Extinction of Contextual Fear with Timed Exposure to Enriched Environment: A Differential Effect

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Abstract

Background: Extinction of fear memory depends on the environmental and emotional cues. Furthermore, consolidation of extinction is also dependent on the environmental exposure. But, the relationship of the time of the exposure to a variety of environmental cues is not well known. The important region involved in facilitation of extinction of fear memory is through diversion of the flow of information leaving the lateral nucleus of amygdala. **Purpose:** The study aimed to address a question to explain how these brain regions react to environmental stimulation during the retention and extinction of fear memory. **Methods:** An enriched environment (EE) is assumed to mediate extinction of fear memory, we examined the apparent discrepancy between the effects of defensive response, the freezing behavior induced by Pavlovian classical fear conditioning by subjecting them to variance in the timing to EE. The different timing of EE exposure was 10 days of EE either before fear conditioning and/or after extinction training to the rats. The local field potentials was recorded from CA1 hippocampus, lateral nu-

cleus of amygdala and infralimbic region of medial prefrontal cortex (mPFC) during the fear learning and extinction from the control rats and rats exposed to EE before and after fear conditioning. **Results:** Exposure to EE before the fear conditioning and after extinction training was more effective in the extinction fear memory. In addition, we also found switching from exploratory locomotion to freezing during retention of contextual fear memory which was associated with decreased theta power and reduced synchronized theta oscillations in CA1-hippocampus, lateral nucleus of amygdala, and infralimbic region of mPFC. **Conclusion:** Thus, we propose that the timing of exposure to EE play a key role in the extinction of fear memory.

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Introduction

Experiences play a major role in determining the ability to cope up with stressful conditions and also vulnerability to ailment [1, 2]. Positive experiences like exposure to enriched environment (EE) which provides increased sensory, social, cognitive, and physical stimulation increases the ability to overcome stress [3]. Also, neurocognitive functions such as spatial learning and fear condi-

tioning abilities are enhanced in EE-reared rats compared to those reared in standard housing (SH) conditions [4–13]. Enhanced neurocognitive functions are supported by the positive changes induced by EE and physical activity, such as the strengthening of synaptic connections and synaptic plasticity and induction of robust neurogenesis [14, 15]. EE exposure may also be neuroprotective, providing protection against insult [16], enhancing post-insult recovery in preclinical models [17] and in human clinical conditions [18]. EE thus affects the structural and functional properties of neurons in the brain to increase the cognitive power. However, our understanding of the effect of EE on connectivity between brain regions is rudimentary. Studies have shown that EE can influence brain plasticity in the hippocampus [19], amygdala [20], and prefrontal cortex [21]. However, these studies have failed to provide information about neuronal oscillations which have functional relevance to the behavior of animals.

Pavlovian fear conditioning is one of the models to study fear memory and its extinction. The role of EE on fear memory and its extinction is well-known [6, 22]. However, the fundamental question is whether interposed exposure to EE such as EE after extinction, EE before and after extinction training on fear memory extinction has not been addressed so far. This apparent discrepancy may be important in understanding the neural mechanisms underlying the environmental modulation of implicit memory. We thus set out to characterize the interactions between EE and their influence on extinction of fear memory.

The synaptic plasticity associated with memory processing was assessed with local field potential (LFPs) recorded from the hippocampus, amygdala, and prefrontal cortex. LFPs are used extensively in identifying alterations in brain network synchrony during learning processes. Evidence from animal studies suggests that Type 1 (6–12 Hz) theta in the hippocampus is associated with voluntary movement and Type 2 with the immobile state [23, 24]. During learning process of a spatial task, the internal representation of the spatial maps would produce an increment in relative power (RP) of CA1 hippocampal neurons in theta frequency range [25]. The role of theta power in fear learning and memory is also supported by the observed increased synchronization between hippocampus and amygdala [26]. In addition, synchronized theta oscillations have also been correlated with arousal and attentional mechanisms [27]. Medial prefrontal cortex (mPFC) which bridges information between limbic and cortical structures plays an important role in the regulation of fear memory facilitating extinction of fear memory [28].

Thus, it is evident that neuronal activities in the hippocampus, amygdala, and mPFC are modulated during learning process. In the present study, we addressed a question to explain how these brain regions react to environmental stimulation during the retention and extinction of fear memory. The hypothesis is that exposure and timing of EE may play a key role in modulating the brain circuitry leading to rapid learning. The role of EE on learning can be evaluated based on time spent in freezing to contextual stimuli. The difference in behavioral responses along with changes in the synchronized theta oscillations in the hippocampus, amygdala, and mPFC enabled the study in understanding cellular processes involved in memory consolidation. In addition, re-exposure to EE was also used as interference during consolidation of fear memory in advancing the extinction of fear memory.

Methods

Experimental Animals

Adult male Wistar rats (60–70 days old) were used in the present study. The animals were housed in polypropylene cages, allowed ad libitum access to food and water, and were maintained on a 12:12-h light/dark cycle. All experiments were carried out in accordance with the guidelines of the Central Animal Research Facility, at the National Institute of Mental Health and Neurosciences (NIMHANS), Bengaluru. Experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC; AEC/30/194/N.P).

Total of 56 rats was used for the behavioral study. The rats were divided randomly into 3 groups: (1) control rats (SH-SH) reared in SH conditions ($n = 16$); (2) enriched environmental (EE-EE) rats, exposed to EE before fear conditioning and with re-exposure after extinction training ($n = 16$); and (3) SH-EE exposed to EE only after extinction training ($n = 24$).

Housing Conditions

SH Conditions

Rats were placed in standard cages (Fig. 1a) measuring $22.5 \times 35.5 \times 15$ cm (SH condition in groups of 3).

EE Conditions

Rats were placed in groups of 10–12, in special cages measuring $102 \times 64 \times 61$ cm (Fig. 1b). EE cages were equipped with various objects such as ladders, tunnels, wooden pieces of different shapes and sizes, interchangeable toys such as balls, building blocks, etc., to provide opportunities for both sensory and physical stimulation. These objects were rearranged every day and different objects were placed in the cages on alternate days for the novelty [29]. Food and water were provided ad libitum. EE exposure was 6 h/day for 10 days. The exposure to EE was before fear conditioning, with re-exposure after extinction training (EE-EE) and single exposure, after extinction training (SH-EE) across the behavioral paradigm. All the behavioral assessment was carried out during the light phase.

Fig. 1. Experimental design: **a** rats reared in standard housing (SH); **b** rats reared in enriched environmental conditions; **c** Context A and Context B; **d** experimental design; **e** experimental protocol for extinction of contextual fear conditioning. pre, preconditioning housing conditions; H, Habituation; FC, contextual fear conditioning; R, retention test; E, extinction training; ET, extinction retention test.

Fear Conditioning Task

Contextual fear conditioning was performed in adult rats (60–70 days old). Contextual fear conditioning protocol followed with slight modifications from the experimental design of a previous study [30]. The protocol was modified in the strength and the duration of the unconditioned stimulus (US), inter-stimulus interval, observation period, and the context design (Fig. 1e) which is detailed below. The entire behavioral procedure involved 4 phases: habituation to 2 different, but similar contexts (Context A and Context B; Fig. 1c); fear conditioning in Context A; extinction training for the extinction of fear memory in Context A and Context B, to examine the generalization transfer of training between contexts [31] and fear extinction in Context A and Context B.

Conditioning Apparatus

The 2 different conditioning chambers were used in both Context A and Context B (Fig. 1c). The conditioning chamber consisted of a chamber (25 × 29 × 28 cm; Coulbourn Instruments) with aluminum and Plexiglas walls and a grid floor with 0.5 cm steel bars spaced at 1.8 cm. In addition, video camera was mounted on the ceiling and tone generator on the sidewalls.

Context A: the chamber was in an isolated room where the lights were turned off and separated from the observers. The conditioning chamber was provided with bright illumination by a sin-

gle overhead light (60 lux) connected to a shock scrambler and generator. The chamber was cleaned with 70% alcohol before placing the rat inside. The floor of each chamber was covered with paddy husk which was used as bedding material (Fig. 1c).

Context B: the conditioning chamber was the same as that used in Context A with overhead light off. The conditioning chamber was placed in a separate room (Context B) with ventilation fans, lights (60 lux), and without white noise. The chamber was cleaned with 70% alcohol solution between each rat before habituation, conditioning, and extinction training/test. The floor of each chamber was covered with paddy husk which was used as bedding material (Fig. 1c).

Behavioral Procedure

Contextual fear conditioning was performed in adult male Wistar rats by placing them individually in the chamber during the different stages of fear learning and extinction (Fig. 1d).

Habituation: in order to habituate the rats to specific and non-specific contextual stimuli, rats were allowed to explore the chamber twice (10 min each), 1 day before training. Habituation was carried out in both Context A and Context B.

Conditioning: during training for the contextual fear conditioning (that is, 24 h after habituation), rats were placed in Context A and allowed to explore the environment freely for initial 2 min

following this, 3 US (electric foot-shocks of 1 mA, 1 s duration) were given with an inter-stimulus interval of 20 s. After the presentation of the last US, the rats were retained in the chamber for another 2 min before returning them to their home cages. The training for the contextual fear conditioning was repeated after 6 h.

Retention

Twenty-four hours after fear conditioning, rats were placed in the retrieval environment (Context B) for 30 min without the US for assessment of fear learning by measuring the contextual freezing response. Throughout the procedure, behavioral responses were videotaped and quantified offline, by an experimenter blind to the procedure. The freezing response was quantified based on immobility of any part of the body except for respiratory movements during the initial 10 min for all session. Freezing during the initial 120 s was considered as a generalized fear response to the context similar to the conditioning context. Fear memory retention was assessed by quantifying the time spent freezing during the 30 min recording session. The percentage time spent in freezing (freezing time%, FT %) was calculated (freezing time $\times$ 100/total observed time) for each animal during the observation period in the retrieval environment.

Extinction Training

Extinction of fear memory was assessed in Context B, in the same set of rats. Twenty-four hours after training, rats were subjected to extinction training in 4 sessions. During extinction training, each rat was placed in Context B and observed for 10 min in the absence of US to determine the contextual extinction of fear memory. The extinction training in Context B, which is similar to but different in few aspects from Context A is to minimize baseline freezing driven by the context, before the onset of extinction training [32]. The extinction training was in 4 sessions, separated with an intersession interval of 8 h (Fig. 1e). Immediately after the extinction training, the SH-SH rats were returned to their home cages, while EE-EE and SH-EE rats were exposed to EE for 10 days.

Extinction Test

Extinction of fear was measured after 10 days of exposure to either SH or EE. This was to measure the effect of environmental exposure on the extinction of fear. The floors of all the chambers were cleaned with 70% alcohol following each test to eliminate odor cues.

Implantation and Recording Procedures for the Recording of LFPs

Rats from SH-SH, SH-EE, and EE-EE group were anesthetized with combinations of ketamine (80 mg/kg body weight) and xylazine (10 mg/kg body weight). In addition, xylocaine (2%) was injected subcutaneously before the surgery. These rats were stereotactically (David Kopf Stereotaxic Instrument, USA) implanted with insulated nichrome wires of 250 μ m diameter in CA1 area of hippocampus (CA1) (antero-posterior, -3.3 mm; medio-lateral, 1.5 mm; dorso-ventral, 3.0 mm), lateral nucleus of amygdala (LA) (antero-posterior, -3.3 mm; medio-lateral, 5.2 mm; dorso-ventral, 8.0 mm), and infralimbic region of the mPFC (ILC) (antero-posterior, $+3.0$ mm; medio-lateral, 0.5 mm; dorso-ventral, 5.0 mm) on the left brain hemisphere. An external screw electrode was implanted above the cerebellum subdurally as a reference electrode.

This entire electrode ensemble was soldered to a 5-pin socket and fixed to the skull using dental acrylic. Healex spray was applied to all the wound edges.

We used the same fear conditioning chamber 5–7 days after implantation surgery to measure the neuronal activities of CA1, LA, and ILC during retention of fear memory and extinction of fear memory. Before training, the rats were habituated to the chamber for 10 min. LFPs recording were carried concurrent with the behavior. The rats were subjected to 2 training sessions, by placing them singly in the chamber. During the initial 2 min, the rat was allowed to explore before the presentation of US (1 mA, 1 s duration). Retention of fear memory was assessed 24 h after the training in the retrieval environment (Context B). During the retention of fear memory, each rat was connected to a swivel commutator and LFPs was recorded for a period of 30 min without the presentation of US for assessment of neuronal activities in the fear context. Context-dependent fear memory and neural activities in LA, CA1, and ILC were recorded and analyzed and compared between SH-SH ($n = 7$), SH-EE ($n = 5$), and EE-EE ($n = 8$) rats. Twenty-four hours after the test for retention of fear memory, rats were assessed in the retrieval environment for 10 min during the extinction training. The extinction training was repeated in 4 sessions with inter-trial intervals of 8 h. The rats were placed once again in the retrieval environment after 10 days of exposure to either SH or EE following 4 extinction training with simultaneous LFP recordings.

Statistical Analysis

All statistical analyses were conducted using SPSS. One- and 2-factor analyses of variance (ANOVA) were used, with post-hoc comparisons of means using Tukey's multiple comparison tests. Student *t* test was used to compare between the 2 groups. Statistical significance was set at $p < 0.05$.

Results

Contextual Fear Conditioning

EE before Fear Conditioning and after Extinction Training Augments Extinction of Fear Memory

To determine the effect of EE on retention of acquired fear memory, freezing responses elicited in the Context B were measured 24 h after contextual fear conditioning. No significant differences in freezing behavior to the Context B was observed between SH-SH and EE-EE rats ($t_{1,54} = 1.192$, $p < 0.238$; Fig. 2a). The conditioned behavioral response was further scrutinized to delineate non-associative factors such as generalization and sensitization by examining behavioral expression during the initial 120 s in Context B. Statistical analysis using *t* test revealed a significant ($t_{1,54} = 5.957$, $p < 0.0001$) effect of EE on the generalized fear response in the retrieval environment. The EE rats showed a reduced generalization between the contexts as compared to the SH-SH rats as shown in Figure 2b.

Fig. 2. Mean percentage of freezing to the Context B after 24 h of contextual fear conditioning. **a** Context-specific fear response by rats housed either in standard (SH = SH) or exposed to EE (EE-EE). Data shown are mean percentage of time spent in freezing ($\pm$ SEM) during a 10-min retention test in the fear context (retrieval environment), 24 h after fear conditioning. No significant differences were observed between the SH-SH and EE-EE. **b** Effect of EE conditions on the generalization of conditioned fear in the retrieval environment during the initial 120 s of retention test. Note a significant reduction in the generalization in EE-EE rats compared to SH-SH rats, *** $p < 0.0001$.

Fig. 3. Effect of EE on extinction of fear memory. **a** Effect of EE on the acquisition of fear extinction. Data shown are Mean Percentage of time spent in freezing ($\pm$ SEM) in the retrieval environment during 10 min of 4 extinction training (E1–E4). **b** Effect of timing of EE conditions on the extinction of fear memory. Significant differences were observed in EE-EE ($n = 16$) and SH-EE ($n = 24$) rats in

comparison with SH-SH ($n = 16$) after 10 days of exposure to EE. ** $p < 0.01$, *** $p < 0.001$ vs. SH-SH. SH-SH, rats reared in standard housing (SH) condition ($n = 40$); SH-EE, rats exposed to EE conditions after extinction training; EE-EE, rats exposed to enriched environment (EE) before fear conditioning and re-exposure after extinction training.

On repeated exposure to retrieval environment in successive 4 sessions of extinction training, freezing behavior was gradually reduced to 10–20% of initial values on the last day of extinction training in both groups (Fig. 3a). Two-factor ANOVA revealed no significant effect on the groups ($F_{1,12} = 0.3511$), indicating that the fear memory eventually extinguished in both group of rats, irrespective of the rats reared either in SH or EE conditions. These results indicate that extinction training outside the conditioning context yielded robust extinction of fear memory in both groups of rats.

To assess timing of EE exposure on the extinction of fear memory in Context B, freezing behavior of the rats were compared between SH-SH, SH-EE, and EE-EE groups (Fig. 3b). Ten days after extinction training, all 3 groups of rats exhibited immediate freezing to the context but gradually reduced at the end. Statistical analysis using one-way ANOVA revealed that EE had significant impact on the fear extinction memory ($F_{2,53} = 9.361$, $p < 0.0003$) showing reduced freezing to the Context B in SH-EE ($p < 0.01$) and EE-EE ($p < 0.001$) group. In addition, the results also suggest that EE before fear condi-

Fig. 4. Histological photographs showing the placement of electrodes in the stratum pyramidal layer of CA1 hippocampus, lateral nucleus of amygdala (LA) and infralimbic cortex of medial prefrontal cortex (ILC).

tioning and subsequently after extinction training was more effective in the fear extinction than that with single EE exposure.

Electrophysiological Assessment during Contextual Fear Learning Processing

Figure 4 shows the histological verification of the electrodes placement in stratum pyramidal layer of the CA1 hippocampus, LA, and ILC of mPFC.

Previous study has shown that differential fear conditioning can affect the neural activities in LA and CA1 neurons at theta frequency range. When testing to the context after 24 h of differential fear conditioning, hippocampal pathways synchronized in theta frequency range with LA neurons [26]. This mechanism may be the basis for the neural networking changes that encode fear memory information during retrieval of fear memory.

To analyze the process involved in the neuronal connectivity between CA1 hippocampus, LA, and ILC with and without an exposure to the EE, we studied the spontaneous changes in LFP activities during contextual fear conditioning. The spontaneous LFPs of CA1, LA, and ILC during epochs of exploratory locomotion and freezing behavior were examined in all 3 group of rats exposed to contextual fear conditioning. Theta activity was visible not only during exploratory walking and also during sniffing, grooming, rearing, and even when the rat was doing other exploratory movements. However, these neurons were discharging at a wider frequency range, that is, at 2–12 Hz. In the present study, the data were compared between groups of rats exposed to SH (SH-SH, $n = 7$) and those exposed to EE before and after fear conditioning (EE-EE, $n = 8$) and EE only after fear conditioning (SH-EE, $n = 5$). When the rat was individually subjected

for habituation in the fear conditioning chamber before training, synchronized theta oscillations was visible not only in the hippocampus but also in the LA and ILC (Fig. 5). The synchronization was observed at the onset of exploratory locomotion and disappeared when the animal was sitting immobile. To objectively characterize the LFPs recorded during various types of behavior, the power spectra were calculated.

Representative example of LFP spectrogram recordings from CA1, LA, and ILC during habituation, retention of fear memory and extinction of fear memory in SH-SH and EE-EE group of rats are shown in Figure 5. Corresponding averaged power spectra showed predominance of the low frequencies within the range of 2–12 Hz in the LFPs of different brain areas and strong peak at 4–8 Hz when the animal was exploring the chamber (Fig. 6).

The RP was extracted from the absolute power at different frequencies and was calculated to discuss the effect of EE on theta activities of CA1, LA, and ILC. The degree of freezing and its relationship with the RP showed a predominant theta power band at 4–8 Hz in all 3 groups of rats (Fig. 6). Accordingly, the statistical analysis was carried out at theta range between 4 and 8 Hz. One-factor ANOVA for the RP of freezing behavior across training conditions did not have significant effect on RP of CA1 of SH-SH rats. The EE-EE rats and SH-EE rats showed a significant reduction in RP during freezing behavior on the day of retention of fear memory ($F_{2,14} = 8.443$, $p < 0.03$), ($F_{2,14} = 9.983$, $p < 0.003$), respectively and on the day of extinction fear memory ($F_{2,8} = 9.983$, $p < 0.001$) in comparison with habituation (Fig. 7a).

LA theta power was compared between fear conditioning stages during freezing (Fig. 7b). Within-group com-

Fig. 5. Representative local field potential (LFP) spectrograms from (a) SH-SH and (b) EE-EE rats during different stages of behavioral testing. Sample LFP spectrograms from CA1, LA, and ILC at habituation, 24 h after fear conditioning (retention test) and 10 days after fear extinction training (extinction test). Theta activity

is predominant in CA1, LA, and ILC during habituation and gradually reduced after the contextual fear conditioning and further increased during extinction memory test in Context B. Black bar indicates exploratory behavior, while red bar for freezing behavior.

parison using one-factor ANOVA results revealed that RP of LA in SH-SH ($F_{2,14} = 9.480, p < 0.002$) and SH-EE rats ($F_{2,11} = 5.167, p < 0.03$) showed reduced theta power on the day of fear retention memory ($p < 0.01$) and fear extinction memory ($p < 0.01$) when compared with that of habituation. However, EE-EE rats did not show significant impact on the theta power of LA both during retention and extinction fear memory test when compared with that of habituation (Fig. 7b). Between-group comparisons did not reveal any significant impact on LA theta power.

The study further compared theta frequency band powers of ILC brain region during fear retention and extinction (Fig. 7c). One-way ANOVA followed by Tukey's multiple comparisons test revealed that the con-

textual fear-induced theta band power at freezing behavior was significantly reduced ($F_{2,23} = 5.807, p < 0.003$) during fear extinction ($p < 0.001$) when compared to habituation and retention stage in SH-SH group. Similarly, EE-EE group ($F_{2,23} = 1.894, p < 0.15$) showed reduced ILC theta power ($p < 0.01$) during fear extinction stage when compared to that of habituation stage. SH-EE group on the other hand showed reduced ($F_{2,23} = 2.423, p < 0.16$) theta power both during retention ($p < 0.01$) and extinction ($p < 0.05$) stages. Between-group comparisons during fear extinction stage using 2-factor ANOVA revealed ($F_{2,51} = 7.100, p < 0.002$) that both SH-EE ($p < 0.01$) and EE-EE ($p < 0.01$) rats showed significant increase in theta power while freezing when compared to SH-SH rats.

Fig. 6. a–i Scatter plot to show the relationship between relative power values (4–8 Hz) of LFPs recorded from CA1, LA, and ILC from SH-SH, SH-EE and EE-EE of freezing behavior of the rats

during the retention of fear memory. Results are in mean $\pm$ SEM indicating predominant frequency was observed during freezing at 4–8 Hz.

Fig. 7. LFP firing rate showing relative power (RP) at theta frequency range (4–8 Hz) from CA1-hippocampus (CA1), lateral nucleus of the amygdala (LA) and infralimbic cortex (ILC) while exhibiting exploratory locomotion and freezing behavior during different stages of contextual fear conditioning. The entire data were compared between SH-SH, SH-EE, and EE-EE group of rats. **a–c** Relative theta power of CA1, LA, and ILC during freezing behavior;

d–f relative theta Power of CA1, LA, and ILC during exploratory locomotion, respectively. Ordinates: relative power of theta band (4–8 Hz). Abscissas: habituation, retention of fear memory and extinction of fear memory. Data represented are in mean $\pm$ SEM. # vs. habituation; * vs. SH-SH. Two factor ANOVA and *t* test was used to compare across the behavioral testing. $p < 0.05$, $p < 0.01$, and $p < 0.001$ was considered as statistically significant.

Freezing Specific Changes in Theta Power in the Fear Circuit

To test changes in theta power was specific to freezing behavior; relative theta power was compared while rat was exhibiting exploratory locomotion. As shown in Figure 7d, theta power of CA1 hippocampal region was significantly increased during fear extinction test ($F_{2,20} = 11.36$, $p < 0.001$) when compared to that of habituation ($p < 0.05$). Both EE-EE ($F_{2,23} = 11.66$, $p < 0.001$) and SH-EE ($F_{2,14} = 16.25$, $p < 0.001$) groups showed reduced theta power during fear retention ($p < 0.01$) and extinction ($p < 0.01$) stages when compared with that of habituation. Between-group comparisons

with 2-factor ANOVA revealed a significant ($F_{4,51} = 6.917$, $p < 0.001$) difference in theta power with reduced theta power in EE-EE ($p < 0.01$) and SH-EE rats ($p < 0.01$) when compared with that of SH-SH rats (Fig. 7d).

Contextual fear conditioning had significant impact on LA showing reduced theta power during fear retention test in SH-SH ($F_{2,20} = 7.152$, $p < 0.009$), EE-EE ($F_{2,23} = 6.124$, $p < 0.01$) and SH-EE ($F_{2,11} = 37.33$, $p < 0.0004$) group. Theta power was also reduced during extinction in SH-SH ($p < 0.05$) and SH-EE rats in comparison with habituation. Two-factor ANOVA was applied to test the interactions between- and within-group which showed

both EE-EE and SH-EE group showed reduced theta power when compared with SH-SH rats ($F_{2,65} = 14.12$, $p < 0.0001$). Tukey's multiple comparison test indicated that SH-EE rats showed significant reduction in theta power during fear retention ($p < 0.01$) and extinction tests ($p < 0.05$; Fig. 7e).

A statistical analysis for theta power of ILC for within-group comparisons revealed no differences in theta power during exploratory locomotion (Fig. 7f) on the day of retention of fear memory in SH-SH rats. One-factor ANOVA ($F_{2,12} = 17.49$, $p < 0.0003$) revealed that RP during exploratory locomotion in SH-SH rats showed increased RP on extinction ($p < 0.05$) in comparison with habituation and retention ($p < 0.001$). In EE-EE rats, we observed a significant reduction in theta power ($F_{2,14} = 6.124$) on the day of retention of fear memory when compared with habituation ($p < 0.05$). SH-EE rats showed decrease in RP on the day of retention ($p < 0.01$) and extinction ($p < 0.001$) in comparison with habituation (Fig. 7f). Between group comparisons were made using 2-factor ANOVA which revealed that ILC theta power showed significant impact on theta power ($F_{2,51} = 17.15$, $p < 0.0001$) during exploratory locomotion showing significant reduction in theta power during fear extinction test in EE-EE ($p < 0.001$) and SH-EE ($p < 0.01$; Fig. 7f).

Further to explain the neural mechanisms involved in increased fear extinction memory (freezing state per se), theta synchrony of ILC was compared with that of LA and CA1 hippocampus. No significant differences in theta synchronization between ILC and LA and CA1 were observed between the groups during habituation (data not shown). Theta synchronization has reduced significantly during retention of fear memory between LA and ILC/CA1 in all 3 groups (Fig. 8a–c). Following extinction training, theta synchronization between these 3 brain regions gradually increased and is more evident in EE-EE group when compared to SH-EE and SH-SH groups (Fig. 8a–c). On the contrary, theta synchrony was not altered during exploratory locomotion indicating that the changes in synchrony were specific to freezing behavior (data not shown). Thus, indicating that increased fear extinction memory was associated with increased synchronized activities between LA/CA1 and ILC region. The confounding factor for volume conduction artifacts can be excluded because (1) the level of correlation between the brain regions was less than 1 and (2) comparisons between groups revealed significant differences in synchronization level during retention and extinction of fear memory.

Discussion

We found that interpolating periods of EE differentially facilitates extinction of generalization of fear conditioning between similar contexts. The present data also provide substantial evidence suggesting that the timing of EE exposure plays an important role in the extinction of fear memory. Consistent with previous reports [33], context-specific fear memory is facilitated by prior exposure to the context before fear conditioning. This facilitation occurs even in rats exposed to EE, presumably by reducing the generalized effect on fear memory retention. EE results in enhanced learning and memory compared to non-EEs [34]. In agreement with Duffy et al. [6], we have also observed that EE exposure before fear conditioning modulated context-specific fear memory. This enhanced fear memory is attenuated more rapidly in EE exposed rats than SH-SH rats by repeated exposure to the retrieval environment. Re-exposure to EE after extinction training revealed that EE-EE rats exhibited less freezing than SH-SH rats during extinction of fear memory, thus indicating that re-exposure to EE after extinction training is beneficial in reducing the return of fear in a retrieval environment.

The effect of EE is more prominent in reducing the generalized response than in facilitating stimulus-specific conditioned fear. The ability of EE-EE rats to discriminate between the contexts was better as these rats showed a reduced generalization in the retrieval context. In addition, EE exposed rats showed a faster extinction than SH-SH rats suggesting that EE accelerates extinction of fear memory. Further re-exposure to EE after extinction training potentiates the extinction indicating the facilitation of extinction of fear, than only the single EE exposure demonstrating the important role timing of exposure to EE. In summary, EE has a beneficial effect on the extinction of fear memory. The EE exposure before the fear conditioning and re-exposure after extinction training played an important role in the extinction of fear memory.

Our data provide substantial evidence suggesting that the timing of EE conditions plays an important role in extinction of fear memory, which is mediated by a network of brain areas and more specifically by the ILC region of the mPFC. There are several evidences to validate the importance of ILC in extinction of fear memory [35, 36]. Consistent with previous report [33], context-specific fear memory is facilitated with prior exposure to the context before fear conditioning. This facilitation occurs even in the rats exposed to EE by reducing generalized effect on fear memory retention. EE exposure is also known to enhance learning and memory than non-EEs [34]. In

Fig. 8. Effect of enriched housing conditions on synchronized theta oscillations between CA1-hippocampus (CA1) versus lateral nucleus of amygdala (LA)/infralimbic cortex (ILC) during retention and extinction of fear memory testing. Averaged cross-correlation data obtained from LA vs. CA1 (**a**); CA1 vs. ILC (**b**), and LA

vs. ILC (**c**) while animals exhibiting freezing behavior. Level of correlation at 1 was considered as absolute correlation between regions. Ordinates: cross-correlation; abscissas: time in seconds. Note an evident reduction in peak correlation of LFP between CA1/LA and ILC in SH-EE and EE-EE rats during freezing.

(For figure 8c see next page.)

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agreement with the previous study [6], we have also observed that EE before fear conditioning enhanced the context-specific fear memory. This enhanced fear memory is attenuated much rapidly in EE rats than SH-SH rats by repeated exposure to the retrieval environment. Re-exposure to EE after extinction training revealed that EE rats exhibited less freezing than SH-SH during the extinction of fear memory, thus indicating that re-exposure to EE condition after extinction training is beneficial in reducing the return of fear in a retrieval environment.

As a step toward the detailed assessment of the cellular mechanisms involved in increased synaptic plasticity following enrichment [12, 37, 38], we present the spontaneous changes in the LFP during baseline conditions. Changes in the synaptic plasticity following exposure to EE can be the primary cause for changes in local networking activities of CA1, LA, and ILC. In line with previous studies [39, 40], the detailed analysis of the LFPs recorded from CA1, LA, and ILC revealed that the ongoing behavioral-related theta rhythm (3–12 Hz) is not only present in the hippocampus, but also in the amygdala and mPFC, thus supporting the earlier findings [41–43] that theta-like rhythmic activity can be found even in structures which do not have the layered organization of cells as is found in the hippocampus. Our data are compatible with the notion that LA, CA1, and ILC are structurally [44, 45] and electrophysiologically [26, 36, 46–50] interconnect-

ed. Prolonged exposure to EE did not reveal changes in theta rhythm and oscillations before learning.

Oscillatory pattern of theta rhythm within and across hippocampal regions indicates the memory formation and retrieval [51, 52]. The physiological changes during theta rhythm may enhance the selective context-dependent retrieval of fear memory without interference from non-predictive cues [26, 53]. However, we have observed reduced synchronized theta oscillatory activities between LA and CA1/ILC during retention of fear memory without impeding the fear memory expression in both NC and EE rats. The asynchronous theta rhythm between CA1, LA, and ILC was based on (i) decreased theta power, (ii) decreased cross-correlation, and (iii) slower theta peak frequency (data not shown) during freezing behavior. Our result differs from previous studies [26, 54] which could be inferred to the parallel state of information processing, due to the previous exposure to retrieval context, which is adequate for retention of fear memory [55–57]. It is likely that pre-exposure to the context before conditioning builds up the cognitive map of the environment in encoding the nature of their context while retrieving the fear memory. When subjected to retrieval environment after 24 h of fear conditioning, the hippocampus, amygdala, and mPFC may process information in parallel (and redundantly) or competitively due to non-specific predictive cues during retrieval of fear memory [55, 58], thus leading into desynchronized theta oscillations.

tions while exhibiting fear. In addition, increased desynchronization of theta rhythm in the local circuitries could equally be attributed to increased involvement of CA1, LA, and ILC during retention of fear memory. Reduction in the synchronized oscillations between LA and CA1 is more robust in EE than NC rats and could be correlated with increased synaptic plasticity in these brain regions.

Further probing into the effect of EE exposure on CA1, LA, and ILC activities with relation to extinction of fear memory in a retrieval environment was found to be robust in EE-EE and SH-EE rats than SH-SH as expected. We found that EE exposure after extinction training not only inhibited the fear memory, but also significantly altered theta power of ILC during extinction of fear memory. Reduction in theta power during extinction fear memory was more pronounced in SH-SH than in EE rats during freezing. In addition, we also observed a gradual recovery in synchronized theta oscillation at a higher frequency range between CA1, LA, and ILC during freezing in EE-EE than SH-SH rats. Reduced return of fear memory in the retrieval environment following extinction training suggests that re-exposure to EE accelerates the extinction fear learning. Previous reports have shown that enriched rats exhibit faster rate of learning in a new context than rats reared under SH conditions [59, 60]. Progressive recovery in theta synchronization between CA1 and LA/ILC during extinction fear memory could be regarded as “positive learning,” indicating rapid habituation to the retrieval environment.

Differences in synchronized theta oscillations between SH-SH, SH-EE, and EE-EE rats during extinction of fear memory could be attributed to heightened synaptic plasticity following exposure to EE [3]. EE-induced physical activity increased cholinergic activity [61], thickness of hippocampus [62], and cortex [38, 63]. Furthermore, re-

ciprocal anatomical connections between CA1, LA, and ILC facilitates cortical activation, which allows the context-specific information flow to the CA1, which is characterized by reduced fear return by EE-EE rats than SH-SH and SH-EE rats. We thus argue that with the gradual recovery in synchronized theta oscillations between CA1 and ILC, it enables the animals to detect the differences in contexts, without impeding the fear memory.

In conclusion, the present data support the hypothesis that EE before fear conditioning and subsequent exposure to enriched housing conditions following extinction training facilitates the extinction of fear memory. Together with electrophysiological findings, we also support the hypothesis that ILC along with CA1 and LA modulates the memory for extinction of contextual fear memory by inhibiting the fear response.

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Authors Contribution

P.H.: this author had substantial contribution in carrying out the study in the data analysis and drafting the manuscript. S.O.: author participated in drafting the manuscript. T.R.L.: authors made substantial contribution in the conception and design; data analysis and interpretation of the study; preparing the manuscript.

Disclosure Statement

The manuscript complied with ICMJE.

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