# To study the role of different subsets of HDACs (Histone Deacetylases) in Fear memory Consolidation and Extinction

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## Certificate

This is to certify that the research work embodied in the thesis entitled **"To study the role of different subsets of HDACs (Histone Deacetylases) in Fear memory Consolidation and Extinction"** has been carried out by **Mr. Sarfraj Ahmad Siddiqui** under my supervision. He has fulfilled all the requirements of Babasaheb Bhimrao Ambedkar University, Lucknow, India 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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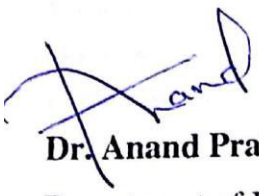

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Sarfraj Ahmad Siddiqui

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# Contents


| Content | Page no. |
|---|---|
| **Title Page** | i |
| **Certificate** | ii |
| **Acknowledgement** | iii |
| **Course work certificate** | iv |
| **Contents** | v-viii |
| **Abbreviations** | ix |
| **List of Figures** | x-xi |
| **List of Tables** | xii |
| **Chapter 1 – Introduction** | 1-4 |
| **Chapter 2 – Review of Literature** | 5-18 |
| 2.1. Fear memory | |
| 2.1.1. Fear consolidation | |
| 2.1.2. Fear extinction | |
| 2.2. Neuroanatomical regions involved in Fear Memory | |
| 2.2.1. Amygdala | |
| 2.2.2. Medial Prefrontal cortex (mPFC) | |
| 2.2.3. Hippocampus | |
| 2.3. The circuitry of fear | |
| 2.4. Molecular biology of fear | |
| 2.5. Epigenetics of fear | |
| 2.6. HDACs in memory consolidation and extinction | |
| 2.7. HDACs and HDAC inhibitors in fear memory | |
| 2.8. Rationale of Study | |
| **Chapter 3 – Aims and objectives** | 19-20 |
| **Chapter 4 – Material and methods** | 21-31 |
| 4.1. Animals | |
| 4.2. Apparatus for behavioral study | |
| 4.3. Fear conditioning | |
| 4.4. Fear extinction | |
| 4.5. Control groups | |
| 4.6. Scoring | |
| 4.7. Details of Brain Sub-Regions under Study | |
| 4.8. Valproic acid/ Drug administration | |
| 4.9. Anxiety measurement for valproic acid | |
| 4.10. Fear conditioning in valproic acid treated animals | |
| 4.11. Fear Extinction in valproic acid treated animals | |
| 4.12. Controls of the valproic acid treated conditioning and extinction group | |
| 4.13. Behavioral test | |
| 4.14. Tissue Preparation for immunohistochemistry | |
| 4.15. Tissue preparation for Real-time PCR | |
| 4.16. Immunohistochemistry | |

4.17. RNA isolation and cDNA preparation
4.18. RNA isolation protocol
4.19. Primers
4.19.1. Primer standardization
4.20. Quantitative real-time PCR
4.21. Statistical analysis
4.22. Image analysis
**Chapter 5 – Results & Discussion: Aim1** 32-59
5.1. Results
5.1.1. Behavior results
5.1.1.1. Fear conditioning
5.1.1.2. Fear extinction
5. 1. 1.3. Control groups
5. 1. 1.4. Test for retention of fear consolidation and extinction
5. 1. 2. Immunohistochemistry
5. 1.2.1. c-fos expression in the amygdala
5. 1.2.2. Histone H3K9 acetylation in the amygdala
5.1.2.3. Histone H4K5 acetylation in the amygdala
5.1.2.4. HDAC1 expression in the Amygdala
5.1.2.5. HDAC2 expression in the Amygdala
5.1.2.6. c-fos expression in Prefrontal Cortex
5.1.2.7. Histone H3K9 acetylation in Prefrontal Cortex
5.1.2.8. Histone H4K5 acetylation in Prefrontal Cortex
5.1.2.9. HDAC1 expression in Prefrontal Cortex
5.1. 2.10. HDAC2 expression in Prefrontal Cortex
5.1. 2.11. c-fos expression in Hippocampus
5.1.2.12. H3K9 acetylation in Hippocampus
5.1.2.13. H4K5 acetylation in Hippocampus
5.1.2.14. HDAC1 expression in Hippocampus
5.1.2.15. HDAC2 expression in Hippocampus
5.1.3. Correlation Results
5.1.3.1. Conditioning correlation
5.1.3.1.1. Amygdala
5.1.3.1.2. Prefrontal Cortex
5.1.3.1.3. Hippocampus
5.1.3.2. Extinction correlation
5.1.3.2.1. Amygdala
5.1.3.2.2. Prefrontal Cortex
5.1.3.2.3. Hippocampus
5.1.4. Real-Time PCR for mRNA expression analysis
5.1.4.1. mRNA expression in Amygdala
5.1.4.1.1. BLA
5.1.4.1.2. CeA
5.1.4.2. mRNA expression in Prefrontal Cortex
5.1.4.2.1. PL
5.1.4.2.2. IL
5.1.4.3. mRNA expression in Hippocampus

5.1.4.3.1. CA1
5.1.4.3.2. CA3
5.1.4.3.3. DG
5.2. Discussion

**Chapter 6 – Results & Discussion: Aim2 – Valproic acid effect on Conditioning** 60-83
6.1. Results
6.1. 1. Behavior results
6.1.1.1. Anxiety measurement for valproic acid
6.1.1.2. Effect of Valproic acid treatment on conditioning
6.1.2. Immunohistochemistry
6.1.2.1. c-fos expression in the amygdala
6.1.2.2. H3K9 acetylation in the amygdala
6.1.2.3. H4K5 acetylation in the amygdala
6.1.2.4. HDAC1 expression in the amygdala
6.1.2.5. HDAC2 expression in the amygdala
6.1.2.6. c-fos expression in Prefrontal Cortex
6.1.2.7. H3K9 acetylation in Prefrontal Cortex
6.1.2.8. H4K5 acetylation in Prefrontal Cortex
6.1.2.9. HDAC1 expression in Prefrontal Cortex
6.1.2.10. HDAC2 expression in Prefrontal Cortex
6.1.2.11. c-fos expression in Hippocampus
6.1.2.12. H3K9 acetylation in Hippocampus
6.1.2.13. H4K5 acetylation in Hippocampus
6.1.2.14. HDAC1 expression in Hippocampus
6.1.2.15. HDAC2 expression in Hippocampus
6.1.3. Real-Time PCR for mRNA expression analysis
6.1.3.1. mRNA expression in Amygdala
6.1.3.1.1. BLA
6.1.3.1.2. CeA
6.1.3.2. mRNA expression in Prefrontal Cortex
6.1.3.2.1. PL
6.1.3.2.2. IL
6.1.3.3. mRNA expression in Hippocampus
6.1.3.3.1. CA1
6.1.3.3.2. CA3
6.1.3.3.3. DG
6.1.4. Correlation
6.1.4.1. Amygdala
6.1.4.2. Prefrontal Cortex
6.1.4.3. Hippocampus
6.2. Discussion

**Chapter 7 – Results & Discussion: Aim2 – Valproic acid effect on Extinction** 84-107
7.1. Results
7.1.1. Behavior results
7.1.1.1. Effect of Valproic acid treatment on Extinction
7.1.2. Immunohistochemistry

7.1.2.1. c-fos expression in the Amygdala
7.1.2.2. H3K9 acetylation the Amygdala
7.1.2.3. H4K5 acetylation in the Amygdala
7.1.2.4. HDAC1 expression in the Amygdala
7.1.2.5. HDAC2 expression in the Amygdala
7.1.2.6. c-fos expression in Prefrontal Cortex
7.1.2.7. H3K9 acetylation in Prefrontal Cortex
7.1.2.8. H4K5 in Prefrontal Cortex
7.1.2.9. HDAC1 expression in Prefrontal Cortex
7.1.2.10. HDAC2 expression in Prefrontal Cortex
7.1.2.11. c-fos expression in Hippocampus
7.1.2.12. H3K9 acetylation in Hippocampus
7.1.2.13. H4K5 acetylation in Hippocampus
7.1.2.14. HDAC1 expression in Hippocampus
7.1.2.15. HDAC2 expression in Hippocampus
7.1.3. Correlation
7.1.4. Real-time PCR
7.1.4.1. mRNA expression in the Amygdala
7.1.4.2. mRNA expression in Prefrontal Cortex
7.1.4.3. mRNA expression in Hippocampus
7.2. Discussion

**Chapter 8 – Conclusion** 108-110

**Publications** 111

**References** 112-120

# Abbreviations

| | |
|---|---|
| AMPA | α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| BA | Basal nucleus of amygdala |
| BDNF | Brain Derived Neurotrophic Factor |
| BLA | Basolateral amygdala |
| CA | Cornu Ammonis |
| CBP | CREB Binding Protein |
| CBT | Combined behavioral therapy |
| 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 |
| DG | Dentate gyrus |
| DH | Dorsal hippocampus |
| EPM | Elevated plus maze |
| Ext | Extinction |
| HAT | Histone Acetyl Transfearse |
| HDAC | Histone Deacetylase |
| IHC | Immunohistochemistry |
| IL | Infralimbic cortex of PFC |
| ITC | Intercalated cell mass |
| LA | Lateral nucleus of amygdala |
| LTM | Long Term Memory |
| LTP | Long Term Potentiation |
| MAPK | Mitogen Activated Protein Kinase |
| NMDA | N-methyl –D-aspartate |
| PBS | Phosphate Buffered Saline |
| PL | Prelimbic cortex |
| PFC | Prefrontal cortex |
| PKC | Protein kinase C |
| PTSD | Post Traumatic Stress Disorders |
| RT-PCR | Real Time Polymerase Chain Reaction |
| SAHA | Suberoylanilide Hydroxamic Acid |
| STM | Short Term Memory |
| TSA | Trichostatin A |
| US | Unconditioned Stimulus |
| VH | Ventral hippocampus |
| VPA | Valproic acid |

# List of Figures


| *Figure No.* | *Title* | *Page No.* |
|---|---|---|
| 1. | Collective data representing the occurrence of PTSD cases in human population | 2 |
| 2. | Diagram representing the training in conditioning (A) and extinction (B) | 8 |
| 3. | Diagram showing the Lymbic system of human (A) and rat (B) involved in fear memory | 9 |
| 4. | Diagram representing the substructure of Amygdala | 10 |
| 5. | Diagram representing the anatomical structure of the Prefrontal cortex of human (A) and rat (B) | 11 |
| 6. | Diagram showing the Hippocampus (A) Immunohistochemistry sections and (B) structure of rat hippocampus | 11 |
| 7. | Diagrammatic representation of (a) Fear conditioning and (b) Fear extinction circuitry involving amygdala, PFC, and Hippocampus | 13 |
| 8. | Diagram showing the model of the molecular mechanism involved in fear memory consolidation and extinction in the amygdala | 15 |
| 9. | Diagram showing the mechanism of HDACs in the regulation of gene expression | 17 |
| 10. | Diagrammatic representation of protocol for fear memory consolidation (left) and extinction (right) | 23 |
| 11. | Structure of valproic acid | 24 |
| 12. | A diagrammatic representation of the protocol for fear memory consolidation (left) and extinction (right) after valproic acid treatment | 25 |
| 13. | Agarose gel electrophoresis of total RNA with intact rRNA bands | 28 |
| 14. | Gel images showing the amplified products of (a) c-fos (b) HDAC1 (c) HDAC2 (d) CBP and (e) GAPDH | 29-30 |
| 15. | Standard amplification plot in real-time PCR showing different phases of the reaction | 30 |
| 16. | The standard melting curve for real-time PCR | 31 |
| 17. | Behavior training of conditioning and Extinction | 34 |
| 18. | c-fos expression in amygdala in conditioning and extinction | 35 |
| 19. | Histone H3K9 acetylation in amygdala in conditioning and extinction | 37 |
| 20. | Histone H4K5 acetylation in amygdala in conditioning and extinction | 38 |
| 21. | HDAC1 expression in amygdala in conditioning and extinction | 39 |
| 22. | HDAC2 expression in amygdala in conditioning and extinction | 40 |
| 23. | c-fos expression in Prefrontal cortex in conditioning and extinction | 41 |
| 24. | Histone H3K9 acetylation in Prefrontal cortex in conditioning and extinction | 42 |
| 25. | Histone H4K5 acetylation in Prefrontal cortex in conditioning and extinction | 43 |
| 26. | HDAC1 expression in Prefrontal cortex in conditioning and extinction | 44 |
| 27. | HDAC2 expression in Prefrontal cortex in conditioning and extinction | 45 |
| 28. | c-fos expression in Hippocampus in conditioning and extinction | 46 |
| 29. | Histone H3K9 acetylation in Hippocampus in conditioning and extinction | 47 |

30. Histone H4K5 acetylation in Hippocampus in conditioning and extinction 48
31. HDAC1 expression in Hippocampus in conditioning and extinction 49
32. HDAC2 expression in Hippocampus in conditioning and extinction 50
33. Correlation for the conditioning 51
34. Correlation for Extinction 53
35. EPM test 61
36. Valproic acid effect on fear memory consolidation 62
37. c-fos expression in amygdala in valproic acid treated conditioning group 63
38. Histone H3K9 acetylation in amygdala in valproic acid treated conditioning group 64
39. Histone H4K5 acetylation in amygdala in valproic acid treated conditioning group 65
40. HDAC1 expression in amygdala in valproic acid treated conditioning group 66
41. HDAC2 expression in amygdala in valproic acid treated conditioning group 67
42. c-fos expression in Prefrontal cortex in valproic acid treated conditioning group 68
43. Histone H3K9 acetylation in Prefrontal cortex in valproic acid treated conditioning group 69
44. Histone H4K5 acetylation in Prefrontal cortex in valproic acid treated conditioning group 70
45. HDAC1 expression in Prefrontal cortex in valproic acid treated conditioning group 71
46. HDAC2 expression in Prefrontal cortex in valproic acid treated conditioning group 72
47. c-fos expression in Hippocampus in valproic acid treated conditioning group 73
48. Histone H3K9 acetylation in Hippocampus in valproic acid treated conditioning group 74
49. Histone H4K5 acetylation in Hippocampus in valproic acid treated conditioning group 75
50. HDAC1 expression in Hippocampus in valproic acid treated conditioning group 76
51. HDAC2 expression in Hippocampus in valproic acid treated conditioning group 77
52. Correlation drug + conditioning 81
53. Valproic acid effect on fear memory extinction 85
54. c-fos expression in amygdala in valproic acid treated extinction group 87
55. Histone H3K9 acetylation in amygdala in valproic acid treated extinction group 88
56. Histone H4K5 acetylation in amygdala in valproic acid treated extinction group 89
57. HDAC1 expression in amygdala in valproic acid treated extinction group 90
58. HDAC2 expression in amygdala in valproic acid treated extinction group 91
59. c-fos expression in Prefrontal cortex in valproic acid treated extinction group 92
60. Histone H3K9 acetylation in Prefrontal cortex in valproic acid treated extinction group 93
61. Histone H4K5 acetylation in Prefrontal cortex in valproic acid treated extinction group 94
62. HDAC1 expression in Prefrontal cortex in valproic acid treated extinction group 95
63. HDAC2 expression in Prefrontal cortex in valproic acid treated extinction group 96
64. c-fos expression in Hippocampus in valproic acid treated extinction group 97
65. Histone H3K9 acetylation in Hippocampus in valproic acid treated extinction group 98
66. Histone H4K5 acetylation in Hippocampus in valproic acid treated extinction group 99
67. HDAC1 expression in Hippocampus in valproic acid treated extinction group 100
68. HDAC2 expression in Hippocampus in valproic acid treated extinction group 102
69. Correlation drug + Extinction 103

# List of Tables


| Table number | Title | Page No. |
|---|---|---|
| 1. | cDNA preparation reaction mix | 28 |
| 2. | Thermal profile for cDNA preparation | 28 |
| 3. | Primer details | 29 |
| 4. | SYBR green PCR reaction mixtures | 31 |
| 5. | SYBR green PCR reaction condition | 31 |
| 6. | Correlation conditioning | 51-52 |
| 7. | Correlation Extinction | 53-54 |
| 8. | Correlation of valproic acid treated Conditioning group | 82 |
| 9. | Correlation of valproic acid treated Extinction group | 106 |

***Chapter 1***

# INTRODUCTION

Pavlovian fear conditioning has been extensively used model to study PTSD and its associative symptoms. Fear conditioning and extinction depict an exhaustive paradigm to understand different stages of the traumatic condition in the experimental animal model. The occurrence and impact of these events are more frequent in the early stage of life than later stage, but almost every individual faces some sort of traumatic condition at least in their life (van der Kolk 2000; Beesdo et al, 2009). Individuals exposed to traumatic events exhibit various types of responses including fear, shock, guilt and anxiety which disappear over time in some people, while others develop persistent symptoms of PTSD (Zoellner et al, 2011; Desmedt et al, 2015). The studies on PTSD have witnessed the occurrence of traumatic events at a higher frequency in humans once in their life which may have a reversible or irreversible impact on human life (Careaga et al, 2016). However, at least 10%population suffers from the persistence of long lasting presence of PTSD among human population (Iribarren et al, 2005) (Fig. 1). Therapeutic approaches used for the treatment of PTSD comprise combined behavioral therapy (CBT) which incorporates behavioral and pharmacological approaches together (Morrison, 2009; Kar N, 2011) but are not foolproof. So, in order to find better ways of treatment it becomes important to understand the mechanism involved in the progression of PTSD associated with the traumatic events.

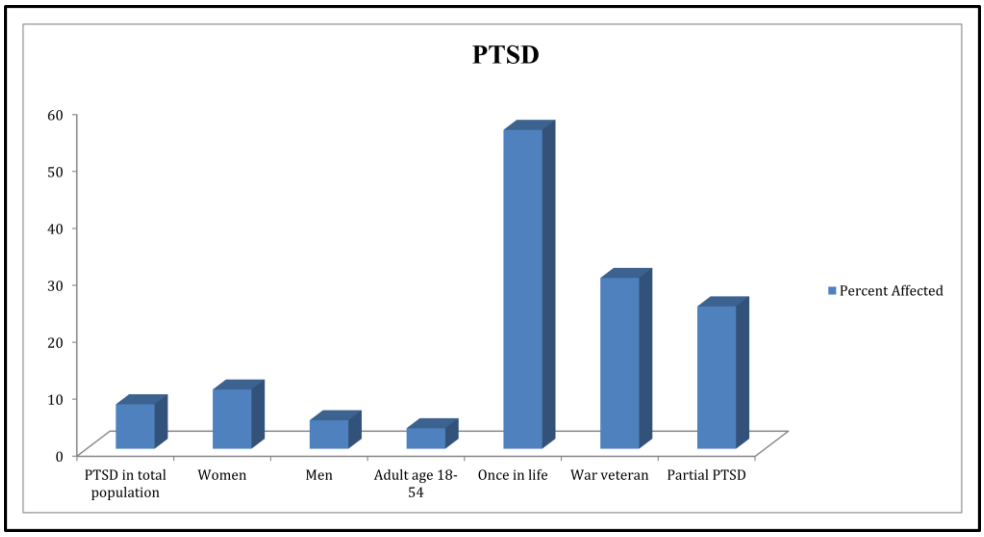



**Fig1.** Collective data representing the occurrence of PTSD cases in the human population (Iribarren et al, 2005).

In Pavlovian fear conditioning animals are allowed to pair a conditioned stimulus (CS) such as tone or context with an unconditioned stimulus (US) such as shock in an experimental condition (Tarpley et al, 2010; Curzon et al, 2009). During fear conditioning animal learns to predict the occurrence of a US on presentation of CS, whereas during extinction, the animal learns to reverse the effect of previous learning of condition by un-pairing of CS and US through CS presentation alone in the absence of US (Calandreau et al, 2007).

Fear memory consolidation and extinction involve different brain region to function in a coordinated manner through interconnected neuronal inputs (Ehrlich et al, 2009). These neuronal inputs connect each other forming a network in a defined and controlled pattern known as circuitry. The circuitry thus formed is responsible for the progression of fear memory consolidation and

extinction and mainly comprises of three brain regions known as PFC, amygdala and hippocampus, interconnected with each other in the modulation of fear circuitry (Peters et al, 2009). Studies have so far evidenced about the connection of PFC and amygdala sub-nuclei in fear conditioning and extinction, but little is known about the exact molecular pattern in association with the fear circuitry (Marek et al, 2013). Amygdala sub region functions in the association of thalamic and cortical information and at the same time functions as a gateway to express fear responses (Phelps and LeDoux, 2005). It is composed of different subregions where LA and BA is the site that receives sensory inputs which in turn control CeL and CeM subregion of the amygdala in the regulation of fear (Janak and Tye, 2015; Yang and Wang, 2017). The prefrontal cortex is composed of PL and IL sub regions whose function is to modulate the function of amygdala and hippocampus during fear memory consolidation and extinction respectively in rodents (Giustino and Maren, 2015). The IL cortical sub region of PFC has direct innervations to vITC within amygdala whereas PL centre of PFC innervates BLA sub region of the amygdala (Adhikari et al, 2015). Hippocampal region is known for its association with the acquisition and processing of contextual memory which coordinates with amygdala and PFC during fear memory consolidation and extinction (Stevenson, 2011). The function of these brain parts depends on cellular and molecular mechanisms such as synaptic connections, synaptic strengthening, MAPK activation, NMDA receptor activation as well as epigenetic mechanisms (Kandel, 2014; Johansen et al, 2011).

Recent studies have shown that the epigenetic regulation of chromatin is amongst one of the important molecular mechanisms involved in learning and memory, which includes histone acetylation, methylation, phosphorylation, sumoylation and DNA methylation (Bannister and Kouzarides, 2011; Sultan and Day, 2011; Kim and Kaang, 2017). Among various types of epigenetic regulation histone acetylation is one of the prominent epigenetic mechanisms involved in molecular processing for learning and memory (Peixoto and Abel, 2013). Histone H3 and H4 acetylation at specific lysine residue are key molecular switches which lead to the activation of a specific set of the gene causing different behavioral outcomes (Siddiqui et al, 2017). Both contextual and cued fear memories are governed by increased histone H3 and H4 acetylation which could further be strengthened by HDAC inhibitors (TSA, SAHA) (Itzhak et al, 2012, 2013). Histone acetylation in amygdala, hippocampus and prefrontal cortex has been found to be associated with fear memory consolidation and extinction (Ranjan et al, 2015; Siddiqui et al, 2017; Kritman and Maroun 2013, Debiec et al., 2010; Duvarci et al. 2005;Herry et al. 2008; Zimmerman and Maren 2010).Increased histone acetylation in hippocampal subregion has a strong association with the progression of fear memory consolidation and extinction (Graff and Tsai, 2013; Bousiges et al, 2013; Fujita, 2012; Matsumoto, 2013). Furthermore increased histone acetylation in PFC at BDNF IV promoter is found to be associated with the extinction of fear whereas increase histone acetylation at BDNF promoter I and IV have been found to be associated with the conditioning of fear memory (Bredy et al, 2007; Stafford, 2012). Altered histone acetylation in LA, BA, CeL and CeM subregion of amygdala showed an association with the conditioning (Siddiqui et al, 2017; Ranjan et al, 2015).

Histone modifications involve modification of various N-terminal residues which play an important role in gene regulation through histone acetylation, influence gene transcription (Bannister and Kouzarides, 2011; Kouzarides, 2007). Acetylation and deacetylation of histones at lysine residues is regulated by the opposing actions of HATs (Histone acetyltransferases) and

HDACs (Histone deacetylases) (Seto and Yoshida, 2014). Depending upon their structural and functional properties HDACs have been classified into 4 classes (Class I - IV) (Seto and Yoshida, 2014). Class I HDACs are generally localized to the nucleus except for HDAC3, which can shuttle out to the cytoplasm. Out of all, class I histone deacetylases HDAC1, HDAC2, and HDAC3 are widely expressed throughout the brain (Seto and Yoshida, 2014). Class II HDACs can shuttle out of the nucleus to modify non-histone proteins (Setoand Yoshida, 2014; Delcuve et al, 2011). However little is known about the role of Class IV HDAC, HDAC11 in the brain. At present class I HDACs especially HDAC1 and HDAC2 have been shown to exhibit extensive involvement in learning and memory (Whittle and Singewald,2014; Bahari jawan et al, 2012; Zhou et al, 2009).

Most of the studies till date have confirmed that HDACs mainly acts as a negative regulator of fear memory consolidation and extinction (Valiati et al, 2017) and is mainly associated with the gene silencing (Gregoretti et al., 2004; Fischer et al., 2010). It also regulates c-fos expression (Yang et al., 2001; Usenko et al., 2003; Renthal et al., 2008), an immediate early gene following contextual fear conditioning (Radulovic et al., 1998; Peleg et al., 2010).A recent study have confirmed that HDAC1 subtype is mainly associated with the promotion of fear extinction (Bahari-jawan et al, 2012) while other HDAC subtypes mainly function in suppression of fear extinction (Morris et al, 2013; Whittle and Singewald, 2014). Thus different subsets of HDACs might be playing an important role in fear memory consolidation and extinction.

The present study looked at the association of histone acetylation and HDAC 1 and 2 during the consolidation of fear and extinction memory in the amygdala, PFC and Hippocampus. The outcomes of this study will enhance our knowledge of the molecular players especially the HDACs in controlling the fear circuitry during fear and extinction learning especially in the network connecting PFC, amygdala and hippocampus and will be helpful in finding out newer targets for targeting bad memories.

***Chapter 2***

# REVIEW OF LITERATURE

Memories are highly dynamic entities and may be good or bad depending on the consequences. Dreadful events form persistent memory and if they remain for a longer period of time then may get converted into fear memory and its related disorders. This condition is known as a Post-traumatic disorder. The prevalence of PTSD is around 10-11% in overall population worldwide and it belongs to the fourth most common psychiatric disorder in the USA (Breslau et al., 1991; Kessler et al., 1995; Schlenger et al., 2002). The key mechanism known to be responsible for the progression of PTSD includes the formation of associative learning occurred due to the environmental cues at the onset of the traumatic event. To study memory based alterations at laboratory level the Pavlovian fear conditioning model is proved to be the best paradigm.

Being a survival factor (fear memory) the incidence of a fearful event is predicted by the animals and a response is generated in turn to overcome such condition. The extinction learning, on the other hand, includes the therapeutic part, where extinction of conditioned fear has direct parallels with cognitive-behavioral treatments such as exposure therapy for anxiety disorders in humans (Bouton, 1988; Rothbaum and Schwartz, 2002; Mineka and Oehlberg, 2008). During extinction, a previously conditioned stimulus (CS) is repeatedly presented in the absence of an unconditioned stimulus (US) (Pavlov, 1927), resulting in suppression of learned fear behavior, often measured as freezing. Although the extinction learning opposes the effect of conditioned learning the effect is caused by creating inhibitory learning instead of erasure of conditioning (Bouton, 1993; Maren, 2011; Bouton et al. 2006; Ji and Maren 2007) as fear return following the passage of time or context.

In other words, extinguished fear shows association with the specific context. The return of fear memory after extinction is a substantial problem for maintaining long-lasting suppression of fear through exposure-based therapies (Rodriguez et al. 1999; Hermans et al. 2006; Effting and Kindt 2007; Quirk and Mueller 2008). The difference in response to extinction shows the effect of environment on extinction which at molecular level controlled by various mechanism including epigenetic changes (Bredy and Barad, 2008).

On analyzing the consolidation and extinction process in detail it was identified that the region-specific molecular changes lead to such phenomenon. At the molecular level it was identified that chromatin modification has a pivotal role in consolidation and extinction of fear. Amongst widely associated molecular mechanism epigenetic regulation through histone acetylation proved to be critical for synaptic plasticity and memory formation (Guan et al. 2009; Levenson et al. 2004). Furthermore, it was found that consolidation and extinction of fear memory are facilitated by the HDAC inhibition (Guan et al, 2009). These studies confirmed that the global HDAC inhibitor may act as a target for improvement in learning and also to the memory deficits in animal models of neurodegenerative disorders (Levenson et al., 2004; Fischer et al., 2007; Lattal et al., 2007; Vecsey et al., 2007; Barrett and Wood, 2008; Morris et al., 2013; Monsey et al., 2011). HDACs promote a transcriptionally inactive chromatin state by removing acetyl groups from histone tail lysine residues. Mice treated with an HDAC inhibitor affected long-term memory formation but exhibited less effect on short-term memory (Stefanko et al. 2009).

HDACs are typically classified into four classes based on their structural homology, sub-cellular localization, and tissue-specific expression patterns. These HDAC subtypes have been found to be associated with varied biological processes such as cellular differentiation, apoptosis, development, and synaptogenesis (Haberland et al., 2009).

Recent investigation has focused the functional roles of different HDACs in various types of brain functions (Guan et al., 2009; Montgomery et al., 2009; Kim et al., 2012). Most of the studies using HDAC inhibitor have revealed the effect of HDAC inhibition on cognitive function using different behavioral paradigms (e.g., Morris Water maze, fear conditioning), without clarifying whether the effect was global for all HDAC classes or was HDAC subtype specific (Levenson et al., 2004; Fischer et al., 2007; Lattal et al., 2007; Vecsey et al., 2007; Barrett and Wood, 2008; Bredy and Barad, 2008; Stefanko et al., 2009; Morris et al., 2010; Monsey et al., 2011). In different subsets of HDACs, HDAC1 has been found to be required for extinction learning through the regulation of histone acetylation (Bahari-Javan et al, 2012). Working memory and extinction learning are favored by the loss of HDAC2 (Michael et al 2013). Although HDAC3 negatively regulates the formation of long-term memory (Susan et al, 2011), HDAC4 positively regulate the learning and memory (Kim et al, 2012). Recent work on HDAC6 proved that it functions in the elimination of protein aggregates in oxidative stress, in mitochondrial transport; and act as a negative regulator for associative and spatial memory formation (Simões-Pires et al., 2013). Therefore it becomes necessary to study the role of HDAC in memory formation and extinction so as to reveal the more specified pathway for treatment of disorders like PTSD.

## 2.1. Fear memory

Learning is an active process which involves various brain regions to function together for acquisition, processing, and storage. Animals receive different pieces of information from the environment and process this information in such a way that it can be recalled at any stage of life. Fear is a form of emotional response which is generated in a traumatic stressful condition and such response differs in the impact it creates on individuals (LeDoux, 2014). In breif, it can be regarded as a condition where a conscious state develops due to the occurrence of threat condition or its imagination (Costanz et al, 2011). Although the state of fear we experience seems similar to the animals, our response to fear is more by the thing we perceived in our life by looking other animal species (LeDoux, 2012; 2014).

Though fear is a prevalent form of cognitive state in human population, its impact on human life is diverse. It functions as a survival factor at one end but on the other side, it may have the effect towards cognitive dysfunction. The different stages of fear memory include acquisition, storage, and retrieval, for stabilizing the fear memory. In animals, a high level of emotional response following fear learning causes very stable, powerful and long-lasting memories which are difficult to erase (Cahill and McGaugh, 1998; McGaugh, 2004). In both the humans and animals the mechanism for fear memory consolidation and extinction has been worked out.

### 2.1.1. Fear consolidation

To study memory based phenomenon Pavlovian model is used. It involves the association of a conditioned stimulus (CS) such as tone or context with an unconditioned stimulus (US) such as an electrical foot-shock, which results in the formation of stable memory consolidation for fear. Following fear memory consolidation the memory can be enhanced by the second phase for stabilization known as retrieval which involves re-exposure to the CS through reconsolidation process. Prolonged re-exposure to the CS leads to the extinction of fear memory which is a new form of inhibitory learning against the fear memory, where animals learn not to fear in response to the CS (Fig 2.A).

### 2.1.2. Fear extinction

Fear extinction is a training through which the long lasting effect of fear memory can be controlled or suppressed by the behavioral training. The method is widely used as an exposure based therapy to overcome the effect of fear memory (Milad and Quirk, 2012). The exposure therapy using extinction training involves the exposure of the individual to a conditioned stimulus which results in suppression of the effect caused by fear memory related traumatic events. Sometimes after the passage of a long time of the training, fear returns which results in persistent of fear for a long time. This effect can be controlled by the repeated presentation of extinction training with intervals of time which results in strong persistent suppression of fear.

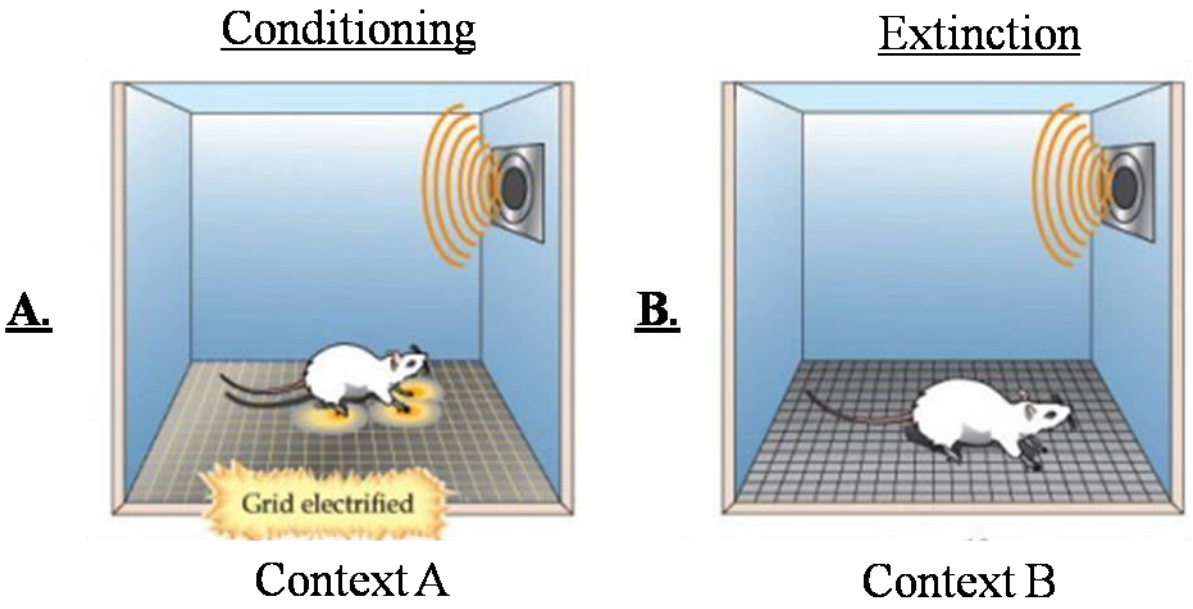



**Fig 2.** Diagram representing the training in conditioning (**A**) and extinction (**B**)

During extinction learning in laboratory condition, the animals are exposed to the repeated presentation of CS (conditioning stimulus such as a tone) in a context that is different from the conditioning context. The animals thus learn to dissociate the associative memory formed between CS and US by the creation of new memory which allows animals to learn that the CS is not associated with the US (Fig 2.B). The experimental use of extinction training intensifies the requirement for comprehending the mechanism involved in fear extinction at cellular and molecular level together with the studies that involve pharmacological intervention (Myers and Davis, 2002; Mueller et al., 2008, 2009; Holmes and Quirk, 2010; Davis, 2011).

### 2.2. Neuroanatomical regions involved in Fear Memory

Memory formation is a continuous process that involves different cortical regions of the brain. Tripartite realms involved are (Maren et al, 2013; Quirk and Mueller, 2008) amygdala, PFC and hippocampus (the limbic system) which function in a coordinated manner for fear memory consolidation and extinction (Johansen et al, 2011). These brain parts function for the different phases of fear memory consolidation and extinction and are interconnected through synaptic connections (Izquierdo et al, 2016) (Fig 3).

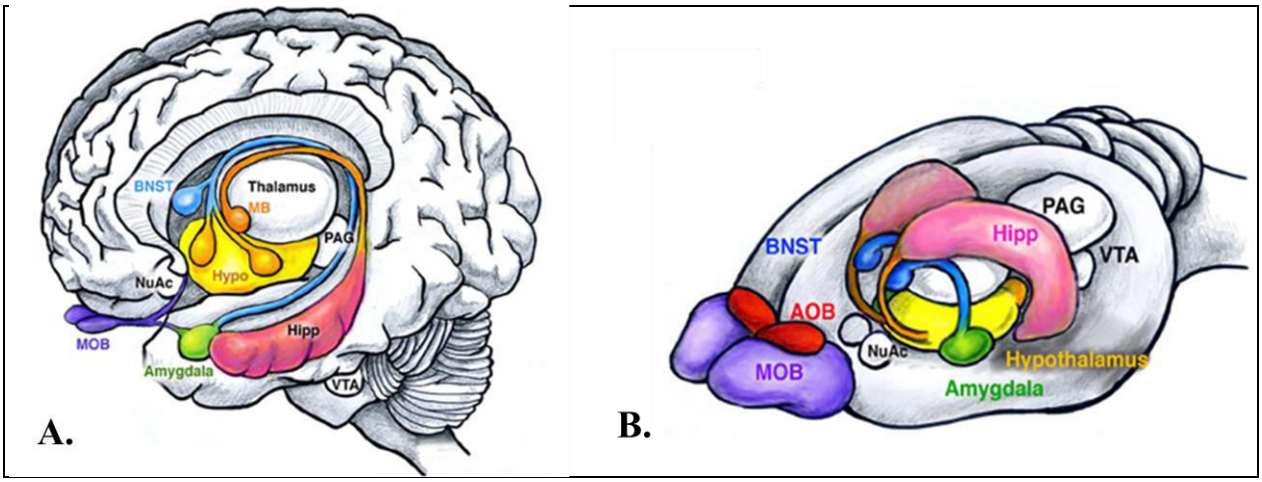



**Fig 3.** Diagram showing the Lymbic system of human (A) and rat (B) involved in fear memory.

### 2.2.1. Amygdala

The amygdala is an almond-shaped structure located deep on both the sides of the temporal lobe in the brain. It is composed of almost 13 different subregions or nuclei formed by the cluster of neurons. The BLA (basolateral amygdala) is the largest subregion which is made up of the lateral nucleus (LA), basolateral nucleus (BA), and accessory basal subregion (McDonald 1998; Turner and Herkenham 1991; Krettek and Price 1978a; Petrovich and Swanson 1997; Veening et al. 1984). The BLA subregion of the amygdala gets sensory inputs from the thalamus, hippocampus, and cortex, which receives sensory information from the environment (Davis and Whalen, 2001). The LA center of BLA functions as the entry point for the sensory information of the auditory, visual, olfactory and taste system (LeDoux, 2007). Sensory information of the CS and US congregate in the LA during Fear learning (Wilensky et al., 2006). The activated LA subregion activates the CeA (Central amygdala) which serves as the output center of the amygdala for the expression of fear (Davis and Whalen, 2001). The CeA, in turn, innervates brainstem for the expression of fear responses which results in specialized behavior and physiological response (LeDoux, 2007). The CeA (the Central nucleus of the amygdala) is composed of CeL (centrolateral nucleus) and CeM (Centromedial amygdala) that are involved in the regulation and expression of the fear and emotional memory. The three clusters (lITC- lateral intercalated cell masses, dITC- dorsal intercalated cell masses and vITC- ventral intercalated cell masses) of GABAergic neurons also known as intercalated cell masses (ITCs) regulate the activity of BLA and CeA subregion of amygdala through inhibitory connections. The other two subregions of amygdala the cortical nuclei and the medial nucleus receive information through olfactory centers. Amygdala also shows its projections from the hypothalamus and brainstem. The hypothalamus functions in the regulation of emotional responses through the regulation endocrine secretion of the pituitary gland (Fig 4).

Amygdala is one of the most important part of the limbic system which is involved in regulation of various types of emotions such as fear, pleasure, and anger as well as in the processing and storage of the emotional memories. It is involved in the regulation of the responses associated with fear. The function is associated with the presence of fear neurons in the amygdala regulating during fear conditioning. The primary role of the amygdala is to form and store the memories that

are associated with emotional experiences. Fear memories are considered to be stored through synaptic connections between neurons in the brain.

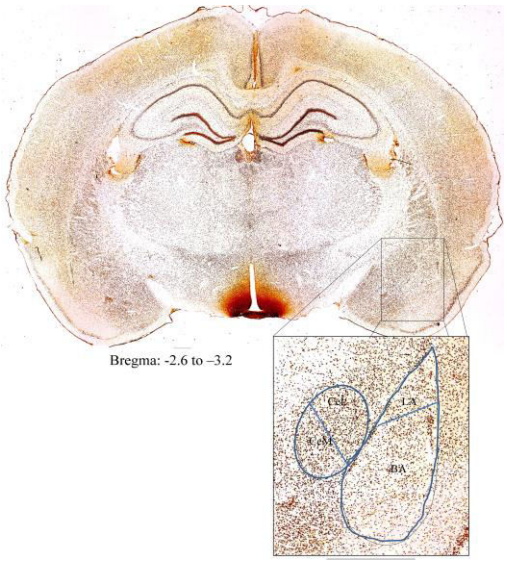



**Fig 4.** Diagram representing the substructure of Amygdala

## 2.2.2. Medial Prefrontal cortex (mPFC) or Prefrontal Cortex

Prefrontal cortex or medial prefrontal cortex is located in the frontal lobe of the mammalian brain. In rodents, the prefrontal cortex includes three parts- the medially located medial prefrontal cortex (mPFC), a ventrally located orbital prefrontal cortex and a lateral or sulcal prefrontal cortex (l-PFC) (Divac and Mogenson, 1985; Groenewegen, 1988). The medial prefrontal cortex region is proved to be involved in the regulation of fear memory consolidation and extinction. It is divided into four distinct subregions, the prelimbic area, the infralimbic area, the medial precentral area (PrCm) or area Fr2 and the anterior cingulate area (Krettek and Price, 1977). The prefrontal cortex is involved in the implementation of psychological functions that involves the capability to differentiate conflicting thoughts such as good and bad, better and best, same and different, effects of present actions on future, expectation based on actions etc.

The PFC receives innervations for a number of sensory information by the cortices, and it also receives connections with the mediodorsal thalamic nucleus (Miller et al, 2017). The lateral prefrontal cortices got connections with temporal and parietal regions correlated with the cognitive functions (Siddiqui et al, 2008). The orbitofrontal and anterior cingulate cortical regions receive inputs from the amygdala and medial temporal structures involved with the emotional responses and memory functions (Bonelli, 2007). The dorsal subregion is innervated with the brain region associated with the cognition and attention while the ventral subregion is associated with the emotional circuitry (Fanselow and Dong, 2010) (Fig 5).

The role of Prefrontal cortex has been found to be associated with the emotional regulation as mPFC lesion shows impaired emotional and cognitive responses (Bonelli, 2007). The studies using Pavlovian fear learning shows the involvement of PFC in cued fear learning although its activity was not associated with the CS-US association. Also in contextual extinction learning the input from the hippocampus to PFC are required for the expression of fear responses in a new

context. Furthermore, the recent works have shown that the PFC is also involved in initial acquisition stages of fear learning.

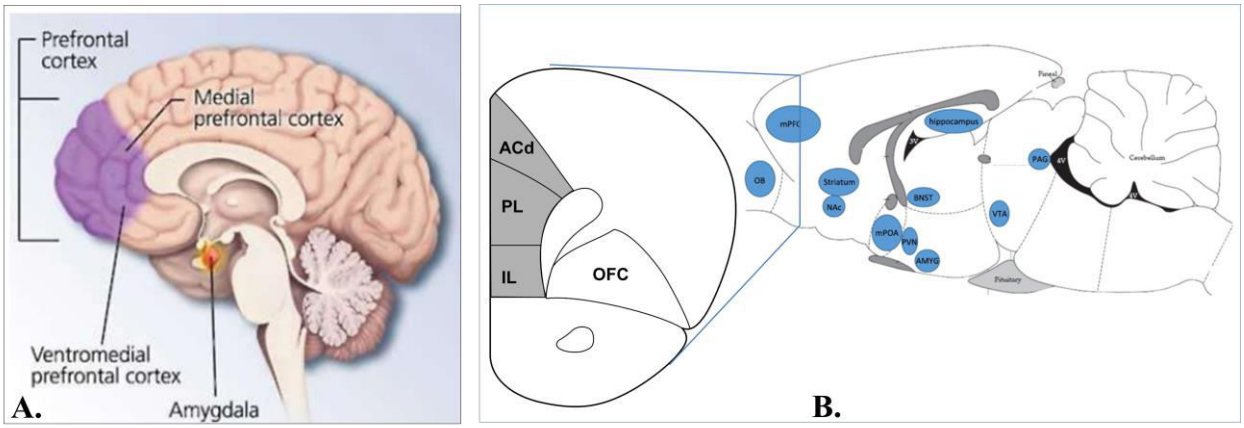



**Fig 5.** Diagram representing the anatomical structure of the Prefrontal cortex of human (A) and rat (B).

## 2.2.3. Hippocampus

The hippocampus is well known small area of the brain of vertebrates and is a component of the limbic system mostly associated with the contextual memory and spatial navigation (Orsini et al, 2012). It is an arched shape structure located in medial temporal lobe in primates. The hippocampus functions in strengthening of short-term memory to long-term memory as well as for spatial memory processes. Its main function is to store the spatial information of the surroundings in the environment. The dorsal hippocampus (DH) processes spatial, verbal and conceptual information. Ventral hippocampus (VH) functions to promote fear and the intermediate hippocampus contain the function of both dorsal and ventral hippocampus (Fig 6).

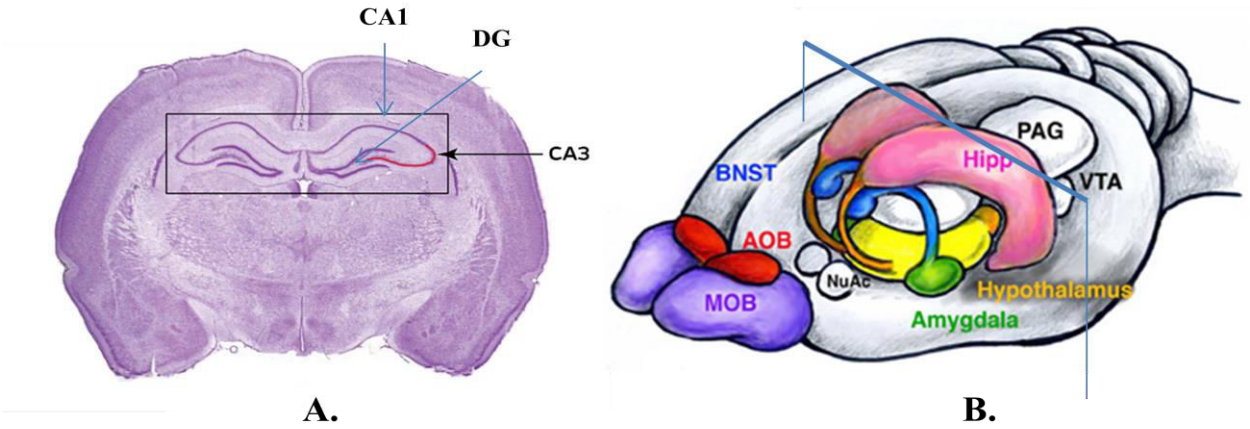



**Fig 6.** Diagram showing the Hippocampus (A) Immunohistochemistry sections and (B) structure of rat hippocampus.

Researchers have worked out the role of the hippocampus in the formation of new memories through previously experienced events which help in the finding of new events, places and stimuli from previous ones. Furthermore, the hippocampus is considered to be involved in the

learning of environmental factors associated with the fearful learning. It has been observed that hippocampus functions in contextual fear learning, as lesion to the hippocampus creates amnesia of contextual fear conditioning. However, lesions of the hippocampus following a time interval do not impair contextual fear conditioning (Sanders et al., 2003; Young et al., 1994).

## 2.3. The circuitry of fear

The acquisition, storage, processing and expression of information are the basic functional domains of the learning and memory processes in animals. This highly organized process involves coordination between different brain regions. Such an arrangement involves brain parts to interconnect with each other and form a circuitry. The amygdala, prefrontal cortex, and hippocampus are at the center of the fear circuit and form a system known as limbic system (Maren, 2001a; Fanselow and LeDoux, 1999). Practically memories do not emerge by the activity of individual brain part but formed from the interactions of a number of other subregions compositely forming the neural circuit. The neural circuit stands for the input, output, and processing of the information which results due to the effect of environmental condition.

Amygdala is involved in acquisition and expression of fear which was confirmed by the lesion studies resulting in disruption of both the acquisition and expression of fear conditioning in rodents (Maren et al., 1996a; LeDoux et al., 1990). It is evidenced by the studies that BLA subregion of the amygdala is the site for CS-US association during fear memory consolidation (Goosens and Maren, 2001). BLA then convey this information to CeA which is acting as a gateway for the expression of resulting fear response (Maren, 2008; Zimmerman and Maren, 2010, 2011). Different subregions of the amygdala function differently in the regulation of fear memory during conditioning and extinction. During conditioning, the LA subregion receives sensory information for the CS and US association from the cortical region and activates BA and dITC in the amygdala (Kim et al, 2006). The BA region activates CeM which is the output center for the fear memory while dITC which is the GABAergic neuron center, sends inhibitory connections to the vITC and inhibits its activity. The vITC has inhibitory GABAergic projections to the CeM of the amygdala which is restricted in conditioning. LA also sends the glutamatergic connection to the CeL of the amygdala and activates PKC zeta-on neurons which in turn inhibit PKC zeta-off neurons which are involved in inhibition of the CeM activity (Pare and Duvarci, 2012). During extinction, the LA subregion receives CS inputs but the lITC with GABAergic inputs to LA inhibit its activity. However, the extinction neurons in BA are activated by the contextual inputs to BA (Pare and Duvarci, 2012), extinction neurons, in turn, inhibit fear neurons (Lee et al, 2013). The activated extinction neurons project to the vITC which upon activation through GABAergic connection inhibit the activity of CeM (Pare and Duvarci, 2012). The vITC during extinction do not receives inputs from dITC and inhibit CeM activity directly through its inhibitory connection to enhance extinction (Pare and Duvarci, 2012). The PKC zeta-off neurons get activated as it is not inhibited by both dITC neurons and PKC zeta-on neurons, the PKC zeta-off neurons then sends inhibitory connections to CeM during extinction learning (Pare and Duvarci, 2012).

The mPFC contain two subdivisions namely PL and IL in rodents, and their human homologs, which exhibited distinct roles for the activity of fear circuit. The dorsally located Prelimbic cortex (PL) is considered to regulate fear expression, while the ventrally located Infralimbic cortex (IL) mediates the suppression of fear (Quirk and Beer, 2006; Sotres-Bayon and Quirk, 2010; Milad and Quirk, 2012; Riga et al., 2014). The PL has innervations bi-directionally

from the BA where it activates the fear neurons as well as modulates the activity of BA (Lee et al, 2013). The IL furthermore innervates extinction neurons in BA and vITC neurons during extinction learning to enhance extinction (Lee et al, 2013).

The hippocampus receives contextual information and conveys this information further to the BLA for further processing (Maren, 2001; Orsini and Maren, 2012; LeDoux, 2014). Furthermore, the direct monosynaptic connections of the hippocampus to BLA also exist via mPFC and entorhinal cortex (Saunders et al., 1988). Tronson et al. (2009) found in their studies that CA1 activity was increased in conditioning and extinction as evident by the c-fos and pERK expression (Tronson et al., 2009), while optogenetic inhibition of CA1 results in long lasting inhibition of fear retention (Goshen et al, 2011). Hippocampus is mainly divided into three main regions as CA1, CA3 and DG, the brain parts associated with the fear learning. The CA1 is the output of the hippocampus from the information which projects to subiculum and entorhinal cortex (Cenquizca and Swanson, 2007). Both the CA1 and CA3 have been found to be essential for acquisition and retention of the fear memory in contextual learning paradigm (Jinzhao and Maren, 2008). Moreover, in BL, the most important subregion of BLA has been found to innervate the hippocampal CA1 and CA3 regions (Pikkarainen et al., 1999). The hippocampal innervations to mPFC modulate the activity of mPFC which in turn regulate the activity of the amygdala during conditioning and extinction of fear memory (Godsil et al, 2013). The hippocampal innervation to IL is involved in regulation of the extinction of fear memory (Gilmartin et al, 2014) (Fig 7).

The coordinated activity of the amygdala, PFC and Hippocampus regulate the extent of fear memory consolidation and extinction. This is interesting that the brain regions involved in the acquisition of fear memory are also associated with the extinction of fear memory and in some brain region, the fear and extinction circuitry shows common overlapping pathways. However, some areas associated with the acquisition and consolidation of fear memory, are commonly not involved in extinction learning and vice-versa (Myskiw et al., 2010). The current status of the findings also shows that in the amygdala (Herry et al., 2008) and hippocampus (Tronson et al., 2009) there are different types of neurons getting activated during conditioning and extinction. So, to answer the question how different types of neuronal population and set of genes are involved in fear memory consolidation and extinction, a detailed study is in need.

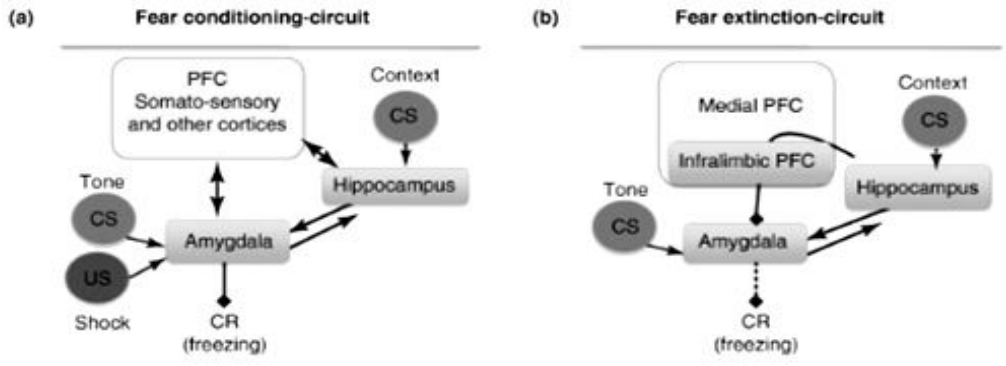



**Fig 7.** Diagrammatic representation of **(a)** Fear conditioning and **(b)** Fear extinction circuitry involving amygdala, PFC, and Hippocampus.

## 2.4. Molecular biology of fear

The consolidation and extinction of fear is a dynamic process which requires the synthesis of a number of proteins such as transcription factor, neurotransmitters and cytoskeleton proteins (Alberini, 2009; McGaugh, 2000). Recent research showed that immediate early genes c-fos and JunB are required for the consolidation and extinction in contextual fear memory and show different response during conditioning and extinction learning (Huff et al., 2006; Strekalova et al., 2003).

The synthesis and function of these proteins are under the control of inter and intracellular signaling cascades in the amygdala, PFC, and hippocampus. The activated signaling in fear memory consolidation and extinction initiates neural plasticity in these subregions which involves NMDA receptor activity in postsynaptic neurons (Johansen et al, 2011; Zimmerman and Maren, 2010). The NMDA receptor activity in synapses conveys calcium ion signaling which is important for synaptic plasticity as well as for the formation of memory (Johansen et al, 2011). Activated NMDA receptor results in elevation of intracellular calcium level which further increases the auto-phosphorylation of CaMKII (Ca2+/Calmodulin (Cam)-dependent protein kinase II) (Silva, 2003; Rodrigues et al., 2004a). Moreover, the CaMKII activates the phosphorylation of AMPA receptor (GluA1 subunit) which results in enhanced synaptic plasticity in LA in fear conditioning and extinction (Malinow and Malenka, 2002; Yeh et al., 2006). The CaMKII then activates PKA (Protein kinase A) which then further activates MAPK/ERK pathways. The activated MAPK/ERK then activates the phosphorylation of CREB (cAMP response element binding), a factor required for transcription of plasticity-related genes (Orsini and Maren, 2012). Although the role of CREB in conditioning and extinction is well known but some results showing CREB phosphorylation (Hall et al., 2001; Mamiya et al., 2009) while others showing its inhibition in consolidation and extinction of fear memory (Lin et al., 2003). The CREB activity was not found to be associated in the hippocampus for contextual fear extinction (Tronson et al., 2009), however, the involvement of protein synthesis has been extensively studied in the hippocampus during extinction. The studies using intra-hippocampal administration of the protein synthesis inhibitor anisomycin has been found to disrupt learning in inhibitory avoidance paradigm (Cammarota et al., 2005; Vianna et al., 2001). The inhibitory neurotransmission in LA subregion has been found to be associated with the conditioning and regulate the activity of neurons (Pare´ et al., 2003; Ehrlich et al., 2009). Neural plasticity during fear learning is also regulated by the metabotropic glutamate receptor activity (Nakanishi, 1994). The formation of LTM for conditioning and extinction requires synaptic strengthening and activates secondary messenger proteins for further signaling (Kandel, 2001; Alberini, 2009; Herry et al., 2006).

The activated transcription factors thus activate RNA which translates into proteins associated with the memory formation (Hernandez and Abel, 2008; McGaugh, 2000; Davis and Squire, 1984). It has been observed that inhibition of protein synthesis by the infusion of protein synthesis inhibitor anisomycin in LA disrupts the LTM formation (Schafe and LeDoux, 2000; Duvarci et al., 2008). The broad spectrum RNA inhibitors also have been found to disrupt LTM stabilization through inhibition of RNA transcription (Duvarci et al., 2008, Hoeffer et al., 2011). Overall, the transcription and translation processes are required for fear memory consolidation and extinction (Jarome et al, 2011). Although transcription events happen in cyton but the translation takes place both in soma and dendrites that results in synaptic strengthening (Helmstetter et al., 2008). The Arc/Arg3.1 protein (Activity-regulated cytoskeletal-associated protein) is one of the transcript products at synapses which are found to be up regulated during conditioning in LA (Guzowski et al., 2000; Ploski et al., 2008). Recently the researchers have also shown the

association of glucocorticoids in fear memory consolidation and extinction (Rodrigues et al., 2009) (Fig 8).

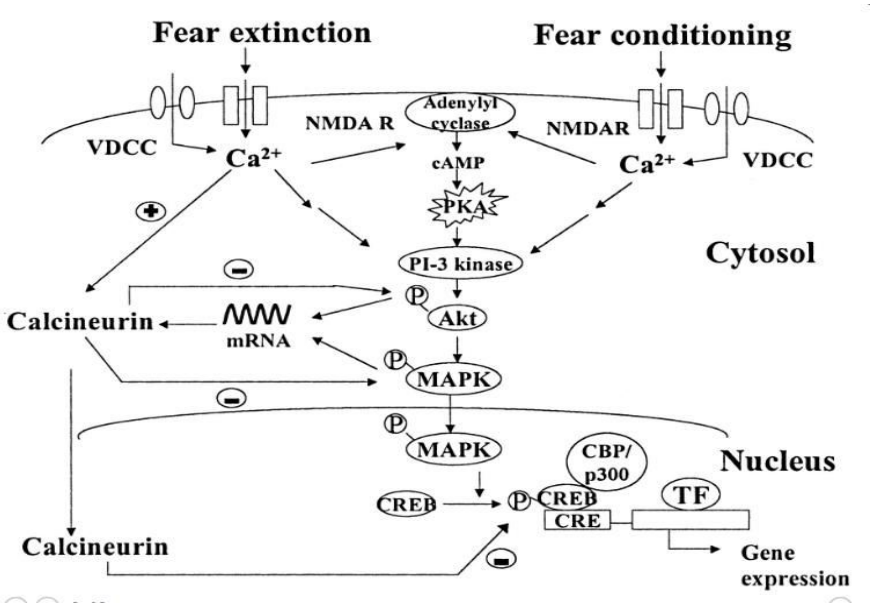



**Fig 8.** Diagram showing the model of the molecular mechanism involved in fear memory consolidation and extinction in the amygdala (Lin et al, 2003).

## 2.5. Epigenetics of fear

The epigenetic mechanism involves a change in the state of chromatin and its expression without posing a change in underlying DNA sequence. The mechanism includes histone acetylation, methylation, phosphorylation, sumoylation and DNA methylation which alter the expression profile (Ruthenburg et al., 2007; Peixoto and Abel, 2013). By the last few decades, epigenetic associations of fear memory and other cognitive processes have been well established by a number of studies in rodents and primates. Of which histone acetylation is the most studied epigenetic mechanism associated with the fear memory consolidation and extinction. Histone acetylation at specific lysine residue on N-terminal histone tail promotes transcription of genes (Ruthenburg et al., 2007; Grayson et al., 2010). Interestingly, the formation of memory has been found to be associated with an increase in histone acetylation in the different brain regions of the rodents (Alarcon et al., 2004; Guan et al., 2002; Levenson et al., 2004; Vecsey et al., 2007). The histone acetylation at various H4 and H3 residues promotes accessibility of the DNA for various transcription factors resulting in expression of the gene (Clayton, 2006). Both the cued and contextual fear memory are regulated by increased histone H3 and H4 acetylation which could further be strengthened by the use of HDACi (e.g. TSA, SAHA) (Peixoto and Abel, 2013).

The acetylation of histone is regulated by the activity of histone acetyltransferases (HATs) and histone deacetylases (HDACs). Moreover, the mutations or deregulation of different KATs or HDACs results in various neurological and neurodegenerative dysfunction. The histone acetylation is found to be associated with the active gene transcription, whereas reduced or no histone acetylation represses gene transcription (Fischer et al, 2010). Researchers have shown that the

enhanced histone acetylation is an essential molecular mechanism involved with fear memory consolidation and extinction (Guan et al, 2009, Bredy et al 2007).

The role of histone acetylation was also confirmed by the studies showing enhanced histone H3K14 acetylation in CA1 following contextual fear conditioning (Levenson et al, 2004). Though histone acetylation is associated with the memory but different types of learning and learning paradigm shows different association with the epigenetic changes (Levenson et al, 2004). This increased histone acetylation during memory formation regulates the expression of genes such as c-fos, CREB, BDNF, Erg1, involved in different phases of memory (Lubin et al, 2008; Bredy et al, 2007). Recent evidence has shown that extinction learning is associated with the altered HAT activity. Following extinction, there is enhanced expression of the HAT (p300/CBP-associated factor, PCAF) in the rodent IL, and intra-IL infusion of the PCAF activator (SPV106) facilitates extinction and inhibits the renewal of fear (Wei et al., 2012). The evidence has shown that the histone acetylation is associated with the consolidation and extinction of fear memory (Whittle and Singewald, 2014), and increased histone H4 acetylation at the promoter region have been studied at bdnf exon IV following extinction learning (Bredy et al, 2007). Furthermore, the acetylation of histone 3 (H3) in CA1 is found to be associated with the consolidation of contextual fear memory which was enhanced by the use of histone deacetylases (HDAC) inhibitors when given prior to the training experiment (Wood et al., 2005; Vecsey et al., 2007; Levenson et al., 2004)

## 2.6. HDACs in memory consolidation and extinction

Histone deacetylases (HDACs) are the class of enzymes that catalyze the removal of acetyl groups from the lysine residues of histone and non-histone proteins and suppress the activity of the gene. There are 18 different HDAC subtypes, and based on sequence similarities have been classified into four classes from class I to class IV. The class I, II and IV are known as classical HDACs as their activity is inhibited by the TSA (Trichostatin A) and shows zinc-dependent activity, while Class III HDACs, known as sirtuins are not affected by the trichostatin A and are NAD+-dependent proteins. Class I HDACs include HDAC1, HDAC2, HDAC3 and HDAC8 isoforms. Of these HDACs, HDAC1 and HDAC2 are known for their extensive involvement in memory like functions (Seto and Yoshida, 2014).

The Class I HDACs are generally localized into the nucleus except for HDAC3, which shuttle between nucleus and cytoplasm, and HDAC8 which is present both in cytoplasm and nucleus. Class II HDACs include HDAC5, HDAC6, HDAC7, HDAC9 and HDAC10 which are able to shuttle from nucleus to cytoplasm to target nonhistone proteins (Seto and Yoshida, 2014). Class III HDACs includes sirtuins (SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, and SIRT7) which are localized in cytosol and nucleus both. There is little information about the Class IV HDAC, HDAC11 which is localized in nucleus and cytoplasm both. HDAC11 is considered to regulate the stability of the protein CDT1 which is involved in DNA replication (Seto and Yoshida, 2014).

Recent findings in research have shown that Class I HDACs, and particularly HDAC1 and HDAC2, are actively involved in CNS development and synaptic transmission (Montgomery et al. 2009). Studies using HDAC1 and HDAC2 deletions in embryonic stages showed abnormal development in the cortical and Hippocampal region (Montgomery et al. 2009). However, the deletion of HDAC2 results in improvement of learning and memory processes (Guan et al, 2009) while it's overexpression results in impaired fear memory. The knockout studies for HDAC2 again

show enhanced extinction learning in fear conditioning paradigm (Morris et al. 2013). Likewise, the class I HDAC, HDAC3 is also known to be a negative regulator of the fear memory, and its deletion improved memory in an object recognition task (McQuown et al. 2011). As compared to the class I HDACs less information is available about the functions of Class II HDACs. As compared to other HDACs of class I HDAC, the deletion of HDAC4 in the forebrain of mice results in impaired learning in the Morris water maze experiment (Kim et al. 2012).

The immense involvement of HDAC inhibitors in the promotion of fear memory consolidation and extinction has raised a number of suggestions to decipher the role of HDACs in the treatment of various cognitive dysfunctions (Fig 9). Thus it has become a novel target to study the epigenetic involved in memory formation and extinction.

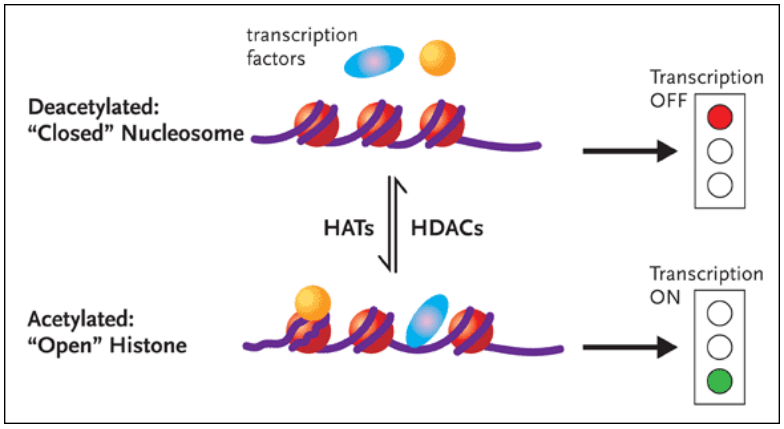



**Fig 9.** Diagram showing the mechanism of HDACs in the regulation of gene expression

## 2.7. HDACs and HDAC inhibitors in fear memory

After analyzing the role of HDACs and its inhibitors in memory formation and extinction the scientists turned their focus towards them as a newer therapeutic approach. The valproic acid (VPA) a neuroprotective drug and potent HDAC inhibitors have been used in mice with Alzheimer's disease (Qing et al. 2008). The Vorinostat (suberoylanilide hydroxamic acid or SAHA) promotes fear extinction through enhanced histone acetylation in the promoter region of NMDA receptor and in the P4 promoter region of BDNF exon IV which is involved in synaptic plasticity and long-term memory (Bredy et al, 2007; Fujita et al, 2012). The widely used HDAC inhibitors selectively affect class I HDACs which affects the stabilization of learning and memory, establishing HDAC class I as a potential target to control fear learning and cognitive functions (Haggarty and Tsai, 2011).

Later it was shown that the impairment of HDAC2 promotes the synaptic plasticity and related genes involved in LTP during fear consolidation and extinction (Guan et al, 2009; Morris et al, 2013). These results suggest that HDAC2 can be a potential target molecule in the regulation of fear memory and target this HDAC might be helpful for PTSD conditions through CBT. Later, the role of HDAC1 on fear extinction promotion has been studied through HDAC1 overexpression studies in mice (Bahari-Javan et al, 2012). Another study has shown that HDAC1 siRNA introduction or HDAC inhibitor introduction promotes histone acetylation at BDNF exon IV promoter (Yasuda et al, 2009). Similar to HDAC2, HDAC3 is known for its association with inhibition of long-term memory in fear learning as its ablation enhanced the memory for spatial learning and CPP

(conditioned place preference) task (McQuown et al, 2011; Malvaez et al, 2010). Furthermore, HDAC8 functions as an inhibitor for the extinction of fear memory through inhibition CREB pathway suggesting CREB as a potential target for enhancing extinction learning (Kida et al, 2002; Gao et al, 2009).

The use of HDAC inhibitors in cognitive enhancement is extensively studied; the use of systematic or in local introduction promotes the conditioning and extinction of fear memory (Morris et al, 2013; Maddox and Shafe, 2011). Similarly, intrahippocampal HDAC administration enhanced the extinction learning for a number of conditioning paradigm (Lattal and Wood, 2013; Lattal et al., 2007; Bredy and Barad, 2008). The types of HDAC isoform as in case of HDAC1 and HDAC2 subtypes affect the extinction memory differently as the gene silencing of HDAC2 but not HDAC1 promotes extinction learning (Grayson et al, 2010; Morris et al, 2013). Thus the studies converge with the use of individual HDAC subtype inhibitors in the enhancement of extinction learning and cognitive functions. The global HDAC inhibitor such as valproic acid has advantageous over other HDACs as it is widely used as a mood stabilizer with no side effect (Peterson and Naunton, 2005). The Valproic acid in brain targets mainly the class I HDACs and also target GABAergic signaling (Mimaki et al, 1984).

## 2.8. Rationale of Study

The drawback of exposure therapy in the treatment of anxiety disorder is the limitations caused by the therapy as the psychological conditions of anxiety often relapse with the passage of time and the change in context. The extinction memory caused by the exposure therapy is generally labile which raise a question for the development of the new effective method. The use of pharmacotherapy together with exposure therapy is a current method for treatment of anxiety disorders. Of which the use of HDAC inhibitors is commonly used cognitive enhancers to promote extinction learning. Although the HDAC inhibitors are the promising target for the therapy but the use of HDAC subtype selective target are still in demand as all HDACs are not an inhibitor of extinction learning (e.g. HDAC1).

***Chapter 3***

# AIMS & OBJECTIVES

**Specific aim 1**

**To find out the role of histone acetylation/histone deacetylation during fear memory consolidation and extinction in PFC, Hippocampus and Amygdala and correlate it to the expression of immediate early genes involved in LTP.**

**Experiment no.1:** Behavioral training for fear Conditioning and Extinction.

**Experiment no.2:** Immunohistochemistry.

**Specific aim 2**

**To find out the effect of HDAC inhibition on Consolidation and extinction of fear memory.**

**Experiment 1:** HDAC inhibitor administration during fear memory consolidation and extinction.

**Experiment 2:** Immunohistochemistry for HDAC1/HDAC2 and c-fos in PFC, Amygdala and Hippocampus.

***Chapter 4***

# MATERIAL & METHODS

## 4.1. Animals

For study male Sprague-Dawley rats (250-300 gm) were used. Rats were housed individually with access to food and water *ad libitum*. Rats were handled for 15 days up to 2-5 minutes each day on a 12-h light/dark cycle, 23°C temperature. All experiments were carried out under strict compliance with Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Environment and Forests, Government of India (Approval number 853/AC/04/ CPCSF).

## 4.2. Apparatus for behavioral study

For behavior training, two identical observation chambers were used each of which was made up of plexiglass (VJ instrument) kept in sound attenuated cabinets for fear conditioning and extinction training. The floor of the chamber which consisted of stainless steel rods (4 mm, diameter), 1.5 cm spaced apart was used for the delivery of foot-shock (US). The first chamber named Context A was used for conditioning, consisted of transparent plexiglass walls with stainless steel rods floor with the arrangement of sound and light, all kept inside the white sound isolated chamber. Whereas another chamber named Context B was used for extinction. This consisted of white black strips on wall and floor covered with white paper and provided with vanilla essence for change in context. For delivery of acoustic CSs, the speaker was arranged outside the wall of the chamber. For the supply of background noise, ventilation fans were arranged in the acoustic chambers.

## 4.3. Fear Conditioning

Fear learning in the rats was performed by providing a paired neutral stimulus/conditioned stimulus (CS) such as tone with a noxious unconditioned stimulus (US) such as a mild foot shock. At day first rats were exposed to context A for 3 min followed by fear conditioning in the context A (V. J. Instruments). Fear learning involves five paired trials of CS (tone 80 dB, total duration 10 s) with US (1 s foot shock 0.7 mA, inter-trial interval: 60 s), provided to the animals. The US co-terminated with the CS. The freezing was measured by calculating freezing percent during conditioned fear response as absent of all body movement except respiration, non-awake, and rest body procedure. Freezing response was videotaped and calculated offline by recording the overall time which was spent in 10-second tone CS. All the animals were then returned to their home cages following experiment. The control naïve group included in this study receives no experimental conditions. Animals from each group ($n$ = 8-10) were sacrificed 2 h following experiment (Fig 10).

## 4.4. Fear Extinction

24 hours following conditioning, fear extinction was performed in animals. Starting with 3 minutes of acclimatization period extinction training was performed in a context B (V. J. Instrument). All the animals were presented with 30 trials of CS (tone, 80 dB total duration 10 s, intertrial interval: 10 s) without presentation of US. Animals ($n$ = 10 per group) were sacrificed for immunohistochemical analysis 2 hr after extinction training. An additional group of the same-aged animals did not receive any experimental manipulations, were used as naïve control in all experiments (Fig 10).

Conditioning, extinction as well as control groups underwent retention test 24 h following training. Testing involves the presentation of the CS (5 tones, total duration 10 s, tone, 80 dB, inter-

trial interval: 10 s) in context B in the absence of foot shock. The animals, which were used for IHC (immunohistochemistry) did not experience retention test.

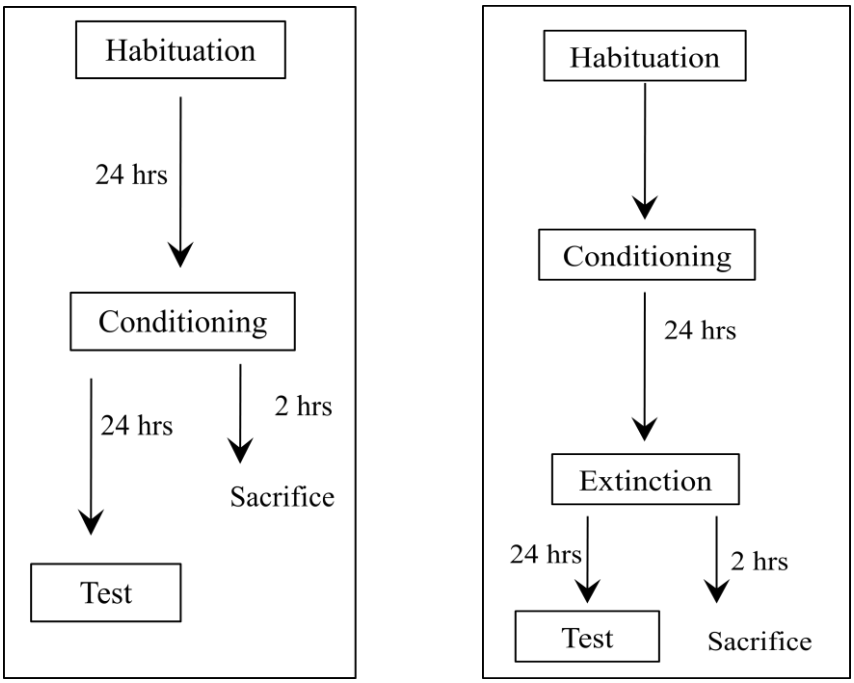



**Fig 10:** Diagrammatic representation of protocol for fear memory consolidation (left) and extinction (right)

## 4.5. Control groups

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

## 4.6. Scoring

The freezing response was measured as the absence of all non-respiratory movements. The scoring was performed for the session as '1' for the movement and '0' for immobility. Freezing was scored for every 2 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 get 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.

## 4.7. Details of Brain Sub-Regions under Study

Three brain regions (Amygdala, Hippocampus and Prefrontal cortex) involved in fear memory consolidation and extinction were taken into the study. Amygdala consists of LA (lateral

amygdala), BA (basal amygdala), CeL (centrolateral amygdala), and CeM (centromedial amygdala) (McDonald 1998; Turner and Herkenham 1991; Krettek and Price 1978a; Petrovich and Swanson 1997; Veening et al. 1984). The prefrontal cortex (PFC) includes PL-PFC (prelimbic prefrontal cortex) and IL-PFC (infralimbic prefrontal cortex), which regulate the expression as well as suppression of fear in rodents, respectively (Giustino and Maren 2015).

## 4.8. Valproic acid/ Drug administration

Sodium valproate (VPA, valproic acid) and valproate semisodium form are used in medications commonly for treating epilepsy, bipolar disorder and in the prevention of migraine headaches. It is also used in the prevention of seizures and can be introduced intravenously or by mouth. HDAC inhibitor Valproic acid was dissolved in 0.1M PBS solution and injected intraperitoneally at a concentration of 100mg/kg of body weight (Bredy and Barad, 2008). The time selected for the drug or vehicle administration was 2 hr before the behavior experiment as indicated in previous studies (Bredy and Barad, 2008; Tremolizzo et al 2002) (Fig 11).

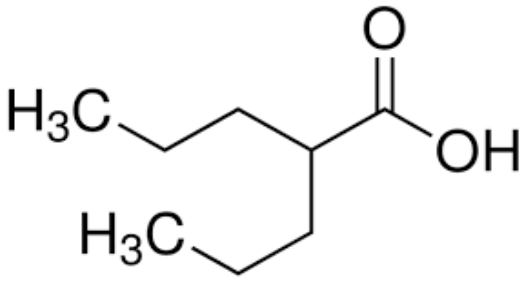



**Fig 11.** Structure of valproic acid

## 4.9. Anxiety measurement for valproic acid

For understanding the effect of Valproic acid on anxiety in rats EPM (Elevated Plus Maze) test was performed. EPM includes two open and two closed arms (40x10 cm) connected at the center so as to form plus-shaped structure. The EPM maze is elevated above 50cm from the ground and was placed in the dim lightroom. Rats were placed at the center of the maze facing closed arms. Rats were observed after placing them in the center for up to five minutes and the entries for the rats entered into open arms were recorded as time spends on each arm. The percent of entries in open arm was recorded during five minutes of exposure in EPM. The entries were calculated as

**% Time in open arm = Time spend in open arm x 100/**
**Total time spend in open and closed arms**

(Rats usually are nocturnal and prefer to stay in closed arm more than open arm)

## 4.10. Fear Conditioning in valproic acid treated animals

Animals from drug + conditioning and sham + conditioning were exposed to context A for 3 minutes prior to the fear conditioned in the context A (a transparent Plexiglas chamber with metal grids that was cleaned before each session with 70% ethanol) (V. J. Instruments). For seeing the effect of valproic acid on conditioning a weak conditioning protocol was used (Kishioka et al,

2009). Fear conditioning comprises of 5 trials of paired Conditioned Stimulus (CS, total duration 10 s, 80 dB) with the Unconditioned Stimulus ( US, 1 s foot-shock 0.5 mA, inter-trial interval: 60 s). The US was presented at the end of CS where US co-terminated with the CS. The freezing was measured as cessation of all movement except breathing, non-awake and rest body procedure during the conditioned fear response. Freezing was videotaped from video tracking device and scored offline by recording total time spent during tone CS (V.J. instruments, India). The freezing was measured in terms of percent learning which is defined as continuous inactivity lasting for at least 2 seconds. Animals were replaced back to their home cages after the experiment. Sham control and drug only groups were included which had no exposure to the experimental conditions but received vehicle or drug only. Each group includes 6-8 animals and sacrificed 2 hours following training (Fig 12).

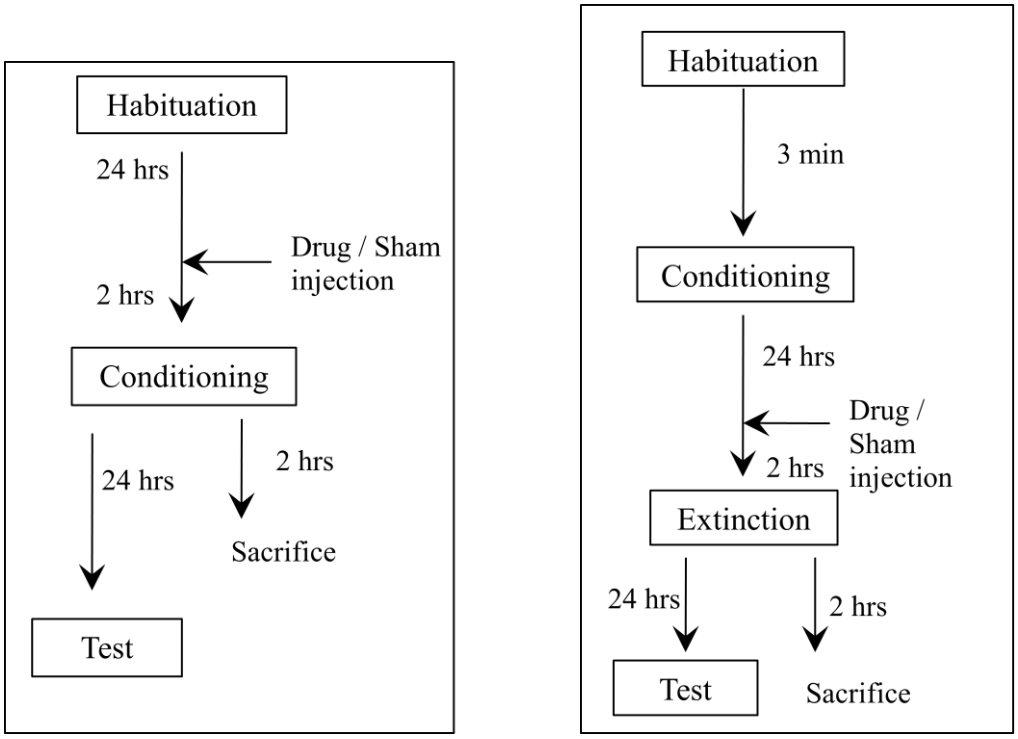



**Fig 12:** A diagrammatic representation of the protocol for fear memory consolidation (left) and extinction (right) after valproic acid treatment

## 4.11. Fear Extinction in valproic acid treated animals

The animals from Drug + Extinction and Sham + Extinction were exposed to context A for 3 minutes prior to the fear conditioned in the context A (a transparent Plexiglas chamber with metal grids that was cleaned before each session with 70% ethanol) (V. J. Instruments). To visualize the effect of valproic acid on extinction a strong conditioning protocol was used which causes a lower level of extinction (Taherian et al, 2014). Fear conditioning comprises of 5 trials of paired Conditioned Stimulus (CS, total duration 10 s, 80 dB) with the Unconditioned Stimulus (the US, 1 s foot-shock 1 mA, inter-trial interval: 60 s). The US was presented at the end of CS where US co-terminated with the CS. The extinction training was performed 24 hours later conditioning. Animals were allowed for 3 minutes of acclimatization period before the extinction training in context B (V. J. Instrument) followed by the presentation with 30 trials of CS (tone, 80 dB total duration 10 s,

intertrial interval: 10 s) without presentation of US. Animals ($n$ = 10 per group) were sacrificed for immunohistochemical and mRNA study. An additional group of same-aged animals did not receive any experimental manipulations, were used as a naïve control in all experiments. The freezing was measured as discussed above. Animals were replaced back to their home cages after the experiment. Sham control and drug only groups were included which had no exposure to the experimental conditions but received vehicle or drug without. Each group included 10-12 animals and sacrificed 2 hours after the experiment (Fig 12).

**4.12. Controls of the valproic acid treated conditioning and extinction group**

For removing the confounding effect of valproic acid or vehicle on behavior two additional groups of animals were included as a sham control and drug only groups. The sham or drug groups ($n$ =10) received vehicle or drug injection intraperitoneally without receiving any behavioral exposure. All the animals were perfused 2 hours following exposure to the drug or vehicle. Animals of the control groups were used for the molecular studies in immunohistochemistry and real-time PCR.

**4.13. Behavioral test**

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

**4.14. Tissue Preparation for immunohistochemistry**

Two hours following a training session, rats were anesthetized with pentobarbital (60 mg/kg, i.p.), perfused transcardially with n-saline, followed by ice-cold 4% paraformaldehyde (in 0.1 M phosphate buffer, pH 7.4). Animals were then decapitated, and the brains were removed and post-fixed in 4% paraformaldehyde for 24 hrs followed by cryoprotection in 10%, 20% and 30% sucrose solutions (in 0.1 M phosphate buffer, pH 7.4) sequentially. Brains were then frozen in isopentane at -30 to -35$^0$C for 30 min and kept at -80$^0$C. Coronal Sections (20 µm thick) containing amygdala and mPFC regions were obtained by cryo-sectioning (Microm HM 525, Germany).

**4.15. Tissue preparation for Real-time PCR**

After behavioral experiments rats were anesthetized using pentobarbital (40 mg/kg, i.p.) and transcardially perfused with chilled normal saline. Animals were then decapitated and brains were removed and cut into three pieces. Brains were then frozen in at -35°C for 30 mins and kept at -80°C until sectioning. Tissues from BLA (basolateral amygdala), CeA (central amygdala), PL, IL, CA1, CA3 and DG subregions were isolated using needles under a dissection microscope. Tissue was chopped from the brain region and kept for mRNA isolation. Tissues from 3 animals were pooled together for each brain region for RNA isolation and Q-PCR studies.

**4.16. Immunohistochemistry**

20 µm coronal brain sections were collected serially from the brain regions containing PFC and Amygdala from all groups for each antibody to have matching sections for each antibody from

each group. The sections were then washed and blocked in PBS (0.01M Phosphate buffer saline) containing 1% normal horse serum (NHS Vecta-stain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA), 0.25% tween 20. Brain sections were incubated overnight at room temperature in anti c-fos (ab7963), acetyl- H3K9 (ab10812), anti-acetyl-H4K5 (H5110-15E2), anti-HDAC1 (H1827-51J) and anti-HDAC2 (H5109-47E) primary antibodies (rabbit monoclonal, 1:1000, 1:1000, 1:200 and 1:1000 dilutions respectively) overnight. Sections were then incubated with a biotinylated secondary antibody (anti-rabbit IgG, 1:500 dilution, Vecta-stain Elite ABC kit,) for 2 h at room temperature followed by Vecta-Stain Elite ABC kit (Vector Laboratories) and DAB staining (DAB peroxidase substrate, Abcam ab64238). Stained sections were mounted on glass slides and images from the sections were acquired from at least three sections per region of the brain using the NS-BR image analysis software from Nikon. Expression was analyzed as the number of positive nuclei in the amygdala, hippocampus and PFC. Number of positive neurons were counted using the Nis-Basic Research image analysis system (Nikon, Tokyo) attached to a Nikon Eclipse Ni microscope (Nikon, Tokyo, Japan).

## 4.17. RNA isolation and cDNA preparation

Total RNA was isolated from rat PFC punches using Stratagene absolute RNA isolation kit (Agilent technologies, Catalog no. #400800) and treated with DNase I (Stratagene) to remove genomic DNA (Section-4.18, RNA isolation protocol). Optical density readings were done. Total RNA was assessed with optical density measurements and DNase-treated total RNA (40-45 ng/µl, OD: 0.045-0.052). Total mRNA was then checked for its purity using spectrophotometer at 260/280nm (O.D. =1.69-1.82) and was run on an agarose gel to check its integrity (Fig 13). Then, Genesure first-strand cDNA synthesis kit (Genetix, PGK-162B) was used to synthesize cDNA from the total tissue mRNA using oligo-dT primers (Table 1, Table 2). Amplified products (6.5 ng/ul) were then used for the real-time PCR amplification.

## 4.18. RNA isolation protocol (Stratagene absolute RNA isolation kit)

1. The frozen tissue stored at –80°C (5 mg) was taken and homogenized with 100ul lysis buffer and 0.7ul βME.
2. Homogenate was transferred to a pre-filter blue spin cup sealed in a 2ml receptacle tube and snap the cap of the tube onto the spin cup.
3. Spin at maximum speed, 5 min.
4. Remove spin cup and discard.
5. The filtrate was added with 100µl 70% ethanol and vortex for 5 min until mixing.
6. The mixture was transferred to RNA binding spin cup seated in a fresh 2ml receptacle tube and cap the spin.
7. Spin at maximum speed 30-60 sec.
8. The spin cup was removed and the filtrate was discarded and replaced the spin cup in receptacle tube.
9. 100µl low salt wash buffer was added, spin at maximum speed for 30-60 sec.
10. The spin cup was removed, the filtrate was discarded, and the spin cup was replaced in receptacle tube.
11. Spin at maximum speed for 2 min, 0.5ul of DNAse was added onto the matrix inside cup and cap spin cup, incubate at $37^0$C for 15 min.
12. 100ul high salt wash buffer was added to spin cup, spin at maximum speed for 30-60 sec.

13. The spin cup removed and retained and the filtrate was discarded, the spin cup was again replaced in a receptacle tube.
14. 100ul high salt wash buffer was added to spin cup, spin at maximum speed for 30-60 sec.
15. The spin cup removed and retained and the filtrate was discarded, the spin cup was again replaced in a receptacle tube. 16. 50ul low salt wash buffer was added to spin cup, spin at maximum speed for 2 min.
17. The spin cup was transferred to 1.5 ml tube and discarded the 2 ml receptacle tube.
18. 30ul elution buffer was added; incubate tube at RT for 2 min.
19. Spin the tube at a maximum speed for 1 min.
20. Steps 18-19 were repeated.
21. Stored at -80$^0$C.

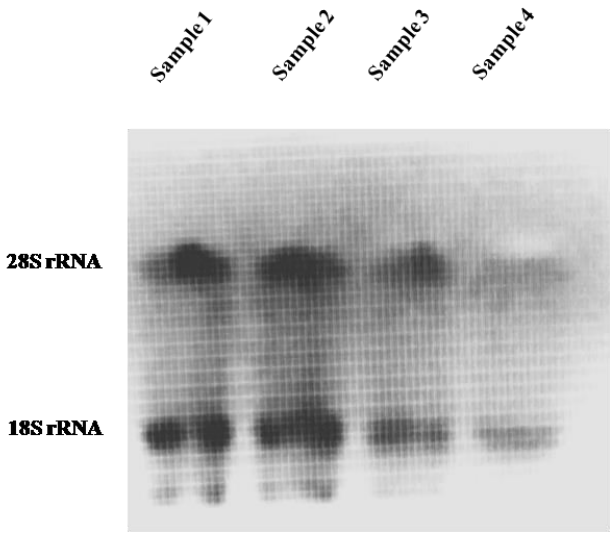



**Fig 13.** Agarose gel electrophoresis of total RNA with intact rRNA bands

**Table 1: cDNA preparation reaction mix**

| Reagent | Volume per reaction (ul/15ul) |
|---|---|
| Template | 3ul |
| Oligo dT primer | 1ul |
| 5x reaction buffer | 3ul |
| RNase inhibitor | 0.75ul |
| dNTP mix | 1.5ul |
| Reverse transcriptase | 0.75ul |
| dd$H_2O$ | 5ul |
| **Total** | 15ul |

**Table 2: Thermal profile for cDNA preparation**

| Temperature ($^0$C) | Time |
|---|---|
| 42$^0$C | 60min |
| 70$^0$C | 5 min |
| 4$^0$C | hold |

## 4.19. Primers

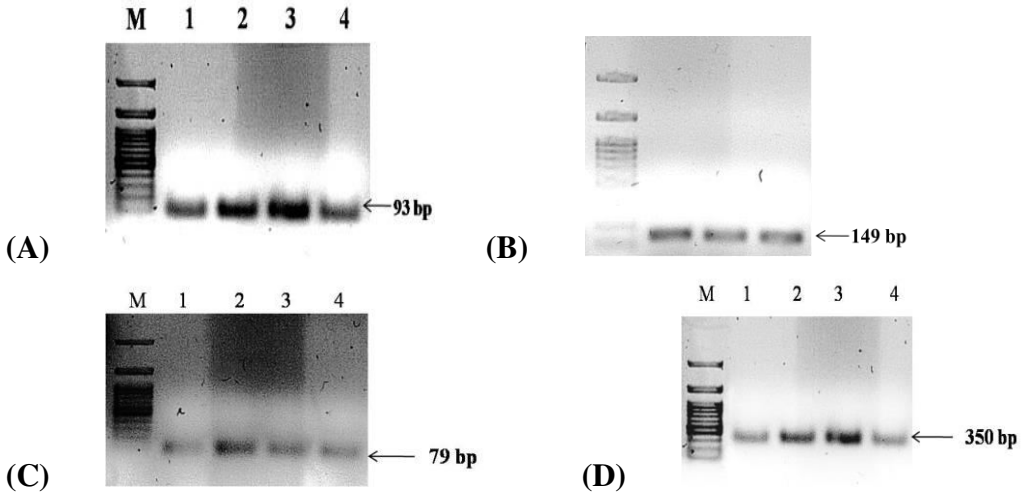
The primers were selected from the literature, checked for their specificity online through primer BLAST by NCBI. The primers were synthesized commercially from Integrated Eurofins, India. Each lyophilized form of primer pair was reconstituted to a final concentration of 100 pmol/µl by dissolving it in TE buffer. The details of the primers used in PCR amplification with their annealing temperatures and expected product sizes are shown in Table 3.

**Table 3: Primer details**

| Primer | Sequence | Annealing temperature | Product Length |
|---|---|---|---|
| c-fos | 5'-CCGACTCCTTCTCCAGCAT-3' (forward)<br>5' –TCACCGTGGGGATAAAGTTG-3' (reverse) | 57.8<br>57.3 | 93bp |
| CBP | 5'-GCTCCTTGCACAGAGAGTGAG-3'(forward)<br>5'-GGAGCAGCAGACTAGGGGTA-3' (reverse) | 60.67<br>60.40 | 350bp |
| HDAC1 | 5'-TCACCGAATCCGAATGACTCATAA-3'(forward)<br>5'-CTGGGCGAATAGAACGCAAGA-3' (reverse) | 59.3<br>59.8 | 149bp |
| HDAC2 | 5'-CGACTGTGAGACTGGAGCAT-3'(forward)<br>5'- AGCCAGTAAGCACGTACAGA-3'(reverse) | 65.5<br>67.9 | 79bp |
| GAPDH | 5'-GGCACAGTCAAGGCTGAGAATG-3' (forward)<br>5'-ATGGTGGTGAAGACGCCAGTA-3' (reverse) | 62.1<br>59.8 | 143 |

### 4.19.1 Primer standardization

Primers were standardized for different Tm values from the template cDNA and the optimal Tm for each primer was used for the amplification. There was no primer dimer obtained in the amplification (Fig 14).



**(E)**

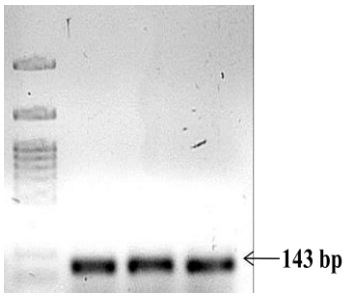



**Fig 14:** Gel images showing the amplified products of **(A)** c-fos (M- DNA ladder, Lane1- Tm 54$^0$C, Lane 2 - Tm 55$^0$C, Lane1- Tm 56$^0$C, Lane1- Tm 57$^0$C) **(B)** HDAC1 (M- DNA ladder, Lane1- Tm 56$^0$C, Lane 2 - Tm 57$^0$C, Lane1- Tm 58$^0$C) **(C)** HDAC2 (M- DNA ladder, Lane1- Tm 54$^0$C, Lane 2 - Tm 55$^0$C, Lane1- Tm 56$^0$C, Lane1- Tm 57$^0$C) **(D)** CBP (M- DNA ladder, Lane1- Tm 54$^0$C, Lane 2 - Tm 55$^0$C, Lane1- Tm 56$^0$C, Lane1- Tm 57$^0$C) and **(E)** GAPDH (M- DNA ladder, Lane1- Tm 56$^0$C, Lane 2 - Tm 57$^0$C, Lane1- Tm 58$^0$C).

## 4.20. Quantitative real-time PCR

Quantitative RT-PCR was used for comparison of mRNA level. Real-time-PCR was accomplished using the Stratagene Max-Pro Real-Time PCR detection System and SYBR green system (Agilent). Relative quantities or fold change of mRNAs were calculated through the comparative Ct method by using the $2^{-\Delta Ct}$ equation. ΔCt is the difference in the Ct values obtained from the comparison of HDAC1 and HDAC2 with the GAPDH control from Q-PCR values. Primers used for cDNA amplification were HDAC1, HDAC2, CBP, c-fos, and GAPDH. For each group, 3-5 animals were used and the real-time PCR was performed in a set of triplicate. Q-PCR reactions were performed in a 20-µl volume containing 10µl SYBR green PCR master mix (2x), 1µl forward and reverse primers both, 3µl cDNA, total volume with ddH2O 20µl. Samples were made ≤ 20 ml with RNase, DNase-free water. Reactions were carried out using a Quant Tect SYBR Green kit (Qiagen) in a Stratagene Max Pro PCR machine as follows: an initial denaturation step at 95$^0$C for 10 min, denaturation at 95$^0$C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 30 s repeated 45 cycles (Table 4, Table 5) (Fig 15, 16).

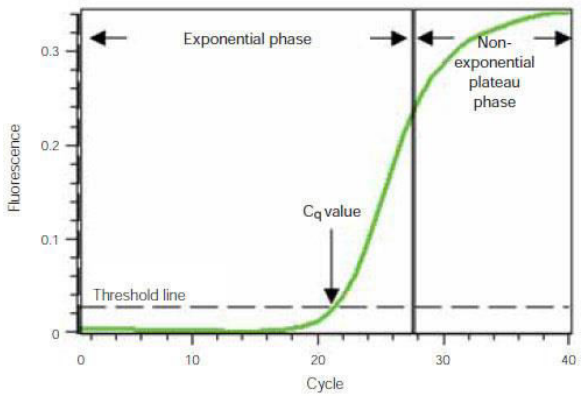



**Fig 15.** Standard amplification plot in real-time PCR showing different phases of the reaction.

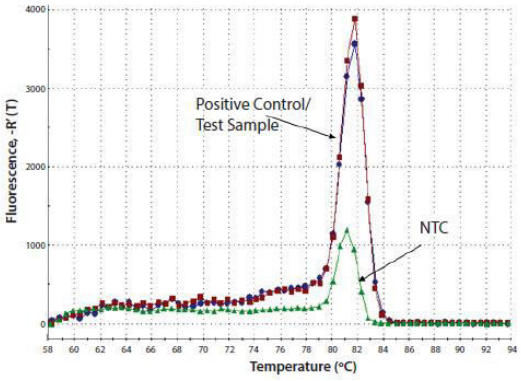



**Fig 16:** The standard melting curve for real-time PCR.

**Table 4: SYBR green PCR reaction mixtures**

| Reagents | Volume per reaction (ul/20ul) |
|---|---|
| SYBR mix (2x) | 10 ul |
| Forward Primer (100mM) | 1ul |
| Reverse Primer (100mM) | 1ul |
| $ddH_2O$ | 5ul |
| Template (cDNA) (~20ng) | 3ul |
| **Total** | 20ul |

**Table 5: SYBR green PCR reaction condition** (40-45 cycles)

| Steps | Temperature ($^0$C) | Time |
|---|---|---|
| Initial Denaturation | 95$^0$C | 10min |
| Denaturation | 95$^0$C | 30sec |
| Annealing | 56-59$^0$C | 30sec |
| Extension | 72$^0$C | 30sec |
| Hold | 4$^0$C | - |

## 4.21. Statistical analysis

Data from behavioral experiments were expressed as means and standard error of the means (±SEM, a measure of the statistical accuracy of an estimate, which is equal to the standard deviation of the theoretical distribution of a large population of such estimates) and were analyzed with one-way analysis of variance (ANOVA). For fear learning the freezing data were transformed into the percent learning. Student *t*-test was used to compare freezing scores across all groups. The mRNA level was compared to fold change using one-way analysis of variance (ANOVA) among all groups.

## 4.22. Image analysis

The sections were analyzed for the positive neurons of different Immunostaining under the up-right microscope from Nikon using NIS Element NS-BR image acquisition software, Nikon. All the images were obtained at 4x and 40x and immune positive neurons were counted manually as well as with the help of NS-BR image acquisition software.

***Chapter 5***

# RESULTS & DISCUSSION: Aim 1

**Aim 1: To find out the role of histone acetylation/histone deacetylation during fear memory consolidation and extinction in PFC, hippocampus and amygdala and correlate it to the expression of immediate early genes involved in LTP.**

## 5.1. Results

### 5.1. 1. Behavior results

#### 5.1.1. 1. Fear Conditioning

The animals exhibited increased freezing response during each successive trial of fear learning. The last trial witnessed enhanced freezing response in both the groups during fear learning as compared to the first trial ($p<0.001$). Both the conditioning and extinction group exhibited similar freezing behavior ($p>0.05$). Overall the two-way ANOVA analysis revealed no significant difference between the groups [$F(1, 80) = 0.456$ ($p>0.05$)] while significant difference in between the trails [$F(4, 80) = 401$, ($p<0.0001$)] during conditioning was further confirmed by the Tukey's Post hoc analysis ($p<0.01$). (Fig 17.A)

#### 5. 1. 1.2. Fear Extinction

On day two, 24 hrs post conditioning, rats from the extinction group were trained in a context B for fear extinction learning by providing tone (CS) in the absence of shock (US). Before the training session rats were allowed to explore the context for 3 mins of habituation period. For entire session percent freezing was measured and the graphs were plotted against the trials. The animals were presented with 30 tones alone in context B and all 30 trials were divided into 6 trial blocks. The animals from the extinction group exhibited a reduction in fear response in each successive trial block and the last trial exhibited a lower level of freezing response when compared to the first trial block of fear extinction ($p<0.0001$). This was confirmed by the two-way ANOVA [$F(4, 25) = 299$, $p<0.0001$] and Tukey's post hoc analysis ($p<0.01$). (Fig 17.B)

#### 5. 1. 1.3. Control groups

Control groups as context only, tone only and shock only to remove any confounds associated with fear and extinction training. The animals from Tone only and Context only groups were allowed to explore the context and remained in the fear conditioning chamber for a time equal to that spent by the fear and extinction learning groups during fear / extinction learning. The tone only group was exposed to the tone and the context the only group was exposed to the context. Shock only (Shock) group was exposed to 5 shock trials without providing time to explore the context so as to remove the association of context with shock. For the overall session the mean % freezing in these control groups was significantly lower as compared to the conditioning group ($p<0.0001$). (Fig 17.D)

#### 5. 1. 1.4. Test for retention of fear consolidation and extinction

Half of the rats from both the conditioning and extinction group underwent retention test following conditioning and extinction training respectively. These animals were not used for molecular analysis. Retention test involved the presentation of five tones (CS) in context B without any foot shock. The conditioned group exhibited a higher level of freezing during the retention test when compared to all the control groups ($p< 0.0001$). (Fig 17.C)

24 hour after extinction training, the remaining half of the rats which did not undergo retention test were perfused and used for molecular analysis in immunohistochemistry and real-time

PCR. The rats from conditioning group ($n$=10), which had not undergone extinction training, were taken as extinction control to compare the extinguished CR of extinction group ($n$=10). All the animals from extinction group exhibited lower freezing response as compared to conditioning group during the retention test.

All the control groups of context only, shock only and tone only exhibited lowest freezing response as compared to the conditioning group for overall trial ($p$<0.0001). The result was also confirmed by Two-way ANOVA for controls which showed significant change for control groups as compared to the conditioning group [F (3, 20) = 150 ($p$<0.0001)] which was also confirmed by Tukey's post hoc analysis ($p$<0.01).

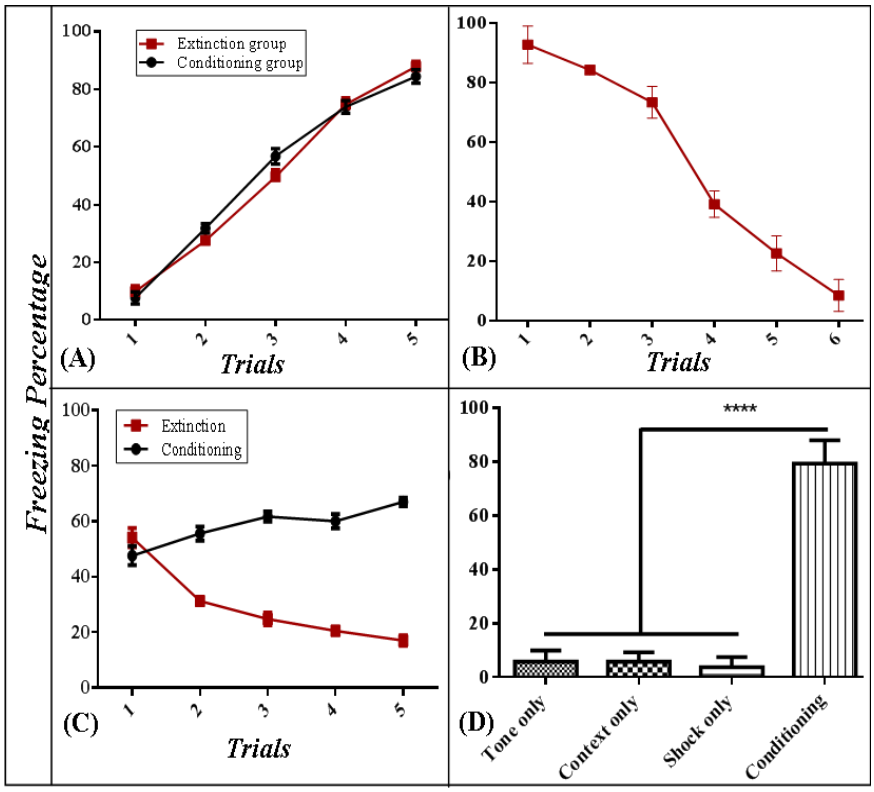



**Fig 17.** Behavior training of conditioning and Extinction **(A)** Conditioning **(B)** Extinction **(C)** Retention test **(D)** Control group freezing.

## 5. 1. 2. Immunohistochemistry

### 5. 1. 2.1. c-fos expression in the Amygdala

c-fos expression was analyzed first to understand the activation mechanism of the amygdala, hippocampus and prefrontal cortex subregions during fear memory consolidation and extinction. It was observed that c-fos expression increased significantly following fear memory consolidation in LA ($p$<0.001), BA ($p$<0.0001), CeL ($p$<0.0001) and CeM ($p$<0.0001) as compared to the naive control group. (Fig 18)

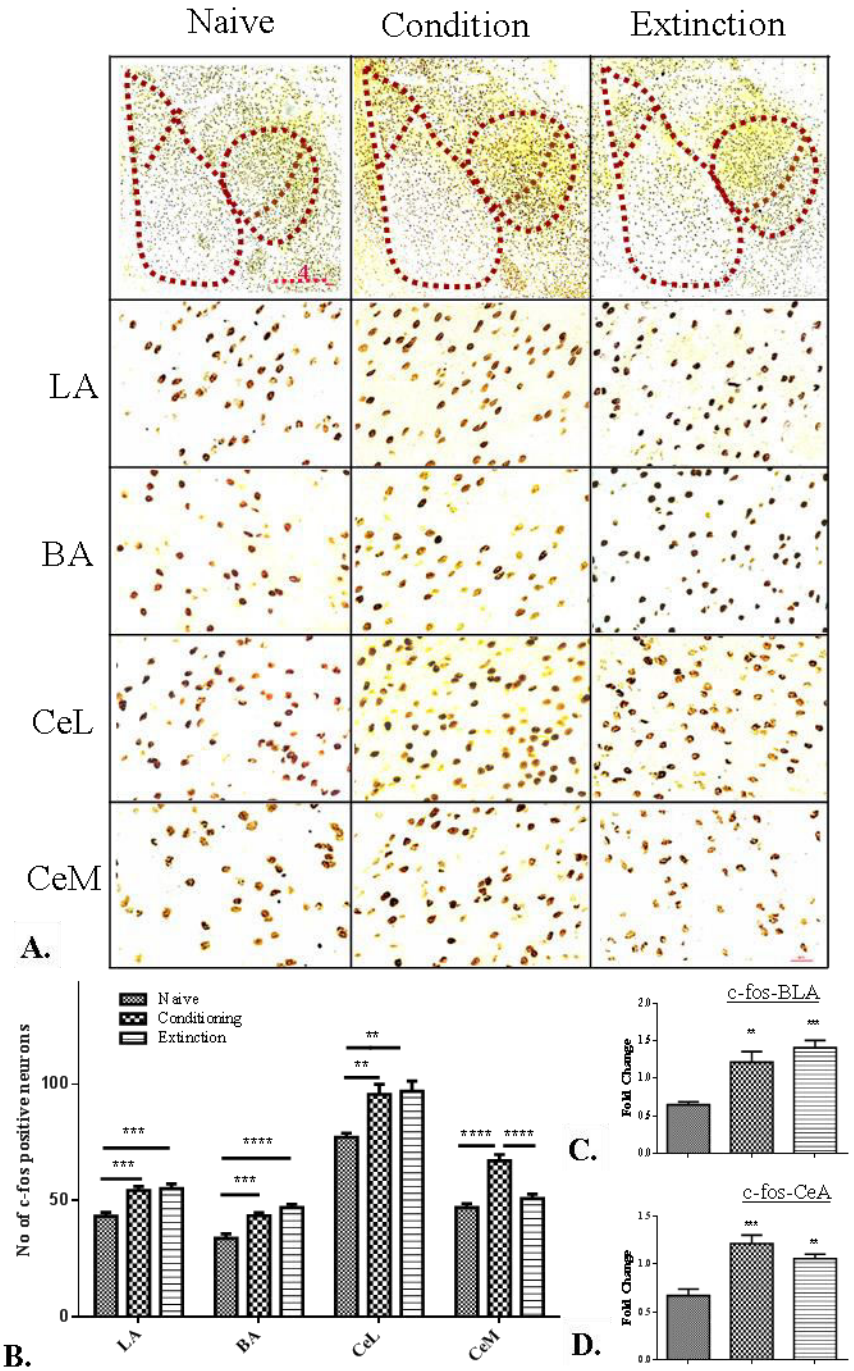



**Fig 18.**c-fos expression in the amygdala in conditioning and extinction. **A.** and **B.** Immunohistochemistry of c-fos expression in LA, BA, CeL and CeM following conditioning and extinction. **C.** and **D.** c-fos mRNA expression in BLA and CeA, respectively.

Following extinction however the c-fos expression increased significantly in LA ($p$<0.0001), BA ($p$<0.001) and CeL ($p$<0.001) but not in CeM when compared to the naive control group ($p$>0.05). The results were confirmed by one-way ANOVA analysis in LA [F(2,27) = 19.96, $p$<0001], BA [F(2,27) = 16.42, $p$<0001], CeL [F(2,27) = 16.79, $p$<0001] and CeM [F(2,27) = 21.36, $p$<0001]. The c-fos expression which is a neuronal activity marker was found to be positively correlated with the fear memory consolidation in LA (p<0.001), BA (p<0.05), CeL (p<0.05) and CeM (p<0.001). A negative correlation of c-fos expression in LA ($p$<0.001), BA ($p$<0.001) and CeL

($p$<0.01) with freezing response were observed following extinction learning which means a positive correlation of activity of these brain region with the extinction learning.

**5. 1.2.2. Histone H3K9 acetylation in the Amygdala**

Further, the study focuses on the participation of histone acetylation in fear memory consolidation and extinction. It was observed that histone acetylation of H3 at K9 increased significantly in LA ($p$<0.0001) [F (2,24) = 19.55, $p$<0.0001], BA ($p$<0.0001) [F (2,27) = 31.61, $p$<0.0001], CeL ($p$<0.001) [F (2,33) = 15.04, $p$<0.0001] and CeM ($p$<0.0001) [F (2,33) = 74.94, $p$<0.0001] following conditioning as compared to the naive control group. While following extinction the histone acetylation of H3 at K9 increased significantly in LA ($p$<0.001) [F (2, 24) = 19.55, $p$<0.0001], BA ($p$<0.0001) [F (2,27) = 31.61, $p$<0.0001] and CeL ($p$<0.001) [F (2,33) = 15.04, $p$<0.0001] but not in CeM following extinction learning as compared to the naive control group. (Fig 19)

**5.1.2.3. Histone H4K5 acetylation in the Amygdala**

Similarly, the acetylation of histone H4 at K5 increased significantly in LA ($p$<0.001) [F (2,45) = 11.75, $p$<0.0001], BA ($p$<0.05) [F (2,44) = 6.251, $p$<0.01], CeL ($p$<0.0001) [F (2,44) = 17.33, $p$<0.0001] and CeM ($p$<0.0001) [F (2,44) = 40.45, $p$<0.0001] following fear learning as compared to the naive control group. However, the acetylation of histone H4 at K5 increased significantly in LA ($p$<0.001) [F (2,45) = 11.75, $p$<0.0001], BA ($p$<0.01) [F (2,44) = 6.251, $p$<0.01] and CeL ($p$<0.0001) [F (2,44) = 17.33, $p$<0.0001] but not in CeM following extinction learning as compared to the naive control group ($p$>0.05). (Fig 20)

**5.1.2.4. HDAC1 expression in the Amygdala**

The HDAC1 expression increased significantly in LA [F (2, 27) = 53.20, $p$< 0.0001], BA [F (2, 27) = 13.14, $p$< 0.001] and CeL [F (2, 27) = 38.82, $p$ < 0.0001] following conditioning as compared to the naïve control group. However in CeM, the HDAC1 expression decreased significantly following fear memory consolidation when compared to the naïve control group [F (2, 21) = 55.88, $p$< 0.01]. HDAC1 expression exhibited a positive correlation in LA ($p$<0.001), BA ($p$<0.001) and CeL ($p$<0.001) with the freezing response observed during conditioning (Table. 1) while in CeM ($p$<0.001) a strongly negative correlation was observed.

Following fear extinction, the HDAC1 expression increased significantly in LA [F (2, 27) = 53.20, $p$< 0.0001], BA [F (2, 27) = 13.14, $p$< 0.01], CeL [F (2, 27) = 38.82, $p$< 0.0001] and CeM [F (2, 21) = 55.88, $p$< 0.0001] as compared to the naïve control group. The LA ($p$<0.001), BA ($p$<0.01), CeL ($p$<0.001) and CeM ($p$<0.001) exhibited a negative correlation for HDAC1 expression with the freezing response observed during extinction learning. (Fig 21)

**5.1.2.5. HDAC2 expression in the Amygdala**

HDAC2 expression decreased significantly in LA [F (2, 24) = 10.98, $p$< 0.01], BA [F (2, 24) = 12.06, $p$< 0.01], CeL [F (2, 21) = 15.03, $p$< 0.001] and CeM [F (2, 21) = 11.22, $p$< 0.001] following conditioning as compared to the naive control group. Following fear learning the HDAC2 expression was found to be negatively correlated with the freezing in LA ($p$<0.001), BA ($p$<0.05), CeL ($p$<0.001) and CeM ($p$<0.001) (Table. 1). (Fig 22)

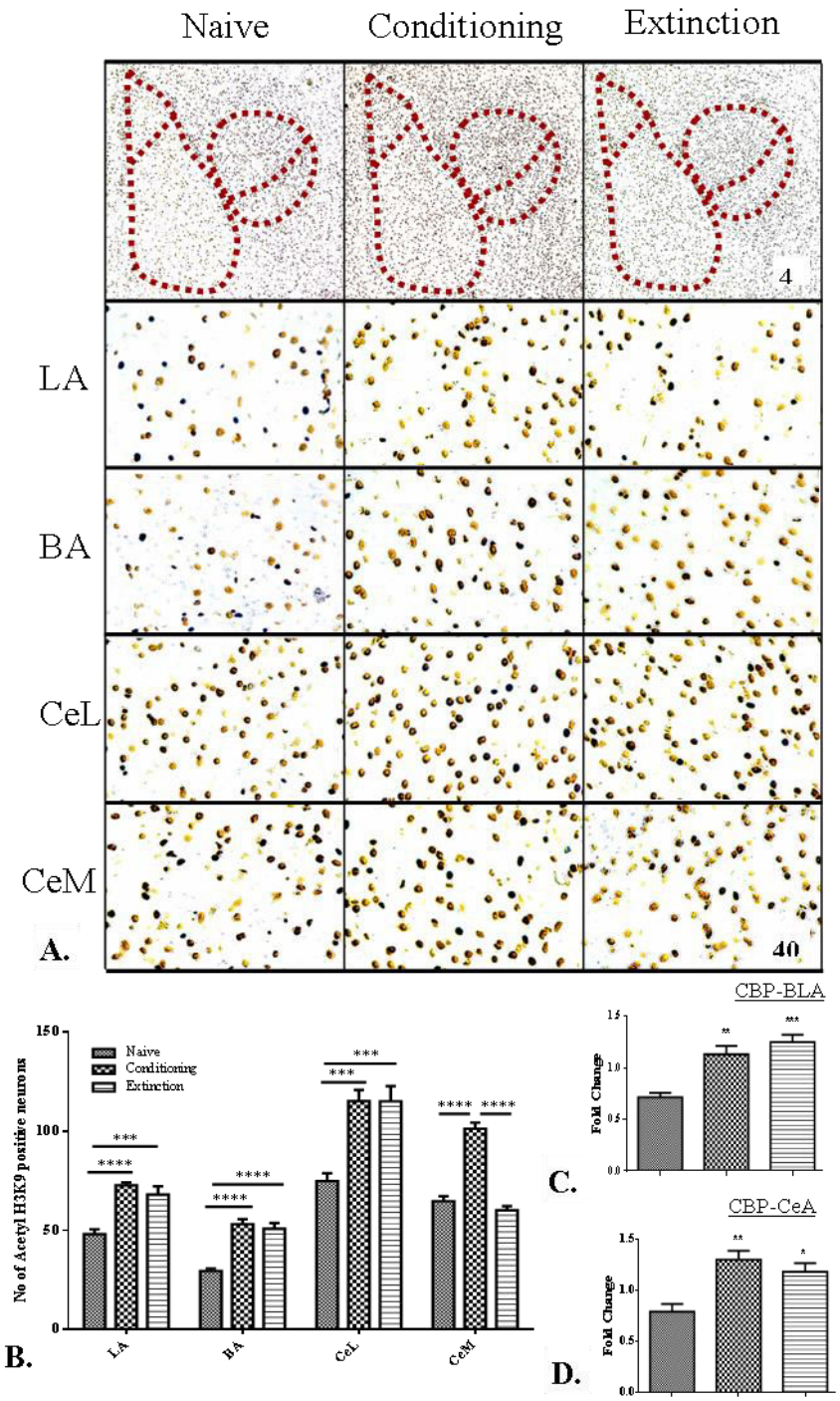



**Fig 19.** Histone H3K9 acetylation in the amygdala in conditioning and extinction. **A.** and **B.** Immunohistochemistry of histone H3K9 acetylation in LA, BA, CeL and CeM following conditioning and extinction; **C.** and **D.** CBP mRNA expression in BLA and CeA, respectively.

Following extinction learning HDAC2 expression decreased significantly in LA [F (2, 24) = 10.98, $p< 0.001$], BA [F (2, 24) = 12.06, $p< 0.001$] and CeL [F (2, 21) = 15.03, $p< 0.001$] but not in CeM [F (2, 21) = 11.22, $p> 0.05$] as compared to the naïve control group. HDAC2 expression in LA ($p<0.001$), BA ($p<0.001$) and CeL ($p<0.001$) exhibited a positive correlation with the freezing response during extinction learning while in CeM no significant correlation was observed.

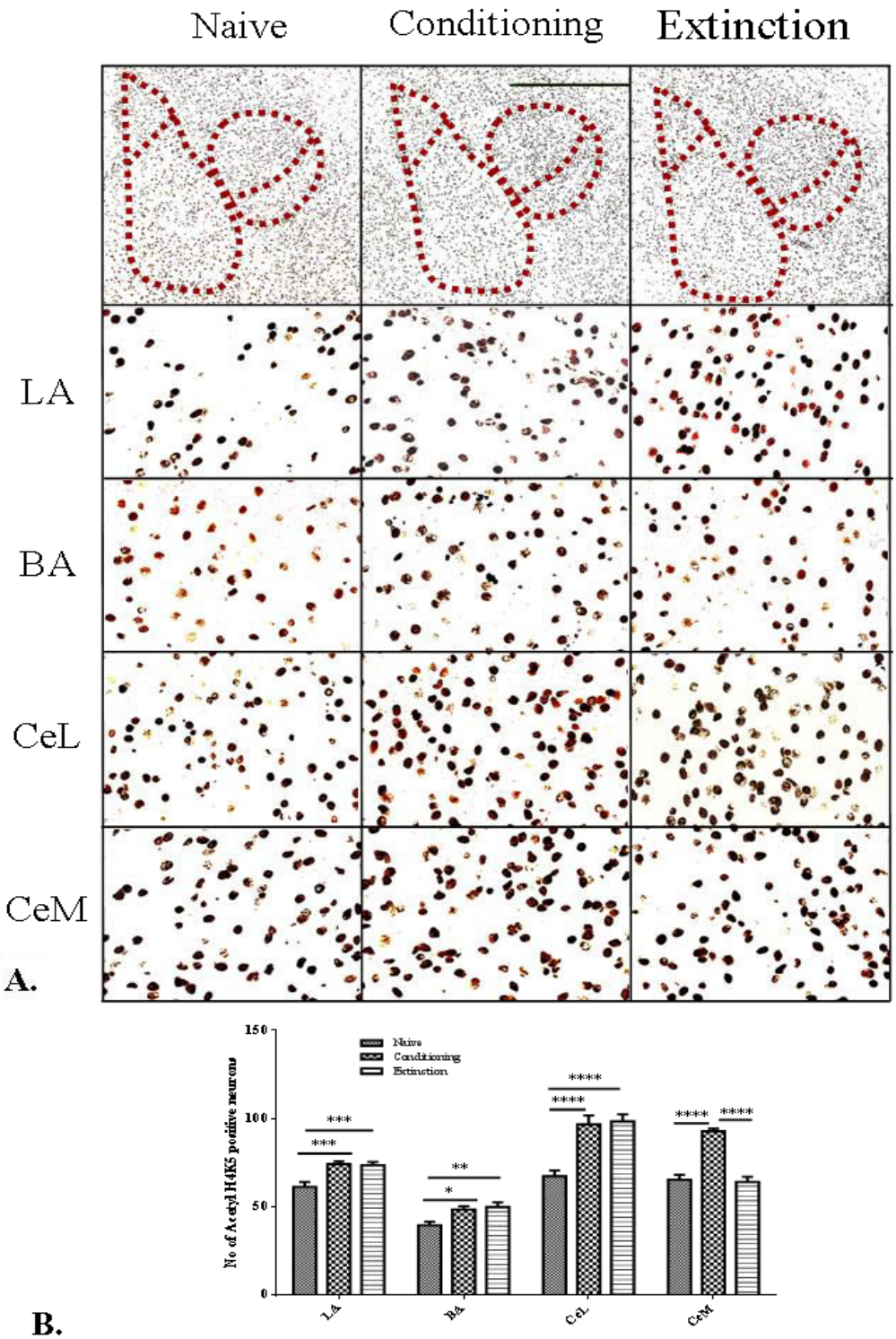



**Fig 20.** Histone H4K5 acetylation in the amygdala following conditioning and extinction. **A.** and **B.** Immunohistochemistry of histone H4K5 acetylation in LA, BA, CeL and CeM following conditioning and extinction

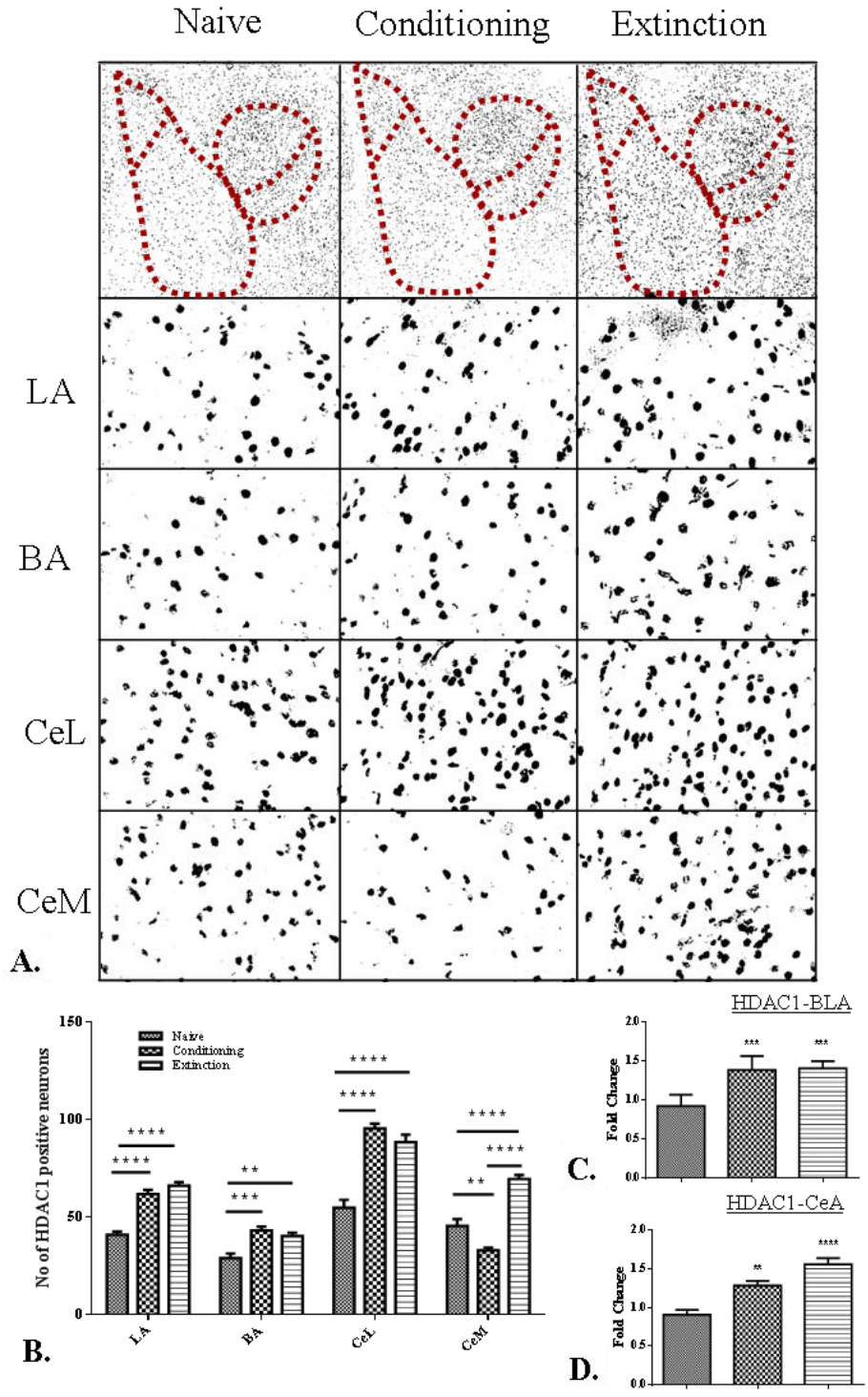



**Fig 21.** HDAC1 expression in the amygdala following conditioning and extinction. **A.** and **B.** Immunohistochemistry of HDAC1 expression in LA, BA, CeL and CeM following conditioning and extinction. **C.** and **D.** HDAC1 mRNA expression in BLA and CeA, respectively.

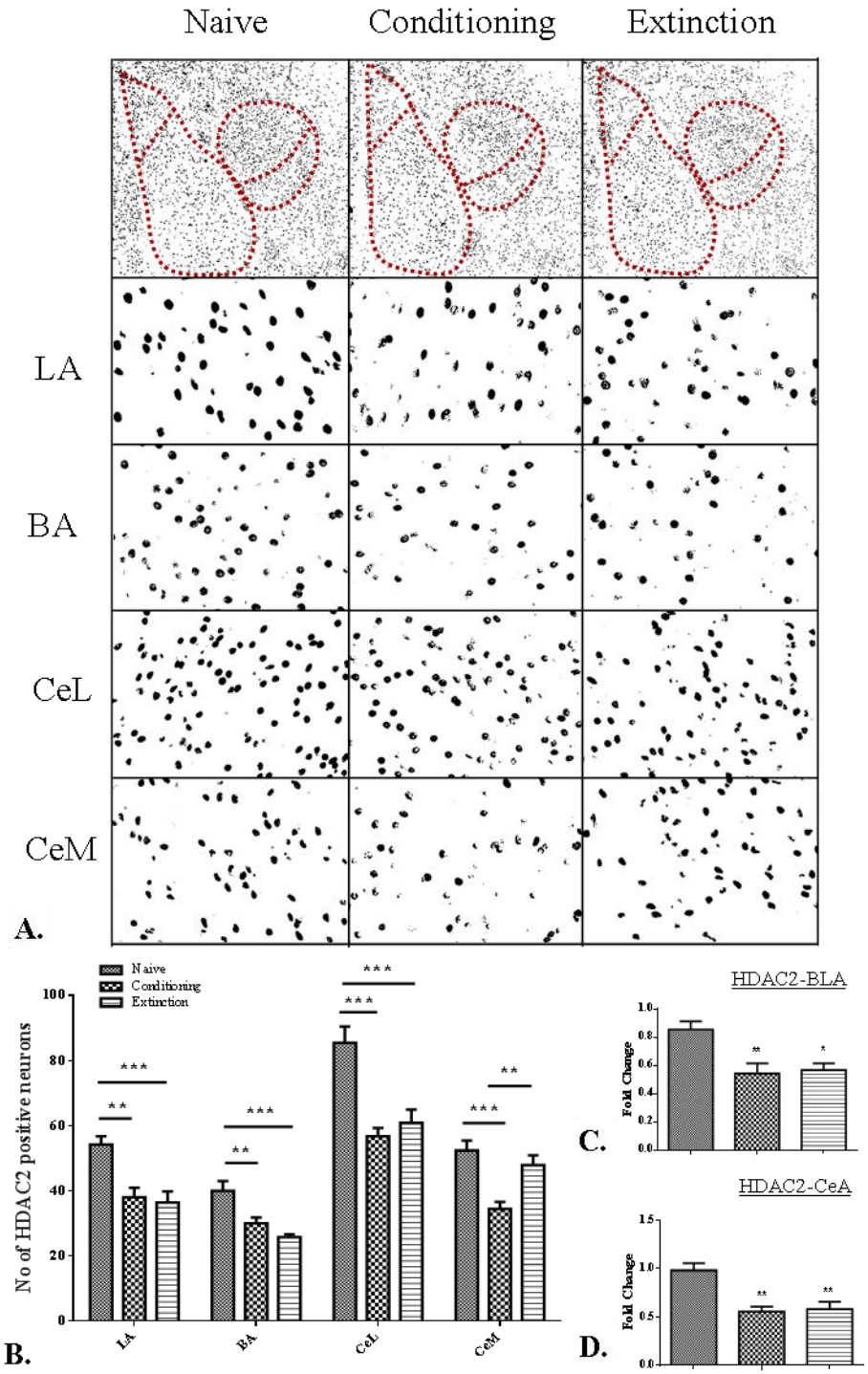



**Fig 22.** HDAC2 expression in the amygdala following conditioning and extinction. **A.** and **B.** Immunohistochemistry of HDAC2 expression in LA, BA, CeL and CeM following conditioning and extinction. **C.** and **D.** HDAC2 mRNA expression in BLA and CeA, respectively.

### 5.1.2.6. c-fos expression in Prefrontal Cortex

Similarly, the c-fos expression was studied in PFC for understanding the activity of PFC subregions following conditioning and extinction of fear memory. The PFC subregions, PL and IL

exhibited different activation pattern following consolidation and extinction of fear memory. In PL, the c-fos expression increased significantly following conditioning of fear memory as compared to the naive control group, whereas no significant change was observed in PL following extinction learning as compared to the naive control group [F(2, 27) = 18.31, $p$<0001]. In IL, however the c-fos expression increased significantly following extinction learning but not following conditioning of fear memory [F (2, 27) = 46.22, $p$<0001] as compared to the naive control group. As PL has the role in fear memory consolidation, IEG c-fos shows activation of PL following conditioning and IL has the role in extinction learning, c-fos expression exhibited enhanced activity following extinction (Sierra-Mercado et al, 2011). (Fig 23)

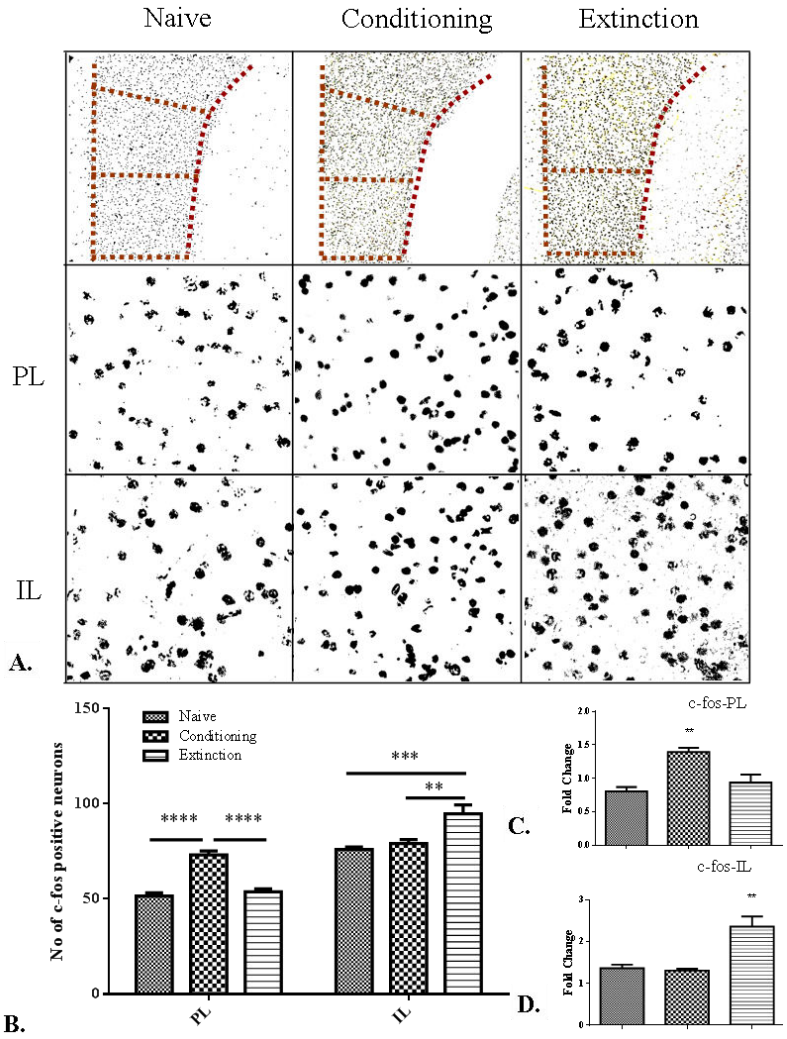



**Fig 23.**c-fos expression in Prefrontal Cortex following conditioning and extinction. **A.** and **B.** Immunohistochemistry of c-fos expression in PL and IL of PFC following conditioning and extinction. **C.** and **D.** c-fos mRNA expression in PL and IL, respectively.

Overall, there was a positive correlation for c-fos expression in PL with the freezing response following fear memory consolidation ($p$<0.001). Differently in IL, a negative correlation was observed for c-fos expression with the freezing response, which means a positive correlation of c-fos expression in IL with the extinction ($p$<0.01).

### 5.1.2.7. Histone H3K9 acetylation in Prefrontal Cortex

Histone H3K9 acetylation increased significantly in PL following conditioning ($p$<0.001) while no significant difference was observed following extinction learning ($p$>0.05) as compared to the naïve control group [F (2, 15) = 14.39, $p$<0.001]. In IL however, the histone H3K9 acetylation increased significantly following extinction learning ($p$<0.01) but no significant change was observed following conditioning ($p$>0.05) as compared to the naïve control group [F (2, 15) = 13.29, $p$<0.001]. (Fig 24)

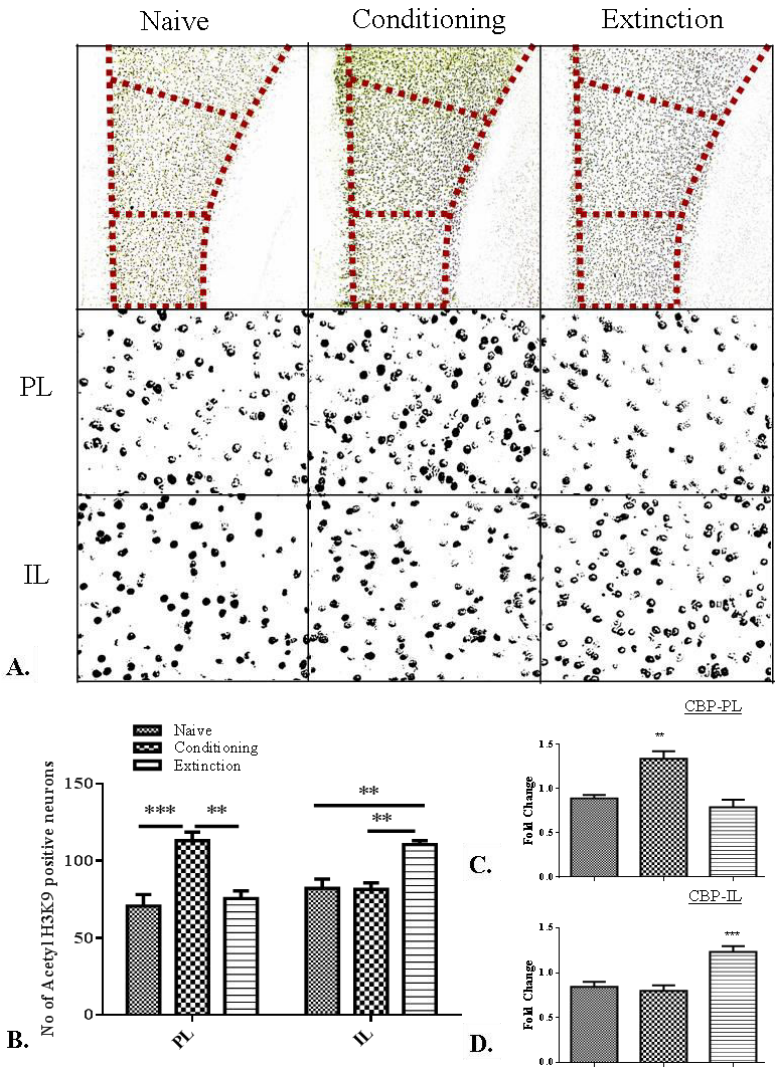



**Fig 24.** Histone H3K9 acetylation in Prefrontal cortex following conditioning and extinction. **A.** and **B.** Immunohistochemistry of histone H3K9 acetylation in PL and IL of PFC following conditioning and extinction. **C.** and **D.** CBP mRNA expression in PL and IL, respectively.

### 5.1.2.8. Histone H4K5 acetylation in Prefrontal Cortex

Histone H4K5 acetylation in PL increased significantly following conditioning ($p$<0.001) as compared to the naïve control group whereas no significant change was observed following extinction ($p$>0.05) compared to the naive control group [F (2, 33) = 13.60, $p$<0.0001]. Histone H4K5 acetylation in IL increased significantly following extinction learning (p<0.01) as compared to the naïve control group while no significant change was observed in IL following conditioning

($p$>0.05) [F (2, 33) = 6.847, $p$<0.01]. In conclusion, the histone acetylation in PL is associated with the conditioning while in IL it is associated with the extinction learning which is collinear with the activity of the corresponding region with acetylation. (Fig 25)

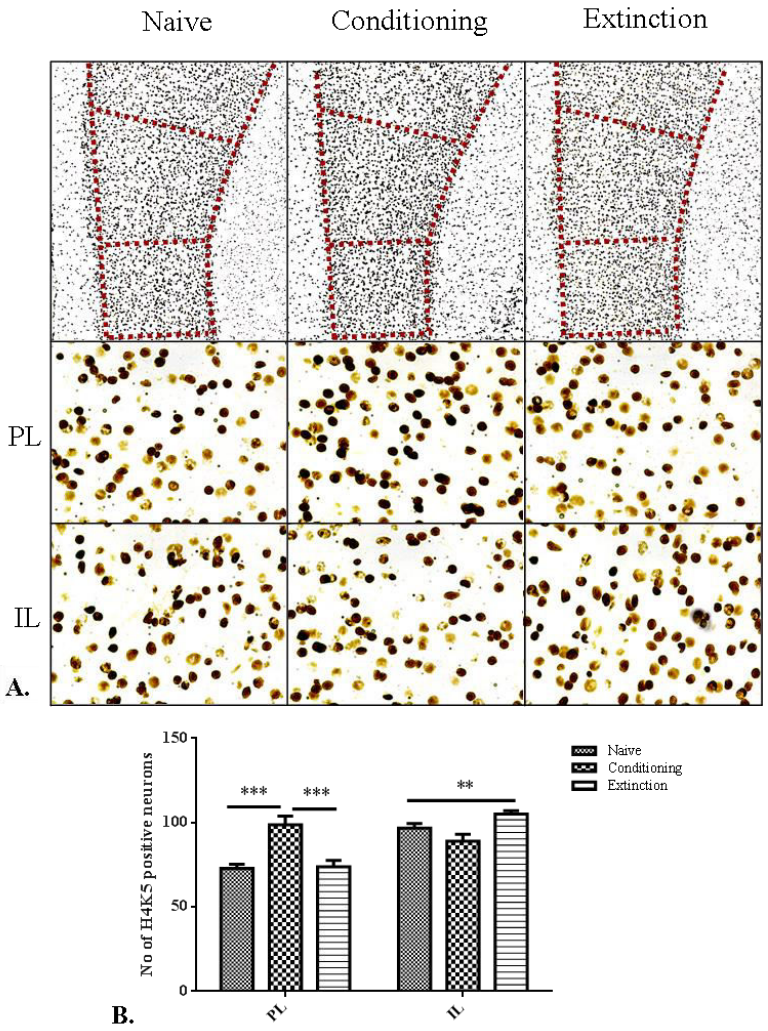



**Fig 25.** Histone H4K5 acetylation in Prefrontal cortex following conditioning and extinction. **A.** and **B.** Immunohistochemistry of histone H4K5 acetylation in PL and IL following conditioning and extinction.

## 5.1.2.9. HDAC1 expression in Prefrontal Cortex

HDAC1 expression decreased significantly in PL [F (2, 27) = 74.55, $p$< 0.0001] following fear memory consolidation whereas there was no significant change in IL [F (2, 27) = 25.16, $p$>0.05] was observed following conditioning as compared to the naïve control group. The PL neurons of conditioning group exhibited a negative correlation ($p$<0.001) for the HDAC1 expression when compared with the freezing response, while IL ($p$>0.05) exhibited no correlation (Table. 1).

The HDAC1 expression in IL [F (2, 27) = 25.16, $p< 0.0001$] decreased significantly following fear extinction as compared to the naïve control group. Whereas, there was no change in PL following extinction as compared to the naive control group. Similarly, as observed in conditioning group, the HDAC1 expression in PL of extinction group exhibited no correlation ($p>0.05$) with the freezing response, while a positive correlation in IL ($p<0.001$) were observed during extinction learning. (Fig 26)

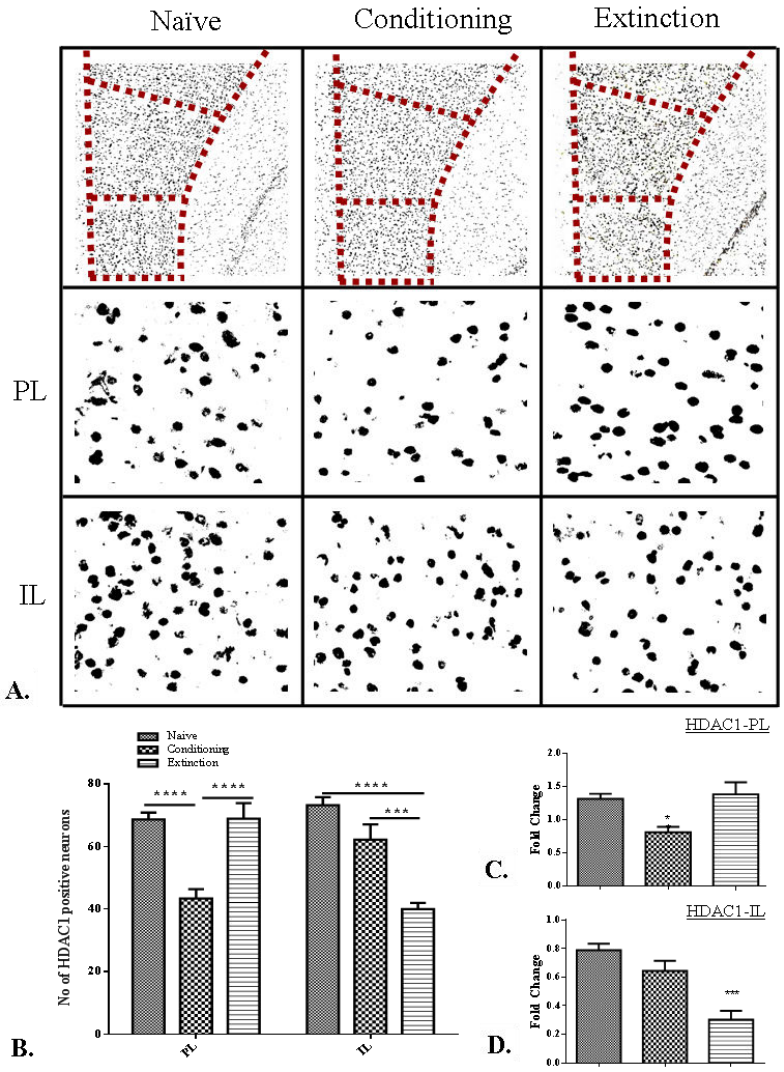



**Fig 26.** HDAC1 expression in Prefrontal cortex following conditioning and extinction. **A.** and **B.** Immunohistochemistry of HDAC1 expression in PL and IL following conditioning and extinction. **C.** and **D.** HDAC1 mRNA expression in PL and IL, respectively.

## 5.1. 2.10. HDAC2 expression in Prefrontal Cortex

In PL, [F (2, 27) = 24.55, $p<0.05$] the HDAC2 expression decreased significantly following fear memory consolidation while following fear extinction the HDAC2 expression increased significantly in PL [F (2, 27) = 24.55, $p< 0.001$] as compared to the naïve control group.

In IL [F (2, 27) = 45.92, $p<0.01$], the expression of HDAC2 decreased significantly following fear memory consolidation whereas no significant change was observed [F (2, 27) = 45.92, $p <0.0001$] following fear extinction as compared to the naïve control group. The HDAC2

expression in PL ($p$<0.01) and IL ($p$<0.001) was found to be negatively correlated with the freezing response during conditioning (Table. 1 and 2). The HDAC2 expression exhibited a negative correlation with the freezing response in PL during extinction learning ($p$<0.01), whereas no correlation was observed in IL during extinction learning. (Fig 27)

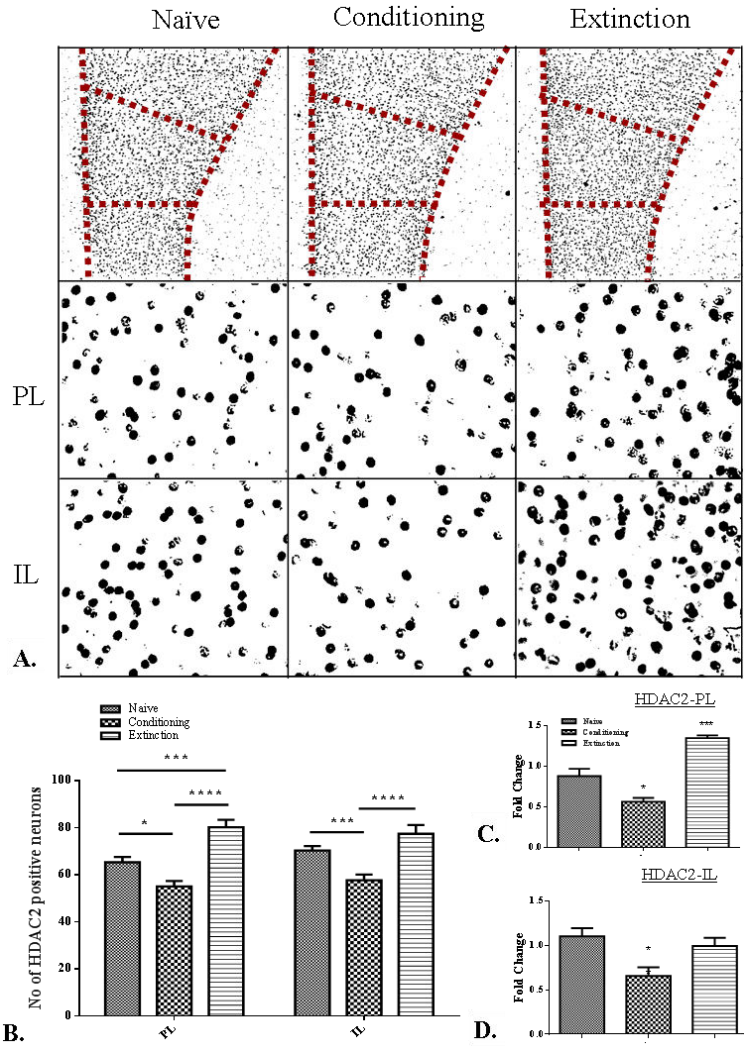



**Fig 27.** HDAC2 expression in Prefrontal cortex following conditioning and extinction. **A.** and **B.** Immunohistochemistry of HDAC2 expression in PL and IL following conditioning and extinction. **C.** and **D.** HDAC2 mRNA expression in PL and IL, respectively.

## 5.1. 2.11. c-fos expression in Hippocampus

c-fos expression was also analyzed for understanding the activity of hippocampal subregion following fear memory consolidation and extinction. In CA1, the c-fos expression increased significantly following conditioning ($p$<0.001) and extinction ($p$<0.0001) when compared to the naïve control group [F (2, 27) = 16.12, $p$<0.0001]. Similarly in CA3, the expression of c-fos positive neurons increased significantly following conditioning ($p$<0.0001) and extinction ($p$<0.0001) as compared to the naïve control group [F (2, 27) = 53.61, $p$<0.0001]. DG exhibited similar pattern as in CA1 and CA3 where c-fos expression increased significantly following

conditioning ($p$<0.01) and extinction ($p$<0.001) as compared to the naïve control group [F (2, 27) = 11.43, $p$<0.001]. Finally, it is clear from above result of IEG c-fos that CA1, CA3 and DG actively participate in fear memory consolidation and its extinction both. (Fig 28)

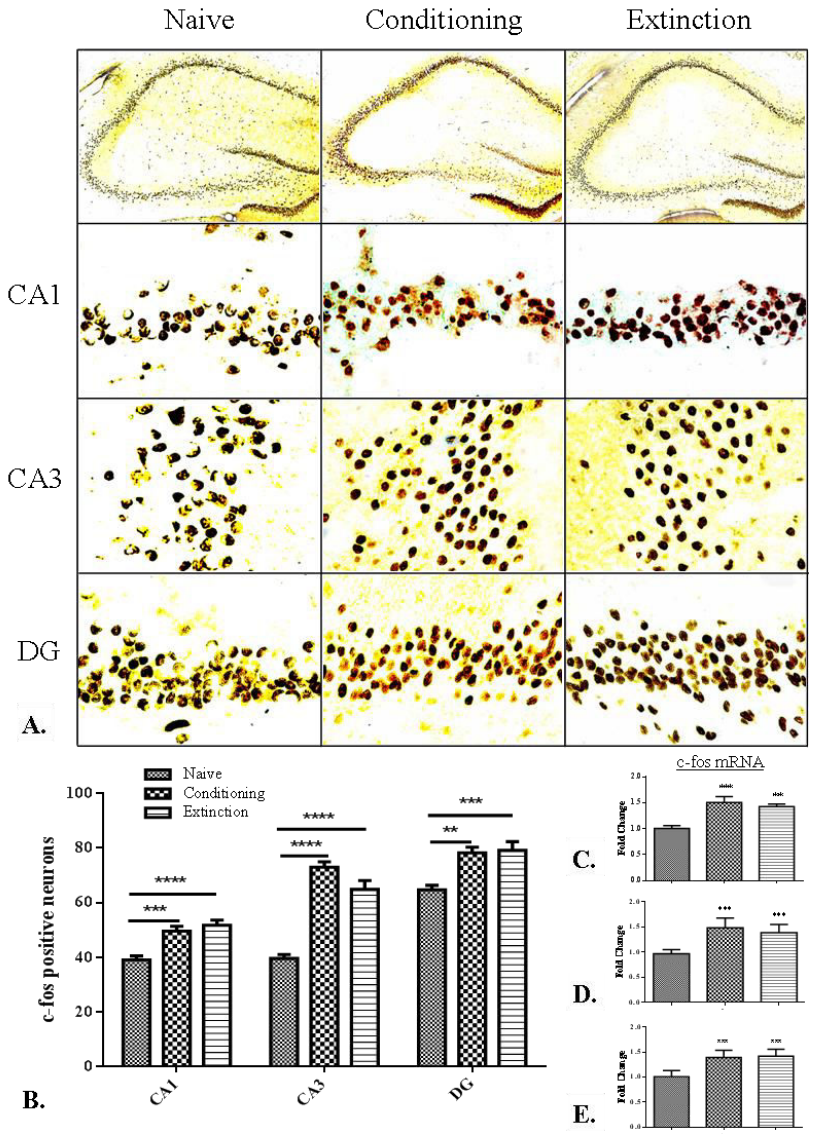



**Fig 28.** c-fos expression in Hippocampus following conditioning and extinction. **A.** and **B.** Immunohistochemistry of c-fos expression in CA1, CA3 and DG following conditioning and extinction. **C., D.** and **E.** c-fos mRNA expression in CA1, CA3 and DG, respectively.

## 5.1.2.12. Histone H3K9 acetylation in Hippocampus

Histone H3K9 acetylation increased significantly in CA1 following conditioning ($p$<0.01) and extinction ($p$<0.001) as compared to the naïve control group [F (2, 15) = 13.97, $p$<0.001]. In CA3, the acetyl H3K9 increased significantly following conditioning ($p$<0.01) but not following extinction training as compared to the naïve control group [F (2, 15) = 8.767, $p$< 0.01]. The DG of hippocampus exhibited similar expression pattern as in CA1, where acetyl H3K9 increased significantly following conditioning ($p$<0.05) and extinction ($p$<0.01) as compared to the naïve control group [F (2, 15) = 8.694, $p$< 0.01]. The histone H3K9 acetylation thus exhibited similarity

with the activity of Hippocampal subregion following conditioning and extinction, which suggests acetylation as a crucial mechanism for controlling conditioning and extinction in the hippocampus. (Fig 29)

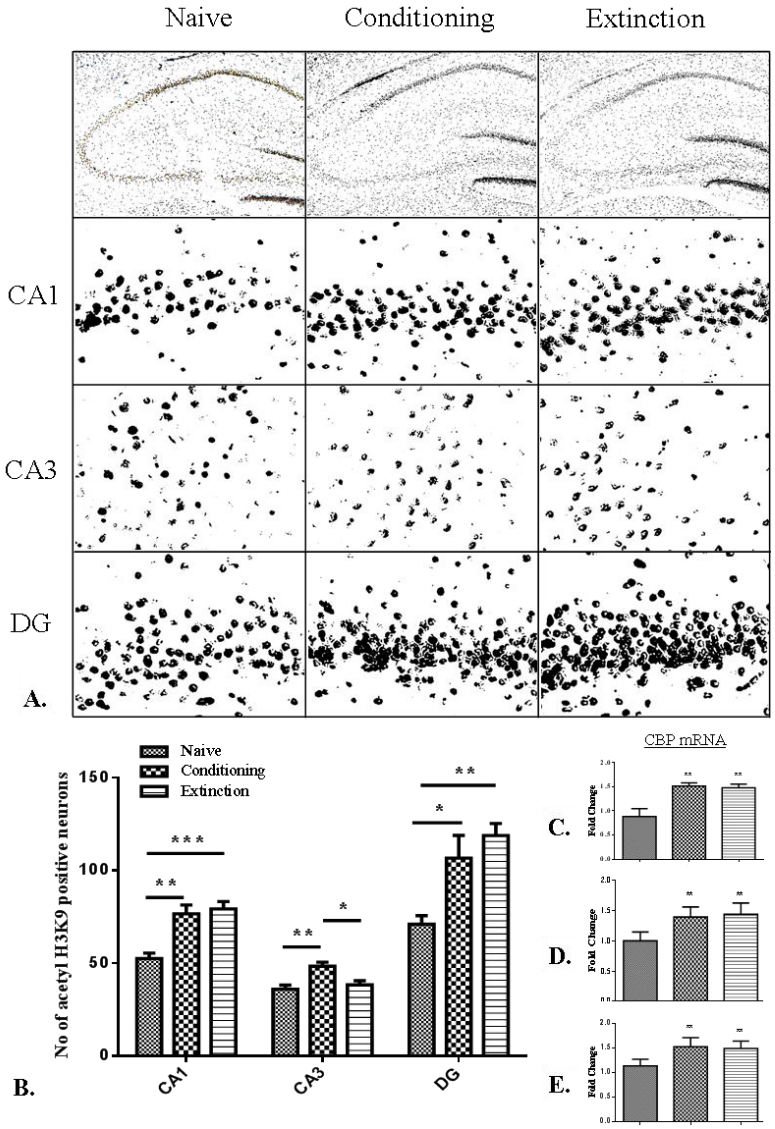



**Fig 29.** Histone H3K9 acetylation in Hippocampus following conditioning and extinction. **A.** and **B.** Immunohistochemistry of histone H3K9 acetylation in CA1, CA3 and DG following conditioning and extinction. **C., D.** and **E.** CBP mRNA expression in CA1, CA3 and DG, respectively.

## 5.1.2.13. Histone H4K5 acetylation in Hippocampus

The histone H4K5 acetylation increased significantly in CA1 following conditioning ($p$<0.01) but not following extinction training as compared to the naïve control group [F (2, 15) = 11.60, $p$< 0.001]. The CA3 exhibited increased H4K5 acetylation following conditioning ($p$<0.01) and extinction ($p$<0.001) as compared to the naïve control group [F (2, 15) = 13.15, $p$< 0.001]. In DG the histone H4K5 acetylation increased significantly following conditioning ($p$<0.0001) and extinction ($p$<0.001) as compared to the naïve control group [F (2, 15) = 20.83, $p$< 0.0001]. (Fig 30)

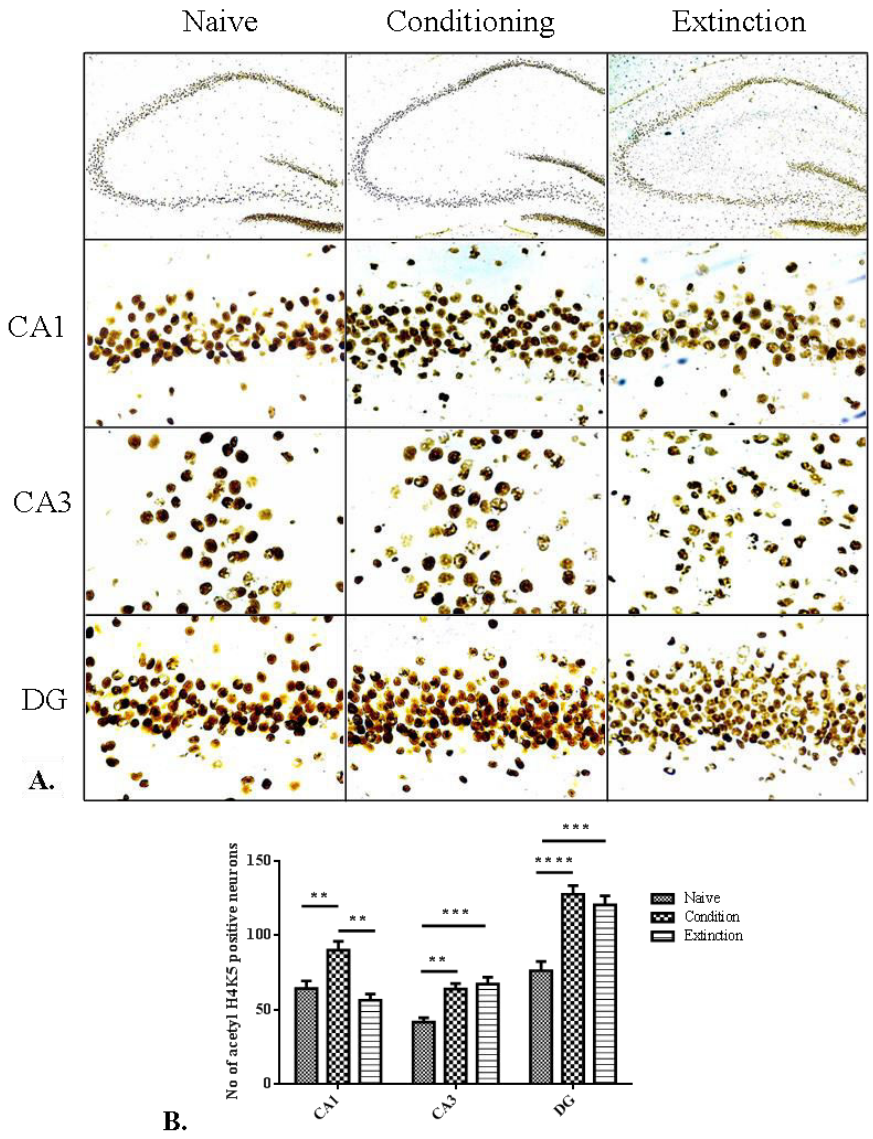



**Fig 30.** Histone H4K5 acetylation in Hippocampus following conditioning and extinction. **A.** and **B.** Immunohistochemistry of histone H4K5 acetylation in CA1, CA3 and DG following conditioning and extinction.

**5.1.2.14. HDAC1 expression in Hippocampus**

In CA1, the HDAC1 expression decreased significantly following conditioning ($p$<0.05) and increased following extinction ($p$<0.0001) as compared to the naive control group [F (2, 27) = 29.39, $p$<0.0001]. In CA3 similarly, the HDAC1 expression decreased significantly following conditioning ($p$<0.01) and increased significantly following extinction ($p$<0.0001) as compared to the naive control group [F (2, 27) = 45.59, $p$<0.0001]. In DG, the HDAC1 expression increased significantly following extinction ($p$<0.01) but there was no change in HDAC1 expression following conditioning when compared with the naive control group [F (2, 27) = 7.7, $p$<0.01]. (Fig 31)

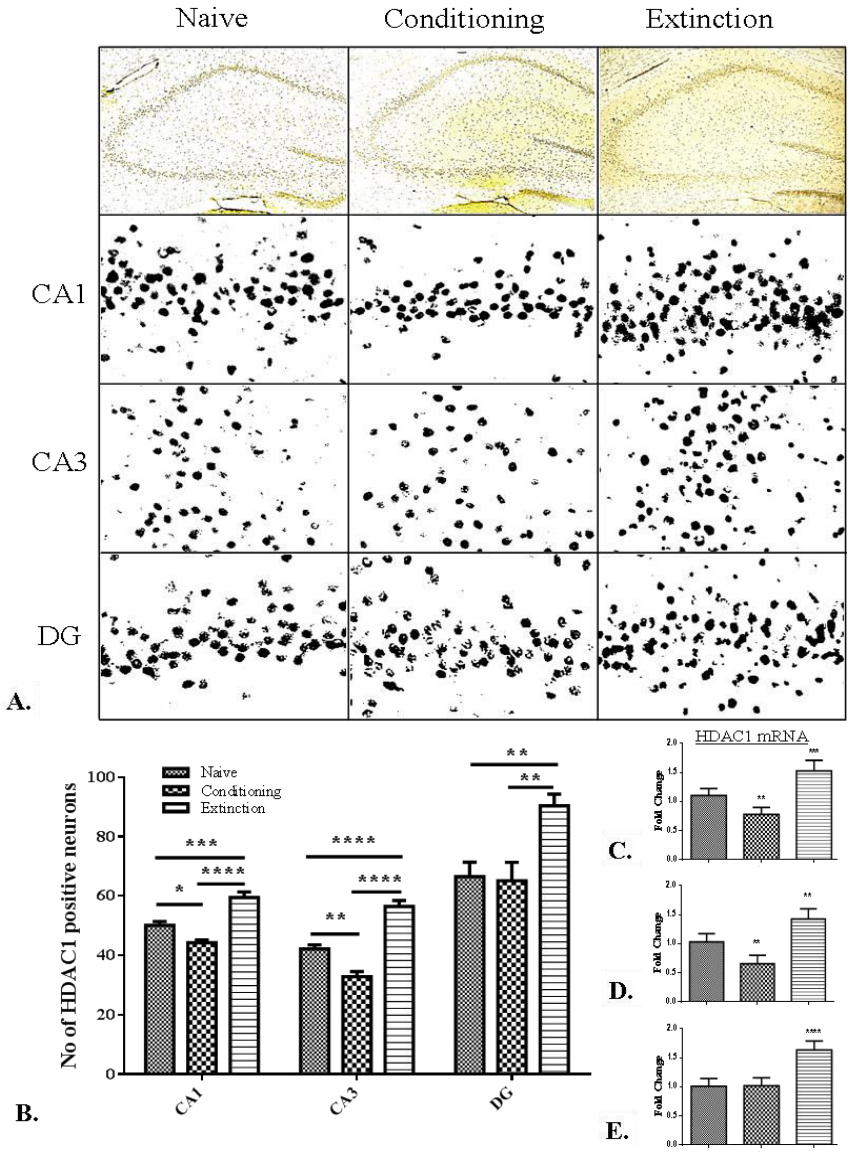



**Fig 31.** HDAC1 expression in Hippocampus following conditioning and extinction. **A.** and **B.** Immunohistochemistry of HDAC1 expression in CA1, CA3 and DG following conditioning and extinction. **C., D.** and **E.** HDAC1 mRNA expression in CA1, CA3 and DG, respectively.

**5.1.2.15. HDAC2 expression in Hippocampus**

In CA1, the HDAC2 expression decreased significantly following extinction learning ($p$<0.01) but not following conditioning as compared to the naïve control group [F (2, 15) = 8.857, $p$<0.01]. CA3 exhibited similar expression pattern as in CA1, where HDAC2 expression decreased significantly following extinction learning ($p$<0.01) but not following conditioning as compared to the naïve control group [F (2, 15) = 8.386, $p$<0.01]. In DG, the HDAC2 expression increased significantly following conditioning (p<0.01) and decreased significantly following extinction learning ($p$<0.01) as compared to the naïve control group [F (2, 15) = 26.82, $p$<0.0001]. (Fig 32)

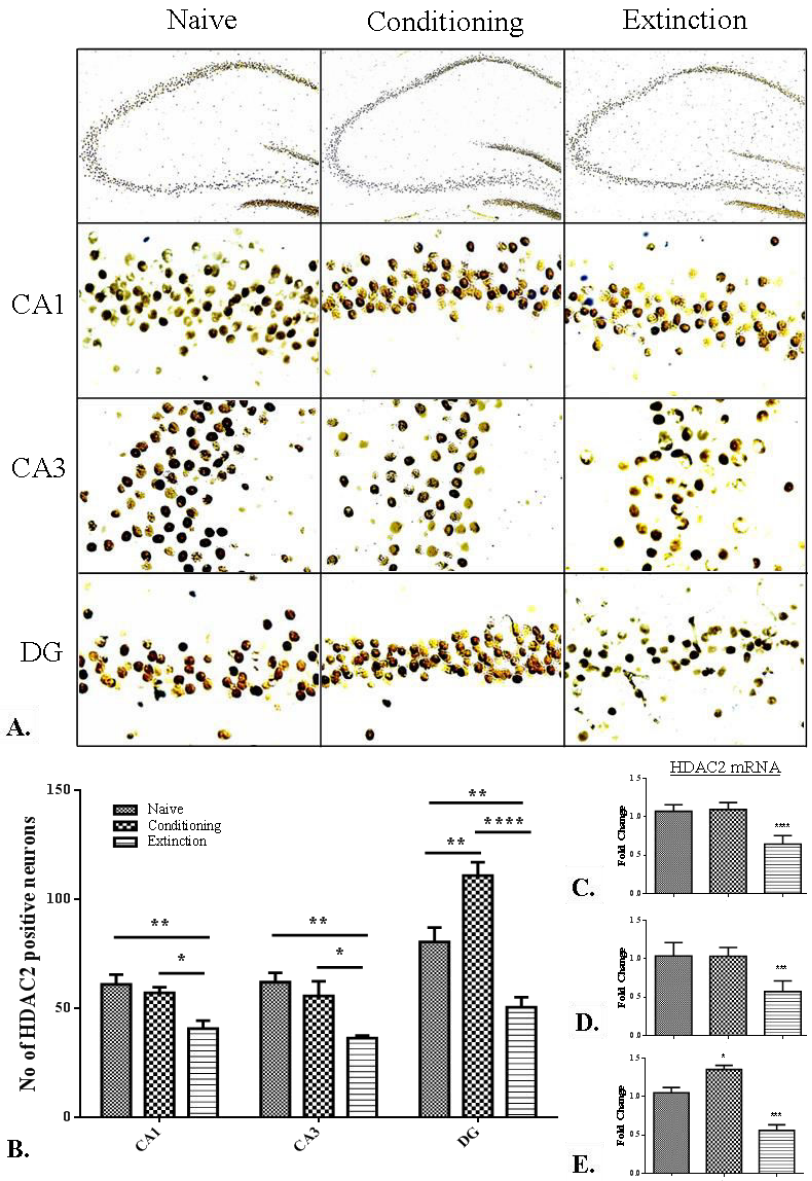



**Fig 32.** HDAC2 expression in Hippocampus following conditioning and extinction. **A.** and **B.** Immunohistochemistry of HDAC2 expression in CA1, CA3 and DG following conditioning and extinction. **C., D.** and **E.** HDAC2 mRNA expression in CA1, CA3 and DG, respectively.

## 5.1.3. Correlation Results

### 5.1.3.1. Correlation for Conditioning in Amygdala, Prefrontal Cortex and Hippocampus

#### 5.1.3.1.1. Amygdala

In LA, a positive correlation was observed for Acetyl H3K9 ($p$<0.001), acetyl H4K5 ($p$<0.05), c-fos ($p$<0.001) and HDAC1 ($p$<0.001) with the freezing response during retention test following conditioning while HDAC2 ($p$<0.001) expression exhibited a negative correlation with the freezing response. Moreover, in BA, a positive correlation for acetyl H3K9 ($p$<0.001), c-fos ($p$<0.01) and HDAC1 ($p$<0.001) while a negative correlation for HDAC2 ($p$<0.05) with the freezing response in retention test was observed following conditioning. In CeL, a positive correlation for Acetyl H3K9 ($p$<0.01), acetyl H4K5 ($p$<0.05), c-fos ($p$<0.01) and HDAC1 ($p$<0.001) expression while a negative correlation for HDAC2 ($p$<0.001) expression was observed with the freezing response of retention test following conditioning. In CeM, a positive correlation for Acetyl H3K9

($p$<0.001), acetyl H4K5 ($p$<0.001) and c-fos ($p$<0.001) expression while a negative correlation for HDAC1 ($p$<0.001) and HDAC2 ($p$<0.001) expression with the freezing response during retention test was observed following conditioning. (Fig 33)

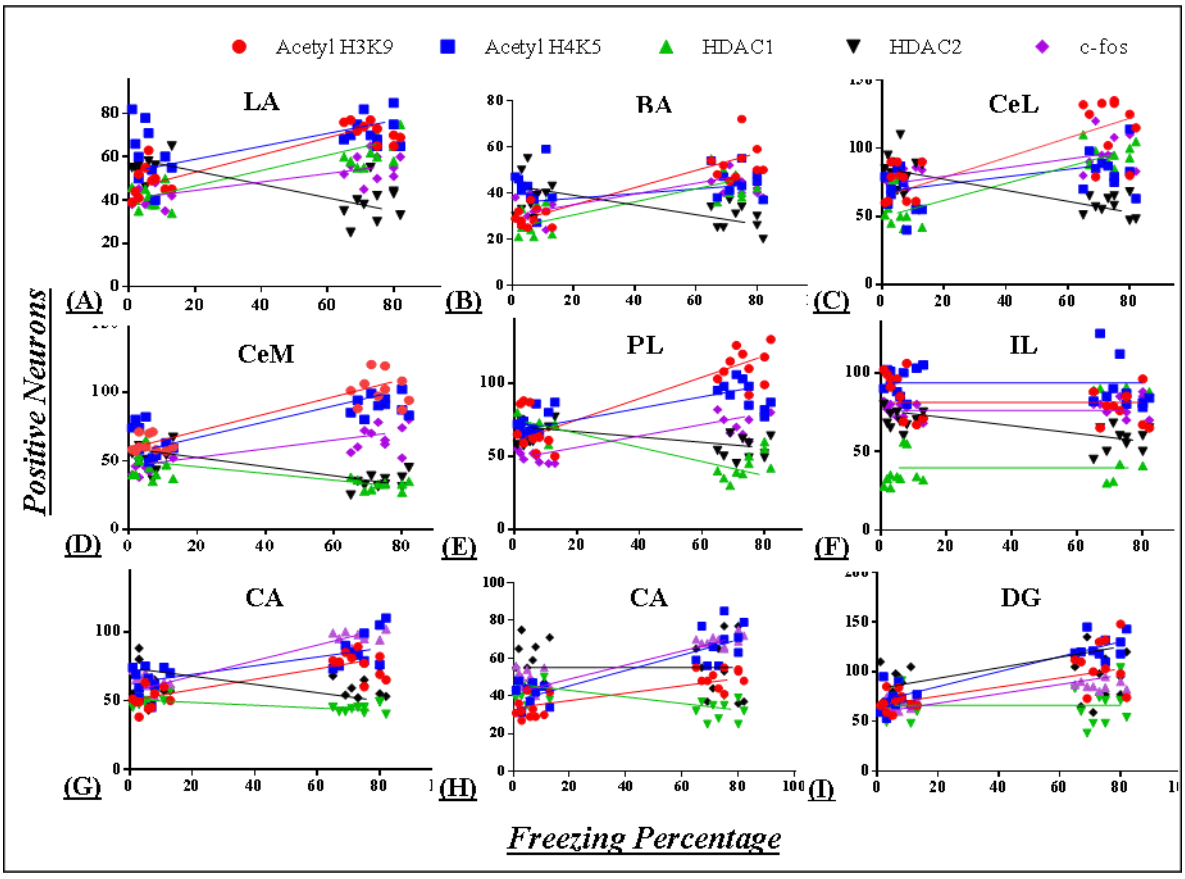



**Fig 33.** The Correlation for the fear conditioning. Correlation of c-fos, acetyl H3K9, acetyl H4K5, HDAC1 and HDAC2 with freezing response following conditioning in amygdala Hippocampus and PFC from immunohistochemistry.

### 5.1.3.1.2. Prefrontal Cortex

In PL, a positive correlation for acetyl H3K9 ($p$<0.001), acetyl H4K5 ($p$<0.001) and c-fos ($p$<0.001) levels, while a negative correlation for HDAC1 ($p$<0.001) and HDAC2 ($p$<0.01) with the freezing behavior of retention test was observed. In IL, a positive correlation for HDAC1 expression ($p$<0.01) while a negative correlation for HDAC2 ($p$<0.001) expression with the freezing response of retention test following conditioning was observed while acetyl H3K9, acetyl H4K5 and c-fos exhibited no correlation with the freezing response. (Fig 33)

**Table 6: Correlation conditioning**

| | Acetyl H3K9 | Acetyl H4K5 | HDAC1 | HDAC2 | c-fos |
|---|---|---|---|---|---|
| LA | r = 0.877 (**)<br>p < 0.001 | r = 0.453 (*)<br>p < 0.05 | r = 0.898 (**)<br>p < 0.001 | r = - 0.696 (**)<br>p < 0.001 | r = 0.817 (**)<br>p < 0.001 |
| BA | r = 0.897 (**)<br>p < 0.001 | - | r = 0.703 (**)<br>p < 0.001 | r = - 0.614 (*)<br>p < 0.05 | r = 0.655 (**)<br>p < 0.05 |

| | | | | | |
|---|---|---|---|---|---|
| CeL | r = 0.755 (**)<br>p < 0.01 | r = 0.498 (*)<br>p < 0.05 | r = 0.881 (**)<br>p < 0.001 | r = - 0.813 (**)<br>p < 0.001 | r = 0.633 (**)<br>p < 0.05 |
| CeM | r = 0.904 (**)<br>p < 0.001 | r = 0.789 (**)<br>p < 0.001 | r = - 0.710 (**)<br>p < 0.001 | r = - 0.753 (**)<br>p < 0.001 | r = 0.827 (**)<br>p < 0.001 |
| PL | r = 0.854 (**)<br>p < 0.001 | r = 0.723 (**)<br>p < 0.001 | r = - 0.819 (**)<br>p < 0.001 | r = - 0.574 (**)<br>p < 0.01 | r = 0.839 (**)<br>p < 0.001 |
| IL | - | - | r = 0.620 (**)<br>p < 0.01 | r = - 0.684 (**)<br>p < 0.001 | - |
| CA1 | r = 0.834 (**)<br>p < 0.001 | r = 0.756 (**)<br>p < 0.001 | r = - 0.605 (**)<br>p < 0.01 | - | r = 0.951 (**)<br>p < 0.001 |
| CA3 | r = 0.861 (**)<br>p < 0.001 | r = 0.878 (**)<br>p < 0.001 | r = - 0.686 (**)<br>p < 0.001 | - | r = 0.920 (**)<br>p < 0.001 |
| DG | r = 0.737 (**)<br>p < 0.001 | r = 0.918 (**)<br>p < 0.001 | - | - | r = 0.902 (**)<br>p < 0.001 |

**5.1.3.1.3. Hippocampus**

In CA1, histone acetyl H3K9 ($p$<0.0001), acetyl H4K5 ($p$<0.0001) and c-fos ($p$<0.0001) exhibited a positive correlation while HDAC1 ($p$<0.01) a negative correlation with the freezing response for retention test following conditioning. Furthermore, in CA3, histone acetyl H3K9 ($p$<0.0001), acetyl H4K5 ($p$<0.0001) and c-fos ($p$<0.0001) exhibited a positive correlation while HDAC1 ($p$<0.001) a negative correlation with the freezing response during retention test following conditioning. In DG, the histone acetyl H3K9 ($p$<0.001), acetyl H4K5 ($p$<0.0001) and c-fos ($p$<0.0001) exhibited a positive correlation with the freezing response while no correlation was observed for HDAC1 and HDAC2 with the freezing response during retention test following conditioning. (Fig 33)

**5.1.3.2. Correlation for Extinction in Amygdala, PFC and hippocampus**

**5.1.3.2.1. Amygdala**

The correlation study was performed on expression profile and freezing behavior of extinction learning which was done by correlating molecular expression profile with the freezing behavior observed during retention test of extinction learning. For extinction learning, in LA, acetyl H3K9 ($p$<0.01), acetyl H4K5 ($p$<0.05), c-fos ($p$<0.001) and HDAC1 ($p$<0.001) exhibited a negative correlation with the freezing behavior which means a positive correlation with extinction learning whereas HDAC2 ($p$<0.001) expression exhibited a positive correlation with the freezing response which means a negative correlation with the extinction learning following extinction when compared with the freezing response during retention test. In BA, acetyl H3K9 ($p$<0.001), c-fos ($p$<0.001) and HDAC1 ($p$<0.01) exhibited a negative correlation with the freezing response which means a positive correlation with extinction learning while HDAC2 ($p$<0.001) expression exhibited a positive correlation with the freezing response and a negative correlation with the extinction learning following extinction when compared with the freezing response during retention test. In CeL, acetyl H3K9 ($p$<0.001), acetyl H4K5 ($p$<0.001), c-fos ($p$<0.01) and HDAC1 ($p$<0.001) exhibited a negative correlation with the freezing response and a positive correlation with the extinction learning while HDAC2 ($p$<0.01) expression exhibited a positive correlation with the freezing response and a negative correlation with the extinction learning when compared with the freezing response during retention test. In CeM, HDAC1 ($p$<0.001) exhibited a negative correlation with the freezing response which means a positive correlation with the extinction learning while

acetyl H3K9, acetyl H4K5, c-fos and HDAC2 exhibited no correlation when compared with the freezing response during retention testing. (Fig 34)

**5.1.3.2.2. Prefrontal Cortex**

In PL, a negative correlation with the freezing response and a positive correlation with the extinction learning for HDAC1 ($p$<0.001) and HDAC2 ($p$<0.01) expression was observed when compared with the freezing response during retention test. However there was no correlation was observed for acetyl H3K9, acetyl H4K5 and c-fos level.

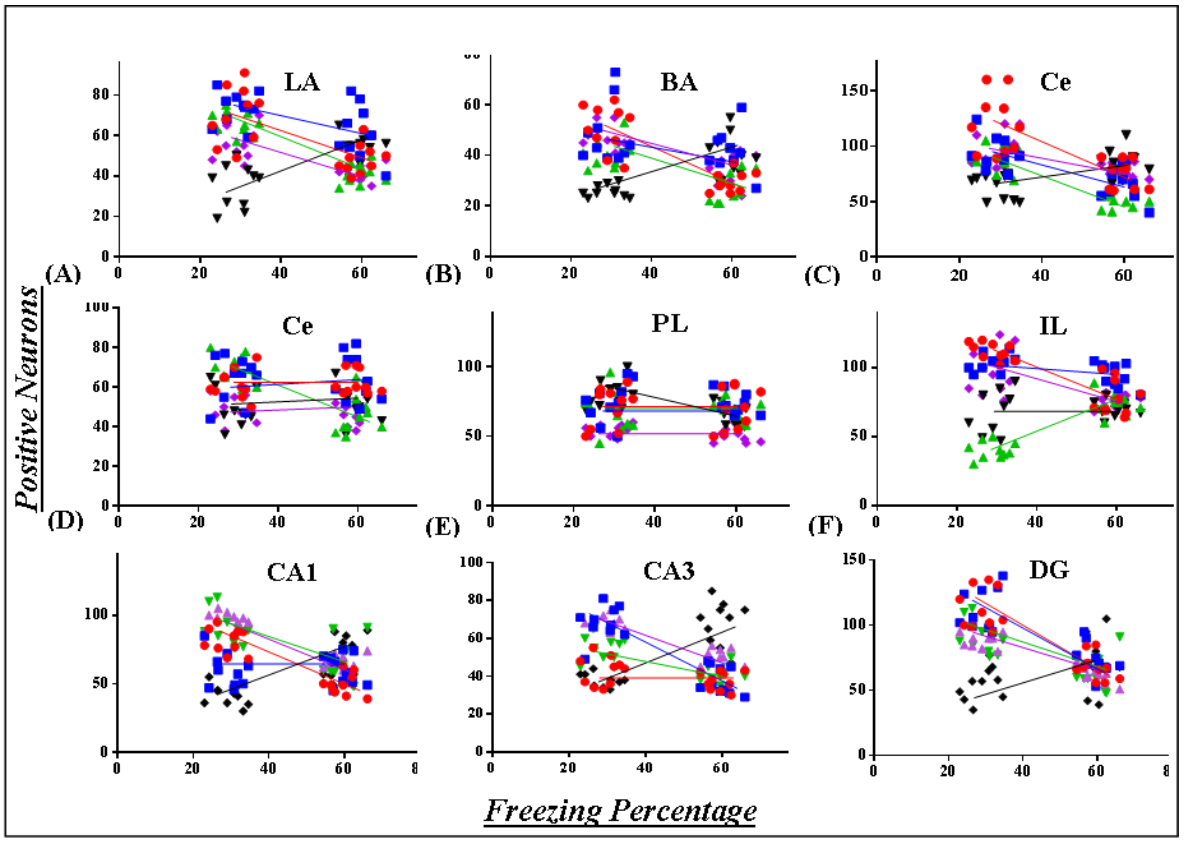



**Fig 34.** The Correlation for fear Extinction. Correlation of c-fos, acetyl H3K9, acetyl H4K5, HDAC1 and HDAC2 expression with the freezing response following extinction in the amygdala, Hippocampus and PFC from immunohistochemistry.

In IL, a negative correlation with the freezing response and a positive correlation with the extinction learning for acetyl H3K9 ($p$<0.001), acetyl H4K5 ($p$<0.05), c-fos ($p$<0.01) and HDAC2 ($p$<0.001) expression was observed while a positive correlation with the freezing response and a negative correlation with the extinction learning for HDAC1 ($p$<0.001) expression was observed when compared with the freezing response during retention test. (Fig 34)

**Table 7: Correlation Extinction**

| | Acetyl H3K9 | Acetyl H4K5 | HDAC1 | HDAC2 | c-fos |
|---|---|---|---|---|---|
| LA | r = - 0.659 (**)<br>p < 0.01 | r = - 0.523 (*)<br>p < 0.05 | r = - 0.907 (**)<br>p < 0.001 | r = 0.748 (**)<br>p < 0.001 | r = - 0.730 (**)<br>p < 0.001 |

| | | | | | |
|---|---|---|---|---|---|
| BA | r = - 0.831 (**)<br>p < 0.001 | - | r = - 0.579 (**)<br>p < 0.01 | r = 0.743 (**)<br>p < 0.001 | r = - 0.776 (**)<br>p < 0.001 |
| CeL | r = - 0.734 (**)<br>p < 0.001 | r = - 0.717 (**)<br>p < 0.001 | r = - 0.794 (**)<br>p < 0.001 | r = 0.655 (**)<br>p < 0.01 | r = - 0.612 (**)<br>p < 0.01 |
| CeM | - | - | r = - 0.822 (**)<br>p < 0.001 | - | - |
| PL | - | - | r = - 0.786 (**)<br>p < 0.001 | r = - 0.647 (**)<br>p < 0.01 | - |
| IL | r = - 0.874 (**)<br>p < 0.001 | r = - 0.542 (*)<br>p < 0.05 | r = 0.896 (**)<br>p < 0.001 | r = - 0.816 (**)<br>p < 0.001 | r = - 0.600 (**)<br>p < 0.01 |
| CA1 | r = - 0.885 (**)<br>p < 0.001 | - | r = - 0.676 (**)<br>p < 0.001 | r = 0.798 (**)<br>p < 0.001 | r = - 0.918 (**)<br>p < 0.001 |
| CA3 | - | r = - 0.868 (**)<br>p < 0.001 | r = - 0.755 (**)<br>p < 0.001 | r = 0.841 (**)<br>p < 0.001 | r = - 0.892 (**)<br>p < 0.001 |
| DG | r = - 0.870 (**)<br>p < 0.001 | r = - 0.802 (**)<br>p < 0.001 | r = - 0.676 (**)<br>p < 0.001 | - | r = - 0.917 (**)<br>p < 0.001 |

#### 5.1.3.2.3. Hippocampus

In the CA1 of hippocampus, a negative correlation with the freezing response and a positive correlation with the extinction learning for acetyl H3K9 ($p$<0.0001), c-fos ($p$<0.0001) and HDAC1 ($p$<0.001) was observed while a positive correlation with freezing response and a negative correlation with extinction learning for HDAC2 ($p$<0.001) expression was observed when compared to the freezing response during retention test. In CA3, a negative correlation with the freezing response and a positive correlation with the extinction learning for acetyl H4K5 ($p$<0.0001), c-fos ($p$<0.0001) and HDAC1 ($p$<0.0001) was observed while a positive correlation with freezing response and a negative correlation with extinction learning was observed for HDAC2 ($p$<0.0001) expression when compared with the freezing response during retention test between extinction and naïve group. In DG, a negative correlation with the freezing response and a positive correlation with extinction learning for acetyl H3K9 ($p$<0.0001), acetyl H4K5 ($p$<0.0001), c-fos ($p$<0.0001) and HDAC1 ($p$<0.001) level was observed while no correlation with the freezing response during extinction learning was observed for HDAC2 expression. (Fig 34)

## 5.1.4. Real-Time PCR for mRNA expression analysis

### 5.1.4.1. mRNA expression in Amygdala

#### 5.1.4.1.1. BLA

The mRNA expression was analyzed in BLA (Comprise of BA and LA) and CeA (Comprise of CeL and CeM) for c-fos, CBP, HDAC1 and HDAC2 from different experimental groups. In BLA, the c-fos mRNA expression increased significantly in conditioning ($p$<0.01) and extinction groups ($p$<0.001) as compared to the naive control group [F (2, 12) = 19.90, $p$<0.001] (Fig 18). Moreover, CBP mRNA expression increased significantly in conditioning ($p$<0.01) and extinction group ($p$<0.001) as compared to the naive control group [F (2, 12) = 17.13, $p$<0.001] (Fig 19). HDAC1 mRNA exhibited similar expression pattern, where HDAC1 expression increased significantly in conditioning ($p$<0.001) and extinction group ($p$<0.001) as compared to the naive control group [F (2, 12) = 18.16, $p$<0.001] (Fig 21) while HDAC2 mRNA expression decreased significantly in conditioning ($p$<0.01) and extinction group ($p$<0.05) as compared to the naive control group [F (2, 12) = 8.384, $p$<0.01] (Fig 22).

**5.1.4.1.2. CeA**

In CeA, the c-fos mRNA expression increased significantly in conditioning ($p$<0.001) and extinction ($p$<0.01) groups as compared to the naive control group [F (2, 12) = 15.44, $p$<0.001] (Fig 18). Likewise, the CBP mRNA expression increased significantly in conditioning ($p$<0.01) and extinction group ($p$<0.05) as compared to the naive control group [F (2, 12) = 10.41, $p$<0.01] (Fig 19). HDAC1 mRNA expression increased significantly in conditioning ($p$<0.01) and extinction group ($p$<0.0001) as compared to the naive control group [F (2, 12) = 23.41, $p$<0.0001] (Fig 21), whereas HDAC2 mRNA expression decreased significantly in conditioning ($p$<0.01) and extinction group ($p$<0.01) as compared to the naive control group [F (2, 12) = 12.88, $p$<0.001] (Fig 22).

**5.1.4.2. mRNA expression in Prefrontal Cortex**

**5.1.4.2.1. PL**

In PL, the c-fos mRNA expression increased significantly in conditioning group ($p$<0.01) but not in extinction group as compared to the naive control group [F (2, 12) = 12.38, $p$<0.01] (Fig 23). Furthermore, the CBP mRNA expression increased significantly in conditioning group ($p$<0.01) but not in extinction group as compared to the naive control group [F (2, 12) = 16.03, $p$<0.001] (Fig 24). Besides this the HDAC1 mRNA expression decreased significantly in conditioning group ($p$<0.05) but not in extinction group as compared to the naive control group [F (2, 12) = 6.45, $p$<0.05] (Fig 26). In addition the HDAC2 mRNA expression decreased significantly in conditioning group ($p$<0.05) and increased in extinction group ($p$<0.001) as compared to the naive control group [F (2, 12) = 41.22, $p$<0.0001] (Fig 27).

**5.1.4.2.2. IL**

In IL, the c-fos mRNA expression increased significantly in extinction group ($p$<0.01) but not in conditioning group as compared the naive control group [F (2, 12) = 9.8, $p$<0.01] (Fig 23). Similarly CBP mRNA expression increased significantly following extinction learning ($p$<0.001) but not following conditioning as compared to the naive control group [F (2, 12) = 15.38, $p$<0.001] (Fig 24). Besides this, HDAC1 mRNA expression decreased significantly in extinction group ($p$<0.001) but not in conditioning group as compared to the naive control group [F (2, 12) = 16.87, $p$<0.001] (Fig 26). Contrary to HDAC1 expression, HDAC2 mRNA expression decreased significantly in conditioning group ($p$<0.05) but not in extinction group as compared to the naive control group [F (2, 12) = 6.2, $p$<0.05] (Fig 27).

**5.1.4.3. mRNA expression in Hippocampus**

**5.1.4.3.1. CA1**

In CA1, the c-fos mRNA expression increased significantly in conditioning ($p$<0.001) and extinction groups ($p$<0.01) as compared to the naive control group [F (2, 15) = 12.64, $p$<0.001] (Fig 28). Furthermore, the CBP mRNA expression increased significantly in conditioning ($p$<0.01) and extinction group ($p$<0.01) as compared to the naive control group [F (2, 15) = 10.27, $p$<0.01] in CA1 (Fig 29). Besides this the HDAC1 mRNA expression decreased significantly in conditioning ($p$<0.01) and increased significantly in extinction group ($p$<0.001) as compared to the naive control group [F (2, 15) = 40.96, $p$<0.0001] (Fig 31). The HDAC2 exhibited different expression where HDAC2 expression decreased significantly in extinction group ($p$<0.0001) but not in conditioning group as compared to the naive control group [F (2, 15) = 40.71, $p$<0.0001] (Fig 32).

**5.1.4.3.2. CA3**

In CA3, the c-fos mRNA expression increased significantly in conditioning ($p$<0.001) and extinction groups ($p$<0.001) as compared to the naive control group [F (2, 15) = 19.04, $p$<0.0001] (Fig 28). Similarly, the CBP mRNA expression increased significantly in conditioning ($p$<0.01) and extinction groups ($p$<0.01) as compared to the naive control group [F (2, 15) = 12.42, $p$<0.001] (Fig 29). HDAC1 mRNA expression decreased significantly in conditioning ($p$<0.0.01) and increased significantly in extinction group ($p$<0.01) as compared to the naive control group [F (2, 15) = 37.71, $p$<0.0001] (Fig 31). Contrary to HDAC1, HDAC2 mRNA expression decreased significantly in extinction group ($p$<0.001) but not in conditioning group compared to the naive control group [F (2, 15) = 20.57, $p$<0.0001] (Fig 32).

#### 5.1.4.3.3. DG

In DG, the c-fos mRNA expression increased significantly in conditioning ($p$<0.001) and extinction group ($p$<0.001) as compared to the naive control group [F (2, 15) = 17.14, $p$<0.0001] (Fig 28). Moreover, the CBP mRNA expression increased significantly in conditioning ($p$<0.01) and extinction group ($p$<0.01) as compared to the naive control group [F (2, 15) = 10.98, $p$<0.01] (Fig 29). Besides this, HDAC1 mRNA expression increased significantly in extinction group ($p$<0.0001) but not in conditioning group as compared to the naive control group [F (2, 15) = 38.92, $p$<0.0001] (Fig 31) whereas the HDAC2 mRNA expression increased significantly in conditioning ($p$<0.05) and decreased significantly in extinction group ($p$<0.001) as compared to the naive control group [F (2, 15) = 37.65, $p$<0.0001] (Fig 32).

## 5.2. Discussion

The current study involves understanding the role of Histone deacetylases in fear memory consolidation and extinction. In the study, the histone acetylation and deacetylation were found to be playing an important role in fear regulation and the mechanism is controlled differentially by different HDAC subtypes. The basic mechanism of fear memory, functions by regulated control over histone acetylation involved in different stages of fear memory as well as for spatial function in different brain regions (e.g. amygdala, PFC, and hippocampus) (Morris et al, 2010). Basically, the fear memory has its path inside the brain through a complex connection known as fear circuitry which connects amygdala, PFC and hippocampus to produce a fear response (Giustino and Maren, 2015). Though the existence of fear circuitry is well known in animals (e.g. rodents and mammals) the molecular mechanism involved in fear memory is still an enigma (Blackiston et al, 2015). In this study, it has been observed that following fear memory consolidation animals response towards conditioning increased successively from first to the last trial which is similar with the previous studies (Datta and O'Malley, 2013; Siddiqui et al, 2017). However during extinction animal exhibited a reduction in fear response in each successive trial block of behavior as reported in earlier studies (Quirk, 2002; Siddiqui et al, 2017). Earlier studies have figured out that fear memory consolidation and its extinction involves different but overlapping neuronal circuitry known as fear and extinction circuit (Pare and Duvarci, 2012; Sah et al, 2003). The current study similarly signifies the role of different subregions of the amygdala, PFC and hippocampus following fear memory consolidation and extinction.

To understand the association of different brain activity the expression of IEG c-fos, which is a neuronal activity marker protein was analyzed in the amygdala, PFC and hippocampus following fear memory consolidation and extinction. In the amygdala, the activity of LA, BA and CeL shows a positive association with the conditioning and extinction both, however, the CeM activity was associated with the fear memory consolidation but not for the extinction learning.

Similarly, the histone acetylation was associated with the expression of IEG c-fos during conditioning and extinction. Furthermore, the CBP expression shows similarity for the mRNA expression as exhibited by the c-fos mRNA expression in BLA and CeA. These results are showing that the enhanced histone acetylation is associated with the activation of the different subregion of the amygdala in conditioning and extinction. The HDAC1 expression shows a positive association with the histone acetylation and c-fos expression in LA, BA and CeL while in CeM, its expression shows a negative correlation with the c-fos expression in conditioning, but in extinction the HDAC1 expression was higher. Contrary to the HDAC1 expression, HDAC2 expression shows a negative association with the c-fos expression in LA, BA and CeL following conditioning and extinction, while in CeM its expression shows a negative correlation with the c-fos expression. In light of the previous study that describes the presence of fear circuit within the amygdala, it may be hypothesized that these molecular changes observed in the present study are associated with the functionality of the components of fear circuitry (Pare and Duvarsi, 2012; Kim et al, 2015). The presence of different types of neuronal population in LA, BA and CeL might be the reason for such differential molecular mechanism in conditioning and extinction.

The studies have shown that the two types of neuronal populations namely dLAd (dorsal) and vLAd (ventral) exist in LA which response during conditioning and extinction respectively (Repa et al, 2001). So it might be possible that this enhanced expression of c-fos and histone acetylation is associated with these different neuronal populations in LA in conditioning and extinction. Furthermore, the HDAC1 and HDAC2 expression in such LA neurons might have an association with the conditioning and extinction, differentially. Also in LA the mGluR2/3 positive neuronal population respond only during fear extinction (Kim et al, 2015), which make another possibility that the HDAC2 suppression in such mGluR2/3 positive neurons might be associated with the extinction and these neuronal populations most probably confined to vLAd neuronal populations. Although the current work did not involve the study of such neuronal populations, the possible mechanism should be worked out for further clarification.

Furthermore, in BA different neuronal populations known as fear and extinction neurons are present (Pare and Duvarci, 2012) that respond to conditioning and extinction respectively. It is most probable that the increased histone acetylation and activity of BA during conditioning and extinction is associated with the fear and extinction neurons, respectively. Moreover, during conditioning, the increased HDAC1 expression might be restricted to the extinction neurons while decreased HDAC2 expression to the fear neurons which results in enhanced BA response in conditioning via the activity of the fear neuron. During extinction, the increased HDAC1 expression in BA might be confined to the fear neuron while decreased HDAC2 expression with the extinction neurons resulting in enhanced activity of extinction neurons.

In CeL, the PKC-zeta on and off neuronal population exist which respond to the conditioning and extinction respectively (Pare and Duvarci, 2012; Kim et al, 2015). The increased histone acetylation in CeL may be associated with these PKC-zeta on and off neuronal activity during conditioning and extinction. The increased activity through enhanced histone acetylation in PKC-zeta on neurons should be associated with the conditioning while enhanced activity through increased histone acetylation in PKC-zeta off neurons might be associated with the extinction learning. The different HDAC1 and HDAC2 expression in CeL might be associated with the regulation of histone acetylation in these neuronal populations during conditioning and extinction.

The CeM, which is the output center for fear expression showed enhanced activity through increased histone acetylation in conditioning but not in extinction. The activity of CeM might be associated with the enhanced histone acetylation in conditioning. The decreased HDAC1 and

HDAC2 expression in CeM during conditioning suggest that diminished HDAC1 and HDAC2 expression results in increased histone acetylation and the activity of CeM. During extinction, the HDAC1 expression was enhanced in CeM, which results in regulation of the fear memory expression. As both the HDACs are suppressed during conditioning but only HDAC1 retains its activity for the extinction memory and might function to create a break on fear expression. So it may be speculated that HDAC1 alone or in combination with HDAC2 regulate the activity of CeM through regulation of histone acetylation.

As histone acetylation for H3 and H4 exhibited a positive correlation in LA, BA, CeL, and CeM during conditioning with freezing behavior, the effect is mostly due to the activation of fear circuitry involving these brain regions (Siddiqui et al, 2017). During extinction, there is an increase in histone acetylation in LA, BA, and CeL which might be due to the enhanced activity of extinction circuitry while no activity in CeM of the amygdala suggests for its suppression or control by the inhibitory neurons. Fear and extinction circuitry overlaps in terms of brain sub-region but the difference lies in the type of neurons (i. e. fear or extinction neurons, excitatory or inhibitory neurons) (Pare and Duvarci, 2012). Previous studies have focused the role of LA and BA in fear memory consolidation and extinction as lesion prior to consolidation and extinction in these region results in impaired expression of fear during consolidation and extinction respectively (Nader et al, 2001; Anglada-Figueroa and Quirk, 2005; Sierra-Mercado et al, 2011). Kim et al, (2015) in his research suggested that mGluR2/3 receptor is required for retention of extinction learning in LA (Kim et al, 2015) This might be the reason behind differential HDAC1 and HDAC2 expression in LA following conditioning and extinction (Kim et al, 2015) as it is clear from the above discussion as well as with previous studies that LA contain different neuronal populations (Cells that respond during conditioning and cells that respond during extinction). It has been concluded from the studies that LA receives input from IL and MGm to promote extinction by reversing the conditioning-induced potentiation at MGm-LA inputs (Park and Choi, 2010).

The PL and IL of Prefrontal cortex act differentially during fear memory consolidation and extinction (Pelloux et al, 2013; Giustino and Maren, 2015). PL has a potent role for activating fear circuitry while the activity of IL is associated with the activation of fear extinction circuitry (Peters et al, 2009; Knapska and Maren, 2009). The results showed increased activity and histone acetylation in PL during fear memory consolidation while in IL during fear extinction.

The Prefrontal cortex exhibited different patterns of HDAC expression during fear memory consolidation and extinction. In PL, the HDAC1 and HDAC2 expression were negatively associated with the conditioning as exhibited by the conditioning group. As previous studies have focused for the presence of a glutamatergic population in PL (Patton et al, 2013; Marek et al, 2013), the decreased expression of both the HDACs might be involved in enhancement of histone acetylation during conditioning which results in enhanced PL activity. During extinction, the HDAC2 shows enhanced expression which should be associated with the suppression of PL and the most probable mechanism might be through GABAergic inhibitory connections through IL (Saffari et al, 2016; Jones et al, 2005). Overall the result suggests that for conditioning both the HDACs subtype should be suppressed which in turn increases histone acetylation, while for extinction only HDAC2 function is necessary for PL. It may also be concluded from the result that HDAC2 is a potent regulator of PL activity during extinction.

In IL, both the HDACs exhibited different expression following fear memory consolidation and extinction. HDAC2 expression decreased following conditioning while there was no change in HDAC1 expression. As both the PL and IL sends inhibitory GABergic connections to each other

(Saffari et al, 2016; Jones et al, 2005), it might be possible that the activation of these neuronal population in PL and IL is associated with the HDAC subtype activity.

In the current study, the hippocampus subregions CA1, CA3 and DG were activated following fear memory consolidation and extinction. The result shows similarity with the previous studies, showing the role of CA1, CA3 and DG in consolidation (Lee and Kesner, 2004) and extinction (Bernier et al, 2017; Ji and Maren, 2008) using different lesion studies. Although the study showed the involvement of CA1, CA3 and DG in conditioning and extinction but no clear evidence is available showing the presence of different neuronal population in these subregions associated with the conditioning and extinction. Although some studies have pointed that different neuronal population responds during conditioning and extinction in CA1, CA3 and DG (Vlachos et al, 2011). The activation mechanism was associated with the increased histone acetylation and HAT activity (CBP/p300 activity) and increased histone acetylation promotes the activity of hippocampus in conditioning and extinction. Although studies by Tronson et al (2009) confirmed the current result, where, an increased c-fos expression in conditioning and extinction was found to be associated with the contextual learning. Furthermore, Sanders et al (2003) and Maren et al (1997) showed in his experiment the role of the hippocampus in context and tone conditioning both, as pre and post-training Hippocampal lesion of the dorsal hippocampus produced a modest tone conditioning deficits. So it might be possible that conditioning and extinction activate hippocampus for the acquisition of CS part of learning through increased histone acetylation. Extinction similar to the conditioning is a new type of learning which require almost similar molecular and cellular mechanism (Myers et al, 2006) and results in activation of Hippocampal subregion following conditioning and extinction. The information can be further increased by the experiments showing the activity of different sets of hippocampal neurons (e.g. in CA1) in different contexts (Vlachos et al, 2011). Furthermore, the CA3 and DG also include different populations of neurons showing activity in different processes (Leutgeb et al, 2007; Knierim, 2002; Kentros et al, 2004).

In hippocampal subregion, both the HDACs showed different expression during conditioning and extinction. During conditioning the HDAC1 expression was suppressed in CA1 and CA3 while HDAC2 expression was increased in DG. During extinction the HDAC1 expression increased in CA1, CA3 and DG while HDAC2 expression decreased in CA1, CA3 and DG subregion of the hippocampus. Although limited information is available about the neuronal population in hippocampus subregion. It might be possible that some neurons respond to conditioning and others to extinction, and this differential activity of neuronal population is associated with the different activity of HDAC1 and HDAC2 during conditioning and extinction.

***Chapter 6***

# RESULTS & DISCUSSION: Aim2 - Valproic acid effect on Conditioning

## Aim 2:

**To find out the effect of HDAC inhibition on consolidation and extinction of fear memory**

**(A). Effect of valproic acid on Conditioning of fear memory**

# 6.1. Results

## 6.1. 1. Behavior results

### 6.1.1.1. Anxiety measurement for valproic acid

EPM (Elevated plus maze) test was performed to measure the anxiolytic /anxiogenic effect caused by the valproic acid in rats. In EPM test, the animals explore the environment but anxiogenic factors affect this behavior which results in the decreased exploration of the environment in open arm. Overall, the entries in open arm were measured as percent entries in the open arm for both the sham treated and valproic acid treated groups in overall duration of time. Both the groups treated with sham (0.01M PBS) and valproic acid (100 mg/kg) exhibited no significant difference with each other for percent entries in open arms when compared in an overall session of the test. A student's *t*-test was performed to compare the entries in open arms where both the groups exhibited no significant difference for the entries in EPM test ($n$=7 animals in each group) [F (6,6) = 1.068, $p$>0.05]. (Fig 35)

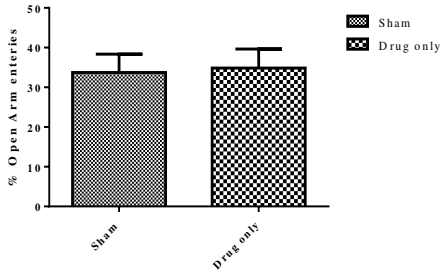



**Fig 35.** EPM test. Anxiety level measured between sham control and valproic acid treated groups exhibited no significant difference with each other ($p$>0.05).

### 6.1.1.2. Effect of Valproic acid treatment on Conditioning

Animals were injected intraperitoneally with the valproic acid (100 mg/kg) or vehicle (0.01M PBS) 2 hrs before the experiment. It was observed that during initial conditioning trial both the drug + conditioning and sham + conditioning groups exhibited a similar level of freezing but overall the freezing was higher in subsequent trials of drug + conditioning group as compared to the sham + conditioning group [F (7,7) = 1.891, $p$< 0.01].

A retention test was performed 24 hrs following conditioning which involves 5 trials of tone in context B. When compared using a*t*-test, drug + conditioning group exhibited significantly enhanced freezing as compared to the sham + conditioning group [F (7,7) = 6.314, $p$< 0.0001]. Initial trials for retention test exhibited similar freezing in both the groups but in later trials drug + conditioning group exhibited enhanced freezing response as compared to the sham + conditioning group. In conclusion, the valproic acid treatment results in an increase in fear learning in drug + conditioning group. (Fig 36)

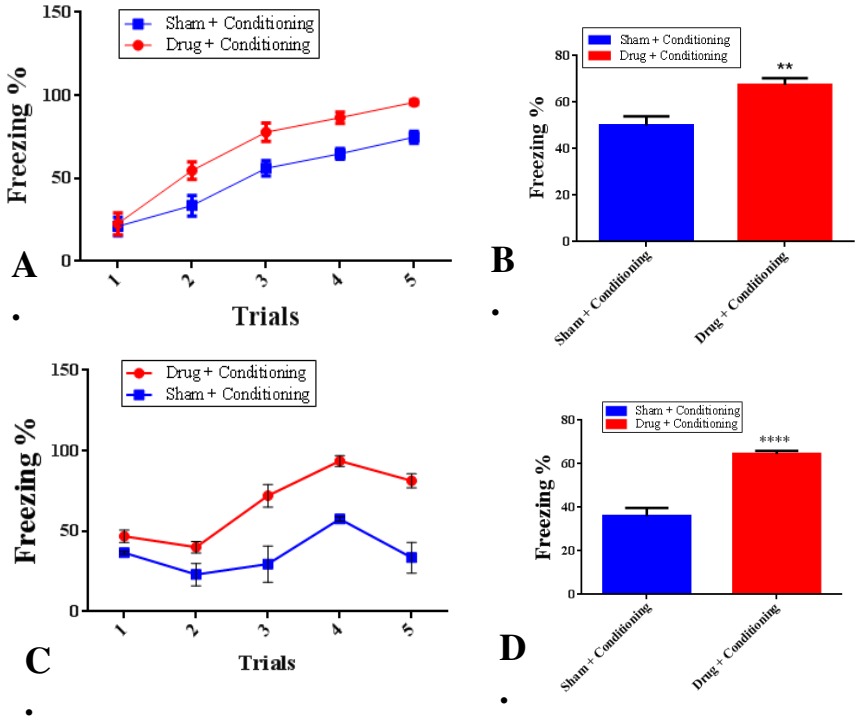



**Fig 36.** Effect of Valproic acid on fear memory consolidation. **(A)** Conditioning training for sham treated (Sham + Conditioning) and valproic acid treated group (Drug + Conditioning), for all trials **(B)** Conditioning training for sham treated and valproic acid treated group, average values of all trials **(C)** Retention test, all 5 trials **(D)** Retention test, average values of all trials.

## 6.1.2. Immunohistochemistry

### 6.1.2.1. c-fos expression in the Amygdala

The c-fos expression in LA increased significantly in drug only ($p$<0.05), sham + conditioning ($p$<0.0001) and drug + conditioning group ($p$<0.0001) as compared to the sham control group. However, the change in expression was significantly higher in sham + conditioning and drug + conditioning group compared to the drug only group in LA [F (3,36) = 34.33, $p$<0.0001]. Furthermore in BA, the c-fos expression increased significantly in drug only ($p$<0.05), sham + conditioning ($p$<0.001) and drug + conditioning ($p$<0.0001) group as compared to the sham control group. The expression in Drug + conditioning group was significantly higher for c-fos expression than drug only and sham + conditioning group which might be due to the effect of valproic acid on enhancement of c-fos expression following conditioning [F (3,36) = 12.94, $p$<0.0001]. The CeL exhibited similar expression for c-fos, where c-fos expression increased significantly in drug only ($p$<0.001), sham + conditioning ($p$<0.0001) and drug + conditioning ($p$<0.0001) groups as compared to the sham control group [F (3,36) = 50.17, $p$<0.0001]. When compared, Sham + conditioning and drug + conditioning group exhibited a significantly higher level of c-fos expression as compared to the drug only group. In CeM, the c-fos expression increased significantly in drug + conditioning group ($p$<0.0001) as compared to the sham control groups while drug only and sham + conditioning groups exhibited no significant change as compared to the sham control group [F (3,36) = 17.17, $p$<0.0001]. (Fig 37)

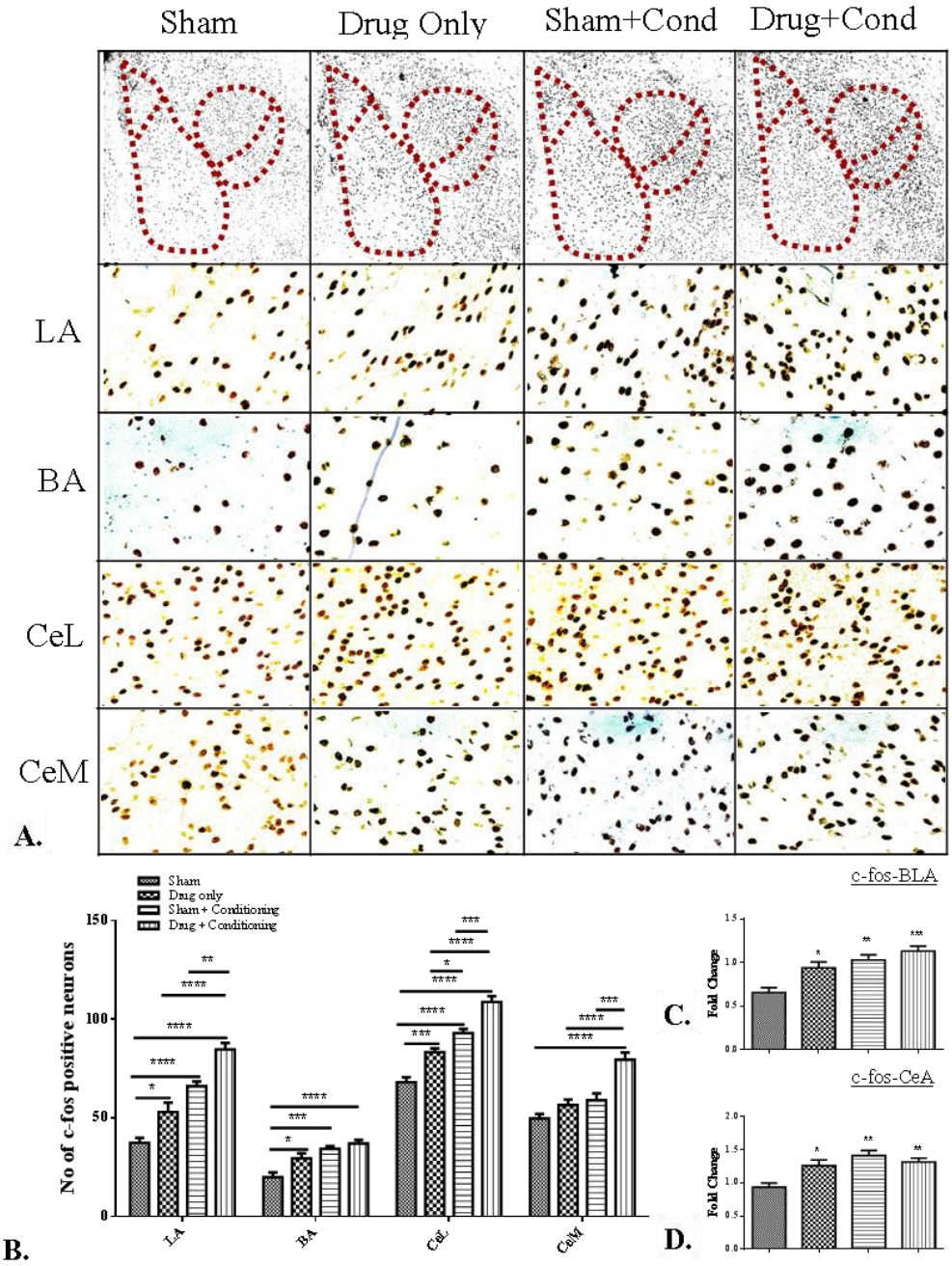



**Fig 37.** c-fos expression in the amygdala in valproic acid treated conditioning group. **A. and B.** Immunohistochemistry of c-fos expression in LA, BA, CeL and CeM following conditioning. **C. and D.** c-fos mRNA expression in BLA (C.) and CeA (D.). (Drug only= valproic acid only, Sham+Cond= vehicle treated conditioning group and Drug+Cond= valproic acid treated conditioning group)

### 6.1.2.2. Histone H3K9 acetylation in the Amygdala

Histone H3K9 acetylation in LA increased significantly in drug only ($p$<0.05), sham + conditioning ($p$<0.0001) and drug + extinction group ($p$<0.0001) as compared to the sham control group [F (3,36) = 45.02, $p$<0.0001]. The acetylation level was significantly higher in sham + conditioning and drug + conditioning groups compared to drug only group following conditioning. Likewise, BA exhibited higher level for H3K9 acetylation in drug only ($p$<0.05), sham +

conditioning ($p$<0.001) and drug + conditioning ($p$<0.0001) groups as compared to the sham control group [F (3,36) = 27.72, $p$<0.0001]. The level was significantly higher in drug + conditioning group as compared to the sham + conditioning and drug only groups. In CeL, the histone H3K9 acetylation increased significantly in drug only ($p$<0.05) sham + conditioning ($p$<0.0001) and drug + conditioning ($p$<0.0001) group as compared to the sham control group [F (3,36) = 16.98, $p$<0.001]. The CeM witnessed with increased acetyl H3K9 in sham + conditioning ($p$<0.001) and drug + conditioning ($p$<0.0001) group as compared to the sham control groups. Both the sham + conditioning and drug + conditioning group exhibited a higher level of H3K9 acetylation as compared to drug only group [F (3,36) = 30.53, $p$<0.0001]. (Fig 38)

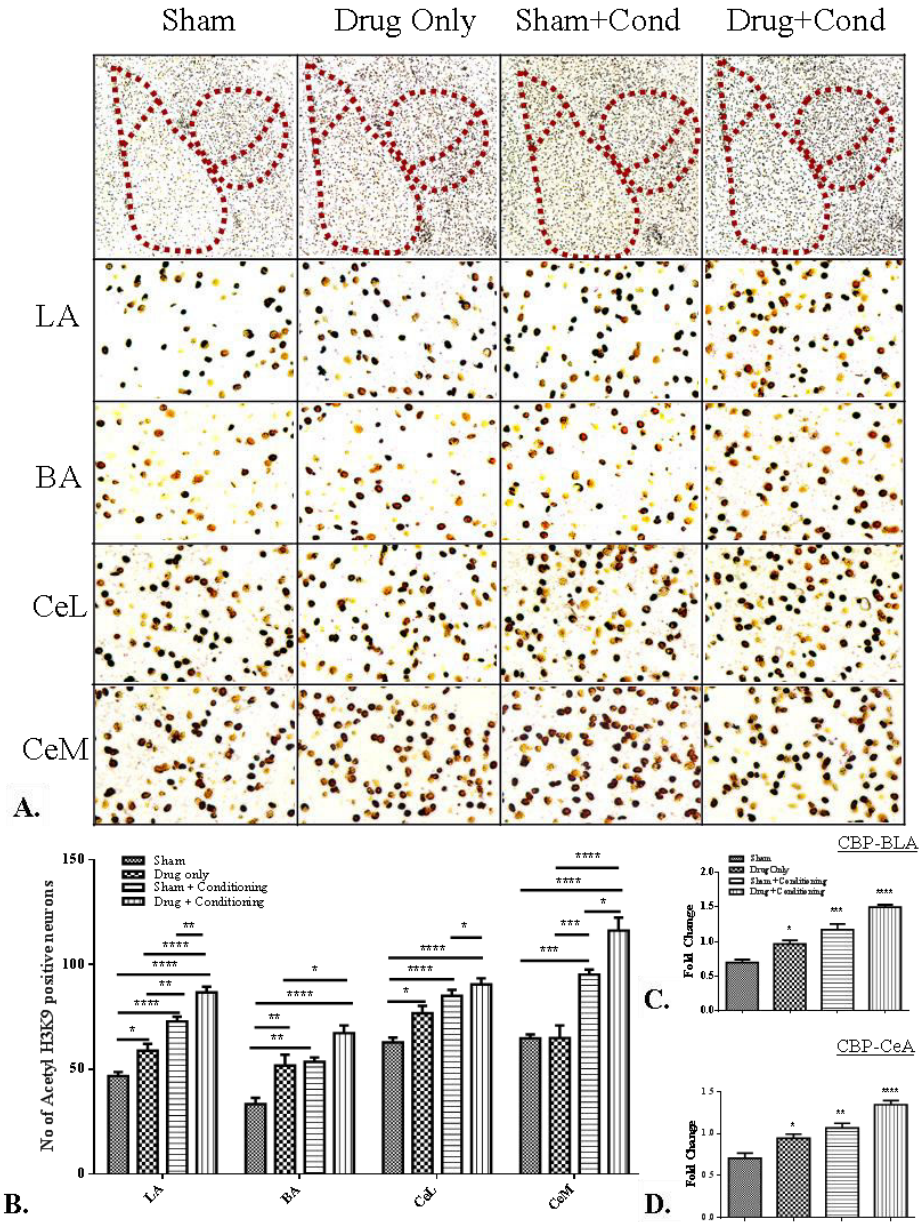



**Fig 38.** Histone H3K9 acetylation in the amygdala in valproic acid treated conditioning group. **A. and B.** Immunohistochemistry of histone H3K9 acetylation in LA, BA, CeL and CeM following conditioning. **C. and D.** CBP mRNA expression in BLA (C.) and CeA (D.). (Drug only= valproic acid only, Sham+Cond= sham treated conditioning group, and Drug+Cond= valproic acid treated conditioning group).

**6.1.2.3. Histone H4K5 acetylation in the Amygdala**

In LA, the histone H4K5 acetylation increased significantly in drug only ($p$<0.05), sham + conditioning ($p$<0.01) and drug + conditioning ($p$<0.0001) group as compared to the sham control group [F (3,36) = 26.45, $p$<0.0001]. Drug + conditioning group exhibit higher level of acetyl H4K5 compared to the sham + conditioning group following conditioning. In BA, the acetyl H4K5 increased significantly in drug only ($p$<0.05), sham + conditioning ($p$<0.05) and drug + conditioning group ($p$<0.0001) as compared to the sham control group [F (3,36) = 12.83, $p$<0.0001]. (Fig 39)

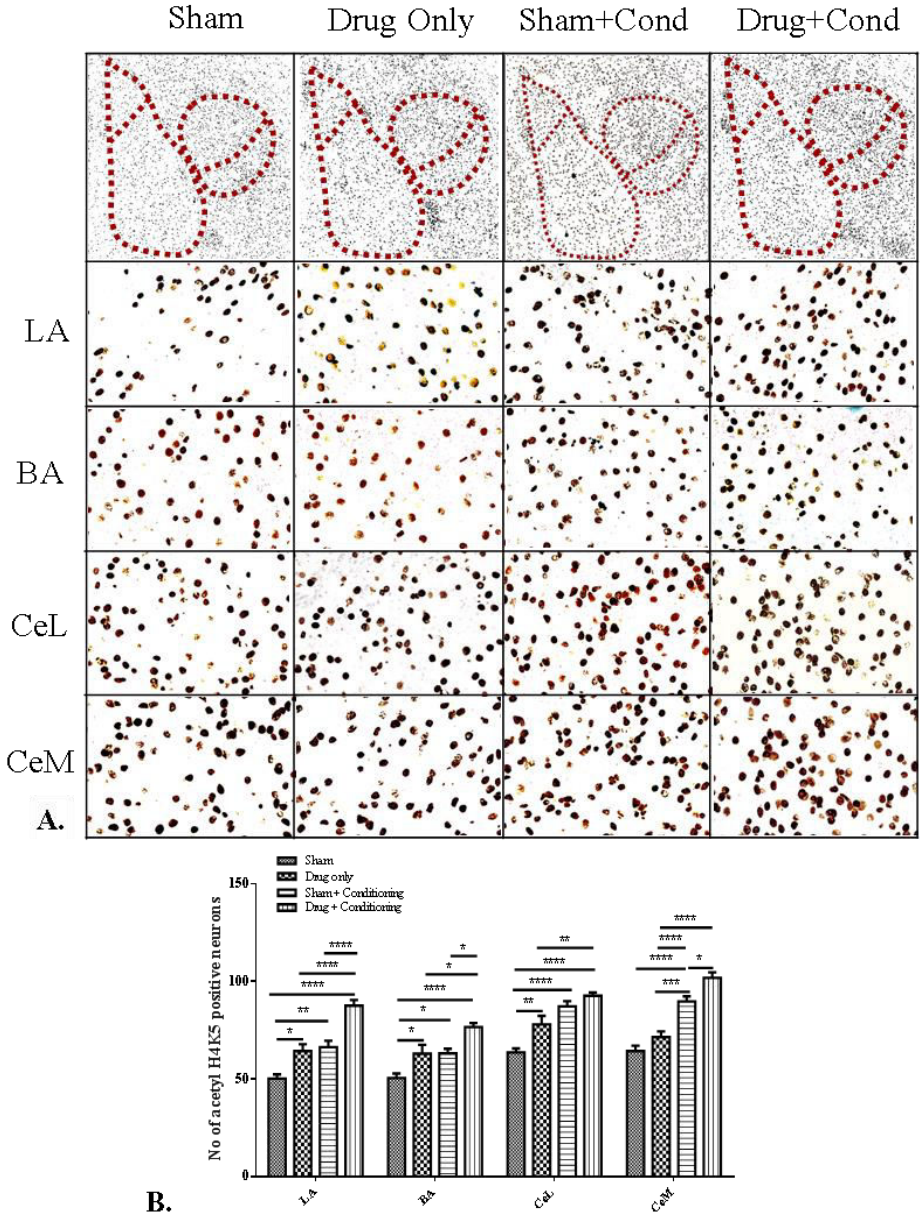



**Fig 39.** Histone H4K5 acetylation in the amygdala in valproic acid treated conditioning group**. A. and B.** Immunohistochemistry of histone H4K5 acetylation in LA, BA, CeL and CeM following conditioning. (Drug only= valproic acid only, Sham+Cond= vehicle treated conditioning group and Drug+Cond= valproic acid treated conditioning group).

The acetyl H4K5 expression was higher in drug + conditioning group than in sham + conditioning group following conditioning. Similarly in CeL, the histone H4K5 acetylation

increased in drug only ($p$<0.01), sham + conditioning ($p$<0.0001) and drug + conditioning ($p$<0.0001) group significantly as compared to the sham control group following conditioning training [F (3,36) = 19.89, $p$<0.0001]. In CeM, the acetyl H4K5 level increased significantly in sham + conditioning ($p$<0.0001) and drug + conditioning group ($p$<0.0001) as compared to the sham control group while no change was observed in drug only group [F (3,36) = 37.45, $p$<0.0001].

#### 6.1.2.4. HDAC1 expression in the Amygdala

The HDAC1 expression in LA, increased significantly in sham + conditioning ($p$<0.0001) and drug + conditioning ($p$<0.0001) group as compared to the sham control group while drug only group exhibited no significant change [F (3,36) = 45.76, $p$<0.0001]. (Fig 40)

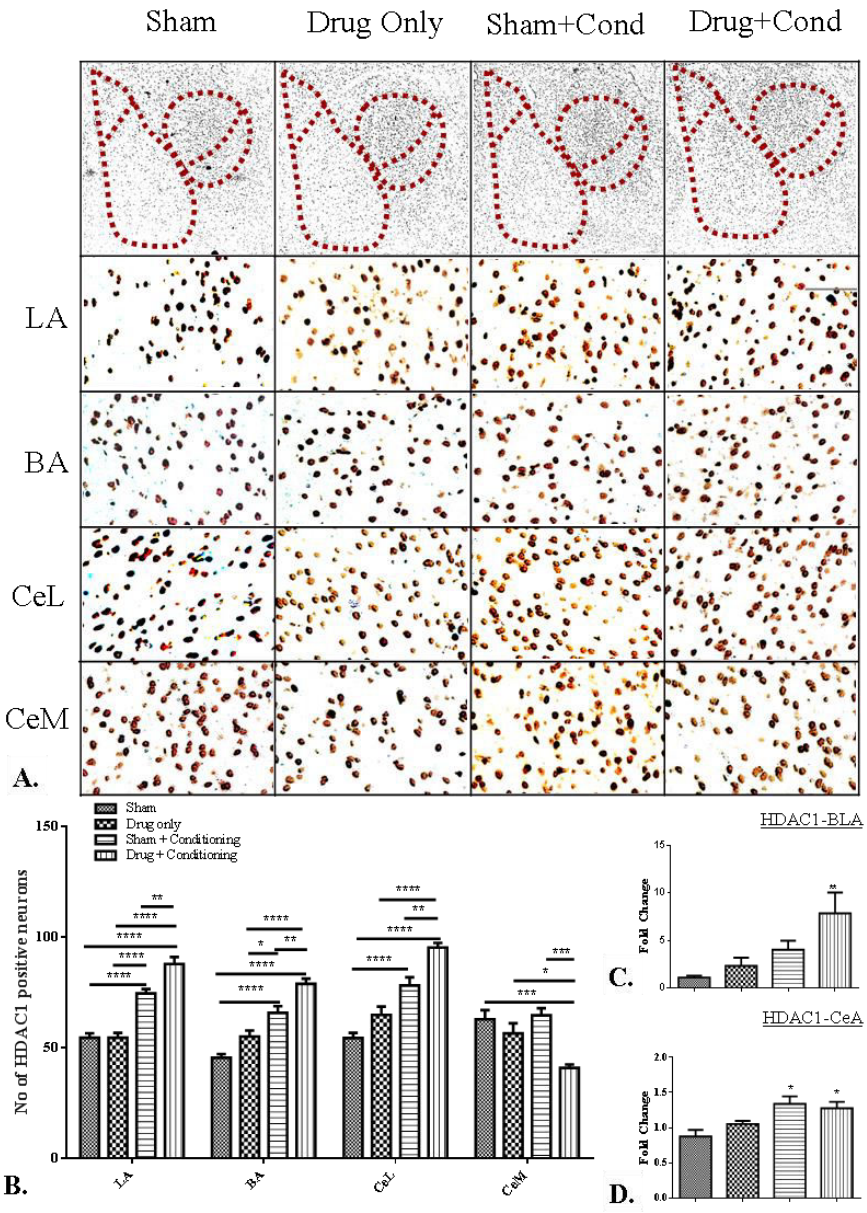



**Fig 40.** HDAC1 expression in the amygdala in valproic acid treated conditioning group**. A. and B.** Immunohistochemistry of HDAC1 expression in LA, BA, CeL and CeM following conditioning. **C. and D.** HDAC1 mRNA expression in BLA (C.) and CeA (D.). (Drug only= valproic acid only, Sham+Cond= vehicle treated conditioning group and Drug+Cond= valproic acid treated conditioning group)

Moreover, in BA, the HDAC1 expression increased significantly in sham + conditioning ($p$<0.0001) and drug + conditioning group ($p$<0.0001) as compared to the sham control group [F (3,36) = 32.59, $p$<0.0001]. Similarly in CeL, the HDAC1 expression increased significantly in sham + conditioning ($p$<0.0001) and drug + conditioning group ($p$<0.0001) as compared to the sham control group [F (3,36) = 32.43, $p$<0.0001]. In CeM, the HDAC1 expression decreased significantly in drug + conditioning ($p$<0.001) as compared to the sham control group while there was no significant change observed in drug only and sham + conditioning group ($p$>0.05) [F (3,36) = 9.140, $p$<0.0001].

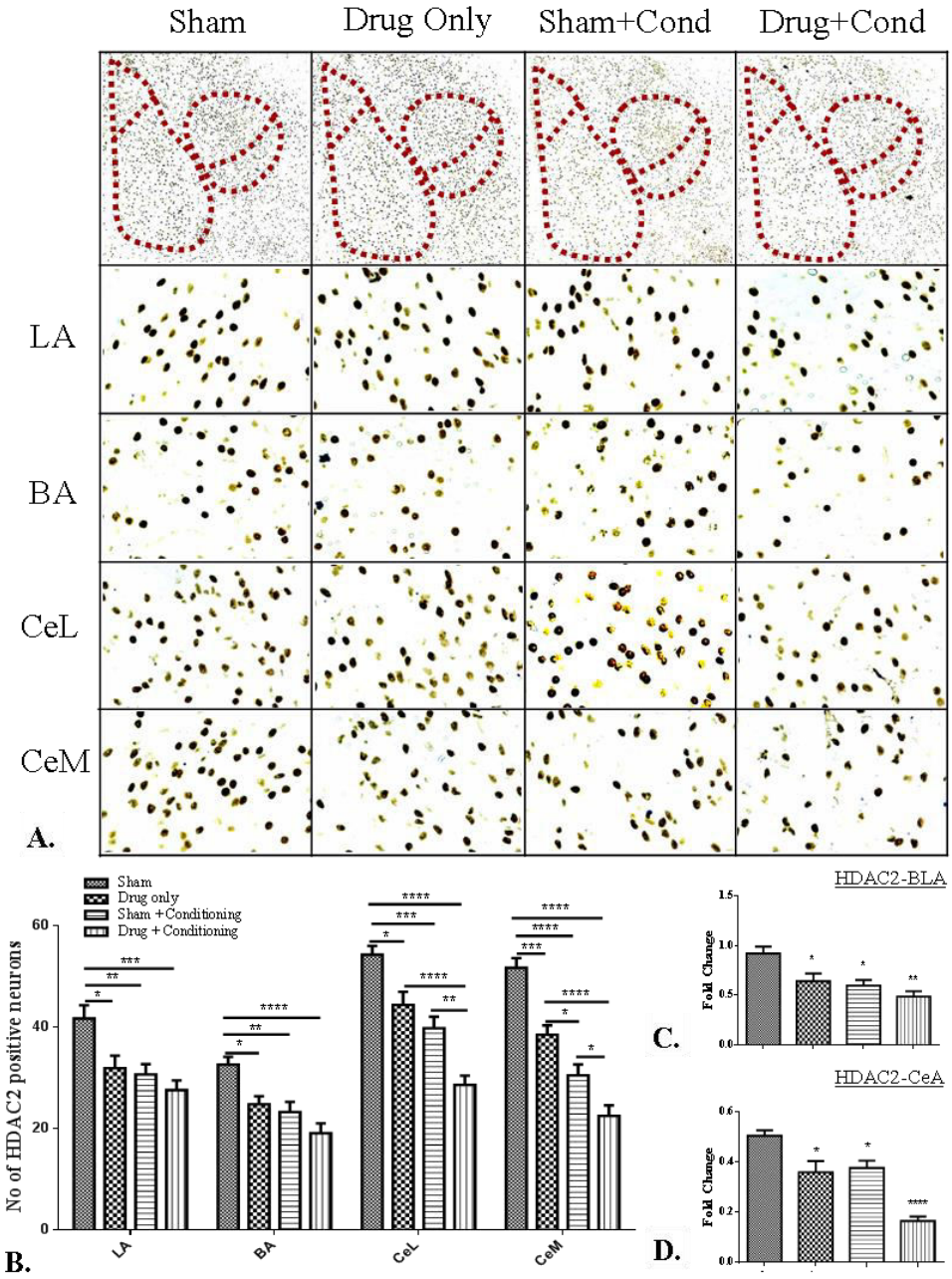



**Fig 41.** HDAC2 expression in the amygdala in valproic acid treated conditioning group**. A. and B.** Immunohistochemistry of HDAC2 expression in LA, BA, CeL and CeM following conditioning. **C. and D.** HDAC2 mRNA expression in BLA (C.) and CeA (D.). (Drug only= valproic acid only, Sham+Cond= vehicle treated conditioning group and Drug+Cond= valproic acid treated conditioning group)

### 6.1.2.5. HDAC2 expression in the Amygdala

HDAC2 expression exhibited different expression than HDAC1 in the amygdala. HDAC2 expression decreased significantly in LA in drug only ($p<0.05$), sham + conditioning ($p<0.01$) and drug + conditioning ($p<0.001$) groups as compared to the sham control group [F (3,36) = 7.224, $p<0.001$]. Drug + conditioning group shows significantly lower expression as compared to the drug only and sham + conditioning group following training. Furthermore in BA, the HDAC2 expression decreased significantly in the entire three groups drug only ($p<0.05$), sham + conditioning ($p<0.01$) and drug + conditioning ($p<0.0001$) group as compared to the sham control group [F (3,36) = 10.04, $p<0.0001$]. The expression of HDAC2 in drug + conditioning group was significantly lower as compared to the drug only and sham + conditioning group. In CeL, the HDAC2 expression decreased in drug only ($p<0.05$), sham + conditioning ($p<0.001$) and drug + conditioning ($p<0.0001$) group as compared to the sham control group [F (3,36) = 25.15, $p<0.0001$]. Drug + conditioning group exhibited significantly reduced freezing as compared to the drug only and sham + conditioning group. In CeM, similarly the HDAC2 expression decreased significantly in drug only ($p<0.001$), sham + conditioning ($p<0.0001$) and drug + conditioning ($p<0.0001$) group as compared to the sham control group [F (3,36) = 38.61, $p<0.0001$]. (Fig 41)

### 6.1.2.6. c-fos expression in Prefrontal Cortex

As a marker for brain activity the c-fos expression was analyzed and it was observed that the c-fos expression was affected by the HDAC inhibitor (Valproic acid) in PFC. (Fig 42)

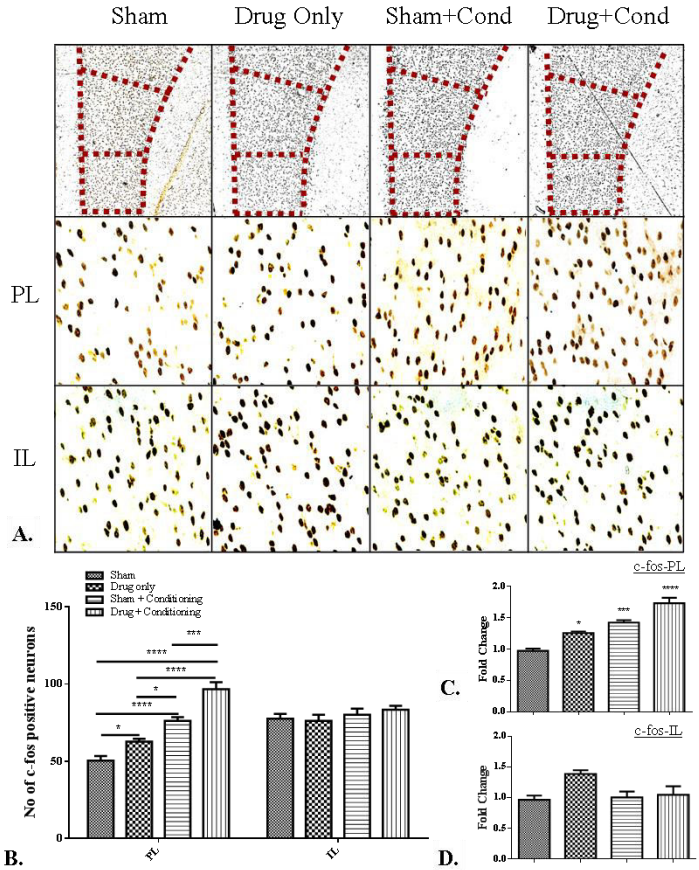



**Fig 42.** c-fos expression in Prefrontal cortex in valproic acid treated conditioning group. **A. and B.** Immunohistochemistry of c-fos expression in PL and IL following conditioning. **C. and D.** c-fos mRNA expression in PL (C.) and IL (D.). (Drug only= valproic acid only, Sham+Cond= vehicle treated conditioning group and Drug+Cond= valproic acid treated conditioning group).

In PL, the c-fos expression increased significantly in drug only ($p$<0.05), sham + conditioning ($p$<0.0001) and drug + conditioning group ($p$<0.0001) as compared to the sham control group [F (3,36) = 41.56, $p$<0.0001]. The c-fos expression was significantly higher in sham + conditioning and drug + conditioning group than drug only group following conditioning. Besides this in IL, no significant change was observed in drug + conditioning and sham + conditioning group for c-fos expression as compared to the sham control group [F (3,36) = 0.8461, $p$>0.05].

### 6.1.2.7. Histone H3K9 acetylation in Prefrontal Cortex

Similar to c-fos, the histone H3K9 acetylation increased significantly in drug only ($p$<0.05), sham + conditioning ($p$<0.001) and drug + conditioning ($p$<0.0001) group as compared to the sham control group. The level of acetyl H3K9 in PL was significantly higher in drug + conditioning group than drug only and sham + conditioning group [F (3,36) = 29.76, $p$<0.0001]. In IL, no significant change was observed in all the three groups, drug only, sham + conditioning and drug + conditioning group as compared to the sham control group [F (3,36) = 0.229, $p$>0.05]. (Fig 43)

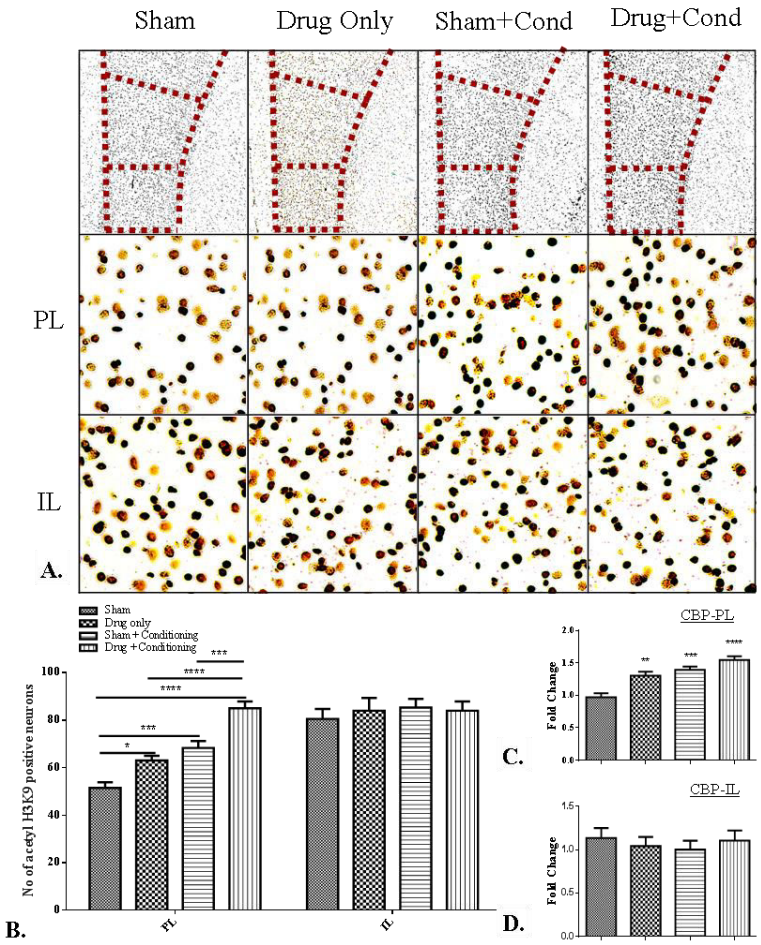



**Fig 43.** Histone H3K9 acetylation in Prefrontal cortex in valproic acid treated conditioning group. **A. and B.** Immunohistochemistry of histone H3K9 acetylation in PL and IL following conditioning. **C. and D.** CBP mRNA expression in PL (C.) and IL (D.). (Drug only= valproic acid only, Sham+Cond= sham treated conditioning group and Drug+Cond= valproic acid treated conditioning group).

### 6.1.2.8. Histone H4K5 acetylation in Prefrontal Cortex

Similar to acetyl H4K5, the histone H4K5 acetylation increased significantly in drug only ($p$<0.0001), sham + conditioning ($p$<0.0001) and drug + conditioning ($p$<0.0001) group as compared to the sham control group in PL [F (3,36) = 35.39, $p$<0.0001]. While in IL no significant change was observed in drug only, sham + conditioning and drug + conditioning group as compared to the sham control group [F (3,36) = 0.369, $p$>0.05]. (Fig 44)

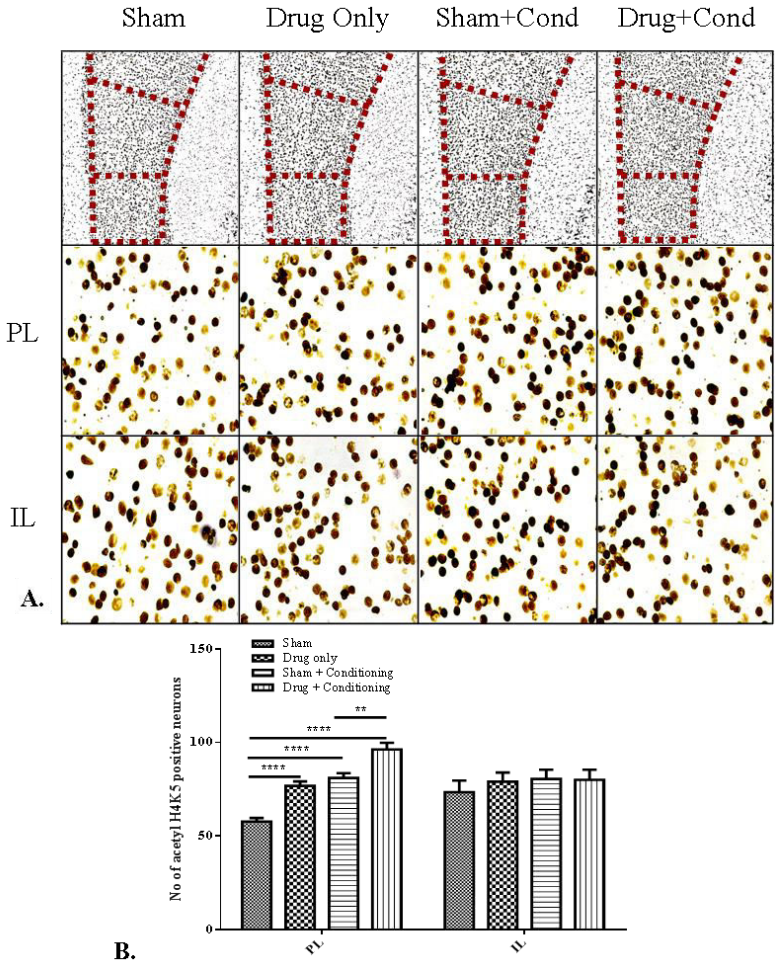



**Fig 44.** Histone H4K5 acetylation in Prefrontal cortex in valproic acid treated conditioning group**. A. and B.** Immunohistochemistry of histone H5K5 acetylation in PL and IL following conditioning. (Drug only= valproic acid only, Sham+Cond= vehicle treated conditioning group and Drug+Cond= valproic acid treated conditioning group).

### 6.1.2.9. HDAC1 expression in Prefrontal Cortex

In PL, the HDAC1 expression decreased significantly in sham + conditioning ($p$<0.05) and drug + conditioning group ($p$<0.0001) but not in drug only group as compared to the sham control group [F (3,36) = 19.37, $p$<0.0001]. Drug + conditioning group exhibited a significantly lower level of expression as compared to sham + conditioning group. Furthermore, no significant change for HDAC1 expression was observed in IL between drug only, sham + conditioning and drug + conditioning group, showing no effect of HDAC1 expression in IL to regulate conditioning [F (3,36) = 0.618, $p$>0.05]. (Fig 45)

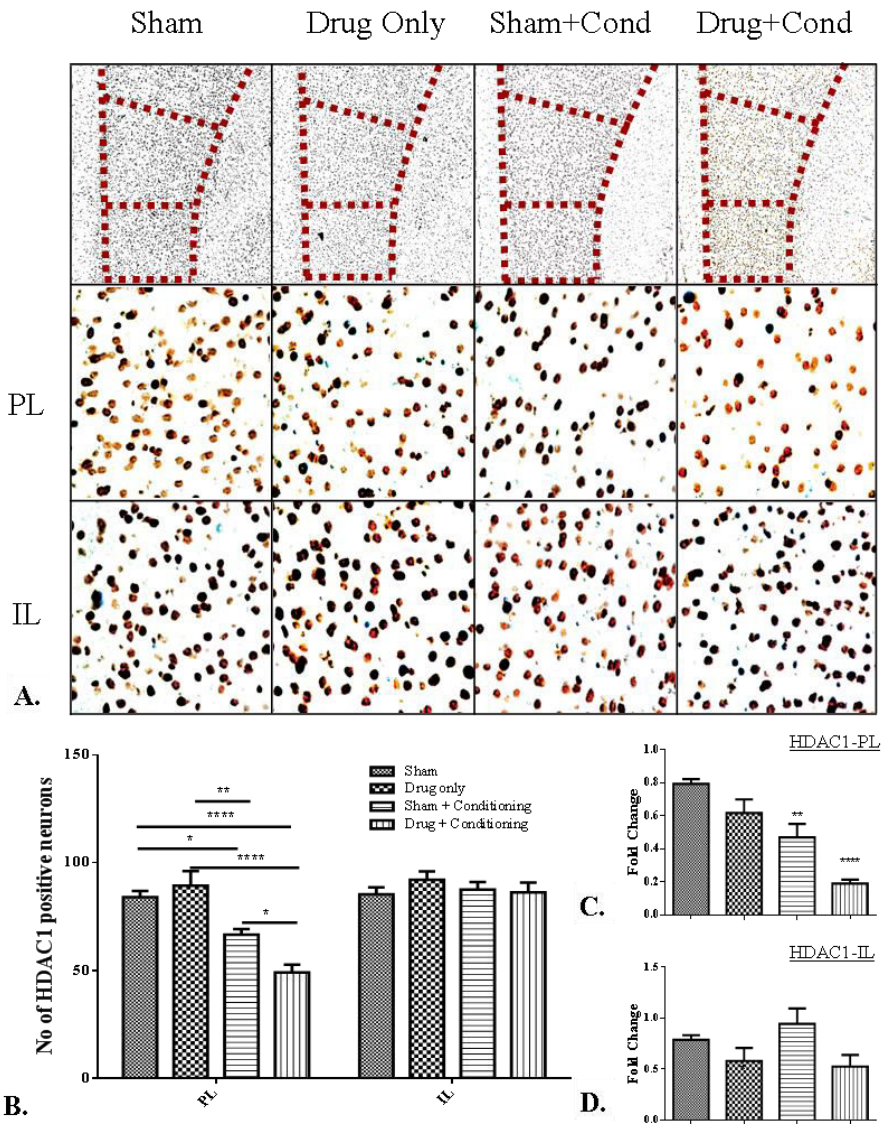



**Fig 45.** HDAC1 expression in Prefrontal cortex in valproic acid treated conditioning group**. A. and B.** Immunohistochemistry of HDAC1 expression in PL and IL following conditioning. **C. and D.** HDAC1 mRNA expression in PL (C.) and IL (D.). (Drug only= valproic acid only, Sham+Cond= vehicle treated conditioning group and Drug+Cond= valproic acid treated conditioning group).

### 6.1.2.10. HDAC2 expression in Prefrontal Cortex

HDAC2 expression similar to the HDAC1 expression decreased in PL in drug only ($p$<0.0001), sham + conditioning ($p$<0.0001) and drug + conditioning ($p$<0.0001) group as compared to the sham control group [F (3,36) = 64.06, $p$<0.0001]. Drug + conditioning group exhibited a lower level of HDAC2 expression as compared to the drug only and sham + conditioning group. In IL, however the HDAC2 expression decreased significantly in drug only ($p$<0.05), sham + conditioning ($p$<0.0001) and drug + conditioning ($p$<0.0001) group as compared to the sham control group [F (3,36) = 35.62, $p$<0.0001]. Drug + conditioning group exhibited a lower level ofHDAC2expression as compared to sham + conditioning and drug only group. (Fig 46)

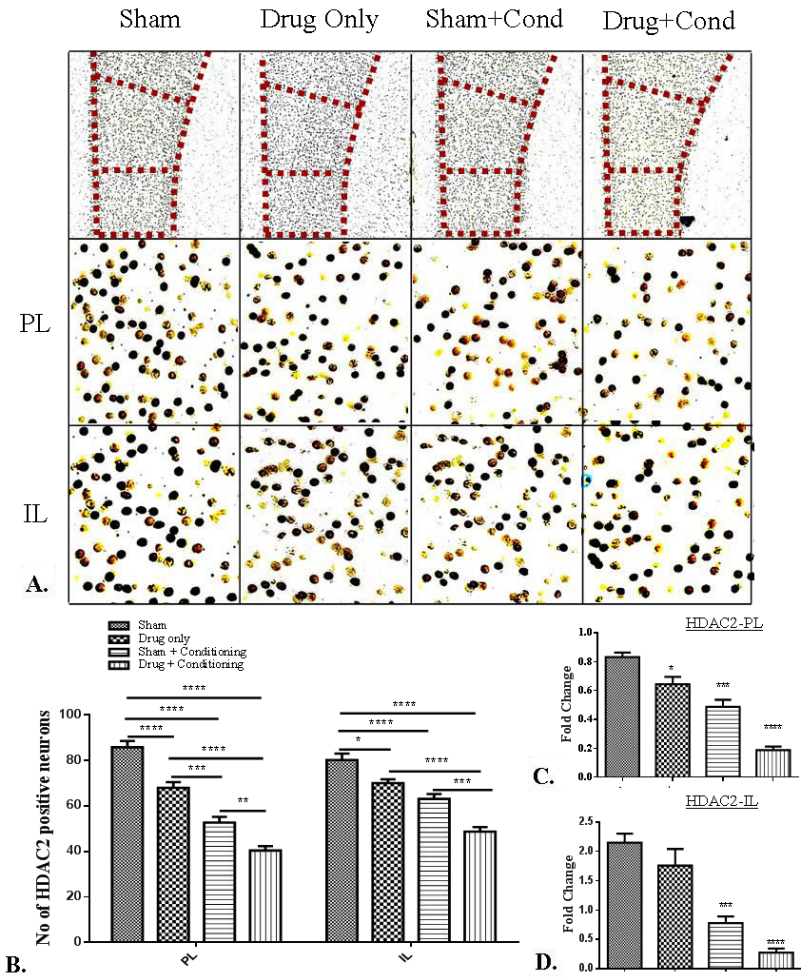



**Fig 46.** HDAC2 expression in Prefrontal cortex in valproic acid treated conditioning group**. A. and B.** Immunohistochemistry of HDAC2 expression in PL and IL following conditioning. **C. and D.** HDAC2 mRNA expression in PL (C.) and IL (D.). (Drug only= valproic acid only, Sham+Cond= vehicle treated conditioning group and Drug+Cond= valproic acid treated conditioning group).

### 6.1.2.11. c-fos expression in Hippocampus

c-fos expression in CA1, increased significantly in drug only ($p$<0.01), sham + conditioning ($p$<0.0001) and drug + conditioning group ($p$<0.0001) as compared to the sham control group [F (3,36) = 97.42, $p$<0.0001]. When compared, drug + conditioning group exhibited a higher level of c-fos expression as compared to the drug only and sham + conditioning group which may be due to an additive effect of valproic acid during conditioning on c-fos expression. Similar to CA1, in CA3, the c-fos expression increased significantly in drug only ($p$<0.05), sham + conditioning ($p$<0.0001) and drug + conditioning group ($p$<0.0001) as compared to the sham control group [F (3,36) = 33.15, $p$<0.0001] whereas drug + conditioning group exhibited similarly higher level of c-fos expression as compared to drug only and sham + conditioning group. In DG, however, the c-fos expression increased significantly in sham + conditioning ($p$<0.0001) and drug + conditioning group ($p$<0.0001) as compared to the sham control group whereas drug only group exhibited no significant change as compared to the sham control group [F (3,36) = 100.7, $p$<0.0001]. When

compared drug + conditioning group exhibited a significantly higher level of c-fos expression as compared to sham + conditioning group, in DG. (Fig 47)

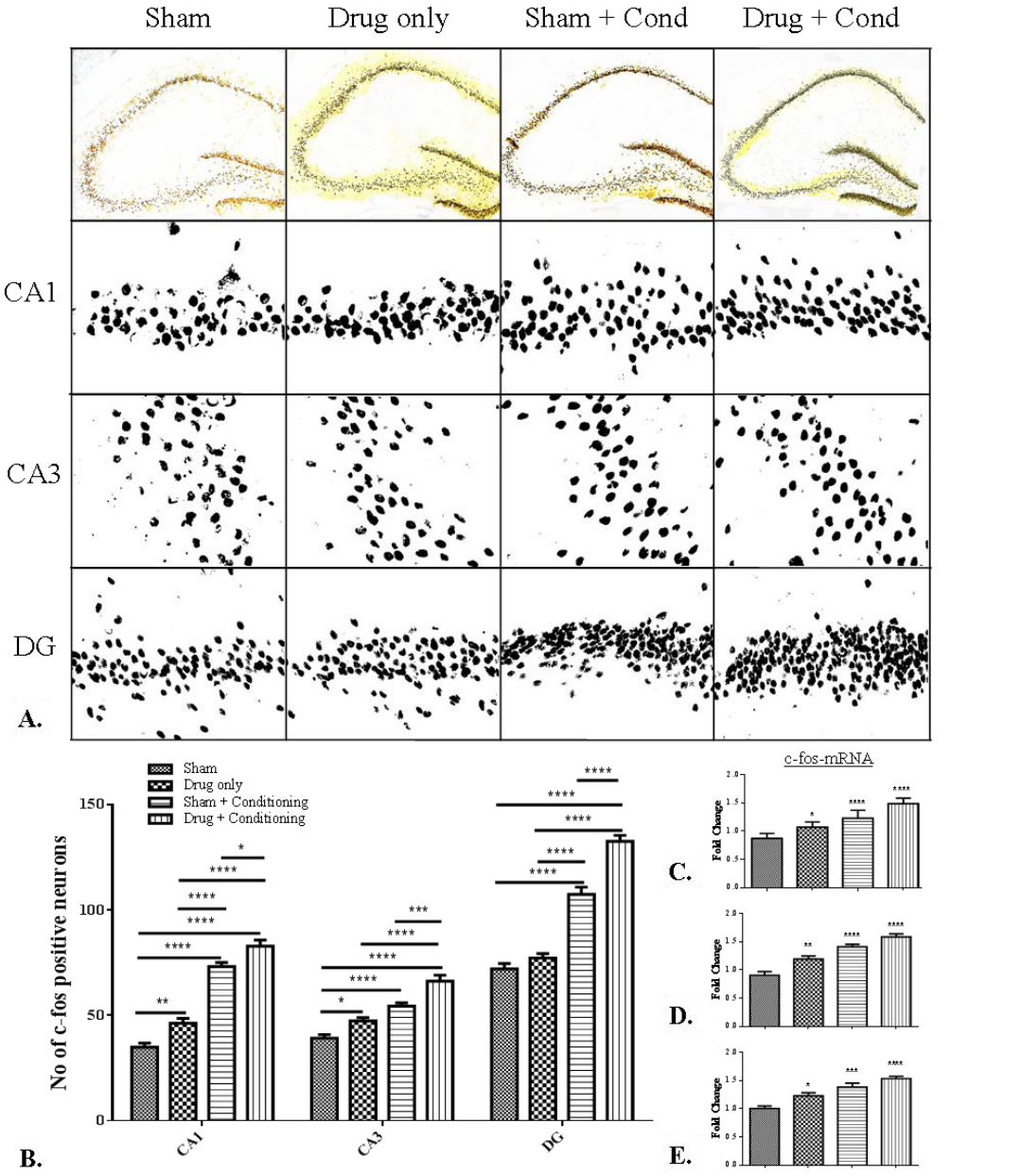



**Fig 47.** c-fos expression in Hippocampus in valproic acid treated conditioning group. **A. and B.** Immunohistochemistry of c-fos expression in CA1, CA3 and DG following conditioning and extinction. **C. D. and E.** c-fos mRNA expression in CA1 (C.), CA3 (D.) and DG (E.) respectively. (Drug only= valproic acid only, Sham+Cond= vehicle treated conditioning group and Drug+Cond= valproic acid treated conditioning group)

### 6.1.2.12. Histone H3K9 acetylation in Hippocampus

Similar to c-fos, the histone acetyl H3K9 level increased significantly in CA1 within drug only ($p$<0.01), sham + conditioning ($p$<0.0001) and drug + conditioning group ($p$<0.0001) as compared to the sham control group [F (3,36) = 52.33, $p$<0.0001]. Drug + conditioning group exhibited a higher level of acetyl H3K9 level in CA1 as compared to the drug only and sham + conditioning group. In CA3, similarly the acetyl H3K9 level increased significantly in drug only

($p$<0.05), sham + conditioning ($p$<0.001) and drug + conditioning group ($p$<0.0001) as compared to the sham control group [F (3,36) = 13.14, $p$<0.0001]. Drug + conditioning group exhibited a higher level of acetyl H3K9 level as compared to the drug only and sham + conditioning group. In DG, the acetyl H3K9 level increased significantly in drug only ($p$<0.0001), sham + conditioning ($p$<0.0001) and drug + conditioning group ($p$<0.0001) as compared to the sham control group [F (3,36) = 52.96, $p$<0.0001]. The additive effect of HDAC inhibitor on conditioning results in higher level of acetyl H3K9 in drug + conditioning group as compared to the drug only and sham + conditioning group. (Fig 48)

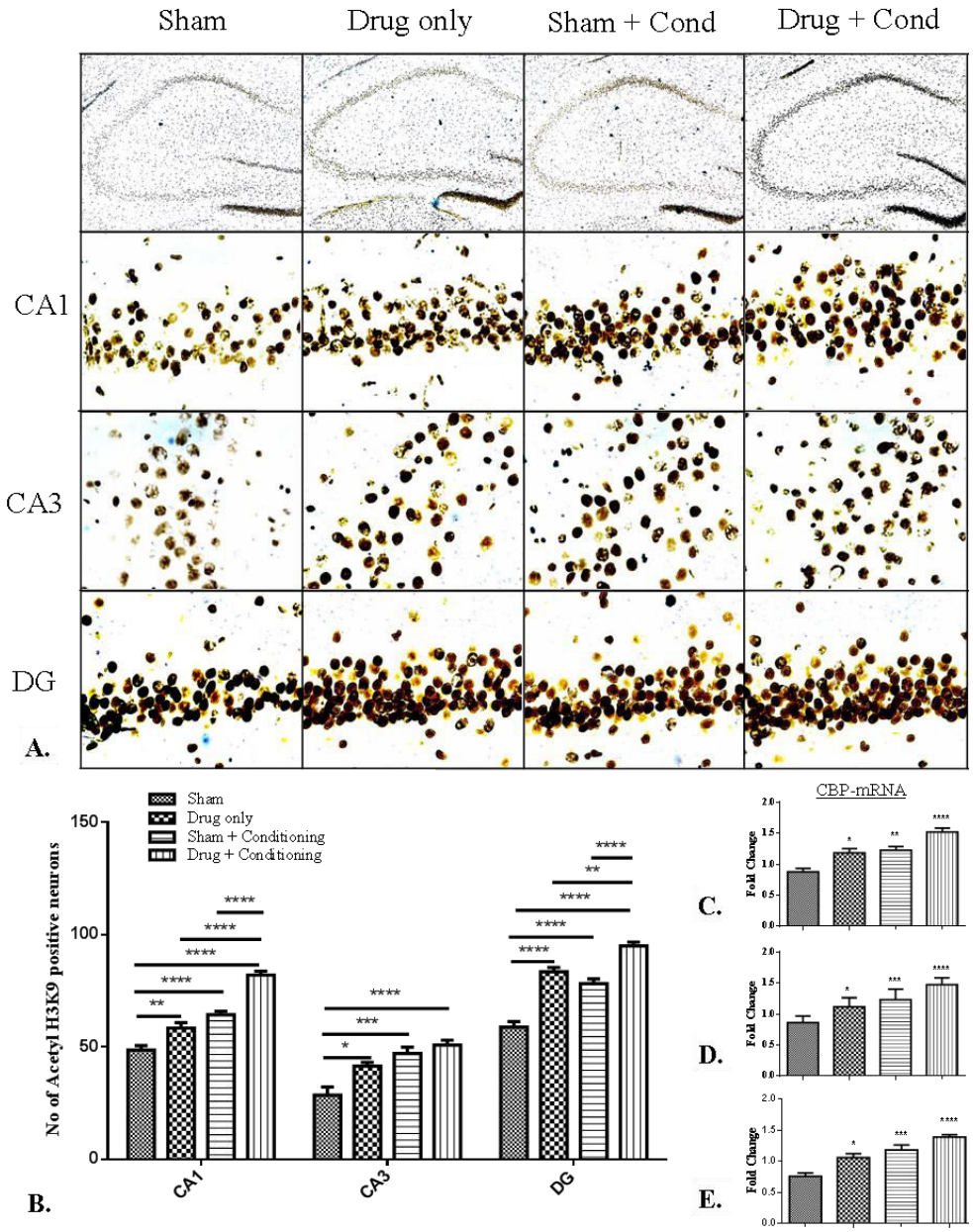



**Fig 48.** Histone H3K9 acetylation in Hippocampus in valproic acid treated conditioning group**. A. and B.** Immunohistochemistry of histone H3K9 acetylation in CA1, CA3 and DG following conditioning. **C. D. and E.** CBP mRNA expression in CA1 (C.), CA3 (D.) and DG (E.). (Drug only= valproic acid only, Sham+Cond= vehicle treated conditioning group and Drug+Cond= valproic acid treated conditioning group)

**6.1.2.13. Histone H4K5 acetylation in Hippocampus**

The histone acetyl H4K5 level in CA1, increased significantly in drug only ($p$<0.05), sham + conditioning ($p$<0.0001) and drug + conditioning group ($p$<0.0001) as compared to the sham control group [F (3,36) = 29.75, $p$<0.0001]. The changes were more significant in drug + conditioning group for histone H4K5 acetylation in CA1 as compared to the drug only and sham + conditioning group. (Fig 49)

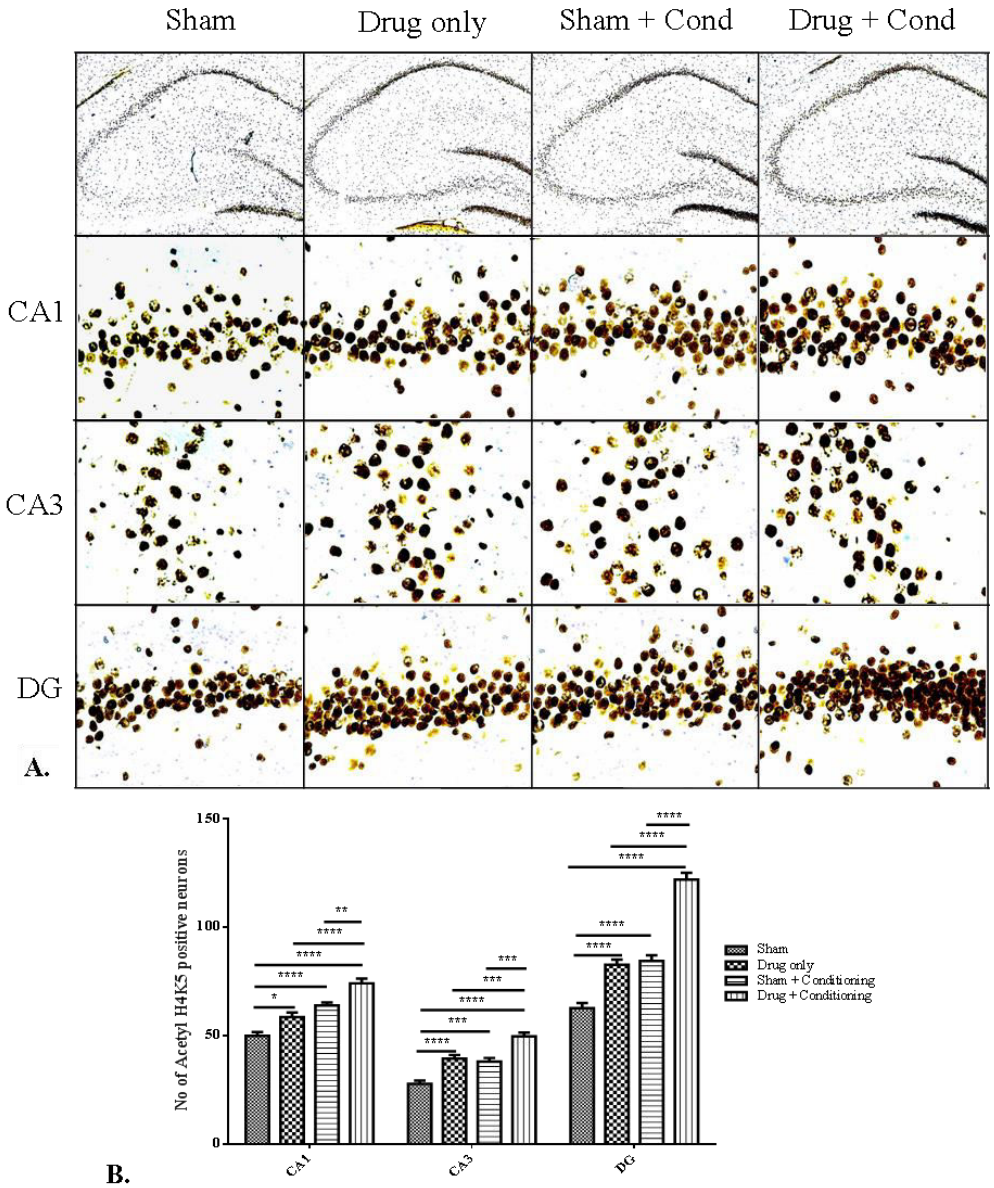



**Fig 49.** Histone H4K5 acetylation in Hippocampus in valproic acid treated conditioning group**. A. and B.** Immunohistochemistry of histone H4K5 acetylation in CA1, CA3 and DG following conditioning. (Drug only= valproic acid only, Sham+cond= vehicle treated conditioning group and Drug+Cond= valproic acid treated conditioning group)

In CA3, the histone acetyl H4K5 increased significantly in drug only ($p$<0.0001), sham + conditioning ($p$<0.001) and drug + conditioning ($p$<0.0001) as compared to the sham control group [F (3,36) = 29.04, $p$<0.0001]. The changes were more significant in drug + conditioning group for acetyl H4K5 level as compared to the drug only and sham + conditioning group. In DG, the acetyl

H4K5 level increased significantly in drug only ($p$<0.0001), sham + conditioning ($p$<0.0001) and drug + conditioning group ($p$<0.0001) as compared to the sham control group [F (3,36) = 86.60, $p$<0.0001]. When compared the changes were more significant in drug + conditioning group for acetyl H4K5 as compared to the drug only and sham + conditioning group.

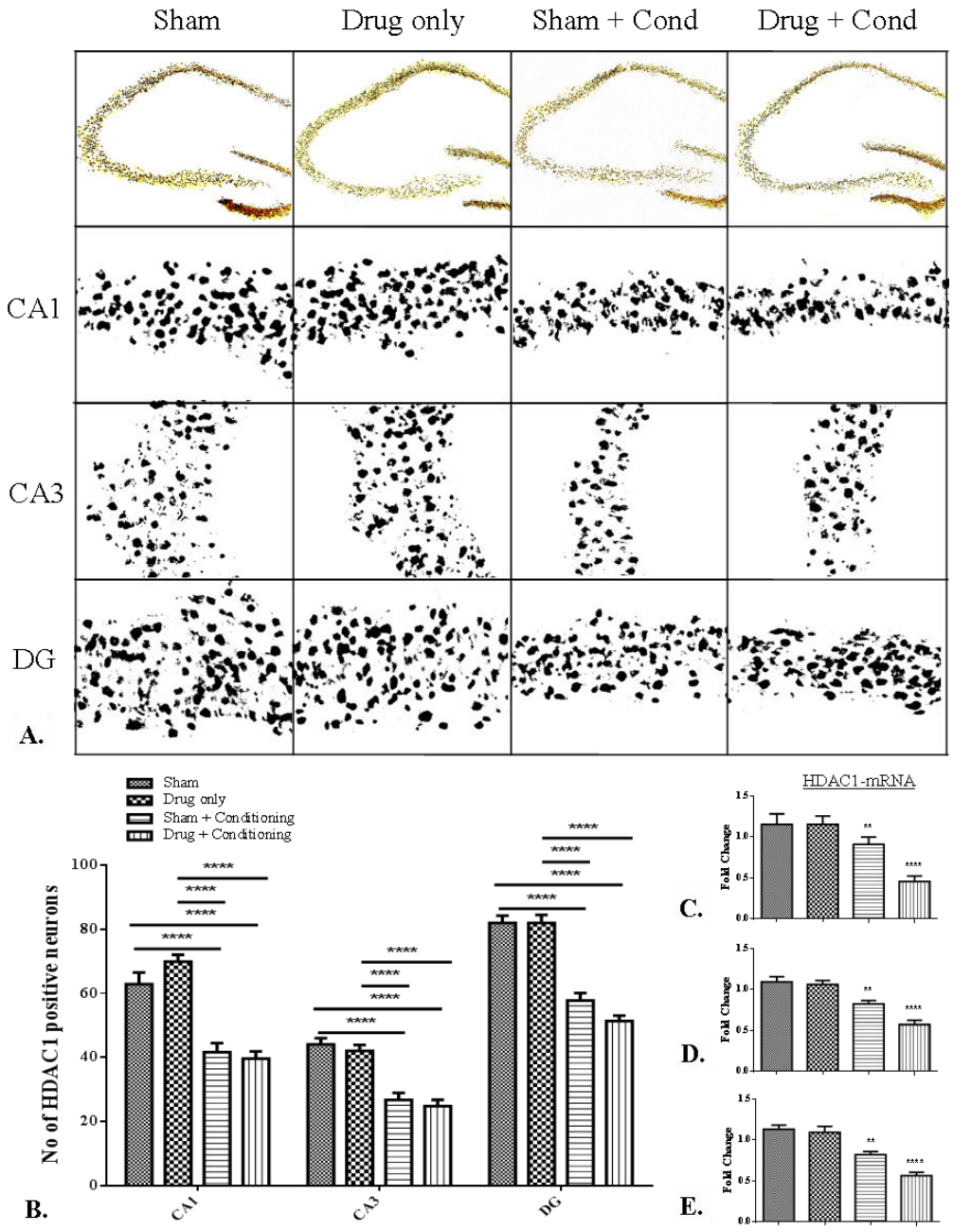



**Fig 50.** HDAC1 expression in Hippocampus in valproic acid treated conditioning group**. A. and B.** Immunohistochemistry of HDAC1 expression in CA1, CA3 and DG following conditioning. **C. D. and E.** HDAC1 mRNA expression in CA1 (C.), CA3 (D.) and DG (E.) respectively. (Drug only= valproic acid only, Sham+Cond= vehicle treated conditioning group and Drug+Cond= valproic acid treated conditioning group)

### 6.1.2.14. HDAC1 expression in Hippocampus

HDAC1 expression decreased significantly in CA1, in sham +conditioning ($p$<0.0001) and drug + conditioning group ($p$<0.0001) as compared to the sham control group while no significant change was observed in drug only group [F (3,36) = 29.38, $p$<0.0001] for HDAC1 expression. In CA3, HDAC1 expression decreased significantly in sham + conditioning ($p$<0.0001) and drug + conditioning group ($p$<0.0001) but not in drug only group as compared to the sham control group [F (3,36) = 24.54, $p$<0.0001]. In DG, the HDAC1 expression decreased significantly in sham + conditioning ($p$<0.0001) and drug + conditioning ($p$<0.0001) group as compared to the sham control group while no significant change was observed in drug only group [F (3,36) = 52.05, $p$<0.0001]. (Fig 50)

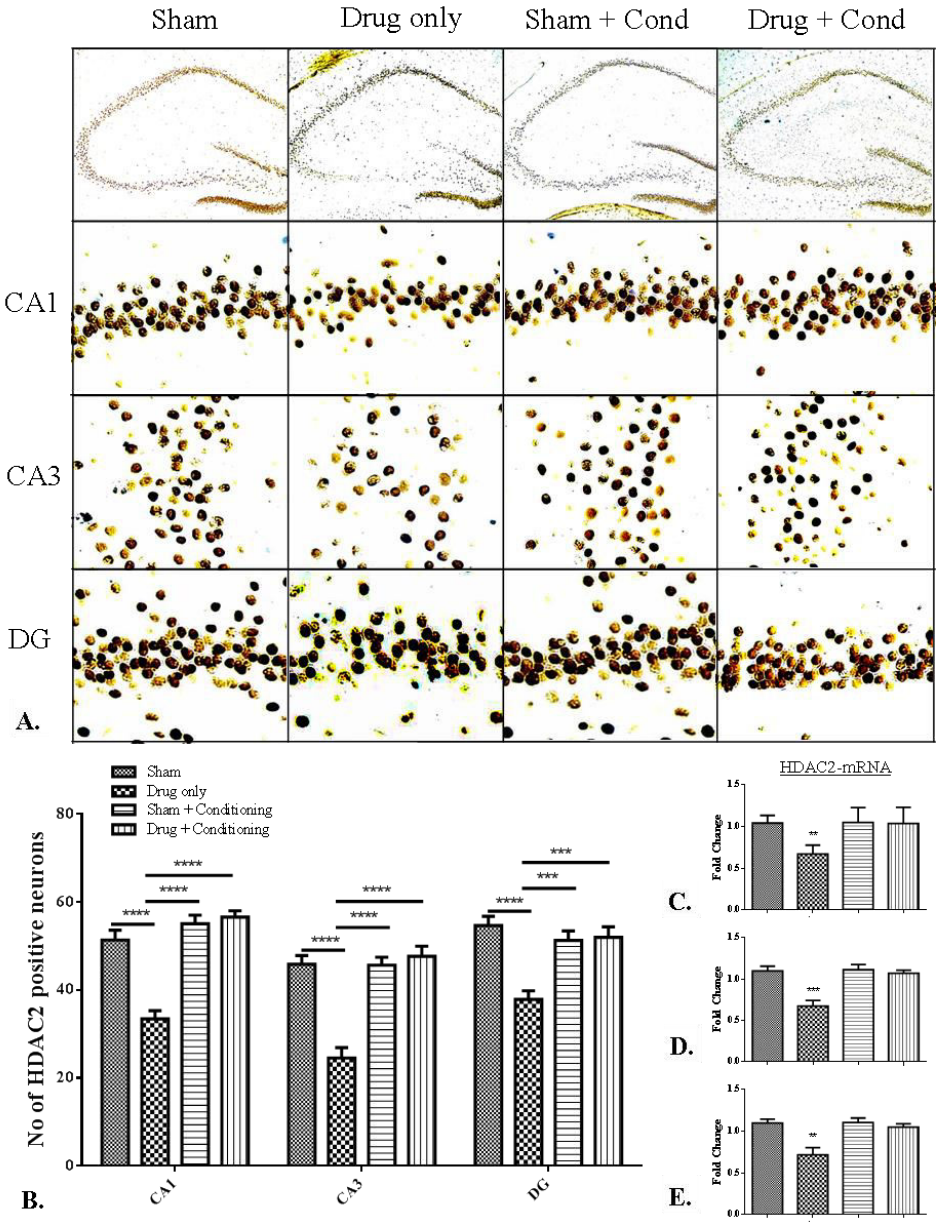



**Fig 51.** HDAC2 expression in Hippocampus in valproic acid treated conditioning group. **A. and B.** Immunohistochemistry of HDAC2 expression in CA1, CA3 and DG following conditioning and extinction. **C. D. and E.** HDAC2 mRNA expression in CA1 (C.), CA3 (D.) and DG (E.) respectively. (Drug only= valproic acid only, Sham+Cond= vehicle treated conditioning group and Drug+Cond= valproic acid treated conditioning group)

#### 6.1.2.15. HDAC2 expression in Hippocampus

HDAC2 expression exhibited different expression in the hippocampus than HDAC1. In CA1, HDAC2 expression decreased significantly in drug only group ($p$<0.0001) but not in sham + conditioning and drug + conditioning group as compared to the sham control group [F (3,36) = 31.35, $p$<0.0001]. Likewise in CA3, HDAC2 expression decreased significantly in drug only group ($p$<0.0001) but not in sham + conditioning and drug + conditioning group as compared to the sham control group [F (3,36) = 26.88, $p$<0.0001]. Similarly in DG, HDAC2 expression decreased significantly in drug only group ($p$<0.0001) but not in sham + conditioning and drug + conditioning group as compared to the sham control group [F (3,36) = 12.02, $p$<0.0001]. (Fig 51)

## 6.1.3. Real-Time PCR for mRNA expression analysis

### 6.1.3.1. mRNA expression in the Amygdala

#### 6.1.3.1.1. BLA

In BLA, c-fos mRNA expression increased significantly in drug only ($p$<0.05), sham + conditioning group ($p$<0.01), and drug + conditioning group ($p$<0.001) as compared to the sham control group [F (3,16) = 11.62, $p$<0.001]. Drug + conditioning group exhibit higher level of c-fos expression as compared to the drug only and sham + conditioning group. Likewise CBP mRNA expression increased significantly in drug only ($p$<0.05), sham + conditioning ($p$<0.001) and drug + conditioning group ($p$<0.0001) as compared to the sham control group [F (3,16) = 34.97, $p$<0.001] (Fig 37). The CBP mRNA was significantly higher in drug + conditioning group than drug only and sham + conditioning group which may be the additive effect of valproic acid on CBP expression (Fig 38). HDAC1 mRNA expression in BLA increased significantly in drug + conditioning group ($p$<0.01) but not in drug only and sham + conditioning as compared to the sham control group [F (3,20) = 5.320, $p$<0.01] (Fig 40). HDAC2 on the other hand showed different expression than HDAC1, in BLA subregion of the amygdala. HDAC2 mRNA expression decreased significantly in drug only ($p$<0.05), sham + conditioning ($p$<0.05) and drug + conditioning group ($p$<0.01) as compared to the sham control group [F (3,16) = 18.86, $p$<0.0001]. In drug + conditioning group the HDAC2 mRNA expression was significantly lower as compared to the drug only and sham + conditioning group. (Fig 41)

#### 6.1.3.1.2. CeA

In CeA, the c-fos mRNA expression increased significantly in drug only ($p$<0.05), sham + conditioning ($p$<0.01) and drug + conditioning group ($p$<0.01) as compared to the sham control group [F (3,16) = 8.469, $p$<0.01]. The expression was significantly higher in sham + conditioning and drug + conditioning group as compared to the drug only group (Fig 37). CBP expression in CeA subregion of amygdala increased significantly in drug only ($p$<0.05), sham + conditioning ($p$<0.01) and drug + conditioning group ($p$<0.0001) as compared to the sham control group [F (3, 16) = 23.65, $p$<0.0001]. The additive effect of valproic acid results in a significantly higher level of CBP mRNA expression in Drug + conditioning than drug only and sham + conditioning group (Fig 38). HDAC1 exhibit similar expression of mRNA in CeA as shown by c-fos and CBP. HDAC1 expression increased significantly in sham + conditioning ($p$<0.05) and drug + conditioning group ($p$<0.05) but not in drug only group as compared to the sham control group following conditioning [F (3,16) = 5.613, $p$<0.01] (Fig 40). Besides this, HDAC2 mRNA expression decreased significantly in drug only ($p$<0.05), sham + conditioning ($p$<0.05) and drug + conditioning group ($p$<0.0001) as compared to the sham control group [F (3,28) = 22.13, $p$<0.0001]. A significantly

lower level of HDAC2 expression was observed in drug + conditioning group than drug only and sham + conditioning group. (Fig 41)

#### 6.1.3.2. mRNA expression in Prefrontal Cortex

##### 6.1.3.2.1. PL

The mRNA expression analysis revealed that in PL, c-fos mRNA expression increased significantly in drug only ($p<0.05$), sham + conditioning ($p<0.001$) and drug + conditioning group ($p<0.0001$) as compared to the sham control group [$F(3,16) = 35.35$, $p<0.0001$]. In drug + conditioning group a significantly higher level of c-fos mRNA expression was observed as compared to the drug only and sham + conditioning group which might be the result of HDAC inhibitor on conditioning behavior (Fig 42). CBP which is a HAT activator shows similar expression in PL as c-fos expression where CBP expression increased significantly in drug only ($p<0.01$), sham + conditioning ($p<0.001$) and drug + conditioning group ($p<0.0001$) as compared to the sham control group [$F(3,16) = 17.39$, $p<0.0001$].Though drug + conditioning group shows significantly higher expression for CBP mRNA, as compared to the drug only and sham + conditioning group (Fig 43). Contrary to c-fos, HDAC1 expression decreased significantly in sham + conditioning ($p<0.01$) and drug + conditioning group ($p<0.0001$) as compared to the sham control group in PL [$F(3,16) = 17.77$, $p<0.0001$]. Drug + conditioning group represent significantly lower HDAC1 expression as compared to sham + conditioning group (Fig 45). Likewise, HDAC2 expression decreased significantly in drug only ($p<0.05$), sham + conditioning ($p<0.001$) and drug + conditioning group ($p<0.0001$) as compared to the sham control group in PL subregion of medial PFC [$F(3,16) = 45.48$, $p<0.0001$]. Drug + conditioning group exhibited a significantly lower level of HDAC2 expression in PL-PFC compared to the drug only and sham + conditioning group. (Fig 46)

##### 6.1.3.2.2. IL

In IL, there was no significant difference in drug only, sham + conditioning and drug + conditioning group for HDAC1, CBP and c-fos expression as compared to the sham control group ($p>0.05$). However, the HDAC2 mRNA expression decreased significantly in sham + conditioning ($p<0.001$) and drug + conditioning group ($p<0.0001$) group as compared to the sham control group while no significant change was observed in drug only group [$F(3,16) = 24.55$, $p<0.0001$] (Fig 46). The expression in drug + conditioning group was significantly lower as compared to the sham + conditioning group.

#### 6.1.3.3. mRNA expression in Hippocampus

##### 6.1.3.3.1. CA1

In CA1, the c-fos mRNA expression increased significantly in drug only ($p<0.05$), sham + conditioning ($p<0.0001$) and drug + conditioning group ($p<0.0001$) as compared to the sham control group [$F(3,20) = 34.32$, $p<0.0001$]. The expression in drug + conditioning group was significantly higher as compared to the drug only ($p<0.0001$) and sham + conditioning group ($p<0.01$) (Fig 47). Similar to c-fos, the CBP expression increased significantly in drug only ($p<0.05$), sham + conditioning ($p<0.01$) and drug + conditioning group ($p<0.0001$) as compared to the sham control group [$F(3,20) = 17.92$, $p<0.0001$]. The CBP expression in CA1 was significantly higher in drug + conditioning group than drug only ($p<0.01$) and sham + conditioning group ($p<0.05$) (Fig 48). HDAC1 mRNA expression exhibited different expression pattern than c-fos and CBP in CA1. The HDAC1 mRNA expression decreased significantly in sham + conditioning

($p$<0.01) and drug + conditioning group ($p$<0.0001) but not in drug only group [F (3,20) = 66.54, $p$<0.0001]. HDAC1, mRNA expression was significantly lower in drug + conditioning group as compared to sham + conditioning group ($p$<0.0001) (Fig 50). Contrary to HDAC1, HDAC2 mRNA expression exhibited no significant change in sham + conditioning and drug + conditioning group, however, HDAC2 expression decreased significantly in drug only group ($p$<0.01) suggesting no role of HDAC2 in CA1 following valproic acid administration on conditioning of fear memory [F (3,20) = 9.361, $p$<0.001] (Fig 51).

#### 6.1.3.3.2. CA3

In CA3 similar to CA1, the c-fos mRNA expression increased significantly in drug only ($p$<0.01), sham + conditioning ($p$<0.0001) and drug + conditioning group ($p$<0.0001) as compared to the sham control group [F (3,20) = 27.96, $p$<0.0001] (Fig 47). Moreover, CBP showed similar expression pattern in CA3 where CBP expression increased significantly in drug only ($p$<0.05), sham + conditioning ($p$<0.001) and drug + conditioning group ($p$<0.0001) as compared to the sham control group [F (3,20) = 21.25, $p$<0.0001] (Fig 48). Drug + conditioning group exhibited a higher level of CBP mRNA expression in CA3as compared to the drug only ($p$<0.01) and sham + conditioning group ($p$<0.05). HDAC1 mRNA decreased significantly in CA3 in sham + conditioning ($p$<0.01) and drug + conditioning group ($p$<0.0001) but not in drug only group as compared to the sham control group [F (3,20) = 21.68, $p$<0.0001]. HDAC1 mRNA expression exhibited a significant reduction in drug + conditioning group as compared to sham + conditioning group ($p$<0.05) (Fig 50). HDAC2 mRNA was not altered in sham + conditioning and drug + conditioning groups as compared to the sham control group while drug only group exhibited reduced expression ($p$<0.001) of HDAC2 mRNA in CA3 [F (3,20) = 13.17, $p$<0.0001] (Fig 51).

#### 6.1.3.3.3. DG

In DG, similarly, the c-fos mRNA expression increased significantly in drug only ($p$<0.05), sham + conditioning ($p$<0.001) and drug + conditioning ($p$<0.0001) group as compared to the sham control group [F (3,20) = 18.07, $p$<0.0001]. There was no significant difference between sham + conditioning and drug + conditioning for c-fos mRNA expression (Fig 47). The CBP mRNA showed similar expression pattern as c-fos in DG, where its expression increased significantly in drug only ($p$<0.05), sham + conditioning ($p$<0.001) and drug + conditioning group ($p$<0.0001) as compared to the sham control group. No significant difference was observed between sham + conditioning and drug + conditioning for CBP mRNA expression [F (3,20) = 18.45, $p$<0.0001] (Fig 48). HDAC1 expression decreased significantly in DG in sham + conditioning ($p$<0.01) and drug + conditioning group ($p$<0.0001) as compared to the sham control group whereas no significant change was observed in drug only group [F (3,20) = 25.96 $p$<0.0001]. Drug + conditioning group expressed a higher level of HDAC1 mRNA as compared to sham + conditioning group (Fig 50). HDAC2 mRNA expression exhibited no significant difference in drug + conditioning and sham + conditioning group whereas drug only group ($p$<0.01) shows a reduction in HDAC2 mRNA expression as compared to the sham control group [F (3,20) = 9.801, $p$<0.001] (Fig 51).

### 6.1.4. Correlation

#### 6.1.4.1. Amygdala

The overall result suggests that HDAC inhibitor valproic acid enhanced the conditioning through regulation of histone acetylation. The regulation by such HDAC inhibitors correlates the levels of acetyl H3K9, acetyl H4K5, c-fos, HDAC1 and HDAC2 in the amygdala, PFC and

hippocampus with the freezing response. A correlation study was performed between sham + conditioning and drug + conditioning groups, for acetyl H3K9, acetyl H4K5, c-fos, HDAC1 and HDAC2 level with the freezing response so as to understand the role of valproic acid on fear learning. In LA, histone acetyl H3K9 ($p$<0.001), acetyl H4K5 ($p$<0.01), c-fos ($p$<0.001) and HDAC1 ($p$<0.05) were positively correlated with the freezing response following valproic acid mediated enhanced conditioning whereas HDAC2 ($p$>0.05) exhibited no correlation. In BA, the acetyl H3K9 ($p$<0.01), acetyl H4K5 ($p$<0.01) and HDAC1 ($p$<0.001) were positively correlated with the freezing response following valproic acid mediated conditioning whereas HDAC2 exhibit no correlation. In CeL, c-fos ($p$<0.01) and HDAC1 ($p$<0.01) expressions were positively correlated with the freezing response following valproic acid mediated enhanced conditioning while HDAC2 ($p$<0.05) exhibited a negative correlation with the freezing response when compared sham + conditioning with drug + conditioning. In CeM, acetyl H3K9 ($p$<0.05), acetyl H4K5 ($p$<0.05) and c-fos ($p$<0.01) were positively correlated with the freezing response following valproic acid mediated conditioning whereas HDAC1 ($p$<0.0001) and HDAC2 ($p$<0.05) exhibited a negative correlation when compared sham + conditioning with the drug + conditioning. (Fig 52)

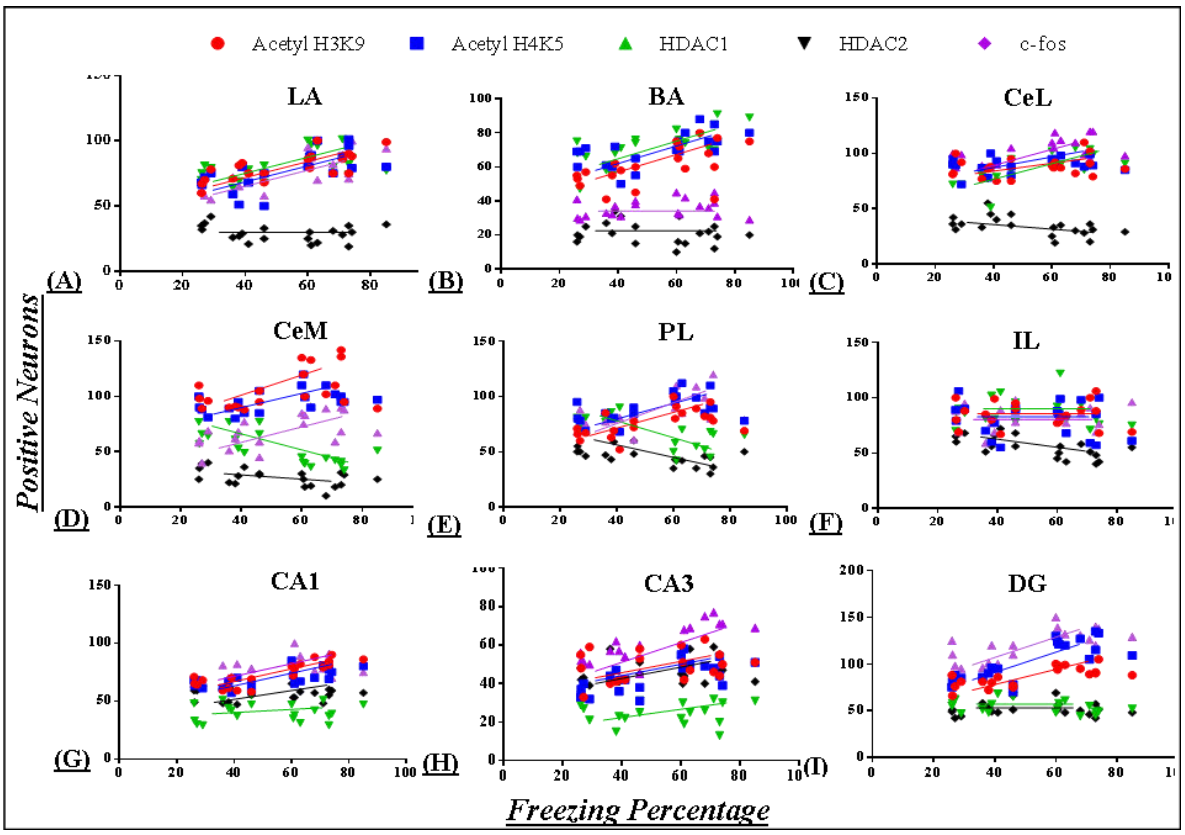



**Fig 52.** Correlation of molecular expression with the freezing response in drug treated conditioning. Correlation of c-fos, acetyl H3K9, acetyl H4K5, HDAC1 and HDAC2 level with freezing response following valproic acid treated conditioning in the amygdala, hippocampus and PFC from immunohistochemistry (compared between sham + conditioning and drug + conditioning group).

### 6.1.4.2. Prefrontal Cortex

In PL, acetyl H3K9 ($p$<0.001), acetyl H4K5 ($p$<0.001) and c-fos ($p$<0.001) were positively correlated with the freezing response following valproic acid mediated enhanced conditioning,

whereas HDAC1 ($p$<0.001) and HDAC2 ($p$<0.01) exhibited a negative correlation when sham + conditioning was compared with the drug + conditioning group. In IL, the acetyl H3K9, acetyl H4K5, c-fos and HDAC1 exhibited no correlation with the freezing response following valproic acid mediated conditioning whereas HDAC2 ($p$<0.01) exhibited a negative correlation when compared sham + conditioning with drug + conditioning group. (Fig 52)

#### 6.1.4.3. Hippocampus

In CA1, acetyl H3K9 ($p$<0.0001), acetyl H4K5 ($p$<0.01) and c-fos ($p$<0.05) were positively correlated with the freezing response following valproic acid mediated conditioning, while HDAC1 and HDAC2 exhibited no correlation with the freezing response. In CA3, acetyl H4K5 ($p$<0.01) and c-fos ($p$<0.001) were positively correlated with the freezing response following valproic acid mediated conditioning. In DG, the acetyl H3K9 ($p$<0.001), acetyl H4K5 ($p$<0.0001) and c-fos ($p$<0.001) were positively correlated with the freezing response following valproic acid mediated conditioning whereas HDAC1 and HDAC2 exhibited no correlation when compared sham + conditioning with the drug + conditioning. (Fig 52)

**Table 8: Correlation of valproic acid treated Conditioning group**

| | Acetyl H3K9 | Acetyl H4K5 | HDAC1 | HDAC2 | c-fos |
|---|---|---|---|---|---|
| LA | r = 0.717 (**)<br>p < 0.001 | r = 0.638 (**)<br>p < 0.01 | r = 0.468 (*)<br>p < 0.05 | - | r = 0.733 (**)<br>p < 0.001 |
| BA | r = 0.559 (*)<br>p < 0.01 | r = 0.623 (**)<br>p < 0.01 | r = 0.707 (***)<br>p < 0.001 | - | - |
| CeL | - | - | r = 0.632 (**)<br>p < 0.01 | r = - 0.540 (*)<br>p < 0.05 | r = 0.594 (**)<br>p < 0.01 |
| CeM | r = 0.452 (*)<br>p < 0.05 | r = 0.478 (*)<br>p < 0.05 | r = - 0.753 (***)<br>p < 0.0001 | r = - 0.515 (*)<br>p < 0.05 | r = 0.587 (**)<br>p < 0.01 |
| PL | r = 0.567 (**)<br>p < 0.001 | r = 0.471 (*)<br>p < 0.001 | r = - 0.668 (**)<br>p < 0.001 | r = - 0.521 (*)<br>p < 0.01 | r = 0.562 (**)<br>p < 0.001 |
| IL | - | - | - | r = - 0.694 (**)<br>p < 0.01 | - |
| CA1 | r = 0.826 (****)<br>p<0.0001 | r = 0.695 (**)<br>p < 0.01 | - | - | r = 0.512 (*)<br>p < 0.05 |
| CA3 | - | r = 0.650 (**)<br>p < 0.01 | - | - | r = 665 (**)<br>p < 0.001 |
| DG | r = 0.702 (***)<br>p < 0.001 | r = 0.793 (****)<br>p < 0.0001 | - | - | r = 0.711 (***)<br>p < 0.001 |

## 6.2. Discussion

The conditioning of fear was influenced by the valproic acid activity. The result suggests that valproic acid given prior to the conditioning promotes conditioning of fear. Furthermore, the enhancement of conditioning learning was followed by the molecular changes associated with the formation of memory. When analyzed the activity in the amygdala, PFC and hippocampus it was observed that valproic acid mediated HDAC inhibition enhanced the activity of subregions of the brain as well as histone acetylation differentially, following conditioning and extinction. During conditioning, the activity of LA, BA and CeL were enhanced by the HDAC inhibitor valproic acid which leads to the activation of the fear circuitry resulting in downstream activation of CeM. This activity might be under the influence of increased histone acetylation and showing similar activity

within the LA, BA, CeL and CeM. The HDAC1 expression exhibited a positive correlation with the histone acetylation and activity of amygdala subregion, while HDAC2 exhibited a negative correlation. In brief the result suggests that the histone acetylation promotes the activation of fear circuitry and this activity is supported by the enhanced HDAC1 expression and reduced HDAC2 expression. The differential expression of both the HDACs in amygdala might be associated with the activation and suppression of different component of fear circuitry. Although valproic acid promotes histone acetylation and fear learning, its target might be the HDAC2 but not HDAC1 as shown by the HDAC2 inhibition during conditioning. The CeM, which shows increased histone acetylation and c-fos expression during conditioning, was associated with the decreased expression of both the HDACs (e. g. HDAC1 and 2). As the HDAC inhibition causes suppression of HDAC2 but not HDAC1, the suppressed expression of HDAC1 in CeM following conditioning might be the result of other inputs to CeM. So it may be concluded that the major inhibitory mechanism is caused by the HDAC2 on histone acetylation in the amygdala, and HDAC2 inhibition enhanced histone acetylation in the amygdala during conditioning.

HDAC inhibitor showed different activity in PFC subregion in conditioning. Although the HDAC inhibitor is associated with the enhanced PL activity in conditioning, it did not affect the IL activity. In PL, HDAC inhibitor showed its role to enhance the activity and histone acetylation which results in enhanced conditioning response through strengthening PL activity. Although HDAC inhibitor shows no effect on HDAC1 expression in PL, its expression was suppressed in HDAC inhibitor treated conditioning group which might be due to the activation through indirect inputs in PL. Moreover, the expression of HDAC2 was inhibited by the HDAC inhibitor in PL and IL both, and this inhibition was straight on HDAC2 expression. In PL, the histone acetylation was under the inhibition of both HDAC1 and HDAC2, whose expression was inhibited by the HDAC inhibition followed by the increased histone acetylation in conditioning learning. However, the decreased HDAC2 expression in IL might be for controlling competitive activity with PL.

The result overall suggests that hippocampus activity was enhanced by the HDAC inhibition for the strengthening of conditioning. The enhanced activity was supported and regulated by the increased histone acetylation through HDAC inhibitor in conditioning. The HDAC1 expression was inhibited by the HDAC inhibitor in conditioning, however, this inhibition was indirect on HDAC1 expression. Although, the HDAC2 expression was suppressed by the HDAC inhibitor the major inhibition for conditioning was caused by the HDAC1 inhibition in the hippocampus. Finally, the result suggests that in hippocampus the major inhibitory mechanism may be associated with the HDAC1 on histone acetylation. The only target molecule during conditioning is HDAC1 whose inhibition promotes the histone acetylation in HDAC inhibitor enhanced conditioning in the hippocampus.

***Chapter 7***

# RESULTS & DISCUSSION: Aim2 – Valproic acid effect on Extinction

**Aim 2**

**(B). Effect of valproic acid on Extinction of fear memory**

## 7.1. Results

### 7.1.1. Behavior results

#### 7.1.1.1. Effect of Valproic acid treatment on Extinction

Animals were trained first for conditioning as mentioned in conditioning protocol except a strong conditioning was performed which results in weak extinction for fear memory by which the effect of the drug on enhanced extinction activity is easy to comprehend. All the animals exhibited similarly enhanced freezing in each successive trial similar to the conditioning experiment when conditioned for strong conditioning. Next day, 24 hours following fear conditioning animals were introduced with either vehicle/sham (0.01M PBS) or drug (Valproic acid) intraperitoneally followed by 2 hrs gap and 24 hours later with the extinction training.

6.1.4.2. PFC

In PL, acetyl H3K9 ($p<0.001$), acetyl H4K5 ($p<0.001$) and c-fos ($p<0.001$) were positively correlated with the freezing response following valproic acid mediated enhanced conditioning, whereas HDAC1 ($p<0.001$) and HDAC2 ($p<0.01$) exhibited a negative correlation when sham + conditioning was compared with the drug + conditioning group. In IL, the acetyl H3K9, acetyl H4K5, c-fos and HDAC1 exhibited no correlation with the freezing response following valproic acid mediated conditioning whereas HDAC2 ($p<0.01$) exhibited a negative correlation when compared sham + conditioning with drug + conditioning group. (Fig 53)

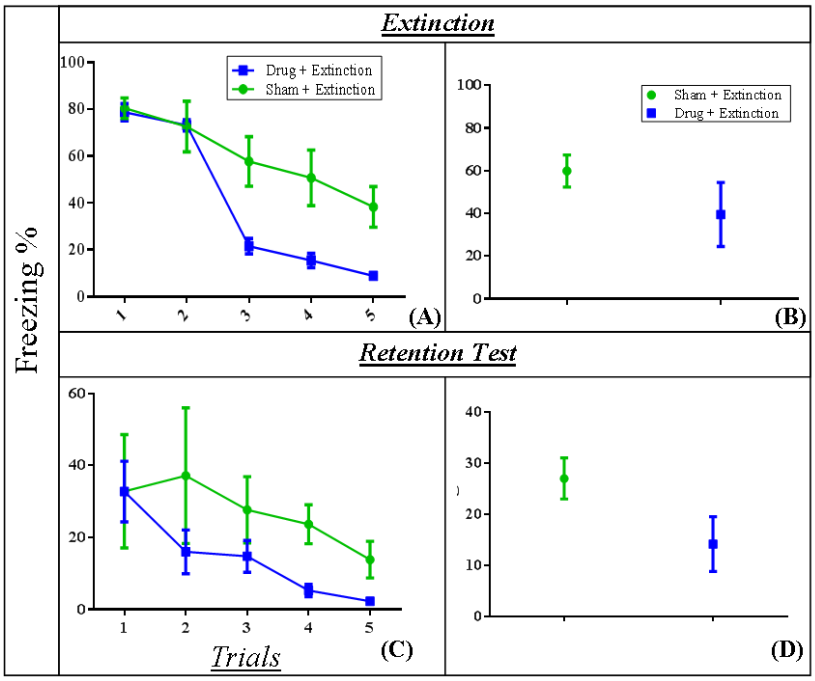



**Fig 53. Valproic acid mediated enhancement of fear memory extinction. (A)** and **(B)** Initial trial of extinction exhibit no significant change between them **(C)** and **(D)** however in retention trial shows the significantly lower level of freezing as compared to extinction and sham + extinction group.

Overall, there was no significant difference observed for freezing response during fear extinction learning in sham + extinction and drug + extinction groups ($p>0.05$) for initial two trials when compared for the five trial blocks of the extinction. In later trials from 3rd to the last trial the freezing was significantly lower in drug + extinction group as compared to sham + extinction

group. The last trial of drug + extinction group exhibited more significant reduced freezing as compared to the sham + extinction group [F (6,6) = 462.5, $p$<0.0001]. In all the groups freezing decreases in each consecutive trial block during extinction learning and the last trial exhibited the lowest freezing as compared to their first trial [F (4,24) = 42.22, $p$<0.0001]. During retention test the freezing decreased significantly from the first trial to the last trial. In first trial drug + extinction and sham +extinction group exhibited similarly lower freezing [F(5,5) = 4.5; $p$>0.05] but in successive trials the freezing decreased significantly in drug + extinction group as compared to the sham + extinction [Last trial, F(5,5) = 65.42; $p$<0.001]. The animals from sham + extinction and drug + extinction group were further divided into two groups; one group was used for molecular study through immunohistochemistry and real-time PCR experiments while other undergone for retention test.

## 7.1.2. Immunohistochemistry

### 7.1.2.1.c-fos expression in the Amygdala

The activity of amygdala as governed by the c-fos expression shows a differential activity for extinction when treated with the valproic acid. The c-fos expression in LA, increased significantly in drug only ($p$<0.05), sham + extinction ($p$<0.0001) and drug + extinction group ($p$<0.0001) as compared to the sham control group [F (3,36) = 28.49, $p$<0001]. The c-fos expression in LA was significantly higher in drug + extinction group as compared to the drug only ($p$<0.0001) and sham + extinction group ($p$<0.01). Likewise, in BA, the c-fos expression increased significantly in drug only ($p$<0.05), sham + extinction ($p$<0.01) and drug + extinction group ($p$<0.0001) as compared to the sham control group [F (3,36) = 23.62, $p$<0001] although the levels were significantly higher in drug + extinction group as compared to drug only ($p$<0.0001) and sham + extinction group ($p$<0.001). In CeL, the c-fos expression increased significantly in drug only ($p$<0.05), sham + extinction ($p$<0.0001) and drug + extinction group ($p$<0.0001) as compared to the sham control group [F (3,36) = 43.71, $p$<0001], and the changes were significantly higher in sham + extinction group ($p$<0.0001) and drug + extinction group ($p$<0.0001). In CeM, the c-fos expression increased significantly in sham + extinction group ($p$<0.0001) but not in drug only ($p$>0.05) and drug + extinction group ($p$>0.05) as compared to the sham control group [F (3,36) = 56.49, $p$<0.0001]. Overall, the conclusion from c-fos expression shows that the enhanced activity in LA, BA and CeL is required for the effective extinction learning. (Fig 54)

### 7.1.2.2. Histone H3K9 acetylation in the Amygdala

In LA, histone H3K9 acetylation increased significantly in drug only ($p$<0.05), sham + extinction ($p$<0.0001) and drug + extinction group ($p$<0.0001) as compared to the sham control group though the changes in sham + extinction and drug + extinction group were significant than drug only group [F (3,36) = 16.50, $p$<0.0001]. Likewise, in BA, the acetyl H3K9 increased significantly in drug only ($p$<0.01), sham + extinction ($p$<0.0001) and drug + extinction group ($p$<0.0001) as compared to the sham control group, but the change in sham + extinction and drug + extinction group was significantly higher than drug only group [F (3,36) = 31.33, $p$<0.0001]. In CeL, the histone H3K9 acetylation increased significantly in drug only ($p$<0.01), sham + extinction ($p$<0.0001) and drug + extinction group ($p$<0.0001) as compared to the sham control group; the changes in sham + extinction and drug + extinction group were significantly higher than drug only group [F (3,36) = 28.83, $p$<0.0001]. In CeM, however, the histone H3K9 acetylation increased significantly in sham + extinction group ($p$<0.0001) as compared to the sham control group which may be due to the presence of a remnant of fear memory in sham treated extinction group [F (3,36)

= 26.75, $p$<0.0001]. There was no change in drug only and drug + extinction group as compared to sham control group. Overall, the result concluded that the enhanced activity in LA, BA and CeL is associated with the increased histone H3K9 acetylation for increase extinction learning when animals were treated with valproic acid. (Fig 55)

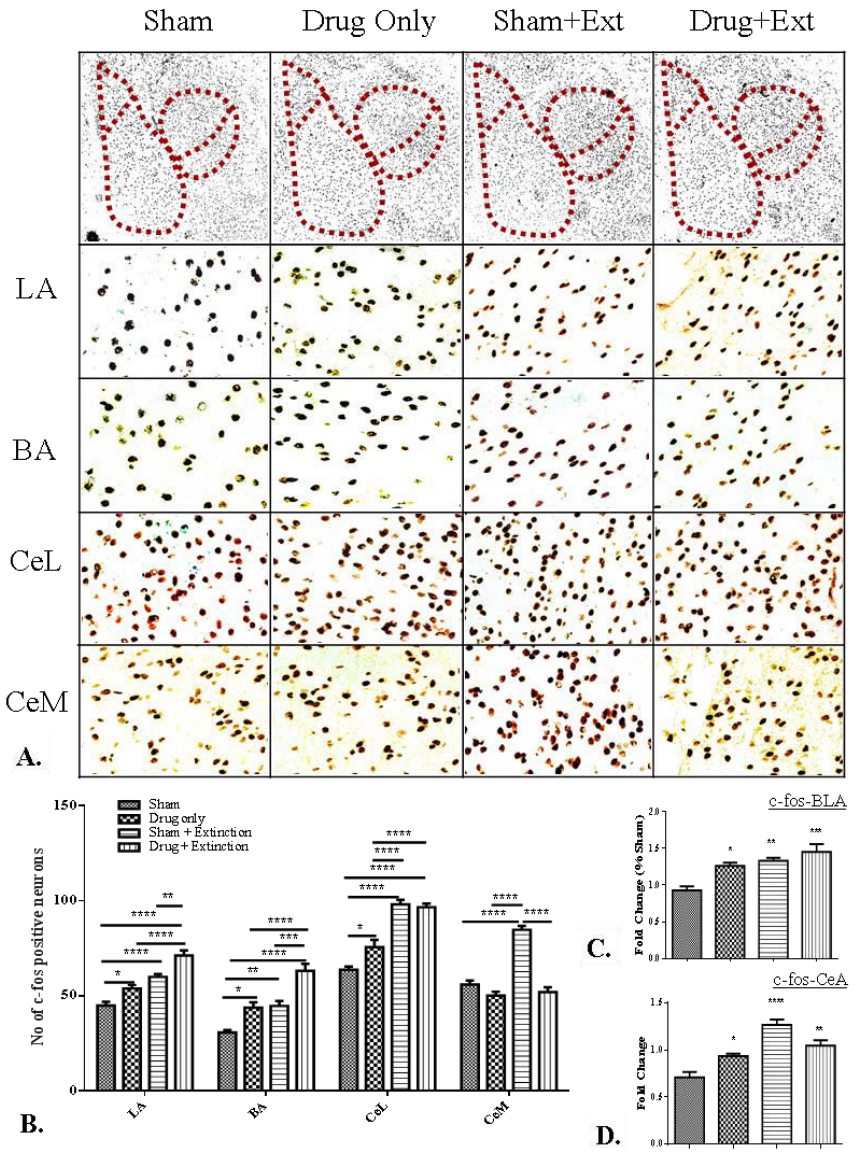



**Fig 54.** c-fos expression in the amygdala in valproic acid treated extinction group**. A. and B.** Immunohistochemistry of c-fos expression in LA, BA, CeL and CeM following extinction. **C. and D.** c-fos mRNA expression in BLA (C.) and CeA (D.). (Drug only= valproic acid only, Sham+Ext= vehicle treated extinction group, and Drug+Ext= valproic acid treated extinction group)

### 7.1.2.3. Hisone H4K5 acetylation in the Amygdala

Histone H4K5 acetylation showed similar expression pattern in the amygdala as for histone acetylation. In LA, histone acetyl H4K5 increased significantly in drug only ($p$<0.0001), sham + extinction ($p$<0.0001) and drug + extinction group ($p$<0.0001) as compared to the sham control group, the change in drug + extinction group was significantly higher as compared to drug only ($p$<0.0001) and sham + extinction group ($p$<0.05) [F (3,36) = 68.55, $p$<0.0001]. In BA, the acetyl

H4K5 level increased significantly in drug only ($p$<0.05), sham + extinction ($p$<0.0001) and drug + extinction group ($p$<0.0001) as compared to the sham control group [F (3, 36) = 15.30, $p$<0.0001]. The change in acetyl H4K5 was significantly higher in drug + extinction group as compared to drug only group ($p$<0.05) but not with sham + extinction group. In CeL, the acetylation increased significantly in drug only ($p$<0.001), sham + extinction ($p$<0.0001) and drug + extinction group ($p$<0.0001) as compared to the sham control group [F (3,36) = 44.37, $p$<0.0001]. The changes in acetyl H4K5 levels were significantly higher in drug + extinction ($p$<0.0001) and sham + extinction group ($p$<0.0001) as compared to drug only group. The CeM, however exhibited different level for acetyl H4K5, where acetyl H4K5 increased significantly in sham + extinction group ($p$<0.0001) as compared to the sham control group while no significant change was observed in drug only and drug + extinction group [F (3,36) = 23.42, $p$<0.0001]. (Fig 56)

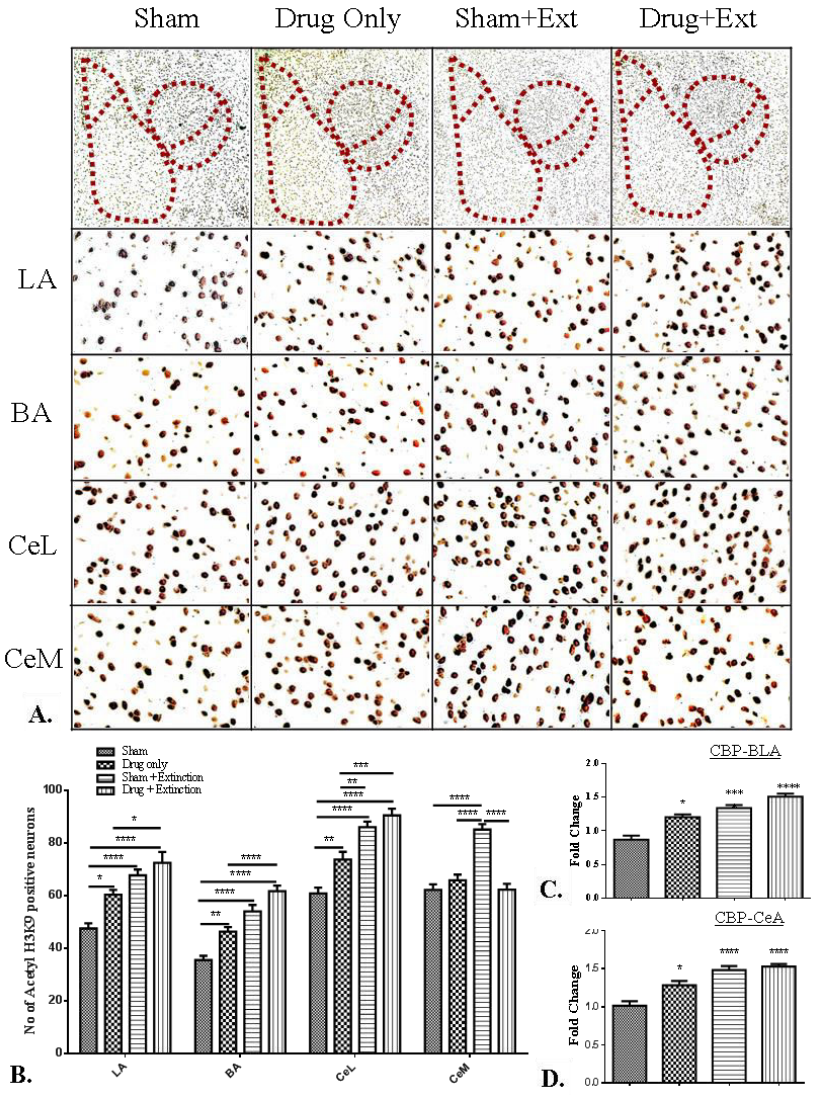



**Fig 55.** Histone H3K9 acetylation in the amygdala in valproic acid treated extinction group**. A. and B.** Immunohistochemistry of histone H3K9 acetylation in LA, BA, CeL and CeM following extinction. **C. and D.** CBP mRNA expression in BLA (C.) and CeA (D.). (Drug only= valproic acid only, Sham+Ext= vehicle treated extinction group and Drug+Ext= valproic acid treated extinction group)

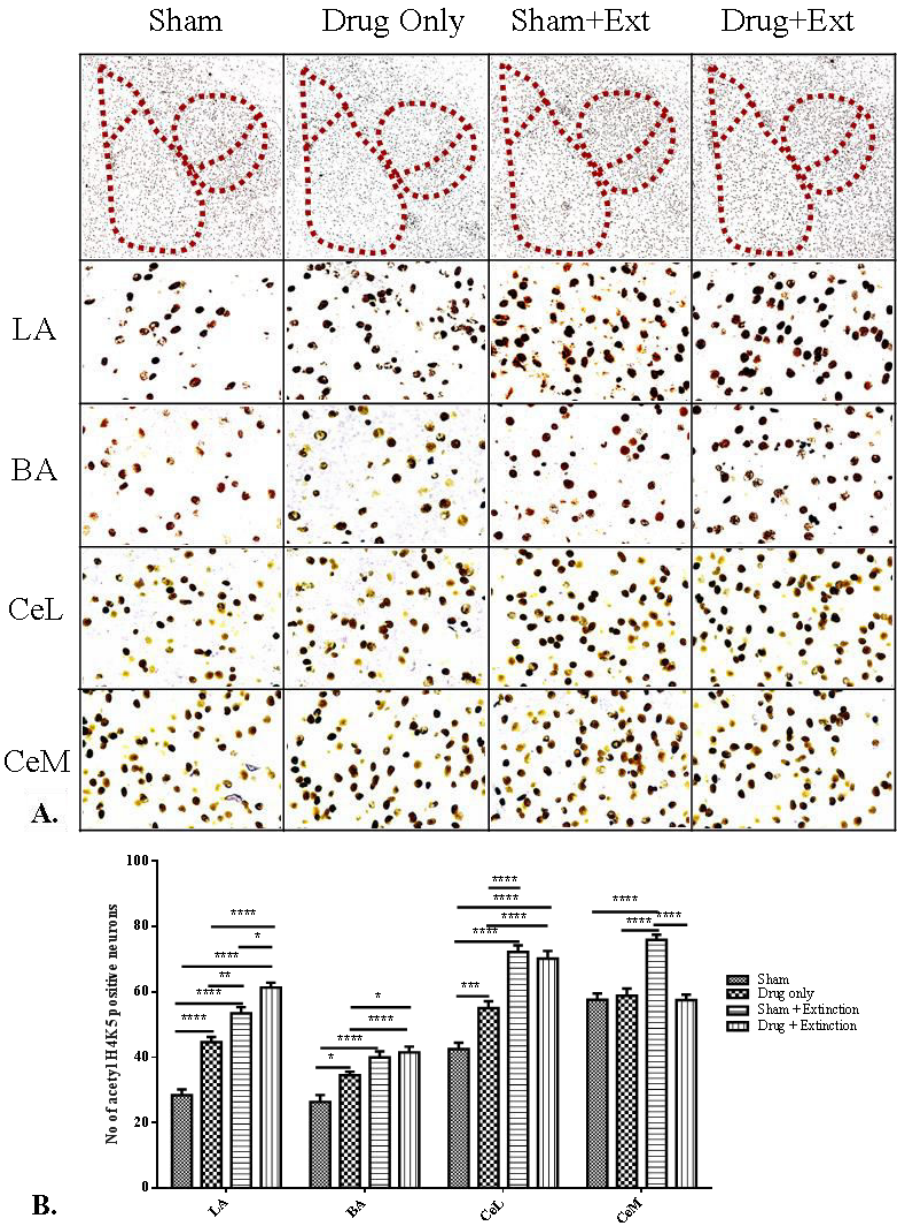



**Fig 56.** Histone H4K5 acetylation in the amygdala in valproic acid treated extinction group**. A. and B.** Immunohistochemistry of histone H4K5 acetylation in LA, BA, CeL and CeM following extinction. (Drug only= valproic acid only, Sham+Ext= vehicle treated extinction group and Drug+Ext= valproic acid treated extinction group)

### 7.1.2.4. HDAC1 expression in the Amygdala

HDAC1 expression in valproic acid treated group following extinction exhibited somehow similar expression profile in the amygdala as shown by acetyl H3K9 and H4K5. In LA, the HDAC1 expression increased significantly in sham + extinction ($p$<0.001) and drug + extinction group ($p$<0.0001) as compared to sham control group while there was no significant change in drug only group [F (3,36) = 23.49, $p$<0.0001]. The change for HDAC1 expression in drug + extinction group was significantly higher as compared to sham + extinction group ($p$<0.05). In BA, the HDAC1 expression increased significantly in sham + extinction ($p$<0.0001) and drug + extinction group ($p$<0.0001) as compared to the sham control group whereas there was no significant change in drug only group [F (3,36) = 84.14, $p$<0.0001]. The change in HDAC1 expression was significantly

higher in drug + extinction group as compared to the sham + extinction group ($p$<0.001). In CeL, the HDAC1 expression increased significantly in sham + extinction ($p$<0.0001) and drug + extinction group ($p$<0.0001) as compared to the sham control group whereas there was no significant change in drug only group [F (3,36) = 66.10, $p$<0.0001]. The change in HDAC1 expression was significantly higher in drug + extinction group as compared to sham + extinction group ($p$<0.01). In CeM, the HDAC1 expression increased significantly in sham + extinction ($p$<0.001) and drug + extinction group ($p$<0.0001) as compared to the sham control group whereas there was no significant change in drug only group [F (3,36) = 22.21, $p$<0.0001]. Overall, the result showing an indirect effect of valproic acid on HDAC1 expression and extinction enhancement, which do not involve targeting HDAC1 in the amygdala. (Fig 57)

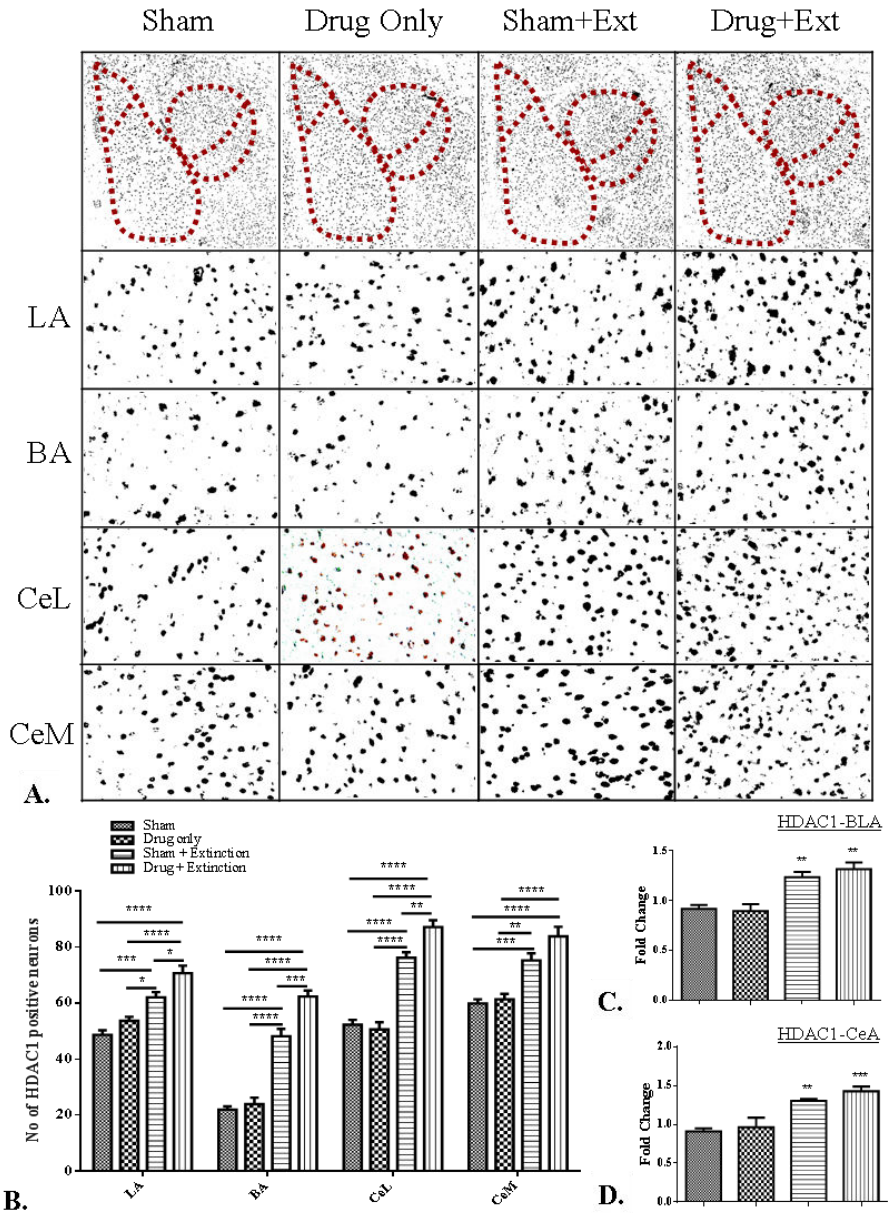



**Fig 57.** HDAC1 expression in the amygdala in valproic acid treated extinction group. **A. and B.** Immunohistochemistry of HDAC1 expression in LA, BA, CeL and CeM following extinction. **C. and D.** HDAC1 mRNA expression in BLA (C.) and CeA (D.). (Drug only= valproic acid only, Sham+Ext= vehicle treated extinction group and Drug+Ext= valproic acid treated extinction group)

### 7.1.2.5. HDAC2 expression in the Amygdala

HADC2 showed different expression than HDAC1 in amygdala where in LA, HDAC2 expression decreased significantly in drug only ($p$<0.01), sham + extinction ($p$<0.0001) and drug + extinction group ($p$<0.0001) as compared to the sham control group [F (3,36), = 37.52, $p$<0.0001]. Sham + extinction ($p$<0.0001) and drug + extinction group ($p$<0.01) exhibited significantly lower HDAC2 expression than drug only group. In BA, the HDAC2 expression decreased significantly in drug only ($p$<0.05), sham + extinction ($p$<0.0001) and drug + extinction group ($p$<0.001) as compared to the sham control group [F (3,36), = 11.82, $p$<0.0001]. (Fig 58)

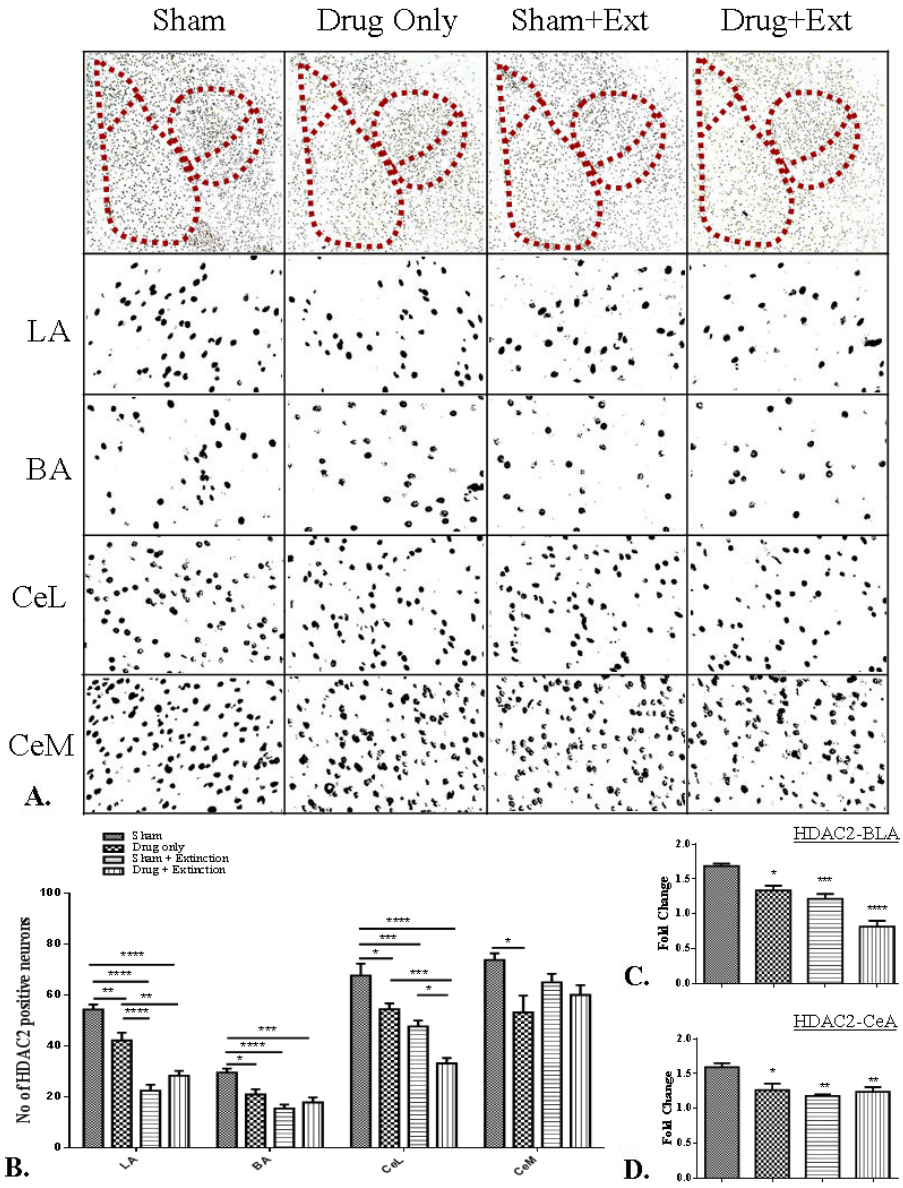



**Fig 58.** HDAC2 expression in the amygdala in valproic acid treated extinction group**. A. and B.** Immunohistochemistry of HDAC2 expression in LA, BA, CeL and CeM following extinction. **C. and D.** HDAC2 mRNA expression in BLA (C.) and CeA (D.). (Drug only= valproic acid only, Sham+Ext= vehicle treated extinction group and Drug+Ext= valproic acid treated extinction group)

In CeL, similarly the HDAC2 expression decreased significantly in drug only ($p$<0.05), sham + extinction ($p$<0.001) and drug + extinction group ($p$<0.0001) as compared to the sham control group [F (3,36), = 22.40, $p$<0.0001]. Drug + extinction group exhibited significantly

reduced HDAC2 expression as compared to the drug only ($p$<0.001) and sham + extinction group ($p$<0.05) which may be due to the additive effect of valproic acid on HDAC2 expression. In CeM, the HDAC2 expression decreased significantly in drug only ($p$<0.05), but showed no change in sham + extinction and drug + extinction group as compared to the sham control group [F (3, 36), = 3.952, $p$<0.05]. Overall, the HDAC2 expression exhibited a negative correlation with the extinction learning in amygdala subregions.

### 7.1.2.6. c-fos expression in Prefrontal Cortex

In PL, the c-fos expression increased significantly in drug only group ($p$<0.05) but not in sham + extinction and drug + extinction group as compared to the sham control group [F (3,36) = 3.821, $p$<0.05]. In IL, however the c-fos expression increased significantly in sham + extinction and drug + extinction but not in drug only group as compared to the sham control group [F (3,36) = 30.58, $p$<0.0001]. In drug + extinction group the c-fos expression was significantly higher as compared to the drug only ($p$<0.0001) and sham + extinction group ($p$<0.001). (Fig 59)

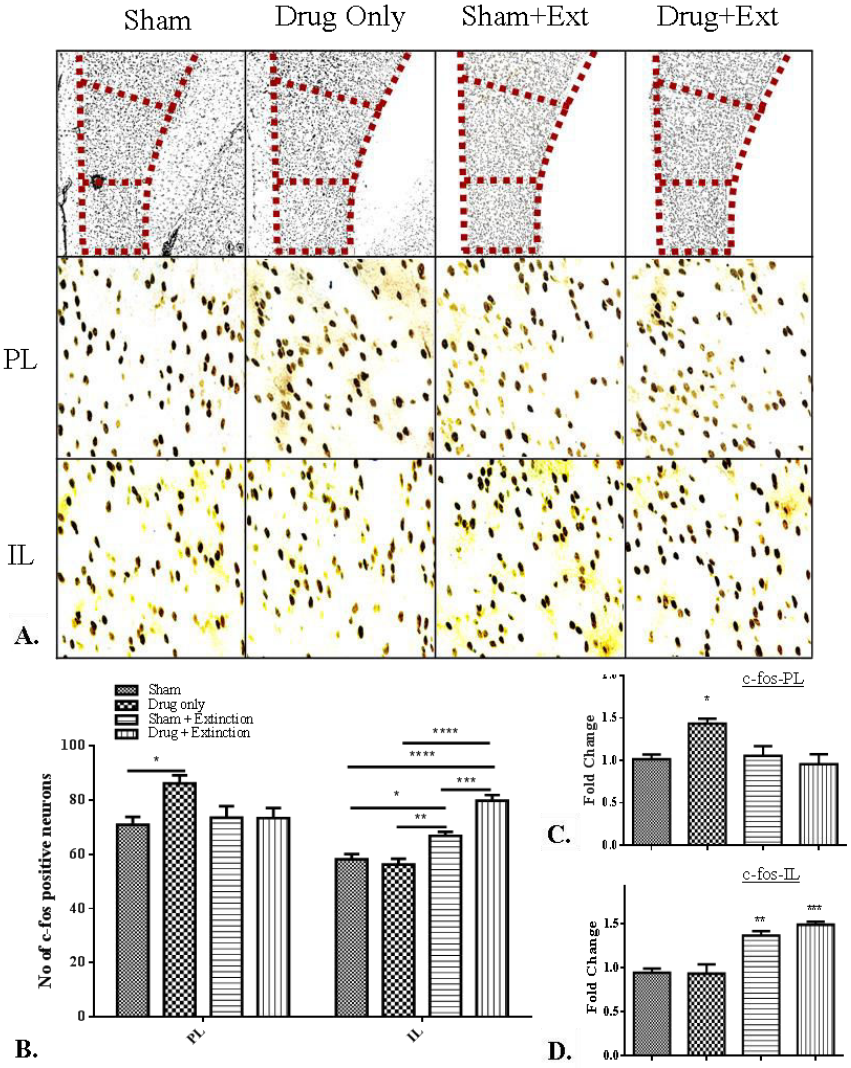



**Fig 59.** c-fos expression in Prefrontal cortex in valproic acid treated extinction group**. A. and B.** Immunohistochemistry of c-fos expression in PL and IL, following extinction. **C. and D.** c-fos mRNA expression in PL (C.) and IL (D.). (Drug only= valproic acid only, Sham+Ext= vehicle treated extinction group and Drug+Ext= valproic acid treated extinction group).

### 7.1.2.7. Histone H3K9 acetylation in Prefrontal Cortex

Similar to c-fos expression, the histone acetyl H3K9 level increased significantly in PL in drug only group ($p$<0.05) but not in sham + extinction and drug + extinction group as compared to the sham control group [F (3,36) = 2.965, $p$<0.05]. In IL, however the H3K9 acetylation increased significantly in drug only ($p$<0.01) and drug + extinction group ($p$<0.0001) but not in sham + extinction group as compared to the sham control group [F (3,36) = 31.18, $p$<0.0001]. Drug + extinction group exhibited significantly higher acetyl H3K9 level as compared to drug only group ($p$<0.0001). (Fig 60)

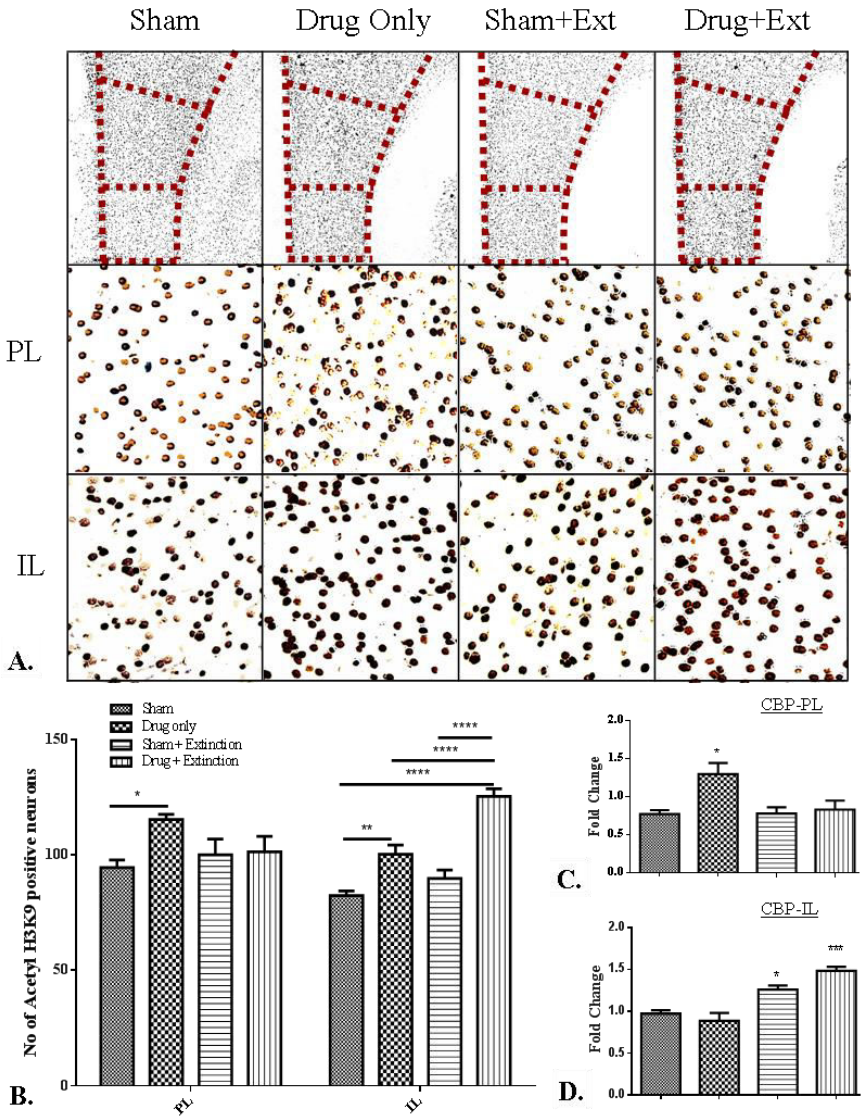



**Fig 60.** Histone H3K9 acetylation in Prefrontal cortex in valproic acid treated extinction group. **A. and B.** Immunohistochemistry of histone H3K9 acetylation in PL and IL following extinction. **C. and D.** CBP mRNA expression in PL (C.) and IL (D.). (Drug only= valproic acid only, Sham+Ext= vehicle treated extinction group and Drug+Ext= valproic acid treated extinction group).

### 7.1.2.8. Histone H4K5 expression in Prefrontal Cortex

Similar to acetylation of H3 at K9, the acetylation of H4 at K5 in PL increased significantly in drug only group ($p$<0.05) but not in sham + extinction and drug + extinction group as compared to the sham control group [F (3,36) = 3.033, $p$<0.05]. In IL, the acetylation of H4 at K5 increased significantly in drug only ($p$<0.0001), sham + extinction ($p$<0.0001) and drug + extinction group ($p$<0.0001) as compared to the sham control group [F (3,36) = 85.94, $p$<0.0001]. Drug + extinction group exhibited significantly enhanced acetylation of H4 at K5 as compared to the drug only ($p$<0.0001) and sham + extinction group ($p$<0.001). In conclusion, increased histone acetylation of H3 at K9 and H4 at K5 is associated with increased in IL activity which was enhanced by the HDAC inhibitor mediated extinction enhancement. While in PL, it was observed that there was no association of HDAC inhibitor on PL activity when treated with extinction training however HDAC inhibitor enhanced the PL activity in drug only group. (Fig 61)

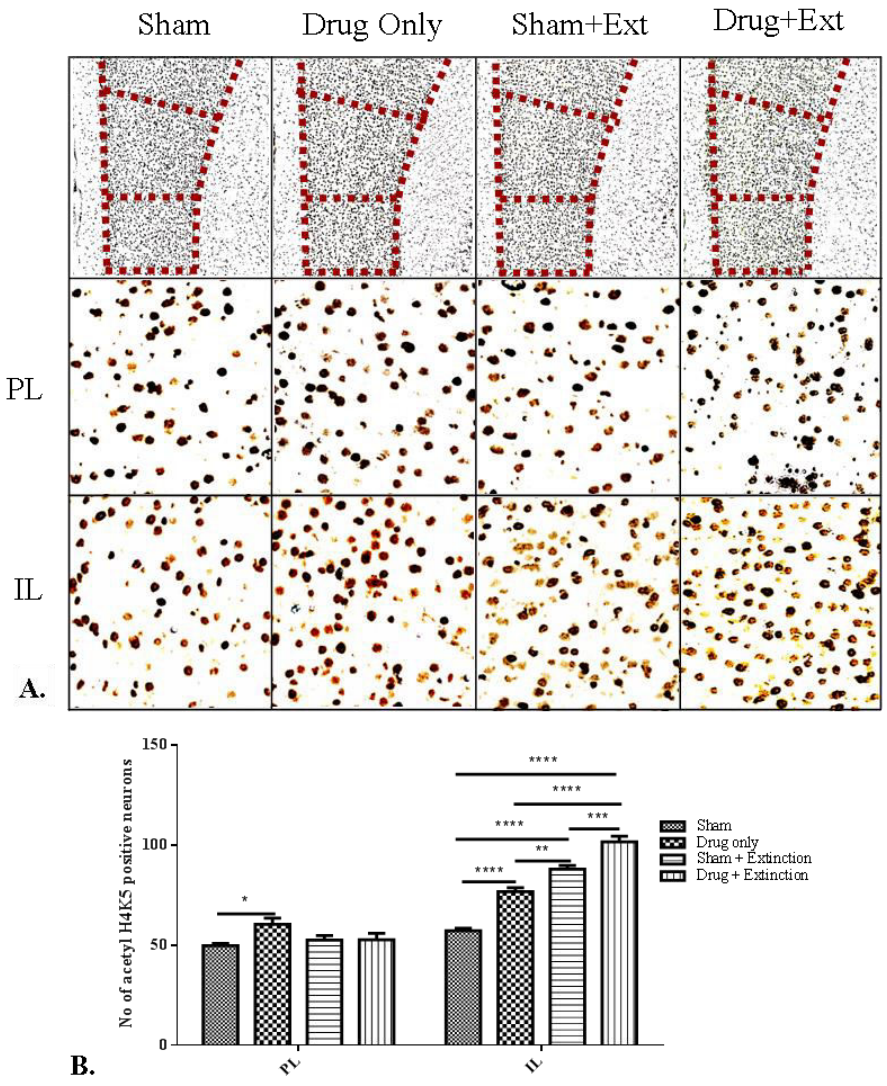



**Fig 61.** Histone H4K5 acetylation in Prefrontal cortex in valproic acid treated extinction group**. A. and B.** Immunohistochemistry of histone H4K5 acetylation in PL and IL following extinction. . (Drug only= valproic acid only, Sham+Ext= vehicle treated extinction group and Drug+Ext= valproic acid treated extinction group).

### 7.1.2.9. HDAC1 expression in Prefrontal Cortex

HDAC1 expression exhibited different expression pattern than IEG c-fos and acetyl histone in PFC. In PL, the HDAC1 expression exhibited no significant change in drug only, sham + extinction and drug + extinction as compared to the sham control group [F (3,36) = 1.347, $p$>0.05]. In IL, however the HDAC1 expression decreased significantly in sham + extinction ($p$<0.001) and drug + extinction group ($p$<0.0001) but not in drug only group as compared to the sham control group [F (3,36) = 21.62, $p$>0.0001]. The drug + extinction group exhibited significantly lower HDAC1 expression as compared to sham + extinction group ($p$<0.01). Overall, the result showed that HDAC1 expression is negatively associated with extinction learning in IL and the effect by valproic acid may be indirect on IL during extinction. (Fig 62)

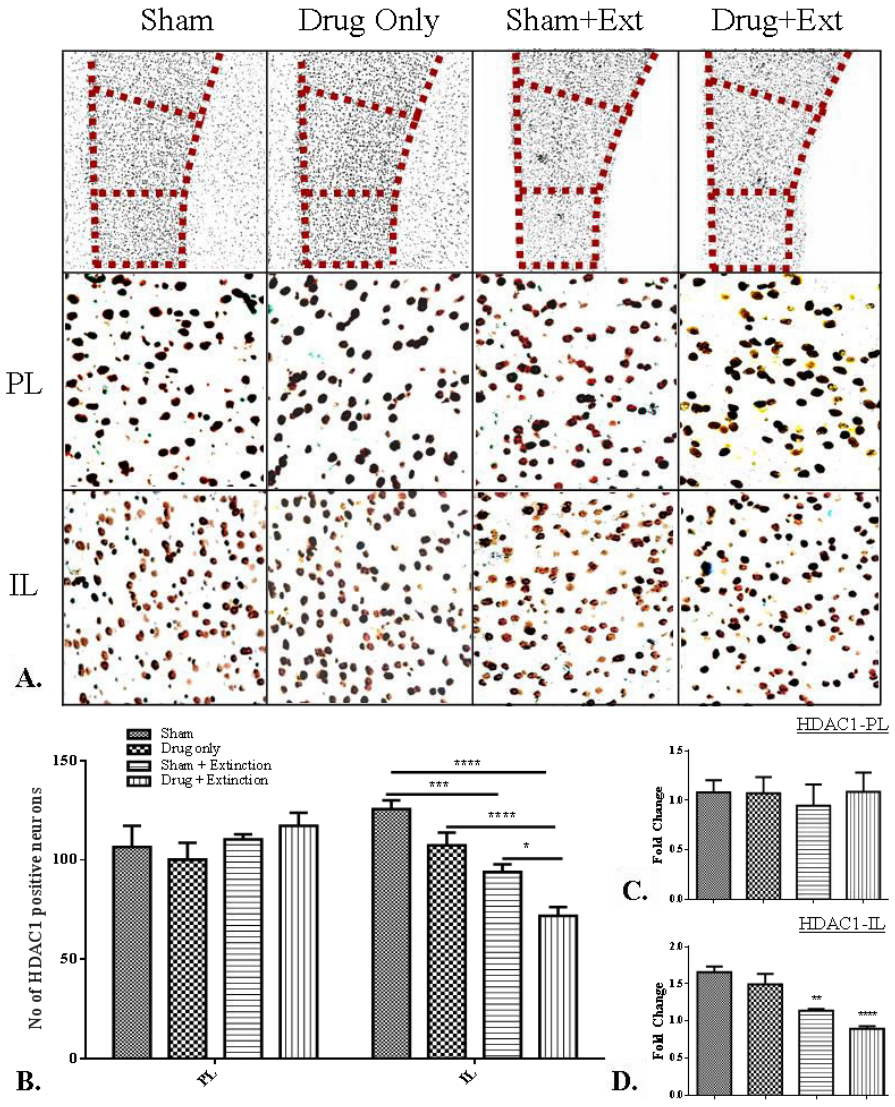



**Fig 62.** HDAC1 expression in Prefrontal cortex in valproic acid treated extinction group**. A. and B.** Immunohistochemistry of HDAC1 expression in PL and IL following extinction. **C. and D.** HDAC1 mRNA expression in PL (C.) and IL (D.). (Drug only= valproic acid only, Sham+Ext= vehicle treated extinction group and Drug+Ext= valproic acid treated extinction group).

### 7.1.2.10. HDAC2 expression in Prefrontal Cortex

HDAC2 exhibited different expression in PFC subregions than HDAC1, acetyl H3K9, H4K5 and c-fos following valproic acid mediated extinction. In PL, the HDAC2 expression decreased significantly in drug only group ($p<0.05$) and increased significantly in drug + extinction group ($p<0.0001$) as compared to the sham control group [$F(3, 36) = 26.86$, $p<0.0001$]. Sham + extinction group showed no significant change for HDAC2 expression in PL ($p>0.05$). In IL, the HDAC2 expression decreased significantly in drug only group ($p<0.05$) and sham + extinction group ($p<0.05$) but not in drug + extinction group as compared to the sham control group [$F(3, 36) = 6.202$, $p<0.01$]. Although there was a direct effect of valproic acid on HDAC2 expression, overall HDAC2 expression increased in PL while in IL the HDAC2 expression was unaffected in drug + extinction group. (Fig 63)

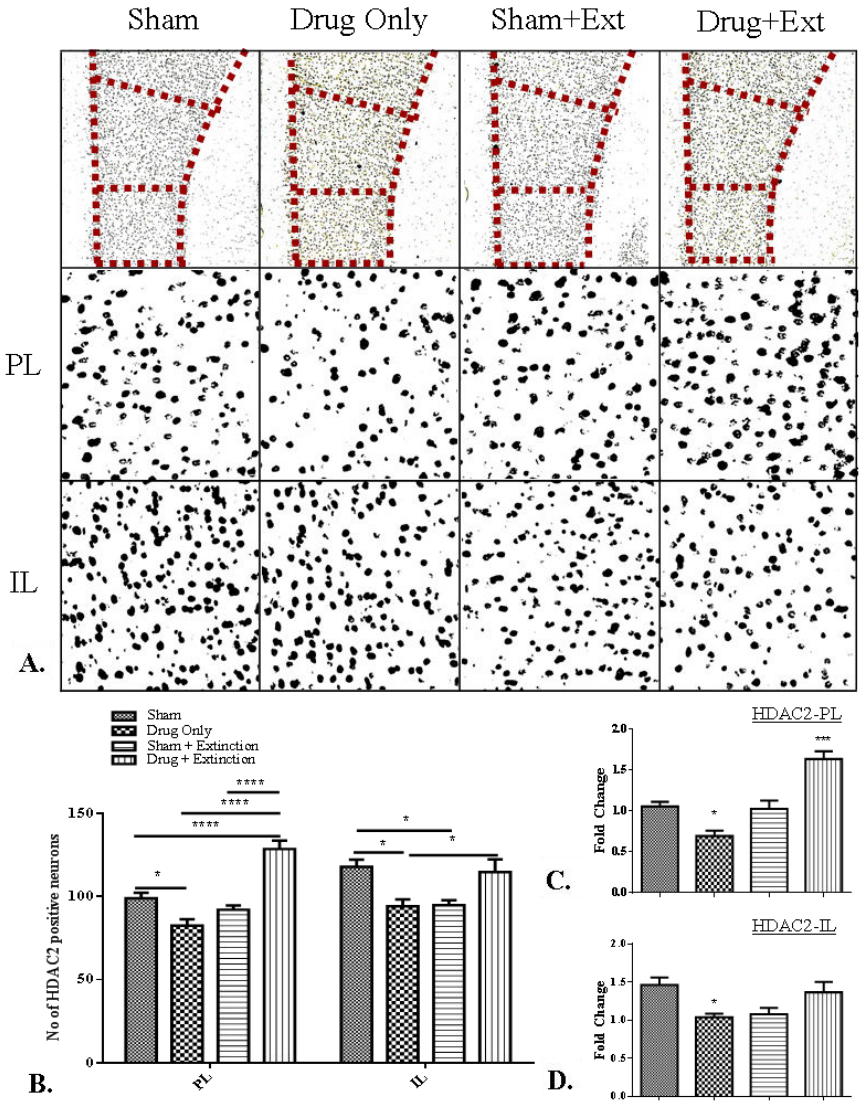



**Fig 63.** HDAC2 expression in Prefrontal cortex in valproic acid treated extinction group. **A. and B.** Immunohistochemistry of HDAC2 expression in PL and IL following extinction. **C. and D.** HDAC2 mRNA expression in PL (C.) and IL (D.). (Drug only= valproic acid only, Sham+Ext= vehicle treated extinction group and Drug+Ext= valproic acid treated extinction group).

**7.1.2.11. c-fos expression in Hippocampus**

As observed in behavior experiment valproic acid increased the memory for fear extinction which results in a change in the expression pattern of brain regions involved in fear circuitry. The c-fos expression in CA1 increased significantly in drug only ($p$<0.01), sham + extinction ($p$<0.0001) and drug + extinction group ($p$<0.0001) as compared to the sham control group [F (3,36) = 105.2, $p$<0.0001]. The change in drug + extinction group was significantly higher as compared to the drug only ($p$<0.0001) and sham + extinction group ($p$<0.05). Likewise in CA3, the c-fos expression increased significantly in drug only ($p$<0.05), sham + extinction ($p$<0.0001) and drug + extinction group ($p$<0.0001) as compared to the sham control group [F (3,36) = 38.09, $p$<0.0001]. The change in c-fos expression in drug + extinction group was significantly higher as compared to the drug only ($p$<0.0001) and sham + extinction group ($p$<0.001). (Fig 64)

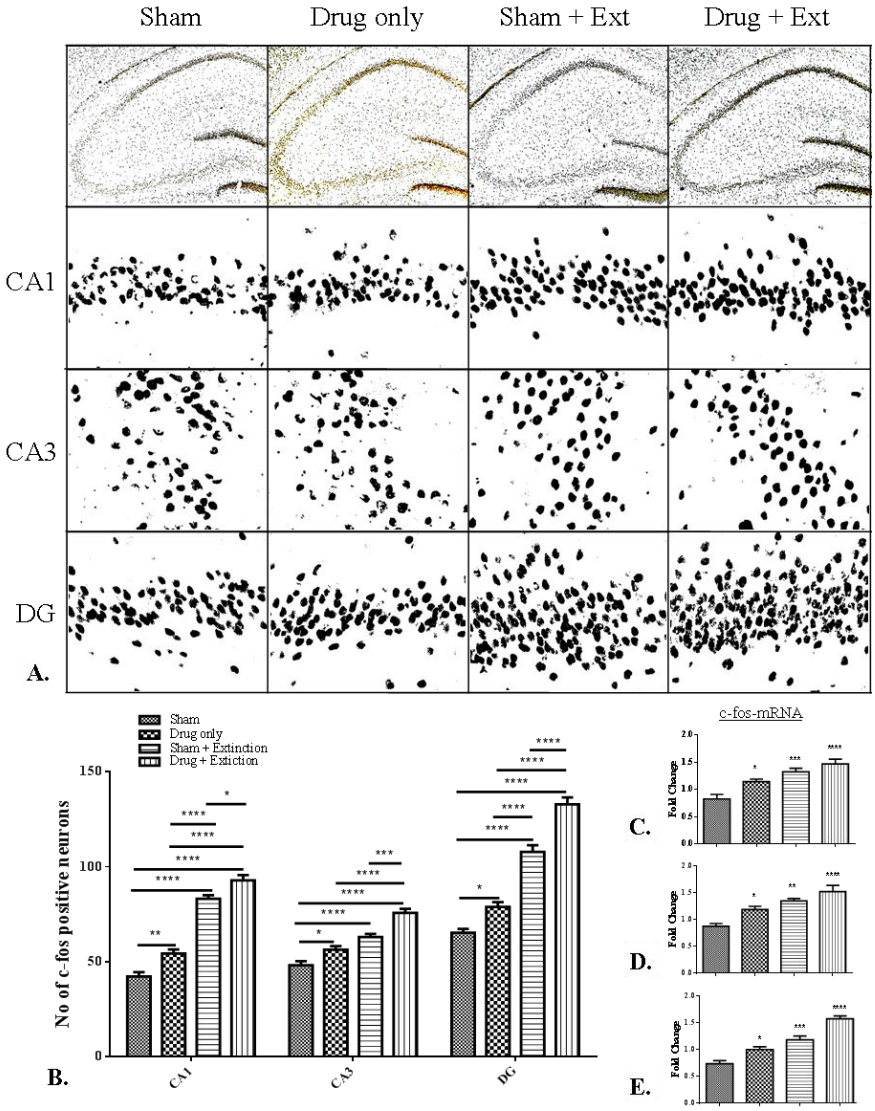



**Fig 64.** c-fos expression in Hippocampus in valproic acid treated extinction group. **A. and B.** Immunohistochemistry of c-fos expression in CA1, CA3 and DG following extinction. **C., D. and E.** c-fos mRNA expression in CA1 (C.), CA3 (D.) and DG (E.). (Drug only= valproic acid only, Sham+Ext= vehicle treated extinction group and Drug+Ext= valproic acid treated extinction group)

DG, exhibited similar activation pattern for c-fos expression as in CA1 and CA3. In DG the c-fos expression increased significantly in drug only ($p$<0.05), sham + extinction ($p$<0.0001) and

drug + extinction group ($p$<0.0001) as compared to the sham control group [F (3,36) = 99.23, $p$<0.0001]. The change in c-fos expression was significantly higher in drug + extinction group as compared to the drug only ($p$<0.0001) and sham + extinction group ($p$<0.0001). The result suggested that increased activity of hippocampus is important for fear memory extinction which was enhanced by the valproic acid.

**7.1.2.12. Histone H3K9 acetylation in Hippocampus**

Similar to c-fos expression, the acetyl H3K9 levels in CA1, increased significantly in drug only ($p$<0.01), sham + extinction ($p$<0.001) and drug + extinction group ($p$<0.0001) as compared to the sham control group [F (3,36) = 21.47, $p$<0.0001]. (Fig 65)

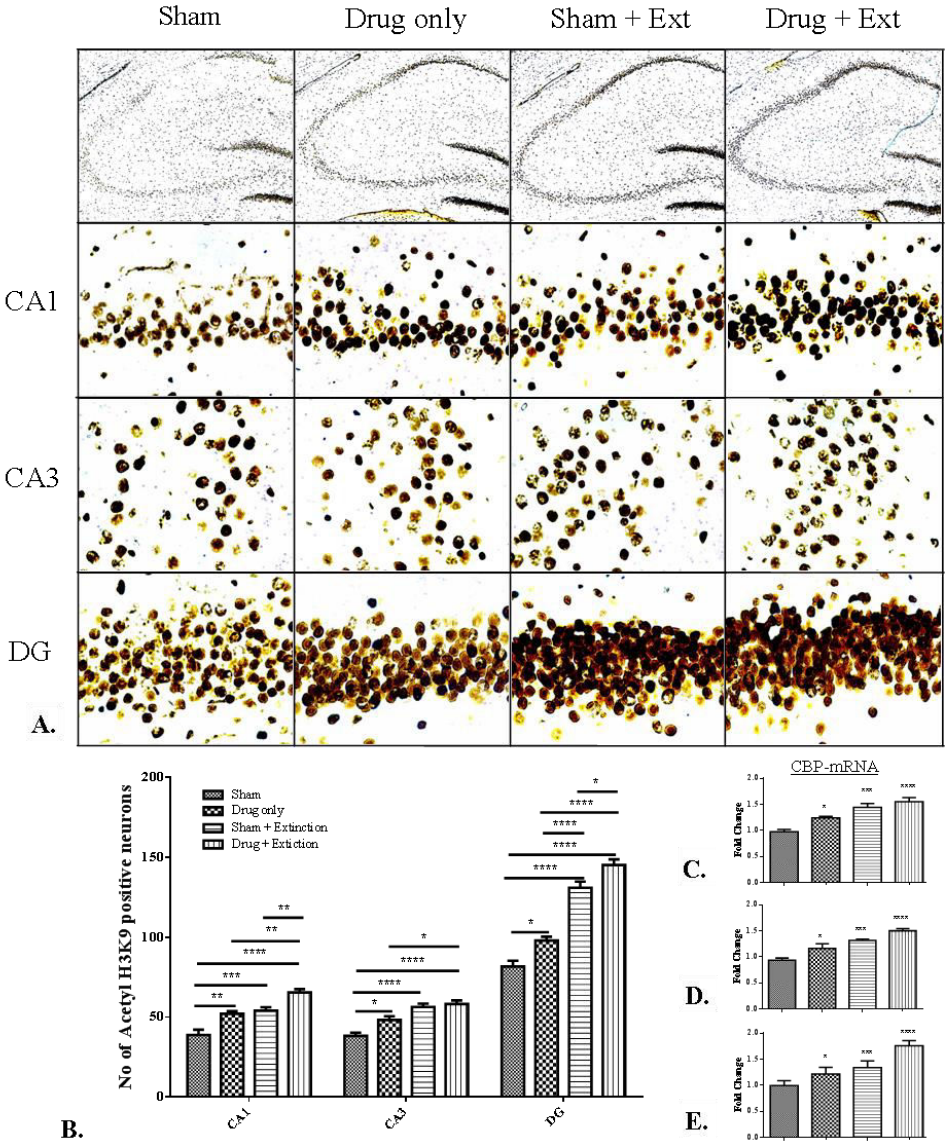



**Fig 65.** Histone H3K9 acetylation in Hippocampus in valproic acid treated extinction group**. A. and B.** Immunohistochemistry of histone H3K9 acetylation in CA1, CA3 and DG following extinction. **C. D. and E.** CBP mRNA expression in CA1 (C.), CA3 (D.) and DG (E.). (Drug only= valproic acid only, Sham+Ext= vehicle treated extinction group and Drug+Ext= valproic acid treated extinction group)

The change for acetyl H3K9 level in drug + extinction group was significantly higher as compared to the drug only ($p$<0.01) and sham + extinction group ($p$<0.01). In CA3, the acetyl

H3K9 level increased significantly in drug only ($p$<0.05), sham + extinction ($p$<0.0001) and drug + extinction group ($p$<0.0001) as compared to the sham control group [F (3,36) = 17.19, $p$<0.0001]. In DG, the acetyl H3K9 level increased significantly in drug only ($p$<0.05), sham + extinction ($p$<0.001) and drug + extinction group ($p$<0.0001) as compared to the sham control group [F (3,36) = 71.42, $p$<0.0001]. The acetyl H3K9 level in drug + extinction group was significantly higher as compared to the drug only ($p$<0.0001) and sham + extinction group ($p$<0.05), the effect may be due to the additive effect of HDAC inhibitor during extinction. Overall, the result confirms that valproic acid increased the activity of hippocampus subregion as well as histone acetylation for stabilization of extinction memory.

**7.1.2.13. Histone H4K5 acetylation in Hippocampus**

In CA1, the acetylation of histone H4 at K5 increased significantly in drug only ($p$<0.05), sham + extinction ($p$<0.0001) and drug + extinction group ($p$<0.0001) as compared to the sham control group [F (3,36) = 32.20, $p$<0.0001]. (Fig 66)

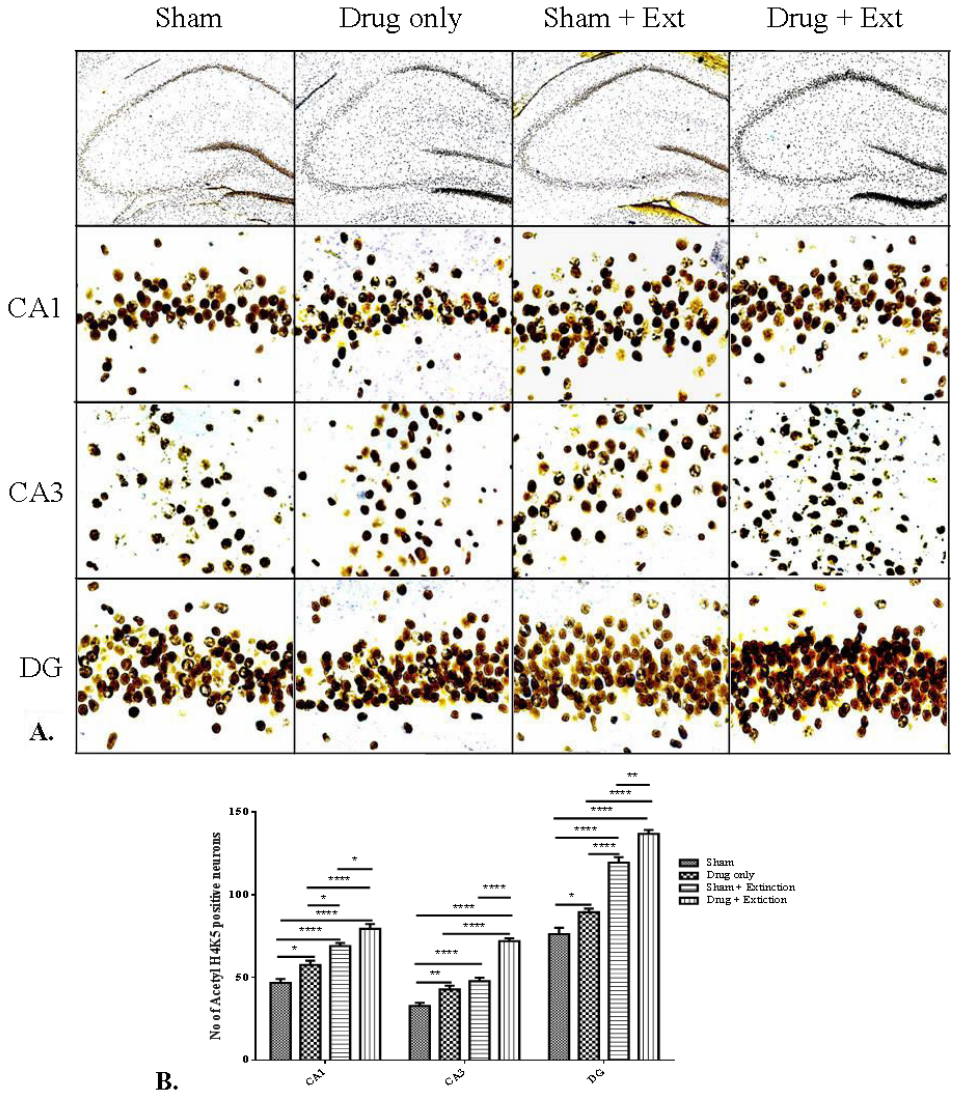



**Fig 66.** Histone H4K5 acetylation in Hippocampus in valproic acid treated extinction group**. A. and B.** Immunohistochemistry of acetyl H4K5 expression in CA1, CA3 and DG following extinction. (Drug only= valproic acid only, Sham+Ext= Vehicle treated extinction group and Drug+Ext= valproic acid treated extinction group)

In drug + extinction group the acetylation of H4 at K5 was significantly higher than drug only ($p$<0.0001) and sham + extinction group ($p$<0.05). In CA3, the acetylation of H4 at K5 increased significantly in drug only ($p$<0.01), sham + extinction ($p$<0.0001) and drug + extinction group ($p$<0.0001) as compared to the sham control group [F (3,36) = 70.30, $p$<0.0001. In drug + extinction group the acetylation of H4 at K5 level was significantly higher than drug only ($p$<0.0001) and sham + extinction group ($p$<0.0001). In DG, the acetyl H4K5 level increased significantly in drug only ($p$<0.05), sham + extinction ($p$<0.0001) and drug + extinction group ($p$<0.0001) as compared to the sham control group [F (3,36) = 83.23, $p$<0.0001. In drug + extinction group the acetyl H4K5 level was significantly higher than drug only ($p$<0.0001) and sham + extinction group ($p$<0.01).

### 7.1.2.14. HDAC1 expression in Hippocampus

HDAC1 exhibited similar expression pattern in the hippocampus as exhibited by the c-fos, acetyl H3K9 and H4K5 level. (Fig 67)

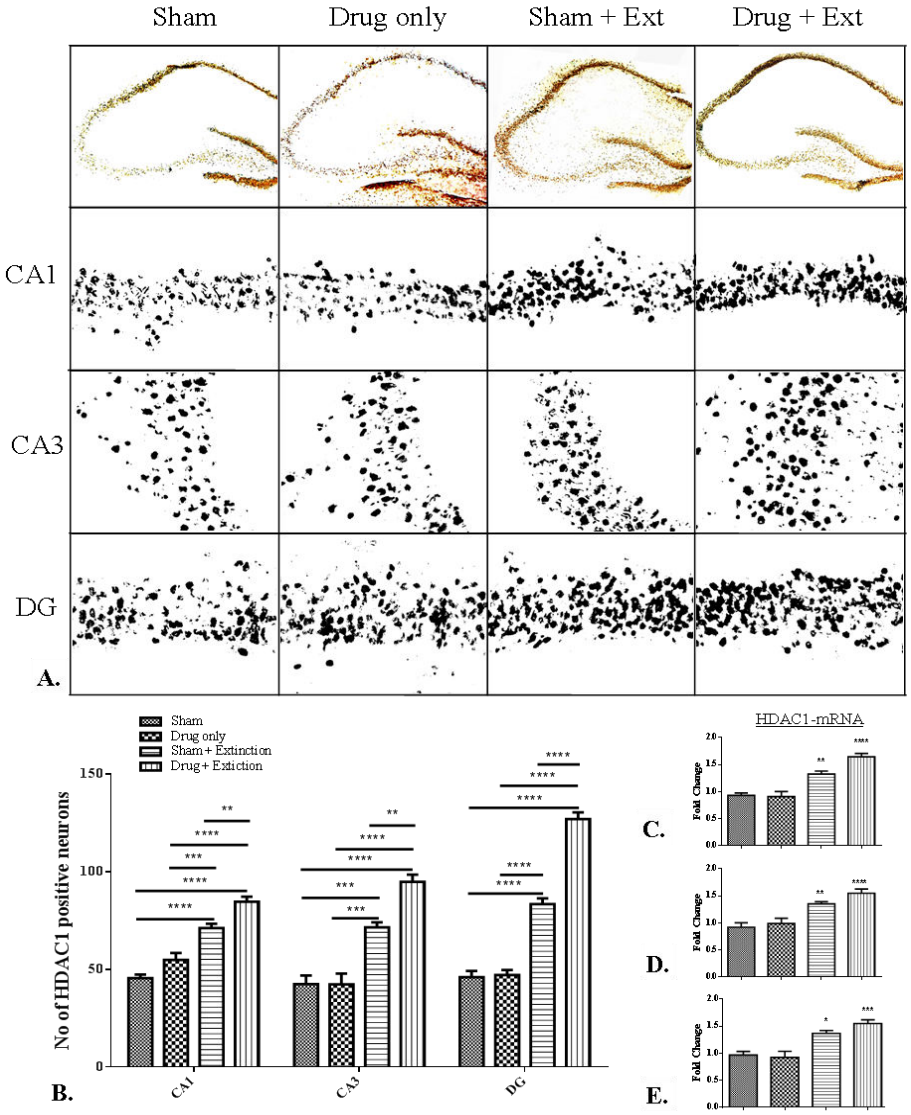



**Fig 67.** HDAC1 expression in Hippocampus in valproic acid treated extinction group. **A. and B.** Immunohistochemistry of HDAC1 expression in CA1, CA3 and DG following extinction. **C. D. and E.** HDAC1 mRNA expression in CA1 (C.), CA3 (D.) and DG (E.). (Drug only= valproic acid only, Sham+Ext= vehicle treated extinction group and Drug+Ext= valproic acid treated extinction group)

In CA1, the HDAC1 expression increased significantly in sham + extinction ($p$<0.0001) and drug + extinction group ($p$<0.0001) but not in drug only group as compared to the sham control group [F (3,36) = 43.84, $p$<0.0001]. The change in drug + extinction group was significantly higher as compared to the drug only ($p$<0.0001) and sham + extinction groups ($p$<0.01). Likewise in CA3,the HDAC1 expression increased significantly in sham + extinction ($p$<0.001) and drug + extinction group ($p$<0.0001) but not in drug only group as compared to the sham control group [F (3,36) = 36.47, $p$<0.0001]. The change in drug + extinction group was highly significant as compared to the drug only ($p$<0.0001) and sham + extinction groups ($p$<0.01). In DG, the HDAC1 expression increased significantly in sham + extinction ($p$<0.0001) and drug + extinction group ($p$<0.0001) but not in drug only group as compared to the sham control group [F (3,36) = 161.9, $p$<0.0001]. Similarly the change in drug + extinction group was highly significant as compared to the drug only ($p$<0.0001) and sham + extinction groups ($p$<0.0001). Overall the result confirmed that HDAC1 expression in the hippocampus is required for fear extinction but its expression is not regulated by the HDAC inhibitor valproic acid.

#### 7.1.2.15. HDAC2 expression in Hippocampus

HDAC2 exhibited different expression pattern than c-fos, acetyl H3K9, acetyl H4K5 and HDAC1 expression in the hippocampus. In CA1, HDAC2 expression decreased significantly in drug only ($p$<0.0001), sham + extinction ($p$<0.0001) and drug + extinction group ($p$<0.0001) as compared to the sham control group [F (3,36) = 67.02, $p$<0.0001]. The HDAC2 expression in drug + extinction group was significantly lower as compared to the drug only ($p$<0.0001) and sham + extinction group ($p$<0.05). Likewise in CA3,the HDAC2 expression decreased significantly in drug only ($p$<0.01), sham + extinction ($p$<0.0001) and drug + extinction group ($p$<0.0001) as compared to the sham control group [F (3,36) = 46.73, $p$<0.0001]. The change for HDAC2 expression in drug + extinction group was significantly lower as compared to the drug only ($p$<0.0001) and sham + extinction group ($p$<0.001). In DG, the HDAC2 expression decreased significantly in drug only ($p$<0.0001), sham + extinction ($p$<0.0001) and drug + extinction group ($p$<0.0001) as compared to the sham control group [F (3,36) = 56.68, $p$<0.0001]. The change for HDAC2 expression in drug + extinction group was significantly lower as compared to the drug only ($p$<0.0001) and sham + extinction group ($p$<0.05). Overall, the HDAC2 expression is suppressed in the hippocampus for better extinction learning and valproic acid target HDAC2 in the hippocampus for increasing extinction learning. (Fig 68)

### 7.1.3. Correlation

Valproic acid enhanced the extinction learning by controlling histone acetylation in the amygdala, PFC and hippocampus. A comparison between sham + extinction group and drug + extinction group showed that in LA, acetyl H4K5 ($p$<0.01) and c-fos ($p$<0.05) were negatively correlated with the freezing response and positively correlated with the extinction learning following valproic acid treatment prior to the extinction training whereas HDAC1 and HDAC2 exhibited no correlation with the freezing response. In BA, the HDAC1 ($p$<0.01) and c-fos ($p$<0.05) expression was negatively correlated with the freezing response and positively correlated with extinction learning when compared sham + extinction with drug + extinction group. In CeL, the HDAC1 ($p$<0.01) exhibited a negative correlation while HDAC2 ($p$<0.05) a positive correlation with the freezing response following valproic acid treated extinction learning. In conclusion, the HDAC1 ($p$<0.01) expression was positively correlated with the extinction learning while HDAC2 ($p$<0.05) was negatively correlated with the extinction learning in CeL. In CeM, the acetyl H3K9

($p$<0.01), acetyl H4K5 ($p$<0.01) and c-fos ($p$<0.001) exhibited a positive correlation with the freezing behavior and a negative correlation with the extinction learning; while HDAC1 ($p$<0.05) exhibited a negative correlation with the freezing behavior and a positive correlation with the extinction learning when compared sham + extinction group with drug + extinction group. In conclusion, the HDAC1 expression was positively correlated with the extinction learning and HDAC2 negatively correlated with extinction learning in the amygdala. (Fig 69)

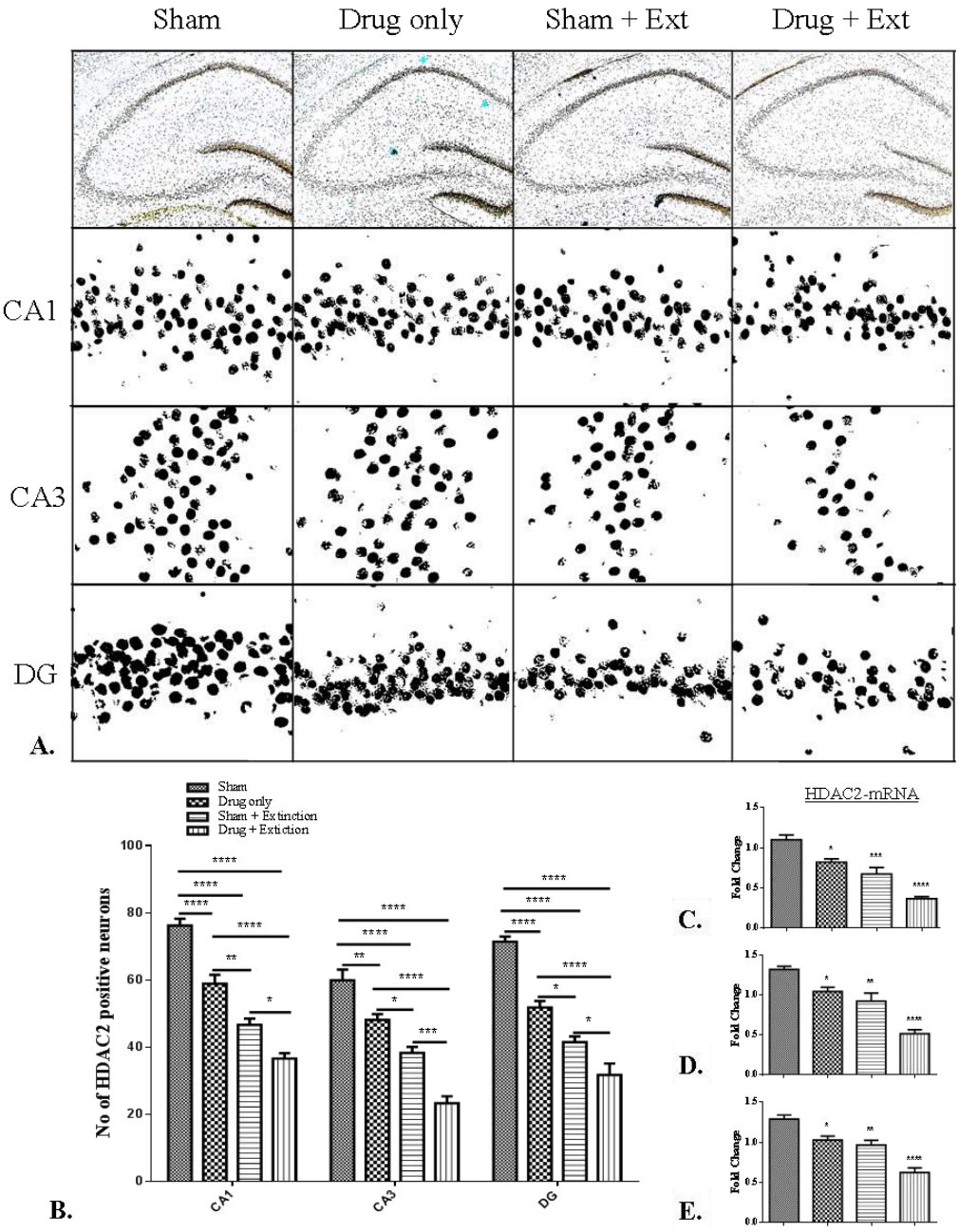



**Fig 68.** HDAC2 expression in Hippocampus in valproic acid treated extinction group**. A. and B.** Immunohistochemistry of HDAC2 expression in CA1, CA3 and DG following extinction. **C. D. and E.** HDAC2 mRNA expression in CA1 (C.), CA3 (D.) and DG (E.). (Drug only= valproic acid only, Sham+Ext= vehicle treated extinction group and Drug+Ext= valproic acid treated extinction group)

In PL, the levels of acetyl H3K9, acetyl H4K5 and c-fos/ HDAC1 exhibited no correlation while HDAC2 ($p$<0.0001) exhibited a negative correlation with the freezing response and a positive correlation with the extinction learning when compared sham + extinction group with drug + extinction group. In IL, however the acetyl H3K9 ($p$<0.001), acetyl H4K5 ($p$<0.05) and c-fos ($p$<0.001) exhibited a negative correlation with the freezing response and a positive correlation with the extinction learning; while HDAC1 ($p$<0.001) exhibited a positive correlation with the freezing response and a negative correlation with the extinction learning when compared sham + extinction group with drug + extinction group. It is clear from the results that HDAC2 suppression in PL and HDAC1 suppression in IL is important for fear extinction.

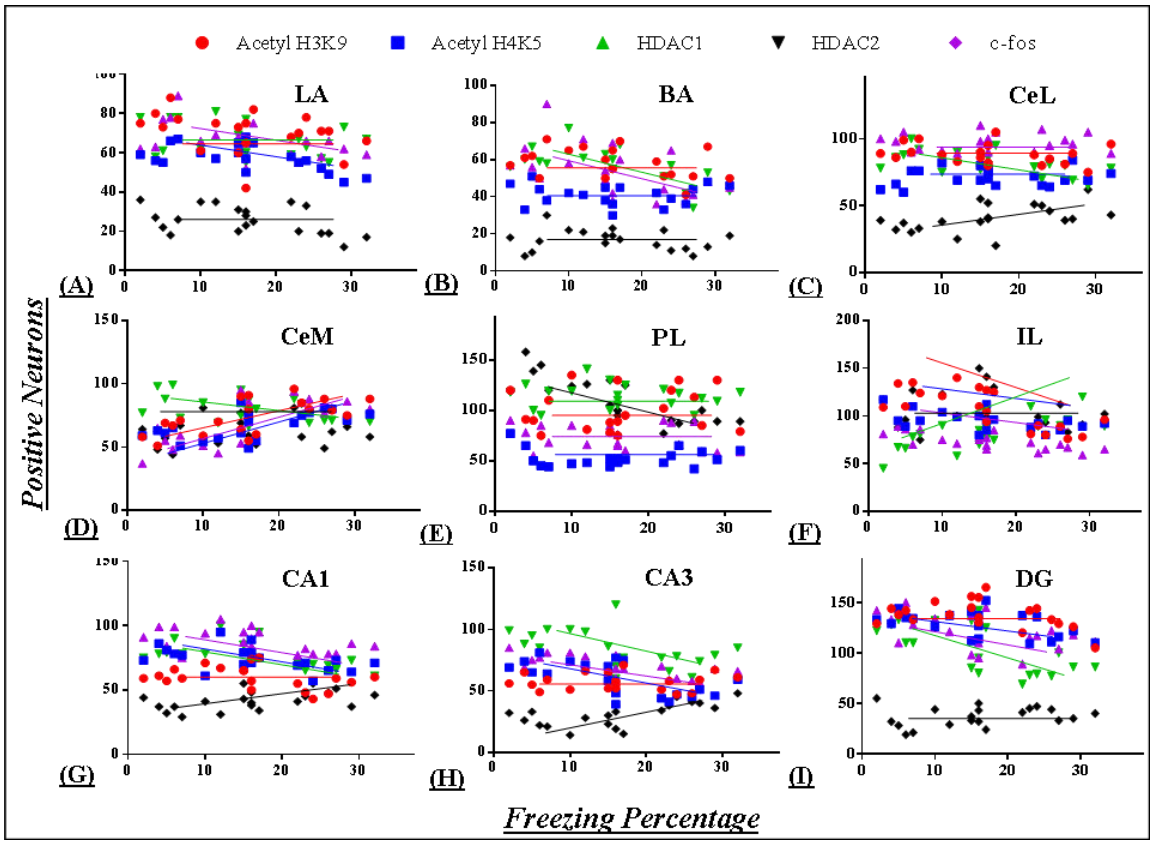



**Fig 69.** Correlation of molecular expression with the freezing response in drug treated Extinction learning. Correlation of c-fos, acetyl H3K9, acetyl H4K5, HDAC1 and HDAC2 with freezing response in valproic acid treated extinction group in the amygdala, hippocampus and PFC from immunohistochemistry (compared between sham + conditioning and drug + conditioning group).

In CA1, acetyl H4K5 ($p$<0.05), c-fos ($p$<0.05) and HDAC1 ($p$<0.05) exhibited a negative correlation with the freezing response and a positive correlation with the extinction learning while HDAC2 ($p$<0.05) exhibited a positive correlation with the freezing response and a negative correlation with the extinction learning when compared sham + extinction group with drug + extinction group. Likewise in CA3, acetyl H4K5 ($p$<0.01), c-fos ($p$<0.001) and HDAC1 ($p$<0.05) exhibited a negative correlation with the freezing response and a positive correlation with the extinction learning while HDAC2 ($p$<0.01) exhibited a positive correlation with the freezing response and a negative correlation with the extinction learning when compared sham + extinction group with drug + extinction group. Similarly in DG, acetyl H4K5 ($p$<0.05), c-fos ($p$<0.05) and

HDAC1 ($p$<0.01) exhibited a negative correlation with the freezing response and a positive correlation with the extinction learning while HDAC2 exhibited no correlation with the freezing response when compared sham + extinction group with drug + extinction group. Overall it is clear that histone H4K5 acetylation, c-fos and HDAC1 expression are positively correlated with the extinction learning while HDAC2 is negatively correlated with the extinction learning. The HDAC1 expression is necessary for fear extinction for fear extinction while HDAC2 expression negatively regulates extinction learning.

## 7.1.4. Real-time PCR

### 7.1.4.1. mRNA expression in Amygdala

When compared, in BLA, the c-fos mRNA expression increased significantly in drug only ($p$<0.05), sham + extinction ($p$<0.01) and drug + extinction ($p$<0.001) group as compared to the sham control group [F (3,16) = 11.15, $p$<0.001] (Fig 54). Likewise, CBP mRNA expression increased significantly in drug only ($p$<0.05), sham + extinction ($p$<0.001) and drug + extinction ($p$<0.0001) group as compared to the sham control group [F (3,16) = 23.10, $p$<0.0001] (Fig 55). Results showed that HDAC1 mRNA increased significantly in sham + extinction ($p$<0.01) and drug + extinction ($p$<0.01) group but not in drug only group as compared to the sham control group [F (3,16) = 13.46, $p$<0.0001] (Fig 57). However, the HDAC2 mRNA decreased significantly in drug only ($p$<0.05), sham + extinction group ($p$<0.001) and drug + extinction group ($p$<0.0001) as compared to the sham control group [F (3,16) = 28.91, $p$<0.0001]. In drug + extinction group the level of HDAC2 expression was significantly lower as compared to drug only ($p$<0.001) and sham + extinction group ($p$<0.01) (Fig 58).

In CeA, the c-fos mRNA expression increased significantly in drug only ($p$<0.05), sham + extinction ($p$<0.0001) and drug + extinction group ($p$<0.01) as compared to the sham control group [F (3,16) = 20.31, $p$<0.0001] (Fig 54). Likewise, the CBP mRNA expression increased significantly in drug only ($p$<0.05), sham + extinction ($p$<0.0001) and drug + extinction group ($p$<0.0001) as compared to the sham control group [F (3,16) = 20.96, $p$<0.0001] (Fig 55). The HDAC1 mRNA expression increased significantly in sham + extinction ($p$<0.01) and drug + extinction group ($p$<0.001) but not in drug only group as compared to the sham control group [F (3,16) = 12.44, $p$<0.001] (Fig 57). However, HDAC2 expression decreased significantly in drug only ($p$<0.05), sham + extinction ($p$<0.01) and drug + extinction group ($p$<0.01) as compared to the sham control group [F (3,16) = 8.07, $p$<0.001] (Fig 58). Overall, the result shows that though the HDAC1 expression is required in the amygdala for fear extinction valproic acid did not affect its expression and some indirect mechanism should be responsible for its function in valproic acid treated extinction learning. However, the HDAC2 is a negative regulator of extinction learning and valproic acid targeting HDAC2 in the amygdala for improvement of extinction learning.

### 7.1.4.2. mRNA expression in Prefrontal Cortex

In PFC, the c-fos expression in PL, increased significantly in drug only group ($p$<0.05) but not in sham + extinction and drug + extinction group as compared to the sham control group [F (3,16) = 5.532, $p$<0.01] (Fig 59). Likewise, the CBP mRNA expression increased significantly in drug only group ($p$<0.05) but not in sham + extinction and drug + extinction group as compared to the sham control group [F (3,16) = 5.718, $p$<0.01] (Fig 60). However, HDAC1 mRNA exhibited no significant change in PL in drug only, sham + extinction and drug + extinction group as compared to the sham control group ($p$>0.05) [F (3,16) = 0.144, $p$>0.05] (Fig 62). When compared, the HDAC2 mRNA was found to be decreased significantly in PL for drug only group ($p$<0.05) and

increased significantly in drug + extinction group ($p$<0.001) but not in sham + extinction group as compared to the sham control group [F (3,16) = 22.82, $p$<0.0001] (Fig 63).

In IL, the c-fos mRNA expression increased significantly in sham + extinction ($p$<0.01) and drug + extinction group ($p$<0.001) but not in drug only group as compared to the sham control group [F (3,16) = 19.06, $p$<0.0001] (Fig 59). Furthermore, the CBP mRNA expression increased significantly in sham + extinction ($p$<0.05) and drug + extinction group ($p$<0.001) but not in drug only group as compared to the sham control group [F (3,16) = 19.77, $p$<0.0001] (Fig 60). HDAC1 mRNA however decreased significantly in sham + extinction ($p$<0.01) and drug + extinction ($p$<0.0001) groups as compared to the sham control group [F (3,16) = 40.22, $p$<0.0001] (Fig 62). The HDAC2 mRNA expression decreased significantly in drug only group ($p$<0.05) but not in sham + extinction and drug + extinction groups as compared to the sham control group [F (3,16) = 4.677, $p$<0.05] (Fig 63). The result shows that the extinction requires an increased HDAC2 expression in PL, and decreased expression of HDAC1 in IL.

#### 7.1.4.3. mRNA expression in Hippocampus

Hippocampus subregion CA1, CA3 and DG are involved in regulation of fear memory consolidation and its extinction. In CA1, it has been found that c-fos mRNA expression increased significantly in drug only ($p$<0.05), sham + extinction ($p$<0.001) and drug + extinction group ($p$<0.0001) as compared to the sham control group [F (3,20) = 14.43, $p$<0.0001] (Fig 64). Similarly, the CBP mRNA expression increased significantly in drug only ($p$<0.05), sham + extinction ($p$<0.001) and drug + extinction group ($p$<0.0001) as compared to the sham control group [F (3,20) = 18.08, $p$<0.0001] (Fig 65). Furthermore, the HDAC1 mRNA expression increased significantly in sham + extinction ($p$<0.01) and drug + extinction group ($p$<0.0001) but not in drug only group as compared to the sham control group [F (3,20) = 27.14, $p$<0.0001] (Fig 67). The changes in HDAC1 mRNA expression was significantly higher in drug + extinction ($p$<0.0001) and sham + extinction group ($p$<0.05) as compared to the sham control group. However, HDAC2 mRNA in CA1, decreased significantly in drug only ($p$<0.05), sham + extinction ($p$<0.001) and drug + extinction group ($p$<0.0001) as compared to the sham control group [F (3,20) = 30.84, $p$<0.0001] (Fig 68). The change in HDAC2 mRNA expression was more significant in drug + extinction group ($p$<0.0001) and sham + extinction group ($p$<0.01) than drug only group.

In CA3, the c-fos mRNA expression increased significantly in drug only ($p$<0.05), sham + extinction ($p$<0.01) and drug + extinction group ($p$<0.0001) as compared to the sham control group [F (3,20) = 13.96, $p$<0.0001] (Fig 64). Similarly, the CBP mRNA expression increased significantly in drug only ($p$<0.05), sham + extinction ($p$<0.001) and drug + extinction group ($p$<0.0001) group as compared to the sham control group [F (3,20) = 18.86, $p$<0.0001] (Fig 65). The HDAC1 mRNA expression increased significantly in sham + extinction ($p$<0.01) and drug + extinction group ($p$<0.0001) but not in drug only group as compared to the sham control group [F (3,20) = 15.13, $p$<0.0001] (Fig 67). However, HDAC2 mRNA expression decreased significantly in drug only ($p$<0.05), sham + extinction ($p$<0.01) and drug + extinction group ($p$<0.0001) as compared to the sham control group [F (3,20) = 25.92, $p$<0.0001] (Fig 68). The change in HDAC2 mRNA was significantly lower in drug + extinction group as compared to drug only ($p$<0.0001) and sham + extinction groups ($p$<0.01).

In DG, the c-fos mRNA expression increased significantly in drug only ($p$<0.05), sham + extinction ($p$<0.001) and drug + extinction group ($p$<0.0001) as compared to the sham control group [F (3,20) = 35.13, $p$<0.0001] (Fig 64). The changes in c-fos mRNA were significantly higher in drug + extinction group as compared to the drug only ($p$<0.0001) and sham + extinction groups

($p$<0.001). Similarly, the CBP mRNA expression increased significantly in drug only ($p$<0.05), sham + extinction ($p$<0.001) and drug + extinction group ($p$<0.0001) as compared to the sham control group [F (3,20) = 46.92, $p$<0.0001] (Fig 65). The changes in CBP mRNA were significantly higher in drug + extinction group as compared to the drug only ($p$<0.0001) and sham + extinction groups ($p$<0.0001). The HDAC1 mRNA expression in CA3, increased significantly in sham + extinction ($p$<0.05) and drug + extinction group ($p$<0.001) but not in drug only group as compared to the sham control group [F (3,20) = 15.21, $p$<0.0001] (Fig 67). However, the HDAC2 mRNA expression decreased significantly in drug only ($p$<0.05), sham + extinction ($p$<0.01) and drug + extinction group ($p$<0.0001) as compared to the sham control group [F (3,20) = 25.20, $p$<0.0001] (Fig 68). The change in HDAC2 mRNA was significantly lower in drug + extinction group as compared to the drug only ($p$<0.001) and sham + extinction groups ($p$<0.01). Overall, it is obvious that hippocampus activity was associated with increased c-fos and CBP; however the HDAC2 expression was negatively associated with extinction learning when valproic acid introduced prior to the extinction learning.

**Table 9: Correlation for valproic acid treated Extinction group with the molecular expression profile**

| | Acetyl H3K9 | Acetyl H4K5 | HDAC1 | HDAC2 | c-fos |
|---|---|---|---|---|---|
| LA | - | r = - 0.595 (**)<br>p < 0.01 | - | - | r = - 0.484 (*)<br>p < 0.05 |
| BA | - | - | r = - 0.625 (**)<br>p < 0.01 | - | r = - 0.516 (*)<br>p < 0.05 |
| CeL | - | | r = - 0.575 (**)<br>p < 0.01 | r = 0.496 (*)<br>p < 0.05 | - |
| CeM | r = 0.634 (**)<br>p < 0.01 | r = 0.618 (**)<br>p < 0.01 | r = - 0.492 (*)<br>p < 0.05 | - | r = 0.705 (***)<br>p < 0.001 |
| PL | - | - | - | r = - 0.813 (****)<br>p < 0.0001 | - |
| IL | r = - 0.735 (***)<br>p < 0.001 | r = - 0.509 (*)<br>p < 0.05 | r = 0.742 (***)<br>p < 0.001 | - | r = - 0.743 (***)<br>p < 0.001 |
| CA1 | - | r = - 0.477 (*)<br>p < 0.05 | r = - 0.544 (*)<br>p < 0.05 | r = 0.488 (*)<br>p < 0.05 | r = - 0.477 (*)<br>p < 0.05 |
| CA3 | - | r = - 0.648 (**)<br>p < 0.01 | r = - 0.487 (*)<br>p < 0.05 | r = 0.631 (**)<br>p < 0.01 | r = - 0.708 (***)<br>p < 0.001 |
| DG | - | r = - 0.500 (*)<br>p < 0.05 | r = - 0.658 (**)<br>p < 0.01 | - | r = - 0.476 (*)<br>p < 0.05 |

## 7.2. Discussion

The use of valproic acid prior to extinction training enhanced the extinction learning in weak extinction training. The global HDAC inhibitor valproic acid together with the enhancement of fear extinction increased the histone acetylation in different subregions of the amygdala, PFC, and hippocampus, as observed in some earlier studies (Whittle et al, 2013; Stafford et al, 2012). During extinction, the HDAC inhibitor enhanced the LA, BA and CeL activity and also the histone acetylation. The activation of these nuclei did not affect the CeM activity, which might be due to the involvement extinction circuit, suppressing the CeM activity. The role of the amygdala in extinction is well known in a number of studies which involved various lesion and genetic manipulation studies (Pare and Duvarci, 2012; Davis and Whalen, 2001; Shechner et al, 2014). In

LA, BA and CeL the HDAC1 expression was increased while HDAC2 expression was suppressed by the HDAC inhibitor. In CeM, however, the expression of HDAC1 was enhanced by the HDAC inhibitor. Although HDAC inhibitor did not inhibit the expression of HDAC1, its main target might be HDAC2 as suggested by the previous studies (Guan et al, 2009), showing that the HDAC2 but not HDAC1 is the main target of HDAC inhibitor during extinction.

The IL which is involved in extinction learning (Quirk and Mueller, 2008) exhibited enhanced activity in HDAC inhibitor-treated extinction group; however, there was no effect of HDAC inhibitor on PL activity in extinction group. HDAC inhibitor enhanced the expression of histone acetylation in IL while in PL the HDAC inhibitor enhanced the activity in drug only group but not in extinction groups. Thus, it may be concluded that enhanced HDAC2 expression in PL and decreased HDAC1 expression in IL is required for the extinction learning, and HDAC inhibitor is doing this by acting on HDACs.

The activity of CA1, CA3 and DG was enhanced by the HDAC inhibitor which results in enhanced extinction learning. Furthermore, this activity might be associated with the influence of increased histone acetylation which was enhanced by the valproic acid significantly. Collinear with the activity and histone acetylation the HDAC1 expression was increased in valproic acid treated extinction group. Although the expression of HDAC1 was enhanced in drug-treated extinction group, its expression was unaffected by the HDAC inhibitor in drug control group. Contrary to the HDAC1 expression, the HDAC2 expression was suppressed by the HDAC inhibitor in drug-treated extinction group. HDAC2 expression showed a negative association with the histone acetylation and might be the main regulator of histone acetylation in the hippocampus for enhancing the extinction learning. However, the HDAC1 shows a positive association with the histone acetylation; its function might not be for the control of histone acetylation in those neurons involved in fear learning in the hippocampus following extinction learning.

As HDAC inhibitor is known for its role in enhancing fear memory consolidation and extinction, it is not well known how spatially these inhibitors affect different subtypes of HDACs. Studies have focused on the association of different HDACs in hippocampus-dependent contextual fear memory consolidation and extinction (Itzhak et al, 2013; Kwapis and Wood, 2014). Our result adds to that knowledge by showing that HDAC1 and HDAC2 have a different spatial association with cued fear memory consolidation and its extinction.

To our knowledge, this is the first study that directly assesses the relationship between histone acetylation and its regulation through HDACs. Our results suggest an alternate mechanism of region-specific regulation of Histone Acetylation through the interplay of HDAC1 and HDAC2 and have important implications for the understanding and treatment of cognitive, psychiatric and neurodegenerative disorders related to impaired histone acetylation.

***Chapter 8***

# CONCLUSION

The current study focused on the role of histone acetylation and the association of HDACs with the acetylation during fear memory consolidation and extinction. The amygdala, PFC and hippocampus showed differential activity for these two distinct processes of fear memory. Though the conditioning and extinction are different learning, same brain regions are shown to be involved in the association of both conditioning and extinction.

Amygdala, which comprises of LA, BA, CeL and CeM showed differential activity during conditioning and extinction of fear. The LA, BA, CeL and CeM were active in conditioning while during extinction all the subregions were active except CeM. This might be the result of overlapping circuitries being engaged following fear and extinction learning. Likewise, the histone acetylation was enhanced in LA, BA, CeL and CeM in conditioning while during extinction the LA, BA, and CeL show enhanced histone acetylation. The result suggests that the histone acetylation in these brain parts is associated with the activity of these brain regions during conditioning and extinction of fear memory. The increased histone acetylation might be associated with the regulation of the activity of these brain regions for consolidation of fear and extinction memory. Both the histone deacetylases (HDACs) HDAC1 and HDAC2 exhibited an association with the activity of these brain regions as well as with the histone acetylation for the fear memory consolidation and extinction. In LA, BA and CeL the HDAC2 shows its association with the histone acetylation during conditioning and extinction. A decreased HDAC2 expression was observed with increased histone acetylation in LA, BA and CeL during conditioning and extinction. In CeM, both the HDAC1 and HDAC2 expression decreased during conditioning together with the increased histone acetylation. This suggests the role of both the HDACs in the regulation of histone acetylation in CeM during conditioning. However during extinction, only the HDAC1 exhibited its association with the regulation of histone acetylation as its expression increased in CeM together with the decreased expression of histone acetylation. So, from these result, it may be speculated that HDAC2 is associated mainly with the regulation of the histone acetylation in LA, BA and CeL in conditioning and extinction while HDAC1 together with HDAC2 is associated with the regulation of histone acetylation in CeM. In LA, BA and CeL the enhanced HDAC1 expression during conditioning and extinction might be associated with the suppression of those neurons that inhibit the activity of fear and extinction circuit during conditioning and extinction respectively.

The PFC activity in conditioning and extinction suggests its association with the histone acetylation. The histone acetylation as well as the activity of PL increased in conditioning while it increased in IL during extinction. The HDAC1 expression decreased in PL and IL, following conditioning and extinction respectively. HDAC2 expression decreased in PL and IL both following conditioning and its expression increased in PL following extinction. From this it may be speculated that increased histone acetylation in PL following conditioning and in IL during extinction regulates the activity of PL and IL in conditioning and extinction respectively. Furthermore, it may also be concluded that in PL both the HDACs, HDAC1 and HDAC2 together regulate the expression of histone acetylation in conditioning while HDAC2 alone regulates the histone acetylation in PL following extinction. But on the other hand the HDAC1 in IL might be associated alone with the regulation of histone acetylation during extinction.

The hippocampus CA1, CA3 and DG exhibited enhanced histone acetylation during conditioning and extinction of fear learning. This suggests the role of histone acetylation in the hippocampus for regulation of consolidation and extinction of fear. HDAC1 expression decreased

in the hippocampus following conditioning while HDAC2 expression decreased following extinction. The result suggests the association of HDAC1 in the regulation of histone acetylation during conditioning while HDAC2 exhibits its regulation of histone acetylation during extinction learning. The enhanced HDAC1 expression in hippocampus in extinction might be associated with the inhibition of the fear memory component to enhance extinction learning.

Overall the above mentioned results suggest that HDAC1 and HDAC2 are involved in the regulation of histone acetylation either alone or in combination in different brain regions during fear memory consolidation and extinction. Furthermore, the use of HDAC inhibitor was found to inhibit the expression of HDAC2 but not HDAC1 leading to enhanced conditioning and extinction learning. HDAC inhibitor enhanced the learning for fear memory consolidation and extinction in a weak learning paradigm which mostly targets HDAC2 to enhance histone acetylation. So it may be speculated that targeting specifically HDAC2 might be important to enhance the conditioning and extinction learning. Further research using HDAC subtype specific HDAC inhibitor may promise to clarify the mechanism associated with conditioning and extinction of fear as well as in therapeutic application in the treatment of behavioral disorders like anxiety and trauma.

# Publications

**Siddiqui S. A**., Singh S., Ranjan V., Ugale R., Saha S., Prakash A. (2017) Enhanced Histone Acetylation In The Infralimbic Prefrontal Cortex Is Associated With Fear Extinction. Cellular and Molecular Neurobiology. doi: 10.1007/s10571-017-0464-6.

Ranjan, V., Singh, S., **Siddiqui, S. A.**, Tripathi, S., Khan, M. Y., & Prakash, A. (2017). Differential histone acetylation in sub-regions of bed nucleus of the stria terminalis underlies fear consolidation and extinction. Psychiatry Investigation, 14(3). http://doi.org/10.4306/pi.2017.14.3.350

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