARASI	:	2012/43
KARAR NUMARASI	:	2012/43-06
ARAŞTIRMA YÜRÜTÜCÜSÜ	:	Doç. Dr. M. Yıldırım Sara
HAYVAN DENEYLERİNDEN SORUMLU ARAŞTIRMACI	:	Dr. İsmail Mikdat Kabakuş
YARDIMCI ARAŞTIRMACILAR	:	-
ONAYLANAN HAYVAN TÜRÜ ve SAYISI	:	40 adet Wistar sıçan

Üniversitemiz Tıp Fakültesi Tıbbi Farmakoloji Anabilim Dalı öğretim üyelerinden Doç. Dr. M. Yıldırım Sara'nın araştırma yürütücüsü olduğu 2012/43 kayıt numaralı ***"Sıçanda REM Deprivasyonu Modelinde SSRI ve SNRI'ların Nöroplastisite ve Hafıza Üzerine Koruyucu Etkisi"*** isimli çalışma Hayvan Deneyleri Yerel Etik Kurulu Yönergesi'ne göre uygun bulunarak oy birliği ile onaylanmasına karar verilmiştir.

Sorumlu araştırmacı deneylere başlangıç tarihini Etik Kurula bildirmekle yükümlüdür

Prof. Dr. Hakan S. ORER
Etik Kurul Başkan