Behavioral acclimatization of fear memory and its relation with histone methylation in correlation with accurate nucleus of the hypothalamus, cAMP response element-binding protein and its target gene.

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Abstract : Fear is psychological and psycho physiological response to a perceived external threat that is consciously recognized as a danger. Failure to extinguish traumatic memories can lead to the development of fear associated with anxiety. This is a major challenge amongst researchers and scientists to come up with newer paradigms of exposure therapies for the effective treatment of fear related anxiety disorders. Fear conditioning in rats is a valuable model for study of cellular/molecular mechanism related to learning as well as memory. Intercalated Cell Mass (ITCs) within the amygdala are key regulatory elements controlling emotional responses by controlling amygdala circuits. Central Amygdala (CeA) is the main output relay for the fear response and has innervations of fear traces from Basolateral Amygdala (BLA) as well as from ITCs. Histone methylation is one of the important mechanisms amongst other histone modifications for controlling behavioral outcome through the regulation of gene. In the present report the expression of histone H3/H4 methylation during fear learning and extinction in rats may change the behavioral outcomes as well as the role of histone modifications especially histone methylation was studied in correlation to ARC, CREB, p-CREB to find out whether fear extinction is a inhibitory learning, erasure of memory or both. We found that the expression of p-CREB was found to be higher in extinction group as compared to their respective control groups in the LA region. However, the changes were insignificant between immediate and delayed extinction group while BA, the expression of p-CREB was significantly higher in delayed extinction group .While the CREB 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 LA region. Similarly the ARC, 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 LA region. Moreover, the level of histone methylation in amygdale is suppressed. The present research highlights the analysis of molecular events in parts of brain and inturn has a great impact on the methods of physiological and psychological researches specially in the field of behavioral science.

Keywords: Behavior, Fear, PTSD, Amygdala, Histone metylation**.**

Introduction: Fear is psychological and psycho physiological response to a perceived external threat that is consciously recognized as a danger. Fear response include increased alertness; concentration on the source of fear; attack and fight-or-flight behaviors and evidence of sympathetic nerve stimulation such as cardiovascular excitation, superficial vasoconstriction and dilation of the pupils. Failure to extinguish traumatic memories can lead to the development of fear associated anxiety disorders viz. Post traumatic stress disorder (PTSD) (**VanElzakker et al., 2014**). These kind of people are mainly cured by exposure therapy based on extinction learning and its retention (**Craske et al., 2008, Rothbaum et al., 2003, Bouton et al., 2001**) and the most importantly reported by Pavlovian based fear conditioning in rats (**Maren, 2005, Pare et al., 2004, LeDoux, 2000**). In healthy subjects, the main brain regions i.e. prefrontal cortex (PFC), hippocampus and amygdala are critical for processing fearful and other kind of emotional stimuli and fear learning to extinguish fear in situation that are no longer threatening.

This is a major challenge amongst researchers and scientists 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**). Any disturbance in it will develop fear related disorders such as post-traumatic Stress disorder (PTSD). The treatment paradigms for these fear related disorders are at par and the wounded have to lead a pathetic life. However, erasure of fear memory is a phenomenon during which there is reversal of molecular events taking place during fear acquisition and extinction.

Previous reports 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**). Interestingly, it was found that extinction training performed

immediately after the fear learning resulted in either "erasure" (**Norrholm et al., 2008**) or fear reduction (**Chang and Maren, 2009**). Signaling cascades in brain structures are reportedly important in fear acquisition and extinction through inhibitory learning. Although histone modification occurs throughout the sequence, the unstructured N-termini of histones (called histone tails) are most extensively modified. These modifications include acetylation, methylation, ubiquitylation, phosphorylation and sumoylation. Thus,-CH3 is found to induce repression of transcription and hence shows to influence a rat in memory consolidation as well as extinction. Fear conditioning in rats is a valuable model for study of cellular/molecular mechanism related to learning as well as memory.

Intercalated Cell Mass (ITCs) within the amygdala is key regulatory element controlling emotional responses by controlling amygdala circuits. Central Amygdala (CeA) is the main output relay for the fear response and has innervations of fear traces from Basolateral Amygdala (BLA) as well as from ITCs. Extinction of fear involves extinction trace innervations into CeA from ITCs directly and from prefrontal cortex (PFC) via ITCs. Histone methylation is one of the important mechanisms amongst other histone modifications for controlling behavioral outcome through the regulation of gene. Amygdala micro-circuitry is integrated with different interconnected nuclei having different neuronal types and connections. Central Amygdala (CeA) is the main output circuitry for the fear response which has innervations of fear traces from basolateral amygdala (BLA) as well as from Intercalated cell mass (ITCs). As CeM (medial nucleus of the central amygdala) is the main output nucleus for the fear responses it is controlled by CeL (lateral nucleus of the central Amygdala) as well as by ITCv (ventral intercalated neurons) in different states of fear conditioning and extinction. During extinction new connections are formed in brain in amygdala, prefrontal cortex, hippocampus and brain stem (**Herry et al, 2010**). Deciphering the signaling pathways and circuitries leading to erasure of fear memory will be of immense importance as the treatment paradigms based on inhibitory learning are not adequate and fear memory gets reactivated even after several rounds of extinction training. Keeping in view the large number of people suffering from these conditions it becomes imperative to come up with newer drug targets which may help in overcoming these conditions.

In the present research, the impact of expression of histone H3/H4 methylation during fear learning and extinction on the behavioral outcomes. It was studied it was hypothesized that the role of histone modifications especially histone methylation was studied in correlation to ARC, CREB, p-CREB and its target gene and also explored whether fear extinction is a inhibitory learning erasure of memory or both.

Materials and Methods: The experiments were conducted on rat as subject for characterizing the neuronal activity in both the Basal Amygdala (BA) and Lateral Amygdala (LA), following immediate and delayed extinction. For the study male Sprague-Dawley rats (250-300 gm) were used. Rats were housed individually with access to food and water adlibitum. Rats were handled for 15 days up to 2-5 minutes each day on a 12-h light/dark cycle, 23°C temperature. All experiments were carried out under strict compliance with committee for the purpose of control and supervision of experiments on animals as per the CPCSF guidelines (853/AC/04/CPCSF).

Subject: Male Sprague-Dawley rats weighing (250-300 gm) were used as subjects for the study. Male Sprague-Dawley rats (3 weeks old) were procured from Indian Institute of Toxicological Research (IITR) Lucknow. They were housed one per cage under standard conditions at temperature of 23°C ± 1°C and 50 % humidity with a 12-h light/dark cycle. Food and water was made available adlibitium to them in due meliu of guidelines established by the Institutional animal care and use committee of BBA University, Lucknow.

Conditioning apparatus: Behavioral fear testing experiments were conducted in two chambers 'A' and 'B' in various contexts. In current study, the experiment was carried out in separate departmental experimental laboratory at biotechnology BBAU. Context 'A' served as conditioning chamber while context 'B' served as extinction chamber. Both contexts had two transparent walls and stainless steel grid floors, however, the grid floor in context B covered with flat white acrylic inserts to minimize context generalization. Context A was wiped down before testing with 10% ethanol and context B with 10% methanol. Personalized video monitoring cameras were mounted in the ceiling of each chamber which are connected with a quad processor to an advanced recorder and monitor for videos and scoring of freezing. Grid floors were connected to a scrambled shock source. Auditory stimuli were delivered via a speaker in the chamber wall. Delivery of stimuli was controlled with a personal computer and was maintained at 80dB throughout behavioral testing.

Fear Conditioning: For acclimatizing the subjects, they were handled for 5-10 min everyday for a week prior to the experiment. After a week, the rats were brought in a separate white cage and were placed directly into the conditioning .Exposure to the room environment was avoided and the subjects were left in the chamber for stabilizing in the conditioning context, Before initiating the fear training session the readings of base line freezing were recorded. Further, the subjects were exposed to five trials of Conditioning Stimulus (CS) – Unconditional Stimulus (US) and the readings of freezing were recorded in the mode when no body movements are shown expect breathing and heart-beat .Each trial lasted for 60 seconds with an inter-trial interval (ITI) of 1 minute at every 10 seconds and than the rats were subjected to the CS. The CS was provided for 10 seconds with the 80 dB intensity tone of 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). One

minute after the completion of the behavioral session, rats were returned to their home cage. Protocol for the study is summarized in Figure 1.

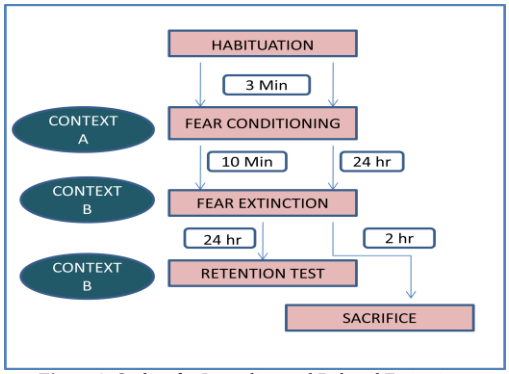


Figure 1. Outline for Immediate and Delayed Extinction.

Fear Extinction: The extinction training was performed after animal behavioral conditioning (fear learning) in a separate room chamber for animal behavior. It was performed at two different stages with duration of 10 min and 24 hours. After fear learning the subjects were placed in a fear extinction chamber for stabilizing the fear extinction context and recorded base line of the freezing. The subjects were placed in the context for 3 min during the extinction process and both the tone and shock were administered on the subjects. The freezing percentage of the subjects were recorded without application of both the conditions after the baseline sessions. 30 CS (tone 80dB for 10 seconds) was administered to the rats with an ITI of 10 seconds. Behavior was measured in the absence of US. In the whole extinction process thirty trials were analyzed and were reported as five trial blocks. Average of every six consecutive trials (six trial = 1 T block, Thus 30 trial = 55 block) was taken. The study included four groups i.e immediate extinction, immediate no extinction, delayed extinction or delayed no extinction to their corresponding two control groups in the absence of tone CS or shock US. Minutes of acclimatization period and extinction training was performed in a context B (V.J. Instruments). All the subjects were presented with 30 trials of CS (tone, 80db total duration 10s, Intertrial interval: 10 s) in the absence of US. Animals (N=10 per cage) were sacrificed for Immunohistochemistry (IHC) analysis 2 hr after extinction training. An additional group of the same aged subjects who has not received any experimental manipulation, were used as naive control in all experimental procedures. Conditioning, extinction as well as control group underwent retention test for 24h. Moreover, the training involved the presentation of the CS (5 tones, total duration 10s and 80db) Inter trial interval: 10 s in context B in the absence of foot shock. The animals which were used for IHC did not experience retention test.

Control groups: For removing the confounding effect of behavior, additional group of animals were included as tone only (CS alone), context only (expose for the same duration to context) and shock only (Shock treated without tone). The context only group (n=10) were subjected to experience the context A and context B in absence of any tone or foot-shock for conditioning and extinction group, respectively. Context only group was allowed to expose the conditioning and extinction context for the same duration as conditioned and extinction group. In tone only group, the rats were confronted with 80 db for 10 sec, ITI, 60sec in conditioning chamber (n=10). The shock only group received 5 trials of shock (0.7mA), ITI 60 sec in the absence of any tone and freezing was measured for the overall duration.

Details of Brain Sub-Regions under Study: Two brain regions (amygdale and prefrontal cortex) involve in fear memory consolidation and extinction were taken into the study. Amygdala consist of LA and BA (**Mc Donald, 1998; Turner and Herkerham, 1991; Krettek and price 1978**) which regulate the expression of fear in rodents respectively (**Giustino and Maren, 2015**).

Behavioral training: Naive subjects were not trained at all and remained in their home cages until sacrifice. Subjects were trained for fear conditioning and extinction inside a conditioning chamber (V. J. Instrument). Before experiments, subjects were allowed to acclimatize in the chamber for 3 minutes. For conditioning of fear, subjects were exposed to five trials of an acoustic signal (80 dB, 10 s) of tone co-terminated with a foot-shock of intensity of 1mA for 1sec. The ITI were used of 10 second after each trial. Subjects were then placed into their home cages. After 24 hours, subjects were re-introduced to the chamber for extinction experiment by providing 30 trials of a tone (80dB, 10sec) in the chamber with context B in the absence of shock and 10sec ITI. Subjects were then again placed into their home cages. Freezing behavior was recorded and analyzed via a video camera connected to video tracking software (V. J. Instrument).

Behavioral test: 24 hours after conditioning, fear retention test was performed by the presentation of the CS (5 tones, 10 s, 80 dB, inter-trial interval: 10 s) in context B for all groups from both the aims. The subjects of the test group were not used for molecular studies while the groups which were used for IHC, did not undergo retention test.

Perfusion: Anaesthetize rat with 50 mg/Kg body weight Sodium pentobarbital (Nembutal) Cut from sternum to expose the heart. Needles was inserted from left ventricle into the right aorta and clip it to keep in place, then right auricle was cut and cold normal saline was passed immediately. After passing approximately 100 ml normal saline, approximately 400 ml ice cold 4% paraformaldehyde was also passed to fix the animal. The brains were than taken out carefully and kept in 4% paraformaldehyde for 24 hours for post fixation.

Cryosectioning: Brains were transferred from paraformaldehyde to 10% sucrose, kept overnight (tissue had to sink fully to the bottom) and transferred to 20% sucrose (for the brain tissue to sink fully, it may take more than 24 hours) then transferred to 30% sucrose (for the brain tissue to sink fully, it took more than 24 hours) followed by freezing of brains (Figure 2).

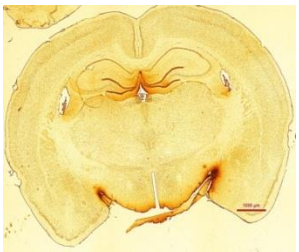


Figure 2. Represents the Bregma of amygdala used in the study.

Immunohistochemistry: Rats from each group were anesthetized using pentobarbital (50 mg/kg) and animals were perfused intracardially with paraformaldehyde. Brains were taken out, followed by sucrose treatment and freezing in isopentane. 3'-Diaminobenzidine (DAB), immunostaining was performed for cAMP response element-binding protein (CREB), Pre- cAMP response element-binding protein (p-CREB. Accurate nucleus of the hypothalamus (ARC) and Dimethyl H3K9me2 coronal brain sections (20 µm) from amygdala region were prepared using cryostat. Brain sections were blocked with normal goat serum (NGS) followed by incubation for overnight at room temperature with-CREB (1:500), p-CREB (1:500), ARC (1:500) and Di-methyl (1:500) antibody. Sections were washed and incubated with biotinylated IgG for 2 hours. DAB staining was performed.

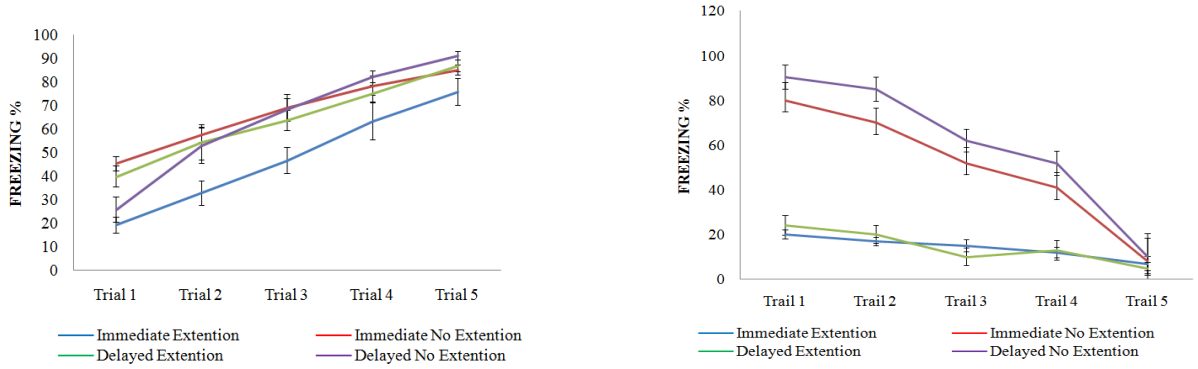
Statistical Analysis: Behavioral data has been presented as means ± standard error (± SEM) and was analyzed using three-way repeated measures followed by Tukey's *post hoc* analysis. For each session, the freezing data was transformed to the percent value. Retention test data and IHC data were analyzed 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 CREB, p-CREB, ARC and histone methylation in the BA and LA Sub region.

Result

Behavioral training for fear Conditioning and Extinction

Behavior: In the present study, The effect of timing of extinction training relative to fear learning on retention of extinction memory was explored .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 increased with each successive trial across the groups and the result was confirmed by three-way repeated measure of conditioning. Data revealed a significant main effect of trials [$F(3,116) = 70.12$, $p<0.0001$] and the interaction of trial [$F(3,116)= 24.10$, $p<0.05$] in the immediate and delayed extinction group during fear learning (Figure 3a).

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. 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 and trial [$F(3,116) = 7.144$, $p<0.0001$] followed by *post-hoc* analysis which confirmed that extinction groups froze more significantly as compared to the 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 (Figure 3b).



(A) (B)

Figure 3. (A) In freezing response of pre-conditioning baseline was very low in all groups of fear learning memory. During experiment of fear learning memory of all the groups is significant percent % is increase after all each consecutive all trials and each trial showed freezing also with no significant percent % difference between the each groups (p>0.05). **(B)** The pre –extinction base line of freezing response of all the groups followed by extinction learning memory .During experiment extinction learning memory, immediate extinction and delayed extinction both groups showed a significant percent % decrease in freezing behavior with each consecutive trial in experiment .However, the percent % freezing during experiment the initial trials was lower in delayed extinction group as compare to immediate extinction group. And also the both control group exhibited low percent % freezing response throughout the trial.

Retention test showed that delayed extinction group exhibited the least level of freezing as compared to the immediate extinction and no extinction group. Two-way ANOVA of retention test showed highly significant association *w.r.t.* time [F (3,116) = 8.20, $p<0.005$] and condition X time interaction [F (3,116) = 17.37, $p<0.0005$] but no significant main effect of condition [F (3, 116) =12.36 p>0.00005] 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 ($p<0.0005$) and delayed no extinction group ($p<0.0005$) Figure 4.

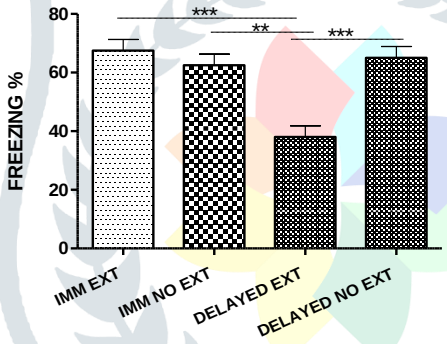


Figure 4. Animal behavior retention test experiment, performed after one day 24 hours of the extinction and no extinction training test .during experiment retention test, group delayed extinction exhibited a significantly percent % low level of freezing than the immediate extinction group and control group. [N=60-100, n=40 animal SD (Sprague Dowly) rat in each group (20 for immunohistochemistry and 20 for retention test)

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 *di-methyl (k3k9me2) expression* has been extensively used as a neuronal activity marker in various studies *a*nd its expression has been correlated to region-specific activity in different brain regions. Our next aim was to examine the region-specific neuronal activity in the BA and LA sub regions of the amygdala following immediate and delayed *extinction*. To accomplish this objective, Coronal brain sections containing the Amygdala were immunostained for di-methyl (h3k9me2) from each group.

p-CREB expression following immediate and delayed extinction: The expression of p-CREB was found to be higher in extinction group as compared to their respective control groups in the LA region however, the changes were insignificant between immediate and delayed extinction group. Two-way ANOVA analysis for p-CREB expression in LA region revealed a significant main effect of extinction condition (extinction vs. no extinction) [F (3, 26) =184, P < 0.0001], Figure 5, However in BA, the expression of p-CREB was significantly higher in delayed extinction group as compared with the immediate extinction group and delayed no extinction group. The expression of p-CREB was further confirmed by two ways ANOVA that revealed a significant main effect of extinction condition (extinction vs. no extinction) [F (3, 26) =184, P < 0.0001] extinction time (immediate extinction vs. delayed extinction) [F (3, 26) = 146, P < 0.0001] as well as extinction condition and extinction time interaction [F (3, 26) = 14.4, P < 0.0001]. In other words, the two sub regions of Amygdala responded differentially to the two extinction paradigms. This differential expression of p-CREB exemplifying the activity in the BA and LA in the immediate and delayed extinction group may be responsible for the deficit in the retention of extinction memory as observed after immediate extinction Figure 5a and 5b.

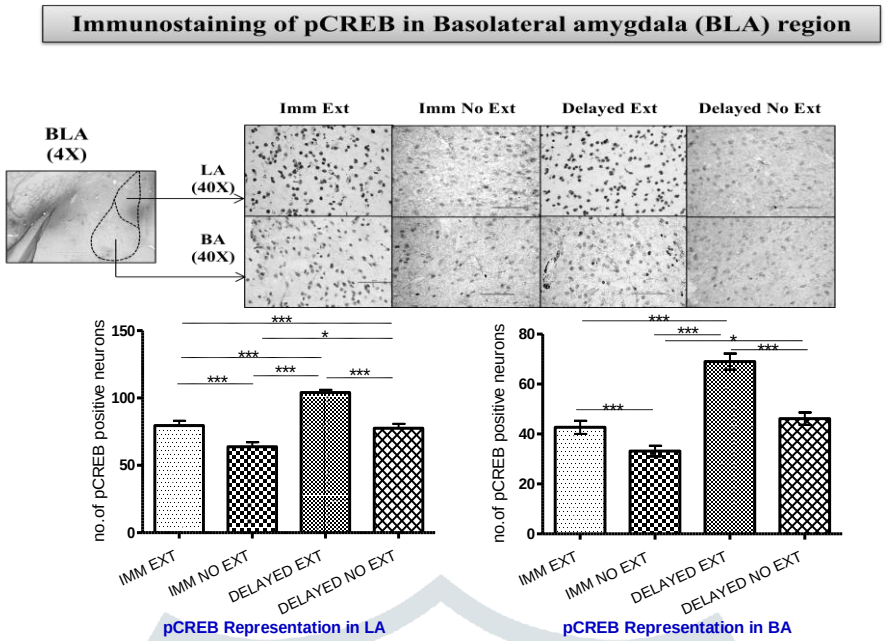


Figure 5. p-*CREB expression* in amygdala following immediate and delayed extinction learning: The p-*CREB expression* was elevated in the BA and LA of both immediate and delayed extinction group when compared to the immediate no extinction and delayed no extinction control groups. However, the p-*CREB* expression was significantly higher in LA of the delayed extinction group as compared immediate extinction group and to the immediate and delayed no extinction controls.

CREB expression following immediate and delayed extinction: The CREB 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 LA region (Figure 6). Two-way ANOVA analysis for CREB expression in LA region revealed significant main effect of extinction condition (extinction vs. no extinction) [$F(3, 26) = 71.2$, $P < 0.0001$] but no effect of extinction time (immediate vs. delayed [$F(3, 26) = 445$, $P < 0.0001$] and condition x time interaction [$F(3, 26) = 5.75$, $P < 0.0001$] Figure 6a. However. In BA, a significantly increased expression of CREB 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 [$F(3, 26) = 94.1$, $P < 0.0001$], extinction time [$F(3, 26) = 9.50$, $P < 0.0001$] (Figure 6b). Expression of CREB seems to be associated with neuronal activity in the BA and LA following immediate and delayed extinction. We next wanted to see whether the increased CREB levels in these regions culminated in methylation of H3 and H4, the reason being studies suggesting methylation of Histone at various lysine residues to be associated with enhanced expression of genes required for synaptic activity and memory consolidation.

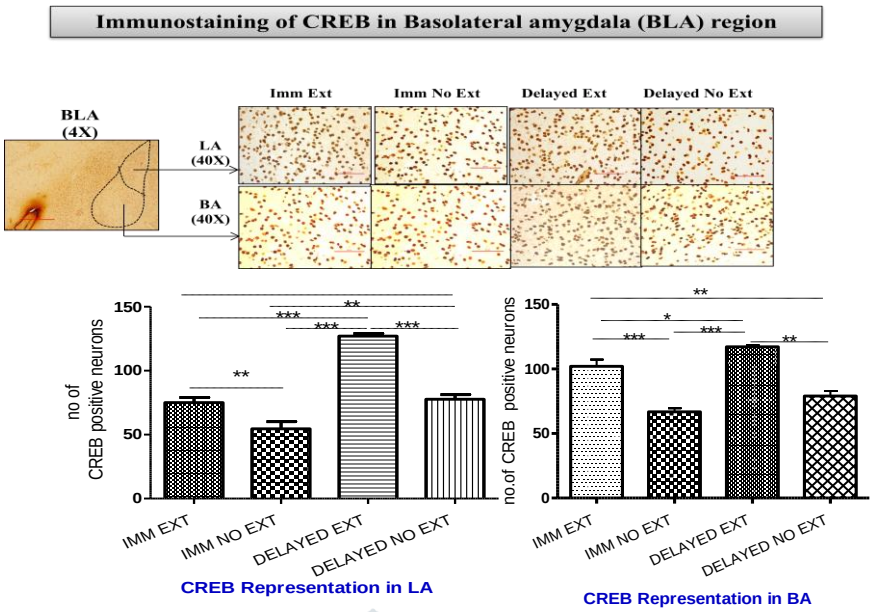


Figure 6. *CREB expression* in amygdala following immediate and delayed extinction fear learning. The *CREB expression* was elevated in the BA and LA of both immediate and delayed extinction group when compared to the immediate no extinction and delayed no extinction control groups. However, the *CREB* expression was significantly higher in BA of the delayed extinction group as compared immediate extinction group and to the immediate and delayed no extinction controls.

ARC expression following immediate and delayed extinction

Similar to the ARC, 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 LA region. Two-way ANOVA analysis for CREB expression in LA region revealed significant main effect of extinction condition (extinction vs. no extinction [F (3, 26) =162.2, P < 0.0001] (Figure 7a) but no effect of extinction time (immediate vs. delayed) and condition x time interaction. However in BA, a significantly increased expression of CREB 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 (3, 26) =195.2, P < 0.0001] (Figure 7b). Expression of ARC seems to be associated with neuronal activity in the BA and LA following immediate and delayed extinction. We next wanted to see whether the increased ARC levels in these regions culminated in methylation of H3 and H4, the reason being studies suggesting methylation of Histone at various lysine residues to be associated with enhanced expression of genes required for synaptic activity and memory consolidation.

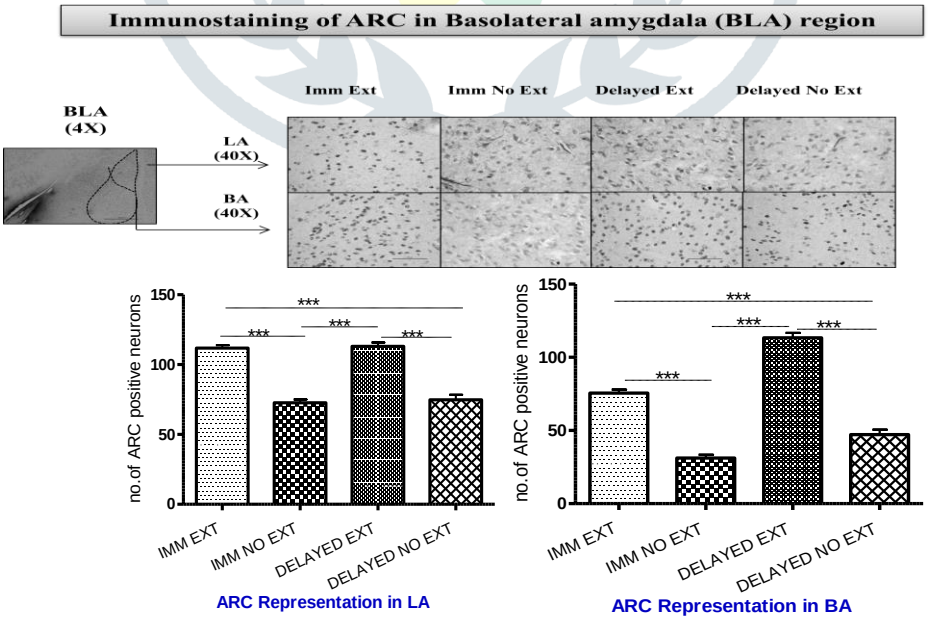


Figure 7. ARC expression in amydala following immediate and delayed extinction fear learning:

The number of ARC positive neurons was elevated in the BA and LA of both immediate and delayed extinction group when compared to the immediate no extinction and delayed no extinction control groups. However, the no. of ARC positive neurons was significantly higher in LA of the immediate extinction group as compared immediate extinction group in BA and to the immediate and delayed no extinction controls.

Histone methylation following immediate and delayed extinction: To correlate the neuronal activation to histone methylation the histone methylation in the LA and BA following immediate and delayed extinction were checked.

We gauged the levels of methyl H3 at lysine residue 9 (K9) and methyl H4 at lysine residue 5 (K5) in LA and BA following the two extinction paradigms. Expression of methyl H3K9 in LA region showed that there was no significant difference between immediate extinction and delayed extinction group, however Immediate and delayed extinction group has a lower level of methyl 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) [$F(3, 26) = 73.3$, $P < 0.0001$], in comparison to the effect of extinction time (immediate vs. delayed) [$F(3, 26) = 8.46$, $P < 0.0001$] (Figure 8a). On the other hand in LA region, the expression of H3K9 was significantly lower in delayed extinction group as compared to the delayed no extinction group and immediate extinction group. Two-way ANOVA analysis of BA [$F(3, 26) = 96.6$, $P < 0.0001$] revealed significant main effect of extinction condition (extinction vs. no Extinction) [$F(3, 26) = 96.6$, $P < 0.0001$] and extinction time (immediate vs. delayed) [$F(3, 26) = 5.35$, $P < 0.0001$] (Figure 8b). These changes in the H3 methylation were linked to the neuronal activity i.e., the ARC, CREB, P-CREB in a region-specific manner. Similar to the H3K9 me2, the Immediate and delayed extinction group exhibited lower level of H3K9 expression, when compared to their respective control groups in BA 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) [$F(3, 26) = 96.6$, $P < 0.0001$]. but no significant difference was observed for extinction time (immediate vs. delayed extinction) [$F(3, 26) = 5.35$, $P < 0.0001$] (Figure 8).

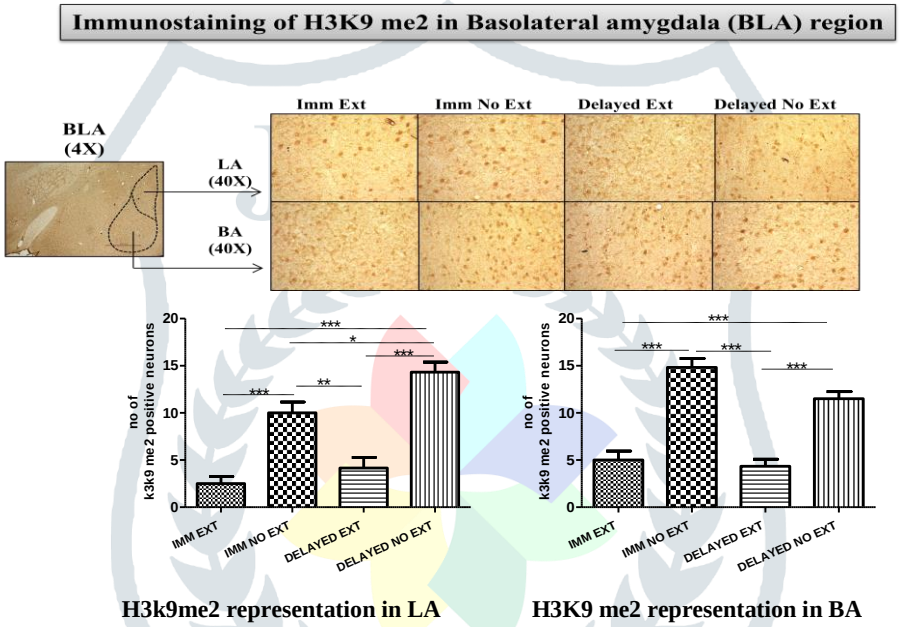


Figure 8. Methyl H3k9 me2 expression in amygdala following immediate and delayed extinction learning: Methylation of histone H3 at the 9th residue of lysine illustrated decrease expression in both immediate and delayed extinction group in both the LA and BA when compared to their respective control groups. Delayed extinction group exhibited significant lower no. of Methyl H3K9 positive nuclei in BA and LA than the control group.

Discussion: In the present study, the effect of immediate and delayed extinction on retention of extinction memory along with neuronal activation and histone methylation in amygdala was examined. 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, 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. Fear circuitry in amygdala poses its effect of fear expression via its interconnected nuclei. An increase in activity of these nuclei leads to different behavior outcomes governed by the stimuli received. Fear learning and extinction induces synapse formation by new neuronal connections, activating different sets of genes within the amygdala which in turn regulate the activity of that part. Acetylation of histone leads to activation of a gene by allowing the access of transcription factors which is the case here also in consolidation and extinction.

This form of learning is characterized by a decrease in a fear response when the contingent relationship between a conditioned stimulus (e.g., a sound) and an unconditioned stimulus (e.g., an electric shock) is compromised. This situation is most commonly implemented when the CS is repeatedly presented in the absence of the shock (**Myers and Davis, 2002**). The resilience of traumatic memories to extinction represents a serious obstacle for treating disorders characterized by abnormal fear and anxiety. Therefore understanding the basic mechanisms underlying fear extinction will lead to improvements in the treatment of this anxiety disorders (**Myers and Davis, 2007**). Studies have pointed out environmental effect on memory formation via the regulation of neuronal histone acetylation (**Levenson et al., 2004**). It has been demonstrated that CREB-binding protein (CBP), a potent HAT and

transcriptional co activator is critical for long-lasting forms of synaptic plasticity (the activity- dependent changes in the strength of neuronal connections) and long-term memory (**Levenson and Sweatt, 2006**).

Firstly it was discovered in the 1960's, histone methylation is a common histone mark that occurs by the addition of a methyl group (-CH3) onto a lysine or arginine amino acid residue (**Murray,1964; Greer and Shi, 2012**). Studies of animals with genetic manipulations of the G9a (**Gupta-Agarwal et al., 2012**) and Mll2/Kmt2b genes (**Kerimoglu et al., 2013**) have firmly defined a role for histone methylation in both learning and memory. A role for HDAC3 has also been identifed for addictions, where inhibition of this enzyme enhanced behavior performance in fear memory formation and the extinction of drug- seeking behavior (**Malvaez et al., 2013**). Effect of epigenetic modifications in addiction is not unidirectional or simple. Histone methylation can activate or repress gene transcription depending on the specific lysine residue modified and as a result can have both negative and positive effects on drug-associated behaviors. Methylation of H3K9 in the NAc inhibits behavioral responses to cocaine and morphine (**Maze et al., 2010**), but methylation of H3K4 in the NAc enhances methamphetamine-induced conditioned place preference (**Aguilar- Valles et al., 2014**). However, histone methylation is necessary for proper long term consolidation of contextual fear memories. However, histone modifications like histone acetylation/phosphorylation facilitate the unraveling of DNA around the histone proteins resulting in the recruitment of the transcriptional machinery which is responsible for mediating gene expression of cell. Apart from this other histone modifications like histone methylation have controversial effects on gene transcription depending on the type of amino acid residue modification and the number of methyl groups present.

As per previous reports histone methylation was implicated in the regulation of chromatin structure present in nervous system (**Tsankova et al., 2006; Huang et al., 2007**). Moreover, till date no such reports of histone methylation regulation in the nervous system functioning is available to represent the process of long-term memory storage. Histone methylation as a potential upregulator of gene transcription in memory consolidation and may be involved in bidirectionally modify gene expression which is depending on the modification of histone residue as well as number of methyl groups present on a given residue. For example, histone tri-methylation is linked to gene repression at H3K9 (H3K9me3), but associated to gene activation at H3K4 (H3K4me3). Histone modifications are catalyzed by various enzymes and the modifications are reversible. As per the previous reports it was found that the enzymes are histone acetyltransferases (HAT) and histone deacetylase complexes (HDACs). HATs transfer an acetyl group to lysine residues, whereas HDACs remove acetyl groups. Histone post-translational modifications are key epigenetic processes controlling the regulation of gene transcription. In recent scenario, it has become apparent that chromatin modifications contribute to cognition through the modulation of gene expression required for the expression and consolidation of memories. Most of the studies on epigenetics in cognition have focused on DNA methylation and histone H3 (de-) acetylation and have been summarized in excellent recent reviews (**Miller et al., 2010**). While DNA methylation is generally considered to inhibit gene transcription and histone acetylation is considered to be activating, histone methylation can achieve both gene activation and repression. Histone methylation initially received less attention, because it was long assumed to be rigid and therefore of little interest to dynamic processes involved in cognition, such as learning and memory. It has been suggested that histone methylation could also be dynamically regulated (**Gräff & Mansuy, 2008; Agarwal et al., 2012**). In recent years, numerous studies have provided evidence that changes in histone lysine methylation, leading to gene expression activation or repression, are also required for the formation and consolidation of long-term memory (LTM) in particular brain regions.

However, 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. Many studies have directly correlated epigenetic changes to memory formation, storage and behavioral outcomes (**Jarome and Lubin, 2014**) as well as epigenetic mechanism for the consolidation of long-term memory was also reported (**Levenson et al., 2004, Guan et al., 2002**) and which is an important consideration when studying epigenetic mechanisms that are involved in cancer formation (**Gong et al., 2016**) Like histone modifications, DNA methylation may constitute an epigenetic code. These complex gene transcription programs initiated during cellular differentiation and division appear to be epigenetically regulated. Epigenetic changes can simply be viewed as one of the final steps (or perhaps the final step) in a long cascade of events that leads to learning-related gene transcription. These epigenetic mechanisms are involved in both biochemical and behavioral responses to drugs of abuse. The first of these studies employed a chromatin immunoprecipitation approach to identify histone modifications at individual gene-targets in the nucleus accumbens following cocaine treatment (**Murray et al., 1964; Lubin at al., 2011; Greer et al., 2012**).

The unwinding story of these two sub-regions viz. LA and BA and hippocampus which regulate the consolidation and retention of fear and extinction in memory (**Penke et al., 2016**). It was found that the expression of p-CREB was found to be higher in extinction group as compared to their respective control groups in the LA region however, the changes were insignificant between immediate and delayed extinction group. However in BA, the expression of p-CREB was significantly higher in delayed extinction group as compared with the immediate extinction group and delayed no extinction group. This predicts that the two sub regions of Amygdala responded differentially to the two extinction paradigms. This differential expression of p-CREB exemplifying the activity in the BA and LA in the immediate and delayed extinction group may be responsible for the deficit in the retention of extinction memory. While the CREB expression was higher in extinction groups as compared to their respective

control groups no significant changes were observed between immediate and delayed extinction groups in LA region. However, in BA,there was a significantly increased expression of CREB in delayed extinction group as compared with the immediate extinction group and delayed no extinction group and it was expressed that CREB seems to be associated with neuronal activity in the BA and LA, following immediate and delayed extinction. Furthermore, the increased CREB levels in these regions culminated in methylation of H3 and H4, the reason being studies suggesting methylation of Histone at various lysine residues to be associated with enhanced expression of genes required for synaptic activity and memory consolidation (**Parkel et al., 2013; Petrovich et al., 1997; Akbarian etr al., 2009; Tzeng et al., 2007; Veening et al., 1984**).

Similar to the ARC, 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 LA region. However, the expression of ARC seems to be associated with neuronal activity in the BA and LA following immediate and delayed extinction. Expression of methyl H3K9 in LA region showed that there was no significant difference between immediate extinction and delayed extinction group however immediate and delayed extinction group has a lower level of methyl H3K9 positive neurons with respect to immediate no extinction and delayed no extinction group and these changes in the H3 methylation were linked to the neuronal activity i.e., the ARC, CREB, P-CREB in a region-specific manner similar to the H3K9 me2, The immediate and delayed extinction group exhibited lower level of H3K9 expression. Our results on behavioral acclimatization of fear memory in relation with histone methylation in correlation with accurate nucleus of the hypothalamus (ARC), cAMP response element-binding protein (CREB), p-CREB as well as its target gene suggest that the activation of neurons in the amygdala region is compromised during fear memory-extinction in both the BA and LA regions. However, the level of histone methylation in amygdale is suppressed.

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