Thesis on

"HISTONE ACETYLATION RELATED ENHANCED EXPRESSION OF CREB TARGETED GENES MAY UNDERLIE ERASURE OF FEAR MEMORY LEADING TO EXTINCTION"

Submitted for the Degree of
Doctor of Philosophy
in Biotechnology

By

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Dedicated to,

My Mother Kaushilya Singh, Father Vinod Singh, brother Ashish Kumar Singh and Pankaj Kumar Singh, and everyone who stood by me through all walks of my life

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CERTIFICATE

This is to certify that the thesis titled "*HISTONE ACETYLATION RELATED ENHANCED EXPRESSION OF CREB TARGETED GENES MAY UNDERLIE ERASURE OF FEAR MEMORY LEADING TO EXTINCTION*" submitted by **Sanjay Singh** is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or other university.

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I hereby declare that thesis entitled ***"HISTONE ACETYLATION RELATED ENHANCED EXPRESSION OF CREB TARGETED GENES MAY UNDERLIE ERASURE OF FEAR MEMORY LEADING TO EXTINCTION"*** is an authentic research work carried out by me under the supervision of **Prof. Dinesh Raj Modi**, Professor, Department of Biotechnology, Babasaheb Bhimrao Ambedkar University, Lucknow and the Co-Supervision of **Prof. Anand Prakash**, Professor, Mahatma Gandhi Central University, Motihari, Bihar. The research work is original, and no part of this work has been submitted for any other degree or diploma.

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List of Publication's

1. Vandana Ranjan, **Sanjay Singh**, Sarfraj Ahmad Siddqui, Sukanya Tripathi, M. Y. Khan and Anand Prakash **"Differential Histone acetylation in sub-regions of BNST underlies fear consolidation and extinction".** Psychiatry Investigation. ISSN 1738-3684 On-line ISSN 1976-3026.

2. **Sanjay Singh,** Sarfraj Ahmad Siddiqui, Sukanya Tripathy, Shiv Kumar, Sudipta Saha, Rajesh Ugale, Dinesh Raj Modi, Anand Prakash, **"Decreased level of histone acetylation in the infralimbic prefrontal cortex following immediate extinction may result in deficit of extinction memory".** Brain Research Bulletin, Volume 140, June 2018, Pages 355-364.

3. Sarfraj Ahmad Siddiqui, **Sanjay Singh**, Vandana Ranjan, Rajesh Ugale, Sudipta Saha, Anand Prakash, "**Enhanced Histone Acetylation in the Infralimbic Prefrontal Cortex is Associated with Fear Extinction**". Cell Mol Neurobiol DOI 10.1007/s10571-017-0464-6.

4. Vandana Ranjan, **Sanjay Singh**, Sarfraj Ahmad Siddiqui, M. Y. Khan and Anand Prakash, "**Differential histone acetylation in the amygdala leads to fear memory consolidation and extinction**". International journal of Science,Technology and Society, ISSN-2395-1605.

5. Sarfraj Ahmad Siddiqui, **Sanjay Singh**, Rajesh Ugale, Vandana Ranjan, Rohit Kanojia, Sudipta Saha, Sukanya Tripathy, Shiv Kumar, Sudhir Mehrotra, Dinesh Raj Modi, Anand Prakash, "**Regulation of HDAC1 and HDAC2 during consolidation and extinction of fear memory**". https://doi.org/10.1016/j.brainresbull.2019.05.011.

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Sincerely,

Sanjay Singh

List of Abbreviations

Amg	Amygdala
ARC	Activity Regulated Cytoskeleton Protein
BA	Basal nucleus of amygdala
BDNF	Brain Derived Neurotrophic Factor
BLA	Basolateral amygdala
CBP	CREB Binding Protein
CeA	Central nucleus of amygdala
CeL	Lateral central nucleus of amygdala
CeM	Medial central nucleus of amygdala
Cond.	Condition/conditioned
CR	Conditioned Response
CREB	Cyclic-AMP Response Element Binding protein
CS	Conditioned Stimulus
Ctx	Context
EC	Entorhinal cortex
Elk-1	ETS-like gene-1
ERK	Extracellular signal regulated Kinase
Ext.	Extinction
GRs	Glutamate receptors
HAT	Histone Acetyl Transfearse
HDAC	Histone Deacetylase
HF	Hippocampal Formation
Hipp	Hippocampus
IHC	Immunohistochemistry
IL	Infralimbic cortex of PFC
LA	Lateral nucleus of amygdala
LTM	Long Term Memory
LTP	Long Term Potentiation
MAPK	Mitogen Activated Protein Kinase
MSK1	NMDA/ERK/ Mitogen activated S6 kinases-1
NCS	Neocortical System
NMDA	N-methyl –D-aspartate
PBS	Phosphate Buffered Saline
PD	Postnatal day
PER	Perirhinal cortex
PFC	Prefrontal cortex
POR	Post rhinal cortex
PTSD	Post Traumatic Stress Disorders

RT-PCR	Real Time Polymerase Chain Reaction
STM	Short Term Memory
Del Renst	Delayed Reinstatement
US	Unconditioned Stimulus
Imm Ext	Immediate extinction
Del Ext	Delayed extinction
Imm no ext	Immediate no extinction
Imm Reinst	Immediate Reinstatement
Del no ext	Delayed no extinction

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Chapter 1:
Introduction

1. Introduction

Ever increasing instances of terrorism and increase in the number of cases of social violence have left some individuals highly depressed, anxious and afraid. The result is an increase in the number of patients with anxiety disorders and post-traumatic stress disorder (PTSD) (Kessler et al., 2005).

"Extinction" is a popular behavioral technique that helps in the suppression of fear memory (Bouton, Mineka, & Barlow, 2001; Craske et al., 2008; Rothbaum & Davis, 2003). This form of learning is characterized by a decrease in a fear response. This situation is implemented when the conditioned stimulus (tone) is repeatedly presented in the absence of the unconditioned stimulus (shock) (Myers & Davis, 2002). In the laboratory, Pavlovian based fear conditioning and extinction in rats is a well-established model for behavioral studies related to memory consolidation like the acquisition, storage, retrieval, and suppression of traumatic fear (LeDoux 2000; Maren 2001, 2005; Kim and Jung 2005). The procedure can be depicted by the diagram underneath (Figure 1)

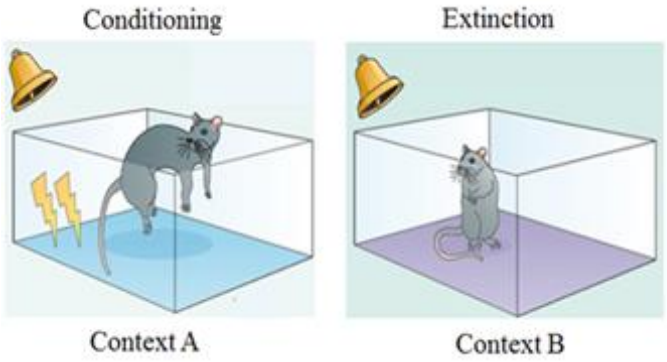

Figure 1: Represents fear conditioning and extinction training

Studies have shown that fear memory returns as a result of inhibitory associations between the now safe cue and the context and its formerly aversive outcome rather than "erasure" (Bouton, 1993). The inhibitory associations acquired during exposure therapy lead to a reduction of fear and have considerable therapeutic benefits (Milad & Quirk, 2012).

In the studies involving both, rodents and humans it has been found that extinction learning recruits much of the same neural circuitry as the initial consolidation of fear memory but with the altered epigenetic modifications of the histone proteins accompanied by the up-regulation and down-regulation of certain genes and transcription factors.

The currently used behavioral therapy is not always effective and there is an urgent need for novel therapies. Therefore, understanding the basic mechanisms underlying fear extinction would be helpful in the treatment of fear related disorders (Myers & Davis, 2007; Quirk & Mueller, 2008). Most theories that appeal to a weakening of some aspect of the original memory emphasize that there is a temporary depression and not a permanent erasure. What is important to recognize is that there are a variety of ways that extinction can be enhanced, and that these processes may act alone or in combination. At a theoretical level, this means that when a manipulation enhances extinction, it may be oversimplified to say that the manipulation enhances the "extinction memory."

Prefrontal cortex, hippocampus and amygdala are the main brain regions involved in the consolidation and extinction of fear memory and any abnormality in the extinction of fear memory makes and individual prone to fear related disorders like PTSD which later on manifest in form of various anxiety related disorders. The signaling cascades in brain structures reportedly important in fear acquisition and extinction through inhibitory learning are well known. 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. Permanent erasure of the fear memory wholly or partially is the only option left for relieving a fear related disorder and studies related to deciphering the erasure phenomenon can lead to sorting out of newer behavioral paradigms and drugs which will cure the patients permanently of fear. Studying the molecular biology of fear erasure will be a novel way to do translational research between the best understood behavioral circuit the fear reaction and fear-related disorders.

Chapter 2:

Review of Literature

2.1 Review of Literature:

The current trends in neurosciences have attracted the interest of neuroscientists initiated to come up with some newer behavioral therapy or pharmacological solution that can reduce or extinct the response generated after traumatic events the interest in theory of learning behavior and memory. Among all the behavior responses fear domain has played a pioneering role in this evolution. The fear domain exhibits a well-established and excellent example of translational research, in which preclinical and clinical research programs play a mutualistic role with each other. By turning the focus on fear memory the connections among learning theory, clinical psychology, psychiatry, affective neuroscience, pharmacology, and genetics also bolstered. Most of the treatments for anxiety and fear based diseases include exposure techniques as a key ingredient (Hofmann & Smits 2008, Mitte 2005). This involves a Pavlovian fear based paradigm which involves repeated exposure of client to the situations that elicit fear response and repeated exposure leads to the reduction of fear. This therapy is proved to be a very effective technique in fear reduction but with certain weakness. Drawbacks include poor retention of fear reduction in the long-term. With advance mechanism of treatment efficacy studies proved that fear reduction may return with a complete relapse (Eddy et al. 2004). Return of fear has been specifically studied following exposure therapy for specific phobias, obsessive compulsive disorder, agoraphobia, and performance anxieties. The existing demographic studies showed the return of fear ranges from 19% to 62% (Craske & Mystkowski 2006). To sort the tangles of behavior related problems Pavlovian based fear is the best model appeared till now (Pavlov 1927).

2.2 Fear Memory:

Memory being the highly dynamic entity is most curious phenomenon of brain to study. Best suited paradigm to study memory is fear memory. Since last 60 years it was investigated using two conditioning procedures two classical conditioning procedures (contextual fear conditioning and fear conditioning to a tone) and one instrumental procedure (one-trial inhibitory avoidance). Memory formation is initiated in three major parts of brain those include hippocampus (contextual conditioning and inhibitory avoidance), in the basolateral amygdala (inhibitory avoidance), and in the lateral amygdala (conditioning to a tone) and the pre- and infra-limbic

ventromedial prefrontal cortex. These fear related are very useful for assessing the behavioral as well as molecular mechanism involved in memory consolidation and extinction. The memory based diseases are noteworthy and essential to be eradicated because any trauma can cause severe effects on human health to the individual being as well as to the society. However to suppress the effect of trauma, extinction training therapy is attributed. Although the effect of a very large number of drugs on fear learning has been intensively studied but their effect was not significant. The extinction of fear learning involves to an extent a reversal of the flow of information and is used in the therapy of posttraumatic stress disorder and fear memories in general.

2.3 Fear Conditioning:

Pavlovian paradigm is the simple and convenient model to represent memory formation and underlying processes. In this method, a tone is preceded by a foot shock results which help in eliciting a fear response. Learning theory favors that this tone- shock pairing leaves a memory trace that consists of three components- a mental representation of the tone (CS), a mental representation of the shock (US), and an associative memory between these two. To estimate the fear memory created in our study we measured freezing response which is considered as a conditioned response. Thus the memory formed by this procedure is then allowed to be extinct by the exposure therapy.

2.4 Fear Extinction:

After consolidation when CS is presented alone without any reinforcement a process called “extinction” takes place (Bouton 2004, Furini 2014, Maren 2001, Milad & Quirk 2012). The process of extinction found its roots in the early 1900s by Pavlov and later studied by Konorski described as a form of inhibitory learning in which animals learn not to fear. It is clearly visible in behavioral experiments: a learned response fades away usually (but not always) gradually once animals realize that the US (the reinforcement) is no longer administered. Reduction in fear response mirrors the disruption of CS-US association (Rescorla & Wagner 1972). If anyhow this association is hindered, confrontations with only the tone will form a new form of memory i.e., “extinction memory." Due to this new memory, the model will not be able to elicit the fear response. This exposure therapy is used in clinical patients to reduce the adverse effects of trauma and anxiety.

2.5 Neurological Model of Fear Extinction:

The exhaustive study on neurological base of extinction memory or other forms of memory have shown the involvement of broadly three main regions of brain: PFC, amygdala and hippocampus (Milad & Quirk 2012). The underlying diagram shows the connections involved in extinction memory (Figure 2.1)

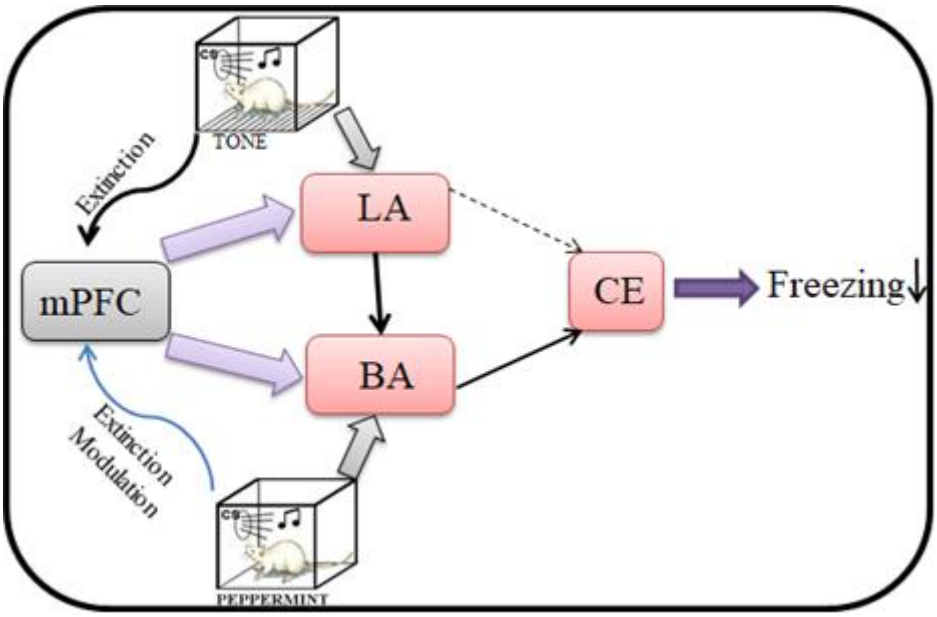

Figure 2.1: Represents the neural model of extinction correlates

2.6 Prefrontal cortex:

This region has gained considerable attention in modern behavioral research. To study the specific activities of a particular region "lesion" studies have played a considerable and fundamental role. Studies in animals showed that frontal lobe damage leads to some dramatic changes in behavior (Bianchi 1895, 1922; Jacobsen et al., 1935). Removal of fibers connecting prefrontal cortex to other brain regions was used to treat emotional disorders as varied as psychosis, depression, and even "criminality." Later due to ethical concerns the surgery was halted, but it showed that gross involvement of PFC in emotional learning. To further explore the specific activity of mPFC it was found that extensive damage to cortical area does not show much impact on conditioning of fear response but leads to the alteration in extinction (LeDoux et al 1989). In a set of studies performed by Morgan and LeDoux, 1995 focused on the infralimbic/prelimbic region as the key mPFC area involved in extinction (Figure 2.2). It was found that PL play a role in fear expression while IL in fear suppression. However findings also

contradict on this point and states that PL and IL have similar projections in amygdala and neuronal activity covary during conditioning and extinction of fear (Gutman et al., 2012; Pinard et al., 2012; Cho et al., 2013; Hübner et al., 2014). They also show a dichotomous activity pattern but rather in the opposite direction predicted by the canonical model (Morrow et al., 1999; Baeg et al., 2001; Frankland et al., 2004; Herry and Mons, 2004; Kim et al., 2010; Holmes et al., 2012; Fitzgerald et al., 2014, 2015a; Halladay and Blair, 2015). But due to varying efferent targets the activity exhibited by them may vary, moreover the neuronal population in these sub regions also are different and may lead to varying effects.

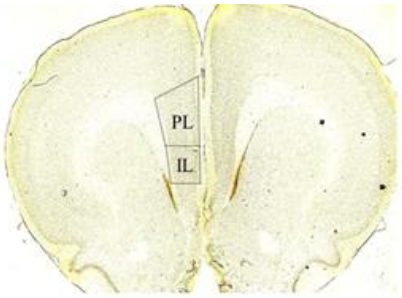

Figure 2.2 Represents the histochemical Diagram showing m-PFC

2.7 Amygdala:

The amygdala plays a central role especially in extinction memory formation. Current electrophysiological (Hobin et al 2003; Quirk et al 1995, 1997; Repa et al 2001; Rogan et al 1997), molecular (Davis 2002; Lin et al 2003b, 2003c; Marsicano et al 2002; Tang et al 1999), and imaging (Gottfried and Dolan 2004; LaBar et al 1998; Phelps et al 2004) studies also implicate its involvement in extinction learning. The LA sub region elicits freezing and other related behavior as CS and US converge in this part. The LA then communicates with the central nucleus (CeL and CeM) which mediate different responses like freezing behavior, autonomic nervous system responses, and endocrine responses (Figure 2.1). The LA is connected with the CeL by basal nucleus and intercalated cell masses (Pare et al., 2004). Later to extinction the ability of CS to control CRs by way of communication between the LA and CE is regulated by the mPFC. Activity between these parts may also be modulated by contextual data in which information is provided by hippocampus (Figure 2.3).

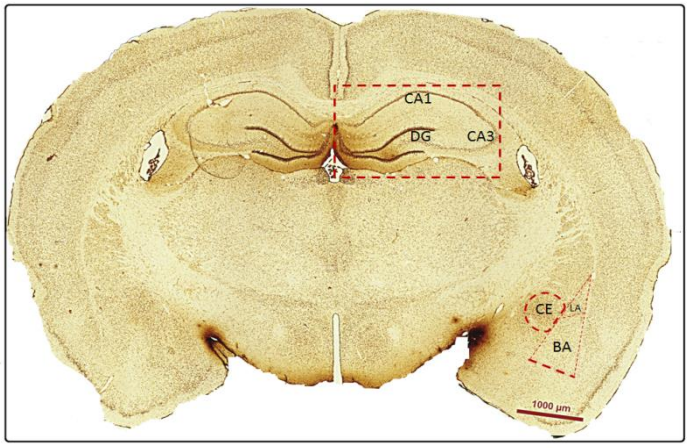

Figure 2.3 Represents the histochemical diagram showing Hippocampus and Amygdala

2.8 Hippocampus:

Contextual correlates of memory leads to the involvement of hippocampus in fear memory consolidation and extinction. It is a minor region in temporal lobe and comprises the major part of the limbic system. Studies performed by Lisman et al., 2017 showed that the hippocampus plays a key role in spatial navigation and contextual memory formation. Researchers have classified it into dorsal and ventral hippocampus. Both have their specific functions like dorsal is responsible for processing of spatial, verbal and logical information whereas ventral in promoting memory formation. The ventral hippocampus (vHPC) is shown to innervate m-PFC and BLA directly thus in a position to modulate fear based responses. Hippocampal inactivation leads to reduced expression of fear (Sierra-Mercado et al. 2011) and also prevent the return of fear after extinction (Hobin et al. 2006, Ji & Maren 2005). During extinction training if hippocampus is inactivated, it leads to poor recall of extinction which suggests that this system normally serves to inhibit fear. Hippocampus is composed of three different subregions that is CA1, CA3 and DG performing their respective functions (Figure 2.3). CA1 and CA3 are found to be involved in the acquisition of memory (Pikkaraeinen et al ., 1999) while DG is found to play a role in context determination and is considered to be the first region having neurogenesis.

2.9 Pathways of Fear Return:

Like the existence of two views positive and negative on the same topic, extinction training also suffers two contradictory thoughts that whether it is an eraser or a new form of learning. Exhaustive studies prove that it is an active learning and does not erase original learning (Myers et al., 2006). Three very influential behavioral paradigms of fear return exist that is renewal (Bouton and Bolles, 1979a; Bouton and King, 1983), spontaneous recovery (Brooks and Bouton, 1993), and reinstatement (Rescorla and Heth, 1975; Bouton 1993, 2002). It was found that in all the three cases conditional response reappears i.e., freezing returns.

2.10 Renewal:

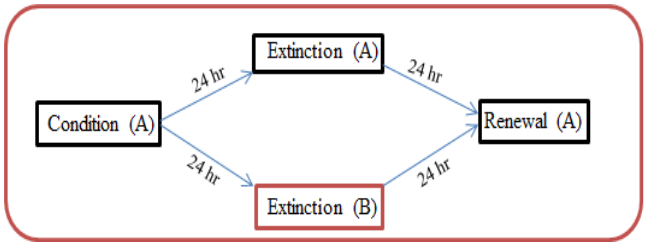

Figure 2.4: Represents the renewal test experiment

Renewal (Figure 2.4) shows the most fundamental paradigm presenting the effect of context on extinction memory. There are different forms of context including ABA, AAB, ABC (where each letter denotes the conditioning, extinction and retention test). In AAB or ABC renewal the retention test is conducted in a context different from that of conditioning as well as context for extinction also varies. However, whether the animal is presented an extinguished CS in the conditioning context (ABA) or in a neutral context (AAB or ABC), renewal is demonstrated by greater conditional response relative to the extinction context (AAA or ABB). Hence, the expression of extinction is context dependent (Bouton, 2004; Bouton et al., 2006). Various models have been developed to illustrate the behavioral mechanism underlying contextual modulation of extinction memory. Studies performed by Bouton (1994) shows that the association between the CS and US is formed during conditioning while in extinction CS-"no US" is formed that blocks activation of previous memory. Thus CS becomes ambiguous and requires an additional factor that is context. It also states that excitatory CS-US association is independent of context and is common to all situations. Rather in extinction the inhibitory CS-no US association is dependent on context and is gated so that it needs repeated exposure of

context. In renewal training CS presentation outside of extinction context will reduce activation of inhibitory link and thus causing a renewal of CR to the CS (Bouton 1994).

2.11 Reinstatement:

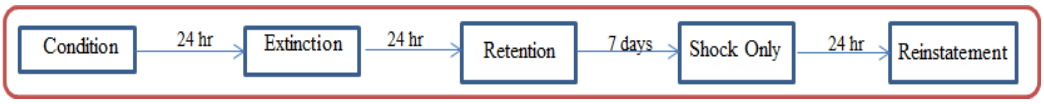

Figure 2.5: Represents the Reinstatement test experiment

In a typical reinstatement task (Figure 2.5) after conditioning and extinction followed by retention test the animals are exposed to few trials of the US alone (Pavlov, 1927; Rescorla and Heth, 1975; Bouton and Bolles, 1979b) and later when reinstatement test is performed the conditional response is restored (Bouton and Bolles, 1979b; Bouton, 1993, 2002). In this case, evidence strongly suggests that the reinstatement effect is due to context conditioning. The context-US associations after extinction promote reinstatement (Bouton, 1993, 2004). Similar to renewal, reinstatement also exhibits the dependence of context on extinction. That is, reinstatement only occurs when the US is presented in the context, in which the extinguished CS is tested but not when reinstating USs are presented in a novel or irrelevant context (Wilson et al., 1995; Frohardt et al., 2000; Bouton, 2004). Not only the context specificity the reinstatement also depends on other properties like strength of reinstatement, attenuation of reinstatement by exposure in same context where initial CS was given (Bouton and Bolles, 1979b; Baker et al., 1991; Bouton, 1993) and third the effect of extinguished CS on non-extinguished CS (Bouton, 1984, 1993).

2.12 Spontaneous Recovery:

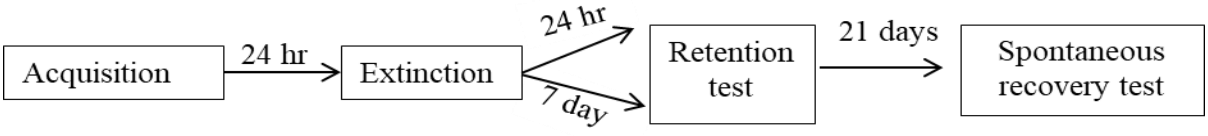

Figure 2.6: Represents the spontaneous recovery test

Though the contextual learning through spontaneous recovery is not a part of this study, but it will be highly irrelevant if it is not discussed here. It was considered to be one of Pavlov's most valuable findings regarding memory formation and its return, which shows that the fear memory may return after the elapse of time (Figure 2.6). As seen for renewal and reinstatement, spontaneous recovery also possesses some pragmatic issues with it. Firstly it had been well established in all the conditioning methods, secondly it has been verified by the studies that it shows a regression type of results that longer the duration between extinction termination and testing greater are the chances of recovery (Quirk 2002). Along with this study Resercola et al., 2004 has found that recovery declines with the repeated exposure of extinction. From various studies by memory theorists it was proved that with the passage of time context also changes which may cause the relapse. Therefore spontaneous recovery also can be considered as a another ‘‘renewal effect’’, that occurs when the CS is tested outside its ‘‘temporal extinction context’’, relative to the ‘‘physical context’’ (Bouton, 1993, 1994).

2.13 Epigenetics:

As learning takes place continuously and life long memory also form every passing moment. Learning and memory are highly dynamic entities and are updated regularly. For these ever changing phenomenon newer synapse have to be formed regularly, but as brain is complex and neurogenesis takes place only during development (except in certain regions like hippocampus) these regular accumulating changes became a point of research. In this line, it was found that the “epigenetics” is a fundamental process involved. It is defined as “study of acquired changes in chromatin structure without changing the DNA sequence underlying it”. Epigenetics involve a change affected by various factors like age, the environment/lifestyle, and disease state. Modifications on N- terminal tail occur by various methods like acetylation, methylation, phosphorylation and ubiquitination. These modifications are included by the protein known as writers, eraser and reader. The underlying table involves the overview of epigenetic machinery (Figure 2.7)

Epigenetic Modification	Writer	Reader	Eraser
Histone Acetylation	Histone Acetyltransferases	Bromodomains; Tandem PHD Fingers; Pleckstrin Homology Domains	Histone Deacetylases
Histone Arginine Methylation	Protein Arginine Methyltransferases (PRMTs)	Tudor Domains (recognize symmetrically dimethylated arginines); WD40 Domains	Histone Demethylases (JMJD6); Peptidyl Arginine Deiminases (putative)
Histone Lysine Methylation	Histone Lysine Methyltransferases	Chromodomains; Tudor Domains; PHD Fingers; MBT Domains; ZF-CW Proteins; WD40 Domains; PWWP	Histone Lysine Demethylases
Histone Phosphorylation	Kinases (JAK2, ATM/ATR, PKC, PKA, Haspin, Aurora B Kinase, RSK2, AMPK, MSK, MEK)	BRCT Proteins	Protein Serine/Threonine Phosphatases; Protein Tyrosine Phosphatases

Figure 2.7 Represents the molecules involved in epigenetics

2.14 Fear Memory and Epigenetics:

As we know arbitrarily about the behavioral approach of fear memory acquisition and extinction the focus of researchers turned towards the molecular alterations underlying these phenomena. Possibly the most fundamental though advance approach lies in the umbrella term “epigenetics”. The modifications added by the reader, writer and eraser to N-terminal of histone tail like acetylation, methylation and sumoylation etc. leads to the altered memory formation and its extinction. This nature of epigenetics has been explored for finding the putative target of memory acquisition and extinction learning (Figure 2.8)

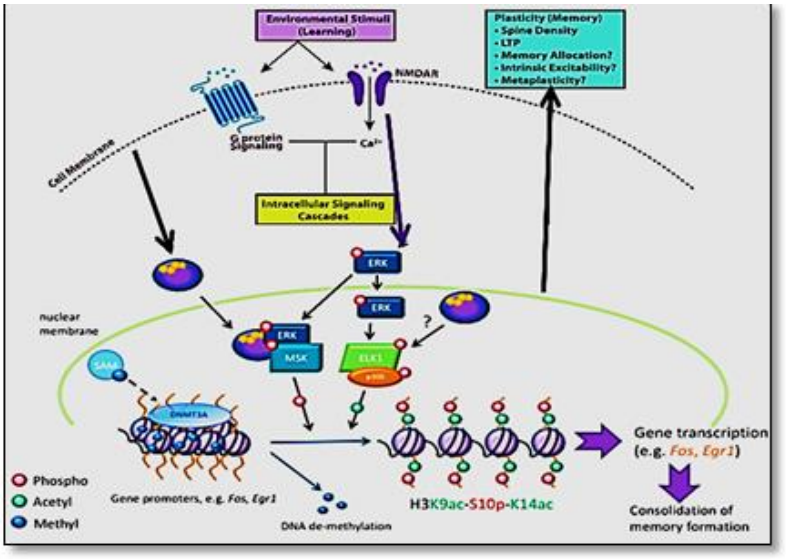

Figure 2.8 Represnts the epigenetic signaling in memory formation

2.15 Fear Conditioning:

Exhaustive studies on auditory fear memory consolidation show that acquisition requires genomic signaling cascades to mediate the transcriptional and translational processes which results in the underlying fear memory formation (Johansen et al., 2011). Particularly it leads to the activation of ERK/MAPK within lateral amygdala (LA) neurons (Schafe et al., 2000). This complex in turn translocate to the nucleus where it leads to the phosphorylation of the transcription factor CREB to mediate downstream transcriptional activation (Josselyn et al., 2001; Ressler et al., 2002; Ploski et al., 2010). Apart from this classical signaling mechanism additional cascade and complexes also influence and regulate synaptic plasticity and memory formation (Levenson and Sweatt, 2005, 2006; Barrett and Wood, 2008; Zovkic et al., 2013; Jiang et al., 2008; Zovkic and Sweatt, 2013).

Among all the modifications studied so far histone acetylation has been most extensively studied within the context of learning and memory (Graff and Tsai, 2013). Lysine carrying positive charge restricts the access of transcriptional machinery to DNA and then addition of an acetyl group relaxes the chromatin structure via Histone acetyltransferases (HATs). To regulate the activity of HATs the enzyme HDAC remove acetyl group and then again subdues the chromatin structure (Varga-Weisz and Becker, 1998; Yang and Seto, 2007).

2.16 Fear Extinction:

Studies on rodents for fear memory extinction based studies have been implemented in treatment of phobias, anxiety disorders, and PTSD (Andero and Ressler, 2012). Epigenetic modifications include HDAC inhibitors which have potential to enhance extinction memory and may also have clinical implications. In studies it has been found that systemic HDAC inhibition is sufficient for facilitating extinction of auditory fear memory (Lattal et al., 2007; Bredy and Barad, 2008; Fujita et al., 2012; Itzhak et al., 2012) and also facilitates extinction of cocaine based conditioning (Malvaez et al., 2010). Study involving intra-hippocampal or intra-ILPFC administration of the HDAC inhibitor sodium butyrate (NaB) induced the contextual fear memory extinction, but only under conditions when extinction training was weak, i.e., NaB administration was not effective in facilitating extinction using more stringent extinction sessions (Stafford et al., 2012). Though HDAC inhibition and extinction suggest the ceiling effect it had not always been prove successful (Kilgore et al., 2010). In a study involving hippocampus it was found that HDAC1 facilitates fear extinction and its inhibition results in impaired extinction (Bahari-Javan et al., 2012). Study involving PCAF shows the impaired activity of IL-PFC during consolidation of extinction for an auditory fear (Wei et al., 2012). While a recent study showed the potential chromatin modification in the vm-PFC occurring with the extinction of previously learned fear alongwith the morphine withdrawal (Wang et al., 2012).

Thus, all the above studies showed that epigenetics also play a noteworthy role in fear memory acquisition and extinction with the key role of acetylation in consolidation while deacetylation in formation of extinction memory. In line with the above performed studies, our study proved that acetylation of histones alters the expression of CREB and its targeted genes in region specific manner during extinction and also during retrieval of memory by taking into consideration various parameters like, context (renewal) and time of extinction (reinstatement).

Chapter 3:

Hypothesis

3. Hypothesis:

Our working hypothesis is that extinction of fear memory occurs both by erasure (unlearning) and inhibitory learning (new learning). Therefore we shall perform initial experiments to examine post extinction retention of fear memory by testing for reinstatement, renewal or spontaneous recovery. If there is erasure, the fear memory would not be susceptible to reinstatement, renewal or spontaneous recovery and if both inhibitory learning and erasure are there would be return of fear but with far less intensity. If it is only through inhibitory learning then the fear memory will return with same intensity every time. To check this hypothesis Reinstatement and Renewal experiments will be performed which will point towards the involvement of type of mechanism underlying the extinction of fear memory during different set ups. From each group half the animals will be sacrificed and perfused after the final tests and half the animals will be sacrificed without the final testing, for immunohistochemical studies.

Chapter 4:

Aims and Objectives

4. Aims and Objectives:

Aim 1. To establish a working model for fear consolidation, extinction for both immediate and delayed, reinstatement and renewal and to find out the effect of delayed and immediate extinction on renewal and reinstatement of fear.

Aim 2. To find out the molecular substrates especially CREB and its target genes which might be playing a role in the reinstatement and renewal as observed following immediate and delayed extinction, in amygdala, hippocampus and prefrontal cortex.

Aim.3: To find out whether histone acetylation has any role to play in the erasure/ inhibitory learning observed during immediate /delayed extinction respectively.

Chapter 5:

Materials and Methods

Materials and Methods

5.1 Model:

All the experiments were conducted on the 2-3 months old adult male Sprague-dawley (SD) rats weight 150-200 grams. The rats were housed individually in separate transparent cage and were maintained on a 12 hours light/dark cycle at optimum temperature of 23±2°C. Access to food and water was made *ad-libitum.* All the experimental rats were handled for 3-5 minutes daily for one week to acclimatize with the handling procedure. All the experiments were strictly performed during morning session and were under the abidance with committee for the purpose of control and supervision of animals, Ministry of Environment and Forests, Government of India (IAEC/UDPS/2016/41).

Apparatus employed for the behavioral study

5.2 Freeze monitor chamber:

To perform auditory behavior training, two observational freeze monitor chambers were used. Among both the chambers, one was dedicated for fear conditioning while other one was used for extinction (VJ instruments). Dimensions and key features of the chambers were as follows:

1. 35.5 cm x 25 cm x 25 cm made of transparent plexiglass.
2. To avoid acoustic cue the provision of the sound attenuating chambers (70 cm x 50 cm x 50 cm) was made inside the outer chamber.
3. 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).
4. A speaker was mounted on the wall of the chamber for the production of conditional stimulus- tone (CS) and ventilation fans were allowed to produce background noise during experiments.
5. A camera was placed to visualize the movement of the animal placed inside the behavioral chamber during conditioning and extinction training .
6. The fear extinction chamber has the context B to discriminate with fear conditioning chamber which have context A.

7. In Fear extinction chamber (context B), walls of the cabinet were surrounded by black and white stripes. The grid floor was fully covered with white sheet to mask the exposure of shock producing grid. The whole cabinet was wiped with vanilla essence to produce a novel olfactory cue during extinction training. Fear conditioning chamber was devoid of such type of modifications.

Behavioral training procedure

5.3 Fear conditioning:

Prior to the commencement of initial fear conditioning training session, freeze monitor chamber was calibrated for shock and tone. The rats were placed inside fear conditioning chamber (context A) and were allowed to acclimatize with the context for the period of 3 min followed by the presentation of 5 training session of tone (CS: 80 dB, 10 sec) associated with the shock (US: 0.70 mA, 1 sec). The inter-trial interval (ITI) between two consecutive trials was 60 sec. After the offset of the conditioning, an additional 1 min was given to rats and then return into their homecage. Freezing response was videotaped by camera and was calculated offline by an observer blind to the experiment.

5.4 Fear extinction:

The fear extinction is a form of non-associative learning resulting in gradual decline of previously generated fear response with the continuous presentation of tone only in extinction chamber (context B). We have chosen two distinct time window to initiate the fear extinction training session after conditioning-

1. Extinction training was initiated after 10 min of fear conditioning- ***Immediate Extinction (context A and B).***
2. Extinction training was initiated 24 hours after fear conditioning- ***Delayed Extinction (context A and B).***

Like fear conditioning, after the acclimatization period of 3 mins, conditioned rats were underwent for extinction training. During onset of extinction training session, 30 trials of same tone (intensity and duration of the tone was kept the same as during conditioning) were presented to the rats in the absence of any foot-shock. All the 30 trials were represented in 5 trial blocks (every 6 consecutive trials = 1 trial block) into the figure. During extinction training paradigm, the timing of ITI was kept 10 sec (Teichner,1952). Following 1 min of extinction training, rats were returned to their home cages.

After receiving extinction training in both the context (context A and B), half of the animals from each group were sacrificed immediately after 2 hrs of extinction training session to observe the molecular changes involved and remaining half were underwent for retention test in context A to observe the recovery of fear memory.

5.5 Renewal test:

During retention test five trials of tone were presented to the animals in the absence of any noxious stimulus (shock) and percent freezing was measured. The intensity of tone was 80 dB for 10 sec. The ITI between two consecutive tones was kept 10 seconds. Retention test was performed to look at the effect of context as well as timing of extinction training on fear memory recovery (Figure 5.1).

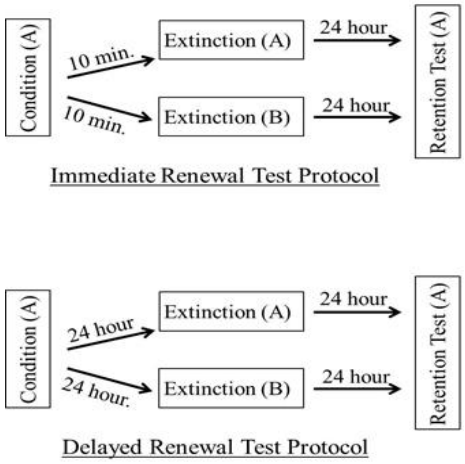

Figure 5.1: Represents the renewal experiment protocol

Further two control groups were also included in the study viz. Immediate no extinction and delayed no extinction. Rats belonging to these two control groups were only exposed to their respective chambers for the same duration as extinction training session without receiving any tone or shock and percent freezing was measured.

5.6 Reinstatement test:

After conditioning (context A), animals were trained for immediate extinction and delayed extinction in context B. Following extinction training session, animals were undergone for retention test (context B). After seven days, a single session of five un-signaled shock (0.70mA, ITI 10sec) was presented to the animals (context A) and next day they were submitted for reinstatement test via the presentation of five trials of tone only. Freezing percent was measured (context B) for whole session (Figure 5.2)

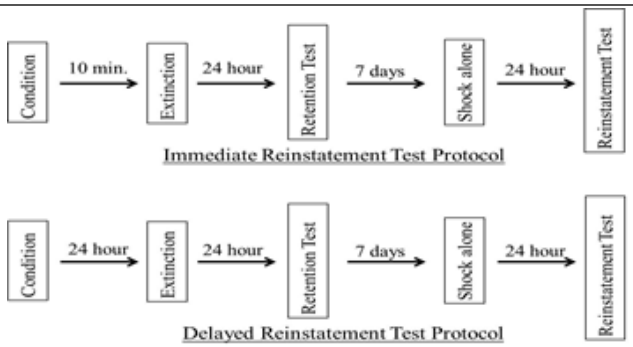

Figure 5.2: Represents reinstatement test protocol

5.7 Scoring:

Freezing response was measured as the cessation of all the locomotor activity except the respiration for every two seconds of time duration. For movement we gave as '1' and for immobility we gave score '0'. Freezing percentage was calculated by a formula-

Freezing percentage = Freezing score / total number of reading x 100

As an alternative, freezing percentage was also recorded automatically by VJ instrument by using video tracking through the CCD camera mounted on the ceiling by a software in a computer attached to freeze monitor.

5.8 Details of Brain region under study:

Present study was conveyed to look out the molecular changes encountered in the primarily three brain regions Hippocampus, Amygdala and Prefrontal cortex. Amygdala is an almond shaped structure consists of a number of groups of neurons viz. lateral amygdala (LA), basal amygdala (BA), centrolateral (CeL) and centromedial region (CeM) (McDonald 1998; Turner and Herkenham 1991; Krettek and Price 1978a; Petrovich and Swanson 1997; Veening et al. 1984). Prelimbic prefrontal cortex (PL) and infralimbic prefrontal cortex (IL) are the two subdivisions of prefrontal cortex (Giustino and Maren 2015). Hippocampus is a part of the limbic system and is mainly involved in the regulation of contextual memories (Raineki et al, 2010, Fournier and Duman, 2013) (Figure 5.3).

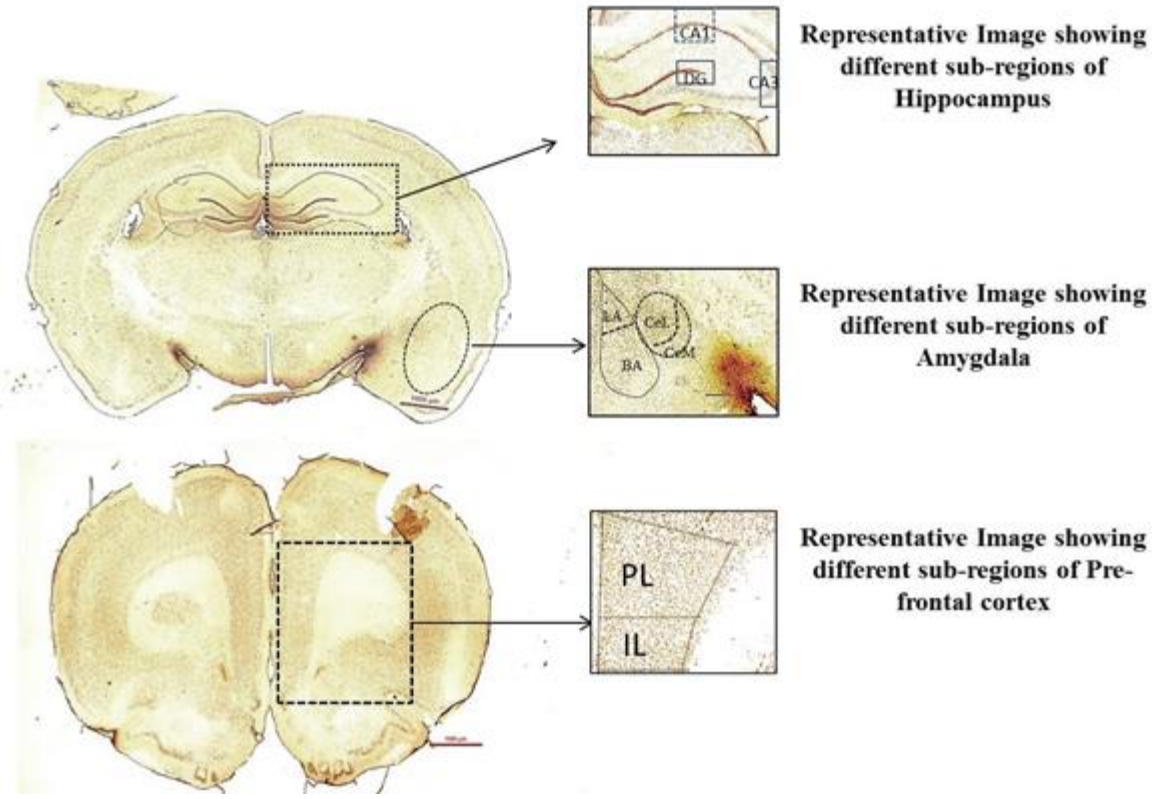

Figure 5.3: Represents the different regions of the Hippocampus, Amygdala and Prefrontal cortex (PFC) involved in fear memory consolidation and extinction.

5.9 Tissue preparation for Immunohistochemistry:

Rats were anesthetized with pentobarbital (25 mg/kg i.p.) with 0.9% n-saline followed by ice cold paraformaldehyde (4% PFA) treatment (prepared in 0.01M phosphate buffer, pH=7.2). Their brains were isolated and allowed to postfix in 4% PFA overnight. Next day brains were transferred to 10% sucrose solution for one day followed by 20% and 30% sucrose solution (sucrose solution were prepared in 0.01M phosphate buffer, pH=7.2) until the brain were settled down in the falcon tube. Following sucrose treatment brains were frozen in isopentane maintaining the temperature of -30° to -40°C for about 30 to 40 minutes on dry ice and brains were stored in -80°C for immunohistochemistry (IHC).

While performing IHC, coronal brain sections were obtained by sectioning with cryostat (Microm HM 525, Germany) maintaining the thickness of 20µm having the region of interest (amygdala, prefrontal cortex and hippocampus).

5.10 Immunohistochemistry:

Trimmed sections from each of the region hippocampus, amygdala and prefrontal cortex were collected serially in 0.01m phosphate buffer saline solution from all the groups to have enough matching sections. Sections were washed with 0.01M phosphate buffer saline (PBS) followed by 3% H_2O_2 treatment to remove any peroxide activity. Sections were then incubated with 1% normal horse serum (NHS, Vecta-stain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA) with 0.25% tween 20 to block non-specific binding followed by incubation with primary antibody (rabbit monoclonal reactivity in rats) viz. acetyl H3K9 (cat. no. ab10812), acetyl H4K5 (cat. no. H5110-15E2), ARC (cat. no. sc-15325), p-CREB (cat. no. sc-101663) and c-fos (cat. no. ab7963) with the dilution of 1:1000, 1:1000, 1.100, 1:100, 1:500 respectively for overnight duration. Next day sections were incubated with biotinylated secondary antibody (anti-rabbit IgG, 1:500 dilution, Vecta-stain Elite ABC kit) for 2 hours of duration followed by the addition of Vecta-Stain Elite ABC kit (Vector Laboratories) and lastly DAB substrate (DAB peroxidase substrate, Abcam ab64238) was applied to the sections to stain. After the appearance of brownish colour sections were repeatedly washed with tap water and then proceed for mounting on the clean gelatinised glass slides.

5.11 Image acquisition:

Image of the immunostained sections were taken from NS-BR image analysis software (Nikon, Tokyo) using a Nikon Eclipse microscope (Nikon, Tokyo, Japan). From each of the brain sections three readings were taken and considered as a single reading. Readings were taken manually by counting the number of neurons immunostained.

5.12 Statistical analysis:

All the data from behavioral experiments were expressed as means and standard error of the mean (± SEM). Behavioral data were analysed using three way ANOVA whereas the molecular analysis by two way ANOVA by using the software graphpad prism and ezANOVA.

Chapter 6:

Results and Discussion

Chapter 6.1

Aim 1.

To establish a working model for fear consolidation, extinction for both immediate and delayed, reinstatement and renewal and to find out the effect of delayed and immediate extinction on renewal and reinstatement of fear.

6.1 Behavioral results for Renewal test (Same context- AAA)

All the experimental animals were handled for 5-8 minutes daily for one week for habituation to the handling process prior to all the behavioral experiments.

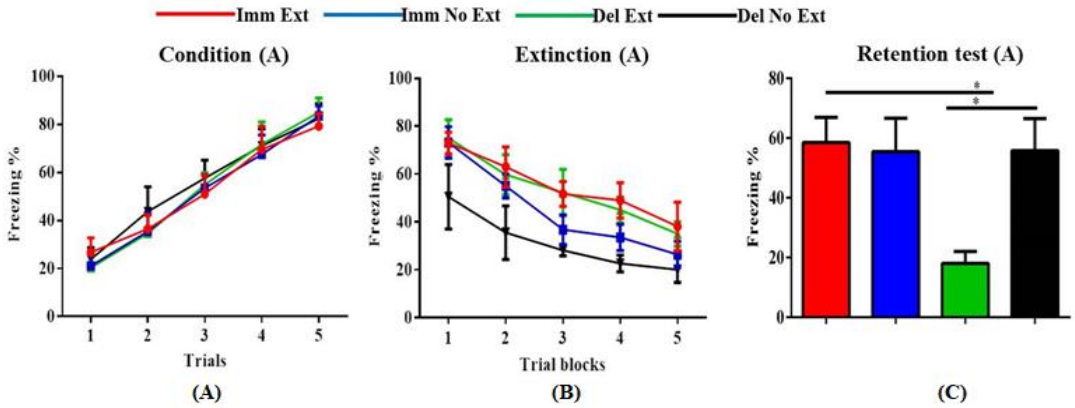

Figure 6.1: (A) Represents the freezing response during fear conditioning (context A), % freezing was similar in all the groups with no significant difference ($p>0.05$) and last trial exhibited highest % freezing (B) Represents % freezing response during extinction training session (context A), the % freezing response was decreased with each successive trial block and last trial block exhibited reduced level of % freezing (C) Represents the % freezing during retention test in the conditioning context (A).

Prior to start of the experiments, animals were randomly divided into the following four groups- immediate extinction (Imm Ext), immediate no extinction (Imm No Ext), delayed extinction (Del Ext) and delayed no extinction (Del No Ext) groups. Animals in all these groups were subjected to conditioning (refer methods section). During the first trial, there was a very low level of % freezing response. There was an increase in % freezing response during successive trial presentation and the last trial exhibited highest % freezing. A three way repeated measures ANOVA revealed significant main effect of trials across the groups [$F(4, 144) = 547$, $p<0.0001$] along with the interaction among the trials, immediate extinction and delayed extinction group [$F(4, 144) = 1.29$, $p<0.05$]. Further Tukey's *post-hoc* test confirmed the difference among the trials (all $p<0.001$) [Figure 6.1 (A)].

After successful conditioning the animals in all the groups were subjected to extinction training in the conditioning context (A) either 10 min (Imm Ext) and 24 hours (Del Ext) after conditioning. For control the was immediate no extinction group and delayed no extinction group.

The animals in these groups were exposed to the context either 10 min (Imm No Ext) or 24 hour (Del No Ext) after conditioning. The % freezing response during extinction training session was analyzed by three way ANOVA. There was a successive decrease in the % freezing across each trial block presentation with significant main effect of trial blocks [F (4, 144) =173, $p<0.0001$], time (imm vs. del) [F (1, 36) =14.7, $p<0.005$] and condition (ext vs. no ext) [F (1, 36) =65.5, $p<0.0001$] with significant interaction between the time x condition [F (1, 36) =9.33 $p<0.05$], time x trial blocks [F (4, 144) =6.57, $p<0.005$] and time x condition x trial blocks [F (4, 144) =10.1 $p<0.0001$]. Tukey's *post-hoc* test for multiple comparison analysis confirmed that the extinction groups froze more than the no extinction group ($p<0.05$) [Figure 6.1 (B)].

Retention test was also performed in the conditioning context (context A) after 24 hours of extinction, to gauge the effect of timing of extinction after conditioning on recovery of fear memory. It was found that the delayed extinction group showed the least amount of % freezing than the other groups. Two way ANOVA analysis for retention test revealed significant main effect of time [F (1, 16) =4.88, $p<0.05$) and also the significant interaction between time x condition [F (1, 16) =5.08, $p<0.05$]. However, the changes were not significant for condition [F (1, 16) =3.67, $p>0.05$). Tukey's *post-hoc* test for multiple comparison analysis confirmed that the delayed extinction group exhibited significantly low level of % freezing response as compared with the immediate extinction ($p<0.05$) and delayed no extinction control group ($p<0.05$) [Figure 6.1 (C)].

6.2 Behavioral results for Renewal test (Different context ABA):

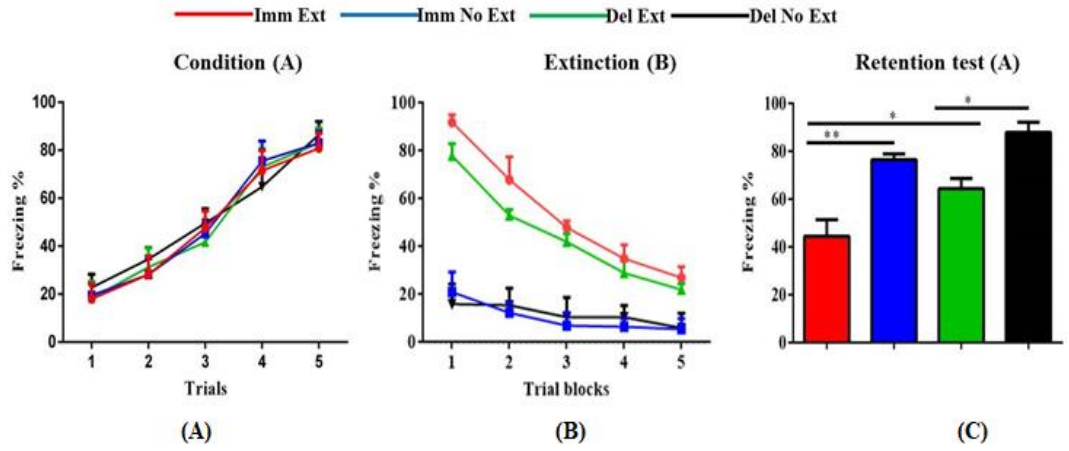

Figure 6.2: (A) Represents the freezing response in (context A). Fear conditioning resulted in the increased % freezing with each successive trial without any significant difference across the groups ($p>0.05$). (B) Represents freezing response during extinction training, % freezing decreased with

each successive trial block. (Context B). (C) Represents the % freezing during retention test in the conditioning context (A).

Firstly the animals in each group underwent fear conditioning as condition training was delivered to the animals of the same context. Three way ANOVA analysis for the conditioning data showed a significant main effect of trials [$F(4, 144) = 862$, $p<0.0001$] and also a significant interaction of trials with the immediate and delayed extinction group [$F(4, 144) = 2.64$, $p<0.05$]. Three way ANOVA followed by Tukey's *post-hoc* test for multiple comparison analysis confirmed the significant difference among trials (all $p<0.001$) [Figure 6.2 (A)].

Extinction training was delivered in context B after 10 min and 24 hour of fear memory acquisition that resulted in the attenuation in the % freezing response with each successive trial block presentation. During the first two trial blocks, the % freezing in the Delayed extinction group was less as compared to that in the immediate extinction group. From the third trial block onwards the freezing response became similar in both the groups (delayed extinction and immediate extinction) without any significant difference ($p>0.05$). Both the control groups (immediate no extinction and delayed no extinction) exhibited decreased level of freezing response throughout the session ($p>0.05$). Three way repeated ANOVA analysis revealed a significant main effect of trial blocks [$F(4, 144) = 205$, $p<0.0001$] and condition (extinction vs no extinction) [$F(1, 36) = 1816$, $p<0.05$]. Significant interaction was also observed between the time (immediate vs delayed) x trial blocks [$F(4, 144) = 5.18$, $p<0.05$], condition x trial blocks [$F(4, 144) = 89$, $p<0.0001$] and time x extinction condition [$F(1, 36) = 7.2$, $p<0.05$] on the freezing response. Tukey's *post-hoc* multiple comparison test confirmed the significant effect of trial blocks on freezing response ($p<0.01$) [Figure 6.2 (B)].

24 hours after extinction, retention test was performed in the conditioning context by the presentation of five trials of tone only. It was found that immediate extinction group showed less recovery of fear memory than the delayed extinction ($p<0.05$). A two way ANOVA analysis revealed significant main effect of time (immediate vs delayed) [$F(1, 8) = 9.42$, $p<0.05$] and condition (extinction vs no extinction) [$F(1, 8) = 40.7$, $p<0.05$]. The interaction between time x condition was not significant [$F(1, 8) = 0.960$, $p>0.05$] [Figure 6.2 (C)].

6.3 Extinction comparison between the same and different context:

The % freezing that was observed during extinction training when compared between the same and different context exhibited no significant difference across the groups. The result was analyzed by

Two way ANOVA that revealed no significant main effect of context (same vs different) [F (1, 16) =0.289, p>0.05] and time (immediate vs delayed) [F (1, 16) =0.363, p>0.05]. Also the interaction between context x time was not significant [F (1, 16) =0.185, p>0.05). The results were further confirmed by Tukey's *post-hoc* test that also exhibited no significant difference across the groups [F (3, 16) =0.2790, p>0.05] (Figure 6.3).

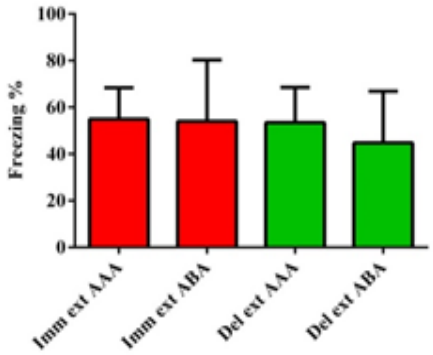

Figure 6.3: Represents extinction comparison between the same and different context

6.4 Retention test comparison between the same and different context:

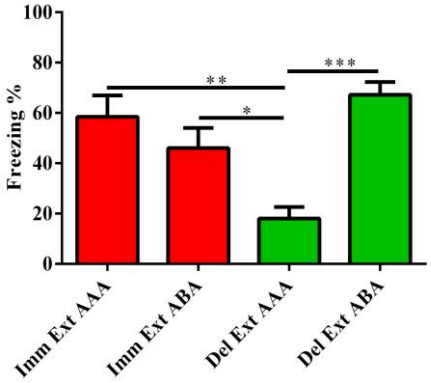

Figure 6.4: Represents the % freezing during retention test comparison between the same and different context

As no significant difference in the % freezing response was observed between the groups of same and different context during extinction training session (Figure 6.3). We then looked for differences in % freezing during retention test. Two way ANOVA analysis revealed significant main effect of context [F (1, 16) =7.35, p<0.01] and context x time interaction [F (1, 16) =20.7, p<0.001] but changes were not significant for time [F (1, 16) =2.03, p>0.05]. Tukey's *post-hoc* test showed significant difference in the % freezing during the retention test in the immediate and delayed

extinction ($p<0.01$) performed in the same context (AAA). The changes were also significant between immediate extinction from different context (ABA) and delayed extinction from same context (AAA) ($p<0.05$). Changes were also significant between delayed extinction (AAA) and delayed extinction (ABA) ($p<0.001$) (Figure 6.4).

6.5 Discussion:

Fear conditioning in rats is a form of associative learning and is an efficient model for studying neurobiology of emotional learning and memory. (Davis, 1992; LeDoux, 2000; Maren, 2001; Sotres-Bayon et al, 2006). In the present study there was an increment in the % freezing response during successive trials of fear conditioning with all animals in each group exhibiting similar high level of % freezing response in last trial. These results are similar to the results shown in other such studies (Singh et al., 2018; Siddiqui et al., 2017, 2019; Ranjan et al., 2015; Mayers et al., 2006). Extinction training delivered either 10 min or 24 hour in context A exhibited that both the immediate and delayed extinction group showed decrement in the level of % freezing response. However, there was a reduction in the % freezing response following delayed extinction when the retention test was performed in the conditioning context A [Figure 6.1(C)]. From behavioral experiments it was found that animals undergo delayed extinction had more time to retain the extinction memory and thus, they were able to differentiate between the non-associated cues i. e. tone and shock although the context was the same in each paradigm. On the other hand, the immediate extinction group underwent extinction training after a very short time gap received less time to retain the extinction memory and due to poorer extinction the retention test was also poor suggesting that the increased freezing behavior during early extinction. Thus from the above experiment we can say that the early intervention after a trauma might not prove to be an effective therapy for patients, if the extinction training was delivered in the same context in which traumatic event occurred.

Once we observed the effect of time (immediate and delayed) on memory, we next tried to look at the effect of change in context. The results showed that extinction training when delivered in a novel context (context B), there was a continual decline in % freezing response. During the early trial blocks the immediate extinction group exhibited significant increased % freezing but later becomes similar to delayed extinction.

Increasing evidence indicates that extinction reverses some of the conditioning-induced processes within the amygdala (Lin et al., 2003b; Lin et al., 2003a; Kim et al., 2007). Findings from these studies suggest that extinction may erase some aspects of fear memory within the amygdala, even though fear can still return at the behavioral level. Myers et al. (2006), have reported less return of fear during immediate extinction. In the present study we found that freezing response during retention test in the immediate extinction was less than the delayed extinction [Figure 6.2(C)]. The probable reasons behind may be the short time window the rats had to learn the conditioning context as the interval between conditioning and extinction training in the immediate extinction group was just 10 mins. However the animals placed in delayed extinction group got enough time to averse the environment during conditioning, and when they were re-exposed to the conditioning context during retention test after fear extinction (context B), the memory associated with the conditioning context reappeared that exhibited increased level of % freezing response. So the lower % freezing response in the immediate extinction group as compared to the delayed extinction group when there was a shift in the context may be because of less or no contextual learning.

No difference in the freezing response was observed between the same context (AAA) and the different context (ABA) groups during extinction training comparison (Figure 6.3) but the differences were evident during the retention test comparison (Figure 6.4). Comparison of freezing response during retention test yielded no significant difference between the immediate extinction of the same context with the immediate extinction of the different context.

The equal level of % freezing response for immediate extinction in both the context suggested no recovery of fear memory at all so it seems that immediate extinction exhibit a kind of "erasure" component. Unlike, delayed extinction which exhibited decreased freezing response in the same context as compared to the different context. It suggested that the fear response reappeared when delayed extinction training was delivered in a novel context explaining that delayed extinction showed inhibitory learning. The behavioral results thus observed here were in line with the Myers et al., 2006, where they also suggested that the extinction of fear memory can be inhibitory learning or 'erasure' of previously acquired memory depending whether the context is the same or different and also on the time lag between the conditioning and extinction training.

6.6 Reinstatement behavioral analysis:

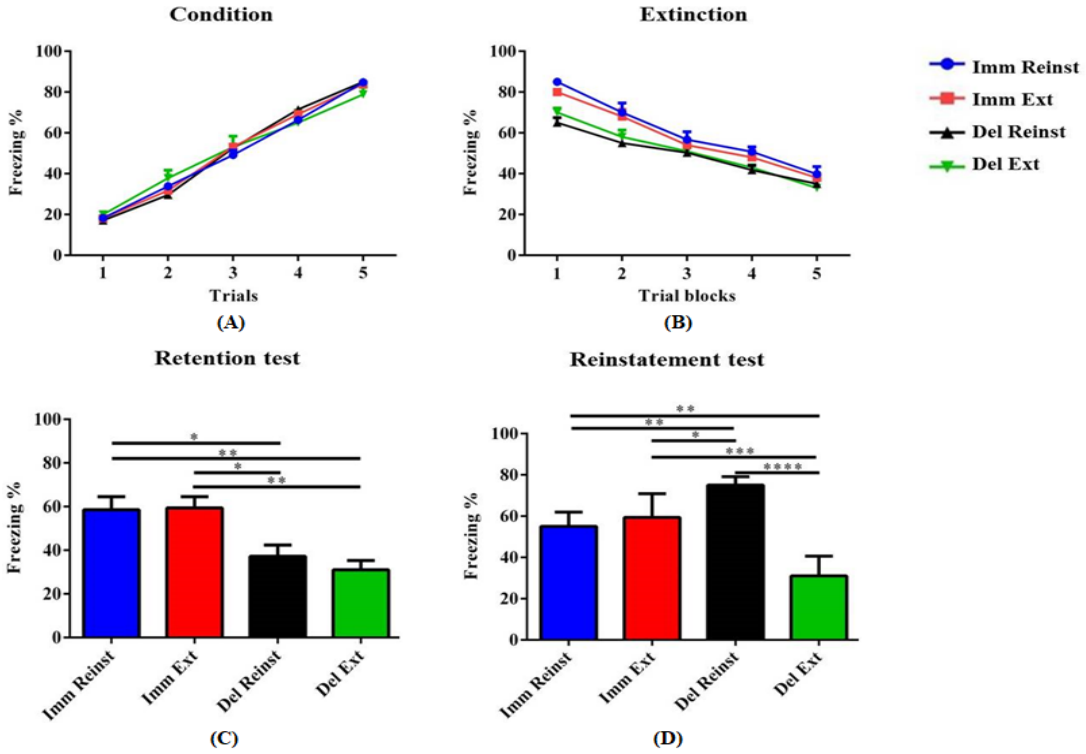

Figure 6.5: (A) Represents the conditioning training session (B) Represents the extinction training session (C) Represents the retention test performed after extinction training session (D) Represents the reinstatement test.

Prior to the beginning of the experiment, all the experimental rats were assigned into the following four groups i.e. Immediate Reinstatement (Imm Reinst), Delayed Reinstatement (Del Reinst), Immediate Extinction (Imm Ext) and Delayed Extinction (Del Ext).

Fear conditioning training was delivered to all the animals placed in groups by presenting the 5 trials of tone coterminated with the shock and graph was plotted between number of trials and freezing % [Figure 6.5 (A)]. Graph showed the increased freezing % response with each successive trial presentation. Initial trial exhibited least level of freezing percent while the last trial exhibited maximum freezing percent response. All the groups exhibited near about equal freezing throughout conditioning session ($p>0.05$). Three way ANOVA analysis was performed to know the effect of time (immediate vs. delayed), condition (reinstatement test vs. retention test) and also the effect of trials along with the interaction between them. Statistical analysis revealed significant main effect of

trials [F (4, 144) = 909, $p<0.0001$] on freezing percent. Significant interaction between condition (reinstatement test vs. retention test) x trial interaction [F (4, 144) =2.67, $p<0.05$] was also observed. Further Tukey's *post-hoc* test confirmed the significant difference among the trials [all $p<0.001$] [Figure 6.5 (A)].

Extinction training was delivered to conditioned animals either 10 min or 24 hour of conditioning session in context B. Extinction training resulted in decreased freezing percent with each successive trial block presentation. Three way ANOVA analysis for extinction data revealed significant main effect of time (immediate vs. delayed) [F (1, 36) =21.5, $p<0.001$] and trial blocks [F (4, 144) =189, $p<0.0001$]. Significant interaction was observed in between trial blocks x time (immediate and delayed) [F (4, 144) =4.64, $p<0.001$]. Further Tukey's *post hoc* analysis confirmed that there was a significant difference across the trial blocks and the last trial block exhibited least level of freezing response than the initial trial block ($p<0.001$) [Figure 6.5 (B)].

Following 24 hours of extinction training, retention test was performed by presenting the 5 trials of tone only. The effect and interaction was measured by Two way ANOVA analysis that revealed significant main effect of time [F (1, 16) =12.1, $p<0.01$] only. Tukey's *post-hoc* analysis confirmed that immediate groups (immediate reinstatement and immediate extinction) froze more than the delayed groups (delayed reinstatement and delayed extinction) ($p<0.05$) [Figure 6.5 (C)].

After 7 days of retention test, rats were allowed to receive five trials of un-signaled foot-shock (without tone) and the next day they were tested by receiving five trials of tone only to gauze the recovery of fear memory (reinstatement test). No difference in the freezing percent was observed in between immediate reinstatement and immediate extinction. However delayed reinstatement group exhibited higher freezing percent than the delayed extinction group. Reinstatement test data was analyzed by Two way ANOVA that revealed significant main effect of condition (reinstatement test vs. retention test) [F (1, 16) =27.1, $p<0.0001$] and time (immediate vs. delayed) x condition interaction [F (1, 16) =40.5, $p<0.0001$]. Tukey's *post-hoc* test also confirmed that there was no significant difference between immediate reinstatement and immediate extinction group ($p>0.05$). But significant difference was observed between delayed reinstatement and delayed extinction ($p<0.001$) [Figure 6.5 (D)].

6.7 Discussion:

The Behavioral experiments of reinstatement test was performed similarly to the renewal experiment as both involved the initiation of extinction training at two different time points i.e. 10 min and 24 hours after the fear memory acquisition. When freezing percent was compared between immediate reinstatement and immediate extinction group, no difference in the freezing percent was observed. In other words, the animals placed in immediate reinstatement group exhibited very little amount of fear recovery or no recovery as compared to the immediate extinction group it suggests that the recovery of fear memory has some "erasure" component. But delayed reinstatement group exhibited robust amount of fear recovery when compared with the delayed extinction group and suggested that the recovery of fear memory is inhibitory learning [Figure 6.5 (D)]. The results shown here are in line with the previous research work performed by another group of scientists Myers et al., 2006. The possible reason behind the non-recurrence of fear memory is that the reinstatement experiment was performed with the animals of entirely different group than the extinction group but they were trained similarly i.e. at the same time and under the same conditions. Another reason might be that immediate extinction group followed somewhat different mechanisms of extinction than the delayed extinction group.

Chapter 6.2

Aim 2.

To find out the molecular substrates especially CREB and its target genes which might be playing a role in the reinstatement and renewal as observed following immediate and delayed extinction, in amygdala, hippocampus and prefrontal cortex.

CREB (cAMP responsive element binding protein) is a transcriptional factor (Yin et al., 1995; Viosca et al., 2009; Sekeres et al., 2010; Suzuki et al., 2011; Martinez, 2015) and has a well-documented role in long term memory formation (Viosca et al., 2009). It is a nuclear protein that becomes activated after the phosphorylation of CREB at serine133 by protein kinase A (PKA) (Shaywitz and Greenberg, 1999). The activated p-CREB binds with the CRE region (cAMP response element) and recruites co-activators CBP (CREB binding protein) thus cause the regulation of certain genes like ARC and c-fos. ARC (activity regulated cytoskeleton) is a gene whose transcription is regulated by p-CREB (Miyamoto, 2006; Alberini and Kandel, 2015, Martinez, 2015). ARC regulates the neuronal plasticity and thus regulating memory (Li et al., 2005). c-fos is an immediate early gene (IEG) and has been widely used in the study of memory related paradigm from last several years. It functions as a neuronal activation marker (Knapska and Maren, 2009). The expression of c-fos suggests the activation of specific brain regions and their subregions during fear learning and extinction learning paradigm. So that the brain region isolated after the behavioral training having the region of interest i.e. hippocampus, amygdala and prefrontal cortex (PFC) were immune-stained for c-fos, p-CREB and ARC.

7. Molecular analysis for same context (AA)

7.1 *c-fos* expression in Amygdala

LA: In the LA region of amygdala delayed extinction group exhibited decreased *c-fos* expression than the other remaining groups. The observed changes were analyzed by two way ANOVA that revealed significant main effect of time [$F(1, 8) = 18.60$, $p<0.01$] and the interaction between time x condition was also significant [$F(1, 8) = 13.60$, $p<0.01$]. However there was no significant main effect of condition [$F(1, 8) = 2.738$, $p>0.05$]. Further Tukey's *post-hoc* test for multiple comparison analysis confirmed that delayed extinction group exhibited decreased *c-fos* expression as compared to the immediate extinction group ($p<0.05$) and delayed no extinction group ($p<0.05$).

BA: Two way ANOVA analysis for *c-fos* expression in BA region of amygdala revealed significant main effect of time [$F(1, 8) = 26.76$, $p<0.001$] and condition [$F(1, 8) = 33.32$, $p<0.001$]. The interaction between time x condition was also found to be significant [$F(1, 8) = 15.26$, $p<0.01$]. Tukey's *post-hoc* test showed significant increased *c-fos* expression in immediate extinction group as compared with the immediate no extinction group ($p<0.001$) and delayed extinction group

(p<0.01). Delayed extinction group showed higher *c-fos* expression than the delayed no extinction group (p<0.05).

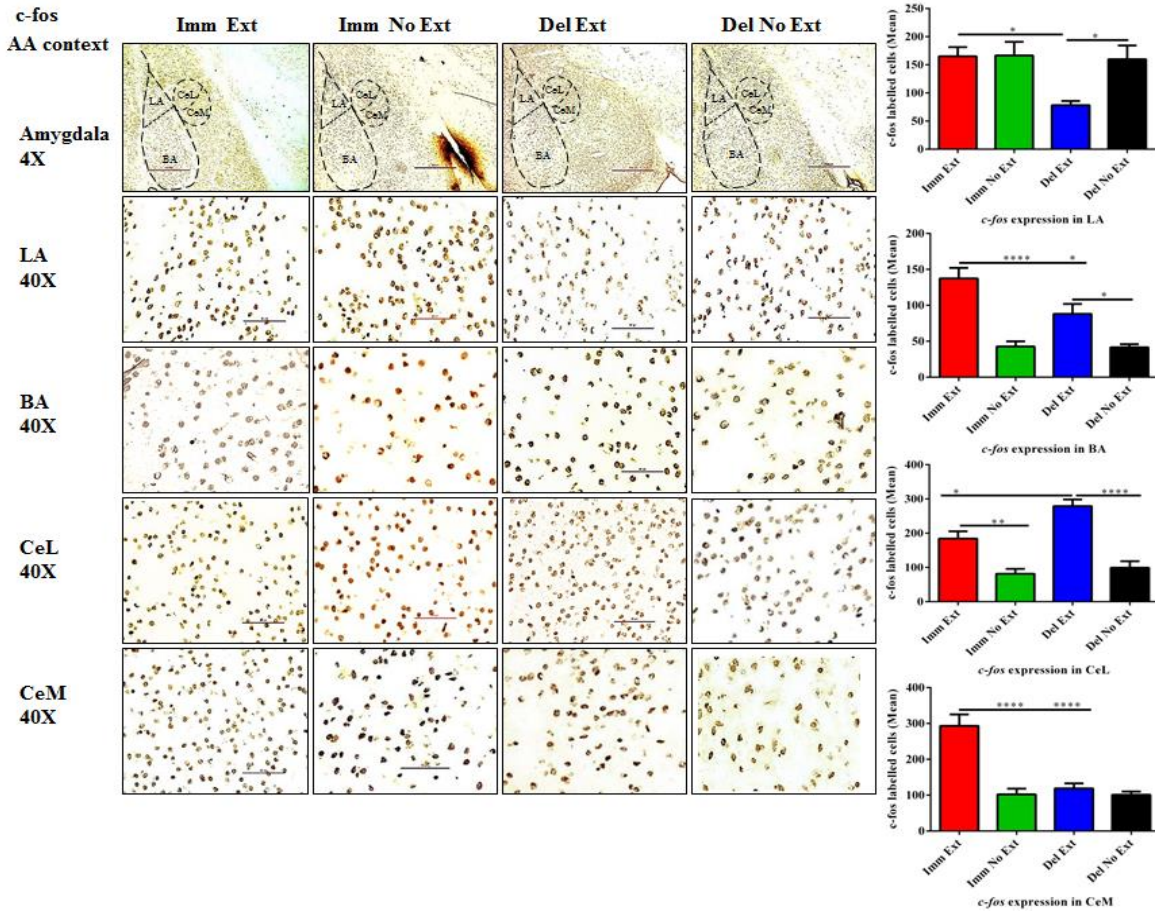

Figure 6.6: Represents the expression of IEG c-fos in the LA, BA, CeL and CeM region of the amygdala

CeL: Two way ANOVA analysis for *c-fos* expression in the CeL region revealed significant main effect of time [F (1, 8) =22.79, p<0.01] and condition [F (1, 8) =35.51, p<0.001]. The interaction between time x condition was also significant [F (1, 8) =10.86, p<0.05]. Tukey's *post-hoc* test confirmed increased *c-fos* expression in immediate extinction (p<0.01) and delayed extinction (p<0.0001) as compared to their respective control groups. However, the expression of *c-fos* was higher in delayed extinction group (p<0.05) as compared with the immediate extinction group.

CeM: *c-fos* expression in CeM region of amygdala exhibited increased expression in immediate extinction group as compared to other remaining groups. Two way ANOVA analysis showed significant main effect of time [F (1, 8) =14.32, p<0.01], condition [F (1, 8) =43.26, p<0.001] and

also the significant interaction between time x condition [F (1, 8) =14.06, p<0.01]. Tukey's *post hoc* test for multiple comparison analysis revealed increased c-fos expression in immediate extinction group than the immediate no extinction group (p<0.001) and delayed extinction group (p<0.001).

7.2 *c-fos* expression in Hippocampus

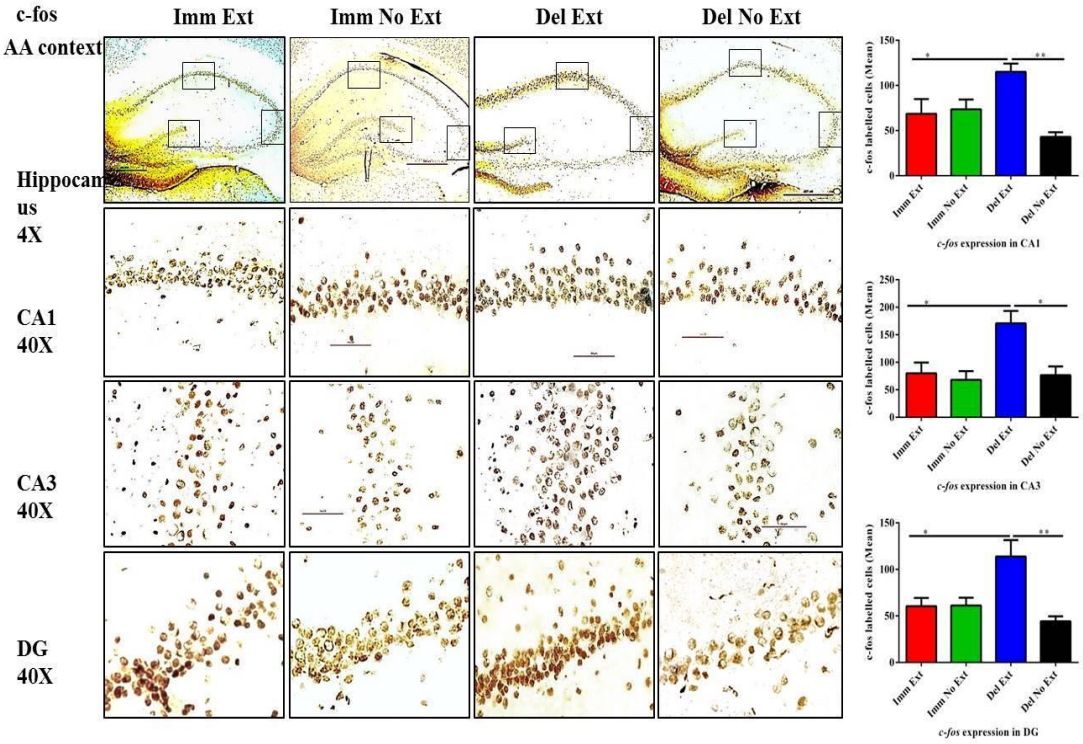

Figure 6.7: Represents the expression of c-fos in the different regions of the hippocampus involved in fear memory consolidation and extinction

CA1: Increased *c-fos* expression was observed in delayed extinction group. Two way ANOVA analysis revealed significant main effect of condition [F (1, 8) =10.66, p<0.05] and also the significant interaction between time x condition [F (1, 8) =10.43, p<0.05]. However there was no significant main effect of time was observed [F (1, 8) =0.4393, p>0.05]. Tukey's *post hoc* test confirmed increased *c-fos* expression in del ext group than the imm ext group (p<0.05) and del no ext group (p<0.01).

CA3: Two way ANOVA analysis for *c-fos* expression in CA3 region revealed significant main effect of condition [F (1, 8) =6.906, p<0.05] also the interaction between time x condition [F (1, 8) =5.839, p<0.05] was significant. However there was no effect of time was observed [F (1, 8)

=1.669, p>0.05]. Tukey's *post hoc* test confirmed the increased *c-fos* expression in del ext than the imm ext (p<0.05) and del no ext (p<0.05).

DG: *c-fos* expression in the DG region showed enhanced expression in delayed extinction group than other remaining groups. Two way ANOVA analysis for *c-fos* expression revealed significant main effect of condition [F (1, 8) =13.61, p<0.01] along with the significant interaction between time x condition [F (1, 8) =7.906, p<0.05]. However no effect of time was observed [F (1, 8) =1.669, p>0.05]. Tukey's *post hoc* test exhibited increased *c-fos* expression in del ext when compared with the imm ext (p<0.05) and del no ext group (p<0.05).

7.3 *c-fos* expression in Prefrontal cortex (PFC)

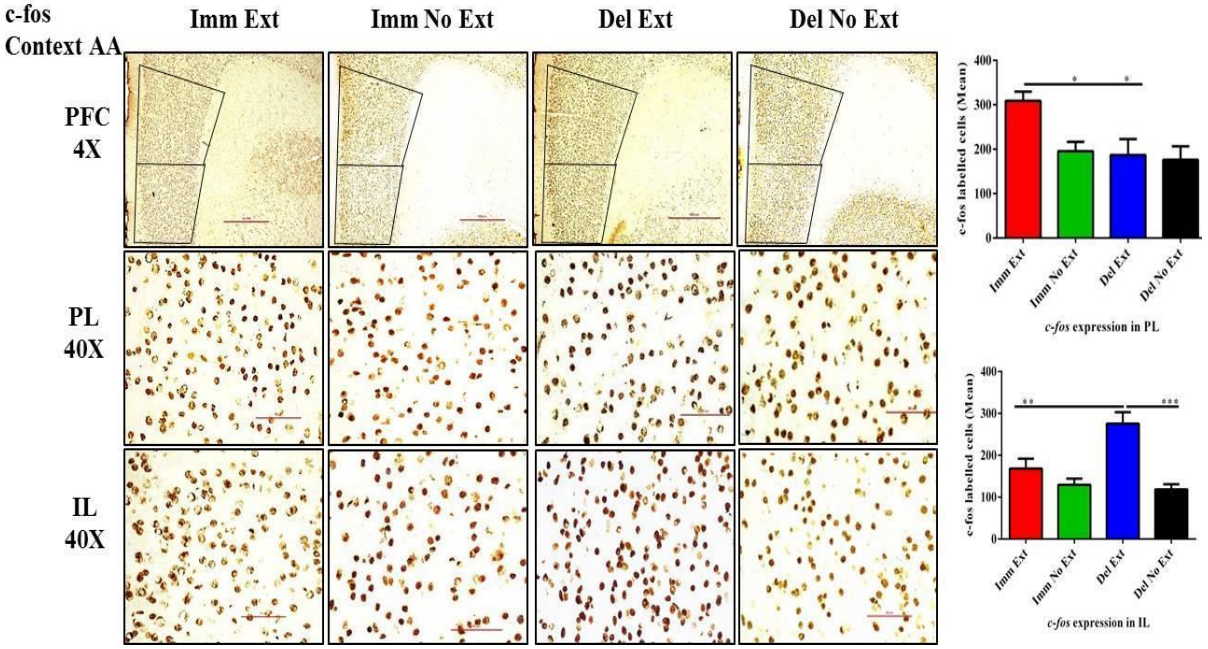

Figure 6.8: Represents the c-fos expression in IL and PL regions of the prefrontal cortex involved in fear memory consolidation and extinction

PL: There was increased *c-fos* expression in PL region of PFC was observed in imm ext than the other remaining groups. Two way ANOVA analysis for above data revealed significant main effect of time [F (1, 8) =5.439, p<0.05] as well as condition [F (1, 8) =6.161, p<0.05]. However the interaction between time x condition [F (1, 8) =2.844, p>0.05] was not significant. Further Tukey's *post-hoc* multiple comparison test exhibited increased *c-fos* expression in imm ext group than imm no ext (p<0.05) and del ext (p<0.05) group.

IL: When *c-fos* expression was observed in IL region of PFC, delayed extinction group exhibited increased expression. Two way ANOVA analysis for *c-fos* expression in IL region showed significant main effect of time [F (1, 8) =8.235, p<0.05] and condition [F (1, 8) =17.02, p<0.01] along with the significant interaction between time x condition [F (1, 8) =12.53, p<0.01]. Further Tukey's *post-hoc* test showed increased *c-fos* expression in delayed extinction group as compared with the immediate extinction group (p<0.01) and delayed no extinction group (p<0.001).

7.4 p-CREB in Amygdala

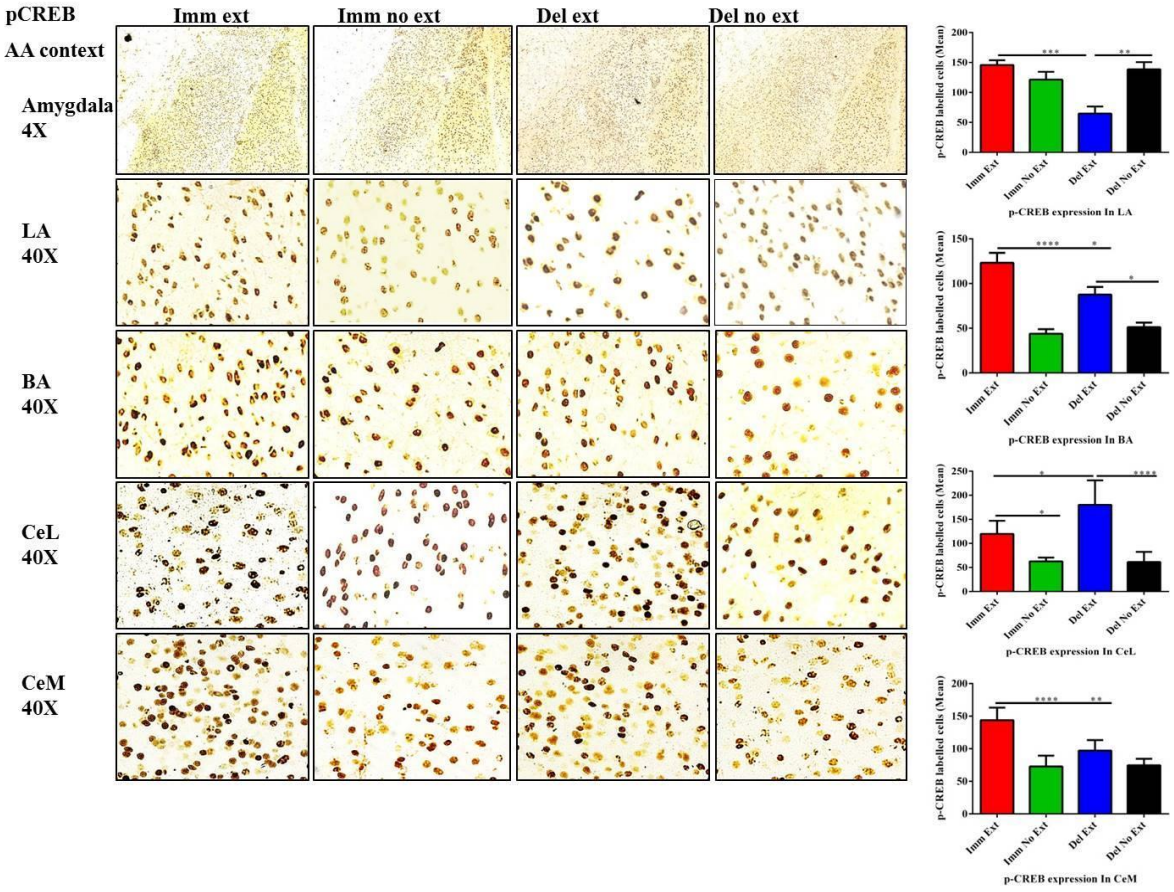

Figure 6.9: Represents the activation of phosphorylated CREB (p-CREB) in the LA, BA, CeL and CeM regions of the amygdala

LA: In the LA region of the amygdala, decreased activation of p-CREB was found in the delayed extinction group. The two way ANOVA analysis revealed significant main effect of time [F (1, 8) =16.69, p<0.01] but no significant effect of condition was observed [F (1, 8) =3.116, p>0.05]. However the interaction between time x condition was significant [F (1, 8) =39.53, p<0.001]. The changes were further confirmed by Tukey's *post-hoc* test that confirmed the decreased activation of

p-CREB in delayed extinction group as compared to immediate extinction ($p<0.001$) and delayed no extinction group ($p<0.01$).

BA: When activation of p-CREB was measured in the BA region of amygdala and was analyzed by two way ANOVA, significant main effect of time [$F(1, 8) = 7.608$, $p<0.05$] and condition [$F(1, 8) = 34.17$, $p<0.001$] was observed. Two way ANOVA analysis also exhibited significant interaction between time x condition [$F(1, 8) = 17.69$, $p<0.01$]. Tukey's *post-hoc* test for multiple comparison analysis confirmed the increased activation of p-CREB in immediate ($p<0.0001$) and delayed extinction ($p<0.05$) group as compared to their respective control groups. The immediate extinction group exhibited higher CREB phosphorylation than the delayed extinction group ($p<0.05$).

CeL: CeL region of amygdala exhibited increased p-CREB activation in delayed extinction group. The two way ANOVA analysis revealed significant main effect of time [$F(1, 8) = 7.041$, $p<0.05$] and condition [$F(1, 8) = 29.97$, $p<0.01$]. The interaction between time x condition was also significant [$F(1, 8) = 7.622$, $p<0.05$]. Tukey's *post-hoc* test further confirmed the increased p-CREB activation in immediate ($p<0.05$) and delayed extinction ($p<0.001$) group as compared to their respective control groups. However delayed extinction group exhibited higher p-CREB activation than the immediate extinction group ($p<0.05$).

CeM: In the CeM region of amygdala, increased activation of p-CREB was observed in the immediate extinction group. The two way ANOVA analysis for p-CREB activation in CeM region revealed significant main effect of time [$F(1, 8) = 9.769$, $p<0.05$] and condition [$F(1, 8) = 44.42$, $p>0.001$]. The interaction between time x condition was also significant [$F(1, 8) = 11.40$, $p<0.01$]. Tukey's *post-hoc* test showed significantly increased activation in immediate extinction group than the immediate no extinction group ($p<0.0001$) and delayed extinction group ($p<0.01$).

7.5 p-CREB in Hippocampus:

CA1: p-CREB activation was also looked in the different sub regions of the hippocampus. In CA1 region of hippocampus, increased p-CREB activation was observed in the delayed extinction group. The two way ANOVA analysis revealed no significant effect of time [$F(1, 8) = 3.425$, $p>0.05$] but the effect of condition was significant [$F(1, 8) = 7.172$, $p<0.05$]. Also the interaction between time x condition [$F(1, 8) = 6.938$, $p<0.05$] was significant. Tukey's *post-hoc* test showed increased activation in delayed extinction as compared to immediate extinction ($p<0.05$) and delayed no extinction control group ($p<0.01$).

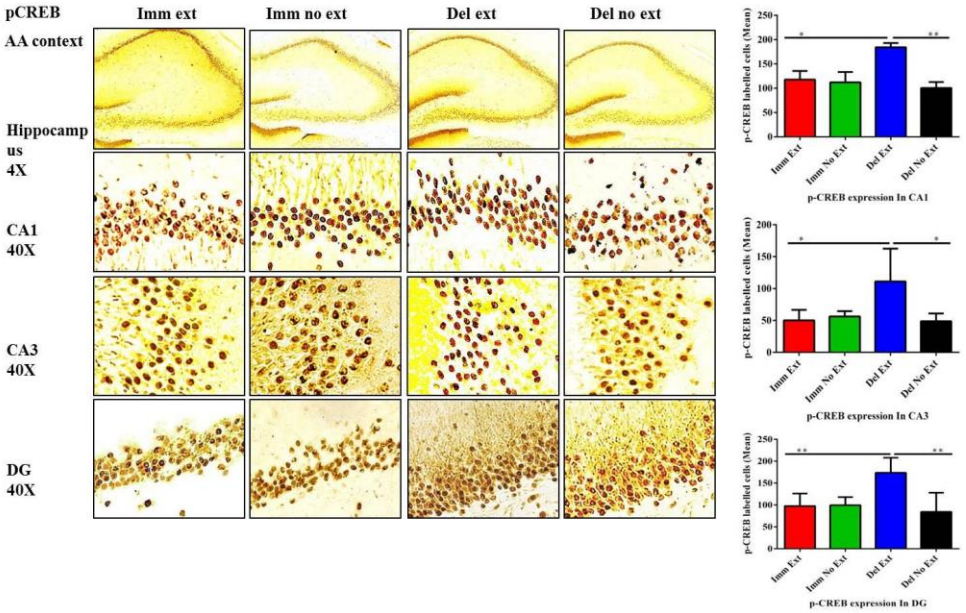

Figure 6.10: Represents the activation of phosphorylated CREB in CA1, CA3 and DG regions of the hippocampus

CA3: Increased p-CREB activation was observed in the delayed extinction group in the CA3 region of the hippocampus. Two way ANOVA analysis revealed significant main effect of condition [$F(1, 8) = 5.648$, $p<0.05$] but the effect of time was not significant [$F(1, 8) = 4.089$, $p>0.05$]. The interaction between time x condition was significant [$F(1, 8) = 6.659$, $p<0.05$]. Tukey's *post-hoc* test exhibited enhanced activation in delayed extinction group as compared to the immediate extinction ($p<0.05$) and delayed no extinction control group ($p<0.05$).

DG: Like CA1 and CA3, in the DG region of the hippocampus increased activation of p-CREB was found in the delayed extinction group. Two way ANOVA analysis revealed no significant effect of time [$F(1, 8) = 4.011$, $p>0.05$] but the main effect of condition was found to be significant [$F(1, 8) = 9.714$, $p<0.05$]. The interaction between time x condition [$F(1, 8) = 9.025$, $p<0.05$] was also significant as suggested by two way ANOVA analysis. Tukey's *post-hoc* test for multiple comparison analysis showed increased activation in delayed extinction group as compared to immediate extinction ($p<0.01$) and delayed no extinction control group ($p<0.01$).

7.6 p-CREB in Prefrontal cortex:

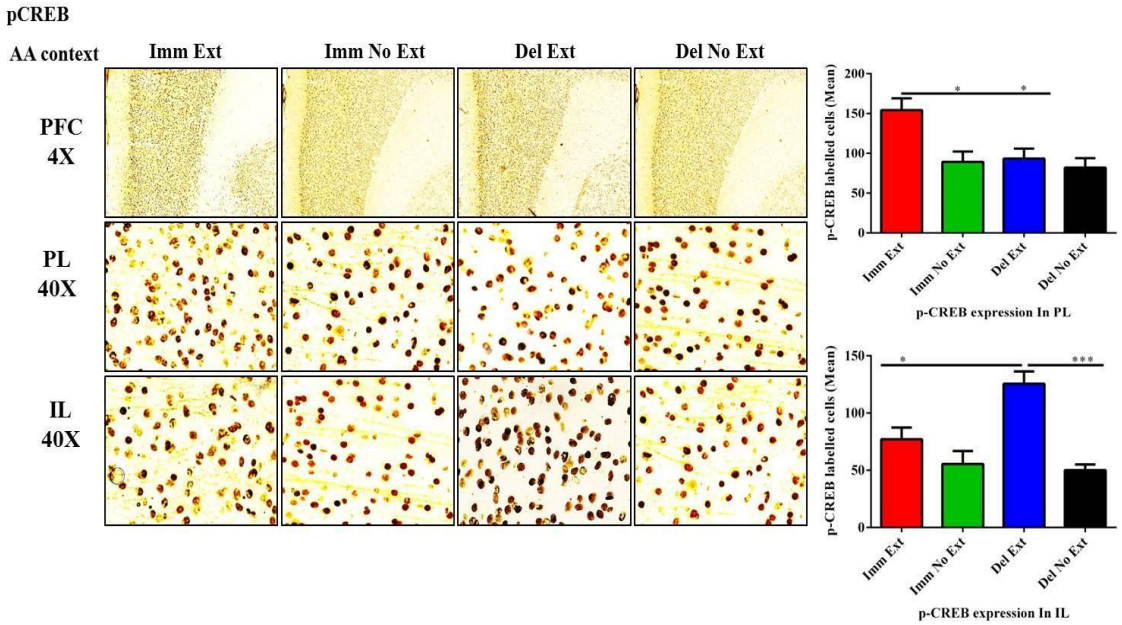

Figure 6.11: Represents the activation of phosphorylated CREB in the PL and IL regions of the prefrontal cortex

PL: Increased p-CREB activation was observed in immediate extinction group than the other groups. The two way ANOVA analysis revealed significant main effect of time [$F(1, 8) = 8.130$, $p<0.05$] and condition [$F(1, 8) = 7.259$, $p<0.05$]. We also looked is there any interaction between time and condition. No significant interaction between time x condition [$F(1, 8) = 5.051$, $p>0.05$] was observed. Tukey's *post-hoc* test confirmed the increased p-CREB activation in immediate extinction as compared to immediate no extinction ($p<0.05$) and delayed extinction ($p<0.05$).

IL: Next p-CREB activation was observed in the IL region of PFC that showed increased activation in delayed extinction group. Two way ANOVA analysis revealed significant main effect of time [$F(1, 8) = 5.372$, $p<0.05$] and condition [$F(1, 8) = 23.55$, $p<0.01$]. The interaction between time x condition was also significant [$F(1, 8) = 8.393$, $p<0.05$] as suggested by two way ANOVA analysis. Tukey's *post-hoc* test confirmed the increased p-CREB activation in delayed extinction group as compared to immediate extinction ($p<0.05$) and delayed no extinction group ($p<0.001$).

7.7 ARC expression in Amygdala:

LA: Decreased expression of ARC was found in delayed extinction group than the immediate extinction and delayed no extinction group. A two way ANOVA revealed significant main effect of time [$F(1, 8) = 21.97$, $p<0.01$] but the main effect of condition was not significant [$F(1, 8) = 2.080$,

p>0.05]. But the interaction between time x condition was significant [F (1, 8) =20.45, p<0.01]. Tukey's *post-hoc* multiple comparison test confirmed the decreased ARC expression in delayed extinction group as compared to the immediate extinction group (p<0.001) and delayed no extinction group (p<0.01).

BA: In the BA region of amygdala increased expression of ARC was found in immediate extinction and delayed extinction group. The two way ANOVA analysis of ARC expression in the BA region revealed significant main effect of time [F (1, 8) =13.53, p<0.01] and condition [F (1, 8) =36.12, p<0.001]. The interaction between time x condition was also significant [F (1, 8) =14.47, p<0.01]. Tukey's *post-hoc* test confirmed the significant increased expression in immediate extinction (p<0.001) and delayed extinction (p<0.05) as compared with their respective control groups. Expression of ARC was highest in immediate extinction than the delayed extinction (p<0.05).

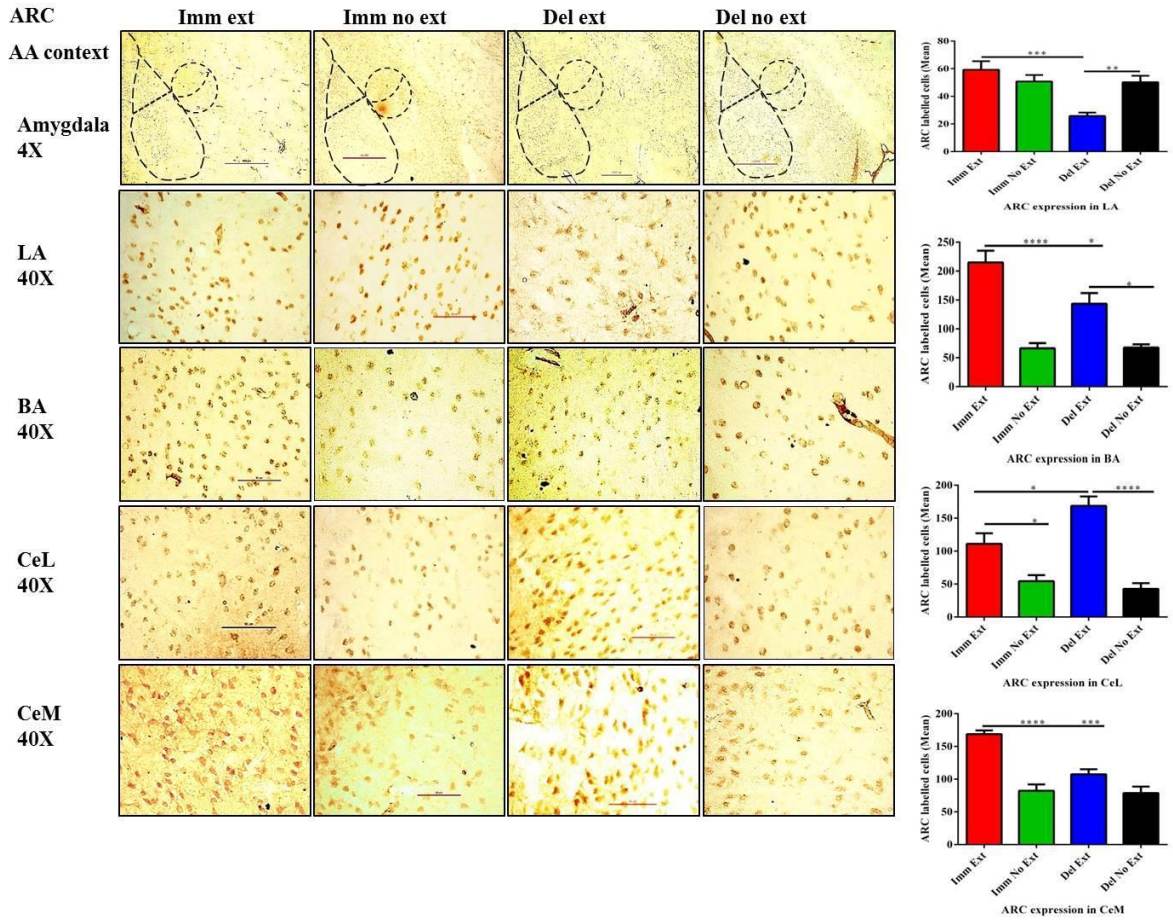

Figure 6.12: Represents the expression of ARC in the LA, BA, CeL and CeM region of the amygdala

CeL: Increased expression of ARC was found in the delayed extinction group. Two way ANOVA analysis for ARC expression in the CeL region of the amygdala revealed significant main effect of time [F (1, 8) =5.952, p<0.05] and condition [F (1, 8) =38.09, p<0.001]. The interaction between time x condition was also significant [F (1, 8) =13.43, p<0.01]. Tukey's *post-hoc* test for multiple comparison analysis confirmed the significant increased expression of ARC was observed in delayed extinction as compared to delayed no extinction (p<0.0001) and immediate extinction (p<0.05). Immediate extinction group showed increased ARC expression than the immediate no extinction group (p<0.05).

CeM: When expression of ARC was measured in CeM region of amygdala, increased expression was observed in immediate extinction group. Two way ANOVA analysis for ARC expression in the CeM region revealed significant main effect of time [F (1, 8) =9.943, p<0.05] and condition [F (1, 8) =95.72, p<0.0001]. The interaction between time x condition was also significant [F (1, 8) =7.966, p<0.05]. Tukey's *post-hoc* test confirmed the increased expression of ARC in immediate extinction group as compared to immediate no extinction (p<0.0001) and delayed extinction group (p<0.001).

7.8 ARC expression in Hippocampus:

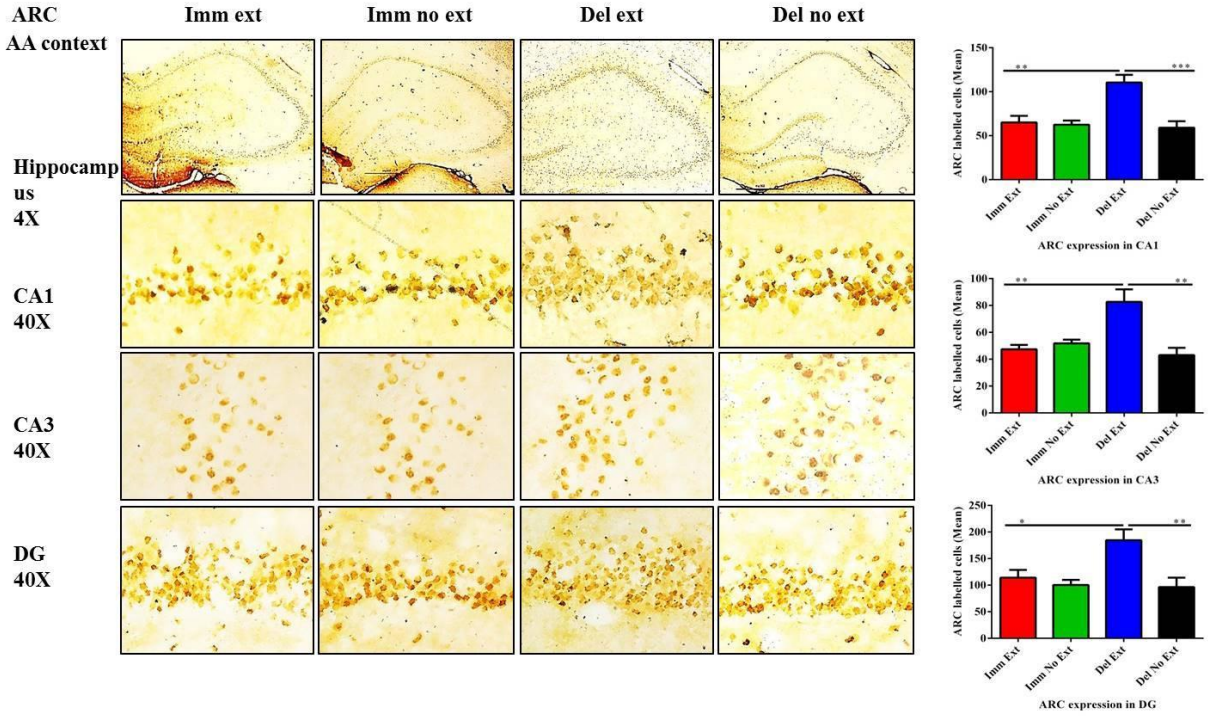

Figure 6.13: Represents the expression of ARC in the CA1, CA3 and DG regions of the hippocampus

CA1: Increased expression of ARC was found in the delayed extinction group in the CA1 region of the hippocampus following two way ANOVA analysis that revealed no significant main effect of time [$F(1, 8) = 4.917$, $p > 0.05$] but the main effect of condition [$F(1, 8) = 43.44$, $p < 0.001$] was significant. The interaction between time x condition was also significant [$F(1, 8) = 6.639$, $p < 0.05$] as evident by two way ANOVA. Tukey's *post-hoc* test confirmed the increased ARC expression in delayed extinction group as compared to immediate extinction ($p < 0.01$) and delayed no extinction group ($p < 0.001$).

CA3: Two way ANOVA analysis for ARC expression in the CA3 region revealed significant main effect of condition [$F(1, 8) = 9.723$, $p < 0.05$] but no effect of time was observed [$F(1, 8) = 4.797$, $p > 0.05$]. However the interaction between time x condition was significant [$F(1, 8) = 13.33$, $p < 0.01$]. Tukey's *post-hoc* test revealed increased ARC expression in delayed extinction as compared to immediate extinction ($p < 0.01$) and delayed no extinction ($p < 0.01$).

DG: DG region of the hippocampus showed increased ARC expression in delayed extinction group. Two way ANOVA analysis for ARC expression in DG region revealed significant main effect of condition [$F(1, 8) = 8.772$, $p < 0.05$] and time x condition interaction [$F(1, 8) = 6.269$, $p < 0.05$]. However, the main effect of time was not significant [$F(1, 8) = 4.993$, $p > 0.05$]. Tukey's *post-hoc* test for multiple comparison analysis confirmed increased expression of ARC in delayed extinction as compared to immediate extinction ($p < 0.05$) and delayed no extinction ($p < 0.01$).

7.9 ARC expression in Prefrontal cortex

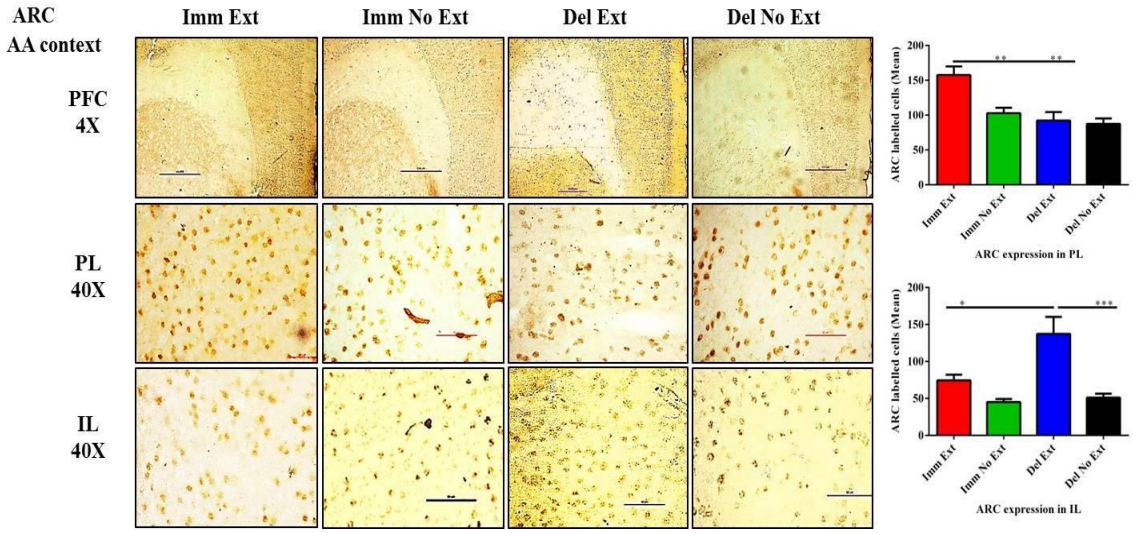

Figure 6.14: Represents the expression of ARC in the PL and IL regions of the prefrontal cortex

PL: Immediate extinction group showed increased expression of ARC in the PL region of PFC. Two way ANOVA analysis in PL region for ARC expression revealed significant main effect of time [F (1, 8) =12.34, p<0.01] and condition [F (1, 8) =11.15, p<0.05]. The interaction between time x condition [F (1, 8) =4.726, p>0.05] was not significant. Further the changes were validated by Tukey's *post-hoc* test that confirmed the increased ARC expression in Immediate extinction as compared to immediate no extinction (p<0.01) and delayed extinction (p<0.01).

IL: ARC expression in IL region of PFC showed increased expression in delayed extinction group. Two way ANOVA analysis revealed significant main effect of time [F (1, 8) =10.20, p<0.05] and condition [F(1,8)=16.33, p<0.01]. The interaction between time x condition was also significant [F (1, 8) =6.951, p<0.05] as suggested by two way ANOVA. Tukey's *post-hoc* test confirmed the increased ARC expression in delayed extinction group as compared to immediate extinction (p<0.05) and delayed no extinction (p<0.01) group.

Immunohistochemistry results for AB context

7.10 *c-fos* expression in Amygdala

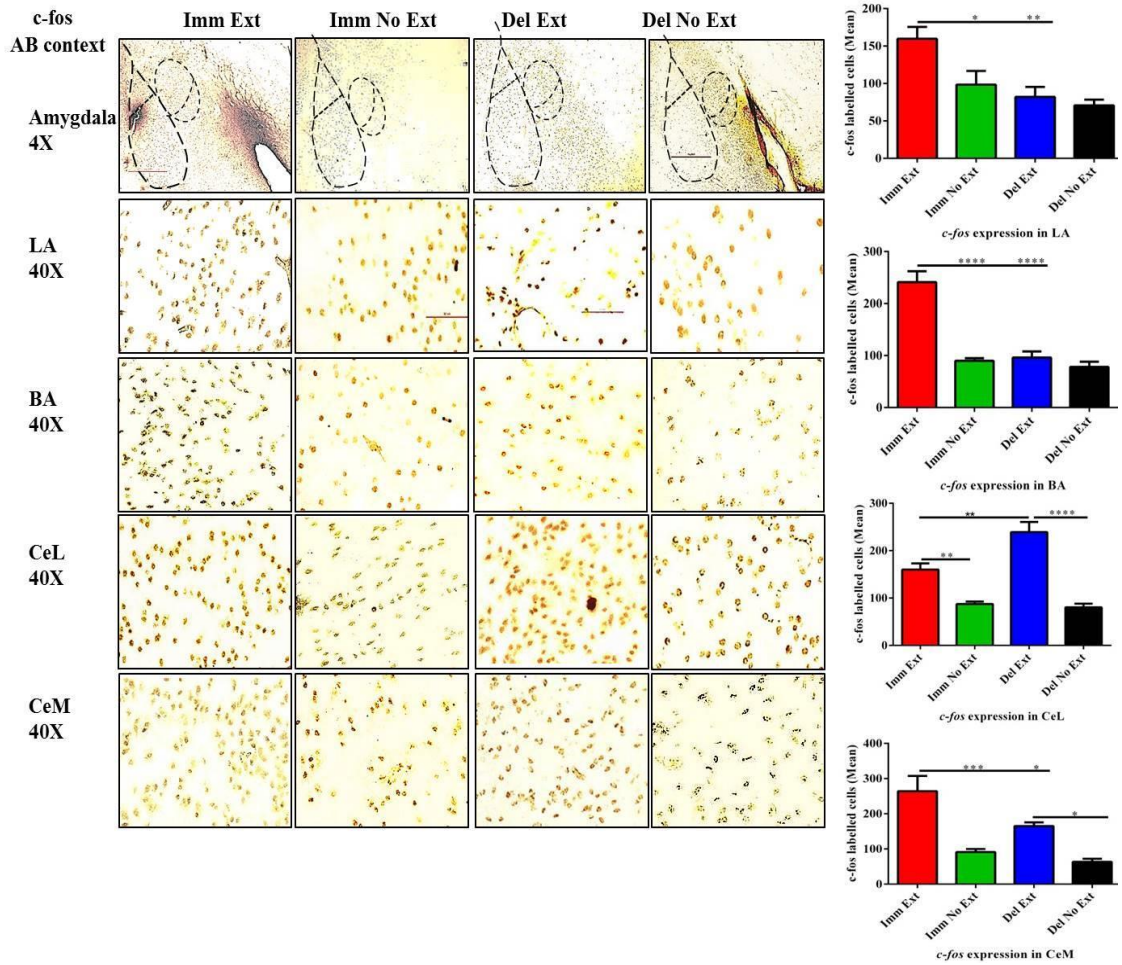

Figure 6.15: Represents *c-fos* expression in the LA, BA, CeL and CeM region of the amygdala

LA: When the expression of *c-fos* was measured in the LA region of amygdala, immediate extinction group exhibited higher expression than the other groups. Two way ANOVA analysis for *c-fos* expression revealed significant main effect of time (immediate vs delayed) [$F(1, 8) = 16.31$, $p<0.01$] and condition (extinction vs no extinction) [$F(1, 8) = 5.409$, $p<0.05$] but interaction between time and condition was not significant [$F(1, 8) = 3.699$, $p>0.05$]. Tukey's *post-hoc* test for multiple comparison analysis confirmed the significantly increased expression of *c-fos* in the immediate extinction group as compared to the immediate no extinction control group ($p<0.05$) and with the delayed extinction group ($p<0.01$).

BA: Immediate extinction group showed increased *c-fos* expression in the BA region of amygdala. Two way ANOVA analysis for the expression of *c-fos* in the BA region revealed significant main effect of time [$F(1, 8) = 81.76$, $p<0.001$] and condition [$F(1, 8) = 25.59$, $p<0.001$]. The interaction between time x condition was also significant [$F(1, 8) = 59.33$, $p<0.001$]. Tukey's *post-hoc* multiple comparison analysis confirmed the significant increased expression of *c-fos* in immediate extinction group than the immediate no extinction group ($p<0.001$) and delayed extinction group ($p<0.001$).

CeL: CeL region of amygdala exhibited higher expression of *c-fos* during immediate extinction and delayed extinction group with their respective control groups. A two way ANOVA analysis revealed significant main effect of time [$F(1, 8) = 9.534$, $p<0.05$] and condition [$F(1, 8) = 68.48$, $p<0.0001$]. The interaction between time x condition [$F(1, 8) = 13.59$, $p<0.01$] was also significant. Two way ANOVA analysis was followed by Tukey's *post-hoc* multiple comparison test that confirmed the significant increased expression of *c-fos* in immediate extinction group with immediate no extinction ($p<0.01$) group. Delayed extinction group also exhibited higher expression of *c-fos* than the delayed no extinction group ($p<0.0001$) while the decreased expression of c-fos was observed in immediate extinction group as compared to the delayed extinction group ($p<0.01$).

CeM: The CeM region of amygdala showed increased *c-fos* expression in immediate extinction and delayed extinction group with their respective control groups but the expression was higher in immediate extinction group than the delayed extinction group. Two way ANOVA analysis revealed significant main effect of time [$F(1, 8) = 12.87$, $p<0.005$] and condition [$F(1, 8) = 24.68$, $p<0.001$]. However the interaction between time and condition was not significant [$F(1, 8) = 4.059$, $p>0.05$].

Tukey's *post-hoc* test confirmed that the significant increased expression of *c-fos* in the immediate extinction group than the immediate no extinction group ($p<0.01$) and delayed extinction group ($p<0.05$). However, significant increased expression was observed in delayed extinction group than the delayed no extinction group ($p<0.05$).

7.11 *c-fos* expression in Hippocampus:

CA1: *c-fos* expression in the CA1 region of the hippocampus did not exhibit any difference among the groups. Two way ANOVA was used to analyze the expression of *c-fos* in the CA1 region that revealed no significant effect of time [$F(3, 24) = 7.399$, $p>0.05$] and condition [$F(1, 8) = 0.01520$, $p>0.05$]. Also the interaction between time and condition [$F(3, 24) = 0.5958$, $p>0.05$] was significant. These results were further confirmed by Tukey's *post-hoc* test that showed no significant difference among them (all $p>0.05$).

CA3: Like CA1 region, we did not find significant main effect of time [$F(1, 8) = 0.9454$, $p>0.05$], condition [$F(1, 8) = 0.5591$, $p>0.05$] and also the interaction between time x condition [$F(1, 8) = 2.519$, $p>0.05$] as evident by two way ANOVA. Further these changes were confirmed by Tukey's *post-hoc* test that yielded no significant difference across the groups (all $p>0.05$).

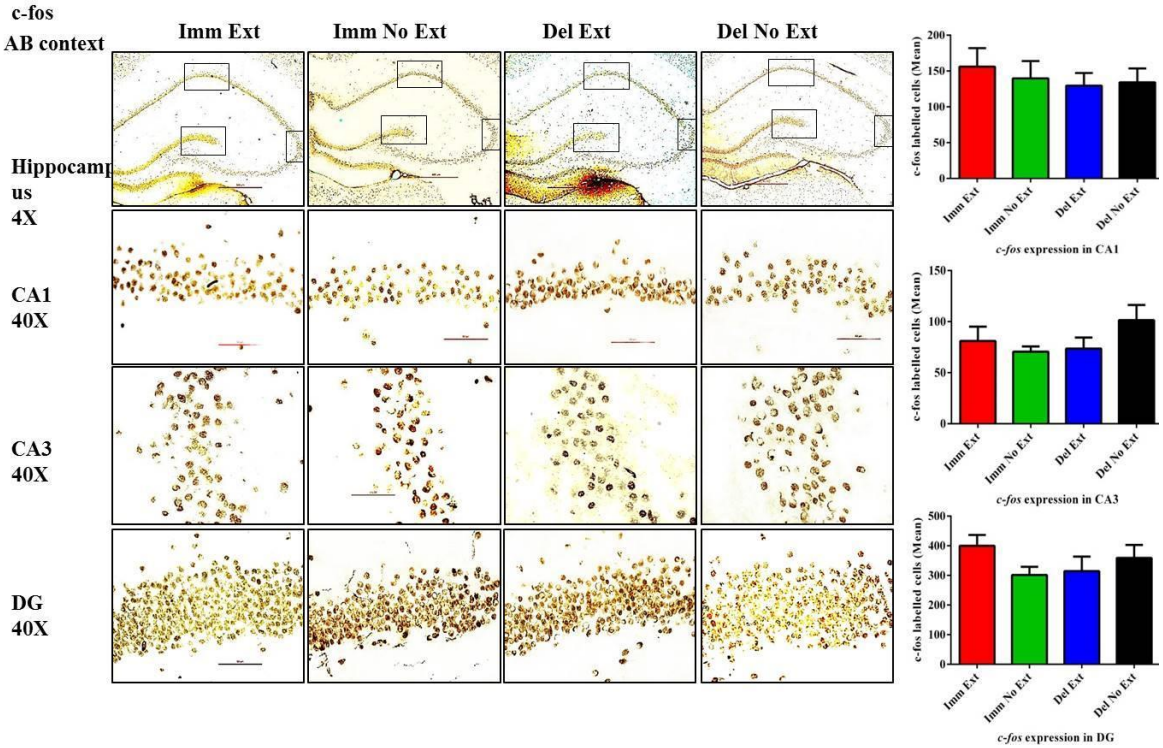

Figure 6.16: Represents the expression of *c-fos* in the CA1, CA3 and DG regions of the hippocampus

DG: When *c-fos* expression was measured in DG region of the hippocampus, no significant difference was observed among the groups. A two way ANOVA analysis exhibited that there was no significant main effect of time [F (1, 8) =0.02986, p>0.05], condition [F (1, 8) =0.04404, p>0.05] and the interaction between time x condition was also not significant [F (1, 8) =4.472, p>0.05]. Tukey's *post-hoc* test confirmed the no difference across the groups (all p>0.05).

7.12: *c-fos* expression in prefrontal cortex

PL: *c-fos* expression in the PL region of the prefrontal cortex was higher in immediate extinction as well as in delayed extinction as compared to their respective control groups. A two way ANOVA analysis for *c-fos* expression in PL region showed significant main effect of condition (extinction vs. no extinction) [F (1, 28) =24.2, p<0.0001], while there was no effect of time (Immediate vs. Delayed) [F (1, 28) =0.183, p>0.05] and condition × time interaction [F (1, 28) =0.018, p>0.05].

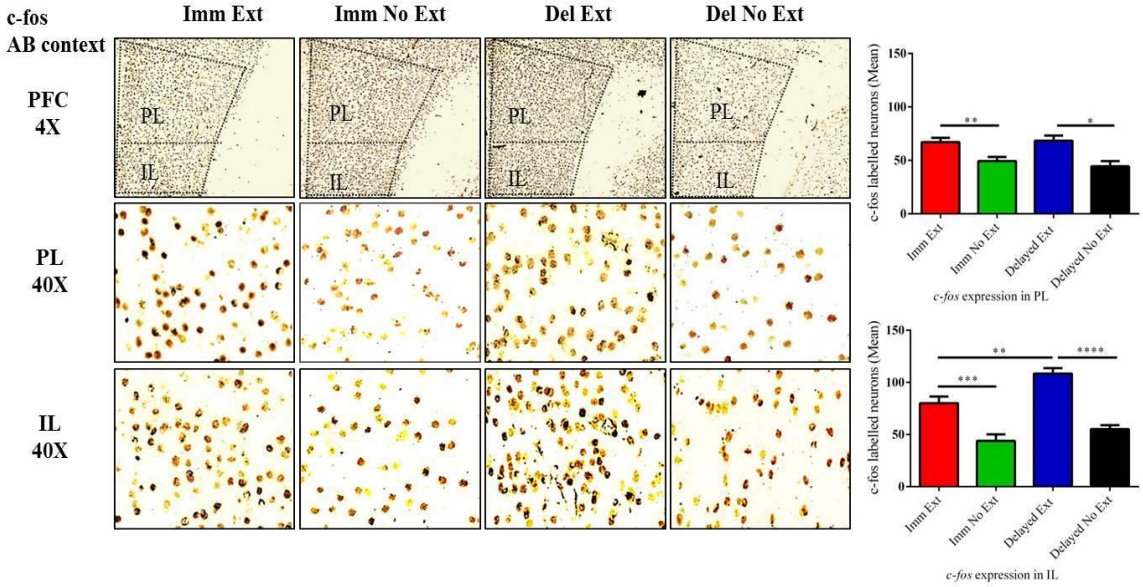

Figure 6.17: Represents the expression of c-*fos* in the PL and IL region of PFC.

IL: *c-fos* expression in IL region was higher in immediate and delayed extinction as compared to their respective control groups. The expression of *c-fos* was further confirmed by two way ANOVA analysis that revealed significant main effect of condition (extinction vs. no extinction) [F (1, 28)

=58.3, p<0.0001], 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].

7.13 p-CREB in Amygdala:

LA: The activation of p-CREB was higher in the immediate extinction group as compared with the other groups. Two way ANOVA analysis for p-CREB activation in the LA region of the amygdala revealed significant main effect of time [F (1, 8) =10.01, p<0.05] and condition [F (1, 8) =15.47, p<0.01]. However the interaction between time x condition was not significant [F (1, 8) =5.233, p>0.05]. These changes were further confirmed by Tukey's *post-hoc* test that revealed immediate extinction group exhibited increased activation of p-CREB than the immediate no extinction (p<0.01) and delayed extinction group (p<0.01).

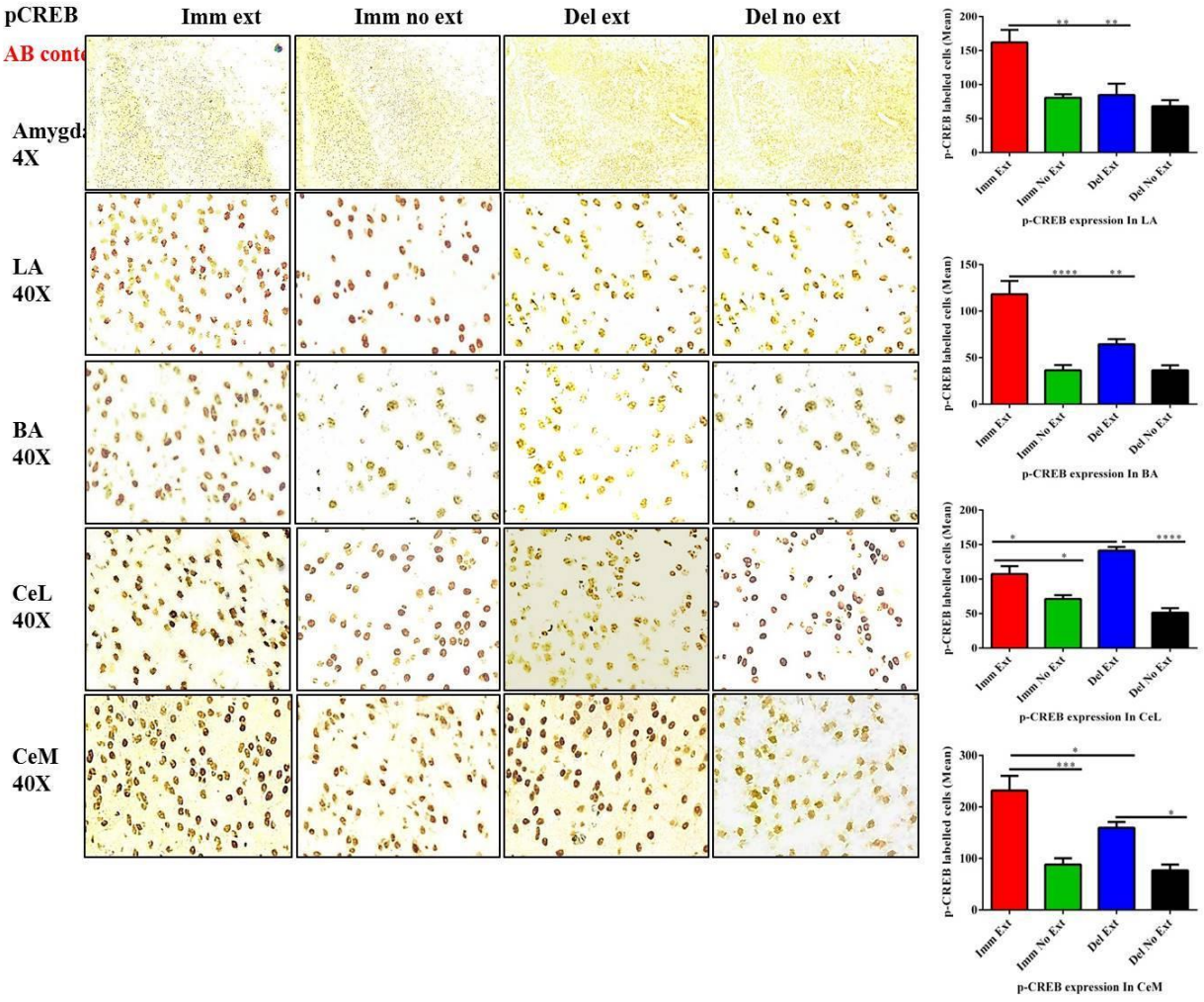

Figure 6.18: Represents the activation of p-CREB in LA, BA, CeL and CeM region of amygdala.

BA: Two way ANOVA analysis for p-CREB activation in the BA region of amygdala exhibited significant effect of time [$F(1, 8) = 21.69$, $p<0.01$] and condition [$F(1, 8) = 26.45$, $p<0.01$]. The interaction between time x condition was also significant [$F(1, 8) = 21.69$, $p<0.01$]. These changes were further confirmed by Tukey's *post-hoc* test that revealed immediate extinction group exhibited increased activation of p-CREB than the immediate no extinction ($p<0.0001$) and delayed extinction group ($p<0.01$).

CeL: In the CeL region of amygdala, there was increased activation of the p-CREB in both the immediate as well as delayed extinction group as compared to their respective controls. Two way ANOVA analysis revealed significant main effect of time [$F(1, 8) = 8.818$, $p<0.05$] and condition [$F(1, 8) = 35.46$, $p<0.01$]. The interaction between time x condition was also found significant [$F(1, 8) = 8.595$, $p<0.05$]. Further Tukey's *post-hoc* test confirmed the increased activation of p-CREB in immediate extinction group as compared to the immediate no extinction ($p<0.05$) group. Delayed extinction group exhibited higher activation of p-CREB than the delayed no extinction group ($p<0.0001$). However, the activation of p-CREB was higher in delayed extinction group than the immediate extinction group ($p<0.05$).

CeM: Two way ANOVA analysis for p-CREB activation in the CeM region of amygdala revealed significant main effect of time [$F(1, 8) = 6.733$, $p<0.05$] and condition [$F(1, 8) = 36.20$, $p<0.01$]. However, the interaction between time x condition was not significant [$F(1, 8) = 3.602$, $p>0.05$]. Tukey's *post-hoc* test confirmed the increased activation of p-CREB in the immediate extinction group as compared to the immediate no extinction ($p<0.01$) and delayed extinction group ($p<0.05$). Delayed extinction group exhibited higher activation of p-CREB than the delayed no extinction group ($p<0.05$).

7.14 p-CREB in Hippocampus:

CA1: CA1 region of the amygdala for p-CREB activation exhibited no difference across the groups. The two way ANOVA analysis revealed no significant main effect of time [$F(1, 8) = 4.878$, $p>0.05$] and condition [$F(1, 8) = 0.01637$, $p>0.05$]. Also the interaction between time x condition [$F(1, 8) = 0.2626$, $p>0.05$] was not significant. Tukey's *post-hoc* test exhibited no significant difference in the activation of p-CREB across the groups ($p>0.05$).

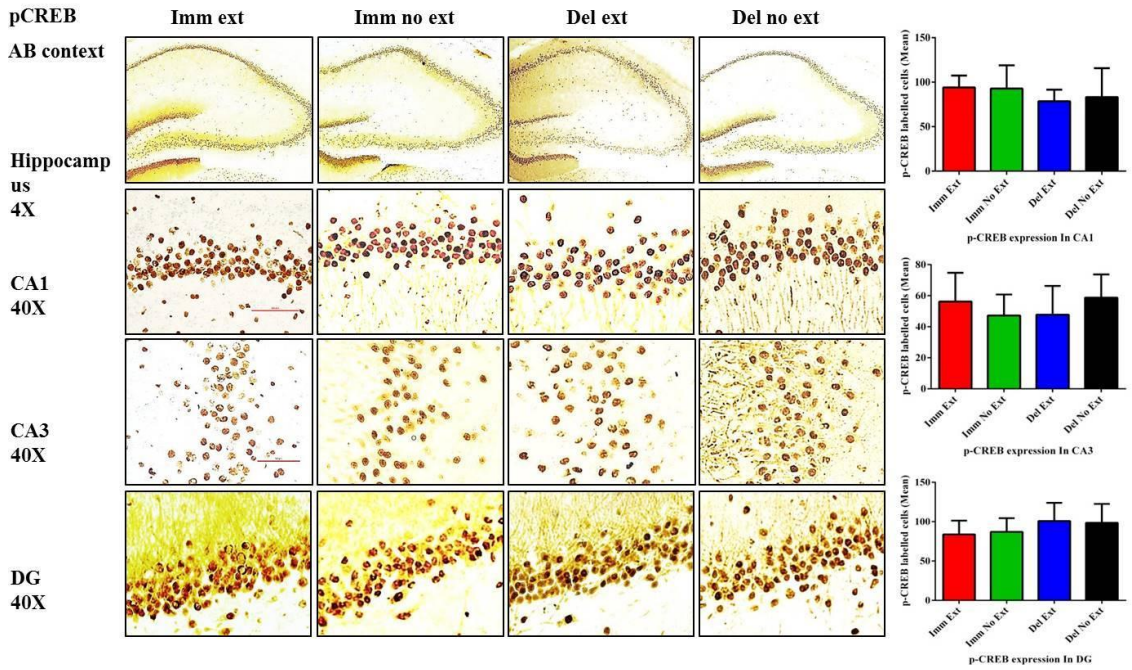

Figure 6.19: Represents the p-CREB activation in the CA1, CA3 and DG regions of the hippocampus.

CA3: In the CA3 region of the hippocampus the activation of p-CREB revealed no significant main effect of time [F (1, 8) =0.04547, p>0.05] and condition [F (1, 8) =0.01881, p>0.05]. The interaction between time x condition was also not significant [F (1, 8) =1.776, p>0.05] as suggested by two way ANOVA. Further Tukey's *post-hoc* test explained that no difference in the activation of p-CREB across the groups (p>0.05).

DG: p-CREB activation in the DG region of the hippocampus was not significant across the groups. Two way ANOVA analysis revealed no significant main effect of time [F (1, 8) =3.361, p>0.05], condition [F (1, 8) =0.002254, p>0.05] as well as time x condition interaction [F (1, 8) =0.1198, p>0.05]. Tukey's *post-hoc* test exhibited no significant difference in the activation of p-CREB across the groups (p>0.05).

7.15 p-CREB in pre-frontal cortex:

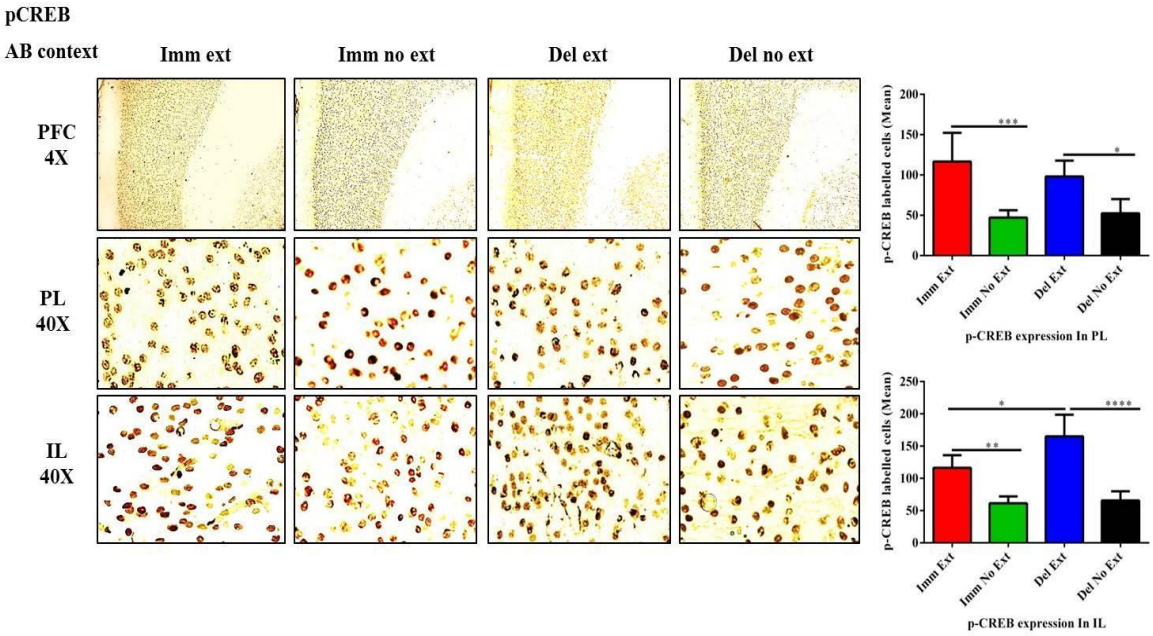

Figure 6.20: Represents the activation of p-CREB in the PL and IL region of PFC.

PL: p-CREB activation was higher in immediate and delayed extinction as compared to their respective control groups in the PL region of PFC without any significant difference between immediate and delayed extinction group. A two-way ANOVA analysis confirmed that there was a significant main effect of condition (extinction vs. no extinction) [$F(1, 8) = 34.11$, $p<0.001$] but no significant main effect for time (immediate vs. delayed) [$F(1, 8) = 0.3998$, $p>0.05$] and condition x time interaction [$F(1, 8) = 1.322$, $p>0.05$] was observed. Further Tukey's *post-hoc* test explained that higher activation of p-CREB was observed in immediate extinction group than the immediate no extinction group ($p<0.001$). Delayed extinction group also exhibited higher p-CREB activation than the delayed no extinction group ($p<0.05$). However, no significant difference in the activation of p-CREB was observed in between the immediate and delayed extinction group ($p>0.05$).

IL: In the IL region of PFC, delayed extinction group exhibited higher activation of p-CREB when compared with the other groups. Two way ANOVA analysis for the p-CREB activation in the IL region showed a significant main effect for condition [$F(1, 8) = 43.71$, $p<0.001$], time [$F(1, 8) = 15.71$, $p<0.01$] as well as condition x time interaction [$F(1, 8) = 10.94$, $p<0.05$]. Tukey's post-hoc test explained that immediate extinction group exhibited higher activation of p-CREB than the immediate no extinction group ($p<0.01$). Delayed extinction group also exhibited higher activation of p-CREB than the delayed no extinction group ($p<0.0001$). Increased activation of p-CREB was observed in delayed extinction group than the immediate extinction group ($p<0.05$).

7.16 ARC expression in Amygdala:

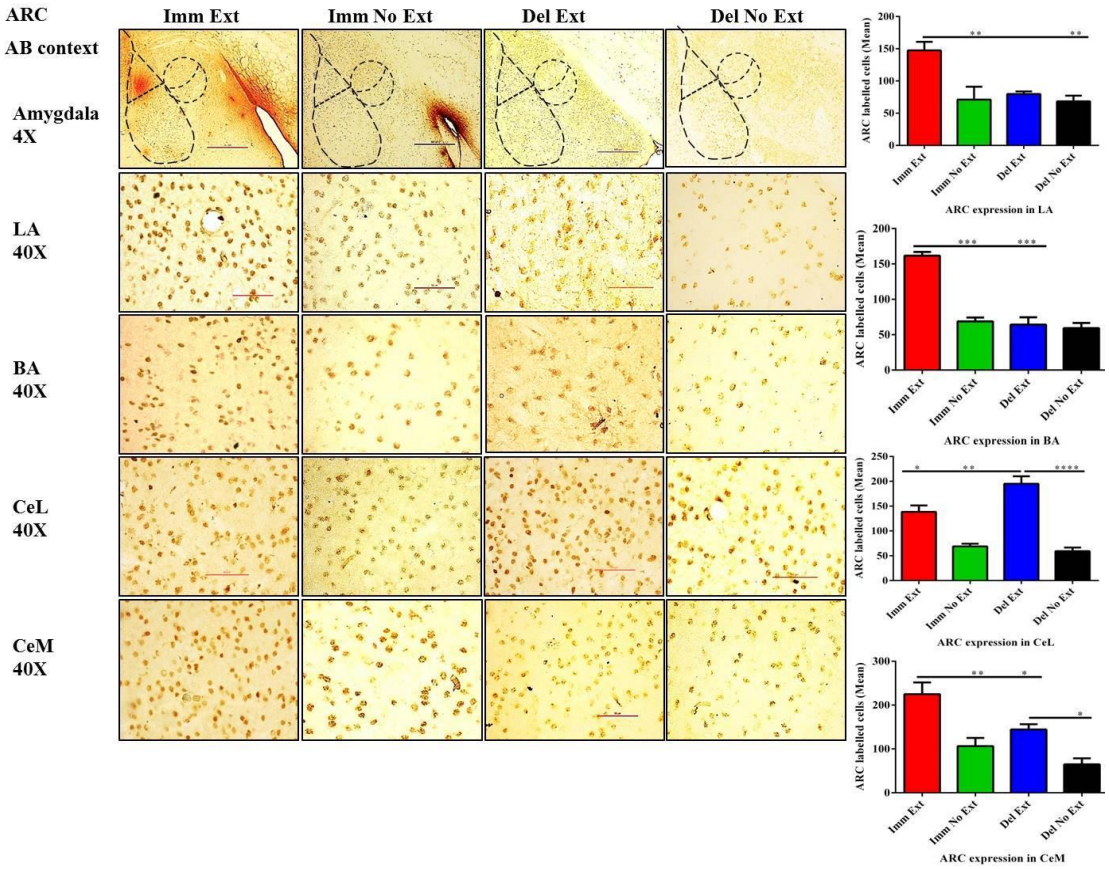

Figure 6.21: Represents the expression of ARC in the LA, BA, CeL and CeM region of amygdala.

LA: Activity regulated cytoskeletal protein (ARC) expression was higher in immediate extinction group as compared with the other groups. A two way ANOVA analysis exhibited significant main effect of time [$F(1, 8) = 7.656$, $p<0.05$] and condition [$F(1, 8) = 12.32$, $p<0.01$]. However the interaction between time x condition was not significant [$F(1, 8) = 4.874$, $p>0.05$]. These changes were further confirmed by Tukey's *post-hoc* test that revealed immediate extinction group showed higher expression of ARC than the immediate no extinction ($p<0.01$) and delayed extinction ($p<0.01$) group.

BA: Two way ANOVA analysis for the ARC expression in the BA region of amygdala exhibited significant effect of time [$F(1, 8) = 71.50$, $p<0.001$] and condition [$F(1, 8) = 34.38$, $p<0.001$]. The interaction between time x condition was also found significant [$F(1, 8) = 48.14$, $p<0.01$]. These changes were further confirmed by Tukey's *post-hoc* test that revealed immediate extinction group

showed higher expression of ARC than the immediate no extinction ($p<0.001$) and delayed extinction group ($p<0.001$).

CeL: When ARC expression was measured in the CeL region of amygdala, increased expression was observed in immediate as well as delayed extinction group as compared to the respective control groups. Two way ANOVA analysis revealed significant main effect of time [$F(1, 8) = 5.757$, $p<0.05$] and condition [$F(1, 8) = 72.77$, $p<0.001$]. The interaction between time x condition was also found significant [$F(1, 8) = 11.42$, $p<0.01$]. Tukey's *post-hoc* test confirmed the increased expression of ARC in the immediate extinction group as compared to the immediate no extinction ($p<0.01$) group. However, delayed extinction group also exhibited the increased ARC expression than the delayed no extinction control group ($p<0.001$) and immediate extinction group ($p<0.05$).

CeM: A two way ANOVA analysis for ARC expression in the CeM region revealed significant main effect of time [$F(1, 8) = 8.255$, $p<0.05$] and condition [$F(1, 8) = 38.67$, $p<0.001$]. However, the interaction between time x condition was not significant [$F(1, 8) = 0.8349$, $p>0.05$]. Tukey's *post-hoc* test confirmed the increased expression of ARC in immediate extinction group as compared to the immediate no extinction ($p<0.01$) and delayed extinction group ($p<0.05$). However delayed extinction group showed higher ARC expression than the delayed no extinction group ($p<0.05$).

7.17 ARC expression in Hippocampus:

CA1: The expression of ARC in the CA1 region of the hippocampus showed no difference for the expression across the groups. Two way ANOVA analysis revealed no significant main effect of time [$F(1, 8) = 0.6897$, $p>0.05$] and condition [$F(1, 8) = 4.105$, $p>0.05$] on the expression of ARC. Also the interaction between time x condition [$F(1, 8) = 0.05322$, $p>0.05$] was not significant as evident by two way ANOVA. Further Tukey's *post-hoc* test exhibited no difference for the ARC expression across the groups ($p>0.05$).

CA3: ARC expression in CA3 region of the hippocampus showed significant main effect of time [$F(1, 8) = 4.359$, $p>0.05$] and condition [$F(1, 8) = 0.04347$, $p>0.05$]. The interaction between time x condition [$F(1, 8) = 0.006616$, $p>0.05$] was also not significant as evident by 2 way ANOVA. Tukey's *post-hoc* test confirmed no difference for ARC expression across the groups ($P>0.05$).

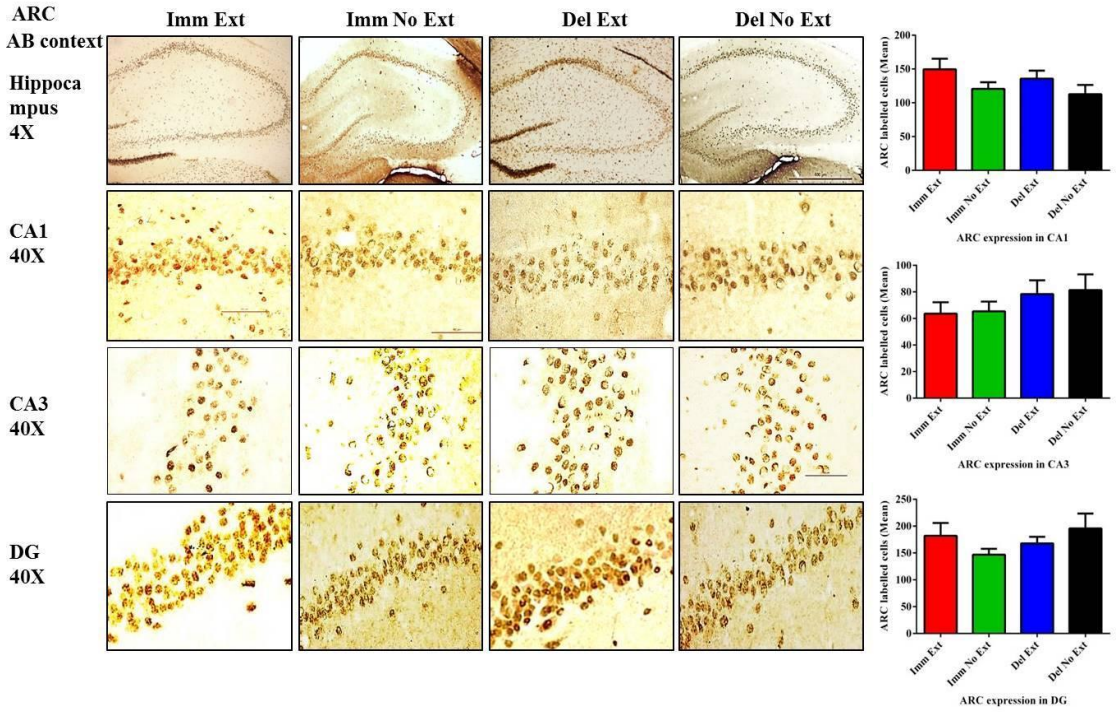

Figure 6.22: Represents the ARC expression in CA1, CA3 and DG regions of the hippocampus.

DG: The expression of ARC in DG region of the hippocampus was not significant across the groups. Two way ANOVA analysis revealed no significant main effect of time [F (1, 8) =0.6025, $p>0.05$] and condition [F (1, 8) =0.04241, $p>0.05$] as well as the interaction between time x condition [F (1, 8) = 1.987, $p>0.05$] was also not significant ($p>0.05$).

7.18 ARC expression in prefrontal cortex:

PL: Immediate and delayed extinction group exhibited higher expression of ARC when compared to their respective control groups in PL the region of PFC but no significant difference between immediate and delayed extinction group was observed. Two way ANOVA analysis confirmed that there was a significant main effect of condition [F (1, 8) =18.51, $p<0.01$] but no significant effect of time [F (1, 8) =0.0001978, $p>0.05$] was observed. The interaction between condition x time was also not significant [F (1, 8) =0.3164, $p>0.05$] for the expression of ARC. Tukey's *post-hoc* test exhibited increased expression of ARC in immediate extinction group than the immediate no extinction group ($p<0.05$). Delayed extinction group also exhibited increased ARC expression than the delayed no extinction group ($p<0.01$). But no significant difference between immediate and delayed extinction was observed ($p>0.05$) for ARC expression.

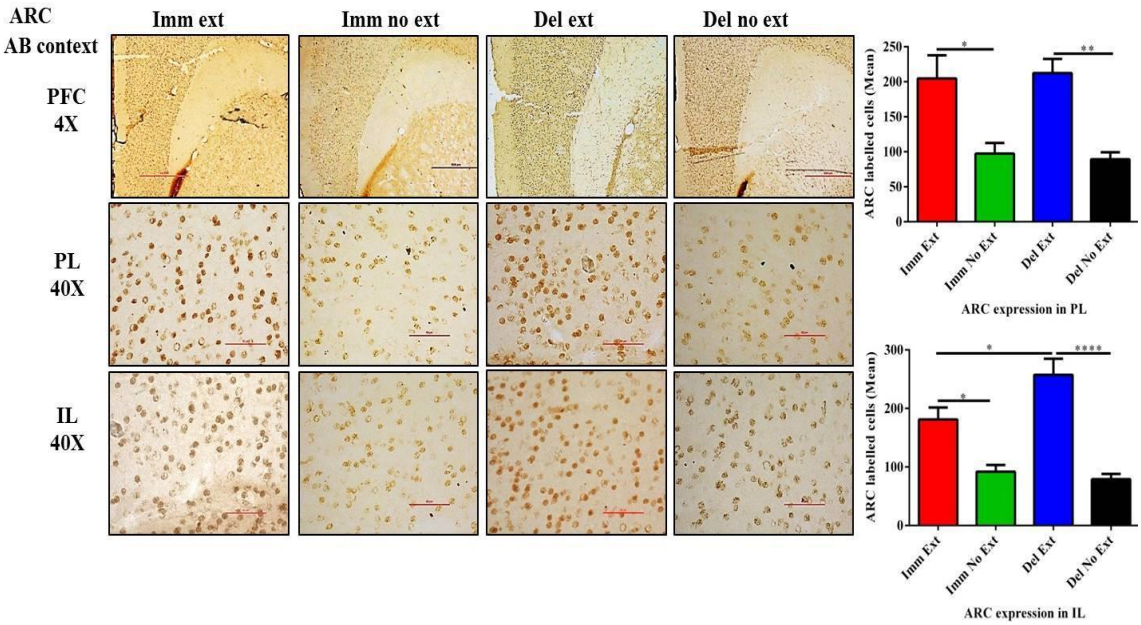

Figure 6.23: Represents the expression of ARC in the PL and IL region of PFC.

IL: In IL region of the PFC, delayed extinction group exhibited higher expression of ARC when compared with other groups in IL region. Two way ANOVA analysis of ARC expression in the IL region exhibited significant main effect for condition [$F(1, 8) = 107.3$, $p<0.0001$], time [$F(1, 8) = 6.131$, $p<0.05$] as well as condition x extinction time interaction [$F(1, 8) = 8.726$, $p<0.05$]. Two way ANOVA followed by Tukey's *post-hoc* test revealed increased ARC expression in the immediate extinction group as compared to the immediate no extinction group ($p<0.05$).While delayed extinction group exhibited increased ARC expression than the delayed no extinction group ($p<0.0001$). Significantly increased expression of ARC was observed in delayed extinction group as compared to the immediate extinction group ($p<0.0001$).

7.19 Discussion:

In this aim, the impact of behavioral alterations at the molecular level were observed in different regions of the brain involved in memory formation. Number of studies have shown that the fear memory consolidation and extinction involves a tripartite region- amygdala, hippocampus and prefrontal cortex (Bordi and Ledoux, 1994; Medina et al., 2002). It was found that the activity exhibited by change in the expression of c-fos, an immediate early gene along with the ARC (Gallo et al., 2018) were parallel to the change in p-CREB. Amygdala, which is a group of at least 13 different nuclei and the most clearly defined regions of it are LA (lateral amygdala), BA (basal amygdala) and CeA (central amygdala). CeA can be further distinguished into CeL (contralateral

amygdala) and CeM (Centromedial amygdala) (Krettek and Price, 1978b). CeM is the output circuitry of the amygdala and its activation exhibits freezing response. CeM region has innervations to the PAG region (Periaqueductal Gray) that ultimately controls the freezing behavioral response (Aggleton JP, 2000). When discussing about the input circuitry there is an initiating role of LA region that becomes activated after receiving the input from thalamic and cortical region and transmit the information into the BA region of amygdala. (LeDoux et al. 1990, Romanski and LeDoux, 1993). Thus, LA is considered as the site of association of CS (tone) and US (shock) and might be responsible for the generation of fear response. Further processing of the acquired information is performed in BA region of amygdala. Then BA sends information to the CeA (CeL + CeM) region (central amygdala). The CeM region had well proven role in the expression of fear acquisition response (Ehrlich et al. 2009).

After the extinction training (context A), firstly different regions of amygdala for c-fos expression were analyzed that exhibited no change in the LA region of immediate extinction but the expression was lowered during delayed extinction when compared with their control groups, suggesting that the decreased c-fos expression during delayed extinction is responsible for lower fear response, however BA region showed increased expression in both the immediate as well as delayed extinction group but expression was higher in immediate extinction suggesting that immediate extinction has higher fear response than the delayed extinction. Expression of c-fos was also observed in CeL and CeM region separately. CeL region exhibited higher expression of c-fos during delayed extinction than the immediate extinction and control group. This suggested that lower fear expression is due to the increased activation of CeL neurons that ultimately suppresses the CeM region to express. So the decreased fear response was also due to the lower c-fos expression in CeM region as compared to immediate extinction. To sum up all these, we can say that if the extinction training was delivered in the same context as of conditioning, the delayed extinction group exhibited lower fear response during retention test and it might be due to the decreased expression in LA and CeM but increased expression in BA and CeL region. The expression exhibited by c-fos was found to be very similar with the expression of ARC and p-CREB. Hippocampus is an important region that is also shown to be involved in memory consolidation and extinction and has a well-established role in contextual learning (Maren et al. 2013). A further region of the hippocampus CA1, CA3 and DG has been analyzed to know how the different regions are participated during these processes (Knapska and Maren, 2009). Statistically no significant change was observed in immediate extinction group when analyzed for the expression of c-fos, ARC and p-CREB as compared to the control group. But when

compared with the delayed extinction group the expression of all the antibodies (c-fos, ARC and p-CREB) was found to be increased in CA1, CA3 and DG region. The increased expression demonstrated that CA1, CA3 and DG regions of the hippocampus has a role in learning only if the extinction training was delivered in the same context and 24 hours after fear memory acquisition i.e., delayed extinction that supports better extinction learning instead of immediate extinction. The possible reason might be that the delayed extinction group got enough time to learn the context while in case of immediate extinction they were not allowed to learn the context as they immediately undergone for extinction training. PFC is differentiated in two regions, IL- PFC and PL-PFC (Liu and Carter, 2018; Giustino and Maren, 2015), also played a significant role in the consolidation of fear and extinction memory. Present study revealed that there is an increased expression of memory related genes (p-CREB, c-fos and ARC) in PL region during immediate extinction than the control as well as delayed extinction group. However IL-PFC shows increased expression during delayed extinction only than other remaining groups. This may be attributed to the fact that during immediate extinction, memory majorly affects the PL regions and thus playing a role in memory consolidation. However, the IL region is involved in delayed extinction and related to the extinction of consolidated memory. The expression pattern exhibited by ARC and c-fos was in line with the p-CREB.

Earlier the memory related paradigm was analyzed in the same context and found that early interference is not a beneficial therapy for traumatic event suppression or elimination rather than the delayed extinction plays a better role in the suppression of fear memory.

In the next part of the experiment, changes were observed when extinction training was delivered in a novel context other than the conditioning context and expression of different p-CREB targeted genes viz. ARC and c-fos was analysed. . Analysis for c-fos expression revealed the increased expression in LA during immediate extinction but no change in delayed extinction when compared with the control group. Similar expression was observed for ARC and p-CREB. The BA region showed somewhat different expression pattern. Here both the immediate and delayed extinction exhibited increased c-fos expression but immediate extinction had highest expression suggesting higher freezing response in delayed extinction when they were tested in the conditioning context. Thus, we can say that BA region becomes activated when extinction training was delivered either immediately or 24 hours after fear conditioning but more active during immediate extinction. This suggests that the decreased freezing response during immediate extinction might be due to the increased expression in BA region. When CeA region (CeL and CeM separately) was analyzed we

observed CeL region has higher c-fos expression during immediate and delayed extinction but the activation was highest in delayed extinction than the immediate extinction, it explains increased fear response during delayed extinction if tested in conditioning context was due to the higher expression of c-fos in CeL region. Contrary to this CeM region becomes more active during immediate extinction than the delayed extinction suggesting lower fear response during immediate extinction. Overall, increased c-fos expression in LA, BA and CeM supports the immediate extinction behavior i.e. higher fear expression when tested in conditioning context. Similar expression pattern was also exhibited by p-CREB and ARC. Next among the limbic region study is the hippocampus with their different regions. As discussed earlier, it plays a role in contextual learning. So when the contextual factor is changed from conditioning to extinction i.e., context B it was found that all the antibodies (p-CREB, c-fos and ARC) revealed no difference in both the immediate extinction as well as delayed extinction for the CA1, CA3 and DG region as compared to the control group suggesting no role of hippocampus in non-contextual learning. Lastly prefrontal cortex region has been analyzed. The PL-PFC and IL-PFC of Prefrontal cortex act differentially during fear memory consolidation and extinction (Pelloux et al, 2013; Giustino and Maren, 2015). During context change PL and IL exhibited increased expression of c-fos in the immediate and delayed extinction group but delayed extinction group exhibited the highest expression of c-fos in IL region which correlates to the behavioral results which is due to the compromised activation of PL/IL. Similarly, when analyzed for immediate extinction it was found that the increase in expression of c-fos positive neurons in PL/IL ratio is the cause of lower fear response when tested in the conditioning context (context A) following fear extinction which was performed in context B. The expression as observed for c-fos was found to be very similar with the expression of ARC and activation of p-CREB.

Chapter 6.3

Aim 3.

To find out whether histone acetylation has any role to play in the erasure/ inhibitory learning observed during immediate /delayed extinction respectively.

From the renewal and reinstatement behavioral experiment, it was evident that the extinction of fear memory involves both the inhibitory learning along with some 'erasure' components that depend upon the time interval of extinction training initiation. Animals that have undergone immediate extinction exhibited a kind of 'erasure' component while animals that underwent delayed extinction exhibited inhibitory learning. The final aim of the study was to explore the role of histone acetylation in the 'erasure' and inhibitory learning as observed during behavioral analysis. To achieve this aim the brain sections from both the context (AA and AB) were immuno-stained for acetyl H3K9 and acetyl H4K5 and the change in the acetylation level was measured in amygdala, prefrontal cortex and hippocampus and the observed changes were further analyzed by Two way ANOVA analysis followed by Tukey's post-hoc test.

Acetylation in the same context

8.1 Level of acetyl H3K9 in Amygdala:

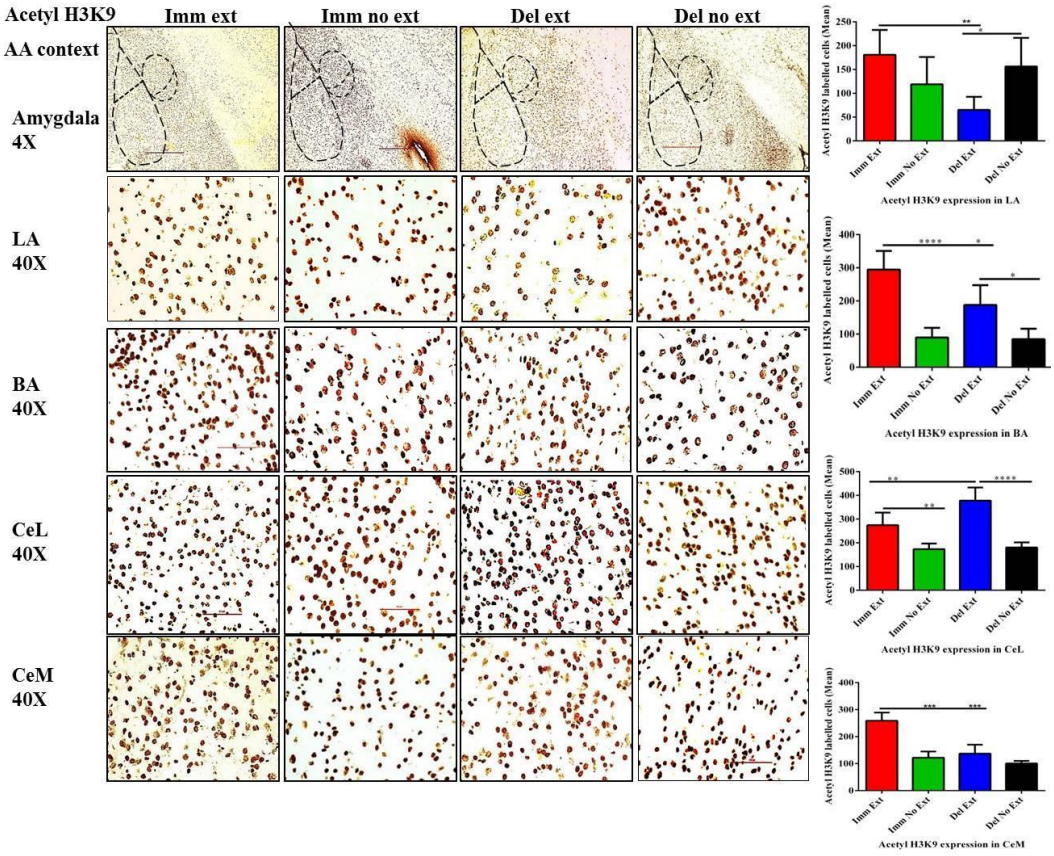

Figure 6.24: Represents the level of acetyl H3 at K9 in the LA, BA, CeL and CeM regions of the amygdala

LA: Decreased level of acetyl H3K9 was observed in delayed extinction group than the immediate extinction group and delayed no extinction group. Two way ANOVA analysis for acetylation of H3K9 revealed significant main effect of time [$F(1, 8) = 9.776$, $p<0.05$] and interaction between time x condition [$F(1, 8) = 37.71$, $p<0.01$]. However, no significant main effect of condition was observed [$F(1, 8) = 0.2422$, $p>0.05$] as evident by two way ANOVA analysis.

BA: Increased level of acetyl H3K9 was observed in immediate and delayed extinction groups as compared to their respective control groups. Two way ANOVA analysis for the level of acetyl H3K9 in BA region revealed significant main effect of time [$F(1, 8) = 15.38$, $p<0.01$] and condition [$F(1, 8) = 36.08$, $p<0.001$]. Also the interaction between time x condition was significant [$F(1, 8) = 12.84$, $p<0.01$]. These changes were further confirmed by Tukey's *post-hoc* test that explained immediate extinction group showed higher level of acetylation than the immediate no extinction group ($p<0.0001$) and delayed extinction group ($p<0.05$). However, the delayed extinction group exhibited higher acetylation than the delayed no extinction ($p<0.05$).

CeL: Two way ANOVA analysis of acetyl H3K9 exhibited significant main effect of time [$F(1, 8) = 7.465$, $p<0.05$], condition [$F(1, 8) = 80.29$, $p<0.001$] and also the significant interaction between time x condition [$F(1, 8) = 5.604$, $p<0.05$]. Tukey's *post-hoc* test revealed that both the immediate extinction ($p<0.01$) and delayed extinction ($p<0.001$) group had increased level of acetyl H3K9 as compared to their respective control groups. However, delayed extinction group had higher acetylation level than the immediate extinction ($p<0.01$).

CeM: Increased level of acetyl H3K9 in CeM region was observed in immediate extinction group than the immediate no extinction group ($p<0.01$) and delayed extinction group ($p<0.01$). Two way ANOVA analysis revealed significant main effect of time [$F(1, 8) = 31.75$, $p<0.001$], condition [$F(1, 8) = 71.90$, $p<0.001$] and also the significant interaction between time x condition [$F(1, 8) = 15.82$, $p<0.01$] was observed.

8.2 Level of acetyl H3K9 in Hippocampus:

CA1: In CA1 region of hippocampus, level of acetyl H3K9 was higher in delayed extinction group as compared to other groups. Two way ANOVA analysis exhibited significant main effect of effect of condition [$F(1, 8) = 12.23$, $p<0.01$] but the effect of time was not significant [$F(1, 8) = 3.058$, $p>0.05$]. But the interaction between time x condition was significant [$F(1, 8) = 6.090$, $p<0.05$].

Tukey's *post hoc* test confirmed significant increased level of acetylation in delayed extinction group as compared to immediate extinction ($p<0.05$) and delayed no extinction control group ($p<0.01$).

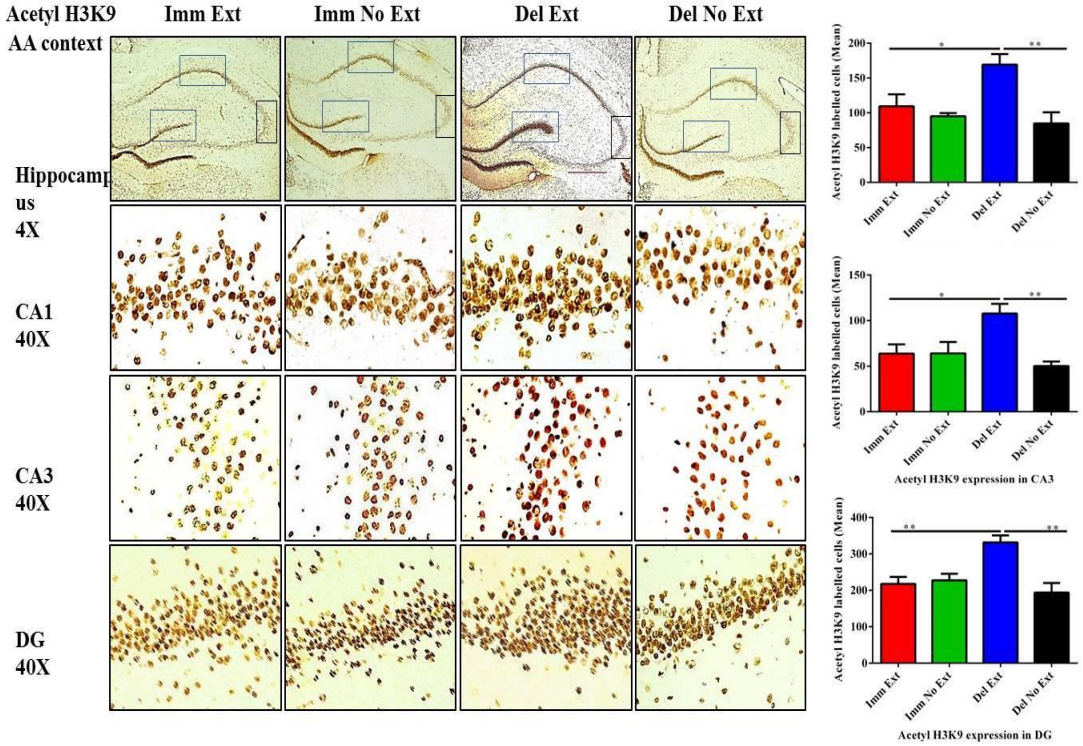

Figure 6.25: Represents the level of acetyl H3K9 in the CA1, CA3 and DG regions of the hippocampus involved in fear memory consolidation and extinction

CA3: When the level of acetyl H3K9 was gauged in CA3 region of the hippocampus, delayed extinction group exhibited increased level of acetylation. Two way ANOVA was used to compare the level of acetylation among the groups, that revealed significant main effect of condition [$F(1, 8) = 13.69$, $p<0.01$] but no effect of time was observed [$F(1, 8) = 1.669$, $p>0.05$]. The interaction between time x condition was found to be significant [$F(1, 8) = 6.115$, $p<0.05$]. Further Tukey's post-hoc test confirmed the increased level of acetyl H3K9 in delayed extinction group than the immediate extinction ($p<0.05$) and delayed no extinction group ($p<0.01$).

DG: Two way ANOVA analysis for acetylation level of H3K9 in DG region of the hippocampus revealed significant main effect of condition [$F(1, 8) = 7.162$, $p<0.05$] but there was no effect of time was observed [$F(1, 8) = 5.148$, $p>0.05$]. However the interaction between time x condition was significant [$F(1, 8) = 17.44$, $p<0.01$]. Tukey's *post-hoc* test for multiple comparison analysis

confirmed the increased level of acetyl H3K9 in delayed extinction group as compared to immediate extinction ($p<0.01$) and delayed no extinction group ($p<0.01$).

8.3 Level of acetyl H3K9 in pre-frontal cortex (PFC):

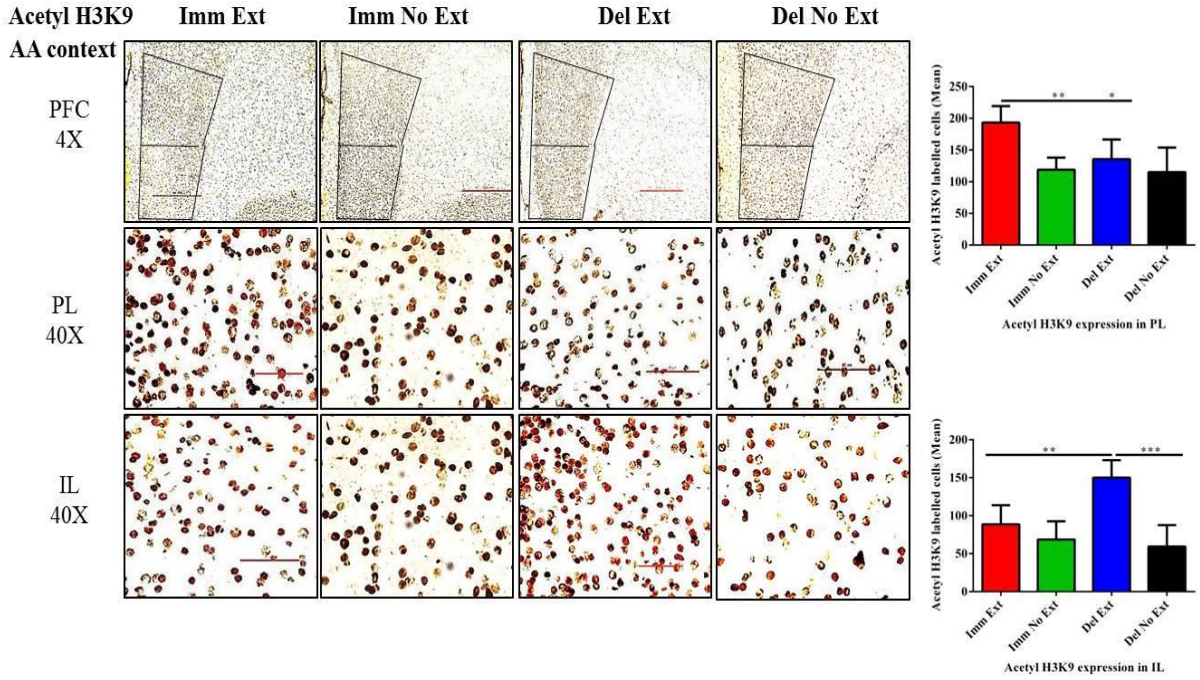

Figure 6.26: Represents the level of acetyl H3K9 in PL and IL region of the Prefrontal cortex

PL: Acetyl H3K9 level in PL region of prefrontal cortex (PFC) analyzed by two way ANOVA analysis revealed a significant main effect of time [$F(1, 8) = 6.453$, $p<0.05$] as well as condition [$F(1, 8) = 10.96$, $p<0.05$] but the interaction between time x condition was not significant [$F(1, 8) = 5.024$, $p>0.05$]. Further Tukey's *post-hoc* test showed that immediate extinction group exhibited increased level of acetyl H3K9 than immediate no extinction group ($p<0.01$) and delayed extinction group ($p<0.05$). However, no significant change was observed in delayed extinction group when compared with the delayed no extinction group ($p>0.05$).

IL: The level of acetyl H3K9 in IL region as analyzed by two way ANOVA revealed significant main effect of time [$F(1, 8) = 7.142$, $p<0.05$] as well as condition [$F(1, 8) = 19.40$, $p<0.01$]. The interaction between time x condition was also found significant [$F(1, 8) = 13.07$, $p<0.01$]. ANOVA analysis followed by Tukey's *post-hoc* multiple comparison test confirmed the increased level of acetylation of H3K9 in delayed extinction group than the immediate extinction group ($p<0.01$) as well as with delayed no extinction group ($p<0.001$).

8.4 Level of acetyl H4K5 in Amygdala:

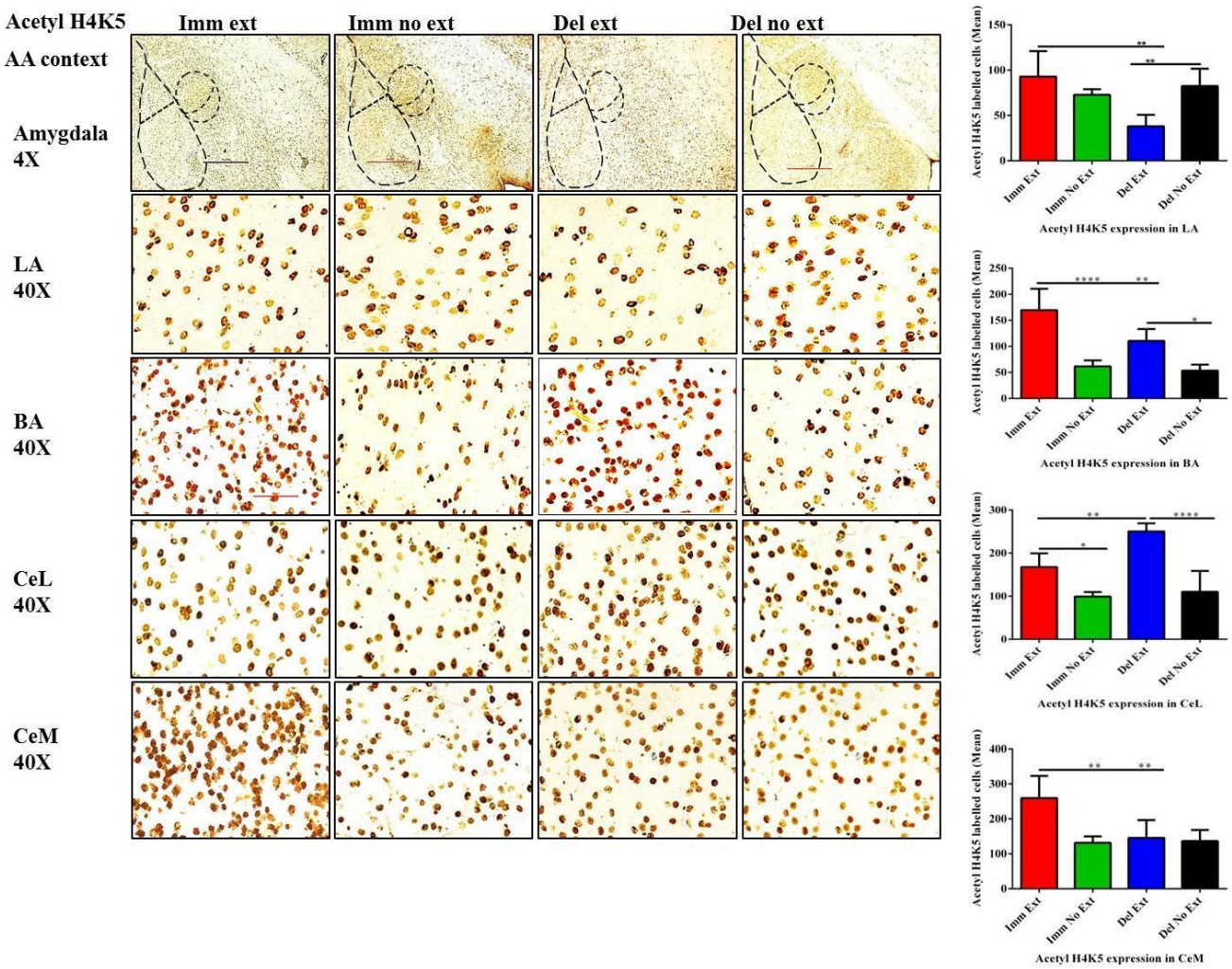

Figure 6.27: Represents the level of acetyl H4K5 in the LA, BA, CeL and CeM regions of the amygdala

LA: Two way ANOVA analysis revealed significant main effect of time [$F(1, 8) = 7.357$, $p<0.05$] but no effect of condition was observed [$F(1, 8) = 2.245$, $p>0.05$]. However the interaction between time x condition [$F(1, 8) = 14.93$, $p<0.01$] was found significant. Tukey's *post-hoc* test confirmed that decreased level of acetyl H4K5 in delayed extinction group when compared with the immediate extinction ($p<0.01$) and delayed no extinction control group ($p<0.01$).

BA: BA region of amygdala showed significant main effect of time [F (1, 8) =26.76, p<0.001] and condition [F (1, 8) =33.32, p<0.001]. The interaction between time x condition was also found to be significant [F (1, 8) =15.26, p<0.01]. Tukey's *post-hoc* multiple comparison test explained that both the immediate extinction (p<0.0001) and delayed extinction (p<0.05) group exhibited increased level of acetyl H4K5 than their respective control groups. When immediate and delayed extinction group was compared, immediate extinction group showed higher acetylation level than the delayed extinction (p<0.01).

CeL: The level of acetyl H4K5 in CeL region of amygdala as analyzed by two way ANOVA revealed a significant main effect of time [F (1, 8) =11.67, p<0.01] and condition [F (1, 8) =56.20, p<0.001]. Along with the main effect of time and condition variables, significant interaction was also observed between time x condition [F (1, 8) =6.838, p<0.05]. Tukey's *post-hoc* test in both the immediate extinction (p<0.05) and delayed extinction (p<0.0001) group showed increased level of acetyl H4K5 with immediate no extinction and delayed no extinction control groups respectively. Delayed extinction had higher level of acetyl H4K5 than the immediate extinction (p<0.01).

CeM: When the level of acetyl H4K5 was observed in CeM region of amygdala, Two way ANOVA revealed significant main effect of time [F (1, 8) =5.386, p<0.05] and condition [F (1, 8) =19.49, p<0.01]. The interaction between time x condition was also found significant [F (1, 8) =6.418, p<0.05]. Further Tukey's *post-hoc* test exhibited increased level of acetyl H4K5 in immediate extinction group as compared to the immediate no extinction (p<0.01) and delayed extinction group (p<0.01).

8.5 Level of acetyl H4K5 in Hippocampus:

CA1: Increased level of acetyl H4K5 in CA1 region of the hippocampus was observed during delayed extinction. Two way ANOVA analysis showed significant main effect of condition [F (1, 8) =5.609, p<0.05] but the effect of time was not significant [F (1, 8) =4.863, p>0.05]. However the interaction between time x condition was significant [F (1, 8) =5.457, p<0.05]. Further Tukey's *post-hoc* test for multiple comparison exhibited increased level of acetyl H4K5 in delayed extinction group as compared to immediate extinction (p<0.05) and delayed no extinction group (p<0.05).

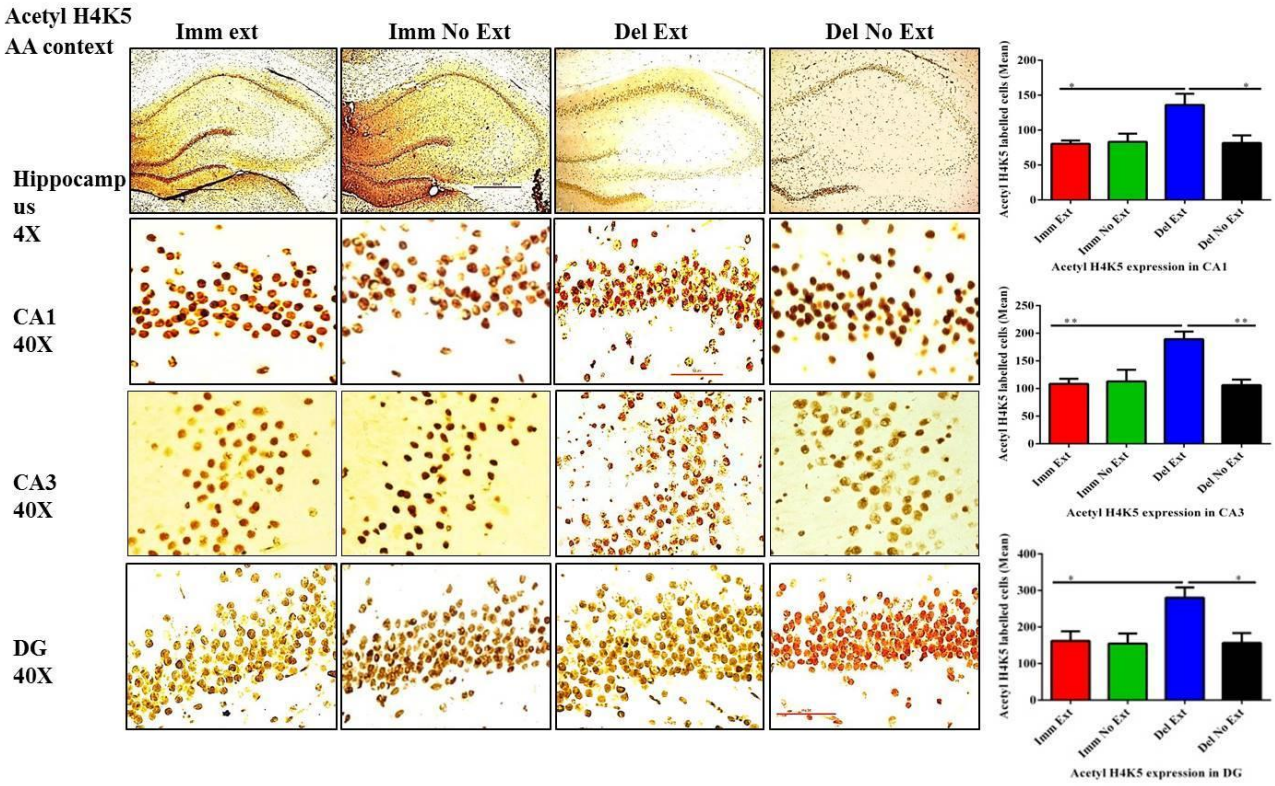

Figure 6.28: Represents the level of acetyl H4K5 in the CA1, CA3 and DG regions of the hippocampus

CA3: Likewise CA1 region, CA3 region also showed significant main effect of condition [F (1, 8) =11.43, $p<0.01$] along with the significant interaction between time x condition [F (1, 8) =7.016, $p<0.05$] but the effect of time was not significant [F (1, 8) =5.057, $p>0.05$] as revealed by two way ANOVA analysis.

Tukey's *post-hoc* test revealed a significant increased level of acetyl H4K5 in delayed extinction group as compared with the immediate extinction ($p<0.01$) and delayed no extinction group ($p<0.01$).

DG: DG region of the hippocampus also showed significant main effect of condition [F (1, 8) =6.373, $p<0.05$] and a significant interaction between time x condition [F (1, 8) =8.684, $p<0.05$]. But the main effect of time [F (1, 8) =5.125, $p>0.05$] was not significant. Tukey's *post-hoc* test confirmed the increased level of acetyl H4K5 in delayed extinction group as compared to delayed no extinction ($p<0.05$) and immediate extinction groups ($p<0.05$).

8.6 Level of acetyl H4K5 in pre-frontal cortex (PFC):

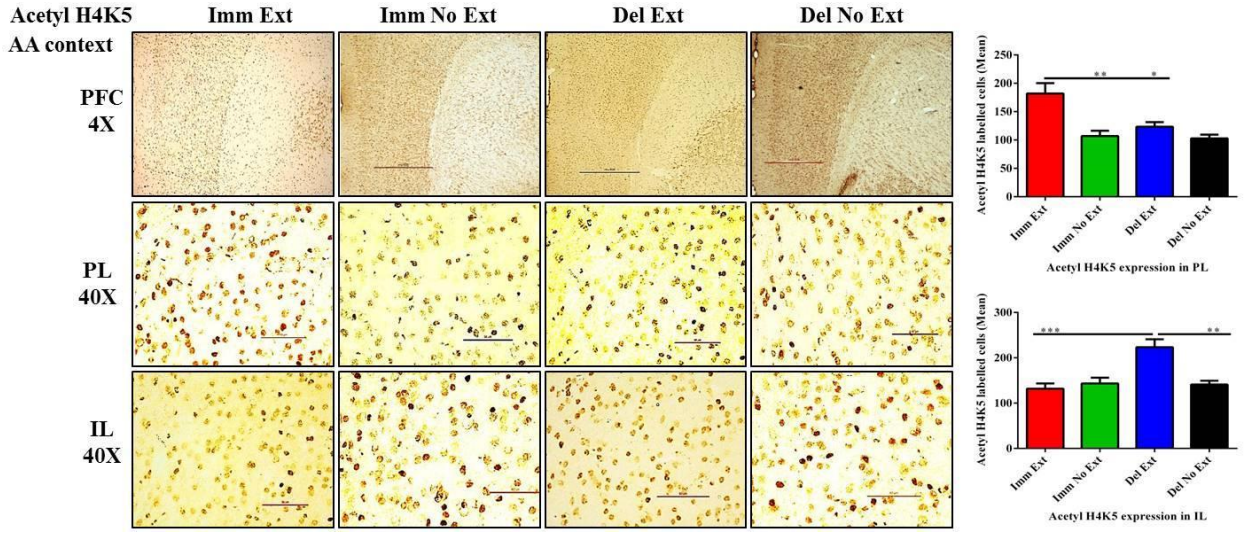

Figure 6.29: Represents the level of acetyl H4K5 in PL and IL regions of the prefrontal cortex involved in fear memory consolidation and extinction

PL: The level of acetyl H4K5 was higher in immediate extinction group than the immediate no extinction and delayed extinction group in the PL region of the PFC. Two way ANOVA analysis revealed significant main effect of time [$F(1, 8) = 7.028$, $p<0.05$] and condition [$F(1, 8) = 18.15$, $p<0.01$]. However the interaction between time x condition was not significant [$F(1, 8) = 5.273$, $p>0.05$]. Further Tukey's *post-hoc* test for multiple comparison analysis showed increased level of acetyl H4K5 in immediate extinction group as compared to immediate no extinction ($p<0.01$) and delayed extinction group ($p<0.05$).

IL: In the IL region of PFC, increased level of acetyl H4K5 was found in delayed extinction group than the immediate extinction group and delayed no extinction group. Two way ANOVA analysis for the acetylation of H4K5 revealed significant main effect of time [$F(1, 8) = 15.36$, $p<0.01$] and condition [$F(1, 8) = 6.007$, $p<0.05$]. The interaction between time x condition was also significant [$F(1, 8) = 17.05$, $p<0.01$]. Two way ANOVA followed by Tukey's *post-hoc* multiple comparison analysis confirmed the significant increased level of acetyl H4K5 in delayed extinction group as compared to immediate extinction ($p<0.001$) and delayed no extinction group ($p<0.01$).

Acetylation in different context

8.7 Level of acetyl H3K9 in Amygdala:

LA: The level of acetylation of H3K9 was higher in immediate extinction group as compared to its control group (immediate extinction group) and delayed extinction group. Two way ANOVA analysis revealed significant main effect of time [$F(1, 8) = 9.403$, $p<0.01$] and condition [$F(1, 8) = 8.939$, $p<0.01$]. However the interaction between time x condition was not significant [$F(1, 8) = 9.131$, $p>0.05$]. Tukey's *post-hoc* test confirmed the increased level of acetylation in the immediate extinction group than the immediate no extinction ($p<0.01$) and delayed extinction group ($p<0.05$).

BA: Increased level of acetyl H3K9 was observed in immediate extinction group as compared to other remaining groups. Two way ANOVA revealed significant main effect of time [$F(1, 8) = 8.102$, $p<0.05$] and condition [$F(1, 8) = 93.26$, $p<0.01$]. Here the interaction between time x condition [$F(1, 8) = 11.04$, $p>0.05$] was significant. Two way ANOVA followed by Tukey's *post-hoc* test confirmed the increased acetylation in immediate extinction group with the immediate no extinction group ($p<0.001$) and delayed extinction group ($p<0.01$).

CeL When acetylation level of H3K9 was observed in CeL region, increased level was observed in immediate extinction and delayed extinction group as compared to their respective control groups. While delayed extinction group has higher level of acetylation than the immediate extinction group. A two way ANOVA analysis revealed significant main effect of time [$F(1, 8) = 6.872$, $p<0.05$] and condition [$F(1, 8) = 39.99$, $p<0.01$]. The interaction between time x condition was also significant [$F(1, 8) = 5.569$, $p<0.05$]. Tukey's *post-hoc* test confirmed that increased level of acetyl H3K9 was observed in immediate extinction group as compared with the immediate no extinction ($p<0.05$) control group and delayed extinction group also has increased acetylation than the delayed no extinction control group ($p<0.001$). However, level of acetyl H3K9 was higher in delayed extinction group as compared to the immediate extinction group ($p<0.05$).

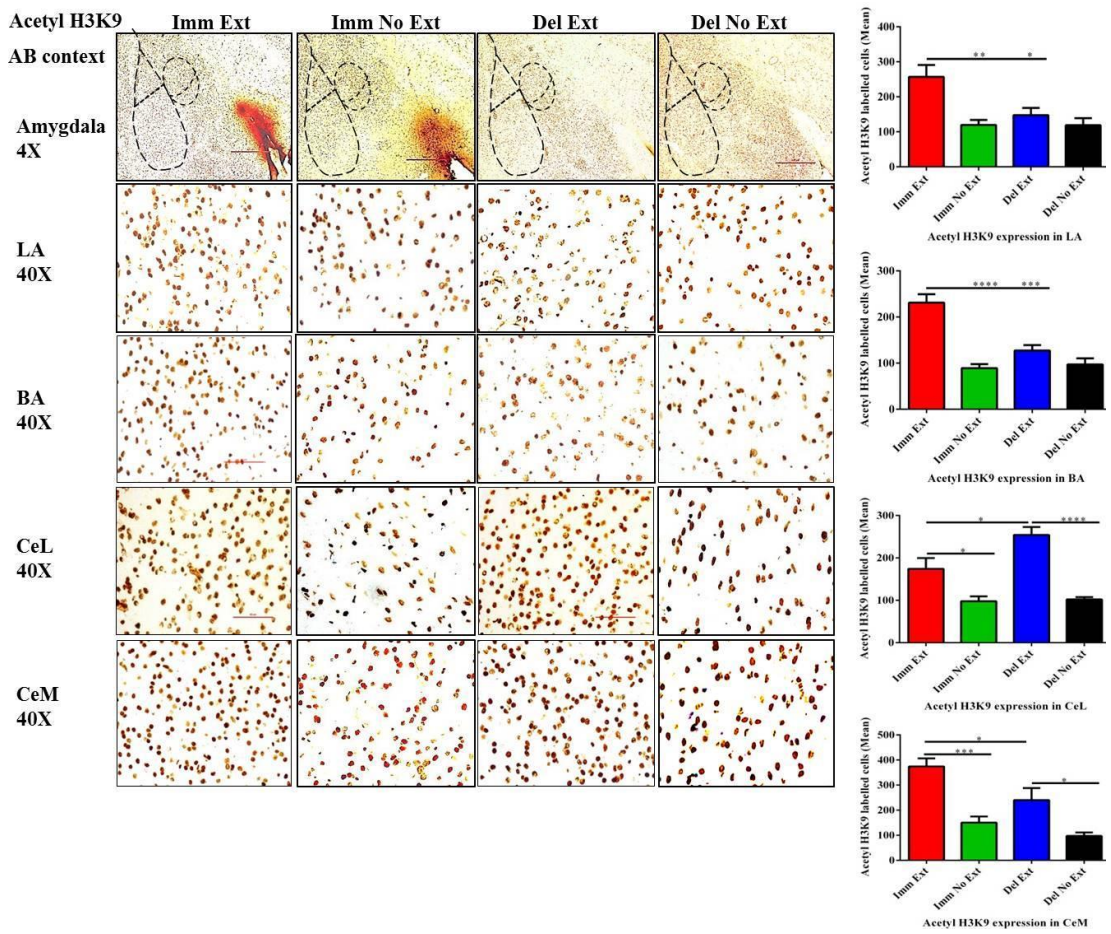

Figure 6.30: Represents the level of acetyl H3K9 in the LA, BA, CeL and CeM region

CeM Two way ANOVA analysis for acetyl H3K9 level in CeM region of amygdala revealed significant main effect of time [F (1, 8) =13.09, $p<0.01$] and condition [F (1, 8) =24.08, $p<0.01$]. However interaction between time x condition [F (1, 8) =2.480, $p>0.05$] was not significant. Tukey's *post-hoc* test showed increased acetylation in immediate extinction group as compared to the immediate no extinction group ($p<0.01$) and delayed extinction group ($p<0.05$). However increased level of acetylation was observed in delayed extinction than the delayed no extinction group ($p<0.05$) but decreased when compared to the immediate extinction group ($p<0.05$).

8.8 Level of acetyl H3K9 in hippocampus:

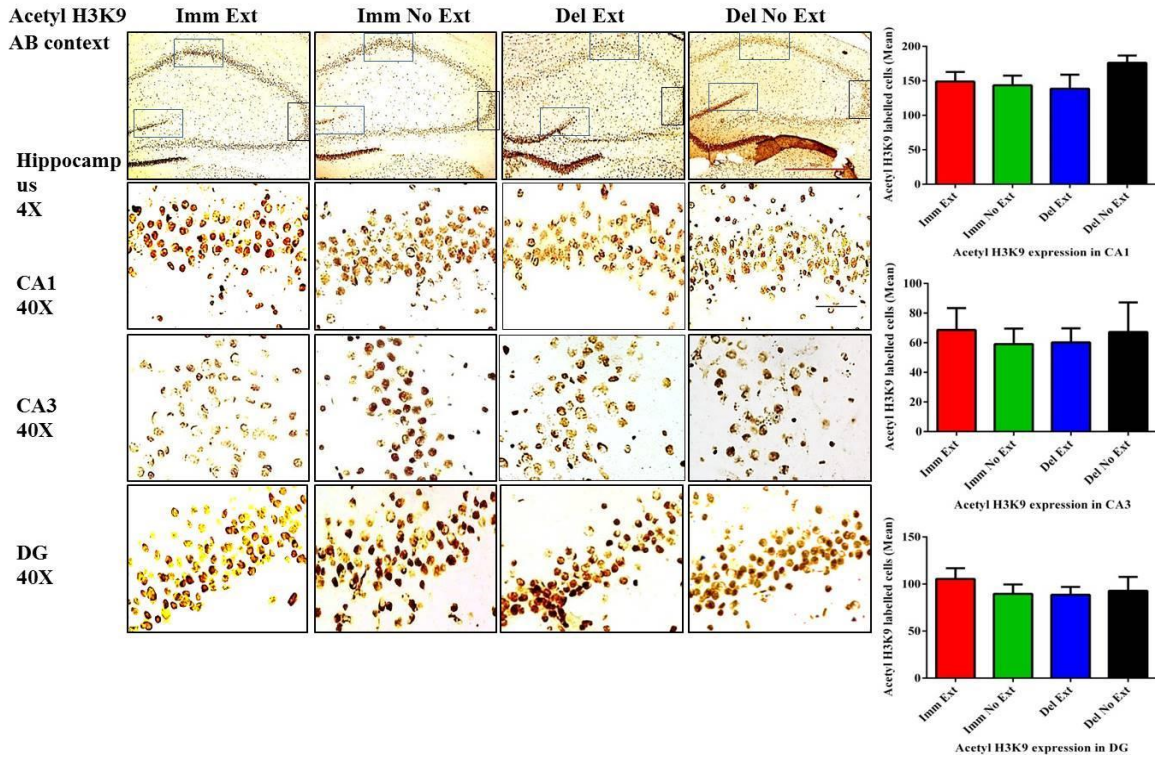

Figure 6.31: Represents the level of acetyl H3K9 in CA1, CA3 and DG regions.

CA1: In CA1 region of the hippocampus no significant difference in the level of acetyl H3K9 was observed across the groups. It was further confirmed by two way ANOVA analysis that revealed no significant main effect of time [F (1, 8) =0.6347, p>0.05] and condition [F (1, 8) =0.9833, p>0.05]. The interaction between time x condition [F (1, 8) =2.381, p>0.05] was also not significant. Further Tukey's *post-hoc* test for multiple comparison analysis exhibited no change in the level of acetyl H3K9 across all the groups (p>0.05).

CA3: CA3 regions of the hippocampus also exhibited no significant difference in the level of acetyl H3K9 across the groups. Two way ANOVA analysis exhibited no significant main effect of time [F (1, 8) =4.296, p>0.05] and condition [F (1, 8) =0.009536, p>0.05]. The interaction between time x condition [F (1, 8) =0.2960, p>0.05] was also not significant. Tukey's *post-hoc* test for multiple comparison analysis also exhibited no significant difference in the level of acetyl H3K9 among the groups (p>0.05).

DG: No change in the level of acetyl H3K9 was observed in DG region of the hippocampus. A two way ANOVA analysis was used as a statistical tool to confirm this. Two way ANOVA analysis revealed no significant main effect of time [F (1, 8) =0.3387, p>0.05] and condition [F (1, 8)

=0.2727, p>0.05] along with no significant interaction between time x condition [F (1, 8) =0.7325, p>0.05]. Tukey's *post-hoc* test also exhibited no significant difference across the group (p>0.05).

8.9 Level of acetyl H3K9 in prefrontal cortex:

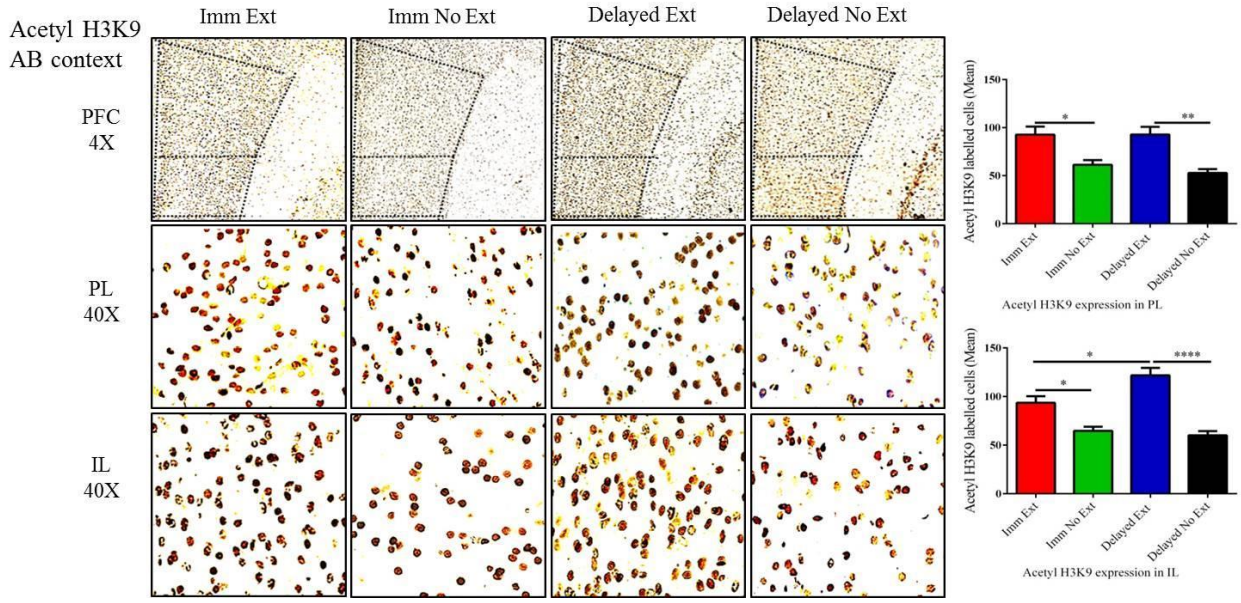

Figure 6.32: Represents the level of acetyl H3K9 in PL and IL region of PFC.

PL: Level of acetyl H3K9 was higher in both the immediate and delayed extinction group as compared to their respective control groups but no change in acetylation level of H3K9 in immediate and delayed extinction groups. The observed changes were confirmed by two way ANOVA analysis that revealed significant main effect of condition [F (1, 28) =28.9, p<0.0001] only. However, the main effect of time [F (1, 28) =0.397, p>0.05] and interaction between time x condition [F (1, 28) =0.421, p>0.05] was not significant.

IL: In IL region of PFC, level of acetyl H3K9 was higher in delayed extinction group and immediate extinction group as compared with the delayed no extinction and immediate no extinction control group respectively. However the acetylation level was higher in delayed extinction than the immediate extinction group. The observed changes were confirmed by two way ANOVA analysis that exhibited significant main effect of time [F (1, 28) =3.92, p<0.05] and condition [F (1, 28) =57.8, p<0.0001]. Along with the main effect, a significant interaction between time x condition [F (1, 28) =7.60, p<0.01] was also observed.

8.10 Level of acetyl H4K5 in Amygdala:

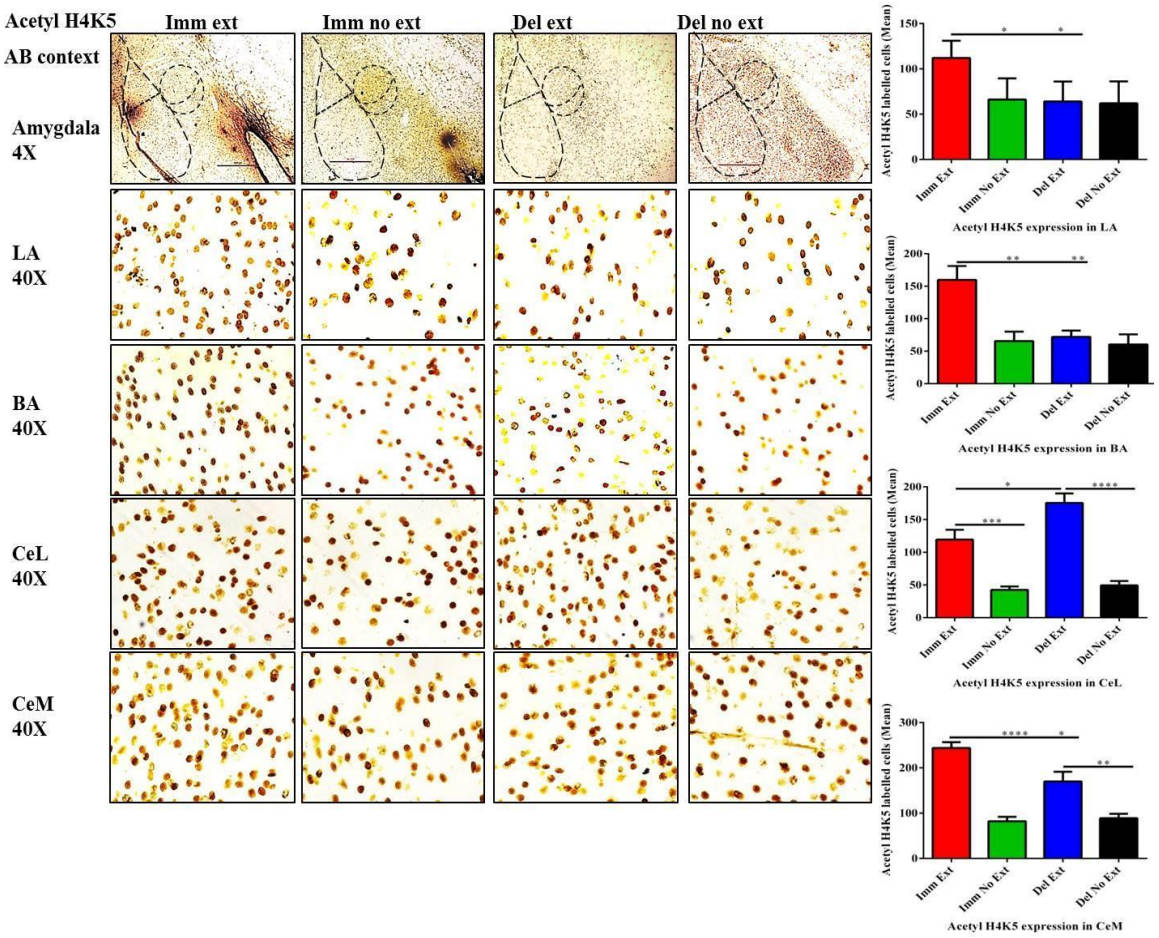

Figure 6.33: Represents the level of acetyl H4K5 in LA, BA, CeL and CeM region.

LA: In LA region of amygdala the level of acetyl H4K5 was higher in immediate extinction group as compared to all other remaining groups. A two way ANOVA was used to observe the changes, that revealed significant main effect of time [$F(1, 8) = 6.621$, $p<0.05$] and condition [$F(1, 8) = 6.001$, $p<0.05$]. However the interaction between time x condition was not significant [$F(1, 8) = 4.662$, $p>0.05$].

BA: Two way ANOVA analysis for the level of acetyl H4K5 in BA region of amygdala revealed significant main effect of time [$F(1, 8) = 7.710$, $p<0.05$] and condition [$F(1, 8) = 12.78$, $p<0.01$]. The interaction between time x condition [$F(1, 8) = 6.082$, $p<0.05$] was also significant. Tukey's *post-hoc* test for multiple comparison analysis further confirmed that immediate extinction exhibited higher level of acetyl H4K5 than the immediate no extinction ($p<0.01$) and delayed extinction group ($p<0.01$).

CeL: In CeL region of amygdala, immediate extinction and delayed extinction showed higher level of acetyl H4K5 when compared to their respective control groups. Two way ANOVA analysis for above data revealed significant main effect of time [$F(1, 8) = 8.960$, $p<0.05$] and condition [$F(1, 8) = 71.04$, $p<0.0001$]. The interaction between time x condition [$F(1, 8) = 5.500$, $p<0.05$] was also significant.

CeM: Two way ANOVA analysis for the acetyl H4K5 level exhibited significant main effect of time [$F(1, 8) = 5.458$, $p<0.05$] and condition [$F(1, 8) = 72.67$, $p<0.0001$]. However the interaction between time x condition [$F(1, 8) = 7.890$, $p>0.05$] was not significant. Further Tukey's *post-hoc* test for multiple comparison analysis exhibited significant increased level of acetyl H4K5 in the immediate extinction ($p<0.0001$) and delayed extinction ($p<0.01$) groups as compared to their respective control groups. However, the acetylation level was higher in immediate extinction than the delayed extinction group ($p>0.05$).

8.11 Level of acetyl H4K5 in Hippocampus:

CA1 No significant change in the level of acetyl H4K5 was observed across the groups and it is confirmed by two way ANOVA analysis that revealed no significant main effect of time [$F(1, 8) = 3.513$, $p>0.05$] and condition [$F(1, 8) = 0.00028$, $p>0.05$]. Along with the non-significant main effect of time and condition, the interaction between time x condition [$F(1, 8) = 3.513$, $p>0.05$] was also not significant. Tukey's *post hoc* test for multiple comparison analysis exhibited no difference in the acetylation of H4K5 was observed among the groups (all $p>0.05$).

CA3 The level of acetyl H4K5 in CA3 region of the hippocampus exhibited no difference among the groups. Two way ANOVA analysis for above data revealed no significant effect of time [$F(1, 8) = 0.2535$, $p>0.05$] and condition [$F(1, 8) = 0.07175$, $p>0.05$]. The interaction between time x condition was also not significant [$F(1, 8) = 0.05907$, $p>0.05$]. Further Tukey's *post hoc* multiple comparison test also exhibited no significant difference in the acetylation level of H4K5 among the groups (all $p>0.05$).

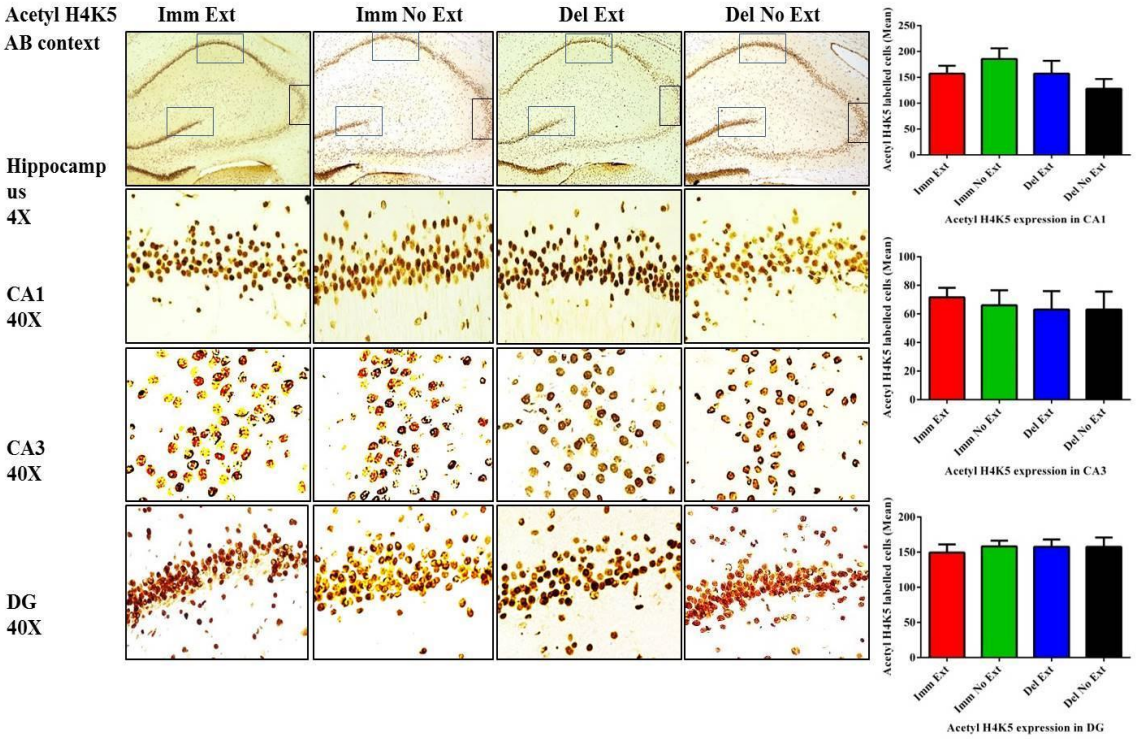

Figure 6.34: Represents the level of acetyl H4K5 in CA1, CA3 and DG regions of the hippocampus.

DG The level of acetyl H4K5 in DG region of the hippocampus exhibited no significant difference across the groups and it was confirmed by two way ANOVA analysis that revealed no significant main effect of time [F (1, 8) =0.09327, p>0.05], condition [F (1, 8) =0.2123, p>0.05] as well as the interaction between time x condition [F (1, 8) =0.1250, p>0.05] was also not significant. Tukey's *post hoc* test also exhibited no significant difference among the groups (all p>0.05).

8.12 Level of acetyl H4K5 in prefrontal cortex:

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

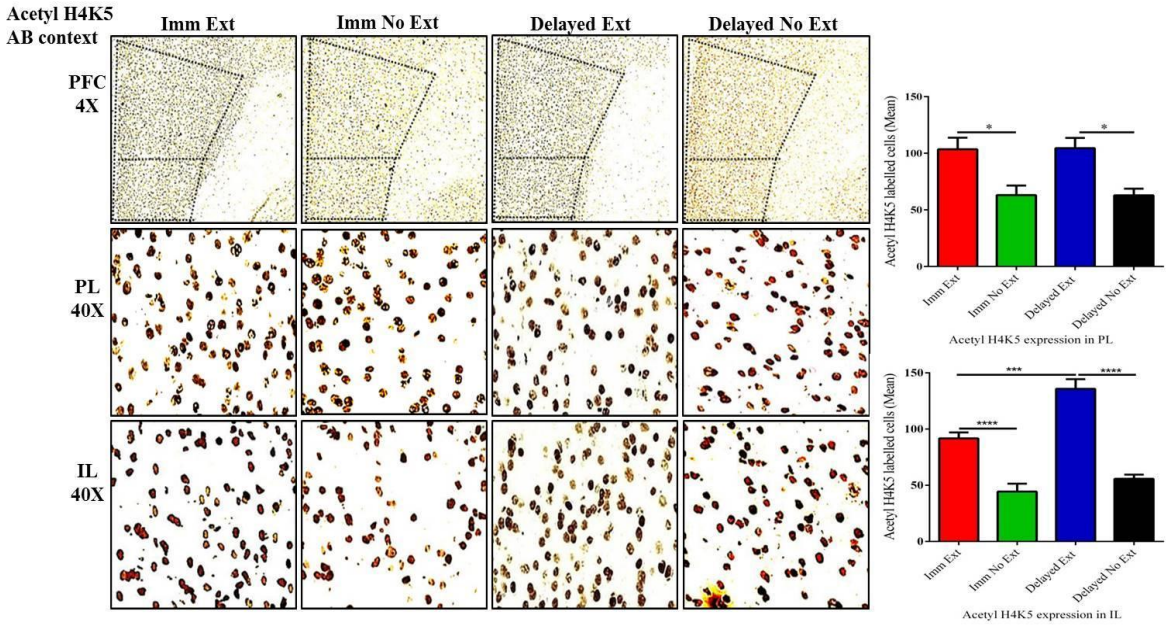

Figure 6.35: Represents the level of acetyl H4K5 in PL and IL region of PFC.

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

8.13 Discussion:

Recent studies suggest the existence of fundamentally different circuits mediating the extinction of learned fear. Moreover, it has been shown reversal of the pathways active during consolidation of fear memory such as through the activation of phosphatases during extinction and dephosphorylate CREB and related targets required towards successful conditioning (Lin et al., 2003). Extinction has also been shown to cause depotentiation of CS inputs (Lin et al., 2003) to the amygdala along with AMPA receptor endocytosis (Kim et al., 2007). Thus the extinction phenomenon may be an amalgamation of an erasure component and inhibitory component. In the present study we found an erasure component in the immediate extinction group with change in context. What exactly controls this phenomenon is still unknown. In the past few decades epigenetics have been shown to play a role in the consolidation and extinction of fear memory. Histone acetylation along with methylation

has been shown to control the expression of genes whose products are required for consolidation of fear and extinction memory.

In the present study we wanted to correlate the level of histone acetylation to the behavioral outcomes in the immediate and delayed extinction paradigms in the same and different context with respect to the context of the fear consolidation. In order to do so we first looked at the acetylation level in different amygdala regions. Amygdala is well known brain structure that is found to be involved in emotional learning response (Phelps and LeDoux., 2005; Pessoa, 2010). The acetylation pattern as speculated was found to be different in the delayed and immediate extinction in the different nuclei of the amygdala. The level of acetyl H3K9 in the LA region of immediate extinction did not change as opposed to the delayed extinction where it was significantly lowered as compared to their respective control groups in similar context. In the AB context, immediate extinction had higher level of acetyl H3K9 than the delayed extinction and its control group. There was a similar increase in H3K9 acetylation in the BA region of the AA context in both the immediate and delayed extinction group as compared to their respective control groups. However in the AB context, the BA region had higher level of acetyl H3K9 in the immediate extinction group as compared to other groups. In the CeL region of the same (AA) context, increased acetylation of H3K9 was observed in both the immediate and delayed extinction group as compared to the control groups but the level was highest in delayed extinction when compared to the immediate extinction. CeL region of the different (AB) context also exhibited similar increased level of H3K9 acetylation pattern. Further in the CeM region of AA context, increased level of H3K9 was observed only in the immediate extinction group than the other groups but in the case of AB context, increased level of H3K9 was observed in both the immediate as well as delayed extinction than their respective control groups, but the immediate extinction had higher acetylation level. The acetylation pattern exhibited by acetyl H3K9 was found very similar with the acetylation pattern exhibited by acetyl H4K5. Overall, there was a general increase in acetylation in the immediate extinction in BA, CeL and CeM but no change in the LA relative to their control groups in the same context. However, in different context there was increased acetylation in LA, BA, CeL and CeM region and this increase in the acetylation might be responsible for the ‘erasure’ of fear memories following immediate extinction. Following delayed extinction there was decreased acetylation in LA and increased acetylation in BA, CeL with no change in the acetylation in CeM as compared to the control group in the same context while increased acetylation in CeL and CeM region with no change in the LA and BA region in different context may be resulting in new learning rather than erasure of the original memory.

We then looked at another very important region of the brain- Hippocampus, which is a part of the limbic system (Rajmohan and Mohandas, 2007) and is involved in the conversion of short term memory into long term memory (Wong, 1997). Earlier studies have reported that the hippocampus is critically involved in contextual learning (Smith and Bulkin, 2014). When the acetylation pattern was checked in different regions of the hippocampus of the same context, we found no change in the acetylation level of acetyl H3K9 in the CA1, CA3 and DG regions during immediate extinction with its control group however the acetylation level of acetyl H3K9 was higher in CA1, CA3 and DG region during delayed extinction as compared to the immediate extinction and delayed no extinction. However in the case of different (AB) context, no significant difference was observed across the groups in the CA1, CA3 and DG regions. Here the pattern exhibited by the acetyl H3K9 was also found to be very similar to the pattern of acetyl H4K5. Overall no significant change in the level of acetyl H3K9 and acetyl H4K5 in CA1, CA3 and DG regions of the hippocampus during immediate extinction with its control groups in the same context as well as in the different context was observed. Thus we can speculate that acetylation in hippocampal subregions is not playing any role in the 'erasure' of fear memory. The higher level of acetyl H3K9 and acetyl H4K5 in the CA1, CA3 and DG regions of the hippocampus during delayed extinction in the same context and no change in these regions in different context might be responsible for inhibitory learning and may be preventing the erasure of fear memory as observed during behavioral analysis.

The third very important region which was studied was PFC, which is another important part of work as relay for buffering the fear and extinction memories during fear memory consolidation and extinction (Hostinar and Gunnar, 2015). In the same context (AA), level of acetyl H3K9 was higher following immediate extinction as compared to other groups in the PL region of PFC, while in the IL region, level of acetyl H3K9 was higher in delayed extinction as compared to other groups. Whereas in case of different (AB) context in the PL region of PFC, higher level of acetyl H3K9 was observed in both the immediate and delayed extinction group as compared to the respective control groups. The IL region of the immediate and delayed extinction group also exhibited higher level of acetyl H3K9 when compared to their respective control groups. Moreover, the delayed extinction group exhibited higher level of acetyl H3K9 as compared to the immediate extinction group. Likewise amygdala and hippocampus, the PFC region also exhibited similar pattern of acetyl H4K5 as exhibited by acetyl H3K9. Overall, it might be concluded that 'erasure' of fear memory might be due to the increased level of acetyl H3K9 and acetyl H4K5 in the PL region but not in the IL region of PFC during immediate extinction in the same context but increased level of acetylation in both

the PL and IL regions in the different context. However inhibitory learning might be due to the higher histone acetylation only in the IL region in the same context and increased acetylation in both the PL and IL region following delayed extinction of the different context.

In conclusion we can say that the different subregions of Amygdala, Hippocampus and PFC work in a synchronised way and might be using the epigenetic machinery especially histone acetylation to sense the learning context and that the timing of extinction training results in different histone acetylation pattern. Thus it could be speculated that histone acetylation may be one of the major factors controlling the behavioral outcomes in the form of less or more fear.

Chapter 7:

Conclusion

Conclusion:

As evident from the results of the aforesaid study it might be concluded that the extinction of fear memory is both inhibitory learning as well as it also involves some 'erasure' component and is dependent on the time interval between memory consolidation and extinction. The behavioral analysis for renewal experiment suggested that the immediate extinction group possessed “erasure” component that represents no recovery of fear at all with the change in context. Whereas delayed extinction exhibited return of fear with the change in context that represents inhibitory learning. Reinstatement experiment when performed to analyze the recovery of fear extinction, it was observed that immediate extinction did not show return of fear with the presentation of un-signaled shock, explaining the 'erasure' phenomenon. While in delayed extinction represents the recovery of fear explaining that it was inhibitory learning.

Following the behavioral analysis, molecular alteration was analyzed for p-CREB and it's target gene ARC and c-fos in different regions of the brain. It was speculated that ‘erasure’ and inhibitory learning during immediate extinction and delayed extinction respectively might be due to the differential activation pattern of p-CREB, ARC and c-fos among the various sub-region of amygdala, hippocampus and PFC region.

The behavioral changes were also analyzed for histone acetylation to know whether histone acetylation had any role to play in the observed behavior. Immunohistochemistry analysis for acetyl H3K9 and acetyl H4K5 showed that the acetylation of histones play a key role in memory consolidation as well as the differential pattern of acetylation may be attributed to the time difference extinction memory.

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Research report

Decreased level of histone acetylation in the infralimbic prefrontal cortex following immediate extinction may result in deficit of extinction memory

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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 min. after fear memory acquisition (Immediate Extinction) had deficits in retention of extinction memory as compared to one which underwent extinction 24 h 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.

1. 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 and Davis, 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, 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 h 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, 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

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Differential Histone Acetylation in Sub-Regions of Bed Nucleus of the Stria Terminalis Underlies Fear Consolidation and Extinction

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Objective The hallmark of anxiety disorders is excessive fear. Previous studies have suggested that selective neural projections from Basal nucleus of stria terminalis (BNST) to amygdala and vice-versa precisely control the fear learning process. However the exact mechanism how the BNST controls fear consolidation and its extinction is largely unknown. In the present study we observed the changes in the BNST sub-regions following fear conditioning and its extinction.

Methods The change in the number of positive neurons was determined by immunohistochemistry for Acetyl H3 (Histone 3), Acetyl H4 (Histone 4), cAMP response element binding Protein (CBP) and c-fos in three sub-regions of the BNST namely the anterio-lateral BNST (STLP) and anterio-medial BNST (STMA), and lateral-ventral BNST (STLV) of rats subjected to auditory fear conditioning and extinction.

Results We found significant increase in the number of CBP, acetyl H3 and acetyl H4 positive neurons in the STMA and STLV but not in the STLP after fear conditioning. However, following fear extinction the number of CBP, acetyl H3 and acetyl H4 positive neurons increased significantly in the STLP but not in the STMA and STLV. Similar changes were observed in the number of c-fos positive neurons after fear consolidation and extinction.

Conclusion The results from this study suggest that the differential histone acetylation in the different sub-regions of the BNST following fear learning and its extinction may be responsible for changes in the neuronal activation patterns resulting in either fear or less fear.

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Key Words Bed nucleus of the stria terminalis, Extended amygdala, Fear memory, Histone acetylation.

INTRODUCTION

The extended amygdala, comprising of the basolateral nucleus of the amygdala (BLA), central nucleus of the amygdala (CeA), and bed nucleus of the stria terminalis (BNST), play important role in the development of fear and anxiety-like behaviors.[1-11] The CeA and BNST, project to various anatomic areas involved in the development of fear or anxiety. The fibers from BLA to the BNST pass through CeA and cells in the lateral division of the CeA project to the BNST.[12]

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There is dissociation in the role of the central amygdala (CeA) and the bed nucleus of the stria terminalis (BNST). CeA is involved in the expression of both cued and contextual fear, while BNST is involved in the expression of contextual fear only.[13,14] The BNST is heterogeneous in structure, and different sub-regions within the BNST appear to make unique contributions to fear and anxiety.[15-22] It shares connections with several important emotion-regulating areas in the brain, including the amygdala, dorsal raphe nucleus, hippocampus, hypothalamus, nucleus accumbens, prefrontal cortex, and ventral tegmental area. It directly influences freezing behavior via its projections to the amygdala and periaqueductal gray.[23-33] Based on cytoarchitecture features, specific neuronal types and their neurochemical make-up, the BNST has been divided into anterio-lateral (BNST-AL/STLP), anterio-medial (BNST-AM/STMA) and anterio-ventral (BNST-AV/STLV) subregions. The hypothalamus-pituitary-adrenal-regulating neurons are concentrated in the ventral (BNST-AV/STLV) and

