

Research report

A study on fear memory retrieval and REM sleep in maternal separation and isolation stressed rats



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HIGHLIGHTS

- Maternal separation stress was done during stress hypo-responsive period.
- We examined changes in sleep and fear memory.
- Increase in the fear memory was negatively correlated with home cage exploration.
- Resistance to the immediate fear extinction and increased REM sleep was observed.
- Altered neuronal activities in hippocampus–amygdala–cortical loop during REM sleep.

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ABSTRACT

As rapid brain development occurs during the neonatal period, environmental manipulation during this period may have a significant impact on sleep and memory functions. Moreover, rapid eye movement (REM) sleep plays an important role in integrating new information with the previously stored emotional experience. Hence, the impact of early maternal separation and isolation stress (MS) during the stress hypo-responsive period (SHRP) on fear memory retention and sleep in rats were studied. The neonatal rats were subjected to maternal separation and isolation stress during postnatal days 5–7 (6 h daily/3 d). Polysomnographic recordings and differential fear conditioning was carried out in two different sets of rats aged 2 months. The neuronal replay during REM sleep was analyzed using different parameters. MS rats showed increased time in REM stage and total sleep period also increased. MS rats showed fear generalization with increased fear memory retention than normal control (NC). The detailed analysis of the local field potentials across different time periods of REM sleep showed increased theta oscillations in the hippocampus, amygdala and cortical circuits. Our findings suggest that stress during SHRP has sensitized the hippocampus–amygdala–cortical loops which could be due to increased release of corticosterone that generally occurs during REM sleep. These rats when subjected to fear conditioning exhibit increased fear memory and increased fear generalization. The development of helplessness, anxiety and sleep changes in human patients, thus, could be related to the reduced thermal, tactile and social stimulation during SHRP on brain plasticity and fear memory functions.

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1. Introduction

The early postnatal period, specifically, stress-hypo responsive period (SHRP) is a critical phase for normal brain development,

characterized by an enormous capacity of structural and functional reorganization of neural circuitry. Bowlby's [1] discovery of attachment theory incited studies on maternal separation stress affecting neuroendocrine and behavioral alterations in different developmental period [2]. In particular, the stress produced by repeated separation from the mother during early postnatal life maternal separation stress (MS) can lead to juvenile and adult mood disorders and reduced cognitive abilities [3].

Exposure to early maternal separation stress often causes pathological changes that principally affect three functionally well

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connected brain regions – amygdala [4], hippocampus [5] and medial prefrontal cortex (mPFC) [6]. Interestingly, the effects of stress on these regions are not the same – while the infralimbic cortex (ILC) showed higher synaptic densities to early maternal separation stress, the anterior cingulate cortex showed a reduced spine density [7]. The present study aimed to understand if early maternal separation together with isolation stress during SHRP affects differentially on the hippocampus (CA1 subregion) (CA1), amygdala (Lateral nucleus of amygdala) (LA) and medial prefrontal cortex (infralimbic cortex) (ILC) in adult rats.

In our previous study, we reported that increased duration of REM sleep was associated with increased synchronized amygdalo-hippocampal theta activities in adult rats exposed to chronic stress [8]. These changes may be related to the profound alteration of the hormonal stress axis and to neurotrophic factor expression in the brain [9] and imbalances in neurotransmitter release in these brain regions [10]. Thus MS-induced brain pathology could result in the development of helplessness, anxiety and sleep changes that reflect symptoms in human patients.

Whether hippocampus and amygdala processes differentially influence the explicit and implicit memory during non-REM and REM sleep still remains controversial [11]. Previous studies have given substantial evidence to suggest that early life stress cause a delayed effect on emotional processing in rats [12]. To address this issue, rats subjected to early maternal separation stress were exposed to differential fear conditioning, and the impact of stress during SHRP of rats on the synchronous firing between hippocampus, amygdala and medial prefrontal cortex during REM sleep was probed.

2. Materials and methods

2.1.1. Rats

Pregnant Wistar rats were bred and raised in the Central Animal Research Facility (CARF) of the National Institute of Mental Health and Neurosciences (NIMHANS). These rats were maintained in polypropylene cages, 1 week before gestation and were provided with ad libitum food and water. It was ensured that rats were maintained on a regular light–dark cycle (12 h:12 h) and was properly ventilated. Experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) and maximum care was taken to minimize the pain and discomfort to the experimental animals during the procedures.

The pregnant rats were first randomly assigned to maternal separation stress (MS) and normal control groups (NC). Polysomnographic recordings and differential fear conditioning was carried out in two different sets of male rats aged 2 months weighing 250–300 g.

MS procedure was carried out under temperature controlled environment different from the normal rearing environment. At first, dam was separated from the pups and placed in a different home cage. Immediately after separation, rat pups were individually placed in a different home cage having six partitions. Thus, there were two types of stress: (1) maternal separation stress, (2) isolation stress for the pups. Maternal separation was carried out during stress hyporesponsive period (SHRP) such as postnatal days 5–7 (P5–P7) (MS) and was carried out during 9 AM to 3 PM for 6 h daily. The room temperature was maintained at 20–22 °C. Rats during the MS procedure (7). Only male offspring were used in the study. The rats left undisturbed during pre-weaning period were considered as normal controls.

2.1.2. Effect of MS stress on the spontaneous behavior of the rat in the familiar home cage

Before subjecting them to differential fear conditioning, rats from control ($n=10$) and MS groups ($n=18$) were tested for their spontaneous behaviors in their familiar home cage for a period of 15 min during the dark phase of the cycle (between 7 PM and 10 PM). The spontaneous locomotor behaviors and quiet behavior of the rat were video-taped and stored for offline analysis. The average time spent on each behavior was classified into exploratory locomotion (exploratory walking and rearing) and quiet behavior (immobility and grooming) during 15 min recording period for each rat which was converted into percentages. The mean values between the NC and MS were subjected to statistical analysis.

2.1.3. Effect of MS on differential fear conditioning

The differential fear conditioning was carried out to evaluate the effect of early maternal separation stress on the associative fear memory and generalization effect in a fear-conditioning chamber (Coulbourn Instruments Inc., USA). The fear conditioning was carried out in context A (foreground context) consisted of aluminum and Plexiglas walls and a grid floor spaced at 1.8 cm. In addition, video camera was mounted on the ceiling and tone generator on the sidewalls. The fear retention test was in context B (background context) which was similar to that of context A but was placed in a different room consisting of ventilation fans, lights-on and without white noise.

3. Fear conditioning procedure

3.1. Habituation

The rats were first habituated to the conditioning chambers of both contexts for 5 min each. During the habituation period, rats were presented with six auditory tones (2.5 kHz, 70 dB intensity) for 10 s (CS–). The inter tone interval was for 20 s. The rats were presented with two habituation protocols first in context A and after 24 h into the context B. The house light remained switched on throughout the protocol.

3.2. Training

After 24 h of habituation (day 3), fear conditioning was carried out in context A (Fig. 4(a)). Each rat was placed into the fear-conditioning chamber (context A) and allowed to explore for 120 s without presentation of any conditioned stimulus (CS). Immediately after 120 s, each rat was presented with three conditioned stimulus (CS+) (10 kHz auditory tone, 10 s, 90 dB) and co-terminated with unconditioned stimulus (US) (0.5 mA scrambled foot shock for a period of 1 s). After the last presentation of CS–US pairings, rat was allowed to stay in the chamber for another 120 s before returning it to their home cage. The conditioning protocol was repeated on the subsequent day with the same CS–US stimulus pairings for the sufficient consolidation of fear memory.

3.3. Testing

Testing for the retention of fear memory was carried out after 24 h of fear conditioning. The retention test was carried out in the context B. The retention protocol comprised of presentation of four CS– tones followed by four CS+ tones without co-terminating with electric footshock stimulus (US). The rats were allowed to remain in the chamber for 120 s after the termination of the last CS+. The entire chamber was wiped with cotton and alcohol to prevent the

presentation of odor cues to the next experimental rat. The behavior of the rat was video-recorded for the offline analysis of freezing behavior to cues and context. The time spent in freezing was considered as an index of fear memory.

3.3.1. Surgery for sleep–wake recording

Rats from both MS ($n=8$) and NC ($n=6$) were anesthetized with the combination of ketamine (80 mg/kg body weight, intraperitoneal injections) and xylazine (10 mg/kg body weight, IP). In addition, xylocaine (2%) was injected subcutaneously before the surgery. These rats were stereotactically (Stoelting Co., USA) implanted with insulated nichrome wires of 250 μ m diameter in CA1 (antero-posterior: -3.3 mm; medio-lateral: 1.5 mm; Dorso-ventral: 2.6 mm), LA (antero-posterior: -3.3 mm; medio-lateral: 5.2 mm; Dorso-ventral: 8.0 mm) and ILC (antero-posterior, +3.0 mm; medio-lateral, 0.5 mm; dorso-ventral, 5.0 mm), on the left hemisphere for measuring the LFP during sleep. Paxinos and Watson [13] rat brain atlas was used to locate all the stereotaxic coordinates in relation to Bregma and Lambda. An external screw electrode was implanted above the cerebellum subdurally as a reference electrode. In addition, electrodes for Electrooculogram (EOG) and electromyogram (EMG) were implanted in external canthus of the eye and posterior nuchal muscle, respectively. This entire electrode ensemble was soldered into a 10-pin socket and fixed on the skull using dental acrylic powder. Healex was applied to all the wound edges and the rat finally placed back into its home cage for 5 days to recover from the surgical effects. Following surgical recovery, polysomnographic recordings were carried out in the rat home cage continuously for 6 h. Once the behavioral experiments were completed, the rats were deeply anesthetized with halothane and transcardially perfused with 10% formaldehyde. The brains were processed for Nissl staining for the histological verification of electrode placement. The rats with confirmed electrodes in the target were only used for the further evaluation of sleep–wake architecture in both control and MS groups.

4. Procedure for sleep recording

After the complete recovery from surgery, the rats were taken to the recording room and left in its home cage for an hour for habituation. Once the rats were familiar with the new environment, they were connected to the swivel commutator first and subsequently basal LFP recordings were carried out continuously for 6 h during light phase of the cycle (12.00–6.00 PM) [14]. The LFP signals were amplified (10 $\times$), band-pass filtered (0.3 Hz–0.3 KHz) and sampled at the rate of 1000 Hz, digitized by a CED A/D converter (CED 1401A/D converter, Cambridge, UK). During this period, the spontaneous behavior of the rat was monitored and was recorded online with the spike 2 software. The rats had free access to food and water during the entire recording session. In order to observe the prolonged effect of early maternal separation and isolation stress on the sleep and associated changes in the amygdalo–hippocampal–cortical network activities, these rats were taken to the recording room and LFP recording was carried out on day 70.

5. Recording and quantification of the sleep architecture

The 6 h sleep–wakefulness cycles were split into 15 s epochs and visually scored on the basis of LFP, EMG and EOG, by two independent judges who were blind to the experimental condition. Each epoch was classified into a particular stage based on predominant sleep stage in a 15 s epoch that belonged [14]. The scoring method described here in detail, is primarily based on five-stage classification of sleep–wake for rats as proposed earlier [15–18].

The wakeful period was classified into active wakefulness (W1) based on the presence of Type 1 theta rhythm in CA1, high frequency LFP in ILC, tonic EMG level and phasic EOG movements; and quiet wakefulness (W2) showing reduced tonic EMG, occasional EOG movements, Type 2 theta rhythm in CA1 and desynchronized ILC LFP. The sleep period was classified into light slow wave sleep (SWS) (S1) characterized by the presence of high amplitude and low frequency LFP activity in CA1 and ILC, reduced EMG activity in the absence of locomotor activity and reduced EOG movements; deep SWS (S2) showing synchronized low frequency (delta range) and high amplitude–low frequency LFP activity in CA1 and ILC, EMG hypotonia and no EOG movements. Figs. 1 and 2 are the representative examples from NC and MS rats showing the presence of synchronized neural activities in CA1 and LA, EOG with rapid bursts of eye movements and EMG showing muscle atonia during different timings of rapid eye movement (REM) sleep. REM onset latency was calculated based on the appearance of the first episode of REM sleep from the beginning of sleep recording.

The percentage distribution of sleep stages was calculated from the total recording time that includes, SWS percentage (TSWS/total recording time); REM percentage (total REM sleep/total recording time); wake time percentage (total waking time/total recording time).

6. Quantification of the changes with local field potentials (LFPs)

The analysis starts with the LFPs recorded (CED analog-to-digital (A/D) converter (Cambridge, UK)) at 1 kHz sampling rate from the three sites. For the quantification, the longest first, middle and the last episode of REM was selected from which a 15 s duration was considered for further analysis. Mathematically, we model these LFPs as a multivariate autoregressive (AR) process of order p [19]. The optimal order p is obtained by using the Akaike Information Criterion [20]. The AR model is fitted to the data using the Levinson–Wiggins–Robinson algorithm [20]. Once the AR model is obtained, the 3×3 spectral density matrix for the process can be obtained directly from the model [21]. This matrix is a function of the frequency. The three diagonal elements of the spectral density matrix give the power spectrum of the three LFPs as a function of the frequency.

Using power spectral data for REM sleep, two major types of analysis were performed: (1) power of the theta frequency range measured from hippocampal, amygdalar and cortical LFP during REM sleep and (2) coherence level between brain regions.

6.1. Granger causality analysis

Granger causality analysis [21] is a leading statistical technique used to quantify causal influences between two time series. This has been used extensively to analyze both LFP [20,22,23] and spike train [24] data. Mathematically, given two time series (say X_t and Y_t), they can be jointly modeled as a realization of a bivariate stationary autoregressive process of order p [19]:

$$X_t = a_1 X_{t-1} + a_2 X_{t-2} + \dots + a_p X_{t-p} + b_1 Y_{t-1} + b_2 Y_{t-2} + \dots + b_p Y_{t-p} + \varepsilon_t$$

$$Y_t = c_1 X_{t-1} + c_2 X_{t-2} + \dots + c_p X_{t-p} + d_1 Y_{t-1} + d_2 Y_{t-2} + \dots + d_p Y_{t-p} + \eta_t$$

Where t denotes the discrete time, ε_t and η_t are the residual errors and the optimal model order p is determined using Akaike Information Criterion [20]. Alternatively, X_t and Y_t can be individually modeled as

$$X_t = \alpha_1 X_{t-1} + \alpha_2 X_{t-2} + \dots + \alpha_p X_{t-p} + \varepsilon'_t$$

$$Y_t = \beta_1 Y_{t-1} + \beta_2 Y_{t-2} + \dots + \beta_p Y_{t-p} + \eta'_t$$

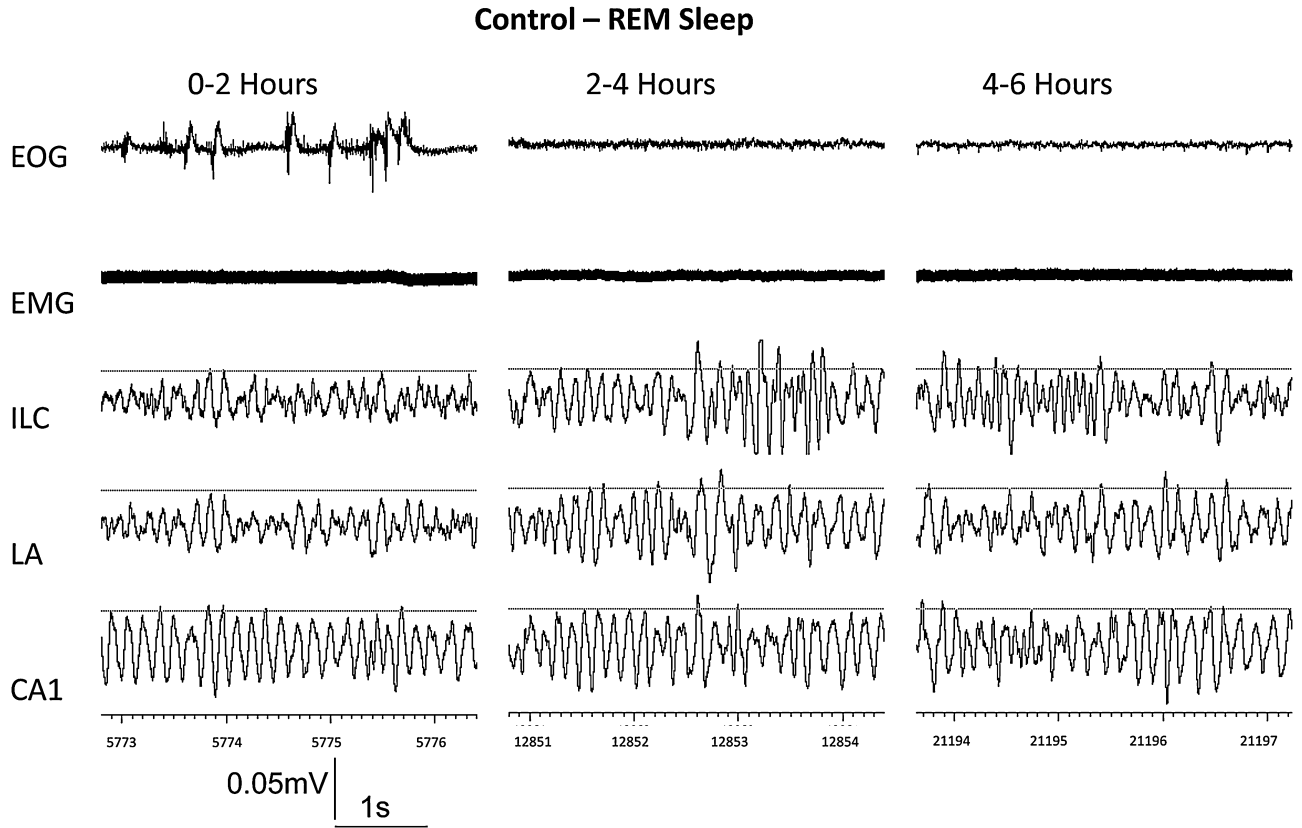


Fig. 1. Representative example of REM sleep showing the spontaneous activities in the ILC, LA and hippocampus (CA1 subregion) from normal control's at three different epochs in 6 h of sleep recording: 0–2 h, 2–4 h and 4–6 h. EOG, Electrooculogram; EMG, Electromyogram; ILC, Infra limbic Cortex; LA, lateral nucleus of amygdala; CA1 hippocampus; x-axis = 1 s; y-axis = 0.05 mV.

If $\text{var}(\varepsilon_t)$ is less than $\text{var}(\varepsilon'_t)$, Y is said to have a causal influence on X . Similarly, if $\text{var}(\eta_t)$ is less than $\text{var}(\eta'_t)$, X is said to have a causal influence on Y . This can be quantified as follows. The Granger causality from Y to X is given by $\ln[\text{var}(\varepsilon'_t)/\text{var}(\varepsilon_t)]$ and Granger causality from X to Y is given by $\ln[\text{var}(\eta'_t)/\text{var}(\eta_t)]$. This gives Granger causality in the time domain.

In the frequency domain, Granger causality from Y to X at a given frequency f can be computed as follows. The spectral power of X at frequency f can be written [25] as a sum of intrinsic power and causal power. Intrinsic power is the power from within the process X and causal power is the contribution from process Y . Granger causality $I_{Y \rightarrow X}(f)$ from Y to X at frequency f is then given by $\ln(\text{power}(f)/\text{intrinsic power}(f))$. A similar definition can be given for Granger causality from X to Y . In order to obtain an explicit expression for Granger causality, we again start with the bivariate autoregressive model, which can be expressed in matrix form as

$$\sum_{i=0}^p A(i)Z_{t-1} = E_t$$

where Z_t is the transpose of the row vector (X_t, Y_t) , E_t is the transpose of the error vector (ε_t, η_t) and $A(t)$ is the 2×2 matrix of coefficients at time t with $A(0)$ being given by the identity matrix. Let Σ denote the error covariance matrix of E_t . The 2×2 transfer function matrix $H(f)$ at a frequency f is given by $H(f) = (\sum_{i=0}^p A(i)e^{-i2\pi f})^{-1}$. Using Σ and H , we can define the 2×2 spectral density matrix $S(f)$ at frequency f as $S(f) = H(f)\Sigma H^*(f)$ where $*$ corresponds to Hermitian conjugate. In terms of S , the total power of the process X is given by $S_{11}(f)$. The intrinsic power from process X is given

by $S_{11}(f) - [\sum_{22} - \sum_{12}^2 / \sum_{11}] |H_{12}(f)|^2$. Hence Granger causality from Y to X at frequency f is given by

$$I_{Y \rightarrow X}(f) = \ln \left[\frac{S_{11}(f)}{S_{11}(f) - [\sum_{22} - \sum_{12}^2 / \sum_{11}] |H_{12}(f)|^2} \right]$$

A similar expression can be obtained for Granger causality from X to Y . The Granger spectrogram is obtained by sliding a short window of fixed length (5 s) across the data and computing Granger causality for each time window.

6.2. Statistics

All the numerical data were presented as mean $\pm$ standard error of the mean (SEM). The statistical analysis was performed using Graph pad Prism software (Graph pad Software Inc.). The data was analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc tests, Mann–Whitney test and Student's t -test.

7. Results

7.1. Spontaneous behavioral assessment in adulthood

The behavior of the MS and NC were recorded and monitored continuously for 30 min in the rat familiar home cage. Spontaneous behavioral data were classified as exploratory locomotor and quiet behaviors. Exploratory locomotor behaviors were referred to as exploratory walking and rearing, while, quiet behavior were immobility and grooming in the rat home cage. Statistical analysis with Student's t -test showed a significant difference between groups

MS – REM Sleep

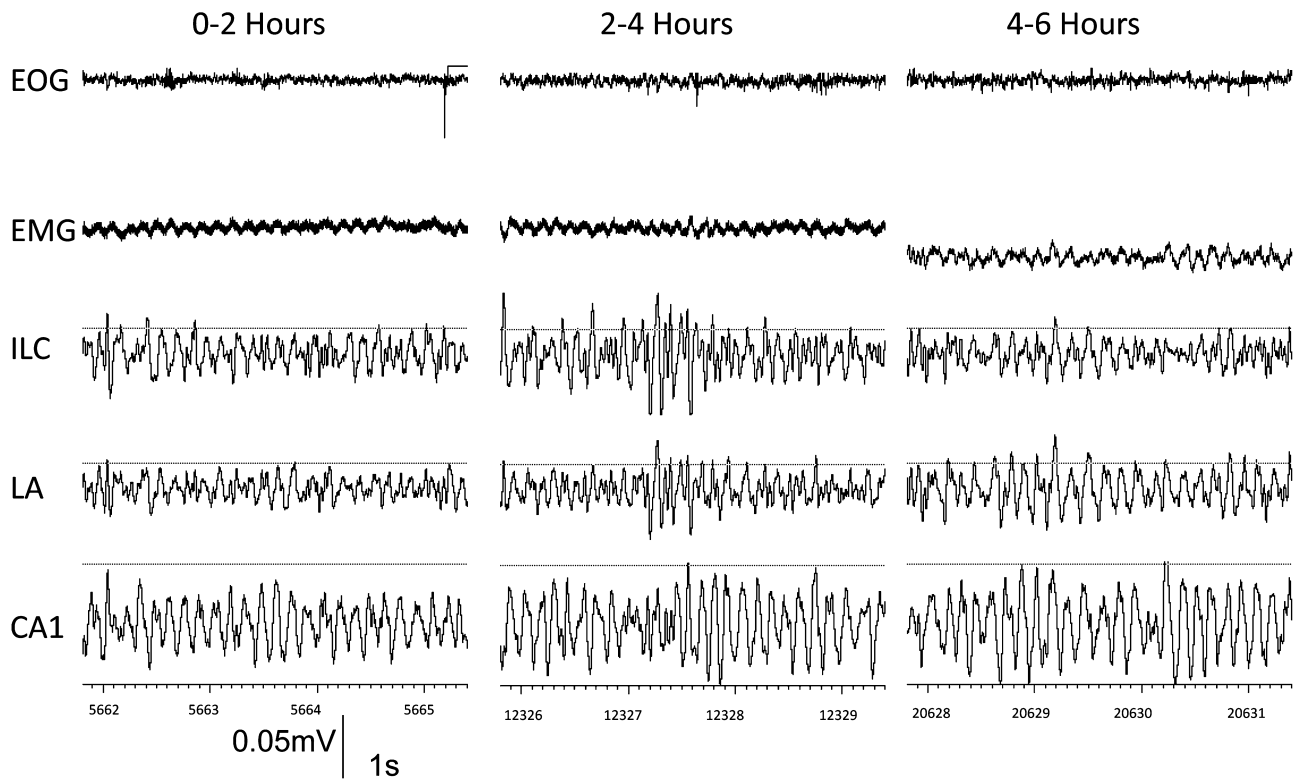


Fig. 2. Effect of MS on paradoxical sleep in the ILC, LA and hippocampus (CA1 subregion) as depicted by representative example, at three different epochs in 6 h of sleep recording: 0–2 h, 2–4 h and 4–6 h. EOG, Electrooculogram; EMG, electromyogram; ILC, Infra limbic Cortex; LA, lateral nucleus of amygdala; CA1 hippocampus; x-axis = 1 s; y-axis = 0.05 mV.

($t_{1,23} = 2.777$; $p < 0.01$) with significant reduction in exploratory locomotion and increase in quiet behavior ($t_{1,23} = 2.777$; $p < 0.01$) when examined in rat home cage (Fig. 3).

7.2. Effects of MS on differential auditory fear conditioning

We investigated the effect of early maternal separation and isolation stress on fear conditioning in adult male Wistar rats. Fig. 4 illustrates the impact of MS on acquisition and retention of classical fear conditioning. Fig. 4 (a) showing outline of differential fear conditioning. During the habituation, both NC and MS rats did not

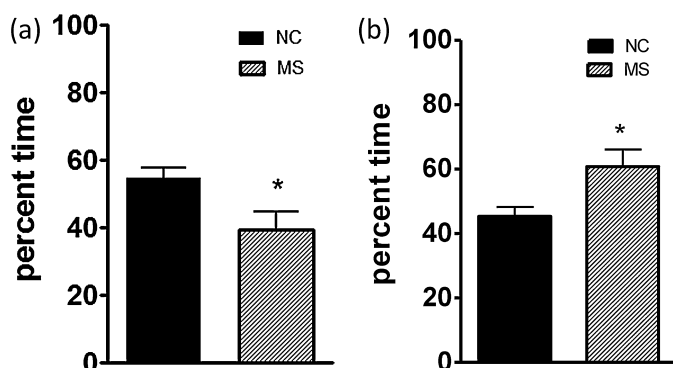


Fig. 3. Behavioral data of the continuous 30 min monitoring in rat home cage. The significant effect of MS stress on exploratory behavior in rats was observed. MS rats showed remarkable reduction in exploratory locomotion and (b) increased quiet behavior (* $p < 0.01$) in the familiar rat home cage. Data represented as Mean $\pm$ SEM from NC ($n = 16$) and MS ($n = 8$). * $p < 0.01$ indicate significant comparisons between NC and MS using t -test. NC, normal control; MS, maternal separation stressed group.

exhibit the spontaneous fear in the fear conditioning chamber. After 24 h of fear conditioning, NC rats did not exhibit significant increase in freezing to the unpaired CS–. But there was a robust increase in the conditioned freezing to the paired CS+, indicating tone-specific fear memory by NC rats ($F_{9,72} = 4.642$, $p < 0.0001$) (Fig. 4 (b)). MS rats, on the other hand, showed significantly higher percentages of freezing behavior before and during the presentation of CS– and CS+ ($F_{17,136} = 12.97$, $p < 0.0001$) (Fig. 4 (b)).

The retention of fear memory was further compared between stimulus conditions using Tukey's multiple comparison test which indicated that the percent freezing behavior of NC rats showed a robust increase in freezing behavior to CS+ and not to CS– when compared to pre-tone conditions ($p < 0.05$). MS group of rats on the other hand, exhibited increased freezing behavior to all tone trials of CS– and CS+ ($p < 0.05$) as compared to pre-tone conditions (Fig. 4 (b)). The increase in freezing to both specific and non-specific stimulus by MS rats is most likely due to the early life stress causing increased fear generalization.

Comparisons between groups were made to study the effect of early MS stress on fear memory to specific and non-specific stimulus in rats. Statistical analysis using Student's t -test revealed that MS rats showed significant increase in freezing behavior during pre-tone conditions ($t_{26} = 2.592$, $p < 0.01$). In addition, the differences in freezing behavior to CS– and CS+ was analyzed and we observed significant differences between groups (2CS– ($t_{26} = 2.111$, $p < 0.04$), 3CS– ($t_{26} = 2.857$, $p < 0.01$), 4CS– ($t_{26} = 2.592$, $p < 0.003$), 1CS+ ($t_{26} = 2.776$, $p < 0.01$), 2CS+ ($t_{26} = 2.531$, $p < 0.01$), 3CS+ ($t_{26} = 3.104$, $p < 0.004$) and 4CS+ ($t_{26} = 3.137$, $p < 0.004$)) (refer to Section 2 for the details).

Finally, the freezing data was compared with the home cage behavior, exploratory locomotion per se. The results showed

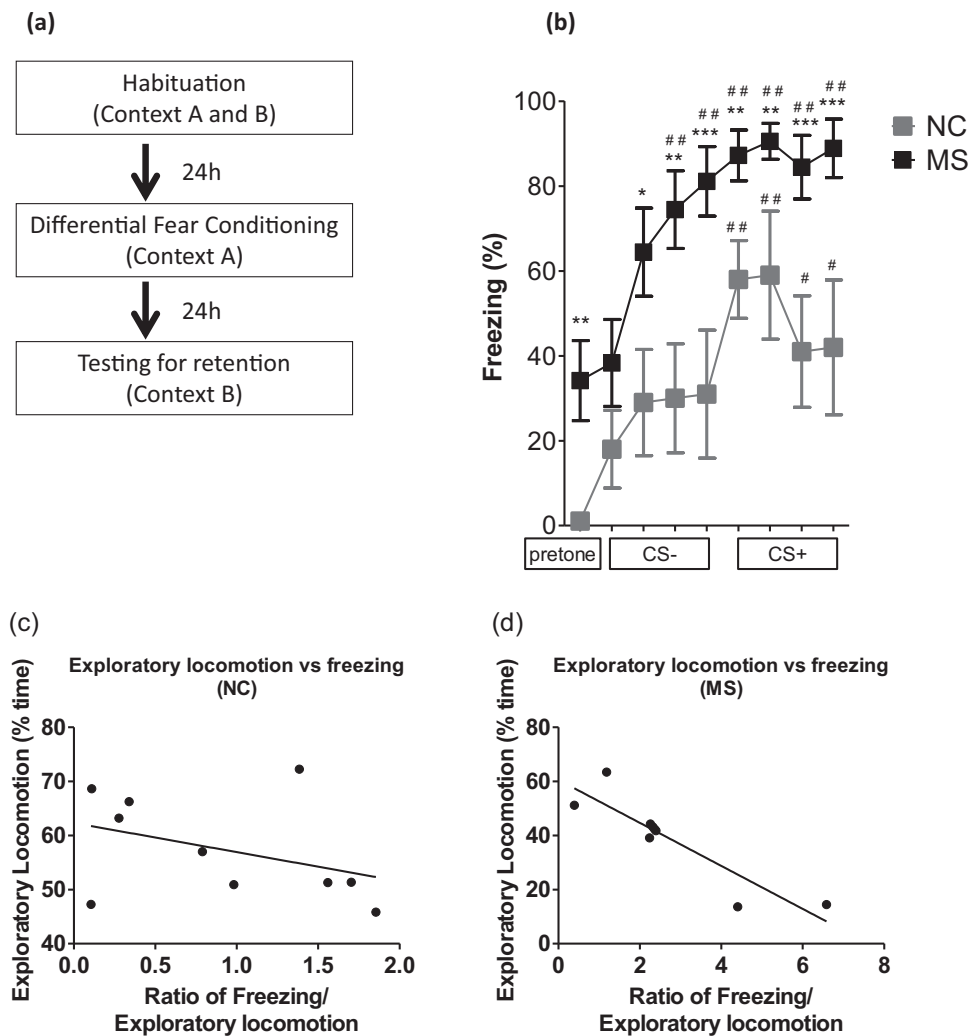


Fig. 4. Freezing to specific and non-specific stimulus after 24 h of classical fear conditioning. (a) Outline of the differential fear conditioning protocol; (b) mean change in freezing behavior to the pre-tone, CS– and CS+ from NC ($n = 10$) and MS ($n = 18$) rats. The spontaneous recovery with reduced freezing after receiving two CS+ was seen in NC, but similar recovery was not observed in MS rats. (c) Histogram representing correlation between freezing and exploratory locomotion in NC and (d) MS group. Data represented are Mean $\pm$ SEM. * $p < 0.04$; ** $p < 0.01$; *** $p < 0.003$ are in comparisons to NC; ## $p < 0.01$; # $p < 0.05$ compared with pre-tone conditions following Tukey's multiple comparison test. r^2 (NC) = 0.15, r^2 (MS) = 0.81. NC, normal control; MS, maternal separation stressed group.

negative correlation between freezing and exploratory locomotion in NC ($r^2 = 0.15$) and MS ($r^2 = 0.81$) rats (Fig. 4c and d).

8. The effect of MS stress on immediate fear extinction

In order to measure the effect of MS stress on the immediate fear extinction, repeated measures of ANOVA was used. The repeated presentation of CS+ during retention testing on fear behavior in NC rats showed increased freezing behavior to the first two CS+ ($p < 0.001$) and gradual reduction in freezing behavior to the subsequent third and fourth CS+ ($p < 0.05$), respectively (Fig. 4(b)). MS rats, on the other hand, failed to show immediate fear extinction as revealed by a persistent increase ($p < 0.001$) in freezing behavior until completion of all the four CS+ (Fig. 4(b)).

8.1. Three days of MS during SHRP not only increased REM sleep but also the total duration of sleep

In addition to fear behavior, the present study also examined the impact of MS on the sleep architecture because repeated physical stressors in adulthood are known to have a predominant effect on rapid eye movement sleep (REM) [8]. Similar changes were

observed in MS group when compared to NC rats as shown with significant increase in REM sleep ($t_{1, 14} = 3.289$; $p < 0.01$) and reduced quiet wake period ($t_{1, 14} = 6.263$; $p < 0.001$) (Fig. 5(a)). The changes in total REM sleep duration were associated with increased number of episodes and not the duration alone. The study also showed a significant decrease in the total duration of wake time ($t_{1, 14} = 2.920$; $p < 0.01$) and significant increase in total sleep time ($t_{1, 14} = 3.064$; $p < 0.008$) (Fig. 5(b)).

8.2. The maternal separation stress during SHRP did not produce significant impact on amplitude of local field potentials of CA1, LA and ILC during REM sleep

Neuronal synchronization in different frequency bands has been previously described in many brain regions during REM sleep. Moreover, the absence or disruption of synchrony between brain areas during REM sleep has been reported in rodent models of cognitive dysfunction [26]. Thus, we examined whether the enhanced theta-gamma frequency bands were accompanied by an increased phase synchrony by means of waveform correlation analysis between CA1, LA and ILC.

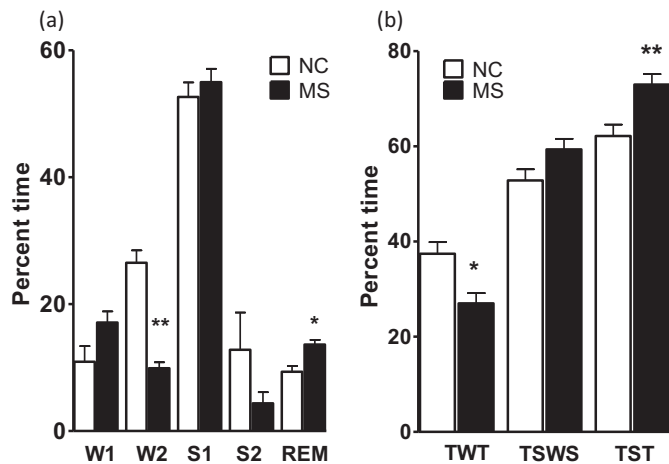


Fig. 5. The effect of MS on sleep architecture. (a) MS during SHRP had significant impact on sleep showing significant (a) reduction in quiet wake (W2) and increase in REM sleep period; (b) reduced total wake time (TWT) and increased total sleep time (TST). Data represented are mean $\pm$ SEM from NC ($n=6$) and MS ($n=8$) rats $^{**}p < 0.001$; $^{*}p < 0.01$.

MS-induced increase in theta–gamma power raises the possibility that these oscillations within the amygdala–hippocampal–infralimbic cortex network may be coupled. Indeed, a previous study has reported bidirectional coupling between the hippocampus, amygdala and infralimbic cortex in the theta frequency bands during sleep [27]. Therefore, we examined how stress modulates the directionality of these oscillations across the LA–CA1–ILC network using Granger-Causality analysis [21] and [25]. This analysis can be performed in the frequency domain and already has been used in previous studies in rats to analyze the directionality of interactions between various brain areas under different behavioral conditions [28] and [29]. The Granger spectral causality within 0–50 Hz was computed using a bivariate autoregressive model among all pairs of structures (see Section 2).

The effect of MS on the averaged absolute power of LFP obtained from ILC and CA1 region of the hippocampus during REM sleep is shown in Fig. 6. We observed a significant reduction in theta power (6–12 Hz) of ILC in Epoch 2 (2–4 h) (Fig. 6(a)). However, there was no significant change in CA1 power and synchronous activity in this 2–4 hour epoch. The absolute power of ILC across the 3 epochs showed a significant reduction at 2–4 h (Fig. 6(b)).

The effect of MS on REM sleep pattern is represented in Fig. 7. MS enhances directional coupling from CA1 to ILC. There was a dominant directional influence from CA1 to ILC in theta (6–12 Hz)

and beta (12–20 Hz) bands (Fig. 7 (a)). We did not observe any significant change in the averaged absolute power of LFP obtained from CA1 (Fig. 7(b)) and ILC region (Fig. 7 (c)) during REM sleep. However, MS enhanced coherence level in theta frequency, but decreases it in beta and gamma frequency between ILC and CA1 in epoch 3 (4–6 h) (Fig. 7(d)), suggestive of increased synchronization at theta frequency relative to others.

Fig. 8 shows that MS enhances directional coupling from CA1 to ILC in a time dependent manner, whereas in control the directional coupling decreases significantly with time. The Granger Causality values in theta bands (6–12 Hz) across the 3 epochs depicts a temporal decrease in directional coupling from CA1 to ILC in theta (6–12) bands at late sleep epoch 3 (4–6 h) (Fig. 8(a)). Coherence data averaged across the 3 epochs in ILC versus CA1 in control and MS rats showed that the synchronous activity in the theta frequency decreased with time in controls compared to MS between ILC and CA1 in epoch 3 (4–6 h) (Fig. 8(b)).

MS enhances directional coupling from LA to ILC as shown in Fig. 9. Population averages of Granger Causality values (Mean $\pm$ SEM) across the frequency bands in epoch 3 (4–6 h) showed a dominant directional influence from LA to ILC in theta (6–12 Hz) and beta (12–20 Hz) bands (Fig. 9(a)). We observed that MS augments directional coupling from LA to ILC in a time dependent manner, whereas in control's the directional coupling decreases significantly with time, suggesting that there is an increased flow of information from LA to ILC in rats subjected to MS in a time dependent manner (Fig. 9(b)). However the ILC $\rightarrow$ LA coupling remained unaffected.

Fig. 10 represents increased directional coupling from CA1 hippocampus to LA in MS rats as represented by Granger Causality values across the frequency bands in epoch 3 (4–6 h). Our data suggests that there is a dominant directional influence from hippocampus to amygdala in theta (6–12 Hz) bands (Fig. 10(a)). Population averages of Granger Causality values (Mean $\pm$ SEM) in theta bands (6–12 Hz) across the 3 epochs. Note the temporal change in directional coupling from CA1 to LA in theta (6–12 Hz) bands at epoch 3 (4–6 h). The LA $\rightarrow$ CA1 coupling remained unaffected across the 3 epochs (Fig. 10(b)).

8.3. Directional coupling from LA and CA1 to ILC is enhanced in late REM sleep phase

In the present study, the changes in neuronal activity in CA1 hippocampus, Lateral nucleus of amygdala and infralimbic cortex were studied during rapid eye movement sleep. Secondly, this study is different from previous study (14) because the study looked into changes in neuronal activities during three stages of sleep—first

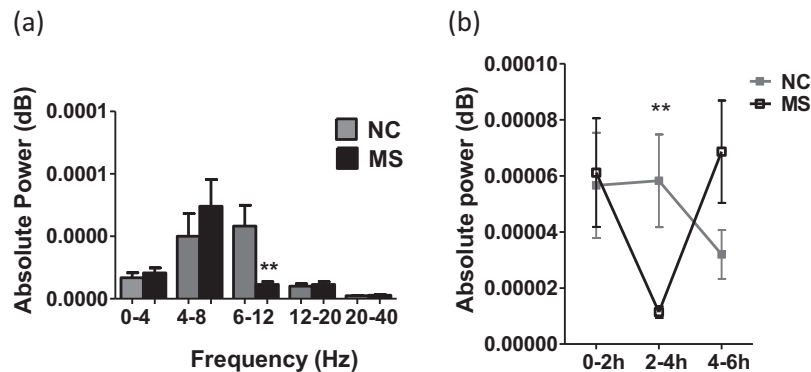


Fig. 6. Effect of MS on theta–gamma oscillations in infralimbic cortex. (a) Averaged absolute power of LFP obtained from ILC during REM sleep at different frequencies. Note the significant reduction in theta power (6–12 Hz) of ILC in Epoch 2 (2–4 h). (b) Represents the absolute power of ILC during REM sleep at 6–12 Hz across the 3 epochs. Data represented as Mean $\pm$ SEM from NC ($n=5$) and MS ($n=7$), $^{**}p < 0.007$ when compared to NC.

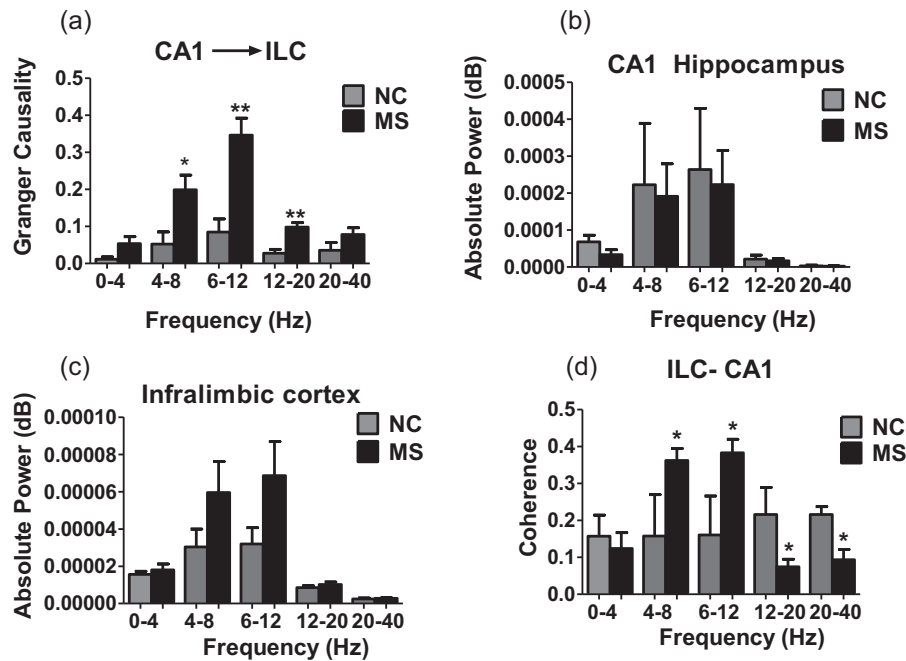


Fig. 7. Averaged data represents the effect of MS on neuronal activities during REM sleep. (a) MS enhances directional coupling from CA1 Hippocampus to ILC. Note the dominant directional influence from CA1 to ILC at theta (4–8 Hz and 6–12 Hz) and beta (12–20 Hz) frequency bands. (b) and (c) shows the averaged absolute power of LFP obtained from CA1 and ILC region during REM sleep respectively. (d) Represents MS-induced changes in coherent activities between ILC and CA1 region during REM sleep. MS enhances synchronous activity in the theta frequency, but decreases it in beta and gamma frequency in epoch 3 (4–6 h). Data represented as Mean $\pm$ SEM from NC ($n=6$) and MS ($n=8$), * $p<0.05$, ** $p<0.01$.

longest episode (0–2 h of sleep), the middle stage of sleep (2–4 h of sleep) and late stage of sleep (4–6 h) (figures in Section 2).

The theta synchronization from CA1 hippocampus and LA to ILC increased gradually as the sleep progresses. The increase in directional flow of information from CA1 hippocampus and LA (Fig. 10(b)) to ILC was observed at 4–8 Hz ($t_{1,9}=2.119$; $p<0.05$) and 6–12 Hz ($t_{1,9}=3.293$; $p<0.01$) (Fig. 8(b)) frequency range and LA to ILC at 4–8 Hz ($t_{1,9}=2.041$; $p<0.05$) and 6–12 Hz ($t_{1,9}=2.114$; $p<0.01$) in late REM sleep. The increase was not significant in the initial phase of REM sleep (Fig. 8(b) and Fig. 10(b)). The coherence analysis, on the other hand, indicated an increased correlation

between ILC and CA1 at 4–8 Hz ($t_{1,9}=2.485$; $p<0.05$) and 6–12 Hz ($t_{1,9}=2.615$; $p<0.05$) frequency range, but reduced correlation at 12–20 Hz ($t_{1,9}=2.664$; $p<0.05$) and 20–40 Hz ($t_{1,9}=2.501$; $p<0.05$) in MS rats when compared to NC (Fig. 8(a) and (b)). However, CA1 to ILC showed increased flow of information at 12–20 Hz range ($t_{1,9}=3.411$; $p<0.01$) (Fig. 8(a)). Based on these findings, it is worthwhile discussing how these brain regions interact during REM sleep.

9. Discussion

The present study has demonstrated the importance of maternal care during SHRP on the sleep and fear memory. Here MS during SHRP has significantly increased REM sleep, increased fear memory and variations in neuronal activities in the hippocampus, amygdala and medial prefrontal cortex during REM sleep. Determining the neural replay in REM sleep has facilitated our understanding of the learning-related functions of REM sleep.

9.1. MS stress and fear memory

Several studies from the literature indicate that the hippocampus matures relatively early in postnatal life, whereas the prefrontal cortex, which is important for the development of an increased memory span matures much later [2]. Although many neural networks of memory develop early in postnatal life, it is not yet known when memory system is fully matured. One hypothesis that we proposed was that the maturation of such memory systems probably depends on the anatomical and functional connectivity of the hippocampus with different cortical regions during SHRP. Hence, the basic assumption in the present study was that MS during this critical period would interfere with the normal connectivity with other brain structures leading to impaired cognitive processes as revealed by increased fear memory to a non-specific stimulus. Supporting

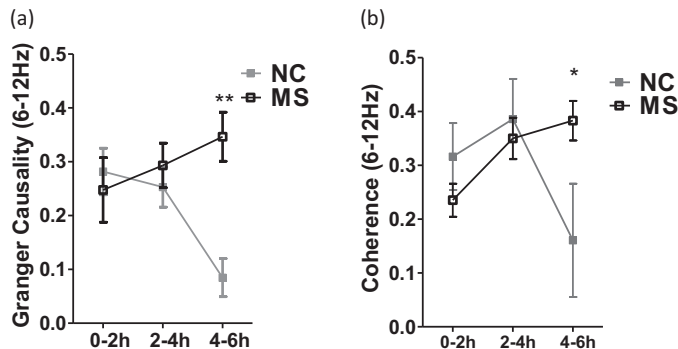


Fig. 8. The neural activities of ILC and CA1 hippocampus during REM sleep. (a) MS enhances directional coupling from CA1 hippocampus to ILC in a time dependent manner, whereas in controls, the directional coupling decreases significantly with time. Population averages of Granger Causality values (Mean $\pm$ SEM) in theta bands (6–12 Hz) across the 3 epochs. Note the temporal decrease in directional coupling from CA1 to ILC in theta (6–12) bands at epoch 3 (4–6 h). (b) MS has also enhanced theta activities between ILC and CA1 region as the sleep advances. Coherence data averaged across the 3 epochs in ILC versus CA1 in control and MS rats. The synchronous activity in theta frequency decreased with time in controls compared to MS between ILC and CA1 in epoch 3 (4–6 h). Data represented as Mean $\pm$ SEM from NC ($n=6$) and MS ($n=8$), Mann Whitney test: * $p<0.05$, ** $p<0.01$.

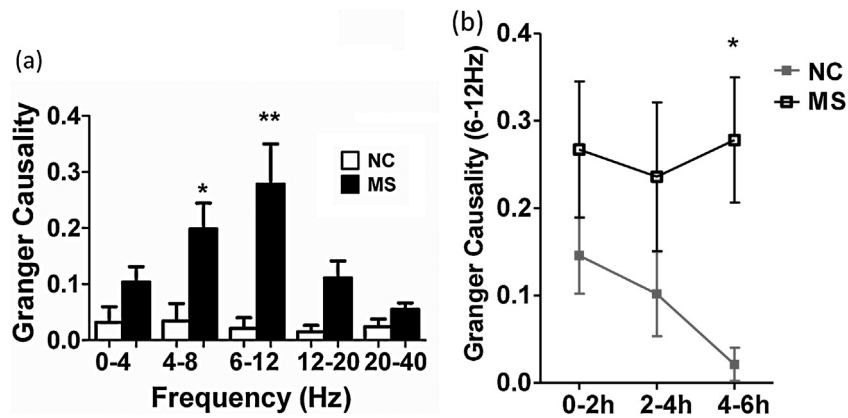


Fig. 9. Neural activities in LA and ILC during REM sleep. (a) MS enhances directional coupling from LA to ILC. Population averages of Granger Causality values (Mean ± SEM) across the frequency bands in epoch 3 (4–6 h). Note the dominant directional influence from LA to ILC in theta (6–12) and beta (12–20) bands. (b) MS enhances directional coupling from LA to ILC in a time dependent manner, whereas in control the directional coupling decreases significantly with time. Population averages of Granger Causality values (Mean ± SEM) in theta bands (6–12 Hz) from NC ($n=6$) and MS ($n=8$) across the 3 epochs. Note the temporal decrease in directional coupling from LA to ILC in theta (6–12) bands at epoch 3 (4–6 h); * $p < 0.05$; ** $p < 0.01$.

evidence from the literature also suggest that increased contextual fear memory in rats experienced MS during SHRP showed increased amygdala c-Fos expression and ACTH release; reduced adrenal corticosterone secretion [30]. These results were attributable to the changes in priming of amygdala fear pathway with lasting consequences for behavioral and endocrine fear-responsiveness.

In the present study, we have demonstrated that maternal separation and isolation stress during SHRP impaired the acquired fear memory retrieval. Here, MS rats showed increased fear memory retrieval as indicated by an increased freezing in a fear conditioning chamber. In both MS and NC rats, conditioning was more specific to the paired tone which was paired with the foot-shock, as the unpaired tone elicited less freezing and paired tone showed increased freezing. However, MS rats showed more freezing to the conditioning chamber during no-tone period as well. The effects of MS on conditioned freezing were further associated with home cage behavior. It was observed that MS rats showing less exploration in its home cage exhibited more freezing to the conditioning environment. Our results were comparable to the previous studies in which maternal separation stress during the early post-natal period impairs the fear conditioning suggesting that it is independent of timing and duration of maternal separation stress

[31]. Thus, these results indicate that fear memory retrieval depends on rearing related stress during SHRP.

9.2. The dynamic processing of hippocampal-amygdala-cortical neural activities during REM sleep provides a starting point to understand the impact of stress during SHRP on fear memory

Second hypothesis was that changes in fear memory in MS rats could be due to the changes in neural synchronization generated by hippocampal-amygdala-cortical loops. Since most of the psychiatric diseases are surfaced either in pubertal [32] or adult life [33], it becomes essential to delineate the kind of stressful experiences they underwent during the early years. In the present study, we tried to understand the neural processing involved in the impairment of cognitive functions by looking into the complex connectivity of the hippocampus with the amygdala and prefrontal cortex.

It is well documented that theta frequency in the hippocampal formation changes with age, psychiatric diseases (Schizophrenia, depression) and it was well correlated with maturation of neural connectivity [34]. The increase in theta power in amygdala and hippocampus during fear memory retention means increased discharge of neurons synchronously [35]. Previous findings from

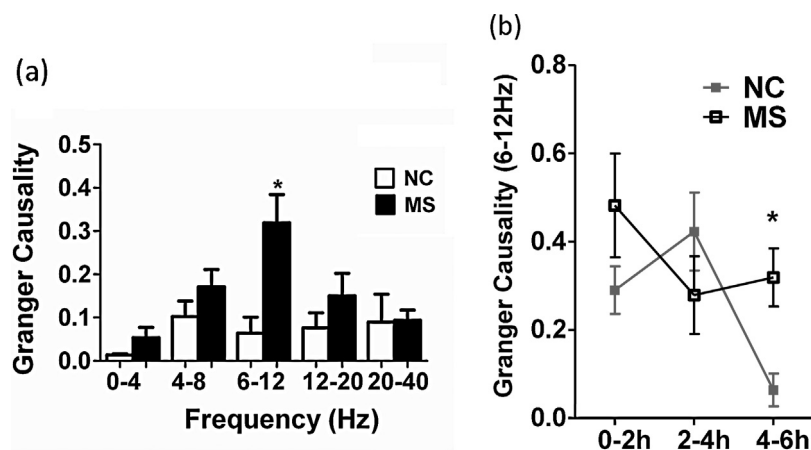


Fig. 10. (a) MS enhances directional coupling from CA1 hippocampus to LA. Population averages of Granger Causality values (Mean ± SEM) across the frequency bands from NC ($n=3$) and MS ($n=8$) in epoch 3 (4–6 h). Note the dominant directional influence from CA1 to LA in theta (6–12 Hz) bands; (b) Population averages of Granger Causality values (Mean ± SEM) in theta bands (6–12 Hz) across the 3 epochs. Note the temporal change in directional coupling from CA1 to LA in theta (6–12) bands at epoch 3 (4–6 h); * $p < 0.05$.

our laboratory showed a direct correlation between increased REM sleep in stressed rats with enhanced theta synchronization in LA and CA1 hippocampus [8].

In the present study, the theta synchronization in amygdalo-hippocampal-prefrontal cortical loops during late phase of REM sleep in MS rats was characterized by: (1) A large increase in unidirectional flow of information from CA1 hippocampus to LA and ILC, LA to ILC at narrow and broad range of theta frequency ranges (4–8 Hz and 6–12 Hz), whereas CA1 hippocampus to ILC showed increased Granger causality ratio at 12–20 Hz and 20–40 Hz reflect an increase in encoding of new information to pre-existing stores. (2) Increased synchronization between ILC and CA1 hippocampus was also seen at a narrow and a broad range of theta frequency (4–8 Hz and 6–12 Hz) with reduced synchronization at 12–20 Hz and 20–40 Hz. This could be related to increased theta synchronization between ILC and CA1, due to increased interactions between these regions during sleep. Enhanced REM sleep together with altered neural activities, such as increased unidirectional flow of information from the amygdala and hippocampus to the ILC could be attributed to the early exposure to stressful environments during SHRP.

REM sleep is an active process [36] and when it predominates, cortisol levels are consistently enhanced due to the hyperactivity of HPA-axis [37] and enhanced amygdala-dependent emotional memory processes [38]. The enhanced corticosterone during REM sleep [39] in such conditions results in the up-regulation of glucocorticoid receptor activation [40] in the amygdala [41] leading to increased fear memory retention processes. Next question raises as to what extent the cortisol feedback [42] during early and late sleep contribute to the emotional memory formation. We found that interactions between the infralimbic cortex, hippocampus and amygdala neurons increased as the sleep progresses. Specifically, a significant enhancement in neuronal interactions was found in late REM sleep at theta frequency range. The functional outcome of these findings is reflected with increased synchronization between ILC and CA1 at theta frequency range. Thus suggesting that increased REM sleep in mammals is an essential function as reactivation of the hippocampal-cortical circuitry in which older information is integrated with the new experience of the organism [43]. The heightened activity in these cortical loops may be a causative factor for the increased fear generalization [44] during the fear retrieval process in a fear conditioning task.

To summarize, the study revealed increased REM sleep associated with enhanced theta synchronization in hippocampal-amygdala-cortical loops in MS rats that provide an ideal niche for understanding the dynamicity of pre-existing information processing to a generalized fear memory. These observations may also form the basis to understand how new information is processed, encoded and reactivated during REM sleep to form a long term memory.

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