ROLE OF HISTONE ACETYLATION & DEACETYLATION DURING FEAR CONSOLIDATION AND EXTINCTION

THESIS

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Certificate

This is to certify that the research work embodied in the thesis entitled **"Role of Histone Acetylation & Deacetylation during Fear consolidation and Extinction"** has been carried out by **Ms. Vandana Ranjan** under our joint supervision. She has fulfilled all the requirements of Babasaheb Bhimrao Ambedkar University, Lucknow for the award of the degree of Doctor of Philosophy in Biotechnology.

The work included in this volume unless otherwise stated is all original and data presented in this thesis are based on author's observations.

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Vandana Ranjan

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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
Tn	Tone
US	Unconditioned Stimulus

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CHAPTER 1

INTRODUCTION

Introduction

Traumatic events generate some of the most persistent forms of memories. Despite the preeminent lifetime occurrence of various fear related anxiety disorders, effective strategies to mitigate long-term traumatic memories are limited. Fear and other anxiety disorders develop after the experience of a traumatic event, which leads to grave physical or psychological harm. Because of a strong emotional underpinning, traumatic memories are extraordinarily robust and difficult to treat as evidenced by an estimated lifetime prevalence of about 29% (Kessler *et al.,* 2005). Among the most efficacious treatments for these disorders are exposure-based therapies (Cukor *et al.,* 2010, Foa, 2000 and Foa and Kozak, 1986), during which a patient is repeatedly exposed to original fear-eliciting stimulus in a safe environment so that the once fearful stimulus can be newly interpreted as neutral or safe (Foa and Kozak, 1986).

Understanding the cellular and molecular mechanisms underlying the formation and maintenance of memories is a central goal of the neuroscience community. One important question is how such temporary changes in the environment can be encoded in a relatively persistent manner by the cell to produce long-lasting memory, such as memory for a fearful event. Identifying the molecular mechanisms of fear memory formation is particularly important in light of the prevalence of post-traumatic stress disorder (PTSD), a debilitating condition characterized by inappropriate fear generalization to safe contexts and stimuli, and other anxiety disorders such as phobias and panic disorders. Learning to avoid cues that signal danger is important to minimize injury, but excessive or persistent responding to non-threatening stimuli (as occurs in PTSD), can also cause harm.

It is well regarded that an organism's ability to lastingly adapt its behavior in response to a transient environmental stimulus relies on the central nervous system's capability for structural and functional plasticity. This plasticity is dependent on a well-regulated program of neurotransmitter release, post-synaptic receptor activation, intracellular signaling cascades, gene transcription, and subsequent protein synthesis. In the last decade, epigenetic markers like DNA methylation and post-translational modifications of histone tails have emerged as important regulators of the memory

process. Their ability to regulate gene transcription dynamically in response to neuronal activation supports the consolidation of long-term memory. Furthermore, the persistent and self-propagating nature of these mechanisms suggests a molecular mechanism for consolidation and maintenance of memory.

The epigenetic mechanisms have recently been implicated in various forms of memory, including fear memory and may represent one important way that transient cell signaling following a brief learning event can produce lasting changes in cellular function and, accordingly, enduring changes in behavior. For fear memory, this means that epigenetic changes may drive the persistent behaviors associated with PTSD, including re-experiencing the event, avoiding cues that trigger memories of the trauma, and continuous hyperarousal.

In rodents, PTSD and general anxiety disorders can be modeled with Pavlovian fear conditioning, a learning task in which an initially neutral conditional stimulus (CS), like a tone or context, is paired with a naturally aversive unconditional stimulus (UCS), usually a footshock. Moreover, Pavlovian fear conditioning as a model of traumatic memory formation has aided in the identification of potential intervention strategies, including extinction and reconsolidation-based memory interventions for the alleviation of traumatic fear memories.

The present work was done to find out epigenetic correlates with reference to the regulation of fear circuit mainly comprised of amygdala being the main brain areas along with hippocampus and prefrontal cortex (PFC). The levels of neuronal and cellular activity marker molecules was determined in amygdala, hippocampus and PFC and was correlated to histone acetylation pattern in the same brain areas following fear memory consolidation and extinction of fear memory.

Taken together, the findings from this study would further authenticate the role of amygdala, Hippocampus and PFC in the consolidation, maintenance and extinction of fear memory. It will shed light on the role of histone acetylation in expression of memory related genes these brain areas and how epigenetics contributes to the distinct aspects of fear conditioning and extinction.

The findings of this study certainly would be helpful to understand and develop behavioral as well as novel drug therapeutic targets for PTSD patient and to provide them opportunity of living a normal life along with society around them. Ultimately, the elucidation of these molecular mechanisms would be fundamental for understanding the memory processes in the brain in general, and should also inform novel therapeutic strategies for psychiatric disorders involving excessive fear responses associated with fear related disorders such as post-traumatic stress disorder and other anxiety disorders.

CHAPTER 2

REVIEW OF LITERATURE

Human beings acquire the top-most position among movable lives when we talk about evolution, developments, progresses, civilizations, cultures and many more. This, for sure, is the human brain, which is gifted with highest and most complex cognitive abilities and skills as well. But with all these facts, some human activities show basic behavioral patterns, prevalent among entire animal kingdom, e.g. hunger, shelter, emotions etc. Fear is one of these basic behavioral patterns and seems indispensible to an organism when its instinct and/or strength to survive against odds, is talked about. Fear makes one alert against any probability of unpleasant situation or threat, whether physical or mental; therefore it is actually a protective strategy, producing multiple complex patterns in behavior that are highly correlated as well as coordinated with each other. Like many of the behavioral as well as physiological factors, the pattern & intensity of fear related behavior shows differences among different species, geological differences among same species and differences in many other ways.

Fear can be described as an emotional state of mind in which an aversive external stimulus is perceived consciously and generate a vigorous response in anticipation of a coming threat which may be life threatening. Fear response includes increased alertness & concentration towards the source of fear; and fight-or-flight behaviors. Physiological consequences include sympathetic nerve stimulation such as cardiovascular excitation; superficial vasoconstriction and dilation of the pupils. Fear is universal emotion prevails through the whole animal kingdom thus making its role vital and establishes it as one of the basic instinct needed for the survival. Fear response, as protective mechanism during experiencing odds, guide the organism how to avoid the unpleasant, harmful or life threatening situations. A unique feature of fear learning is that it can be acquired with as little as one exposure and can persist for a lifetime (Maren, 2005).

Fear is known of two types: Innate that comes with existence of an organism and acquired that comes while interacting with environment. Humans are known to born with two innate fears - fear of loud sound and fear of falling (Gibson & Walk,

1960; Adolph *et al.*, 1997), and rest of the fear types are acquired as experience during life-course. Many studies have been made to understand biological basis of acquired fear that how a cue gets associated with a fear response against a certain object or event, and how associated behavior is reproduced later on by just banking upon memory of that fear even though there is no threat present again.

The same fear related stimulus is modulated differently following same process of cognition and learning in different people, therefore tuning up the level of fear by brain produces different behavioral outputs in people. Proper tuning produces appropriate fear response hence referred as rational behavior but improper tuning of fear response leads to inappropriate fear response, which is categorized as irrational behavior and studied under interdisciplinary fields of Psychology and Neuroscience. The persistence and progression of such irrational behavior leads to behavioral disorders, which further affect physical and mental health of the person. The most common form of fear disorder is anxiety. The anxiety disorders are categorized into Panic disorder, Phobias, and Generalized Anxiety Disorders (GAD). The fear follows any traumatic event, such as terrorism, accident, sexual assault, natural disasters, life threat, war etc, and its memory consequently leads to Post Traumatic Stress Disorders (PTSD), which also includes anxiety disorders. On one hand, PTSD is not developed in every person who has gone through a trauma, and on the other hand, even the witness of traumatic event, who actually has not undergone traumatic experience, can develop PTSD.

The brain areas known to be involved in processing of fear related stimuli are Amygdala, Hippocampus and Prefrontal Cortex (PFC). The fear circuitry in brain operates through well-regulated neuronal activities going on differentially in these regions to tune up the required level of fear (Ingrid Ehrlich *et al.,* 2009) in healthy individuals but impaired in patients of PTSD. The clinical aspects of fear behaviors manifest when fear circuitry does not do its job as required normally. Though the neural and molecular mechanism underlying differences in fear response among different individuals is not fully understood yet, individual variability in fear response among population (Kessler *et al.*, 1995) shows some categorization and can be termed as "behavioral phenotypes", e.g. individuals with less, high or very high fear response (Bush *et al.*, 2007). Extreme phenotypes are of medical concern as they reflect

clinical aspect of fear related behavior and easy victim of harmful/threatening situations due to less fear on one hand and PTSD, Anxiety disorders etc., due to high fear on the other hand. The currently used therapeutic agents have unpleasant side effects and are not always effective. Thus, there is an urgent need for newer therapies. "Extinction" is a popular behavioral therapy to block recurring traumatic memories but success has shown limitations.

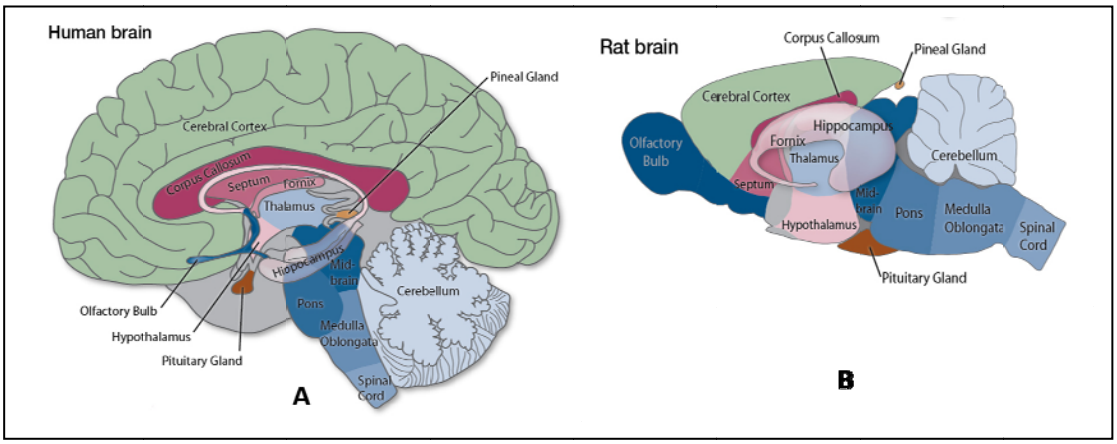

Figure 2.1: General anatomy of brain - (A) human & (B) rat brain

2.1. Fear Memory formation

Fear Memory formation can be described as mental activity that involves simultaneous stimulation of multiple brain areas and recall of information that have been learned or experienced earlier in life. The environmental stimuli from outside world are received through our senses in the form of physical and chemical stimuli and processed in three main stages in order to convert that information into memory. These three stages in memory formation and maintenance are presented in figure 2.2.

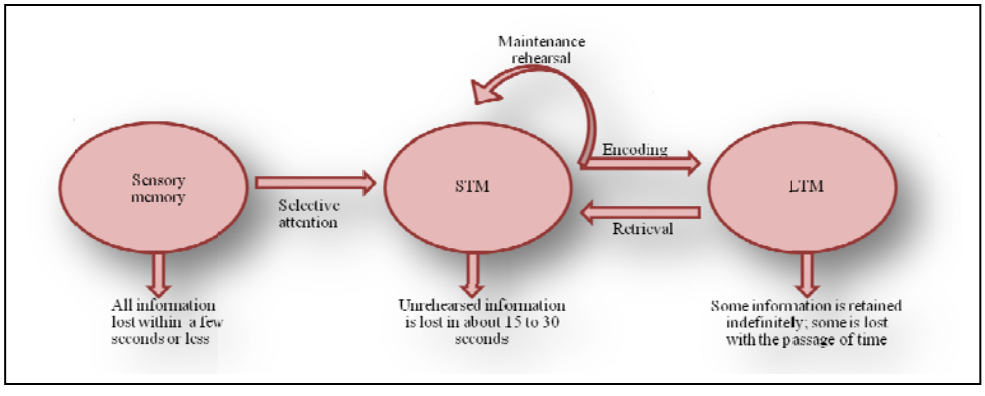

Figure 2.2: Schematic presentation of stages in memory formation

Acquisition/Registration: It is all about receiving, processing and combining received information to make a response. Concentration is limiting factor for this registration stage. This very chain of biological events following a traumatic experience is referred as Fear acquisition.

Consolidation/Storage: This is transformation of the received information from short-term memory (STM) to more stable long-term memory (LTM) over time following fear processing is called **Fear consolidation**.

Retrieval: It is recollecting the information in response to some cue, which activate same neuronal pattern used earlier to store that particular information. Recollecting fear memory is called **Fear recall**.

In 1949, Hebb proposed that when two interconnected neurons fire at the same time, the synapses between them become stronger, and remain so for a long time afterwards. Studies showed that high-frequency stimulation led to a long-term enhancement of synaptic transmission (Bliss & Lomo, 1973), a form of plasticity that has become known as long-term potentiation (LTP). This way the stabilization of the fear associations into long-term memory is referred to as **fear consolidation** (Abel and Lattal, 2001; Tonegawa *et al.,* 2003; Nader, 2003). Fearful experiences are rapidly acquired and thus easily consolidated into long-term memory. Research till date has concluded that the neural activities are same for both either having personal experience or upon being witness of a threat or tragedy.

Extinction of memory is another stage of memory during which part of memory loses its ability to recall, in other words memory of events weakens with time. Some psychological theories have described extinction as an "unlearning" process (Rescorla & Wagner, 1972) but recently extinction has been found to be new inhibitory learning which also depends on gene expression, protein synthesis and synaptic plasticity (Bruel-Jungerman *et al.,* 2007; Alberini, 2009) as in process of consolidation; and does not erase the registered association between conditioned stimulus (CS) – unconditioned stimulus (US), instead **fear extinction** forms a new memory that inhibits expression of conditioned response. Extinction is a necessary mechanism to regulate unnecessary excess fear (Bouton & Moody, 2004, Bouton & King, 1983; Quirk, 2002; Myers & Davis, 2007). From the viewpoint of scientists

working on memory and research work done in this area, memory formation (LTM) is dependent on synaptic activity induced protein synthesis and posttranslational modifications (PTMs), (Kandel, 2001; Pang *et al.,* 2004; Routtenberg & Rekart, 2005). It is also proposed that LTM is feedback regulated to continuously update these modifications in order to make them lasting long.

2.2. Role of Epigenetics in Fear learning

We know that it is organism's genome, responsible for differences among the factors contributing to one's personality. Genetic information while governs biological identity of an organism, Epigenetics confers a particular personality to each individual within same type of organisms e.g. humans. Epigenetics involves study of heritable modifications to chromatin and DNA without changing its sequence. Earlier chromatin was believed to have just structural role in purpose of genome packaging, but later many studies were made and it is shown to be very dynamic component of regulation mechanisms, affecting gene expression (Felsenfeld and Groudine, 2003). It is epigenetics, responsible for differences in phenotype of genetically identical person e.g. monozygotic twins (Keverne *et al.,* 2015), arise from time-acquired changes in epigenome in response to environmental stimuli. Epigenome simply comprise of DNA plus chromatin structure. While DNA sequence within chromatin packing is unchangeable, the histones are subject to many post-translational modifications at its amino-terminal "tails" e.g. acetylation, phosphorylation, methylation, ubiquitylation, sumoylation, glycosylation and ADP ribosylation (Suganuma & Workman, 2008; Peterson & Laniel, 2004). Many of these histone modifications may occur in combinations to make a specific "histone code" creating distinct instructions for gene expression (Strahl & Allis 2000; Turner, 2000). Site-specific covalent modifications of histone tails by chromatin remodeling protein complexes change the transcriptional states; say transcriptional activation and repression (Peterson & Laniel, 2004). Though histone modifications may be transient, stable or permanent yet these epigenetic marks evolve an expression pattern, to facilitate, maintain, or block transcriptional processes, in specific cell or tissue type and such patterns are heritable (Wolffe & Guschin, 2000). Noncoding MicroRNAs (miRNAs) have also been studied for their role as potent epigenetic regulators affecting repression or activation of a broad repertoire of targets through direct binding to the 3'-UTR of target mRNAs and

noncleavage degradation of the target mRNA via deadenylation (Djuranovic *et al.,* 2012; Eulalio *et al.,* 2009; Lim *et al.,* 2005).

It simply means that each organism has a single genome but the same individual has multiple epigenome, which may differ by cell and tissue type as evolved in response to environmental stimuli and may change over the lifetime of the organism. Recent research in the field of behavioral neurosciences has focused on epigenetic basis of gene regulation and emphasized on molecular neural adaptations underlying behavioral changes so as to explore possible therapeutic measure to treat behavioral disorders.

Studies so far in the field of fear associated memory processing have been targeted to find out cellular, biochemical and molecular basis of fear learning. The research works mainly in past decade majorly spotted light on molecular basis of LTP in fear memory formation that governs the dynamic behavior of chromatin structure responsible for epigenetic regulation of synaptic plasticity in fear circuitry. Earlier it had been reported that one of the regulatory processes of chromatin structure is the acetylation and deacetylation of histone proteins. Histone acetyltransferases (HAT) acetylate conserved lysine amino acids on histone proteins by transferring an acetyl group from acetyl CoA to form ε-N-acetyl lysine. Many theories suggest effect of acetylation differently-Acetylation brings in a negative charge, which neutralizes the positive charge on the histones and decreases the interaction of the N termini of histones with the negatively charged phosphate groups of DNA. As a result, the condensed chromatin is transformed into a more relaxed structure, which is associated with greater levels of gene transcription (Jenuwein & Allis, 2001).

Another hypothesis says- differential acetylation makes the site for recruitment of many transcription factors and associated proteins which may regulate level of local gene expression (Verdone *et al.,* 2005). HDACs (histone deacetylases) are class of enzymes that remove acetyl groups; increase the positive charge of histone tails hence the interaction between histone proteins and DNA. The increased DNA binding condenses DNA structure and prevents transcription, or otherwise it can be said as HDACs exert deacetylation as opposed to HATs. It is generally thought that histone acetylation is permissive for gene expression and that inhibition of HAT

activity impairs, whereas inhibition of HDAC activity enhances, synaptic plasticity and memory (Sharma, 2010).

The researchers have established a connection between stimuli induced physiological changes and histone acetylation states (Crosio *et al.*, 2003). The activity dependent changes in epigenome are adapted as form of chromatin structure set in order to register all the synaptic plasticity related alterations (Bird, 2007). Many reports suggests that memory formation involves epigenetic mechanisms that modify the structure of chromatin (Swank & Sweatt 2001; Guan *et al.,* 2002; Alarcon *et al.*, 2004; Korzus *et al.*, 2004; Wood *et al.*, 2005; Levenson & Sweatt 2005) and specific post translational modifications (PTM) of histone proteins, in particular acetylation and phosphorylation (Levenson & Sweatt, 2005) and also methylation (Gupta *et al.,* 2010) regulate long term memory formation by modulating promoters of plasticity-related transcription factors, neurotransmitter receptors, cytoskeletal proteins, adhesion molecules, and metabolic factors (Levenson & Sweatt, 2005; Wood *et al.,* 2006). The work towards mapping the brain areas involved in fear learning - using resting-state functional magnetic resonance imaging showed involvement of vmPFC and amygdala in fear extinction (Feng et al., 2016) and - with transcription active state found that ITC of amygdala, CA1 & CA3 region of hippocampus have role in contextual fear learning (Radwanska et al., 2015). Different or particular level of histone acetylation is managed by HATs and HDACs activity; while level of histone phosphorylation is regulated by combined action of Protein Kinases (PKs) and Protein Phosphatases (PPs). In brain, PKs such as extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) and MSK1 (Mitogen activated S6 kinases-1) led pathways are known to contribute to the epigenetic regulation of long-term memory (Chwang *et al*., 2006, 2007; Atkins *et al*., 1998).

cAMP-response element (CRE) binding protein (CREB), a nuclear transcription factor acts as a master gene that turns on other memory associated genes through functional and/or structural changes underlying long-term synaptic plasticity and memory (Frank & Greenberg, 1994; Yin &Tully, 1996; Silva *et al*., 1998). ERK is known to regulate CREB. CREB co-activation and histone acetylation (HAT) function of CREB Binding Protein (CBP) is required for memory formation (Sterner & Berger, 2000). CBP is critical for in vivo acetylation of lysines on histones

H2B, H3, and H4. Enhanced CBP expression and increased histone acetylation in hippocampus has been suggested to rescue drug induced contextual fear memory impairment; and increased c-fos level along with enhanced H3K12 acetylation is suggested to occur in learning induced active neurons in hippocampus (Zhong et al., 2015). These findings suggest how the changes in epigenome related to memory consolidation and extinction are connected with cellular processes concerned with the phenomena of synaptic plasticity i.e. changing the strength of connections between neurons, in response to either use or disuse of transmission through the particular neurons.

The fear extinction memory is another important aspect of fear learning and its formation has been found to be new inhibitory learning which also depends on gene expression, protein synthesis and synaptic plasticity (Martin *et al.,* 2000; Bruel-Jungerman *et al*., 2007; Alberini, 2009) as the processes in fear memory consolidation do. Several studies have investigated the neural mechanisms of this inhibition, focusing on the amygdala, prefrontal cortex, and hippocampus (Myers & Davis, 2007; Quirk & Mueller, 2008; Radulovic & Tronson, 2010; Herry *et al*., 2010), specifically, the basolateral complex of the amygdala (BLA), comprising the lateral (LA) and the basal (BA) nuclei, actively participates in fear conditioning and extinction (Falls *et al.*, 1992; Lu *et al*., 2001; Goosen *et al*., 2003; Herry *et al*., 2006; Sotres-Bayon *et al*., 2007; Quirk *et al*., 2006; Myers & Davis, 2007).

Formation of long-term contextual fear memory requires N-methyl–D-aspartate (NMDA) receptor dependent synaptic transmission and the ERK/MAPK signaling cascade in the hippocampus (Fanselow *et al*., 1994; Atkins *et al*., 1998; Selcher *et al*., 1999; Rampon *et al*., 2000), interaction of Glutamate receptors (GRs) with the NMDA/ERK/Mitogen activated S6 kinases-1 (MSK1)/ETS-like gene-1 (Elk-1) signaling pathways (Reul *et.al*., 2009), and inhibition of either of these critical cellular processes blocks the memory-associated increase in acetylation of histone H3 (Levenson *et al*., 2004). Levenson also reported a different form of long-term memory - latent inhibition was associated with altered acetylation of histone H4, whereas H3 acetylation was unaltered by this paradigm. Formation of enduring memories of significant events requires changes in the differential BDNF gene expression to allow an optimal epigenomic activation pattern in hippocampus neurons to accommodate their altered neuro-physiological function during contextual fear learning (Lubin *et*

al., 2008). Hormones related to psychological physiology e.g. glucocorticoid hormones via the glucocorticoid receptor (GR) may greatly facilitate the epigenomic mechanisms which leads through NMDA-ERK signaling, to MSK1 and Elk-1 activation (found in dentate gyrus neurons, a part of the hippocampus involved in encoding of memories) resulting in histone H3S10-phosphorylation and K14-acetylation, H4 hyper-acetylation, gene induction and formation of memories of the event (Reul *et.al*., 2009).

Several HDAC inhibitors such as NaB (sodium butyrate), valproic acid, and trichostatin have been shown to increase acquisition and extinction of LTM (Bredy *et al*., 2007; Lattal *et al*., 2007; Levenson *et al*., 2004; Stafford *et al.,* 2012; Wood, Attner, Oliveira, Brindle & Abel, 2006; Yeh, Lin & Gean, 2004). These findings supports that there is a link exist between epigenetic regulation and signaling pathways involved in fear memory formation.

2.3. The fear circuitry

Neural components of behavior related to emotions such as joy, anger, sorrow, hate, love, courage, or fear etc. is unconsciously processed in different brain areas to exert a precise physiological response suitable for that particular emotion. These brain areas together systemized as **limbic system, which** is also responsible for memories of emotional learning (fig.- 2.3 & 2.4). It includes mainly thalamus, hypothalamus, amygdala, and hippocampus. Amygdala is particularly responsible for processing and storage of memories of fear emotions.

The Amygdala: its connections & role

Amygdala is an almond shaped structure, collection of around ten heterogeneous nuclei, located deep within temporal lobe. The anatomist Burdach made its first structural description in 19th century but the functional role was discovered in 20th century. It is known to be involved in conditioning of fear response (Phillips & LeDoux, 1992). The lesion of the amygdala blocks innate or conditioned fear (Davis, 1997) and also blocks conditioned freezing response against cue CS and context both (Abel & Lattal, 2001). The amygdala is mainly divided in basolateral amygdala (BLA), inter-calated cell mass (ITC) and central nucleus of amygdala

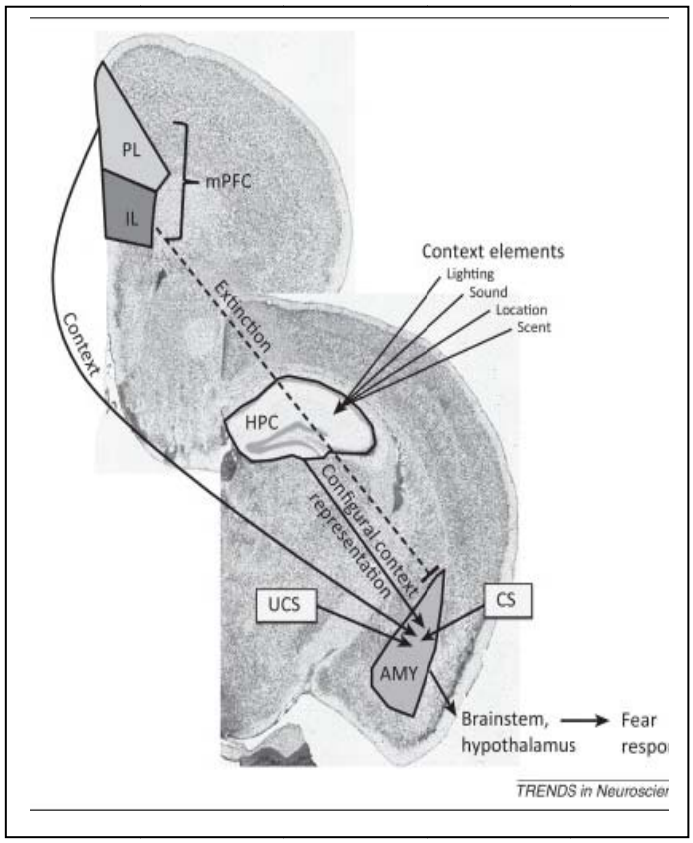

Figure 2.3:

Schematic representation of limbic system in memory processing ; (adapted from Trends in Neurosciences

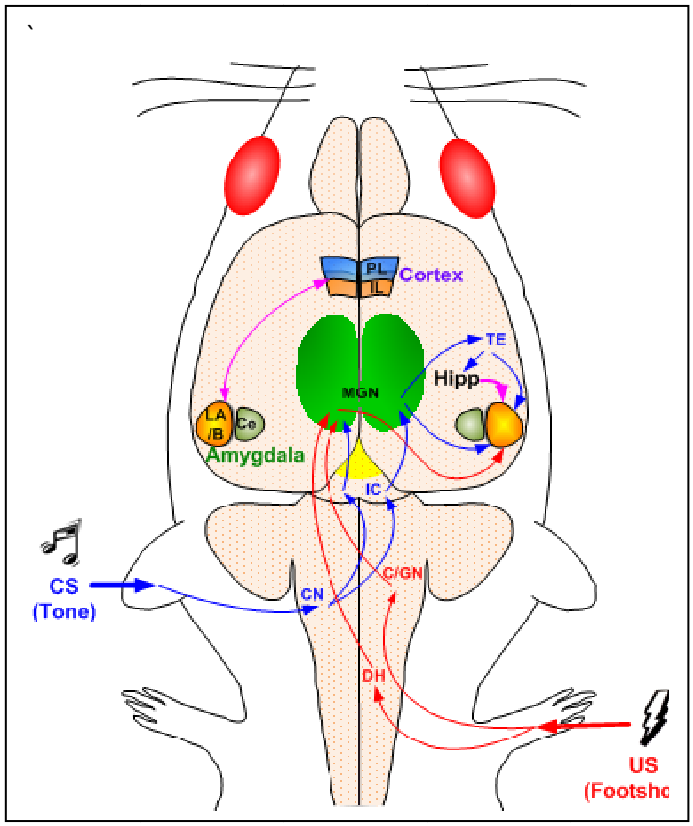

Figure 2.4: Fear circuitry in brain

Pathways involved in auditory fear conditioning. The tone information is delivered to LA via the medial division of medial geniculate body (MGN) and the shock information is delivered to LA via posterior intralaminar nucleus (PIN). The tone input to LA is potentiated when tone and shock are paired. Output from the LA projects to the central nucleus (Ce) through inter-calated cells (ITC, not show in the figure) and BA neurons, eliciting a fear response. LA, lateral nucleus; BA, basal nucleus; CN, cochlear nucleus; DH, dorsal horn of spinal cord; IC, inferior colliculus; PL: prelimbic medial prefrontal cortex, IL: infralimbic medial prefrontal cortex.

(CEA) regions. BLA comprises mainly of the lateral nucleus (LA) and the basal nucleus (BA) of amygdala.

Amygdala is believed to be the center of the Circuitry for fear memory (Le Doux, 2003). LA is the key site for neuronal activities underlying memory storage during fear conditioning (Fendt & Fanselow, 1999) i.e. fear memory acquisition, consolidation and reconsolidation. The US associated specific features appear to be encoded in the amygdala (Debiec *et al*., 2010, Tsvetkov *et al*., 2015). Cued fear conditioning is a hippocampus-independent learning (Kim & Fanselow, 1992). LA receives sensory information via a direct pathway from thalamic nuclei through the internal capsule (LeDoux *et al.,* 1990) and via an indirect thalamo-cortico-amygdala pathway through the external capsule (LeDoux *et al*., 1991).

BLA modulates the elements of memory consolidation in other brain region & has no direct role in memory storage (Cahill & McGaugh, 1998). Destruction of BLA before learning prevents learning (Fanselow & Le Doux, 1999). Afferents to the BLA are known to be arising from cortical and thalamic nuclei and those, arising from hippocampus, rhinal and prefrontal cortices (McDonald *et al*., 1996; McDonald, 1998; Pitkanen *et al*., 2000). The BLA receives sensory inputs through cortical and thalamic projections from all modalities: olfactory, gustatory, somatosensory, auditory and visual (LeDoux *et al*., 1991; Shi & Cassell, 1998; Shi & Davis, 2001). In addition afferents from the prefrontal cortex (Rosenkranz & Grace, 2002), the rhinal cortices and the hippocampus (Canteras and Swanson, 1992; McDonald *et al*., 1996; McDonald, 1998; Pitkanen *et al*., 2000) are the sources of polymodal inputs to the BLA and suggested to be responsible of behavioral flexibility. The afferents from PFC to BLA has been implicated in fear inhibition (Sotres-Bayon *et al*., 2004). Rhinal and hippocampal inputs transmit information about contextual memories (Corcoran & Maren, 2001; Corcoran *et al*., 2005).

Study with neurotoxic lesions of the BLA before fear conditioning was shown to impair the formation of CS-US associations (Cousens & Otto, 1998) i.e. failed to create associative memory for fear cue; and neurotoxic lesions of BLA after fear conditioning prevent retrieval of fear memory (Cousens & Otto, 1998; Campeau & Davis, 1995) means BLA is mainly involved in processing sensory modalities in fear.

Various efferent projections (eg., to brain stem and other) of the central nucleus of amygdala (CEA) are suggested to mediate the behavioral and autonomic concomitants of conditioned fear (LeDoux, 1988; Davis, 2000). There are important intrinsic connections too in the amygdala. During fear expression, the LA engages CEA which projects to areas of the hypothalamus and brainstem that control behavioral (e.g. freezing, startle), endocrine and autonomic conditioned responses (CRs) associated with fear learning (DM14, LeDoux *et al*., 1988). BA targets lateral capsular division (CElc) and the medial division (CEm) of the CEA (Savander *et al*., 1996) while LA targets CElc only (Pitkanen, *et al*., 1995). Amygdala is thought to be the main activity centre for auditory cue processing during fear conditioning and fear extinction (Falls *et al*, 1992; Lu *et al*., 2001; Goosen *et al.,* 2003; Herry *et al*., 2006; Sotres-Bayon*et al*., 2007; Quirk *et al*., 2006; Myers & Davis, 2007). Role of BLA is important in extinction learning (Barad *et al*., 2006). Some of the molecular players in memory processing e.g. PPs such as PP1, a protein Ser/Thr phosphatase, is abundant in brain structure like amygdala and can form a complex with epigenetic players e.g. HDACs to co-regulate histone PTMs (Canettieri *et al*., 2003). Histone acetylation in the LA is critical; also for reconsolidation of fear memories (Maddox & Schafe, 2011).

HDAC inhibition associated with increased H4 histone acetylation, accelerate extinction of cued fear memory in WT mice (Yossef Itzhak *et al.,* 2012). During auditory fear memory retrieval, H3 acetylation is regulated in LA (Maddox & Schafe, 2011) and epigenetic alterations in the lateral amygdala are also required for reconsolidation of a Pavlovian fear memory.

Both auditory and visual stimuli send projections to ipsi- and contra-lateral side (fig.- 2.5). Relatively predominant projections are denoted by thicker lines in the figure. Auditory pathways are divided into thalamo- and cortico-amygdalo pathways. The visual pathways to the amygdala are less well defined but also represented as combined thalamic and cortical inputs. The rest of the sensory inputs, such as olfactory, vestibular and gustatory, form important parts of complex stimuli, and it is represented as contextual stimulus pathways that centers around hippocampus, which receive inputs from various sensory cortical areas. (Kim & Choi, 2002).The essential US pathways to the amygdala are still not very clear. With the help of this fear

circuitry, details of many questions related to fear expression and its regulation are now able to be explained.

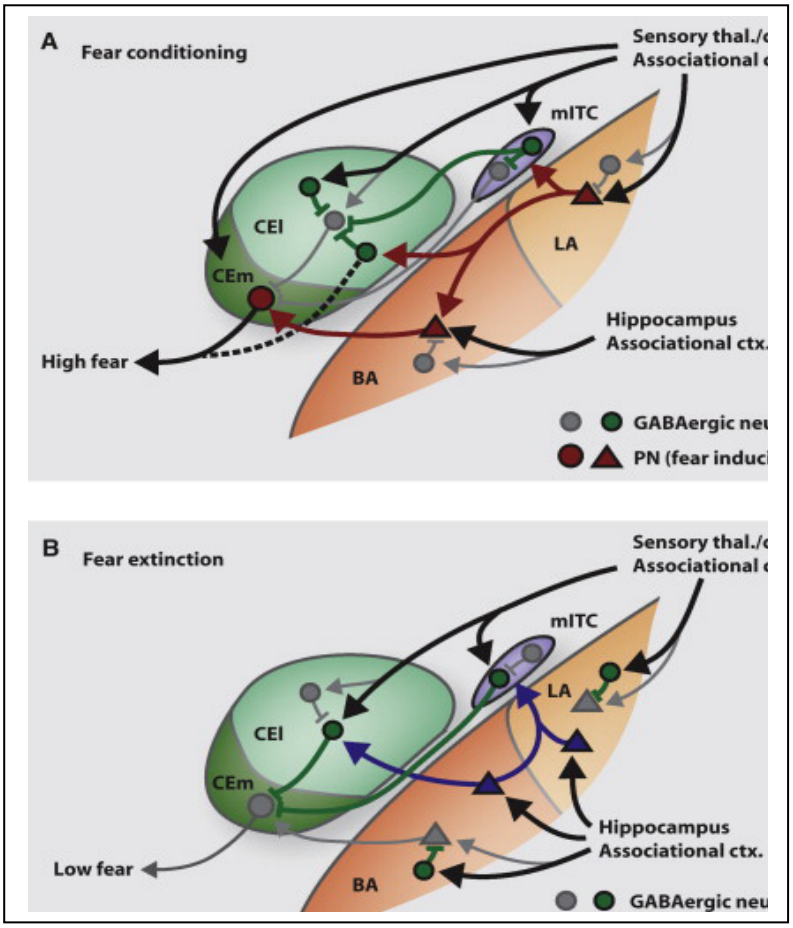

Figure 2.5: Flow of information during Fear Conditioning and Extinction

(A) During fear acquisition, suppression of feedforward inhibition in the LA enables glutamatergic LTP at sensory cortical and thalamic afferents to projection neurons. Fear consolidation and expression may involve a long-term decrease in local GABAergic drive in feedforward and feedback circuits within the BLA, thereby increasing output activity of fear-inducing projection neurons. In parallel processes, fear acquisition and expression can be coded in the CEA - either by increasing sensory drive to CEm output neurons directly or, by increasing excitatory drive to subpopulations of CEl neurons locally inhibiting CEm projecting neurons or increasing mITCs activity, both of which would lead to disinhibition of CEm output.

(B) During acquisition of extinction, plasticity of contextual inputs could lead to increased activity of fear-inhibiting projection neurons in the BLA. During consolidation, long-term enhancement of local GABAergic drive within the BLA occurs, which could serve to suppress activity of fear-inducing projection neurons. Neuropeptide-mediated increases in BLA to mITC transmission result in inhibition of CEA output during extinction learning. During retrieval of extinction memory, mITC inhibitory activity, controlled by several inputs, including those from medial prefrontal cortex, reduces CEm output to suppress fear responses.

[Adapted from - Ingrid Ehrlich et al., Neuron, Volume 62, Issue 6, 2009, 757 – 771]

Hippocampus: its connections & role

Hippocampus is known to process information related to context associated with CS, whereas cue CS and US are processed within the basolateral amygdala (Phillips & LeDoux, 1992, Rudy & Reilly, 1999, Debiec *et al.,* 2010). Hippocampus plays crucial role with some other brain areas to acquire contextual learning (Wiltgen *et al*.,2006). Contextual fear conditioning is a hippocampus-dependent form of memory (Holt & Maren 1999, Kim & Fanselow 1992). Hippocampus become functional for hippocampus dependent contextual learning after postnatal day (PD) 17 in rats but fear association with amygdala develops only after PD 23 (Foster & Burman -2010). The contextual learning is mainly processed in hippocampal formation and supported by afferent and efferent connections with neocortical system (NCS). Amygdala receives afferents from NCS as well hippocampal system (HS). These connections are summarized in following figure –

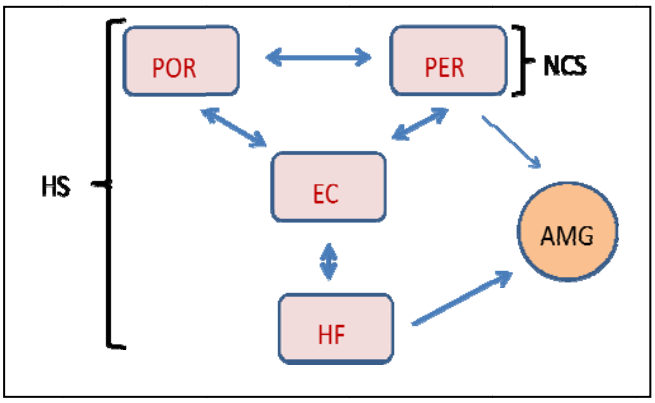

Figure 2.6: Schematic representation of limbic system (HF -Hippocampal Formation, NCS - Neocortical System, POR - Post rhinal cortex, PER - Perirhinal cortex, EC - Entorhinal cortex, HS – Hippocampal system).

Lesion of hippocampus blocks conditioned freezing response against context only and not for CS (Abel & Lattal, 2001). In both hippocampus (Kandel, 1997, Milner *et al.,* 1998, Malenka & Nicoll, 1999) and amygdala (Huang *et al.,* 2000, Huang &Kandel, 1998, Schafe *et al*., 2000, Brambilla *et al.,* 1997) structures, LTP is thought to involve in activation of a variety of protein kinase signaling pathways, either directly or indirectly, by increase in intracellular Ca2+ in the postsynaptic cell at the time of LTP induction. β-adrenergic signaling in the hippocampus is required for contextual memory retrieval (Murchison *et al*., 2004). Contextual information is processed in the hippocampal formation (HF), which plays a critical role in gating the

response of rats to extinguished tone stimuli (Corcoran *et al*., 2005). The route by which the hippocampal cortex (HPC) exerts its effects is thought to be through the mPFC (Hobin *et al*., 2003; Maren & Quirk, 2004). It is well established that contextual information gates behavioral response to conditioned stimuli, especially following extinction (Bouton, 2004).

Pre Frontal Cortex: its connections & role

PFC is comparatively less studied brain area in correlation of fear processes. It is suggested to facilitate fear memory by integrating sensory and emotional signals and to co-ordinate with amygdala based fear network (Gilmartin *et al*., 2014). The infralimbic (IL) component of the mPFC has role in consolidation and recall of extinction (Quirk GJ *et al*., 2006), while pharmacological inactivation of prelimbic (PL) component was found to abolish the expression of conditioned fear (Blum *et al*., 2006; Corcoran & Quirk, 2007). Quirk and colleagues investigated whether the prelimbic (PL) region of the medial prefrontal cortex (mPFC) might be involved in sustaining freezing. In a series of experiments they showed the critical role of PL for the expression of fear over the duration of the tone: (a) Pharmacological inactivation of PL was found to abolish the expression of conditioned fear (Blum *et al*., 2006; Corcoran & Quirk, 2007), (b) micro-stimulation of PL was found to augment conditioned fear (Vidal-Gonzalez *et al*., 2006); and, importantly, that the time course of PL tone responses parallels the time course of conditioned fear (Burgos-Robles *et al*., 2009). This finding is supported by studies examining neuronal activity with cFos, which correlated PL activation with fear expression and extinction failure. What are the structures in PFC that might modulate the memory of auditory fear during extinction? Again, several studies by Quirk and others reveal that the activity in infralimbic (IL) region of mPFC modulates the amygdala during recall of extinction memory and found to facilitate recall of extinction (Quirk *et al.,* 2006; Quirk & Mueller, 2008), moreover deficient IL activity results in failure of recalling the extinction (e.g., Milad & Quirk, 2002). Burgos-Robles *et al*., (2009) also noted that increased neuronal activity in PL following tones in rats was associated with poor recall of extinction, suggesting that these rats had excessive consolidation of fear memory.

2.4. The Behavioral Paradigms in Fear Learning

A well-established model based on Pavlovian classical conditioning is used for behavioral analysis of fear learning in this study. Ivan Petrovich Pavlov a Russian physiologist, winner of Nobel Prize for 'Physiology or Medicine' in 1904, gave his famous classical conditioning model based on associative learning in 1920. Classical fear conditioning behavioral paradigm is based on his model and being used in behavioral research to understand the physiological and molecular events, which occur during fear, associated learning. Pavlovian model is simple in approach yet powerful tool to study & design strategically various components of fear memories and PTSD like consolidation, recall, reconsolidation, extinction, anxiety etc.

The main feature of various classical conditioning paradigms is associative learning in which a cue (CS) is associated with a reward or punishment or threat (US). Gradually the trained animal produces the same psycho-physiological response/behavior against that cue even in absence of US, as made previously associated with US. (Pearce & Hall, 1980).

Fear conditioning paradigm (Training)

In fear conditioning paradigm, the associative learning is created by pairing a harmless cue (sound or light which initially has no effects on animal's behavior) called Conditioned Stimulus (CS) with an aversive stimulus (mild foot shock) called Unconditioned Stimulus (US). During fear conditioning, the acquisition of experiences for CS and US elicits a learned fear response (fig. 2.7 & 2.8), which includes increases in heart rate, blood pressure, and stress hormone release (Maren, 2001; Fanselow & Poulos, 2005). When presented alone, the CS is capable of eliciting the fear response in conditioned animals in anticipation of an expected foot shock (fig. 2.9). The fear response against a harmless cue is generated because during learning, animal has associated it with noxious stimulus. Thus produced response is called conditioned response (CR) and fear against cue is referred as experimental fear.

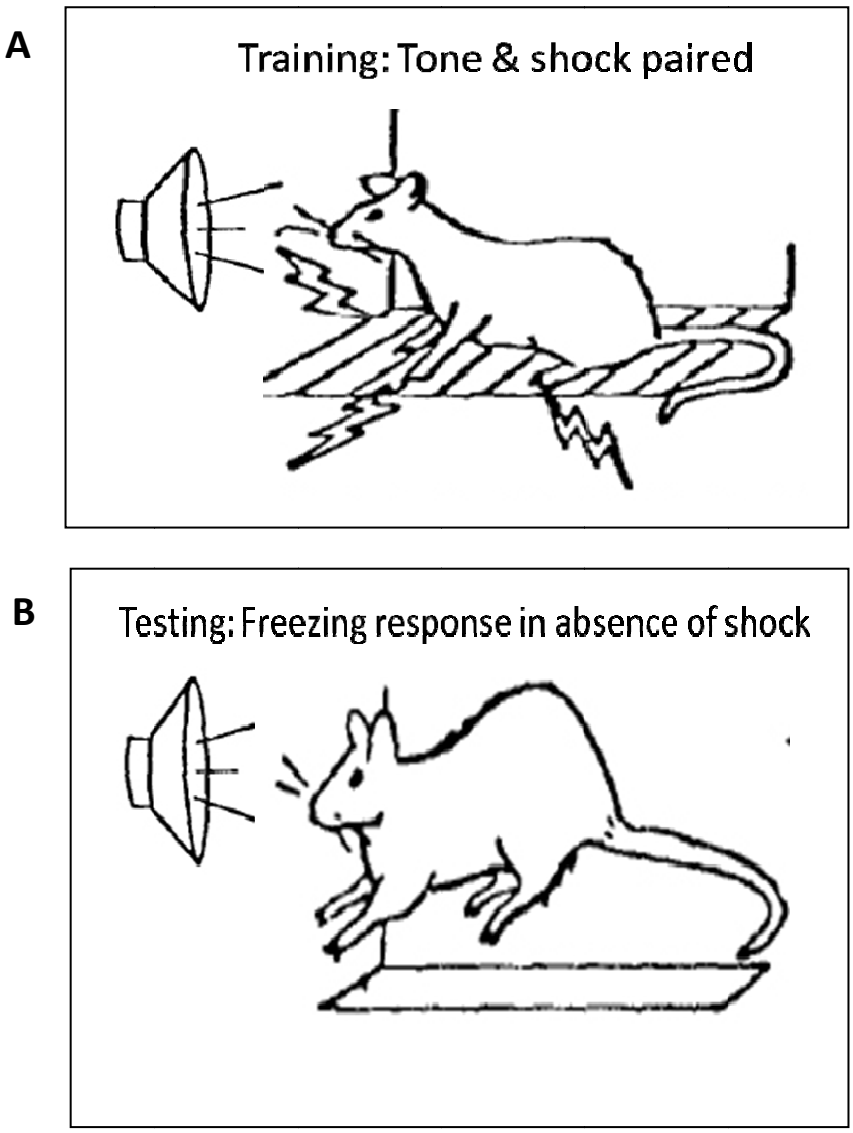

Figure 2.7: Pavlovian Fear Conditioning Paradigm based on classical conditioning model

(A) Fear conditioning – learning of fear occurs through acquisition of harmful sensory stimulus in a context. An auditory cue-CS (neutral stimulus causing no harm) when paired with aversive cue (US) foot shock is acquired by animal as fear stimulus and experience is memorized by brain in a process called fear consolidation.

(B) Test for fear consolidation – The fear conditioned animal shows fear response when presented with fear cue (here it is a sound) in a later stage of life while no shock is given. The fear response against a non-harmful auditory stimulus occurs because of associative learning which was created by fear conditioning. The fear response against CS (sound) is called Conditioned Response, CR

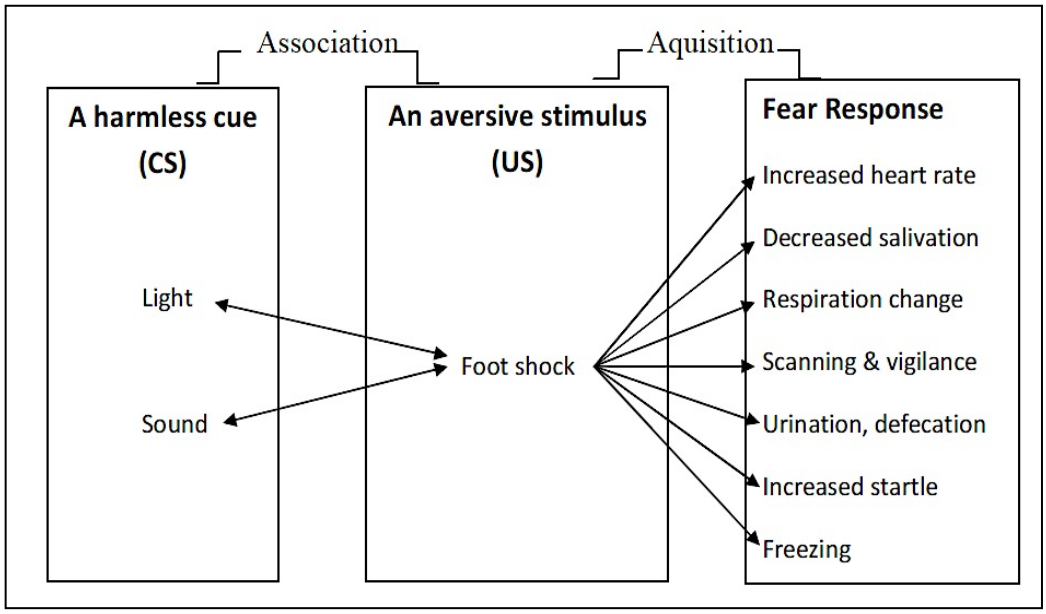

Figure 2.8: Conditioned responses produced during fear acquisition

Measuring the Conditioned Response (Testing)

Memory retrieval is an important aspect of memory processing because this is the only way a memory can be tested and measured for whether consolidation occurred and to which extent (strong or poor consolidation). During test session the defensive responses (fig.2.9) elicited by the CS in animals are characterized by freezing (complete immobility except as required for breathing), reflex expression (characterized by fear-potentiated startle), & autonomic (increase in heart rate and in the mean arterial pressure) and endocrine (stress-related hormone release) responses (LeDoux, 2000, Fanselow,Pavlov-1980).

Test is performed by exposing the fear-conditioned animal to the context or cue (CS), which was used with US during fear conditioning, this time without pairing it with US. The same test procedure is used to test retention of extinction learning. Freezing response is measured in terms of percentage on time scale i.e. duration of cue exposure and higher percentage is associated with strong association between CS & US therefore strong memory for that particular fear on the other hand low or no fear response against context or cue means fear learning process had not coded the association of two memories – one of US with another of context or CS.

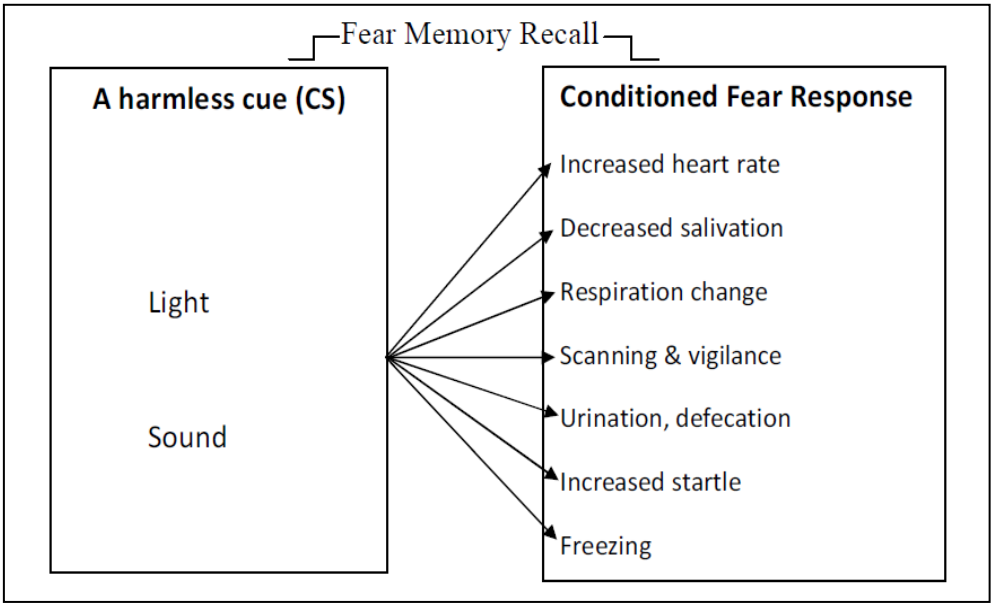

Figure 2.9: Conditioned response against learned conditioned stimulus

Extinction paradigm (Training)

Memory extinction is a process in which a conditioned fear response gradually diminishes over time as an animal learns to uncouple a response from an associated stimulus (Myers & Davis, 2002). The extinction paradigm includes behavioral training comprise of repeated presentation of CS in absence of any foot shock i.e. US. The subjected animal acquires a new learning that the CS (Sound) is no more a prediction of shock and shows gradually reduced conditioned fear response (Myers & Davis, 2002) and ultimately no fear may be produced with repeated CS (fig.2.10). Memory for this learning is called Extinction memory. Extinction is not forgetting instead the original association remains at least partially intact and years of study have established extinction as a distinct learning process (Myers & Davis, 2002). This fact is based on observation that fear memory may return even after very long time in three different ways known as spontaneous recovery, renewal & reinstatement (Bouton**,** 1993). The extinction therapy has been in use for long time to treat patient of PTSD, phobias etc.

CS alone presentation causes memory retrieval during which the original memory becomes labile for a time interval, restabilized afterward in order to reconsolidate following the same order of events as during consolidation (Nader,

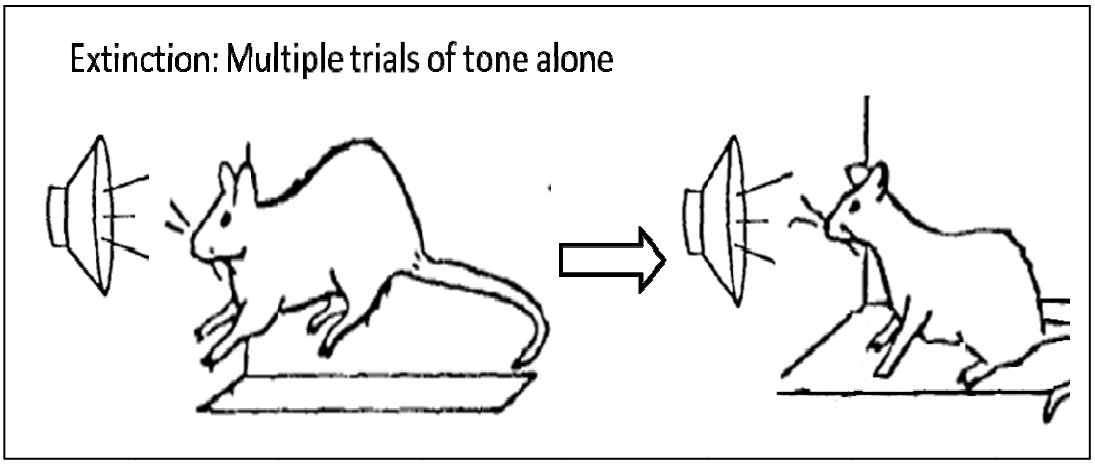

Figure 2.10: Fear Extinction Paradigm – repeated presentation of CS in a novel context which is different from fear conditioning context leads to new learning in which association between sound (CS) and shock (US) is disrupted and CS remains no more capable of eliciting CR therefore gradually reduced fear response ultimately extinguished. & finally no fear generated upon presentation of CS.

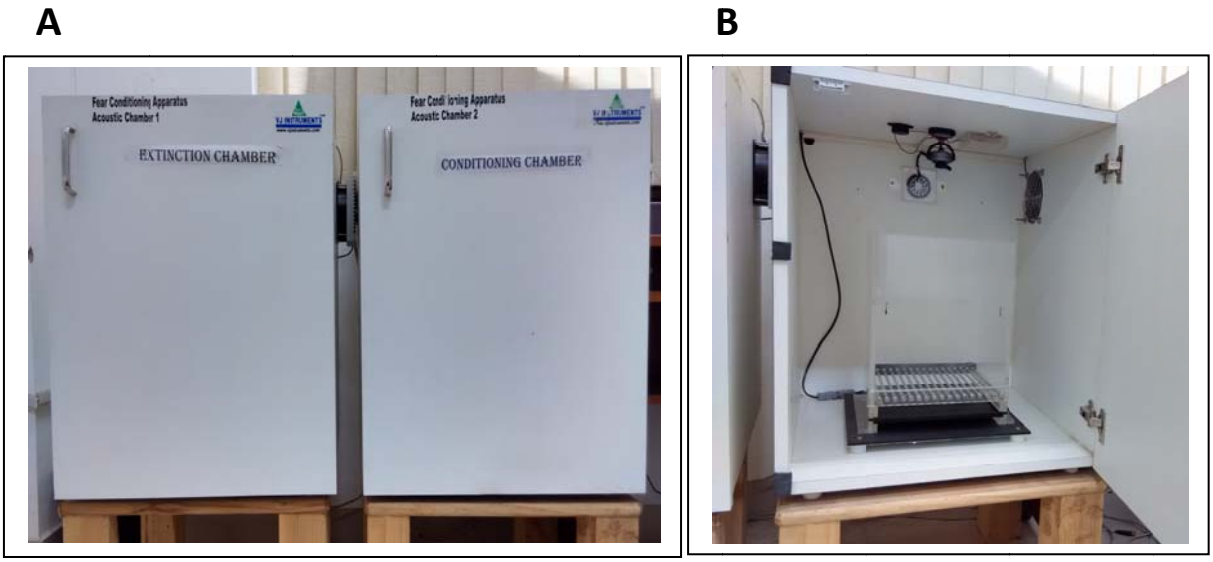

Figure 4.1: Freeze monitor from VJ Instruments.

(A) Separate chambers for fear conditioning and fear extinction training

(B) Interior of Freeze monitor – a plexiglass chamber with electrifiable grid floor is placed in centre of soundproof cabinet. A small ventilation fan is fitted on side wall and a CCD camera on roof of the chamber. The chamber is lighted with LED light source mounted and a speaker for sound source on side wall of cabinet.

2003) to preserve the memory.Whereas opposing to it, extinction of fear memory also is processed during this period (Myers & Davis, 2002), it means extinction depends on memory retrieval. Fear extinction weakens the association between CS and US, an important mechanism to inhibit excess fear. The fear conditioning model used in this study with rodents has become a useful tool to study the memory processing at molecular and systems levels (Abel, 2001; Tonegawa *et al.*, 2003; Nader, 2003) and has been the basis of various mechanism proposed for molecular & cellular outcomes of fear consolidation, fear reconsolidation and fear extinction.

2.5. Towards the work in this thesis

Thalamo-amygdalar network was implicated in cued fear conditioning by processing sensory information for CS & US (Bauer *et al*, 2001; Rogan *et al,* 1997). Pharmacological studies made some insight towards analyzing the role of HAT & HDACs, the major players in maintenance for level of acetylated histone proteins. Blocking HDAC was reported to rescues memory deficit and improves memory formation (Korzus, 2004; Levenson, 2004). Valproic acid induced HDAC inhibition is found to enhance LTM for both acquisition and extinction of cued fear (Breddy, 2008). Treatment with an HDAC inhibitor make better the fear extinction deficits possibly through increased histone acetylation to support the formation of new extinction memories (Matsumoto et al., 2013). HDAC inhibition leads to increased H4 acetylation which is also associated with accelerated extinction of cued fear memory in wild type mice (Itzhak *et al,* 2012). Intra LA infusion of HAT inhibitor impairs fear memory consolidation (Maddox *et al*, 2013). DNMT activity has also been implicated in altered histone acetylation as its inhibition found to blocks the concomitant, memory-associated histone H3 acetylation (Miller & Sweatt, 2007). DNMT inhibition in LA is associated with impaired fear memory consolidation and impaired H3 acetylation which is in contrast with the effect of HDAC inhibition in the same region; an increase in DNMT and H3 acetylation in LA has role in cued fear memory suggesting the two epigenetic components work in concert to regulate cued fear memory consolidation (Monsey *et al*., 2011).

Using specific knockouts some studies has provided further significant observations regarding epigenetic modulations involved in manipulation of behavioral

phenotypes, e.g. Class I HDAC (HDAC2) knockout rodents have shown highly accelerated extinction of conditioned fear response (Morris, 2013).

The role of hippocampus in contextual fear learning was discovered by pharmacological interventions and lesion studies (Ahi *et al,* 2004; Maren & Fanselow, 1995; Philips & LeDoux, 1992). Normal age related memory impairment has also been linked with lack of histone acetylation at H4K12 in hippocampus and its restoration through HDAC inhibition found to reinstate the expression of learning induced genes (Peleg *et al*., 2010). Increased H4K5 acetylation is reported in activity dependent hippocampal memory activation, (Park *et al*., 2013). PP1 is known to negatively regulate the hippocampus dependent long-term memory (LTM) and synaptic plasticity (Koshibu *et al*., 2011). Loss of CBP in dorsal CA1 of the hippocampus has resulted in selective impairments to long-term potentiation and long-term memory for contextual fear, suggesting a necessary role for specific chromatin modifications, selectively mediated by CBP during consolidation of memories in hippopcampus (Barrett *et al*., 2011). Acetylation of hippocampal histone H3 but not H4 significantly increases after an animal is trained with a contextual fear-conditioning paradigm (Levenson *et al*., 2004). NMDAR & ERK dependent histone H3 acetylation in CA1 region of hippocampus has role at initial stage of LTM formation and consolidation of contextual fear memory which can be enhanced by HDAC inhibition (Levenson *et al,* 2004; Dobbin & Tsai, 2007; Wood, 2005, Vecssay, 2007). Dorsal hippocampus has role in contextual fear memory retrieval in a latent inhibition paradigm (Holland &Bouton, 1999). Latent inhibition of conditioned fear also supposed to be epigenetically regulated as it correlates with increased acetylation at H4 in hippocampus (Levenson, 2004). HDAC inhibition study associated with enhanced learning during extinction (Hait *et al,* 2014) and suggested a role of hippocampus in contextual extinction (Lattal *et al,* 2007). The finding is supported by observation that NaB induced increased histone acetylation in hippocampus infralimbic network enhances fear extinction (Stafford, 2012). On the other hand over expression of HDAC1 in dorsal hippocampus significantly facilitates fear extinction and suggested mechanism involves Deacetylation of H3K9 followed by trimethylation on target genes; moreover HDAC1 inhibition showed impaired fear extinction (Bahari-Javan *et al*., 2012). CBP, a HAT, associated with CREB via KIX

domain of CBP has role – imparting selectivity to CREB for its target gene, in activation of genes required for long term storage of hippocampus dependent memory (Wood *et al*., 2006). Reduced histone acetylation at H3K14, H4K5 & H4K12, induced by isoflurane, in CA1 of hippocampus is associated with reduced fear response (Zhong *et al*., 2014).

Breddy *et al*., 2007 suggested from his findings that increased histone H4 acetylation in PFC is associated with strong extinction & with long term memory for extinction of conditioned fear while increased acetylation at H3 is found only in conditioned mice but not in extinction group. HDAC inhibition with NaB infused within mPFC resulted in increased histone acetylation in IL but not PL, which was correlated with enhanced extinction (Stafford *et al*., 2012). Activity of p300 in ILPFC constrains synaptic plasticity and reduction in the function of this HAT is required for formation of extinction memory (Roger Marek *et al*., 2011). The activity of PCAF; p300/CBP associated factor, in IL region of PFC is reported as necessary for fear extinction memory formation and interfere with reconsolidation of original memory trace (Breddy *et al*., 2012). Very recently the epigenetic correlates have also been implicated to investigate the effect of drugs of abuse on consolidation/reconsolidation of fear extinction memories (Pizzimenti & Lattal, 2015; Arias *et al*., 2015; Sillivan, *et al*., 2015) towards designing new therapeutic measures. Keverne has reviewed major epigenetic changes which take its role during brain development and which are suggested to affect the behavior of an organism in view point of behavioral disorders exist from very birth-point of that organism but not acquired during learning course as fear memories do (Keverne *et al.,*2015). It may be concluded that learning induced epigenetic changes responsible for behavioral pathogenesis are susceptible for their extinguished effects by different mechanism of memory extinction while congenital changes are not.

All these research findings done so far suggest that a type of epigenetic code, different in different brain areas and for different learning paradigms might exist for fear related learned memory formation. Also the formation of specific types of memories is associated with specific patterns of histone modifications (Cohen *et al.,* 2008; Gräff & Mansuy, 2008; Wood *et al*., 2006).

CHAPTER 3

AIMS & OBJECTIVES

Aims & Objectives

The working hypothesis towards this study is that both fear consolidation and fear extinction; bring about changes in level of histone acetylation differentially in PFC, amygdala and hippocampus. The same fear stimulus affects distinct neuronal populations in these brain areas at the same time, and expression of fear is dependent on, and related with pattern of expression of important genes known to have role in fear memory processing which in turn is regulated by histone acetylation occur differentially in these brain areas.

3.1. Aims of the thesis

Specific Aim1: To investigate gene expression related to memory formation in Prefrontal cortex, Amygdala and Hippocampus during the consolidation and extinction of fear-related learning in a model of auditory fear conditioning.

1.1 To investigate the changes in the levels of ERK/p-ERK, ARC and CREB/p-CREB in the amygdala, Hippocampus and Prefrontal cortex (PFC) in brains of rats fear conditioned and undergone extinction for fear respectively.

Specific Aim 2: To investigate whether the changes in the gene expression as in aim 1 are under epigenetic control during fear memory consolidation and extinction.

1.2 To investigate changes in the histone H3/H4 acetylation in the Amygdala, Hippocampus and Prefrontal cortex.

1.3 To investigate the expression of CREB Binding Protein (CBP), a HAT commonly involved in CREB mediated gene expression in the regions of amygdala, hippocampus and PFC in the brains of rats fear conditioned and undergone extinction training respectively.

3.2. Plan of Work:

3.2.1. *F*ear Conditioning experiment: to develop experimental fear for a sound (CS) in rats (n=12) by pairing the sound with a shock (US) in Pavlovian classical conditioning paradigm.

3.2.2. Fear extinction experiment: Rats (n=12) undergone fear conditioning are to be trained for 'not to fear' against same sound (CS) by repeated presentation of sound in the absence of the shock (US).

3.2.3. Control experiments: to create three control groups (n=12 each) named Ctx (context) only, Shk (shock) only and Tn (Tone) only in which the Rats to be exposed to either context alone, shock alone or tone alone respectively.

The schematic showing the groups is presented below.

Groups	Conditioning	24 hr	Extinction	Perfusion delay
1. Condition	5 tone-Shock		-	24 hrs post condition
2. Extinction	5 tone-Shock		30 tones	24 hrs post extinction
3. Ctxonly	context A		context B	2 hrs post exposure
4. Tnonly	5 tones		30 tones	2 hrs post exposure
5. Shkonly	5 shocks		-	2 hrs post exposure
6. Naïve	-		-	with experimental groups

3.2.4. **Brain collection and sectioning –** to performintracardial perfusion with 4% ice-cold paraformaldehyde in order to fix tissue and collection of brain for making tissue sections from amygdala, hippocampus and PFC regions to be used in Immunohistochemical analysis.

3.2.5. **Immunohistochemical (IHC) analysis –** to perform IHC with collected brain section to check expression level of ERK/p-ERK, ARC and CREB/p-CREB as mentioned in specific aim 1. And to localized the level and distribution pattern of acetylation at Histone H3 and H4 along with CBP (a HAT) as mentioned in specific aim 2, in brain regions under investigation.

3.2.6. **Image analysis –** to prepare IHC slides for microscopic study and analyze them for immune-positive neuronal cell count using image acquisition software from Nikon.

CHAPTER 4

MATERIAL & METHODS

4.1. Animals:

In present study, young adult, male Sprague Dawley (S.D.) rats (12 - 16 weeks old), weighing 200-250 grams were used for all experimental groups. The rats were housed separately at least five days prior to behavioral training with water and food available *ad libitum*, and handled for 2-3 minutes twice a day. Rats were housed under 12/12 hours light/dark cycle. Room temperature was maintained at 23°C. All experiments with animals were performed during morning sessions. All experiments were approved by CPCSEA.

4.2. Apparatus and Context Setting:

The apparatus: Freeze Monitor (V. J. Instruments) was used for auditory cued fear conditioning, fear extinction and testing. It consists of a removable & transparent Plexiglas square chamber (35.5 cm x 25 cm x 25 cm); with an electrifiable grid floor (0.4 cm diameter, spaced 0.6 cm apart), connected with a calibrated shock generator, to deliver the shock as unconditioned stimulus (US); & covered by a transparent Plexiglas lid. The grid floor was illuminated at 100 lux by the LED lights. The chamber was made soundproof by placing it in a square sound-attenuating cabinet of dimension (70 cm x 50 cm x 50 cm); fitted with Light-emitting diode (LED) light source, a speaker to deliver sound cue - the conditioned stimulus (CS), and a Charge Coupled Device (CCD) camera mounted on ceiling; and a small ventilation fan on its one of the walls adjacent to roof as a source of white noise. All the surfaces in the chamber were white.

Fear conditioning and extinction took place in two different contexts (context A and B). The conditioning and extinction boxes and the floor were cleaned with 70% ethanol or 1% acetic acid before and after each session, respectively. To score freezing behavior, each chamber was equipped with a ceiling-mounted Charge Coupled Device (CCD) camera connected to a computer-installed with software (V. J. Instruments) program to analyze captured image and videos. The animals were considered to be freezing if no movement was detected for 2 s.

In this study the Context setting **ABB** was used i.e. **context A** for fear conditioning, **context B** for extinction training and retention tests. For **Context A** - all the inner walls of the soundproof chamber were white with a black sheet placed on floor beneath the metal grid. A unique olfactory context was created by wiping the floor with 1% acetic acid solution. The **context B** was used in fear extinction training protocol and to test retention of fear or of fear extinction (Retention Test). Context B was different from context A. It had black & white stripes on wall of the cabinet. Also the floor was wiped with 1% ammonium hydroxide solution for different olfactory context. Similarly a white sheet was placed over the metal grid to make the floor of context B different from conditioning context.

Before each test, the acrylic walls and floors were wiped with a towel soaked in distilled water, and the grids were wiped with 70% ethanol to prevent a bias based on olfactory context. The grids were wiped with ethanol instead of water to ensure that the grids do not lessen their electrical conductivity due to rust.

4.3. Behavioral procedures

Fear conditioning – Day 1:

For fear conditioning of rats, a protocol similar to Chang *et al.,* 2009 was used with a few modifications. The rats from experimental groups (n=12 each) were transported to behavioral training room from animal house in their home cages. The fear conditioning chamber was set to 'context A' and freeze monitor system was calibrated for shock intensity & the loudness of auditory cue. A rat from conditioned group (n=12) was placed in the transparent Plexiglas chamber inside sound attenuated chamber (Freeze monitor, VJ Instruments; fig. 4.1), one subject per chamber. The animal was allowed to explore the chamber for three minutes after placement in the chamber to habituate for it. Subsequently it was presented five conditioning trials, each consisting of an auditory CS (conditioned stimulus, a tone of 80 dB & 10 sec duration), paired with an US (unconditioned stimulus - a foot-shock of 0.7 mA & 1 sec duration) coterminous with tone. The conditioning trials were separated by inter-trial intervals (ITI) of 60s sec. The final shock was followed by additional 1min before removal of animal from the chamber. The animals were then transported back to their home cages.

Fear extinction – Day2:

Extinction training session was performed in context B, 24 h after conditioning session. The rats (n=12) previously fear conditioned were transported to behavioral training room in containers with black wall that was different from their home cages used to transport them for fear conditioning on previous day. Rats were placed in transparent plexiglass chamber, one rat per chamber, and allowed to explore the chamber for 3 minutes. This 3 min habituation was followed by presentation of 30 auditory CSs (80 dB, 10sec) with 60 sec ITI, followed by additional 1 minute in chamber. Freezing was measured while tone was delivered during the extinction session. The animals were transported back to their home cages.

Testing – Day2/3:

Retention Test was performed to check the behavioral outcomes produced as a result of learning in which animals learned to associate CS with US (as a result of fear conditioning) and subsequently learn to break this association between CS & US (as a result of fear extinction training). The test was performed on each experimental group (n=4) to check the successful conditioning to an auditory cue (retention of fear consolidation) or successful extinction to the same cue (retention of fear extinction), both in context B. Testing followed 24 hrs of training for each group. Rats in conditioning group (n=4) were tested on day 2 for retention of fear, 24 hrs after fear conditioning and rats from extinction group (n=4) were tested on day 3 for retention of extinction memory, 24 hrs after extinction training. To test fear consolidation, the rats, fear conditioned, were subjected to a 3-min acclimation period followed by 3 auditory CS (80 dB, 10sec, 60 sec ITI) in context B, followed by additional 1 minute in chamber. No US was delivered during test session. The freezing response during tone (CS) delivery was recorded. Likewise to test successful extinction of fear memory, the conditioned response was tested 24 hrs after fear extinction session. The session was same as to test fear consolidation, i.e. consisting of 3 auditory CSs (80 dB, 10sec, 60 sec ITI) with no shock delivery followed by one more minute in chamber. The conditioned response was measured as % freezing during tones. A high % freezing score during test following conditioning signifies the success of standard protocol for Fear conditioning training. All animals of conditioned group were trained by using same protocol. The low or no freezing response during test session following

the extinction training was observed as successful standard protocol set for extinction training, and all the rats in extinction group were subjected to same protocol.

Control groups:

Additional groups of animals apart from naïve control were created as control separately for auditory and contextual experience and shock stimulation. The 'Context only' (Ctx) group (n=12) was subjected to experience the context A & context B in absence of any tone or foot-shock. The rats were first submitted to the context A (Day 1) for same time duration as taken to condition the rats, and then 24 hrs later submitted to context B (Day 2) for same time duration as spend by rats during fear extinction training. No tone and no shock were delivered during both the sessions.

Another group named 'Shock only' (Shk) group (n=12) received 5 foot-shocks immediately after placing the rat in fear conditioning chamber with Context A excluding any tone delivery and removed shortly afterwards. This way any association of shock with either context or tone is controlled; to check any level of unconditioned fear causing biochemical changes due to shock alone.

A third control group named as 'Tone only' (n=12) was submitted to context A and context B separated by 24 hrs for same duration as for conditioning trial and extinction trial, and presented tones only but no shock. Tone only group was included as control for any biochemical changes occurring in response to non associative auditory cue. The rats from 'Naïve' group (n=12) remain in their home cages throughout the experiments and were never exposed to any experimental conditions. The rats of naïve group were perfused with rats from other experimental groups.

Scoring:

Conditioned response, CR, was observed manually as freezing i.e. the absence of all non-respiratory movements. Scores of '0' for immobility and '1' for movement was scored for every 5 sec block during tone delivery in all experimental and control groups except for naïve group. The scores were summed up and divided by the total number of readings to derive a percent freezing score. As a separate measure, freezing was also recorded automatically using video tracking through the CCD camera controlled by software in a computer attached to freeze monitor.

4.4. Immunohistochemical Analysis

Collection of brain and obtaining brain sections

Rats from all groups i.e. conditioned, extinction and naïve group, were anesthetized using pentobarbital (50 mg/kg body weight) and perfused intracardially, with 4% ice-cold paraformaldehyde (prepared in 0.01M phosphate buffer, PB) to fix tissues. The brains were dissected out then post-fixed in same 4% paraformaldehyde for 24 hrs. The post-fixed brains were cryoprotected by sequential transfer in 10%, 20% and 30% sucrose solution prepared in 0.01M PB for 24 hrs in each one or for time till the brains settled down at bottom of the vial containing sucrose solution. In order to preserve the brain samples for future uses the samples were dissected in three parts and freezed using isopentane as cold bath at -30 ^{o}C. The treated brain samples were transferred into labeled vials and stored at -80 ^{o}C. Brain sections to be used for IHC were 20 µm thick coronal sections and obtained from frozen brain samples using Microtome (Thermo-fisher) set at -30 ^{o}C, from PFC, amygdala and hippocampal regions. 4 -6 sections were collected in 0.01M PBS against each antibody (against Acetyl H3, Acetyl H4, ERK, p-ERK, ARC, CREB & CBP) to perform IHC.

Immunohistochemistry (IHC) procedure

To perform IHC, 20 µm thick coronal brain sections from PFC, amygdala and hippocampus region from experimental as well as control groups were collected in sufficient volume of .01M PBS to keep section free – floating. The brain sections were processed for IHC. Firstly washed for 2 X 10min with 0.01M PBS followed by 30min incubation in 3% H_2O_2 prepared in .01M PBS containing 0.25% Triton X-100 (PBST). Again tissue sections were washed for 6 X 5min with 0.01M PBS. The sectioned were blocked with 1% Normal horse serum (NHS Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA) prepared in 0.25% PBST, given 1hr incubation. It was followed by overnight incubation at room temperature after addition of primary antibodies against Acetyl H3 (1:1000 dilution), Acetyl H4 (1:1000 dilution), ERK (1:500), p-ERK1/2 (1:200 dilution), ARC (1:500), CREB (1:1000), p-CREB (1:500 dilution) & CBP (1:500 dilution). Unbound antibodies were washed with 0.01M PBS for 3 X 15min. Secondary antibody (biotonylated anti-mouse IgG, 1:500 dilution, Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA) prepared in 0.25% PBST was applied to these brain sections

and incubated for 2 hrs followed by 3 X 15min washing with 0.01M PBS. Further brain sections were incubated for 1 hr in ABC complex supplied with DAB staining kit (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA) then washed for 3 X 15min with PBS. Thus processed brain sections were then stained with DAB substrate (Vectastain, Vector Laboratories) till the color appeared. Multiple washing with tap water followed repeated washing with PBS. Sections were mounted on slide for study under microscope using Image acquisition software.

Image analysis

The slides were analyzed for positive neurons for different immunostaining under up-right microscope from Nikon using NIS Element NS - BR image acquisition software from Nikon. Images were acquired at 4x & 20x and immunostained cell/nuclei were counted manually as well as with the help of automated software.

4.5. Statistical analysis

The raw data was processed under statistical analysis using software sigmaplot-13 from sigma. For each behavioral training session, the freezing data were transformed to a percentage of observations. The data was expressed as means & standard error of the means (SEM) and were analyzed with one-way analysis of variance (ANOVA). IHC slides were analyzed for immune-positive neurons count for different molecules to be compared among the groups and brain regions. The level of molecules was analyzed by one-way analysis of variance (ANOVA). Post-hoc comparisons in the form of Bonferroni tests were performed after a significant overall F ratio.

CHAPTER 5

RESULTS

5.1. Results of behavioral procedures

5.1.1. Fear conditioning (Day 1):

The fear conditioning session for both experimental groups – condition (Cond.) and extinction (Ext.) groups was performed on day 1 in context A. The experimental fear created by pairing a tone (CS) with mild foot shock (US) resulted in successful fear conditioning as it resulted in high degree of conditioned response (CR), interpreted as absence of all non-respiratory movements also called freezing while CS was being presented. The level of CR was calculated as % freezing. The freezing was observed and recorded for full duration the rat spent in chamber of Freeze Monitor (VJ instrument, India). The duration has comprised of an initial habituation period of 3 minutes during which the rats were allowed to explore the context before presentation of any paired CS and US. Result shows that there was no freezing observed during this acclimatization period. This also confirmed that Context presentation in absence of paired CS-US could not create experimental fear or associative learning. Also result for this period was used to set a reference point to compare the CR produced as result of paired CS-US presentation in fear conditioning session. The results of fear conditioning session carried out in context A in both experimental groups showed that rats has shown freezing response as first CS-US paired trial was presented and freezing increased with each next trial of paired tone-shock delivery. In 5^{th} and last trial, a robust increase in freezing was observed in both groups as compared to acclimatization period and initial conditioning trial during session (figure 5.1**B**). The graph plotted between mean % freezing (n=12) during tone delivery and time of conditioning trials shows a sharp increase in CR in last trial as compared to initial trial ($p<0.0001$). The % freezing was also compared with that of control groups (fig.- 5.2B) in which no paired CS-US trials were presented, therefore set as reference point to compare CR response as a result of associative fear learning in conditioned rats with that of non-associative learning in control groups. The comparison between condition group and control groups was found to have extremely high % freezing in condition group ($p<0.0001$) during training session on day 1 in the same context A.

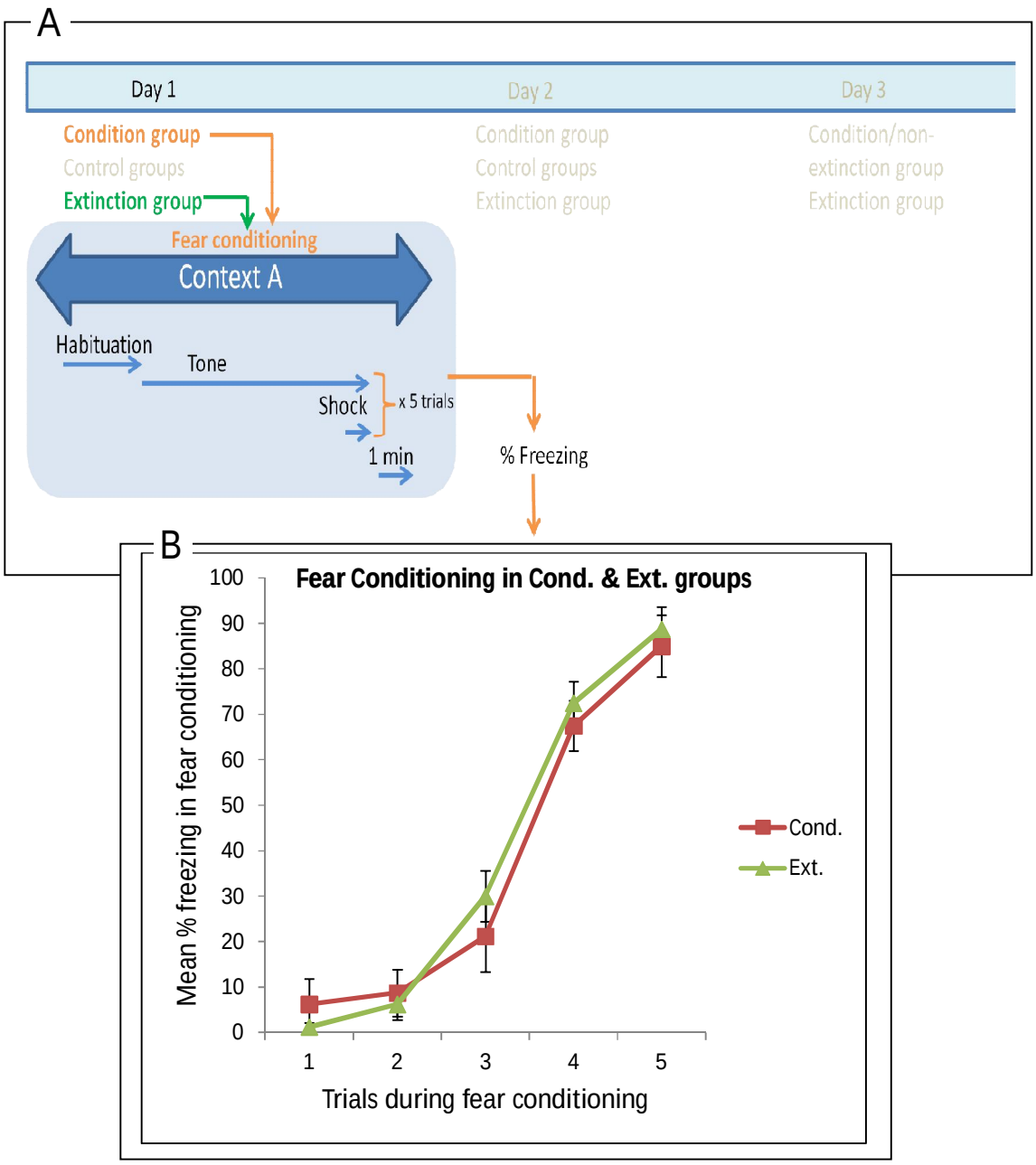

Figure 5.1: Results of fear conditioning behavioral protocol. (A) Experimental design for behavioral experiments on day 1 for experimental groups. Single rats was conditioned at a time in 'Context A' to fear the tone (CS) as per protocol summarized in pictogram. (B) The graph represents behavioral response (CR) during fear conditioning session in both experimental groups. A robust increase in % freezing response by rats was observed with each advancing trial of tone-shock pairing during session in both condition and extinction groups as compared to habituation period and to control groups shown in fig. 5.2B (p<0.0001). Cond., condition, Ext., extinction, CS, conditioned stimulus, CR, conditioned response.

5.1.2. Control groups (Day 1):

Three control groups were submitted to control parameters (context, tone and shock) selected from fear conditioning set-up in which fear conditioning has been carried out on day 1. The % freezing response by animals was recorded during exposure to any one of the control parameters in context A. Rats from Tone only (Tn) and Context only (Ctx) control groups spent same time duration in chamber while exposed to tone and context respectively as spend by rats during fear conditioning; but Shock only (Shk) group were limited to shock presentation only and not given time to explore the context so as to subtract the association of context with shock in shock only group. The Ctx and Tn group showed no change in mean % freezing as compared to habituation period ($p>0.5$). The mean % freezing in these groups was significantly very-very low in final trial as compared to final trial observed in condition and extinction group ($p<0.0001$) during fear conditioning session (fig.-5.2B). The animals from Shk only control group showed a little freezing as compared to habituation period but the difference was not significant. Also when final shock alone trial of Shk group was compared with that of final CS-US paired trial given to condition and extinction group, it was found that the mean % freezing in Shk only group was significantly lower ($p<0.0001$) than experimental groups.

5.1.3. Fear Extinction (Day 2):

The rats from Ext. group which have been fear conditioned along with Condition group in context A on day 1 were trained to extinguish fear against tone CS in context B, 24 hrs post conditioning on day 2. In novel context rats were allowed to explore the context, afterward presented 30 tone trial but no shock during entire session. The change in CR was recorded during tone delivery with each advancing trial. The graph was plotted between the mean % freezing of group (n=12) and trials. The mean % freezing for 5 consecutive trials was averaged to get six observation points out of total 30 trials delivered (fig. 5.3B). Initially very high % freezing (CR) was expressed by rats upon presentation of tone (CS) as the animals have learned to associate the fear memory of shock with particular tone, which gradually reduced with subsequent trial and sharply reduced with some final trials as shown in fig fig. 5.3B. There was significant decrease in mean % freezing response in final trials of extinction session as compared to initial trials ($p<0.0001$) during extinction session.

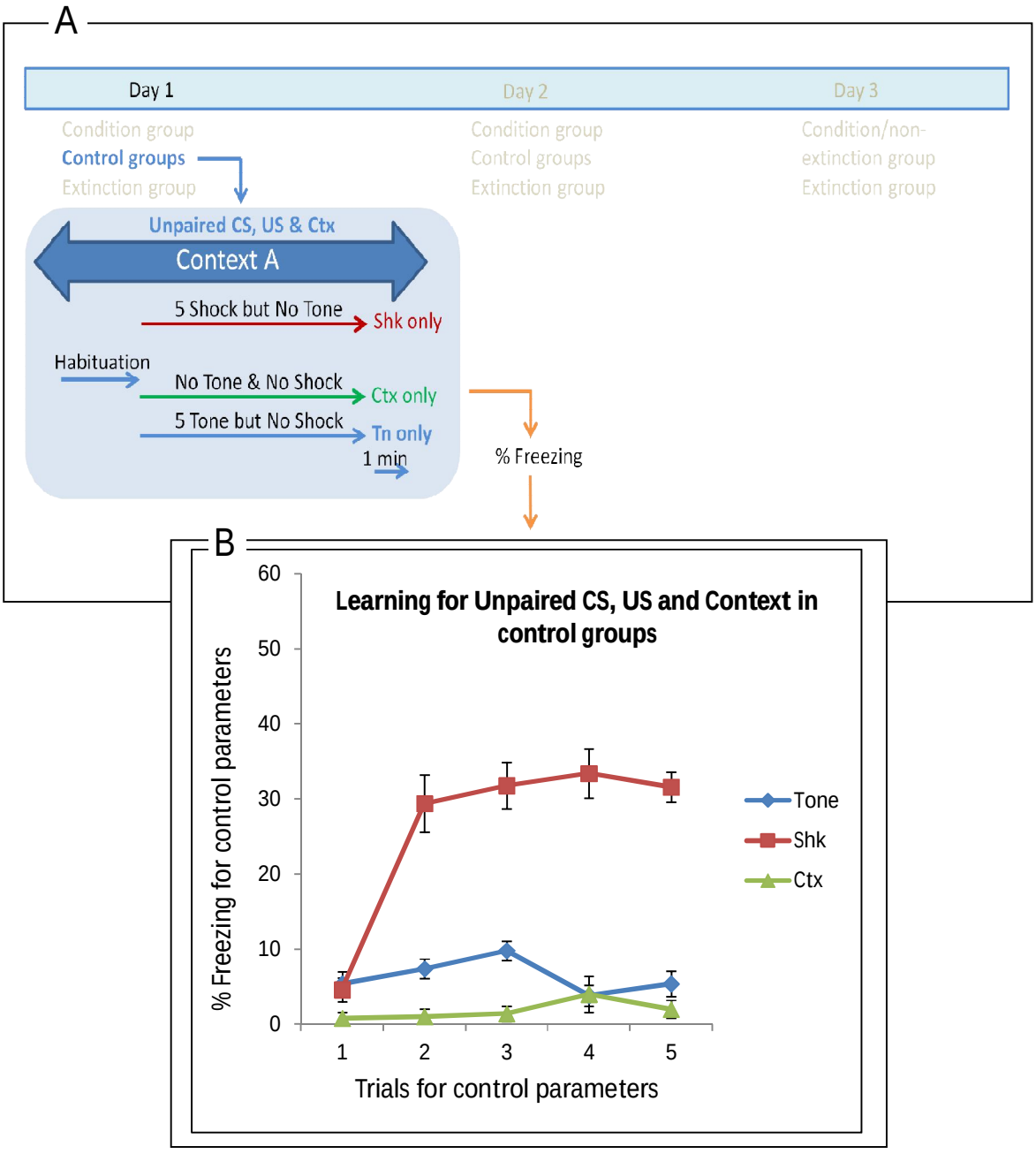

Figure 5.2: Results of behavioral procedures in control groups. (A) Experimental design for behavioral experiments on day 1 for control groups. Rats were exposed to CS, US and CTX not altogether but to unpaired components of fear conditioning for the same duration as rats spent in freeze monitor during fear conditioning. The experiment is summarized in pictogram. (B) The increase in mean % freezing response during session was not as significant as compared between habituation period and final trial which shows that fear memory of aversive stimulus is not associated with unpaired cue. ($p>0.05$). Ctx, context, Tn, tone, Shk, shock.

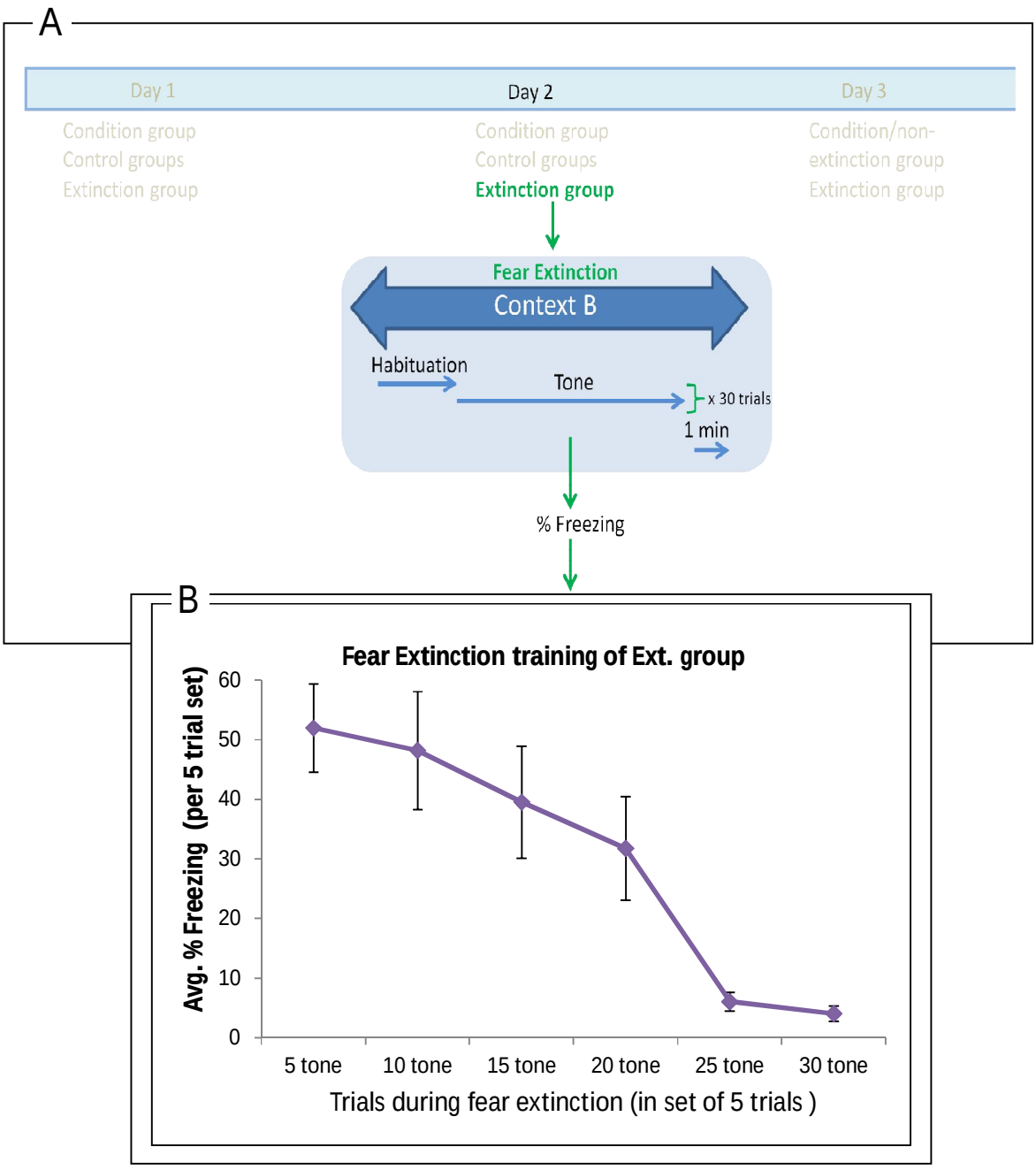

Figure 5.3: Result of fear extinction learning. (A) Experimental design of behavioral procedure on day 2. All rats from extinction group (conditioned on day 1) were extinguished in 'Context B' for not to fear the tone (CS) as per protocol summarized in pictogram. 30 CS and no US were presented. (B) The graph represents CR was produced against tone during fear extinction session. The % freezing response was extinguished with advancing trial of tone alone and no freezing was recorded by the end of session in extinction groups as compared to initial trials during same session (p<0.0001).

The result shows that extinction training has weaken the association of CS with US and CS loosen its capability of eliciting the CR in animals undergone fear extinction.

5.1.4. Test for retention of fear consolidation (Day 2):

On day 2, the rats from experimental groups as well as control groups (n=4) were tested for the retention of fear memory against CS (sound cue) in novel context. Three tone trials without any foot shock were delivered in context B (see details under method section). As expected the rats undergone fear conditioning in the condition and extinction groups produced very high level of freezing response when presented with CS – tone in novel context B. The graph in fig. 5.4B shows the comparison of freezing response in all groups. The freezing response in experimental groups is referred as conditioned response while no such response could be seen from animals of control groups which had not been fear conditioned against the CS (tone). There was a significant difference in % freezing observed during test in experimental groups as compared to control groups during test. There was significantly higher CR (mean % freezing) for CS in the condition ($p<0.0001$) and extinction groups ($p<0.0001$) as compared to the CR in the naïve control as well as to Ctx, Shk and Tn groups respectively. There was no difference in the CR between condition and extinction groups ($p>0.05$).

No significant change in freezing response was observed upon tone delivery between Tn group and Shk group ($p>0.05$) for entire time duration the rats spent in freezing chamber ($p>0.05$). Tone was also not found to produce any CR in Ctx group during test as no CS had been presented to Ctx group on day 1. Similarly no difference was observed between Shk and Ctx group upon CS presentation during test session. The behavioral results of control groups signifies that unpaired presentation of CS and US does not make the CS capable of producing fear response i.e. CS couldn't acquire noxious properties, hence no fear memory is formed for tone therefore no association of CS with any aversive US was created.

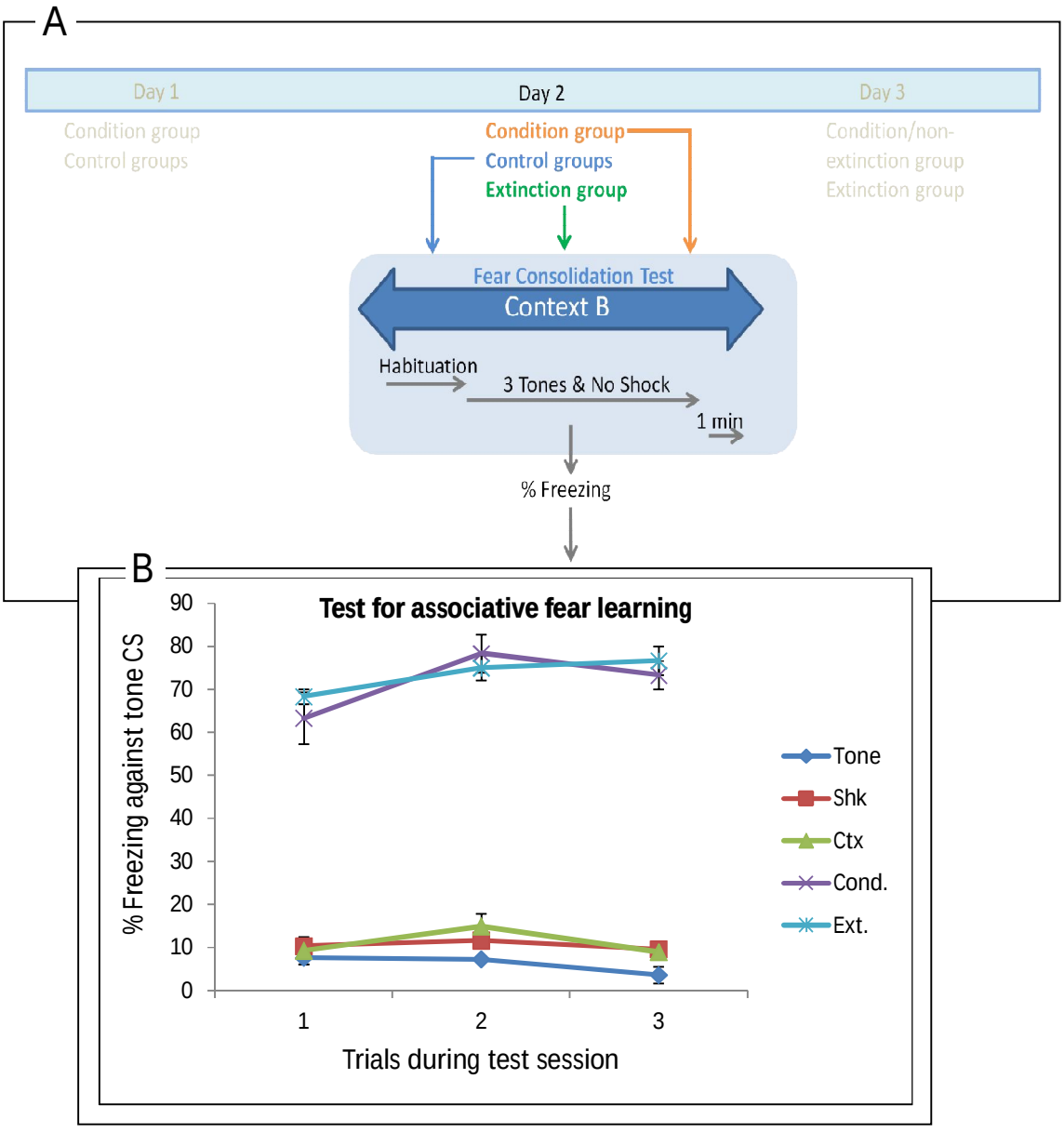

Figure 5.4: Results of tests for associative learning. (A) The rats from experimental as well as control groups were tested for formation of fear memory of US associated with CS on day 2 in 'context B'. The % freezing was recorded during CS presentation. (B) The graph represents different response between experimental and control groups. Rats, fear conditioned on day 1, from cond. and ext. group showed very high CR for CS while no significant change in CR was noticed in control groups during CS presentation. The results are collinear with fact that fear association of US is specific to paired CS only and not with context or unpaired tone and shock.

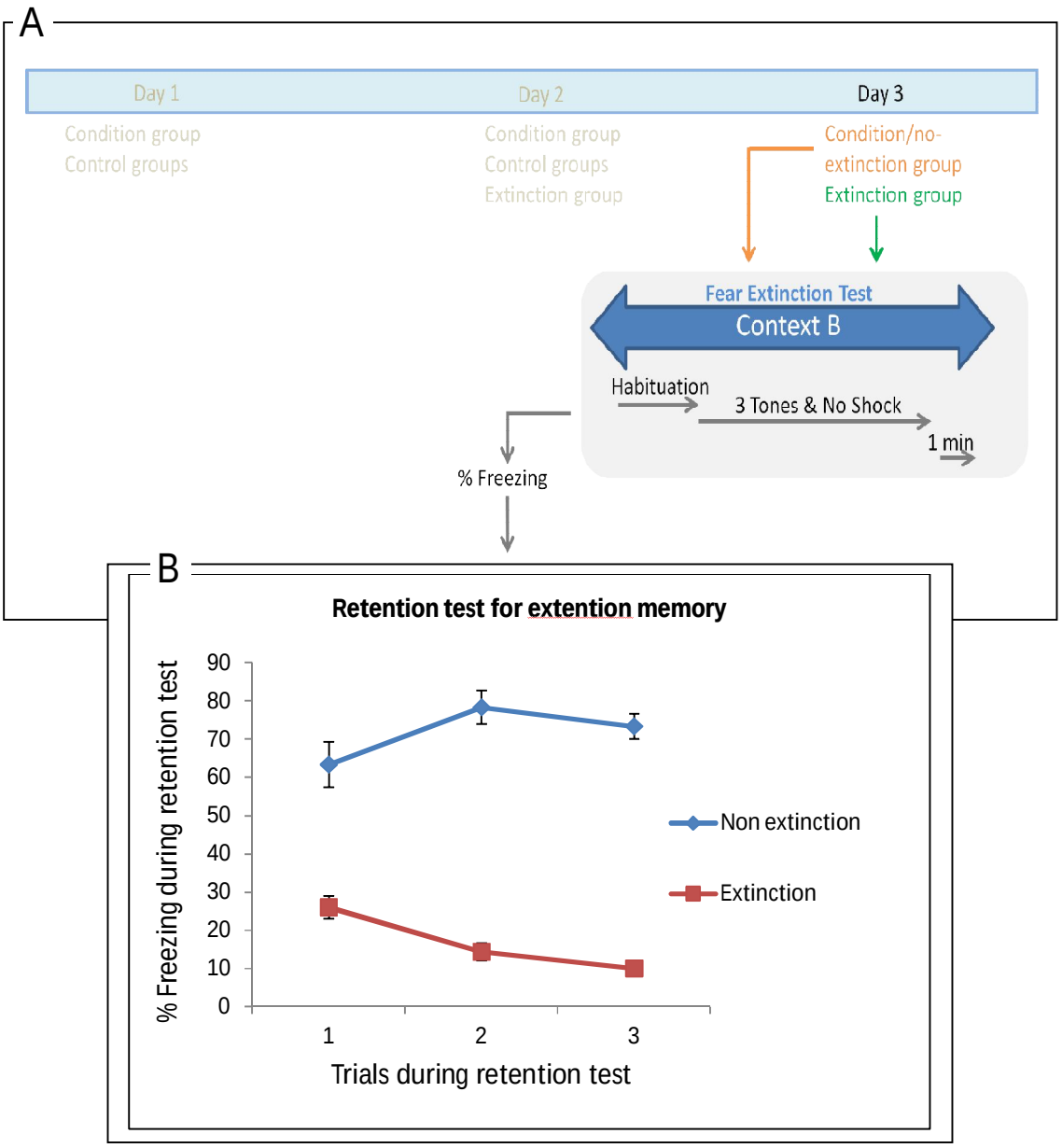

Figure 5.5: Results for retention test for extinction memory. (A) Experimental set up to check for consolidation of extinction memory on day 3 in rats undergone fear extinction on day 2. The protocol summarized in pictogram is same as used to test fear consolidation in all groups on day 2. (B) The graph shows very high % freezing in rats which did not undergone fear extinction training following fear conditioning while little CR was recorded against CS in rats from extinction group ($p<0.0001$). The result explains that extinction training leads to learning for dissociation of paired CS and US.

5.1.5. Test for retention of extinction memory (Day 3):

Consolidation of extinction memory was tested on day 3 i.e. 24 hrs post extinction training. The experimental design of test was same as that used to test the associative learning or fear consolidation in all groups on day 2. Three tone trials were presented in context B and % freezing response was recorded during tone presentation. The rats from condition group (n=4), which had not undergone extinction training, were taken as no extinction control to compare the extinguished CR of extinction group (n=4).

No change in % freezing score was observed during tone presentation in extinction group as compared to pre test habituation period ($p>0.05$). However there was a huge difference in % freezing response – CR between extinction and no – extinction groups as high level of CR was recorded in no-extinction group when compared to extinction group ($p>0.05$), fig.5.5B. The test result is important to refer the successful extinction for molecular and epigenetic studies made afterward.

5.2. Immunohistochemical analysis under specific aim 1:

To investigate the changes in the levels of ERK/p-ERK, ARC and CREB/p-CREB in the amygdala, Hippocampus and Prefrontal cortex (PFC) in brains of rats fear conditioned and undergone extinction for fear respectively.

5.2.1. Immunohistochemical analysis for pERK:

Introduction

Extracellular signal-regulated kinase (ERK) is well known for its role in cell growth and differentiation (Kornhauser and Greenberg, 1997; Impey *et al.,* 1999; Oruban *et al.,* 1999). Its activation in neurons is activity dependent and also found to be activated in hippocampus following LTP (Impey *et al.,* 1999). Inhibition of ERK/pERK led to impaired memory consolidation (Brambilla *et al.,* 1997; Atkins *et al.,* 1998; Selcher *et al.,* 1999). The activation of ERK/MAPK signaling pathway is important in consolidation of both auditory as well as contextual fear consolidation and its activation in time dependent manner in LA has been observed for associative (paired CS-US presentation) learning only in post fear conditioning paradigm (Schafe

et al., 2000). Moreover extinction of auditory fear conditioning also involves activation of ERK/MAPK in BLA and its inhibition in BLA prevents formation of extinction memory (Herry *et al*., 2006). ERK regulate transcription factor CREB that in turn activate expression of many memory related genes (Sweatt *et al*., 2001). ERK/MAPK signaling pathway inhibition in mPFC disrupts consolidation of extinction learning (Hugues *et al.*, 2004). In hippocampus, p-ERK increases in time phases after fear conditioning.

p-ERK level in Amygdala region:

There was increase in the number of p-ERK immuno-positive neurons in amygdala following fear conditioning as compared to naïve control group. There was significant increase observed in the no. of p-ERK positive neurons in the LA following conditioning as compared to naïve control (p<0.001). Similarly, the no. of p-ERK positive neurons increased following fear extinction in LA when compared with naïve control (p<0.05) (fig.5.6A & B) (ANOVA one way, F (2, 15) = 25.90).

Similar pattern was observed for changes in the no. of p-ERK in BA. There was significant increase in number of p-ERK positive neurons following fear conditioning as compared to naïve control (p = 0.0013). The increase in the no.p-ERK positive neurons in BA following extinction was not significant as compared to naive control (ANOVA one way F, (2, 15) = 10.61). In CeL of the central Amygdala there was significant increase in the no. of p-ERK positive neurons following conditioning (p< 0.001) and extinction as compared to naïve group (p<0.001); (F (2, 15) = 11.92, p = 0.0008). Unlike CeL, the CeM was active only during consolidation (fig.5.6B). ERK phosphorylation was observed only after conditioning in CeM. No changes were observed in the extinction group. The no. of p-ERK positive neurons increased significantly following fear conditioning as compared to naïve control (p<0.0001)(F (2, 15), = 12.57, p = 0.0006).

p-ERK level in Hippocampus region:

There was differential ERK phosphorylation level was observed in hippocampal sub-regions CA1, CA3 and DG following different fear learning paradigms (fig. 5.7 A & B). Fear consolidation resulted in significant increase in p-ERK level in CA1 (p=0.0001) & CA3 (p=0.0117) as compared to naïve control in

same region. However, the increase in the number of immune-positive neurons for p-ERK in the CA1 region of extinction group was not significant as compared to naïve group. [F (2, 15) = 34.35]. There was significant increase in the number of p-ERK positive neurons following extinction (p<0.005) and conditioning (p = 0.0117) in CA3 as compared to naïve control (fig.5.7 B), [F (2, 15) = 14.26, p = 0.0003]. A different pattern of p-ERK level in DG was noticed following fear conditioning and fear extinction. There was significant decrease found in the p-ERK positive neurons following conditioning (p<0.005) and extinction (p<0.001) in DG as compared to naïve control group [F (2, 15) = 16.99), p = 0.0001].

p-RK level in PFC region:

Differential changes in phosphorylation of ERK were also observed in the IL and PL sub-regions of the PFC following fear conditioning and fear extinction (fig. 5.8 A & B). There a significant increase in no. of p-ERK positive neurons was found in the PL following conditioning (p = 0.003) as compared to naïve. However no significant changes were observed in the PL following extinction (p = 0.6345) as compared to naïve. The difference between conditioned and extinction group was significant as there were higher no. of p-ERK positive neurons found in conditioned group (p = 0.0041), (ANOVA, one way, F (2, 15) = 14.76, p = 0.0003) fig.5.8B.

Whereas in the IL, the number of p-ERK positive neurons was found to be significantly higher following fear extinction (p<0.001) but not following conditioning (p = 0.7376) as compared to naïve. Condition group also showed significantly high level of p-ERK in IL as compared to extinction group (p<0.01). (ANOVA, one way, F (2, 15) = 12.10, p = 0.0007).

5.2.2. Immunohistochemical analysis for pCREB:

Introduction

CREB, is a transcription factor. In its active form pCREB (phosphorylated CREB), binds a particular base sequence on DNA called cAMP response element (CRE) to regulate the expression of downstream gene. The changes in Ca^{2+} or cAMP level following a wide variety signals during neuronal activity leads to activation of protein kinases that phosphorylate CREB at Ser-133 for its activation (Jensen et al. 1991) and has been linked with contextual memory formation &inhibitory avoidance

in rodents (Impey *et al,* 1998; Taubenfeld *et al,* 1999). More than 300 different stimuli have been reported to activate CREB (Johannessen , Delghandi , Moens , 2004). The type and nature of stimulus as well as of neurons determine which gene or set of genes' expression will be modulated among hundreds of CREB targeted genes. It was reported that inducible activation of a CREB inhibitor blocks consolidation of long-term fear-conditioned fear memories (Kida *et al.,* 2002). Another study suggested that the process of memory extinction could depend on increased CREB activation in forebrain (Wang *et al,* 2004). Many studies in rodents have shown memory and learning deficits when cAMP-PKA pathway was altered at different steps or CREB inhibitors were used (Wu *et al.*, 1995; Huang *et al.*, 1994; Abel *et al.*, 1997). Many other studies have confirmed that CREB dependent gene expression in vertebrates is necessary for Long term memory (LTM) and plasticity (Benito & Barco, 2010). CREB stimulates the expression of neurotrophin BDNF that promotes synaptic growth, which contribute to the enhancement of LTP (Barco *et al.*, 2005). CREB activation has been found to induce the transcription of its target genes in glutamatergic neurons after behavioral training aimed to create fear memory which is amygdala and hippocampus dependent (Taubenfeld -1999, Impey *et al.*,1998, Porte *et al.*,2008)

p-CREB level in amygdala region:

Immunostaining for p-CREB in amygdala shows differential phosphorylation of CREB in different sub-regions of amygdala (fig.5.9 A & B). In LA the number of p-CREB positive neurons increased significantly following conditioning ($p<0.01$), but not following extinction ($p = 0.329$) as compared to naïve control group. (fig. 5.9B). In LA more immune-positive neurons were found following conditioning as compared to extinction group ($p<0.05$). [ANOVA-one way, $F (2, 15) = 9.888$, $p = 0.0018$]. In BA, the increase in the no.of p-CREB positive neurons following conditioning and extinction was not significant ($F (2, 15) = 1.278$, $p = 0.3072$).

In CeL, there was significant increase in number of p-CREB positive neurons following conditioning ($p<0.01$) and extinction ($p<0.0001$) as compared to the naïve control. No change was observed in between conditioned and extinction group ($p = 0.5393$), [$F (2, 15) = 18.83$, $p<0.0001$]. In the CeM, however, there was significant increase in the no. of p-CREB positive neurons following conditioning ($p<0.001$) but

not following the extinction (p = 0.4073) as compared to naïve control. Condition group also noticed for significantly more no. of p-CREB positive neurons in CeM as compared to extinction group. ($p<0.05$), [F (2, 15) = 12.52, p = 0.0006].

p-CREB level in hippocampus region:

The behavioral training modulated the level of p-CREB in all regions from hippocampus under study (fig. 5.10 A & B). The CA1 region was marked with increase in number of p-CREB positive neurons in both the experimental groups as there were significant changes observed following conditioning ($p<0.0001$) and extinction ($p<0.0001$) as compared to naïve control group. But the difference in no.of p-CREB positive neurons within experimental group was not found significant (p = 0.1017), [ANOVA, one way, F (2, 15) = 46.02, $p<0.0001$].

In CA3, there was significant increase in the number of p-CREB positive neurons following extinction ($p<0.0001$) but not following conditioning ($p>0.05$) as compared to naïve control. Significant difference was found between experimental groups as more p-CREB level was detected in extinction group ($p<0.01$) in CA3, [ANOVA-one way, F (2, 15) = 20.10, $p<0.0001$]. In DG there was a significant increase in the number of p-CREB positive neurons following extinction ($p<0.0001$) but not following conditioning ($p>0.05$) as compared to naïve control and extinction group has higher no. of p-CREB positive neurons as compared to condition group ($p<0.0001$), (fig.- 21B). [ANOVA-one way, F (2, 15) = 42.21, $p<0.0001$], fig. 5.10B.

p-CREB level in PFC region:

A differential change in phosphorylation of CREB was observed in the IL and PL sub-regions of the PFC following fear conditioning and fear extinction (fig.-5.11A & B). There was a significant increase in the p-CREB positive neurons in the PL following conditioning (p = 0.0002) as compared to naïve. However there was no significant change observed in no. of p-CREB positive neurons in the PL following extinction ($p>0.9999$). While condition group showed more p-CREB positive neurons as compared to extinction group ($p<0.001$), [ANOVA, one way, F (2, 15) = 19.97, p = 0.0001].

In the IL, the number of p-CREB positive neurons increased significantly following fear extinction (p = 0.0039) as compared to naïve. However no significant

change was observed following conditioning (p = 0.3105), whereas no.of p-CREB positive neurons was much higher following extinction as compared to conditioning (p<0.001), [ANOVA, one way, F (2, 15) = 16.90, p = 0.0001].

5.2.3. Immunohistochemical analysis for ARC

Introduction

Neuronal activity induced synaptic modification includes induction of IEG (immediate early genes) expression for effector molecule Arc/Arg3.1 **(**activity-regulated cytoskeleton associated protein) that is involved in cellular changes at locations such as cytoskeleton or receptors (Besnard *et al.,* 2014). The transcripts of ARC are transported to dendrites and translated there suggesting its possible role in post synaptic changes in memory formation (Steward, 1998; Lonergan *et al.,* 2010). Arc-knockout mice showed impaired spatial learning and disrupted fear memory to context and auditory stimuli (Plath *et al.,* 2006). Memory recall is accompanied by expression of the genes *Zif268* and *Arc* and inhibition of specific proteins synthesis in hippocampus upon memory recall has been shown to interrupt its reconsolidation and permanent loss of recalled memory (Lattal & Abel, 2004) or impair maintenance of LTM without affecting its induction (Guzowski *et al.,* 2000) . A recent study contradict this finding and suggests that under conditions of short-term recall, Zif268 and ARC-dependent cellular processes in the hippocampus act to constrain extinction rather than to mediate reconsolidation (Trent *et al.,* 2015). Memory for inhibitory avoidance task is enhanced by increased ARC expression in CA1 of hippocampus which is dependent on activation of β-adrenergic receptors in BLA (Lovitz & Thompson, 2015). Separate exposure to context and shock trigger Arc expression in the frontal association cortex, FrA, which receives projections from BLA (Nakayama *et al.,* 2015). Chronic exposure to corticosterone induced increase in long lasting ARC expression in LA is found to be associated with enhanced consolidation of a fear memory through enhanced LTM (Monsey *et al.,* 2014). mPFC-dependent fear extinction is impaired and linked to increased ARC expression in this region (Niimi, *et al.,* 2014). There are very few research work notified on neuronal processing of auditory fear memory. A latest work reported that diet enriched with curcumin is capable of impairing fear memory consolidation and reconsolidation processes and associated with prevention of elevated level of ARC

expression in LA in post Pavlovian fear conditioning paradigm (Monsey *et al.*, 2015). Elevated expression of ARC in BLA but not LA and CeA nuclei of amygdala has been associated with enhanced fear memory of trace fear conditioning, a forebrain-dependent fear associative learning paradigm as compared to other fear conditioning paradigm (Chau *et al.,* 2013). Nuclear localization of ARC may recruit a specific HAT complex to modulate learning-induced acetylation of H4K12 suggesting its role in epigenetic regulation of long-term memory formation (Wee et al., 2014). Epigenetic correlates of this IEG – Arc in fear associated processing is very obscure and needs more experimental interventions.

ARC level in amygdala region:

There was increase in the number of ARC immuno-positive neurons in amygdala following fear conditioning as compared to naïve control group. There was significant increase observed in the no. of ARC positive neurons in the LA following conditioning as compared to naïve control ($p<0.0005$). Similarly, the no. of ARC neurons increased following fear extinction in LA when compared with naïve control ($p=0.0038$) (fig.5.12A & B), (ANOVA-one way, $F(2, 15) = 14.11$, $p=0.0004$). In BA sub-region no significant change in ARC level was found amongst all the groups [ANOVA-one way, $F(2, 15) = 0.1192$, $p = 0.8884$].

In CeL of the central Amygdala there was significant increase in the number of ARC positive neurons following conditioning ($p=0.0005$) and extinction ($p<0.05$) as compared to naïve group. No change between experimental groups was found in CeL ($p= 0.5254$), [ANOVA-one way, $F(2, 15) = 13.01$, $p=0.0005$].

Unlike CeL, the CeM was active only during fear consolidation. ARC level increased only after conditioning in CeM. No changes were observed in the extinction group ($p>0.9999$) as compared to naïve group. The no. of p-ERK positive neurons increased significantly following fear conditioning as compared to naïve control ($p<0.01$) and condition group was noticed with significantly high level of ARC as compared to extinction group ($p<0.005$), [ANOVA-one way, $F(2, 15) = 10.42$, $p=0.0015$], fig. 5.12B.

ARC level in hippocampus:

Differential changes have been noticed in hippocampus in ARC level following behavioral training in different sub-regions of hippocampus (fig.5.13A & B). In CA1, similar increase in no. of ARC positive neurons was found in both condition (p=0.0005) and extinction (p=0.0002) groups as compared with naïve control while no difference was detected between two experimental groups (p>0.9999), (ANOVA-one way, F (2, 15) = 18.31, p=0.0001). In CA3 sub-region, there was a significant increase in the level of ARC positive neurons following conditioning (p=0.0001) as compared with naïve control. The increase in extinction group was not as significant as observed in condition group upon comparing it with naïve (p=0.1527) but the difference between condition and extinction group was significant as more no. of ARC positive neurons were observed in condition group in comparison to extinction group (p<0.05), [ANOVA-one way, F (2, 15) = 17.16, p=0.0001]. In DG sub-region, elevated level of ARC was found following conditioning as compared with naïve (p = 0.0020). The changes in extinction group were not found significant upon comparing the no. of ARC positive neurons in it with that of condition group (p = 0.0761) and naïve control group (p = 0.2814), (fig.-5.13B); [ANOVA-one way, F (2, 15) = 9.205, p<0.0025].

ARC level in PFC:

In Pre-frontal cortex, in the PL region, significant increased in the no. of ARC positive neurons in condition group (p=<0.0001) and extinction group ($p\leq$0.0001) respectively as compared with naïve [ANOVA-one way, F (2, 15) = 28.25, p<0.0001]. In IL, there was no significant difference in the no. of ARC positive neurons following conditioning as compared to naïve control (p = 0.9829) observed (fig.5.14B). The difference in ARC level between naive and extinction group was found significant as there were more ARC positive neurons in extinction group (p<0.001) and also higher no. of ARC positive neurons were detected following extinction in IL as compared to condition group (p<0.001). [ANOVA on way, F (2, 15) = 25.84, p<0.0001).

5.3. Results of Immunohistochemical analysis under specific aim 2

To investigate whether the changes in the gene expression as in aim 1 are under epigenetic control during fear memory consolidation and extinction.

Introduction

Cellular response to an environmental stimulus is exerted through various biochemical reactions and cell signaling which are ultimately governed by the level of gene expression. The experience-dependent transcriptional regulation plays a critical role in the stabilization of short-term memory to long-term memory. Post-translational modifications of histones, particularly histone acetylation has been shown to be a mechanism for transcriptional regulation during long-term memory formation (Guan *et al.,* 2009; Alarcon *et al.,* 2004; Levenson *et al.,* 2004a; Colvis *et al.,* 2005; Kumar *et al.,* 2005; Levenson and Sweatt, 2005; Chwang *et al.,* 2006; Wood *et al.,* 2006; Bredy *et al.,* 2007; Fischer *et al.,* 2007; Lubin *et al.,* 2008). It is achieved through the regulation of accessibility of the promoters to the transcription machinery (Barrett & Wood, 2008; Renthal & Nestler, 2008; Day & Sweatt, 2011 and Krishnan *et al.,* 2011). Active state of chromatin was visualized by immunostaining for modified Histone for acetylation at H3 and H4 in brain areas, PFC, Amygdala and Hippocampus, known to be involved in fear processing and together these brain areas constitutes the main fear circuitry.

5.3.1. Results for modulation in H3K9 acetylation

Acetyl H3K9 level in amygdala

Histone acetylation especially acetylation of H3 at lysine residue 9 has been shown to be associated with active transcription (Peixoto & Abel , 2013). To monitor the activity of fear circuit we inspected differential activity pattern of amygdala nuclei IHC images are shown in fig.5.15 A & B. The number of acetyl H3K9 positive nuclei were increased in LA following conditioning as compared to naïve control ($p<0.001$). There was also an increase in acetylation level at H3K9 in LA following extinction as compared to naïve group ($p<0.001$) while the increase in no. of immune-positive nuclei in both the experimental groups was found similar as compared to each-other (ns, $p>0.05$), [$F(2, 27) = 31.71$, $p<0.0001$]. There in BA also the pattern similar to that

of LA was noticed where more acetylated H3K9 level was found during fear conditioning (p<0.0001) and fear extinction as compared to naïve (p<0.0001) but no significant change between condition and extinction group (ns, p>0.05) was observed [Anova-one way, F (2, 33) =44.3, p<0.0001] in CeL sub-region, both fear conditioning and extinction were noticed to make a significant increase in acetyl H3K9 positive nuclei as compared to naïve control (p<0.001). No difference was noticed between condition and extinction group (ns, p>0.05), [Anova-one way, F (2, 33) =15.04, p<0.0001]. in CeM level of acetyl H3K9 positive nuclei increased during conditioning and extinction. Significant changes were observed following conditioning (p<0.0001) as compared to naïve and also following extinction (p<0.001) as compared to naïve control animals. There was difference in acetyl H3K9 positive nuclei between condition &. Extinction group but not significant [Anova-one way, F (2, 33) =58.44, p<0.0001]; (fig. 5.15B).

Acetyl H3K9 level in hippocampus

In hippocampus all the regions under study were found to have an increase in acetyl H3K9 positive nuclei following conditioning (fig.5.16A & B). in CA1 region, highly significant increase was observed in condition group for acetyl H3K9 positive nuclei as compared to both extinction (p<0.0005) as well as naïve control (p=0.0031) respectively. But there was no change found between extinction and naïve group (p=0.9196), [Anova-one way, F (2, 15) = 14.57, p=0.0003]. In CA3 region, significantly increased level of Acetyl H3 positive nuclei was observed in condition group as compared to naïve (p = 0.0044) and also as compared to extinction group (p = 0.001) while extinction group didn't show any change as compared with naïve (p=>0.9999). [Anova-one way, F (2, 15) = 12.25, p = 0.0010]. In DG, significantly higher and similar increase was observed in acetylation level at H3K9 following fear conditioning (p = 0.0046) and fear extinction (p = 0.0091). [F (2, 15) = 11.24, p=0.0019]; while no difference between both the experimental groups was observed (p>0.9999); (fig.5.16B).

Acetyl H3K9 level in PFC

The no. of immune-positive nuclei for acetyl H3K9 increased in the PL of the PFC during conditioning with significant value as compared to naïve control

(p<0.001). The differences between extinction and naïve group were not found significant (p>0.05), however significant difference was observed between condition and extinction group (p<0.001) with more acetylated H3K9 during conditioning. [Anova-one way, F (2, 15) = 16.01, p = 0.0002] (Fig. 5.17A & B). Infralimbic prefrontal cortex (IL) region was observed with a different pattern than that of PL nuclei. Here acetyl H3K9 positive nuclei were increased during extinction as compared to naïve control (p<0.001). The increase in level of acetyl H3K9 following extinction was significantly higher than condition group also (p<0.001). But no significant change was observed in condition group as compared to naïve group (ns, p>0.05). [Anova-one way, F (2, 15) =16.82, p<0.0001]; (fig. 5,17B).

5.3.2. Results for modulation in H4K5 acetylation level

Acetyl H4K5 level in amygdala

The pattern of acetylation at H4K5 has been visualized by immunostaining and IHC images were analyzed to investigate the extent of acetylation in all brain tissues (fig. 5.18A & B). In LA there was significant increase observed in acetyl H4K5 positive nuclei following conditioning (p<0.05) as well as following extinction (p<0.05) as compared to naïve control group. The changes were not significant in acetyl H4K5 level between condition and extinction group in LA (p>0.05), [Anova-one way, F (2, 15) = 9.549, p = 0.0021]. In BA the changes were not found significant for acetyl H4K5 positive nuclei following conditioning as well as following extinction as compared to naïve control and also when experimental groups compared with each other (p>0.05). [ANOVA one-way, F (2, 15) = 0.3399, p = 0.7172]. There in CeL region change in acetylation level at H4K5 was found to increase following extinction in comparison to naïve (p<0.05), while changes following condition was not significant as compared to naïve (ns, p>0.05) in CeL; also the difference between condition and extinction was not significant (p>0.0753), [Anova-one way, F (2, 15) =5.133, p = 0.0200]. The opposite pattern of that in CeL was observed in CeM region. Here there more no. of acetyl H4K5 positive nuclei were found in CeM following conditioning as compared to naïve control group (p<0.01) while no changes were observed in extinction group as compared to naïve group (p>0.05). The difference between extinction and condition groups was found significant (p<0.01); (fig.5.18), [Anova-one way, F (2, 15) =10.46, p=0.0014]; (fig. 5.18B).

Acetyl H4K5 level in hippocampus

In hippocampus a similar pattern of acetylation was observed in regions under study where fear conditioning was marked with increased no. of acetyl H4K5 positive nuclei (fig. 5.19A & B). There significant increase was observed in acetylation at H4K5 in CA1 region following conditioning as compared to naïve control group ($p<0.005$) and also when compared to animals undergone fear extinction ($p<0.01$), [Anova-one way, F (2, 15) =9.975, $p = 0.0018$]. In CA3 region, there too increased level of acetylation was found following fear conditioning as compared to naïve control group ($p<0.001$) and the difference of increase in condition group as compared to extinction group was also significant ($p<0.05$), [Anova-one way, F (2, 15) =11.01, $p = 0.0011$]. There in DG, significantly higher level of acetylation at H4K5 was found following conditioning ($p<0.001$) and also following extinction ($p<0.005$) as compared to naïve control group. The condition and extinction both groups were detected with similar level of increase in acetyl H4K5 positive nuclei and no difference was found between them (ns, $p = 0.2568$), [Anova-one way, F (2, 15) =59.11, $p = 0.0001$]; (fig. 5.19B).

Acetyl H4K5 level in PFC

The sub-regions of PFC, PL & IL were detected with differential pattern for acetylation level at H4K5 (fig.- 5.20A & B). A very significant increase in no. of acetyl H4K5 positive nuclei in PL was observed following conditioning as compared to naïve control group ($p<0.001$). The level of acetylation following extinction was not different with that compared to naïve control ($p>0.05$) but very low as compared to condition group ($p<0.0001$); [Anova-one way, F (2, 15) =26.04, $p<0.0001$].

In Infralimbic prefrontal nuclei there increased level of acetyl H4K5 was detected following condition in comparison to naïve control ($p<0.05$). There were significantly no change found in acetyl H4K5 positive nuclei in extinction group when compared with naïve control group ($p>0.05$). The difference between condition & extinction group was also observed with higher no. of H4K5 positive nuclei following conditioning as compared to extinction group ($p<0.01$), [Anova-one way, F (2, 15) =10.21, $p<0.0016$]; (fig.- 5.20B).

5.3.3. CBP (CREB Binding Protein) expression

CBP expression in Amygdala

Expression of CBP, which is a HAT (histone acetyl transferase), was investigated to correlate changes in acetylation levels at histone H3 & H4 with activity of CBP during neuronal processing of fear conditioning and fear extinction. IHC images were presented in fig. 5.21A & B. There in LA, CBP positive nuclei were increased following both conditioning ($p<0.01$) and extinction ($p<0.01$) as compared to naïve and the changes were significant while no difference was detected in between condition and extinction group ($p>0.05$), [Anova-one way, F (2, 15) =11.68, $p<0.001$]. The no. of CBP positive nuclei increased in BA following extinction significantly as compared to naïve ($p<0.01$) control and condition group ($p<0.05$). Condition group was not observed to have any difference when compared with extinction group (ns, $p>0.05$) in BA, [Anova-one way, F (2, 15) =8.657, $p<0.01$]. There in CeL, CBP expression was found to be lower during conditioning as compared to naïve control ($p<0.0001$). The no. of CBP positive nuclei were not found different in extinction group as compared to naïve control ($p<0.05$), but very significantly higher no. of CBP positive nuclei were found in condition group as compared to extinction group ($p<0.005$) in CeL; [Anova-one way, F (2, 15) =18.44, $p<0.0001$]. In CeM there were higher no. of CBP positive nuclei observed following conditioning as compared to naïve control ($p<0.01$). No change was detected following extinction in CeM as compared to naïve control (ns, $p>0.05$). The condition group was also observed to have more no. of CBP positive nuclei when compared with extinction group ($p<0.01$), [Anova-one way, F (2, 15) = 8.206, $p<0.0039$]; (fig.5.21B).

CBP expression in hippocampus

Expression of CBP was found different in hippocampus in comparison to amygdala (fig.5.22A & B). There in CA1 region, an increased level of CBP was found following conditioning when compared to naïve control ($p = 0.0071$). There was no difference observed in CBP positive nuclei following extinction as compared to naïve control ($p> 0.9999$). The difference between condition and extinction group for CBP level was significant with more CBP positive nuclei following conditioning

as compared to extinction group was observed (p<0.001) in CA1; (ANOVA one way, F (2, 15) = 11.11, p = 0.0015). In CA3 region of the hippocampus, there was significant increase in the CBP level in conditioning group as compared to naïve control (p = 0.0022) and extinction group (p = 0.0001). The difference between extinction group and naïve control was not significant (p = 0.4220) in CA3 region. [ANOVA one way, F (2, 15) = 17.85, p<0.0010)]. In DG sub-region, there was difference between extinction and naïve control was observed with lower number of CBP positive nuclei in extinction group as compared to naïve control (p = 0.0024) and also as compared to condition group (p<0.001), but there was no difference found between condition and naïve control group (p=> 0.9999). [ANOVA one way, F (2, 15) = 12.34, p = 0.0015]; (fig.5.22B).

CBP expression in PFC

In PFC, different regions showed different CBP expression level as shown in IHC images (fig. 5.23A & B). In PL sub-region, highly significant increase in CBP positive nuclei was found following conditioning as compared to naïve control (p<0.001). The increase in extinction group was not found significant as compared to naïve control group (p>0.05). The condition group was observed with more significant increase in CBP level as compared to extinction group (p<0.001) in PL region. In IL sub-region there was an increase in CBP level was found in condition group (p<0.001) but it was not found significant following extinction as compared to naïve control (p>0.05). The no. of CBP positive nuclei were observed higher following conditioning (p<0.001) as compared to extinction group (p<0.001), [Anova-one way, F (2, 15) =25.43, p<0.0001]; (fig. 5.23B).

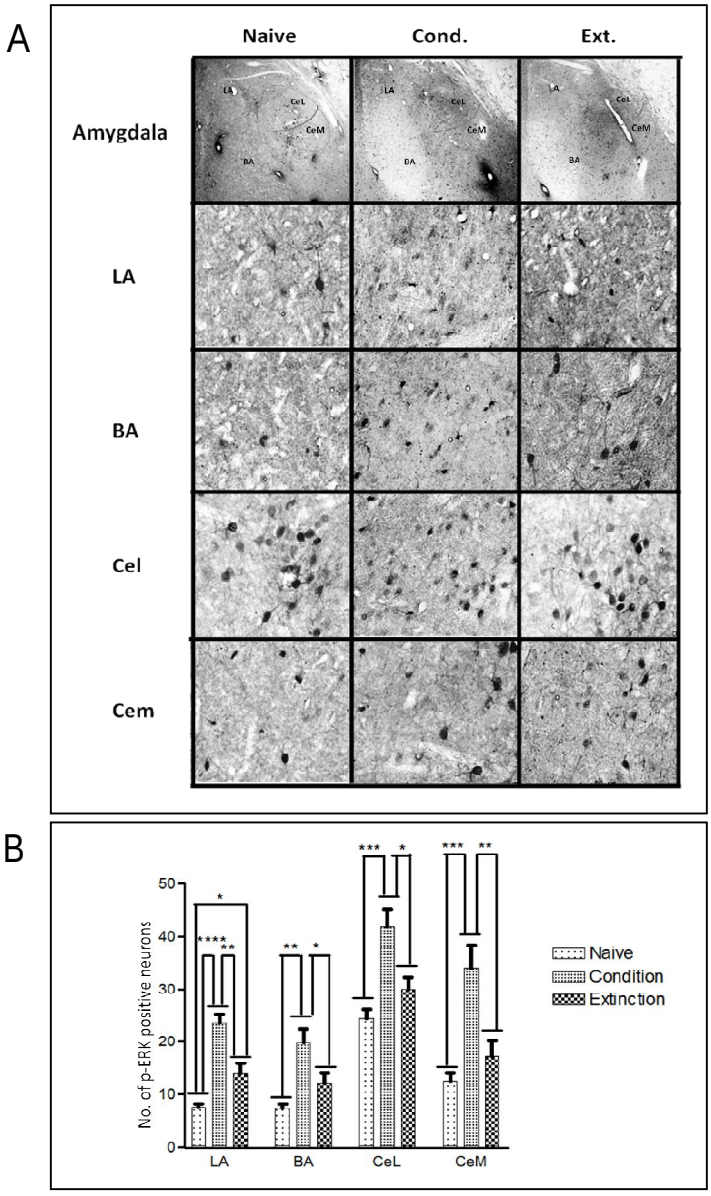

Figure 5.6: (A) Representative images of immunostaining for p-ERK in Amygdala. The images were acquired at 4x (first row) and at 20x (rest of the rows). (B) More activity of ERK was seen in amygdala regions following conditioning but not during extinction except in LA, as compared to naïve control. LA, [F (2, 15) = 25.90, (p<0.001)], in BA [F (2, 15) = 10.61, (p= 0.0013)], in CeL [F (2, 15) = 11.92, (p= 0.0008)] and CeM [F (2, 15) = 12.57, (p= 0.0006)]. All the results are presented as mean +/- s.e.m. (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001); LA, lateral amygdala, BA, basal amygdala, Cel, centre-lateral amygdala, Cem, centre-medial amygdala.

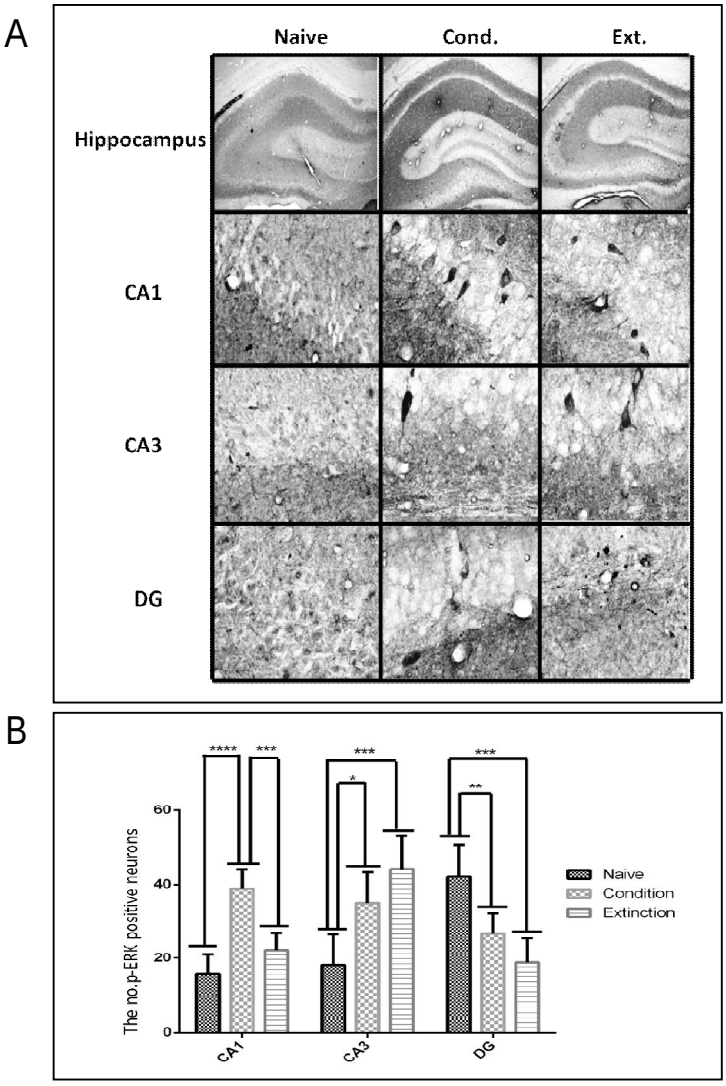

Figure 5.7: (A) Representative images of immunostaining for p-ERK in Hippocampus. (B) The number of p-ERK positive neurons is changed differentially in CA1, [F (2, 15) = 34.35), (p=0.0001], CA3, [F (2, 15) = 14.26), (p=0.0117] & DG sub-regions [F (2, 15) = 16.99), p=0.0001] across the groups. The results are presented as mean +/- s.e.m. CA, cornu ammonis, DG, dentate gyrus.

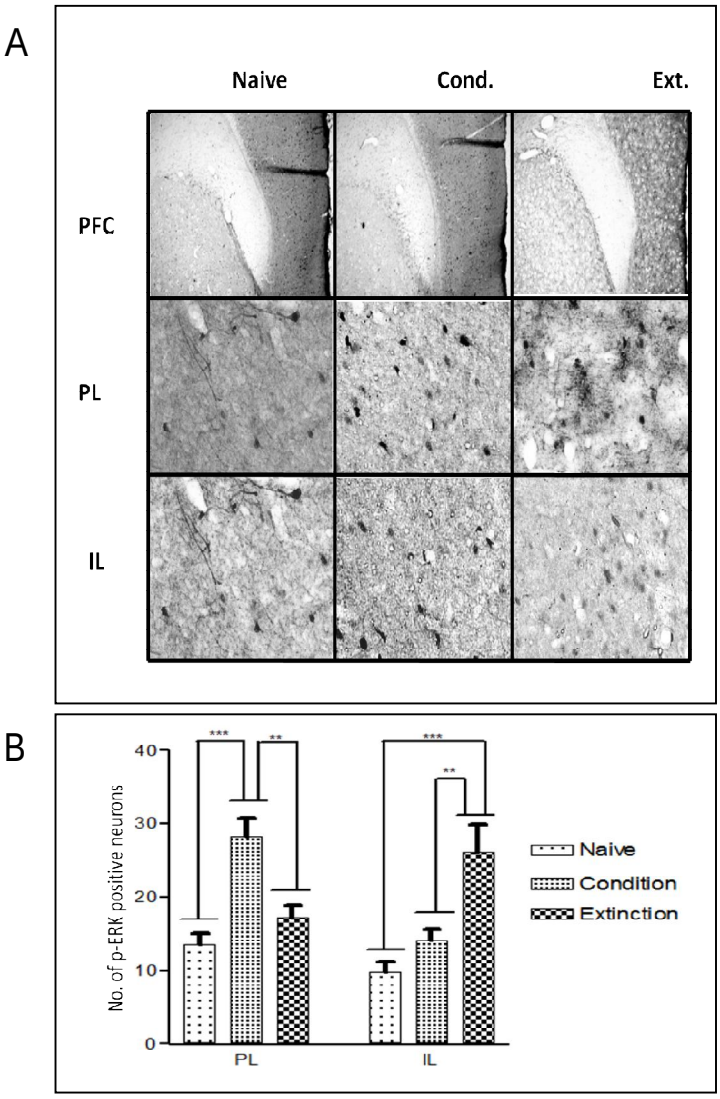

Figure 5.8: (A) Representative images of immunostaining for p-ERK in PFC. (B) The PL region was observed for increased no. of p-ERK positive neurons during conditioning as compared to naïve and extinction group ($P = 0.0003$, $F(2, 15) = 14.76$). Whereas in IL there were more of p-ERK positive neurons found during extinction as compared to naïve and conditioning group ($P = 0.0007$, $F(2, 15) = 12.10$). The results are presented as mean +/- s.e.m . PL, pre-limbic PFC, IL, infralimbic PFC.

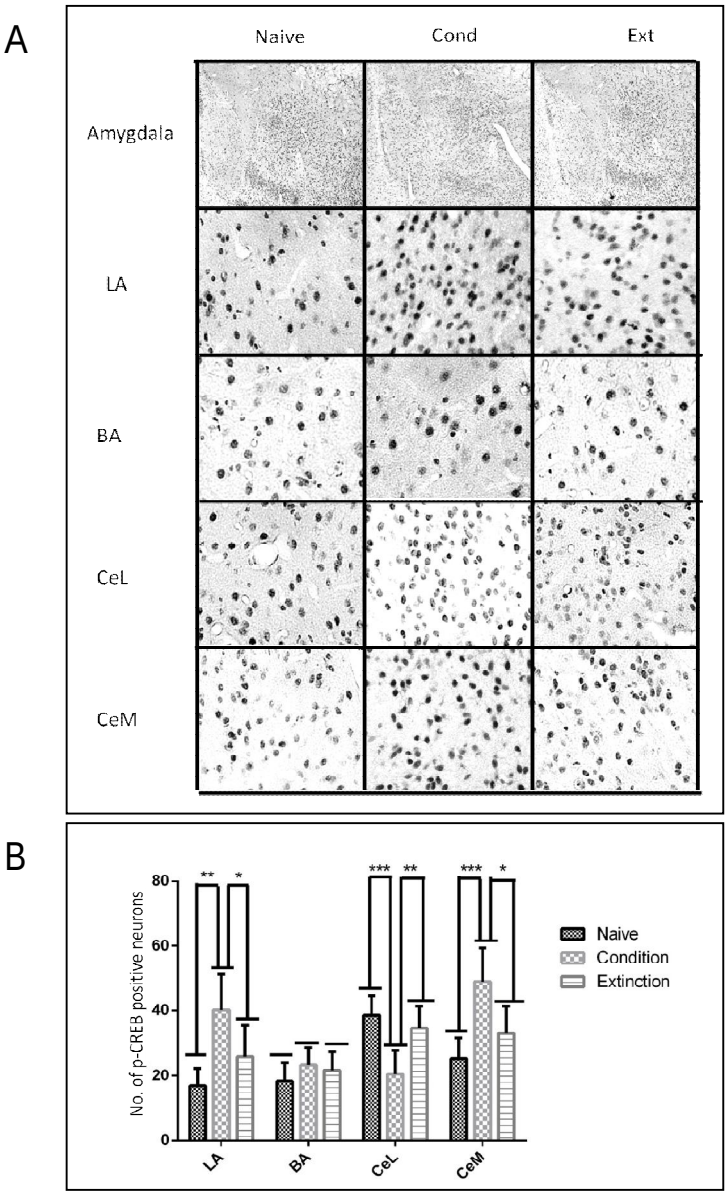

Figure 5.9: (A) Representative images of immunostaining for p-CREB in Amygdala. (B) More p-CREB positive neurons were observed in LA (P = 0.0018, F (2, 15) = 9.888), CeL (P = 0.0014, F (2, 15) = 11.24), CeM (P = 0.0006, F (2, 15) = 12.52) of amygdala following fear conditioning to suggest correlation of gene activity in these regions with that of consolidation of fear memory; whereas BA seems to be non-participating in acquisition or learning of conditioned fear. The results are presented as mean +/- s.e.m.

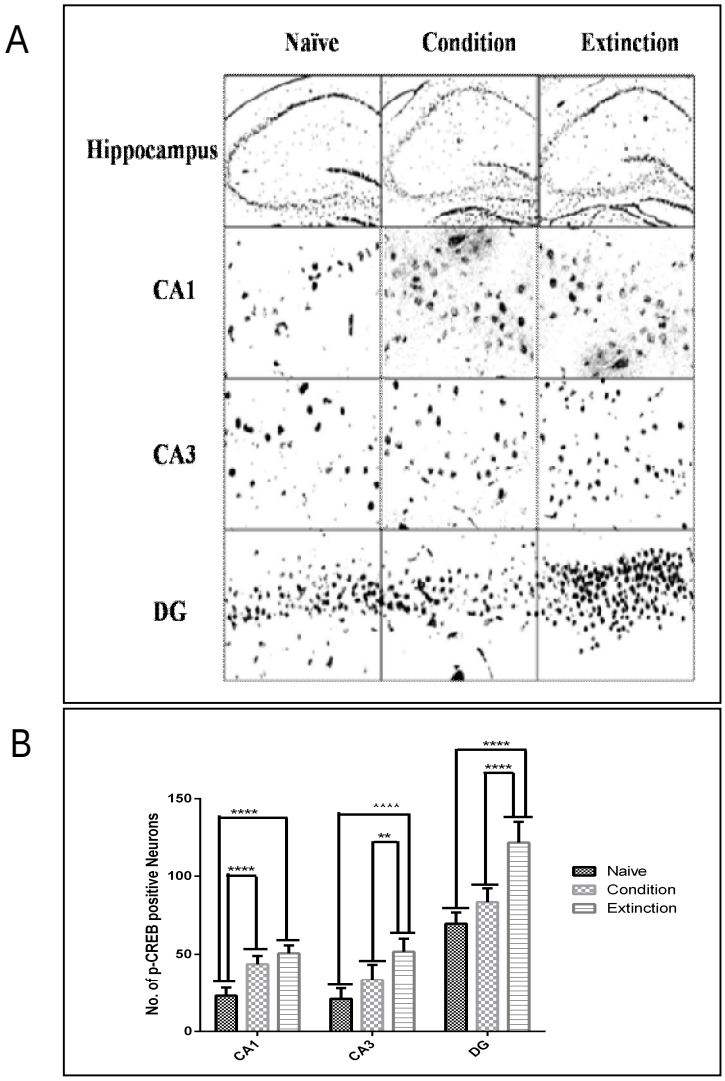

Figure 5.10: (A) Representative images of immunostaining for p-CREB in Hippocampus. (B) Significant increase in number of p-CREB positive neurons was noticed in CA3 ($F(2, 15) = 20.10$, $p < 0.0001$) and DG ($F(2, 15) = 42.21$, $p < 0.0001$) sub-regions in hippocampus following extinction as compared to naïve control and condition group, whereas non significant differences were seen among naïve control and condition group. In CA1 the number of p-CREB positive neurons was high $F(2, 15) = 46.02$, $p < 0.0001$) following both conditioning and extinction as compared to naive.

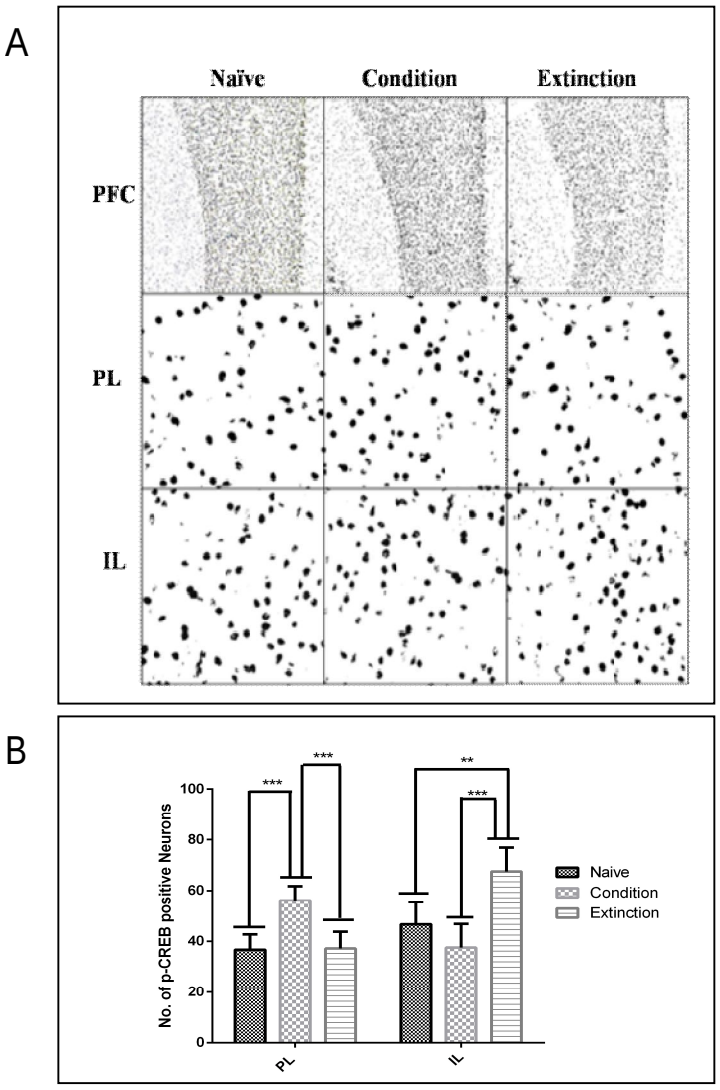

Figure 5.11: (A) Representative images of immunostaining for p-CREB in PFC. (B) The PL region was observed for increased no. of p-CREB positive neurons during conditioning as compared to naïve and extinction group ($F(2, 15) = 19.97$, $p=0.0002$). Whereas in IL, more of p-CREB positive neurons were found following extinction as compared to naïve and conditioning group [$F(2, 15) = 16.90$, $p=0.0001$]. The results are presented as mean +/- s.e.m .

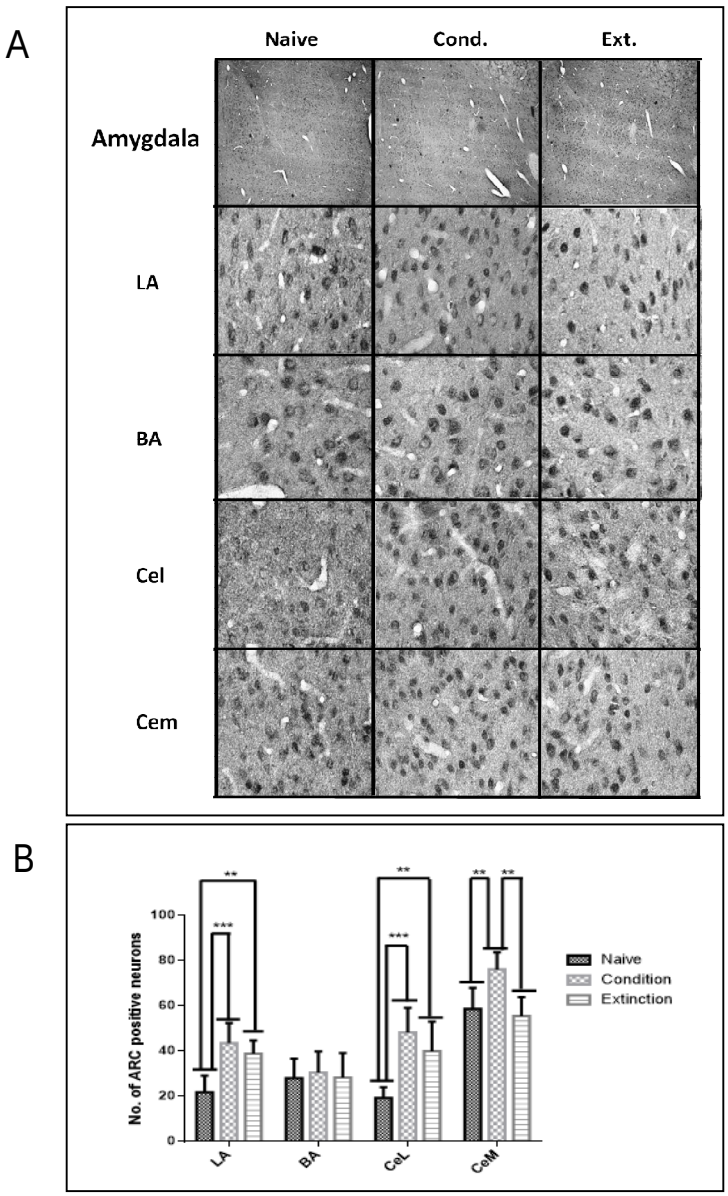

Figure 5.12: (A) Representative images of immunostaining for ARC in amygdala. (B) There were significant changes noticed in number of ARC positive neurons following both conditioning and extinction when compared to naïve in major amygdala regions except for BA. The increase in ARC positive neurons was more significant following conditioning in LA [F (2, 15) = 14.11, p=0.0004] and CeL [F (2, 15) = 13.01), p=0.0091]; and in CeM [F (2, 15) = 10.42, p=0.0021] as compared to naïve. The extinction group showed significant increase in LA and CeL region but not in CeM region. The results are presented as mean +/- s.e.m.

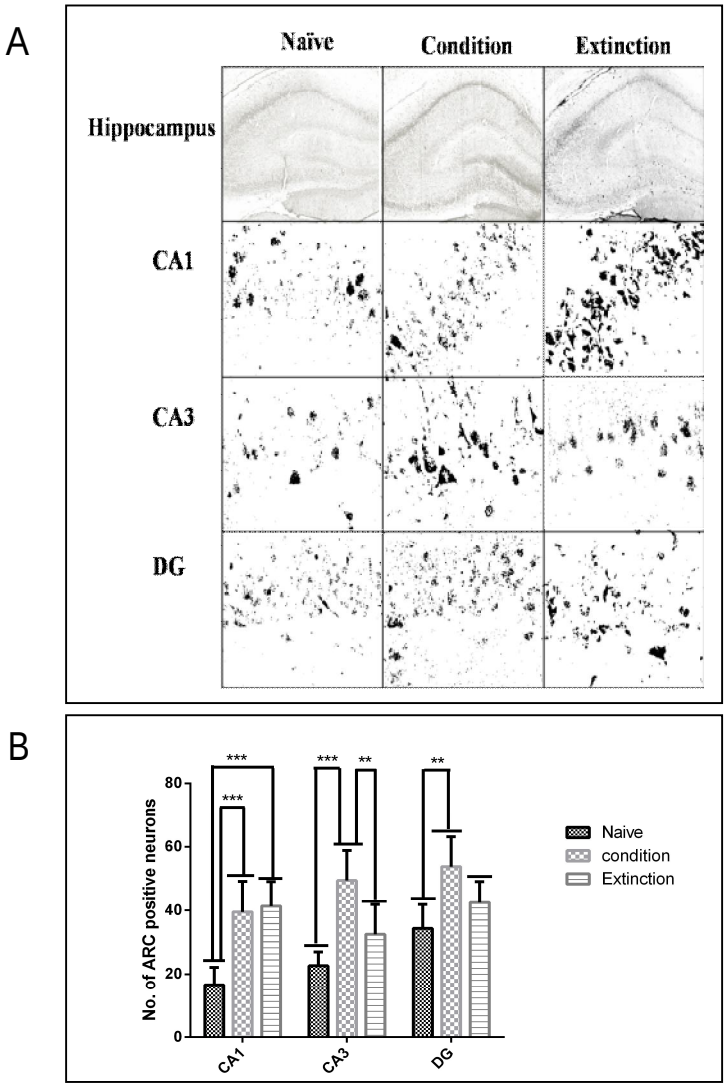

Figure 5.13: (A) Representative images of immunostaining for ARC in Hippocampus. (B) The CA1 region showed substantial difference in the form of increased no. of ARC positive neurons following conditioning (p=0.0005) and extinction (p=0.0002) both as compared to naïve control [F (2, 15) = 18.31]. The CA3 region exhibited more ARC positive neurons in condition group as compared to naïve control (p=0.0001) as well as to extinction group (p=0.0068), [F (2, 15) = 17.16]. Whereas no. of ARC positive neurons were observed for a significant increase in DG following conditioning [F (2, 15) = 9.205, p= 0.0020] but not extinction as compared to naïve. The results are presented as mean +/- s.e.m .

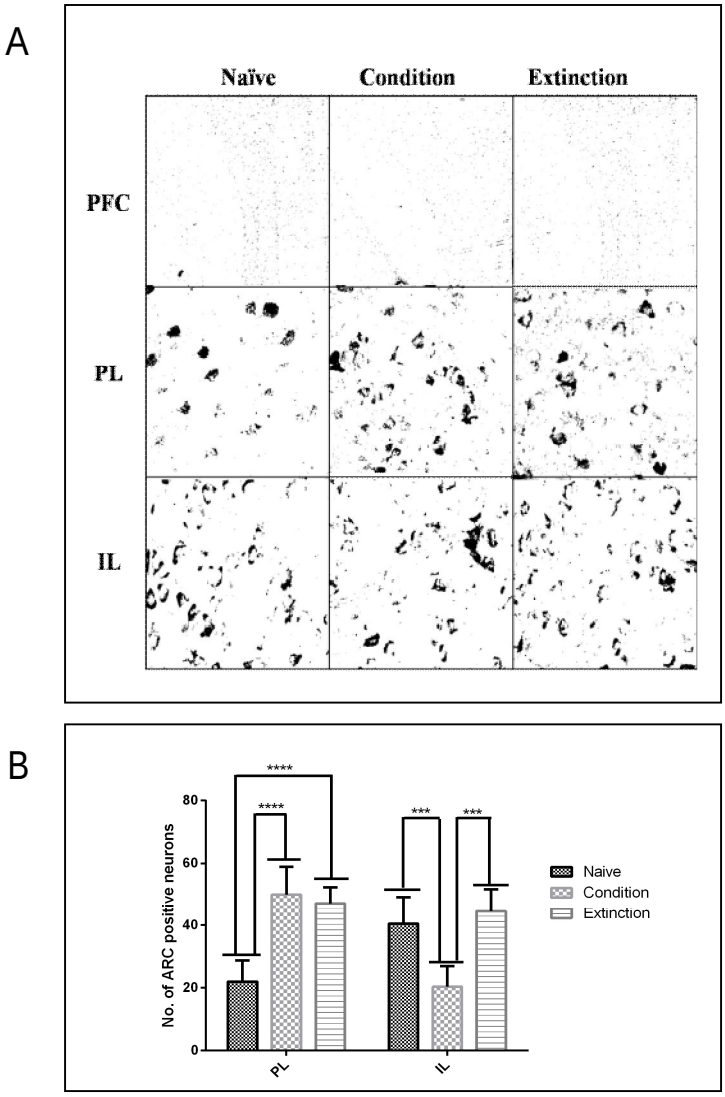

Figure 5.14: (A) Representative images of immunostaining for ARC in PFC, (B) both the experimental groups, condition and extinction, showed a significant increase in ARC positive neurons as compared to naïve in PL region of PFC [F (2, 15) = 28.25, p=<0.0001]. A different pattern in IL was observed with no difference between naïve and extinction group while less of ARC positive neurons were found following conditioning as compared to both naïve and extinction group [F (2, 15) = 17.63, p=0.0010]. The results are presented as mean +/- s.e.m .

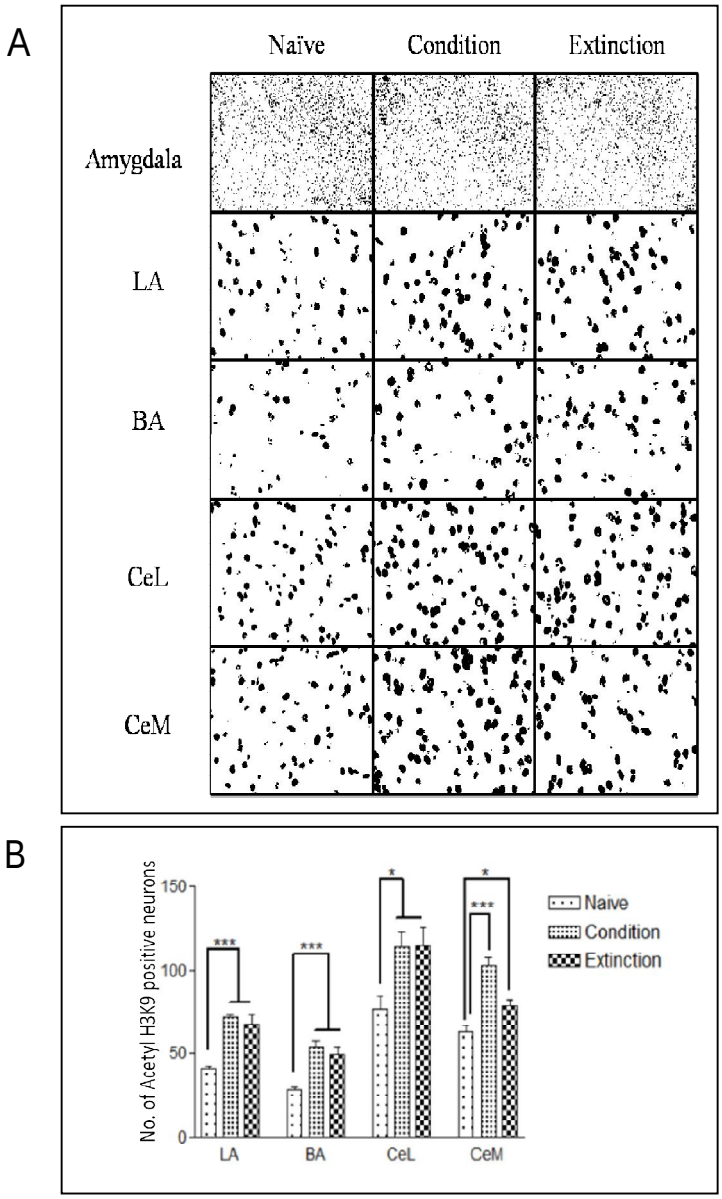

Figure 5.15: (A) Representative images of immunostaining for acetyl H3K9 in amygdala. (B) A similar pattern of increase in H3K9 positive neurons was found in major amygdala regions [LA: $F(2, 27) = 31.71$, $p<0.0001$; BA: $F(2, 33) = 44.3$, $p<0.0001$; CeL: $F(2, 33) = 15.04$, $p<0.0001$ and CeM: $F(2, 33) = 58.44$, $p<0.0001$] in both the experimental groups as compared to naïve, while no difference was noticed among the condition and extinction group except for CeM. In CeM, More H3K9 positive neurons were observed on condition group as compared to naïve control and extinction group both. The results are presented as mean +/- s.e.m.

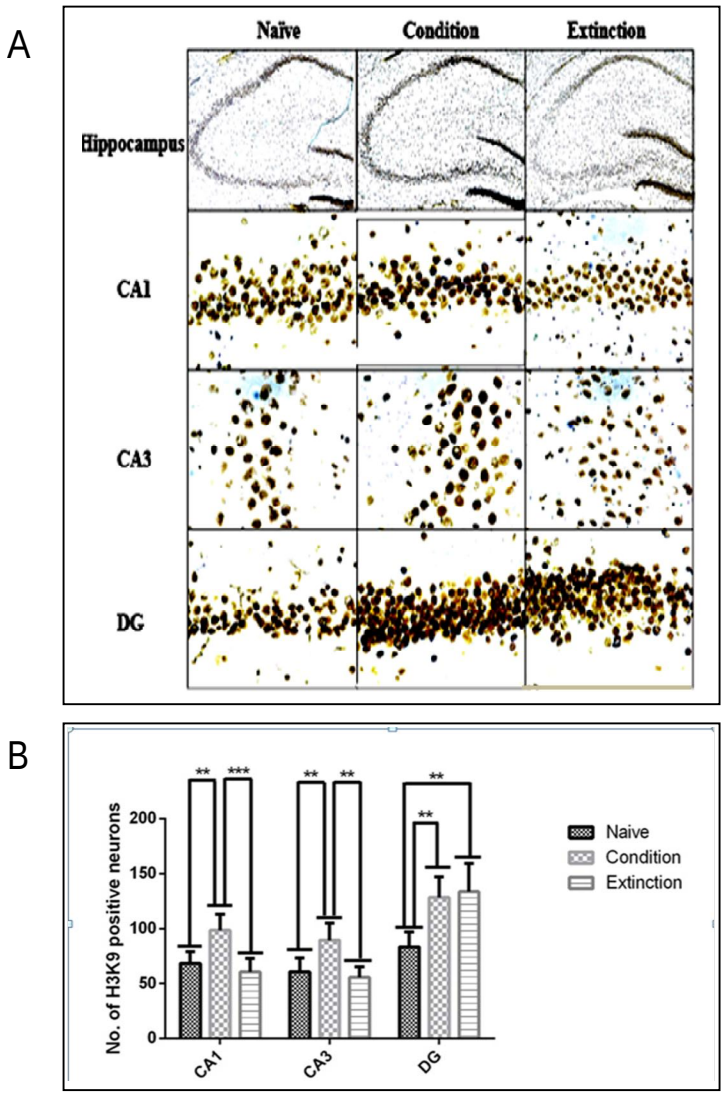

Figure 5.16: (A) Representative images of immunostaining for acetyl H3K9 in hippocampus. (B) The results showed that no. of acetyl H3K9 positive neurons were increased following conditioning with similar trend in CA1 [F (2, 15) = 14.57, p=0.0004] and CA3 [F (2, 15) = 12.25, p=0.0010] regions as compared to both naïve and extinction group. Whereas in DG the no. of H3K9 positive neurons were found to have significant increase in both condition and extinction groups as compared to naïve control but no change among the two groups, [F (2, 15) = 11.24, p=0.0019]. The results are presented as mean +/- s.e.m .

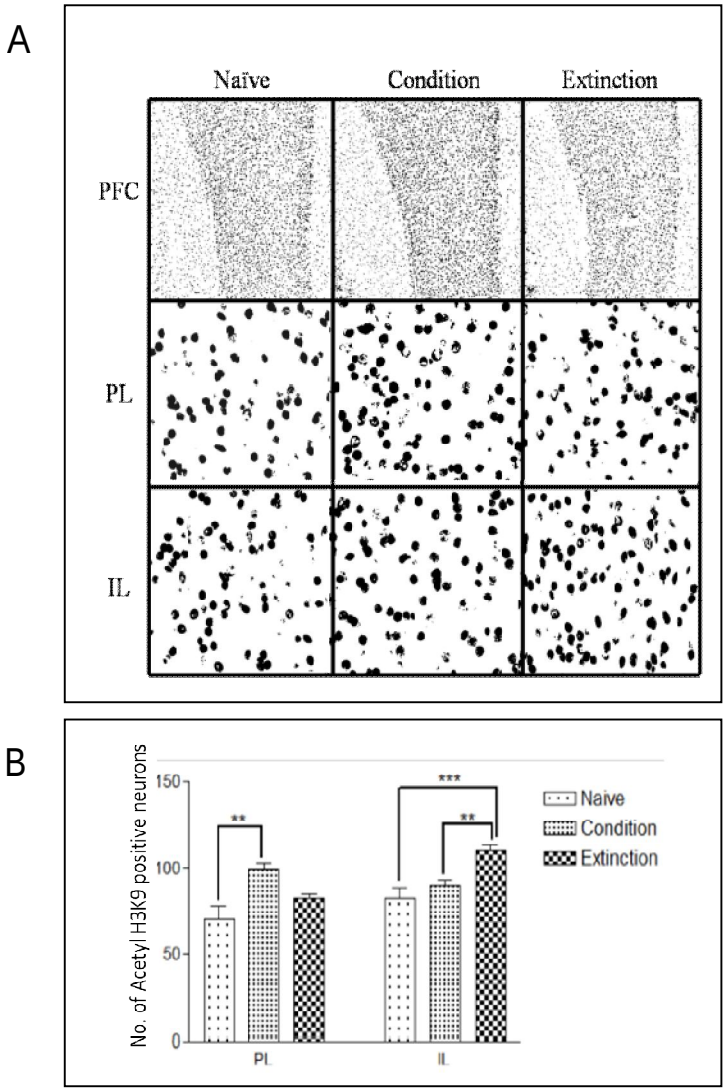

Figure 5.17: (A) Representative images of immunostaining for acetyl H3K9 PFC; (B) There more no. of acetyl H3K9 positive neurons were found in PL in condition group as compared to naïve with no significant differences between condition and extinction or extinction and naïve [F (2, 33) =21.35, p<0.001]. The increase in no. of acetyl H3K9 positive neurons followed different pattern in IL region as significantly more acetyl H3K9 positive neurons were observed following extinction in comparison to both condition and naïve control groups while no change was there in condition group as compared to naïve [F (2, 33) =31.15, p<0.0001]. The results are presented as mean +/- s.e.m.

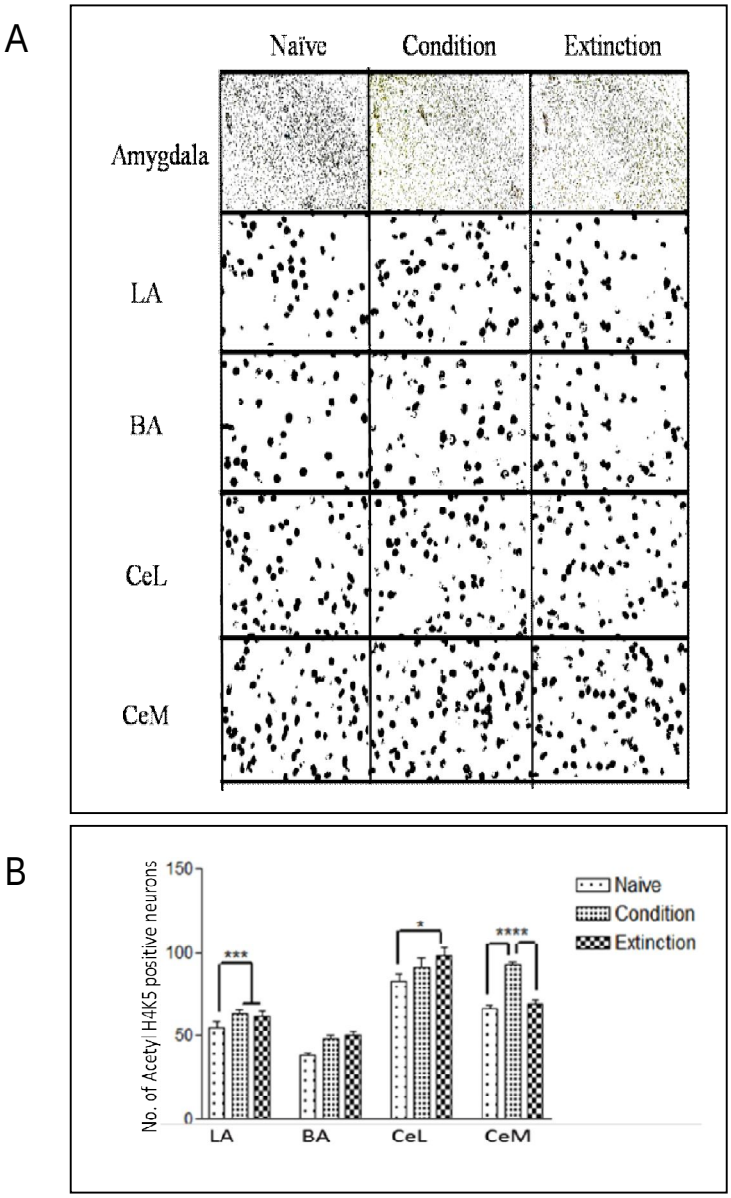

Figure 5.18: (A) Representative images of immunostaining for acetyl H4K5 in amygdala. (B) Fear conditioning was found to make an increased level of acetylation at H4K5 in all regions but more significant increase was observed in CeM as compared to naïve and extinction group [F (2, 48) = 41.9, *p*=0.0001]. In LA the increase was noticed in acetyl H4K5 positive neurons following conditioning and extinction as compared to naïve control (p>0.001). A similar increased was noticed in BA too. There were more acetyl H4K5 positive neurons found following extinction in CeL in comparison to condition and with more significant change when compared to naïve control [F (2, 48) =3.653, *p*=0.0334]. The results are presented as mean +/- s.e.m .

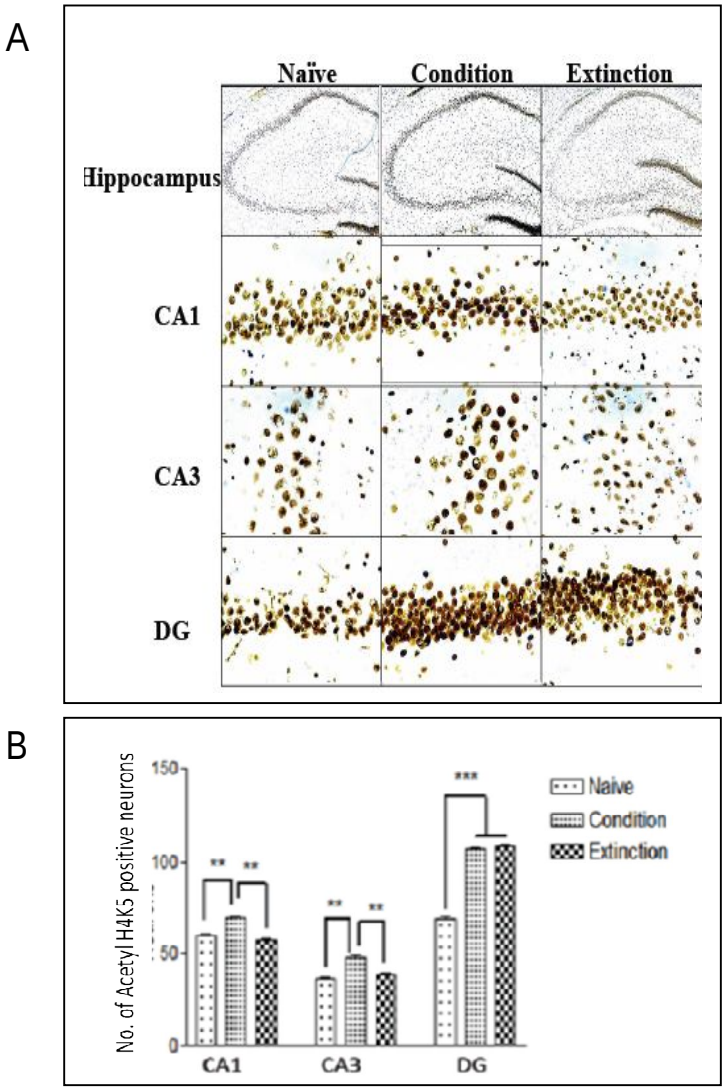

Figure 5.19: (A) Representative images of immunostaining for acetyl H4K5 in hippocampus. (B) Significant increase in acetyl H4K5 positive neurons was found following conditioning in CA1 [Anova-one way, F (2, 15) =9.975, p=0.0018] and CA3 [Anova-one way, F (2, 15) =11.01, p=0.0011] as compared to naïve control and the difference was also significant between condition and extinction group in these regions. The changes were similar in DG region in condition and extinction group.

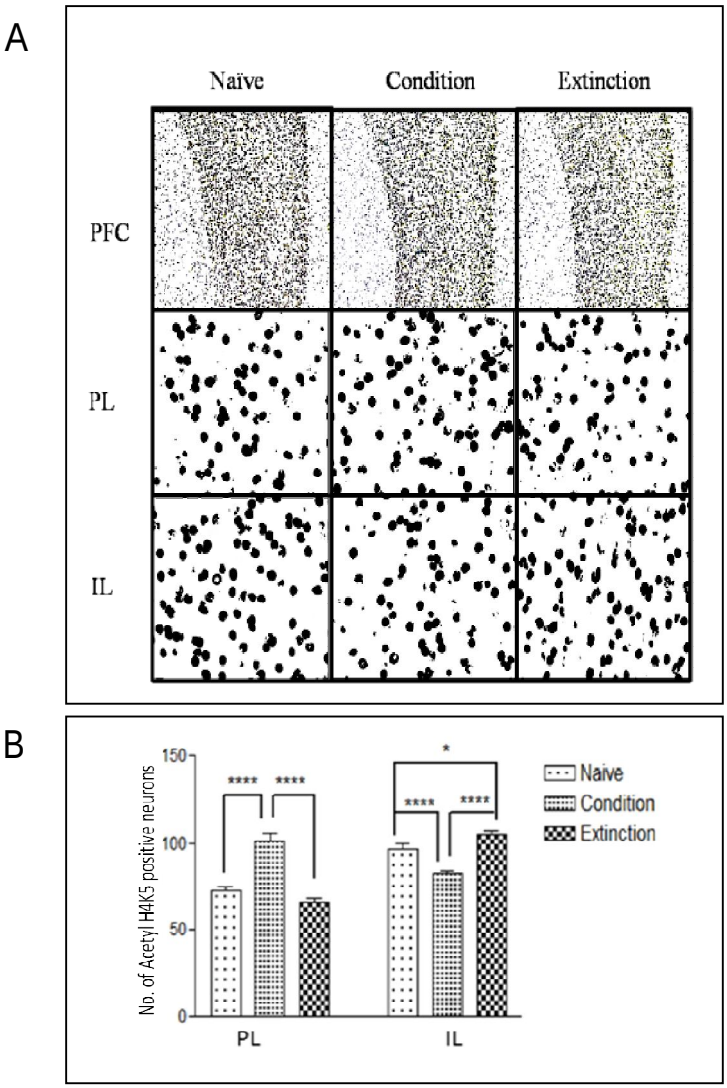

Figure 5.20: (A) Representative images of immunostaining for acetyl H4K5 in PFC. (B) Differential level of acetyl H4K5 positive neurons were found in PL & IL of PFC during conditioning and extinction. Condition group showed significantly higher level of acetylation at H4K5 in PL region as compared to extinction and naïve control [F (2, 33) =29.33, p<0.0001] while it was found significantly lower in IL region in comparison to other groups. There was also higher no. of acetyl H4K5 positive neurons following extinction as compared to naïve control, [F (2, 33) =31.34, *p*<0.0001]. The results are presented as mean +/- s.e.m .

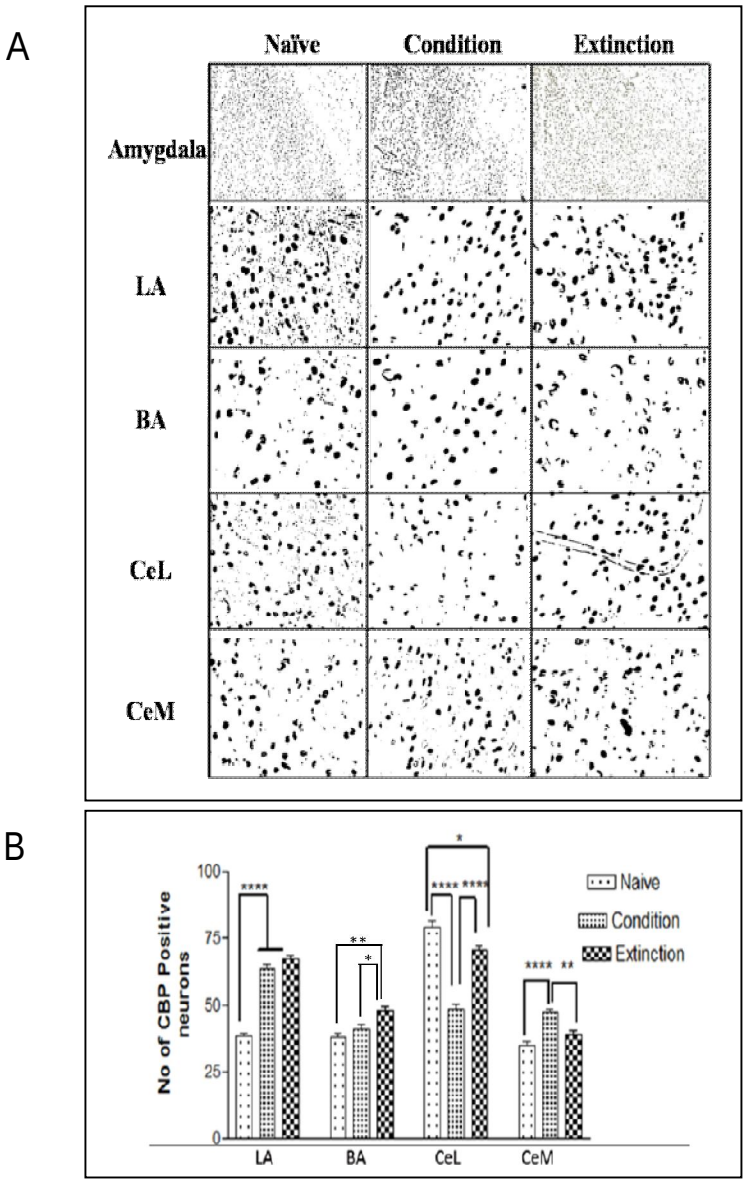

Figure 5.21: (A) Representative images of immunostaining for CBP in amygdala. (B) Differential pattern of CBP level was found in amygdala following conditioning and extinction. Highly significant change in no. of CBP positive neurons were seen in LA for both experimental groups as compared to naïve [F (2, 33) =100.7, p<0.0001], while in BA extinction group was observed to have significant changes as compared to condition and Naïve group [F (2, 33) =7.648, p<0.01]. In CeL region the changes were significant among all the group compared to each one but it was decrease in CBP positive neurons as compared to Naïve control [F (2, 33) =59.65, p<0.0001]. In CeM, there was an increased in no. of CBP positive neurons following conditioning [F (2, 30) =17.08, p<0.0001] as compared to extinction and naïve. The results are presented as mean +/- s.e.m .

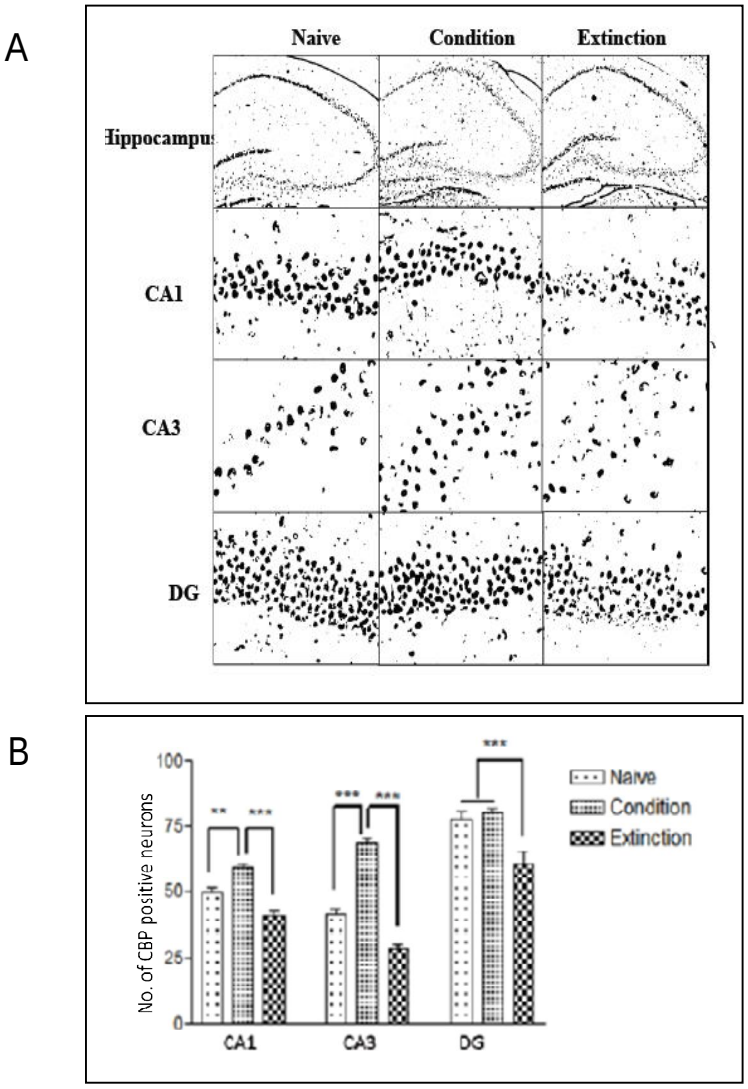

Figure 5.22: (A) Representative images of immunostaining for CBP in hippocampus. (B) A significantly higher no. of CBP positive neurons were found in CA1 region following conditioning in comparison to naïve and extinction [F (2, 15) = 11.11, p=0.0015]. Similar pattern was observed in CA3 but changes were more significant in condition group as compared to naïve and extinction group [F (2, 15) = 17.85, p=0.0001]. Whereas in DG no change was observed between condition and naïve control but significantly lesser no. of CBP positive neurons were found in DG following extinction when compared to both condition and naïve control group [F (2, 15) = 12.34, p=0.0015]. The results are presented as mean +/- s.e.m .

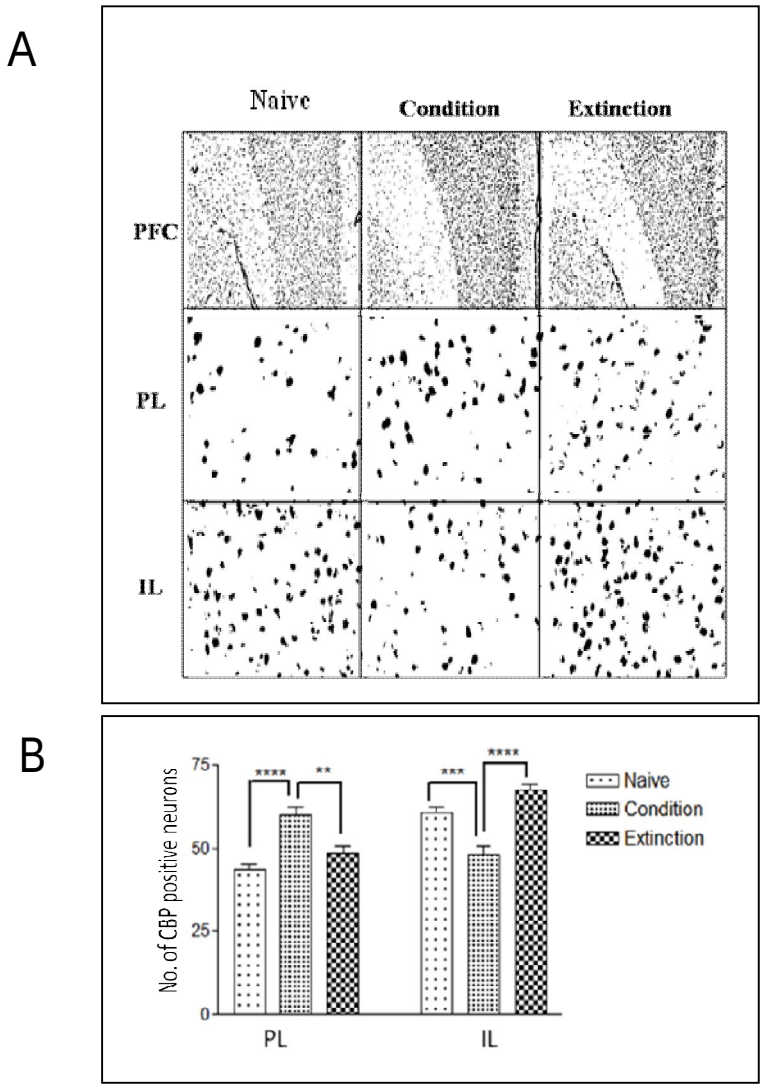

Figure 5.23: (A) Representative images of immunostaining for CBP in PFC. (B) Significant increase was noticed in no. of CBP positive neurons following conditioning in PL region of PFC as compared to extinction and naïve groups, while no difference between naïve and extinction group was observed [F (2, 33) =14.57, p<0.0001] in PL. In IL region too there was no change between naïve and extinction groups. No. of CBP positive neurons was observed to reduced in IL as compared to naïve control as well as extinction group with significant value [F (2, 33) =22.29, p<0.0001]. The results are presented as mean +/- s.e.m

CHAPTER 6

DISCUSSION

Discussion

The present study was an effort to understand the neurobiology of fear in a comprehensive way with emphasis on the molecular machinery operative during a particular phase of learning in the brain parts involved in fear learning and extinction. The experience-dependent plasticity of neuronal circuits during learning was studied in a rat model of classical auditory fear conditioning, which is a robust behavioral paradigm that enables a direct link between plasticity of neuronal circuits and behavioral changes. The study of this learning process is mostly known as classical conditioning, a process in which an organism's innate response to a stimulus becomes conditioned as expressed in response to a previously neutral stimulus (Rescorla, 1988).

For fear conditioning in rats a conditioned stimulus (CS), such as a tone, is paired with an aversive unconditioned stimulus (US), such as electric shock. After several such pairings, the tone elicits autonomic and behavioral fear responses such as freezing (Fanselow, 1980). The association between the tone and shock is robust and long lasting, and it has been suggested that conditioned fear associations are indelible (LeDoux *et al.,* 1988). The fear-conditioning paradigm used in this study produced significant freezing in the rats and it is in line with earlier studies. It was found that the tone shock associations during the fear-acquisition/ learning resulted in significant increase in the freezing with each subsequent trail. The last freezing or the fifth trail produced robust freezing and the rats exhibited considerable freezing on the following day during retention test, a marker for successful fear consolidation.

Our next step was to produce successful fear extinction in our conditioned rats. Fear extinction, which is an adaptive form of inhibitory learning in response to altered environmental contingencies (Herry *et al*., 2006). Repeated presentation of the CS in the absence of the US causes the conditioned responses to extinguish. A single session of un-reinforced tones is sufficient to extinguish conditioned freezing levels from 70% to near 0% (Quirk *et al*., 2002; Herry, 2006). The extinction training in the present study was performed by presenting the CS or the tone in absence of shock in a novel context. This resulted in significant decrease in the % freezing in each consequent session and resulting in negligible freezing levels in the final trail. During

the retention test the rats undergone extinction showed negligible conditioned response to the tone. This is an indication of successful fear extinction and it is in accordance with previous studies, which have demonstrated similar learning outcomes following extinction learning (Ayres et al., 1971; Frey et al., 1977; Rescorla 1967).

After having successful fear consolidation and extinction in rats our next aim was to look at the changes in the phosphorylation of CREB, ERK and expression of immediate early gene ARC in BA, LA, CeL and CeM of Amygdala, IL and PL of PFC and CA1, CA3 and DG of Hippocampus following fear conditioning and its extinction. It was found that memory consolidation following fear conditioning and its extinction lead to significant changes in the expression pattern of ARC in the IL and PL of the PFC and BA, LA and CeA of the amygdala. The conditioning, as reported in several previous studies, in general result from neuronal activation in the LA and BA of Amygdala converging on the CeA, the main output hub of fear. Our results show an increase in the phosphorylation of CREB and ERK in the LA, BA and CeM following conditioning and in the BA, LA, CeL and IL-PFC following extinction. Recent studies have shown that fear consolidation leads to activation of neurons of in the BA, LA and CeM of Amygdala and PL of PFC. It has been shown that the neuronal activation in these sub-regions is necessary for fear learning and consolidation (Akirav et al., 2006; Muller et al., 1997; Lalumiere & McGaugh, 2005;Repaet al., 2001). Likewise CeL and IL-PFC have been reported to be actively involved in extinction learning.

Learning involves changes in the synaptic strength between neurons in the brain's circuitry, which can either be strengthened or weakened over time depending on their activity (Bliss & Collingridge, 1993). This is termed as synaptic plasticity. It is well known that molecular events leading to synaptic plasticity during fear consolidation, extinction learning and retrieval require activation of distinct signaling pathways along with the expression of IEGs (Nader *et al.,* 2000; Hall *et al.,* 2001). The activity-regulated cytoskeletal associated protein (Arc/Arg3.1) is an effector immediate-early gene (IEG) that has been widely implicated in experience-dependent synaptic plasticity and memory formation (Lyford *et al.,* 1995; Steward *et al.,* 1998; Guzowski *et al.,* 2000; Plath *et al.,* 2006; Ploski *et al.,* 2008). The transcriptional characteristics and rapid subcellular trafficking of Arc/Arg3.1 have made it an

attractive tool to map regions of the brain that are involved in memory retrieval (Guzowski *et al.,* 2001). Arc/Arg3.1 has been shown to be regulated within the hippocampus after re-exposure to a familiar environment or retrieval of a spatial memory (Guzowski *et al.,* 1999; Gusev *et al.,* 2005), within the hippocampus and amygdala after retrieval of a contextual fear memory (Zhang *et al.,* 2005; Mamiya *et al.,* 2009), and within the amygdala after retrieval of an appetitive memory (Petrovich *et al.,* 2005).

Similarly, Extracellular signal-regulated kinase (ERK) is well known for its role in cell growth and differentiation (Kornhauser& Greenberg, 1997; Impey *et al.,* 1999; Oruban *et al.,* 1999). Its activation in neurons is activity dependent and also found to be activated in hippocampus following LTP (Impey *et al.,* 1999). Inhibition of ERK/p-ERK led to impaired memory consolidation (Brambilla *et al.,* 1997; Atkins *et al.,* 1998; Selcher *et al.,* 1999). The activation of ERK/MAPK signaling pathway is important in consolidation of both auditory as well as contextual fear consolidation and its activation in time dependent manner in LA has been observed for associative (paired CS-US presentation) learning only in post fear conditioning paradigm (Schafe *et al.,* 2000). Moreover extinction of auditory fear conditioning also involves activation of ERK/MAPK in BLA and its inhibition in BLA prevents formation of extinction memory (Herry *et al*., 2006). ERK also regulates transcription factor CREB that in turn activates the expression of many memory related genes (Sweatt *et al*., 2001). Inhibition of ERK/MAPK signaling pathway in mPFC disrupts consolidation of extinction learning (Hugues *et al*., 2004). In hippocampus, p-ERK increases in time phases after fear conditioning.

In the present study we observed the neuronal activity in the IL and PL of PFC and BA, LA, CeM and CeL of the amygdala through ARC labeling and phosphorylation of ERK and CREB. It was found that fear consolidation resulted in increased ARC labeling and phosphorylation of ERK and CREB in the PL-PFC along with CeM and CeL but not of IL as evident by the ARC expression in these region as compared to the naïve control. On the contrary, extinction of the fear resulted in activation of IL and CeL but not of CeM and PL as revealed by ARC expression and phosphorylation of ERK and CREB. No significant changes were observed in the BA and LA between the conditioned and extinction group. So it seems likely that the

memory consolidation, which results due to fear learning, requires activation of PL of mPFC and both CeL and CeM of central Amygdala. However, extinction learning requires activation of IL and both CeL and CeM but not of PL. These results are similar to the earlier studies where extinction learning appears to be mediated by a network of BA to other brain areas while consolidation recruits different brain areas. It has also been shown that the infralimbic division (IL) of the mPFC projects to inhibitory neurons in the amygdala involved in suppressing fear after extinction (Rosenkranz & Grace 2002; Pare *et al.* 2004; Likhtik *et al*. 2008), whereas the prelimbic division projects to excitatory projection neurons involved in fear expression (McDonald et al. 1996; Likhtik *et al*. 2005; Burgos-Robles *et al.* 2009).

Although most research has examined the processes of the amygdala, considerable evidence has been obtained which indicates that the hippocampus is crucial for the development of emotionally significant fear conditioning in relation to contextual information (Phelps, 2004; Bouton & Moody, 2004; Hobin *et al.,* 2003; Phillips, & LeDoux, 1992).The hippocampus has been the target of several studies investigating the contextual acquisition and extinction of fear conditioning but also the retrieval of the fearful memories. A recent report suggested hippocampus not only responsible for the formation of fear conditioning to a context, but also to a tone (Bast *et al.,* 2001). In the present study we used cued model of fear conditioning in which the retention test or the extinction learning was carried out in a different context than the learning context. This resulted in the nullification of the contextual fear. However the changes in the phosphorylation of CREB and ERK and ARC expression though not in a concerted way as seen in the other two regions under study, it may be possible that apart from contextual learning the hippocampus must be interacting with the cue dependent associative learning in some ways because of which the changes were seen.

After looking at the differential activation of the sub regions following conditioning and extinction as evident by the concerted changes in the phosphorylation of CREB and ERK, the last objective of this study was to find out any links between this differential activation and histone acetylation was took up. Lately epigenetic mechanisms especially histone acetylation has been shown to play a crucial role in consolidation of fear memory and its extinction (Alarcon *et al,* 2004; Park *et al,* 2013; Levenson *et al,* 2004). Histone acetylation has been implicated in

synaptic plasticity and memory consolidation during context dependent learning. A study from David Sweatt's group have shown robust regulation of histone H3 acetylation, but not H4, 60 min following contextual fear conditioning in area CA1 of the hippocampus (Levenson *et al.,* 2004; Lubin and Sweatt, 2007; Miller *et al.,* 2008). HDAC inhibition resulting in increased histone acetylation enhances memory consolidation in a variety of hippocampal-dependent learning paradigms, including object recognition and contextual fear conditioning (Levenson *et al.,* 2004; Stefanko *et al.,* 2009). CBP and p300 are large multidomain proteins that possess intrinsic histone acetyltransferase (HAT) activity. It has been shown that CBP is critical for the in vivo acetylation of lysines on histones H2B, H3, and H4. CBP's homolog p300 is unable to compensate for the loss of CBP function in knockouts. Neurons lacking CBP maintain phosphorylation of the transcription factor CREB, but fail to activate CREB: CBP-mediated gene expression. Loss of CBP in dorsal CA1 of the hippocampus results in selective impairments to long-term potentiation and long-term memory for contextual fear and object recognition. Several studies have shown that stimuli, which usually induce activity-dependent gene transcription, lead to an increase in histone acetylation at the *c-fos* promoter as well as other activity-regulated gene promoters (Tsankova *et_al.* 2004). It has been shown that extinction training leads to induction of IEG c-fos in the BA (Herry& Mons, 2004). This may be predicted as, that Histone acetylation also play a role in extinction. Recently it has been shown that neurons specific to BA are active during extinction training along with another set of neurons, which are active during fear conditioning. The switching of activity of these neurons is very necessary for behavioral outcomes either in form of more fear or less fear. Thus there must be some molecular relay, switching the neurons between these modules. We speculate that it may be the changes in the histone acetylation patterns in these neurons that might be acting as the relay. Also there are distinct extinction pathways connecting BA to mPFC (Herry, 2008) and these pathways may tip the balance between distinct neuronal circuits of PFC leading to states of low or high fear. So the activity in the mPFC especially in the IL and PL of the PFC may also be contributing to changes in the behavioral outcomes either in form of fear or less fear. In the current study we found that BA and LA along with the CeL and IL-PFC showed increase in the H3 and H4 acetylation following extinction

and the LA, BA, CeM and PL-PFC showed increased acetylation following conditioning.

Currently, evidence from a multitude of studies using pharmacological manipulations, electrical stimulation, or lesions suggests that diminishing the activity of CeM attenuates fear and anxiety responses, while increasing CeM output leads to stronger fear responses (Davis, 2000). In the present study it was found that the neurons in the CeM were active following fear learning but not following extinction as evident by CBP expression. The neurons in the CeL were more active as compared to the CeM following extinction. This differential activation was also highlighted by increase or decrease in acetylation of Histone H3 and H4 respectively and followed a similar pattern as ARC. This strongly supports the notion that modification of local, intra-CeA inhibitory gating may be intimately involved in controlling fear and anxiety behavior and fear learning depending on the behavioral state. Furthermore, it will be important to identify neuronal subpopulations, address plasticity mechanisms at the level of identified subtypes, and determine how local interactions control CeL activity and CeM output.

In hippocampus specifically in the CA1 region there was increase in the level of CBP along with the acetylation of H3 and H4. There was also increase in the phosphorylation of CREB and ERK and the immediate early gene (IEG) ARC following conditioning but not following extinction. However in the DG and the CA3 there was increase in the level of CBP, Acetyl H3 and H4 following fear consolidation and extinction both. It is now well known that both hippocampal areas CA1 and CA3 contribute to the acquisition of context-dependent extinction, but that only area CA1 is required for contextual memory retrieval. In the present study we did find changes in the CA1 following cued conditioning, which subdued on extinction training. However, other two regions i.e., DG and CA3 show increased activity in terms of histone acetylation and phosphorylation of CREB and ERK as well as elevated levels of ARC.

CONCLUSION

Conclusion

The results of this study clearly point towards the existence of an epigenetic machinery operative at sub region levels to be responsible for differential activation of neuronal subpopulations in the PFC, Amygdala and Hippocampus following fear conditioning and extinction.

A consistent pattern of molecular expression, distribution and epigenetic modifications were found in neuronal active areas of PFC, hippocampus and amygdala in post conditioning and post extinction paradigms.

The activation pattern of ERK, the upstream signaling molecule in neural processing of fear and also a neuronal activity marker, showed that all nuclei of amygdala active during fear conditioning while LA & CeL region were more active during fear extinction. In hippocampus, CA1 was active during fear consolidation and CA3 during fear extinction with no changes in DG region. PL-PFC seemed to participate in fear conditioning and IL-PFC was active in fear extinction.

So it was evident that different brain areas participated in two fear learning paradigms.

CREB, a downstream transcription factor from ERK pathway, was activated in learning induced active neurons. LA & CeM seemed to be transcriptionally active following fear conditioning. LA & CeL were found to have active transcription state in extinction process. CREB was active in all regions of hippocampus during extinction. Different pattern of ERK and CREB phosphorylation in hippocampus suggested that ERK pathway may not be responsible alone for CREB activation. CREB activation in PFC was found to be collinear with previous studies with more active transcriptional state in PL during fear conditioning and in IL during fear extinction.

More ARC expression throughout amygdala except for BA showed that amygdala actively modulated fear associated learnings via changes at synaptic connections. CA1 region seemed to involve synaptic modulation to facilitate extinction memories while other hippocampal regions were found active with cellular changes at their synaptic connections. Inhibitory interventions of IL-PFC for fear

expression suggested it as one of the possible mechanism underlying extinction memories.

Differential acetylation pattern of histone H3K9 & H4K5 was found across the regions under study in two learning paradigms. All nuclei showed enhanced acetylation at H3K9 except for IL-PFC following fear conditioning. This suggested that neuronal activity induced transcriptional activation involved epigenetic regulation of fear related genes' expression. In central nucleus of amygdala, CeA sub-nuclei had lower CBP level therefore strongly supported lower acetylation level of H3 and H4 during fear extinction; while higher CBP level in LA was collinear with enhanced acetylation of the histones during both of learning paradigms.

The three regions of the hippocampus DG, CA1 and CA3 under observation in this study showed modulations in levels of CBP and acetylation of H3 and H4. However no definitive pattern was observed expect for the CA1 region, which suggested other molecular interventions in the form of HATs or endogenous HDAC inhibitors were player in epigenetic regulation of fear processing and these findings suggest that further studies are required to pin more appropriate epigenetic modulators of fear memories.

The present study showed that expression and distribution pattern of some key molecules, which are known to play crucial role in fear memory associated learning, was different in post learning scenario (conditioning/extinction) in various brain regions making fear circuitry. Thease changes may be the actual link between the changes at terminal synapses and gene expression. It points towards a mechanism where molecules of memory formation pathway are recruited or de-recruited to change the interpretation of cellular event in the molecular language and the outcome being a behavioral outcome in form of fear or less fear.

The findings from this study will add to the existing knowledge in the field of fear biology and would help to develop a broader understanding of the phenomena of fear conditioning and extinction. Further research would be required to specifically trace out the changes taking place at the promoter of the genes implicated in fear conditioning and extinction, which will help in finding newer drug targets for better clinical treatments for phobias and anxiety disorders, such as posttraumatic stress disorders.

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PUBLICATIONS

Publications

1. **"Differential histone acetylation in amygdala leads to fear memory consolidation and extinction",** 2015, Vandana Ranjan, Sanjay Singh, Sarfraj Ahmad Siddiqui, M.Y. Khan, Anand Prakash, International Journal of Science Technology and Society, Vol. 1 (1); Jan-June 2015, 43-50.

2. **"Consolidation of fear memory and its' recall are cue dependent",** 2012**;** Vandana Ranjan, M.Y. Khan, Anand Prakash; International Research Journal of Management Science and Technology (IRJMST); vol 3(1); 661-666.

3. **"Differential Histone Acetylation in sub-regions of BNST underlies Fear Consolidation and Extinction"**, 2016, Vandana Ranjan, Sanjay Singh, Sarfraj Ahmad, Sukanya Tripathi, M.Y. Khan, Anand Prakash; Psychiatry Investigation, manuscript ID PI-2016-0092.R1 (accepted for publication)