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Impaired histone acetylation in the Infralimbic Prefrontal Cortex following Immediate extinction may result in deficit of extinction memory

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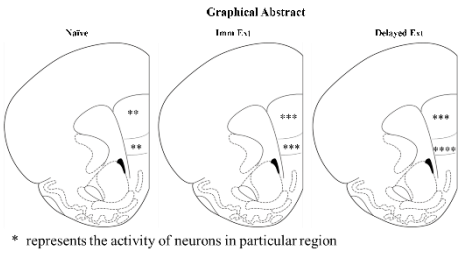
Graphical abstract



Highlights

- Delayed extinction supports better extinction than the extinction performed at early time point (immediate extinction).
- IL region plays a major role in extinction as compared to PL region.
- The level of histone acetylation in IL region was higher in delayed extinction as compared to the immediate extinction.

Abstract

In the last few decades, there has been exponential increase in studies aiming to trace the molecular mechanism of fear extinction with a hope to minimize the return of fear after exposure therapy required for operational treatment of anxiety disorders. The present study explored how the timing of extinction training after developing a specific fear, affects the consequent return of the extinguished fear and the role of histone acetylation in controlling the circuitry, thereof. It was found that rats undergone extinction training 10 mins after fear memory acquisition (Immediate Extinction) had deficits in retention of extinction memory as compared to one which underwent extinction 24 hrs after fear acquisition (Delayed Extinction). When the differences were sorted at the circuitry level the relative activity of the infralimbic prefrontal cortex (IL) to prelimbic cortex (PL) was found to be lower in the immediate extinction group as compared to the delayed extinction group as evidenced by the *c-fos* expression in the mPFC of these groups. Further investigation showed that acetylation of histone H3/H4 along with the levels of CREB binding protein (CBP) which is a histone acetyltransferase (HAT), was associated with neuronal activation and was significantly lower in the IL of the immediate extinction group than the delayed extinction group. In conclusion, the observed deficits in the immediate extinction group may be the result of compromised activation of IL, which in turn may be associated with changes in histone acetylation.

Key words: Immediate extinction; Delayed extinction; Retention test; PTSD; Conditioning

Introduction

Failure to extinguish traumatic memories in some individuals may lead to the development of fear related anxiety disorders such as Post traumatic stress disorder (PTSD) (VanElzakker et al., 2014). Such individuals are mainly treated by exposure therapy based on extinction learning and its retention (Craske et al., 2008, Rothbaum et al., 2003, Bouton et al., 2001). A very good translational model for developing behavioral paradigm for fear related anxiety disorders is Pavlovian based fear conditioning in rats (Maren, 2005, Pare et al., 2004, LeDoux, 2000). In this model, a rat is fear conditioned by presenting it to several rounds of the conditioned stimulus (CS) such as tone with an unconditioned stimulus (US) such as foot shock. 24 hours later when such fear conditioned rat is presented with the tone in the absence of US in a different context, there is a reduction in the fear response by a phenomenon termed Fear extinction. However, the reduction in response to CS is temporary and fear returns with the passage of time and change in context (Myers and Davis, 2007, Bouton et al., 2006, Pavlov, 1927). This poses a major challenge amongst both the basic scientists and psychotherapists to come up with newer paradigms of exposure therapies for the effective treatment of fear related anxiety disorders (Muigg et al., 2008, Wessa and Flor, 2007, Myers and Davis, 2002, Rosen and Schulkin, 1998, Rasmusson and Charney,

1997).

Previous studies in both the rodents and humans suggest that the timing of extinction after fear learning had a varied effect on the strength of extinction (Golkar et al., 2012, Huff et al., 2009, Maren and Chang, 2006, Myers et al., 2006, Norrholm et al., 2008). In one such report, it was found that extinction training performed immediately after the fear learning resulted in either "erasure" (Norrholm et al., 2008) or reduction of fear (Chang and Maren, 2009). However, many studies which followed up onto this topic observed that immediate extinction was not as effective as delayed extinction in inhibiting the return of fear, a phenomenon which has been termed as ''immediate extinction deficit (IED)" (Maren, 2014, Stafford et al., 2013, Long and Fanselow, 2012, Archbold et al., 2010, Kim et al., 2010, Woods and Bouton, 2008). Chang and Maren, 2009, have reported that the reduction in fear observed after immediate extinction is short lived which may be due to short term habituation rather than long term extinction. However the neuronal basis of IED at the molecular level is largely unknown and studies aiming to find out the exact neural correlates of extinction learning at circuitry level will add onto the field (Maren and Quirk, 2004, Pare et al., 2004, LeDoux, 2000).

The altered neural activity in the amygdala and IL subregion of mPFC is reportedly responsible for regulation of fear (Greenberg et al., 2013) and extinction memory (Sotres-Bayon et al., 2006, Quirk et al., 2000). The role of mPFC was further confirmed by the studies where lesions of mPFC in rats resulted in the impairment of recall of extinction memory (Milad and Quirk, 2002, Morgan et al., 1993, Morgan and LeDoux, 1995). The infralimbic prefrontal cortex (IL) and the prelimbic prefrontal cortex (PL) subregions of the mPFC play distinct roles during fear and extinction learning (Quirk and Mueller, 2008). Moreover, the IL activity correlates to the recall of the extinction memory (Milad and Quirk, 2002) and PL activity to the expression of fear response (Burgos-Robles et al., 2009, Likhtik et al., 2005). Furthermore, pharmacological interventions in IL, impaired extinction during retrieval test (Quirk and Mueller, 2008) while other studies involving electrical stimulation (Milad et al., 2004, Milad and Quirk, 2002) of IL enhanced extinction in retrieval test suggesting the role of IL in the promotion of extinction in rodents. The IL showed an increase in CS-evoked single-unit responses (Milad and Quirk, 2002) and in spontaneous bursting (Burgos-Robles et al., 2007) after extinction training. As evident by the studies of Corcoran and Quirk, 2007, the inactivation of PL prior to the start of the conditioning training resulted in reduced fear expression to the conditioned stimulus without disrupting acquisition.

Acetylation of histone at Lysine residues occurs through the action of histone acetyl transferases (HATs), such as CREB-binding protein (CBP/p300) and is associated with the consolidation of memory following fear and extinction learning (Alarcon et al., 2004, Levenson et al., 2004, Guan et al., 2002, Lunyak et al., 2002, Turner, 2002). The expression of immediate early genes (IEGs) such as *c-fos* and Egr1 (Hawk and Abel, 2011, Perez-Cadahia et al., 2011, Dragunow, 1996) is under the control of histone acetylation that occurs in the promoter

region of these genes. Furthermore, the changes in histone acetylation regulate memory consolidation in a range of learning models, including contextual fear learning (Sintoni et al., 2013, Stefanko et al., 2009, Levenson et al., 2004). Enhanced histone H4 acetylation in neurons of IL-PFC has an established role in the storage of fear extinction memories (Ferreira et al., 2015). Acetylation of Histone H3 in area CA1 (field CA1 of the hippocampus) of the hippocampus has been shown to be necessary for contextual fear learning (Miller et al., 2008, Lubin and Sweatt, 2007, Levenson et al., 2004).

In the present study, we worked towards the hypothesis that neuronal activity in the IL is required for the retention of extinction memory and altered neuronal activity in the IL following immediate extinction (Milad et al., 2004) may lead to the deficits in retention of extinction memory. To explore this hypothesis, we characterized neuronal activity in both the IL and PL following immediate and delayed extinction. We found that successful retention of extinction memory following delayed extinction was associated with increased neuronal activity in the IL; this effect was attenuated in the rats undergone immediate extinction. We further hypothesized that the region-specific activation of neurons in the PL and IL of immediate and delayed extinction group may be under the epigenetic control especially histone acetylation and to explore this, the levels of CBP, Acetyl H3 and H4 following immediate and delayed extinction were compared in the IL and PL region of mPFC.

Materials and Methods

Subjects

2-3 months old SD (Sprague-Dawley) rats weight 150-200 gram were used throughout the study. They were maintained on a 12-hour light/dark cycle and had access to food and water *ad libitum*. They were housed individually in separate cages at a temperature of $25^0C \pm 2^0C$. All experiments were performed as per the CPCSEA guidelines (853/AC/04/CPCSEA), Govt. of India, New Delhi. A total of N=80-100 animals were used in the study (n=20-24 animals in each group).

Behavior Apparatus

Two identical observation chambers made up of plexiglass (fear conditioning apparatus VJ instrument) kept in sound-attenuating cabinets were used for fear and extinction training. The floor of each chamber consisted of stainless steel rods (4 mm in diameter) spaced 1.5 cm apart and had provision for the delivery of footshock (US). An arrangement of the speaker was made outside the wall of the chamber for the delivery of acoustic CS. The ventilation fan was mounted on the acoustic chambers to supply the background noise. One chamber with Context A was used for conditioning and the other chamber with Context B was used for extinction. Context B was made different from context A. Context B was provided with Vanilla scent to change the olfactory cues,

white sheets with black strip were placed around the walls and grid floor was fully covered to hide the stainless steel rods.

Conditioning

All the rats used in the study were handled for one week for 5-10 minutes per day prior to the start of the experiment. For fear learning, the rats were brought into a separate black cage and directly placed inside the conditioning chamber without exposure to the surrounding environment. They were allowed to acclimatize to the conditioning context (Context A) for 3 minutes prior to the start of the fear training session and baseline freezing was measured. After acclimatization, five sessions of paired CS-US were presented to rats and freezing was recorded as the time during which the rats did not show anybody movement except breathing and heartbeat during total duration of the trial. The CS (tone) was provided for 10 seconds with the intensity of 80 dB coterminous with US (shock) of 0.70 mA (for 1 second). The inter-trial interval between shock and the next tone was 60 seconds and the freezing behavior was calculated by both the observer and the software (Fig. 1C) (V.J. instruments, India). 1 min after the completion of the behavioral session, rats were returned to their home cage. Protocol for the study is summarized in Fig. 1(A).

Extinction

The extinction training was performed in a novel context (Context B) at two different time points 10 mins and 24 hours after fear learning. Prior to the commencement of extinction training, the baseline freezing was recorded across the groups by exposing the rats to the extinction context for 3 minutes. No tone or shock was delivered during this period. Following the baseline session, 30 CS (tone 80 dB for 10sec) were administered to the rats with an intertrial interval of 10 seconds in the absence of US and the freezing behavior was measured (Fig. 1D). For analysis, 30 trials were represented as 5 trial blocks by taking the average of every 6 consecutive trials (6 trials = 1 trial block, thus 30 trials = 5 trial blocks).

Two types of controls were included in the study viz., immediate no extinction and delayed no extinction to overcome the confounding effects. Rats in the control groups were only exposed to the extinction context for the same duration of time of extinction to their corresponding immediate and delayed extinction group, in the absence of any tone (CS) or shock (US).

Retention test

From each group (n=20-22 animals), half of the rats underwent retention test 24 hours after extinction training, while the remaining half were directly sacrificed after 2 hours of extinction training for molecular study (Immunohistochemistry). During retention test, the animals were presented with 5 trials of tone alone (80 dB for 10 seconds) in the extinction context (Context B). The inter-trial interval between two tones was maintained 10

sec and freezing score was calculated so as to measure the retention of extinction learning (Fig. 1E).

Brain Sub-Regions under Study

Medial Prefrontal cortex (mPFC) encompasses PL (Prelimbic prefrontal cortex) and IL (Infralimbic prefrontal cortex) (Giustino and Maren, 2015) (Bregma: 2.76- 3.24) (Fig. 1B). 4-5 brain sections were collected for each antibody from each animal of the group and the data were represented as means of the number of positive neurons from each animal.

Tissue processing

2 hours following extinction training, rats were anesthetized with pentobarbital (50 mg/kg i.p.) and perfused transcardially with normal saline (0.9%) followed by ice-cold 4% paraformaldehyde (PFA). Animals were then decapitated and their brains were then kept in 4% PFA for 24 hours. Next day the brains were transferred to 10% sucrose solution for one day followed by 20% and 30% sucrose solution serially until settled down in the sucrose solution. Brains were then frozen in isopentane for 30-40 minutes on dry ice between -30 to -40°C and stored at -80^0C until immunohistochemistry was performed.

Immunohistochemistry

Coronal brain sections of 20 µm thickness containing mPFC were collected using cryostat (Thermo-scientific Microm HM 525). The sections were blocked with 1% NHS (NHS Vecta-stain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA) and 0.25 % tween 20 and then incubated overnight with primary antibody for c-fos (1:500, cat. no. ab7963), acetyl H3K9 (1:1000, cat. no. ab10812), acetyl H4K5 (1:1000, cat. no. ab51997) and CBP (1:1000, cat. no. ab2832) (all antibodies from Abcam). Next day, the sections were incubated with the biotinylated secondary antibody (1:500 dilutions, Vecta-stain Elite ABC kit, PK 6200) for 2 hours followed by ABC complex (ABC kit, Vector Laboratories) for 2 hours at room temperature. Finally, DAB substrate (Himedia, cat.no. RM2735) was added. The immunostained sections were then mounted on clean and frosted slides. Three readings were taken from PL and IL subregions and their average was counted as one reading. A total of three corresponding brain sections from each animal were analyzed for each antibody. Readings were estimated by manual counting and also by NS-BR image analysis software (Nikon, Tokyo) using a Nikon Eclipse microscope (Nikon, Tokyo, Japan).

Statistical Analysis

Behavioral data has been presented as means and standard error of the means (± SEM) and was analyzed using three-way repeated measures followed by Tukey's *post hoc* analysis. For each session, the freezing data was transformed to the percent value. Retention test data and IHC data were analyzed using two-way ANOVA. All the

statistical calculations were performed by the ezANOVA software. Pearson correlation was performed between % freezing observed during retention test versus expression of *c-fos*, CBP and histone acetylation (Fig. 6) in the IL subregion.

Results

Behavior

In the present study, we explored the effect of timing of extinction training relative to fear learning on retention of extinction memory. The rats were subjected to extinction training either 10 mins (Immediate extinction) or 24 hrs after fear learning (delayed extinction). Before the onset of conditioning, during acclimatization period of 3 min, baseline freezing was recorded indicating that all the rats possess similar low level of freezing response with no significant difference across the groups. During fear learning, the freezing response increases with each successive trial across the groups (Fig. 1C), and the result was confirmed by three-way repeated measure of conditioning data which revealed a significant main effect of trials [F (4,144) = 862, $p<0.0001$] and the interaction of trial [F (4,144)= 2.64, $p<0.05$] in the immediate and delayed extinction group during fear learning. Tukey's *posthoc* test confirmed significant differences between the trials (between 5 trials) in conditioning (all $p<0.001$).

Prior to the commencement of extinction learning, the baseline freezing was low across all the groups with no significant difference. During extinction learning, both the immediate and delayed extinction groups showed attenuation in the freezing response with each consecutive trial (Fig. 1D). However**,** immediate extinction group exhibited a comparatively higher level of freezing as compared to the delayed extinction group during the initial trials. The freezing during the last trial was similar in both the groups ($p>0.05$). The immediate no extinction and delayed no extinction groups maintained low freezing throughout the session. The result was confirmed by three-way Repeated measure which showed significant main effect of extinction condition [F (1,36)=1816, $p<0.05$] and trial [F (4,144)= 205, $p<0.0001$] as well as for interaction extinction condition x trial [F (4,144)= 89, $p<0.0001$], extinction time x extinction condition [F(1,36)=7.2, $p<0.05$], extinction time x trial [F (4,144)= 5.18, $p<0.05$] on freezing response. The *post-hoc* analysis confirmed that extinction groups froze more significantly than no-extinction groups ($p<0.001$) and there was a significant effect of trials on freezing response ($p<0.0.01$).

24 hours after successful extinction learning, retention test was performed to gauge the retention of fear. Retention test showed that delayed extinction group exhibited the least level of freezing as compared with the immediate extinction and no extinction group. Two-way ANOVA of retention test showed significant main effect of time [F (1,16) = 8.89, $p<0.01$] and condition x time interaction [F (1,16) = 10.8, $p<0.01$] but no significant

main effect of condition [F (1,16) = 3.32, $p>0.05$] was observed on freezing response. Tukey's *post-hoc* analysis confirmed that delayed extinction had a low level of freezing response as compared to the immediate extinction group ($p<0.01$) and delayed no extinction group ($p<0.01$) (Fig. 1E).

Overall the immediate extinction group had deficits in the retention of fear extinction memory whereas delayed extinction group had stable long-term retention of fear extinction.

The *c-fos* expression has been extensively used as a neuronal activity marker in various studies and its expression has been correlated to region-specific activity in different brain regions (Siddiqui et al., 2017, Hoffman et al., 1993, Bullitt, 1990). Our next aim was to examine the region-specific neuronal activity in the IL and PL subregions of the mPFC following immediate and delayed extinction. To accomplish this objective, coronal brain sections containing the mPFC were immunostained for c-fos from each group.

***c-fos* expression following immediate and delayed extinction**

The expression of *c-fos* was found to be higher in extinction group as compared to their respective control groups in the PL region however, the changes were insignificant between immediate and delayed extinction group. Two-way ANOVA analysis for *c-fos* expression in PL region revealed a significant main effect of extinction condition (extinction vs. no extinction) [F (1, 28) = 24.2, $p<0.0001$], while there was no effect of extinction time (Immediate vs. Delayed) [F(1,28) = 0.183, $p>0.05$] and condition x time interaction [F(1,28) = 0.018, $p>0.05$].

However in IL, the expression of *c-fos* was significantly higher in delayed extinction group as compared with the immediate extinction group and delayed no extinction group. The expression of *c-fos* was further confirmed by two way ANOVA that revealed a significant main effect of extinction condition (extinction vs. no extinction) [F (1, 28) = 58.3, $p<0.0001$], extinction time (immediate extinction vs. delayed extinction) [F(1,28) = 15.2, $p<0.005$] as well as extinction condition and extinction time interaction [F(1,28) = 4.06, $p<0.05$] (Fig. 2).

In other words, the two subregions of mPFC responded differentially to the two extinction paradigms. This differential expression of *c-fos* exemplifying the activity in the PL and IL in the immediate and delayed extinction group may be responsible for the deficit in the retention of extinction memory as observed after immediate extinction.

Our second major aim of the present study was to correlate epigenetics especially histone acetylation to the region-specific differential activation of neurons. For this the expression of CBP, which is a histone acetyltransferase mainly attributed to histone acetylation at different lysine residues of histone H3 and H4 in the promoter region of genes resulting in enhanced expression of memory-related genes, was gauged (Korzus et al., 2004).

CBP expression following immediate and delayed extinction

Similar to the c-fos, the CBP expression was higher in extinction groups as compared to their respective control groups but no significant changes were observed between immediate and delayed extinction groups in PL region. Two-way ANOVA analysis for CBP expression in PL region revealed significant main effect of extinction condition (extinction vs. no extinction) [F (1,28) = 21.3, $p<0.0001$] but no effect of extinction time (immediate vs. delayed) [F(1,28) = 0.099, $p>0.05$] and condition x time interaction [F(1,28) = 0.050, $p>0.05$]. However in IL, there was a significantly increased expression of CBP was observed in delayed extinction group as compared with the immediate extinction group and delayed no extinction group. This information was further supported by two way ANOVA analysis that exhibited significant main effect of extinction condition [F (1,28) = 48.3, $p<0.0001$], extinction time [F(1,28) = 6.73, $p<0.01$] and extinction condition x extinction time interaction [F(1,28) = 4.03, $p<0.05$) (Fig. 3). Therefore, the expression of CBP seems to be associated with neuronal activity in the PL and IL following immediate and delayed extinction.

We next wanted to see whether the increased CBP levels in these regions culminated in acetylation of H3 and H4 the reason being studies suggesting acetylation of Histone at various lysine residues to be associated with enhanced expression of genes required for synaptic activity and memory consolidation (Lubin, 2011, Siddiqui et al., 2017, Ranjan et al., 2015).

Histone acetylation following immediate and delayed extinction

To correlate the neuronal activation to histone acetylation we next looked at histone acetylation in the PL and IL following immediate and delayed extinction. We gauged the levels of Acetyl H3 at lysine residue 9 (K9) and Acetyl H4 at lysine residue 5 (K5) in PL and IL following the two extinction paradigms.

Expression of acetyl H3K9 in PL region showed that there was no significant difference in between immediate extinction and delayed extinction group however immediate and delayed extinction group has a higher level of acetyl H3K9 positive neurons with respect to immediate no extinction and delayed no extinction group. Two-way ANOVA analysis confirmed that there was significant main effect of extinction condition (extinction vs. no extinction) [F (1, 28) = 28.9, $p<0.0001$]. However the effect of extinction time (immediate vs. delayed) [F (1, 28) = 0.397, $p>0.05$] and extinction condition x extinction time interaction was not significant [F (1, 28) = 0.421, $p>0.05$].

On the other hand in IL region, the expression of H3K9 was significantly higher in delayed extinction group as compared to the delayed no extinction group and immediate extinction group. Two-way ANOVA analysis of IL revealed significant main effect of extinction condition (extinction vs. no extinction) [F (1, 28) = 57.8, $p<0.0001$] and extinction time (immediate vs. delayed) [F (1, 28) = 3.92, $p<0.05$] as well as extinction

condition and extinction time interaction [F(1, 28)= 7.60, $p<0.01$]. These changes in the H3 acetylation were linked to the neuronal activity i.e., the *c-fos* expression in a region-specific manner (Fig. 4).

Similar to the H3K9, the immediate and delayed extinction group exhibited higher level of H4K5 expression when compared to their respective control groups in PL region but no significant difference between immediate and delayed extinction group was observed. Two-way ANOVA analysis confirmed that there was significant main effect of extinction condition (extinction vs. no extinction) [F (1, 28) = 22.9, $p<0.0001$] but no significant difference was observed for extinction time (immediate vs. delayed extinction) [F (1, 28) = 0.003, $p>0.05$] and extinction condition x extinction time interaction [F (1,28) = 0.004, p>0.05].

Delayed extinction group exhibited higher level of acetyl H4K5 expression when compared with other groups in IL region. Two way ANOVA analysis of Acetyl H4K5 positive neurons in the IL showed a significant main effect for extinction condition (extinction vs. no extinction) [F (1,28)= 99.1, $p<0.0001$], extinction time (immediate vs. delayed) [F(1,28)= 18.8, $p<0.0001$] as well as extinction condition x extinction time interaction [F(1,28)= 6.54, $p<0.01$] (Fig.5).

Correlation between Retention memory and *c-fos*, CBP and histone acetylation expression

Overall there was a negative correlation between the no. of positive neurons expressing *c-fos* in the IL of the delayed extinction group as compared to immediate extinction group ($r = -0.6591$; $p<0.05$) with the freezing % during retention test. Similarly the CBP expression ($r = -0.7442$; $p<0.05$) and acetylation of H3 ($r = -0.6861$; $p<0.05$) and H4 ($r = -0.7603$; $p<0.05$) in the IL of the delayed extinction group was negatively correlated with the freezing % when compared with the immediate extinction group (Fig. 6) (Table 1).

In summary, the IL activity following immediate extinction was significantly less than that observed following delayed extinction. In other words, the IL activity is essential for the consolidation of extinction memory and the resultant inactivity in IL following immediate extinction may be responsible for extinction deficit.

Discussion

In the present study, we examined the effect of immediate and delayed extinction on retention of extinction memory along with neuronal activation and histone acetylation in mPFC. A deficit in the retention of extinction memory was observed in the immediate extinction group when compared to that in the delayed extinction group. Many previous studies have also reported such deficit in retention of extinction memory after immediate extinction (Chang and Maren, 2009, Chang and Maren, 2011, Thompson et al., 2010, Kim et al., 2010). However, there are conflicting reports about the outcomes of immediate extinction. Most of the studies have reported deficit in the extinction memory except one which reports “erasure” of fear memory following

immediate extinction (Myers et al., 2006). These observations were totally based on the decrements that were observed in renewal, reinstatement and spontaneous recovery in the early extinction groups. Moreover, lack of assessment of within session extinction and absence of no extinction controls in the study makes it difficult to really prove that "erasure" has occurred. Thus our conclusion along with others that immediate extinction is less effective than delayed extinction in long term retention of fear is in disagreements with the reports of Myers et al., 2006. In fact, Chang and Maren, 2009, did find short term context independent suppression of fear after immediate extinction which was more likely because of habituation rather than extinction. Although in the present study we did not use probe CS to test the extinction retention after 15 minutes of extinction, the deficit in retention of extinction observed after 24 hours during the retention test was similar to that reported by the above study. Further investigation of the circuitries altered in the immediate extinction group as compared to the delayed extinction group will shed light on the molecular events responsible for short term habituation after immediate extinction.

To ascertain this, the changes occurring in the two subregions viz. PL and IL of mPFC were gauged, which along with the amygdala and hippocampus regulate the consolidation and retention of fear and extinction memory (Marek et al., 2013, Preston and Eichenbaum, 2013, Corcoran and Maren, 2001, Maren et al., 2013). Both the delayed and immediate extinction groups exhibited activation of neurons in PL and IL subregion of mPFC as exemplified by the *c-fos* expression following extinction training. However, the increase in the IL neuronal activity for the delayed extinction group was relatively higher as compared to the immediate extinction group. Congruent with this study some previous studies have also shown enhanced neuronal activation in the IL following extinction learning and in the PL following fear learning (Thompson et al., 2010). Moreover, the fear behavior is reported to be differentially regulated by PL and IL (Sotres-Bayon et al., 2006, Milad et al., 2004, Courtin et al., 2013) via an increase or decrease of activation of neurons in these regions (Sierra-Mercado et al., 2011, Sotres-Bayon and Quirk, 2010) and our study paralleled with these studies. Electrophysiological studies suggest a correlation between the firing of IL neurons during extinction training to the extent of fear suppression (Giustino and Maren, 2015) and further authenticate the role of IL and the results of this study. The deficit in retention of extinction memory observed in the immediate extinction group in the present study and many other studies (Chang and Maren, 2009, Chang and Maren, 2011, Thompson et al., 2010, Kim et al., 2010) might be a result of compromised activation of IL neurons and electrical stimulation of mPFC results in the elimination of this deficit (Kim et al., 2010). In other words, the altered neuronal activation in the IL may be contributing to the failure of the rats undergone immediate extinction, to maintain fear suppression.

Besides *c-fos* expression, we also looked at the CBP expression which was found to be relatively higher in IL than that of immediate extinction group which correlates with the c-fos expression. CBP is a histone

acetyltransferase and its enhanced activity results in acetylation of histones H3 and H4 often modulating the gene expression during memory formation (Peixoto and Abel, 2013). In either case, our data suggested that there is compromised neuronal activity in the IL of rats that fail to extinguish fear relative to those that extinguish fear normally.

Epigenetics is an umbrella term used for all the changes that affect gene expression without changing the underlying gene sequence. It includes modifications of histones mostly at lysine residues by addition of acetyl group, methyl group etc. or modification of DNA by addition of mainly methyl residues (Waddington, 2012). Many studies have directly correlated epigenetic changes to memory formation, storage and behavioral outcomes (Jarome and Lubin, 2014). Growing evidences are there that suggest histone acetylation as an important epigenetic mechanism for the consolidation of long-term memory (Levenson et al., 2004, Guan et al., 2002). It occurs at various lysine residues present within the core histone (Roth et al., 2001) and different forms of learning induce different patterns of acetylation at specific gene promoters. In the present study, the activity of the neurons in the IL and PL was associated with the histone acetylation. The acetylation of H3 and H4 at residues K9 and K5 respectively were elevated in the IL and PL following immediate and delayed extinction and it was associated with the changes in CBP levels in these two groups. This shows the possible involvement of CBP as histone acetyltransferase during extinction and the resulting histone acetylation may be controlling the neuronal activation via *c-fos* expression. The activation of neurons, as well as the CBP expression and acetylation of H3 and H4, was compromised in the IL of immediate extinction group and may be one of the reasons behind immediate extinction deficit.

One another important reason for immediate extinction deficit could be the high level of fear observed during the initial trials of the extinction training when extinguished shortly after state of fear there is a failure in encoding the extinction contingency or there is a failure to associate the extinction memories to context. Such animals fail to retrieve the extinction memory during retention test (Maren, 2014).

Similarly, stress has been shown to compromise the function of IL and impairs extinction [Maren and Holmes, 2016). In the immediate extinction group, the extinction training is being done at a very short interval after the fear learning and the unconditioned stimulus (shock) may be acting as a stressor. Therefore, it could be speculated that the deficit seen in the immediate extinction group may be resulting from the stress. The release of stress hormones such as cortisol reportedly change the consolidation of memory and intervene in the formation associative learning such as conditioning and extinction memory by compromising the function of the neural circuits and the molecular substructures involved [Maren and Holmes, 2016, Shors, 2004, Schwabe et al., 2010). Merz et al., 2014 have reported a deficit in fear extinction learning on the administration of cortisol in healthy men. Further studies will be required to test the changes resulting from the stress.

Myers et al., 2006 reported that immediate extinction prevents the return of fear might even erase the fear memory. On the contrary, present study, along with some other studies both in animals (Maren, 2014, Stafford et al., 2013, Long and Fanselow, 2012, Chang and Maren, 2009, Chang and Maren, 2011, Chang et al., 2010, Kim et al., 2010, Woods and Bouton, 2008, Maren and Chang, 2006) and humans (Huff et al., 2009, Schiller et al., 2008, Alvarez et al., 2007) suggest that immediate extinction does not erase fear memory, but rather leads to deficit in extinction retention and correlates to neuronal activation in the PL and IL of mPFC. This in turn seems to be under epigenetic control through acetylation of H3 and H4. Our results are consistent with the growing evidence from animal and human studies proposing an immediate extinction deficit (Maren, 2014) and that the differential region-specific activation in the PL and IL may be under epigenetic control.

The medial prefrontal cortex through its connections with amygdala and hippocampus is reported to play an important role in the suppression of fear response. PL subregion of mPFC through its innervations to basal amygdala (BA) and to the dorsal intercalated cell masses (dITC) controls the centromedial nucleus (CeM) during fear response while the IL innervates centromedial region of amygdala via ventral intercalated cell masses (vITC) that activates centrolateral nucleus of amygdala (CeL) thus suppressing the fear response (Duvarci and Pare, 2014). The present study looked at the changes in the neuronal activity in the PL and IL and associated it to the retention of extinction memory after immediate and delayed extinction. The infralimbic and prelimbic neurons responded differentially to immediate and delayed extinction paradigms. Overall the results of this study correlate that the hypoactivity IL and hyperactivity of the PL of mPFC during the immediate extinction training to the deficit observed during the retention test. Amygdala hyperexcitability that accompanies fear training may hamper mPFC activity along with the hippocampal mPFC circuits thus interfering with the formation of extinction memory and its retrieval. In other words, during immediate extinction the increased PL activity in immediate extinction group may be resulting in hyperexcitability of BLA and dITC neurons which later affect the encoding of extinction memory and its retrieval during retention test. Furthermore, the higher activity of IL in delayed extinction group might be activating CeL and vITC subregion resulting in the proper encoding of extinction memory and its retrieval during retention test.

Our findings from the present study suggest long-term extinction is minimal when extinction is conducted shortly after fear learning in rats and this deficit may be due to the compromised activation of neurons in IL of rats undergone immediate extinction. This deficit in long-term extinction appears to be related to the level of histone acetylation in the IL of the immediate extinction group. Our results suggest that attempts to reduce fear memory shortly after a traumatic event may not be effective and psychological interventions along with the use of specific epigenetic intervention may help in better extinction memory.

Conclusion

In the present study, immediate extinction was found to be less effective than delayed extinction in terms of retention of extinction memory. To sum up, our data for the first time suggests that there may be an association between the activation of IL and PL in the mPFC to the timing of exposure therapy after the traumatic event and this might be under epigenetic control through differential histone acetylation in these regions. The data from the current study may be helpful in planning psychological as well as pharmacological interventions during exposure therapy after traumatic events.

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Conflict of Interest

Authors declare no conflict of interest.

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Legends

Figure 1: Freezing behavior during fear memory consolidation and extinction.

A. Outline for Immediate and Delayed Extinction. **B.** Represents the bregma of Prefrontal cortex used in the study. **C.** Pre-conditioning baseline freezing response was very low and similar in all groups followed by fear learning During Fear learning all the groups exhibited significant increment in % freezing after each consecutive trial and last trial showed robust freezing with no significant difference between the groups (p>0.05). D. Represents pre-extinction baseline freezing among all the groups followed by extinction learning. During extinction learning, both the immediate extinction and delayed extinction group showed a significant decrease in freezing behavior with each consecutive trial. However, the % freezing during the initial trials was higher in immediate extinction group as compared to the delayed extinction group. Both the control groups exhibited low freezing response throughout the trial. **E.** Represents the retention test, performed after 24 hours of the extinction and no extinction training. During retention test, delayed extinction group exhibited a significantly low level of freezing than the immediate extinction and control groups. [N=80-100, n=20 animals in each group (10 for IHC and 10 for retention test)]

Figure 2: *c-fos* expression in PFC following immediate and delayed extinction learning:

The *c-fos* expression was elevated in the PL and IL of both immediate and delayed extinction group when compared to the immediate no extinction and delayed no extinction control groups. However, the *c-fos* expression was significantly higher in IL of the delayed extinction group as compared immediate extinction group and to the immediate and delayed no extinction controls.

Figure 3: CBP expression in PFC following immediate and delayed extinction learning:

The no. of CBP positive neurons was elevated in the PL and IL of both immediate and delayed extinction group when compared to the immediate no extinction and delayed no extinction control groups. However, the no. of CBP positive neurons was significantly higher in IL of the delayed extinction group as compared immediate extinction group and to the immediate and delayed no extinction controls.

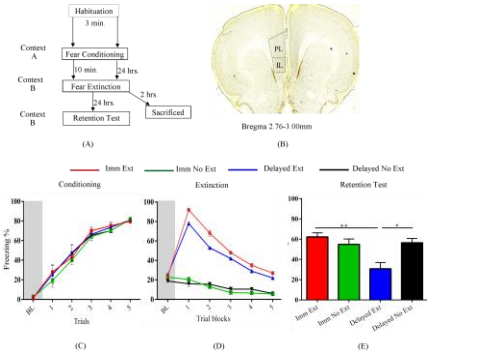
Figure 4: Acetyl H3 expression in PFC following immediate and delayed extinction learning:

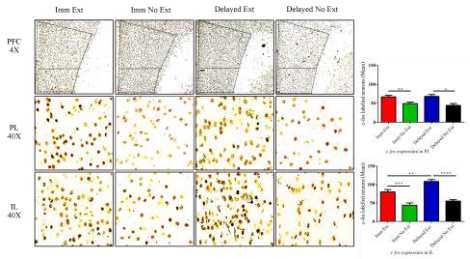
Acetylation of histone H3 at the 9^{th} residue of lysine illustrated increased expression in both immediate and delayed extinction group in both the PL and IL when compared to their respective control groups. Delayed extinction group exhibited significant higher no. of Acetyl H3K9 positive nuclei in IL than the immediate extinction group.

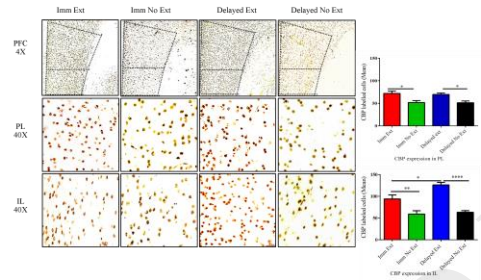
Figure 5: Acetyl H4 expression in PFC following immediate and delayed extinction learning:

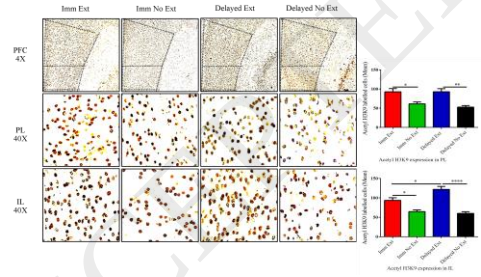
Acetylation of H4 at K5 increased in both the PL and IL region of immediate and delayed extinction group as compared to their respective control groups (immediate no extinction and delayed no extinction). The increase in the IL of the delayed extinction group was significantly higher than that of the immediate extinction group.

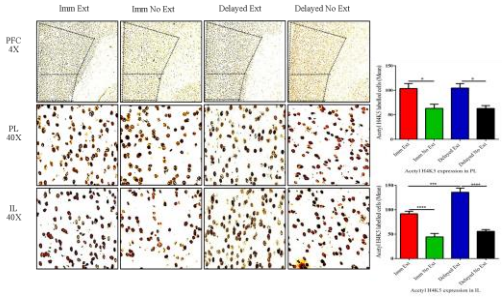
Figure 6: A negative correlation was observed between freezing % in extinction retention group and neuronal expression (positive correlation between extinction learning and neuronal expression) for histone acetylation at H3 k9, H4 k8 along with the expression of *c-fos* and CBP in the IL of delayed extinction group as compared to the immediate extinction group.











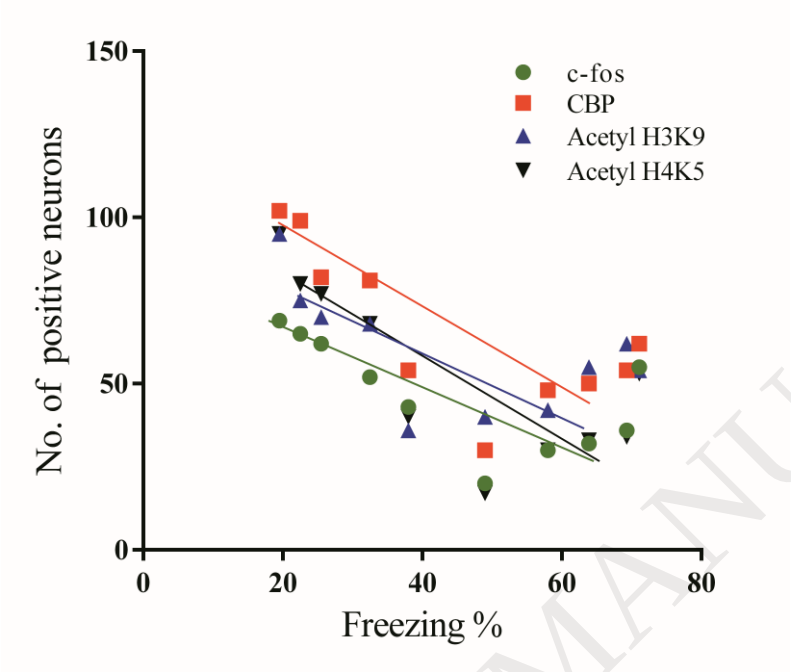


Table 1. Represents the correlation values between freezing % and no. of positive neurons between immediate extinction vs. delayed extinction group in IL region of mPFC.

Freezing %	**No. of positive neurons in IL region of m-PFC**			
	c-fos	**CBP**	**Acetyl H3K9**	**Acetyl H4K5**
***r*-value**	-0.6591	-0.7442	-0.6861	-0.7603
***p*-value**	0.0382 *	0.0136 *	0.0285 *	0.0107 *