# "Dysregulation of a set of miRNAs by Ethanol leads to Alcoholism"

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**BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY**
**LUCKNOW**

**FOR THE DEGREE OF**
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**IN**
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**2018**

# DECLARATION

I hereby declare that thesis entitled "**Dysregulation of a set of miRNAs by Ethanol leads to Alcoholism"** is my own research work carried out under the Joint supervision of **Prof. M. Y. Khan** and **Dr. Anand Prakash**, Department of Biotechnology, School for Biosciences and Biotechnology, Babasaheb Bhimrao Ambedar University, Lucknow U.P., India The research work is original and no part of this work has been submitted for any other degree or diploma in any other university. All the above given information is true to the best of my knowledge.

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## CERTIFICATE

This is to certify that the thesis titled "**Dysregulation of a set of miRNAs by Ethanol leads to Alcoholism**" submitted by **Ms. Sneha** is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university.

The thesis submitted to Babasaheb Bhimrao Ambedkar University Lucknow satisfies all the requirements as stipulated in the Doctor of Philosophy (Ph.D.) regulations – 1999 as amended in 2008/2010/2013 and it is fit for submission and evaluation for the award of the degree of Doctor of Philosophy of the University.

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## Ph.D. Course Work Certificate

This is to certify that **Ms. Sneha**, Enrollment No. 003/12 Ph.D. Research Scholar, Department of Biotechnology of this University has successfully completed her Ph.D. Course work in the examination held during November, 2012.

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Sneha

Sneha

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## *Abbreviations*

| | |
|---|---|
| ARC | Activity Regulated Cytoskeleton protein |
| ANOVA | Analysis of Variance |
| AUDs | Alcohol Use Disorders |
| °C | Degree Celsius |
| µg | Microgram |
| µl | Microliter |
| BLA | Basolateral Amygdala |
| BAC | Blood Alcohol Concentration |
| bp | Base pair |
| cDNA | Complementry Deoxy Nucleic Acid |
| CeA | Central Amygdala |
| Ct | Threshold Cycle |
| CBP | CREB-Binding Protein |
| CREB | cAMP Receptor Binding Protein |
| ChIP | Chromatin Immunoprecipitation |
| DAB | Di-Amino Benzidine |
| DNA | Deoxyribo Nucleic Acid |
| dNTP | Deoxynucleotide |
| DPX | Dibutyl Phathalate Xylene |
| EPM | Elevated Plus Maze |
| gm | Gram |
| HAT | Histone Acetyl Transferase |
| HDAC | Histone De- Acetyl Transferase |
| i.p. | Intra-peritonial |
| IHC | Immunohistochemsitry |
| LDB | Light Dark Box |
| M | Molar |
| mm | millimeter |
| mRNA | messenger-RNA |
| miR/miRNA | microRNA |
| MeA | Medial Amygdala |
| NAc | Nucleus accumbens |
| NAcS | Nucleus accumbens Shell |
| NacC | Nucleus accumbens Core |
| NHS | Normal Horse Serum |
| NPY | Neuropeptide Y |
| ng | Nanogram |
| n-Saline | Normal Saline |
| PBS | Phosphate Buffer Saline |
| PFA | Paraformaldehyde |
| PFC | Pre-Frontal Cortex |
| PCR | Polymerase Chain Reaction |
| Pri-miRNA | Primary microRNA |
| qRT-PCR | Quantitative Real Time PCR |
| RNA | Ribonucleic Acid |
| sec | Second |

| | |
|---|---|
| S.E.M. | Standard Error of Mean |
| *SD* | *Sprague dawley* rat |
| VTA | Ventral Tegmental Area |
| WHO | World Health Organization |

Alcohol addiction is a chronic relapsing disorder and is characterized by repetitive alcohol drinking patterns leading to a loss of control over alcohol consumption. Alcohol is a psychoactive substance consumed from centuries in society. The harmful use of alcohol depends on the volume of alcohol consumed, quality of alcohol and drinking pattern. According to Global status report on alcohol and health by WHO-2014, alcohol consumption caused about 3.3 million deaths which are 5.9% of all global deaths. Therefore it is important to decipher the molecular mechanisms underlying alcohol addiction. Most of the studies suggest the involvement of mesolimbic reward circuitry in initiating the drug-seeking behavior. These changes are long-lasting and cause changes in synaptic plasticity and neuroadaptation.

Understanding of the cellular and molecular mechanisms underlying the changes occur during addiction is crucial for the development of effective drugs of treatment. The various effects of addiction range from mood disorders, depression, anxiety, memory disorders to many neurological diseases. The aspiration to raise the pleasure or to alter mood motivates preliminary drug use. The drug habituated brain circuitry demands more drug use to achieve the same euphoric effect, which could be attained by a lesser dose of substance or drug. The frequent drug use dominates the 'liking' response of drug, over intense and passionate urge towards drug or 'wanting'. However, the researchers observed that these physiological processes of 'liking' and 'wanting' are mediated by distinctive circuitries, still are associated with each other.

The term 'epigenome' was first defined by Waddington (1942), refers the histone proteins wrapped around the genetic material DNA and control the expression of genes through chemical modifications via different mechanisms. The various epigenetic modifications include histone acetylation, histone methylation, histone phosphorylation, histone sumoylation, ubiquitylation, ADP-ribosylation, DNA-methylation and microRNA mediated changes. Among these, histone acetylation, histone methylation, DNA methylation and modifications through microRNA have been widely studied by researchers. Studies observed a very strong role of these epigenetic changes in the development of an organism, its traits and also development of various diseases including alcohol addiction and disorders associated with alcoholism.

The role of histone acetylation in the neurological disorders has been extensively studied. Histone acetyltransferases (HATs) and histone de-acetyltransferases (HDACs) are the two type of key enzymes, which dynamically regulate the remodeling of chromatin structure and gene expression. A mechanism which involves cyclic-AMP responsive element binding (CREB) protein, get activated by phosphorylation and this phosphorylated-CREB (pCREB) recruits co-factor CBP (CREB binding protein). The intrinsic HAT activity of CBP facilitates the relaxation of chromatin through the transfer of acetyl groups to histone protein and results in increased gene expression. The studies related to CREB, CBP and its targeted genes, such as *Arc* (activity regulated cytoskeleton protein), *Npy* (neuropeptideY), *Bdnf* (brain derived neurotrophic factor) and *c-fos*, identified these as crucial molecules in many neurological processes, diseases and particularly in the development of alcoholism. Several studied have implicated the significant role of *Creb* and its target genes role in limbic reward circuitry, mainly in nucleus accumbens (shell and core) and in amygdala subregions (basolateral, central and medial amygdala) in the regulation of ethanol induced anxiety behavior.

In extension, the recent findings evidenced the role of small molecules (19–24 nt) of microRNAs in neurodegeneration, neurotoxicity, synaptogenesis, synaptic plasticity and many other important neurological processes which suggest that they could be involved in regulating structural and functional aspects of long term potentiation during synapse formation, memory and addiction. MicroRNAs have also been implicated in conciliating the effects of many drugs of abuse viz. cocaine, nicotine, heroine, alcohol and several other classes of drugs. The addictive drugs can manipulate a number of genes only by targeting single miRNA which in turn modulate addiction related neuronal mechanisms. These small molecules regulate gene expression at thepost-transcriptional level by altering the translation of their target mRNAs. The exposure of ethanol on the brain regions (specifically involved in the reward feeling) influence the expression of miRNAs and interfere with the normal function of brain. Several miRNAs, for example, miR-9, miR-34a, miR-132, miR-124 and many others displayed their complex interactions with epigenetic machinery. These miRNAs alter the gene expression and are also the

influence of these genes expression in a feedback loop mechanism to maintain homeostasis.

The present work was done to find the effect of acute ethanol exposure and chronic ethanol exposure on the expression level of a selected set of miRNAs, which also can target transcription factor *Creb* and immediate early genes viz. *Arc*, *Npy*, and *c-fos* along with the *Cbp* genes. The changes during the ethanol withdrawal after the chronic exposure of ethanol was also assessed to better understand the underlying mechanism related to withdrawal symptoms. The differential effect of ethanol on expression of miR-9, -124, -132, -181a and -212, were studied in nucleus accumbens shell and core, basolateral amygdala (BLA), central amygdala (CeA) and in medial amygdala (MeA), along with expression of the target genes involved in alcohol dependence. The changes found in the expression of miRNAs and target genes mRNA and protein expression during different ethanol treatment conditions will shed some light on the role of microRNA.

The outcome of the study will help to relate the molecular changes in the behavioral responses. Though the pathways involved in the addiction circuitry, are also associated with the memory formation, the finding of the project will help to develop newer drug targets for rehabilitation and also shed some light on the molecular events and the pathways involved in memory formation and addiction.

## 2.1. Addiction

The word “addiction” is derived from a Latin term for “enslaved by” or “bound to.” A powerful influence of addiction by hijacking the brain regions had three distinct hallmarks: uncontrolled use of substance or behavior, craving and continual use regardless of negative consequences. Practice of certain drugs like nicotine, ethanol, cocaine, heroin, marijuana and also involvement in gambling, shopping, video gaming, eating, cell phones, internet and sex are some of the pleasurable activities, individual get tangled and become an addict and also challenge the survival in its absence. Recent researches found the repetitive use of substances or behavioral activities opt the neuronal processes and exert a potent effect on the brain processes. The occupation of addictive drugs or activity influences the natural ability of brain to adapt and survive. The rewiring of brain guided by the addictive drugs or pleasurable activity leads to change in brain activity and establish a strong association with drug or activity related stimuli/surroundings.

### 2.1.1. Reward

Some physiologists consider reward as a positive reinforcer because of its property to achieve the behavioral outcome, goal of action and finally pleasure. The evolutionary development helps to identify the objects, events or situations which best suit the organism. The neurological processes of the brain, guide to identify the objects for survival and reproduction, through the value of reward (Berthoud and Morrison, 2008; Singh et al., 2010). The acquisition of reward related subject directs through the learning, decision-making and emotions and caused by the sensory perception and regulated movements mediated by neurological processes (Balleine and Dickinsion, 1998). The prime role of the brain favors the evolutionary fitness in animals including humans (Dawkins R, 1976; Bentham J, 2000; Schroeder T, 2004).

Researchers categorized the reward as primary and secondary rewards. Primary rewards are considered to be innate and related to the survival and reproduction to maintain the homeostatic equilibriums (Hull CL, 1943). Most of the primary rewards are also associated with the learning like food. Conversely, the secondary reward also known as nonprimary rewards are typically extrinsic and influence the

elementary rewards like survival, reproduction and evolutionary fitness selection. Nonprimary rewards are not homeostatic and can be material, non-material or social. Materials influencing reward can be money, expensive cars, jewelry or food. The non-material and non-physical rewards are gambling, gaming, exercise, music or attractiveness towards novelty. In addition to the secondary rewards, the social rewards comprise of societal activities, interactions, friendship, attention which also have no homeostasis basis and also don't add nutrient values or direct benefit for reproduction. Nonetheless, these non-primary rewards do not directly affect the homeostasis, nutrient value and reproduction, but enhance the functions of primary rewards by increasing the evolutionary fitness, indirectly.

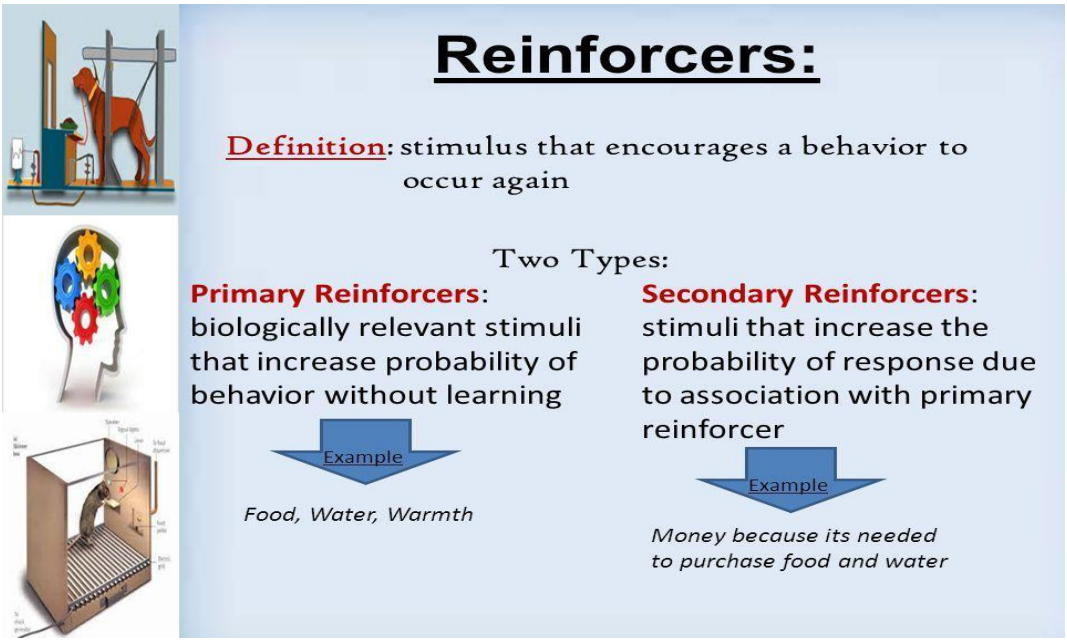



**Figure 2.1: Types of Reinforcers**

The pleasurable activities for their own sake, without being the means for getting extrinsic rewards are referred as the intrinsic rewards. These intrinsic and extrinsic rewards collectively motivate the animal for a particular behavior and pleasurable activity (Barto et al., 2004; Singh et al., 2010). Psychologists suggest that the decision we take, may generate our own rewards like reading, traveling, winning position and power over people, own beauty and looks, and many other behaviors.

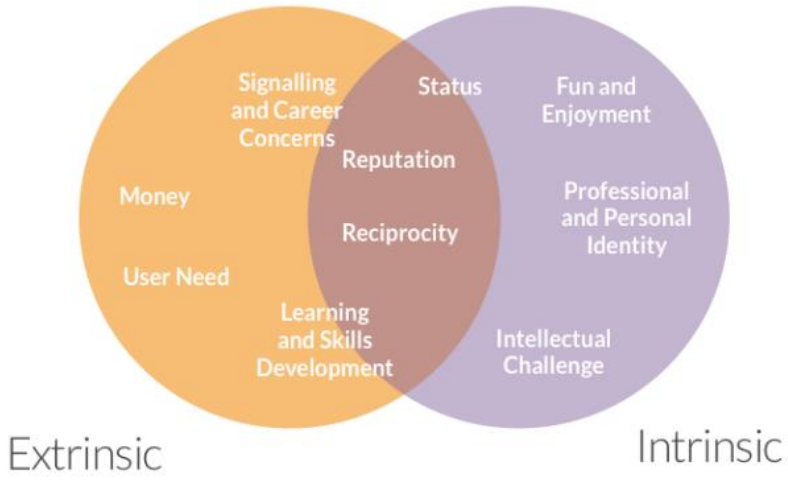



**Figure 2.2: Traits of extrinsic and intrinsic rewards.**

### 2.1.2. Drugs of abuse

Drug abuse or substance abuse is characterized by the compulsive and continuous use of substance despite negative consequences. These negative effects include physical, social, psychological and economic harm. The harmful effects of drug abuse not only affect the person with addiction, but also suffer the family members (American Psychiatric Association, 2013). The abuse of drugs causes the anti-social behavior and causes long term personality changes and sometimes criminal acts too (Stone et al., 2012). Commonly abused drugs include alcohol, cannabis, barbiturates, benzodiazepines, cocaine, methaqualone, opioids and some substituted amphetamines (Everitt and Robbins, 2005; Baler and Volkow, 2006; Volkow et al., 2016). The genetic predisposition of addiction and addiction through the learning or habit is still a debatable topic for researchers and psychologists. The compulsive and continuous use of the substance leads to the tolerance to the drug effect and causes withdrawal related symptoms when the dose is reduced or stopped (Koob GF and Le Moal M, 1997; Volkow et al., 2016). These mood altering substances influence person's thinking and decision making capability, including health risks. Multiple scientific evidences proved the negative effects of drug abuse during the pregnancy potentially harm an unborn baby. Scientists reported that the effect of addiction depends on a variety of factors: age, sex, dose, duration of consumption of drug, drug abuse in combination and also

mode of administration (Tschann et al., 1994; Fergusson et al., 1997; Wills et al., 2001; Hyman et al., 2005; Fishbein et al., 2006).

Alcohol is one of the most commonly abused drugs used worldwide and has strong effect on the people. From ancient time, people used alcohol in societal interactions and meetings, in celebrations and to relax. The effects of alcohol vary from person to person and depend on the variety of factors such as the quality and quantity of drink, age, health status and also family history (Koob GF and Le Moal M, 1997).

Cocaine, an addictive stimulant is also abused by the users. At initial the use of cocaine causes euphoria, alertness and anxiolytic response, but the long term use of cocaine affects appetite, infection in bowel tissue, insomnia, headache, blood pressure and increase risk of infectious diseases. Withdrawal from cocaine after a long term use causes depression, tiredness, vivid unpleasant dreams, slowed thinking and movement, restlessness and insomnia. So far, there is no medication approved to cure the cocaine addiction completely (Mello NK and Mendelson JH, 1997; Pierce RC and Kalivas PW, 1997; Mendelson et al., 1998).

Another drug, morphine, which is extracted from seed pods of the Asian opium poppy plant. Morphine is an opioid drug and includes heroin. Heroin can be injected, smoked or snorted by its users and causes pleasurable feelings. The long term use of heroin affects liver, kidney, and valves of the heart, along with increased risk of HIV, hepatitis and other infectious diseases due to the shared syringe needles. The withdrawal symptoms include insomnia, restlessness, muscle and bone pain and vomiting after the cessation of drug (Wikler A, 1948; Rossetti et al, 1992; Marinelli M, 1994; Kreek MJ, 1997; Marinelli et al., 1998)

These days the use of inhalants as a mood altering substance has been remarkably increased due to the ease in availability and less cost. Inhalation of spray paints, markers, fuels and many household products causes the short term relaxation and reward because of solvents (eg. amyl nitrite), aerosols (eg. butanol, propanol) and gases, but the continuous use of inhalants cause problems with thinking, movement, dizziness, drowsiness, suffocation, convulsions or seizures, coma and even sudden sniffing causes death due to heart failure. The effective medication or treatment for the inhalant addiction is not yet available (Embleton et al., 2013; van Amsterdam et al., 2015; Nguyen et al., 2016)

Another drug LSD (scientific name lysergic acid diethylamide) is a hallucinogen and manufactured from lysergic acid is a potent drug of abuse. This drug has a powerful effect on brain and causes Hallucinogen Persisting Perception Disorder (HPPD) characterized by frightening flashbacks and paranomia. Marijuana or cannabis contains a psychoactive chemical delta-9-tetrahydrocannabinol, or THC made from the hemp plant, Cannabis sativa. Smoking of marijuana causes boosted sensory awareness and euphoria followed by drowsiness and problems with learning and memory. A drug of abuse MDMA (3,4-methylenedioxy-methamphetamine) generally known as ecstasy has similarities with amphetamine and hallucinogen mescaline and causes long term negative effects such as long-lasting confusion, depression, alertness, memory, increased anxiety, impulsiveness, aggression and organ damage and heart failure (Kouri et al., 1999; Siqueira et al., 2001; Lee et al., 2007; Windle M and Wiesner M, 2010)

The abuse of steroids in young population is tempting to achieve the physical appearance in less time and also for enhancing the athletic and sexual performance. These anabolic steroids are easily available in the commercial stores in the form of tablets, capsules, cream and injectable solutions. An unregulated and misuse of drug effects include serious consequences such as kidney damage or failure, liver damage, changes in cholesterol level, aggression, lowered sperm count, infertility, and increased risk for prostate cancer. The shared syringe needle also increases the risk of HIV, hepatitis and other infectious diseases (Maharaj et al., 2000; Stergiopoulos et al., 2008; Omar et al., 2017; Zarghami A and Nazari P, 2017).

Tobacco is one of the most popular addictive drugs, and can be divided into two parts as smoked (Bidi, cigarette) and smokeless tobacco (betel quid, gutkha). Commercially this is available in multiple forms like cigarettes, cigars, bidis, hookahs and as smokeless tobacco such as loose dry oral snuff or moist snuff packed in small pouches. The use of tobacco increases the risk of oral cancer in an independent manner. Tobacco consumption is recognized as a behavioral risk factor in 75–95% cases of oral cancer in India (Varshitha A, 2015). The risk of oral cancer is 20 times higher in tobacco abusers compared to the non-users. Nicotine cause some other health related issues are lung cancer, chronic bronchitis, emphysema, heart disease, leukemia, cataracts and pneumonia (Wilkins et al., 1982; Cinciripini et al., 1989).

## 2.2. Alcoholism

Alcoholism, also known as alcohol use disorder (AUD) occurs due to the consumption of alcohol in any form of beverage or drink that results in mental and physical health issues. In last few decades, the global alcohol consumption has increased, mainly in the developing countries. The first sip of alcoholic drink immediately starts increasing the blood alcohol concentration (BAC) and the BAC level corresponds with the amount of consumed alcohol, which results in more impaired control in decision making, memory and alertness and in severe conditions risky, violent behavior and sometimes suicidal. The widespread effects of alcohol use on brain, heart, liver, pancreas and immune system have been widely studied by many researchers. The long term use of alcohol results in mental illness, Wernicke–Korsakoff syndrome, irregular heartbeat, liver cirrhosis, and an increase in the risk of cancer. Many studies revealed the significant role of environmental factors and genetics associated with the development of alcohol addiction (NCETA Consortium, 2004).

### 2.2.1. Effects of Alcoholism

Alcohol is a lipid-soluble substance and can directly damage brain and affect the central nervous system by penetrating blood brain barrier (Rubio-Araiz et al., 2017). Excessive alcohol drinking can seriously harm health and damage to liver, kidney, heart, brain and central nervous system (Koob GF, 2003; Manta et al., 2016; Lidal et al, 2013; Tawa et al., 2016; Rehm et al., 2016; Hagström et al., 2017). Liver is the organ which is particularly vulnerable, because it metabolizes alcohol and other toxins. Drinking over a long period causes nausea, vomiting, fever, loss of appetite, abdominal pain and jaundice which are symptoms of inflammatory liver and alcoholic hepatitis. Alcoholic hepatitis prominently develops liver cirrhosis in upto 70% patients. Alcohol as reaches the stomach, irritates the internal lining of the stomach and intestines, causing vomiting, nausea and ultimately ulcers (Hagström et al., 2017). The chronic alcohol use also leads to pancreatitis and affect the release of insulin and glucagon hormone which regulated the metabolism. Research indicates that chronic alcohol drinking

increases the risk of cancers of the mouth, throat, larynx and esophagus (Ormel et al., 2017; Gu et al., 2017; Rattray et al., 2018; Gandini et al., 2018).

As discussed earlier that the variable effects of alcohol depend on the age, the effects of alcohol are evident in aged individuals in comparison to younger adults. The absorption of alcohol in females is less in comparison to males, because of the more fat in female body (Tschann et al., 1994; Fergusson et al., 1997; Wills et al., 2001 Hyman et al., 2005; Fishbein et al., 2006). The use of alcohol when combined with certain medications, such as painkillers, tranquilizers and antihistamines can be fatal (Altamura et al., 2013; Pujol et al., 2018). The various brain areas get affected by the alcohol are: Cerebral cortex, controls thought process and consciousness; Limbic system, controls emotions and memory (Justinova et al., 2009; Meruelo et al., 2017, Craske et al., 2017); Cerebellum, coordinates the movement of muscles (Harrison et al., 2017; Moreno-Rius et al., 2017); Hypothalamus and Pituitary Gland, control and influence sexual behavior and urination through secretions of sex, thyroid and growth hormones (Pavlov et al., 2017; Golden A, 2017; Zhu et al., 2017; Faehrmann et al., 2017); and Medulla or Brainstem, which directly or indirectly control the breathing, heart rate, temperature and consciousness (Winklewski et al., 2017; Lippert et al., 2018)

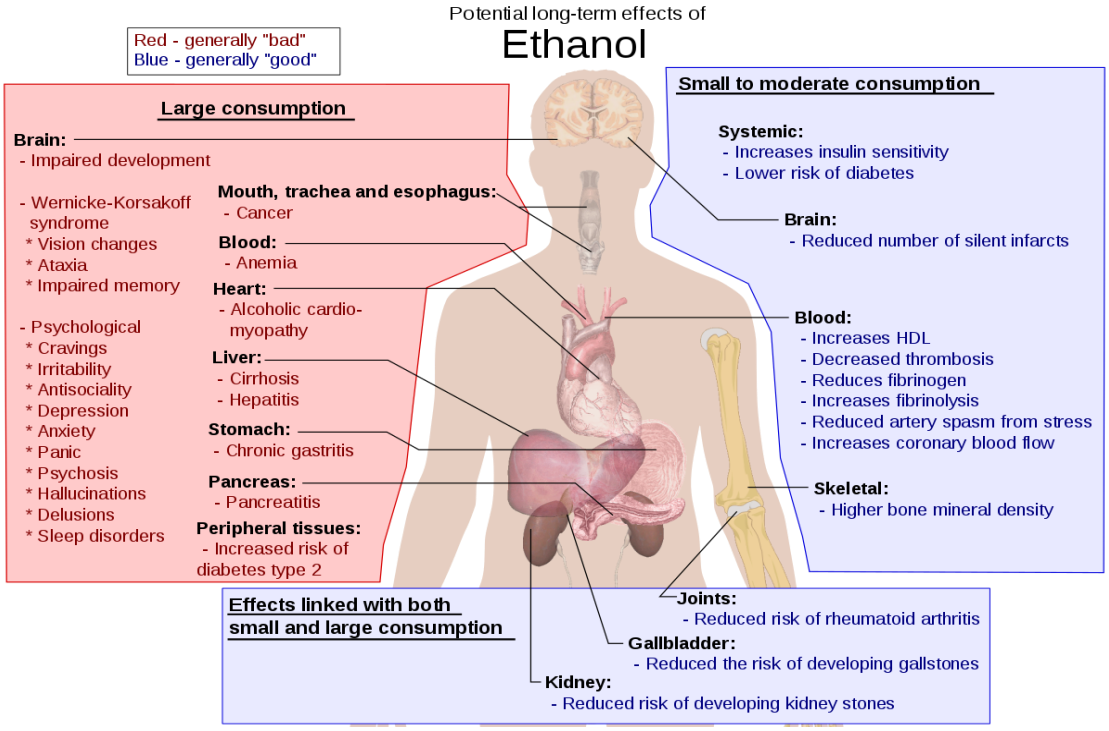



**Figure 2.3: Long term effects of ethanol consumption**

Neurotransmitters, the chemical messengers present in the synaptic terminals of the neurons, transmit the signals through electrical activity. These signals may enhance the brain activity by excitatory neurotransmitters or reduce brain activity by inhibitory neurotransmitters. Alcohol escalates the effects of the inhibitory neurotransmitter GABA in the brain, leads to sluggish movements and slurred speech. At the same time, another excitatory neurotransmitter glutamate inhibits by the alcohol results in diminished physiological activities. In addition, alcohol increases the amount of dopamine in reward circuitry, responsible for the feeling of pleasure when a person takes a drink (Valenzuela CF, 1997).

**Fetal Alcohol Syndrome:** Exposure to alcohol during pregnancy can seriously harm the developing fetus by damaging embryonic cell that will ultimately form the brain structures such as basal ganglia (responsible for spatial memory and other cognitive functions), the cerebellum (involved in balance and coordination) and the corpus callosum (assistances communication between the right and left hemispheres of the brain). Exposure of alcohol at any stage of pregnancy affects the developing baby in learning, memory and attention at the later stage of life (Vorgias D and Bernstein B, 2017; Lange et al., 2017).

### 2.2.2. Reward circuitry

The Brain Reward System is a specific limbic circuit that generates the feelings of pleasure. This coordination originates in a group of neurons that are located in the mid brain (called the ventral tegmental area, or VTA). These neurons then connect to a variety of places within the limbic system, but the important connection is to the nucleus accumbens in the basal ganglia. The basal ganglia are a large, complex set of structures within the limbic system that functions in generating movements, cognitive functions and emotional as well as motivational activities. When a drug activates the VTA neurons, these neurons release dopamine into the nucleus accumbens and the person feels pleasure (Koob GF, 1998; Rossi MA and Stuber GD, 2018).

The Limbic System is a heterogeneous array of brain structures of the cerebral hemisphere, in a particular the hippocampus, amygdala, and fornicate gyrus. The limbic system is responsible for creating feelings and motivation. These feelings

supply the contexts for sensory and motor activities and can alter the perception towards the world and behave in it. This portion of the brain physically connects the survival oriented brain stem with the cognitively oriented cortex. All drugs that people abuse change the way, limbic system works. Drugs disrupt the vigilant modulation of feelings and motivations that underlie normal behavior. When these feelings lose touch with reality, the person receives relief, pleasure, contentment, and relaxation take over (Nestler EJ and Malenka RC, 2004; Lee et al., 2015; Baldo BA, 2016; Salamone et al., 2016).

A multifaceted relationship of biological, psychological and socio-environmental factors governs the etiology and pathology of alcohol dependence. Besides hippocampus, amygdala, and fornicate gyrus the other important parts of reward reinforcing limbic system includes ventral tegmental area (VTA), nucleus accumbens (NAc), ventral striatum, bed nucleus of the stria terminalis (BNST) and extended regions of amygdala. Food and water intake, reproduction and survival are the primary physiological functions controlled by the reward system. The crucial role of dopamine in the reward system studied under the effect of psychoactive substances including alcohol, opioids, cocaine and amphetamine. The brain regions participate in the limbic reward circuitry overlap with the learning and memory formation circuitries. It also articulates the fact that the brain centers related to memory and learning help in receiving the rewarding experience in future (Ron D and Barak S, 2016; Nandrino et al., 2017).

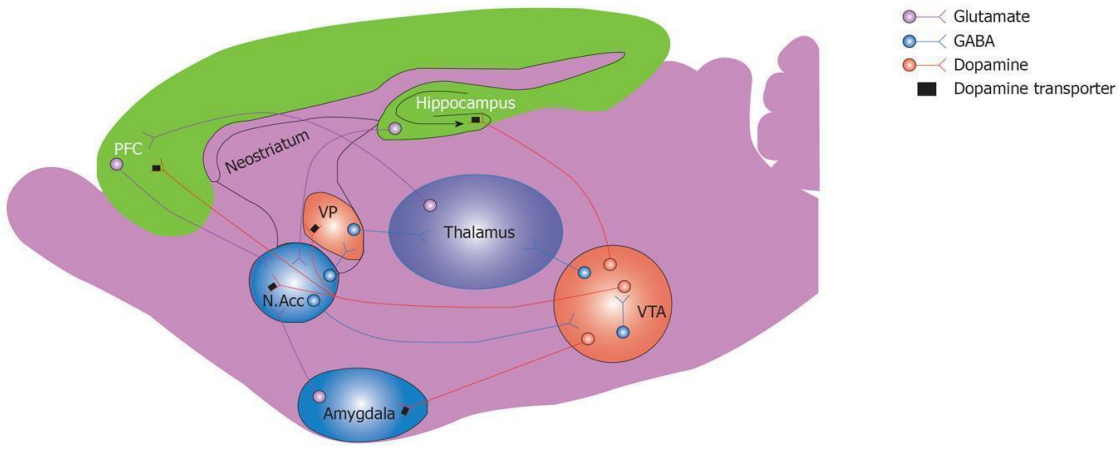



**Figure 2.4: Schematic diagram of Limbic reward circuitry**

The ventral tegmental area (VTA) is the site of dopaminergic neurons, which take part in the differentiation of environmental stimulus whether rewarding or aversive. The NAc, also known as ventral striatum targeted by the dopaminergic neurons of VTA region and mediates the pleasure effect of natural rewards and drugs of abuse (Koob et al., 1998, Koob GF, 2003; Gonzales et al., 2004). Amygdala is particularly considered as emotional center of the brain reward circuitry. It also participates in the conditioned forms of learning which assist the associations of environmental cues and interact with VTA-NAc pathway to distinguish the rewarding and aversive stimuli and in remembering of type of experience (Koob et al., 1998; Pandey, 2004; Gonzales et al., 2004). Hippocampus is the region associated with declarative memory, the memory related to persons, places, or things. Hippocampus and amygdala together establish reward memories related to drug use and crucial for the relapse. Another important region of brain reward circuitry is hypothalamus accompanies the physiological needs of the organism (Dees et al., 2015). The findings of many studies explained the role of prefrontal cortex region in the reward seeking role. The medial prefrontal cortex, anterior cingulate cortex, and orbitofrontal cortex have been widely studied to understand the reward seeking behavior in an addicted patient (Klenowski PM, 2018). Undoubtedly, all these brain regions and many more function in a highly organized and interrelated manner and control the phenotypic response to an array of environmental stimuli.

## 2.3. MicroRNA

### 2.3.1: Overview

MicroRNAs are a class of short ~22 nt long RNA molecules, which act as regulators of gene expression (Ambros, 2004). Since the discovery of miRNAs in 1993, a huge number of evidences indicate the essential role of microRNAs and other non-coding RNAs in various biological processes (Lee et al., 1993). Typically, miRNA target the 3' untranslated region (UTR) of mRNA at the complementary sequence and cause mRNA silencing or degradation (Filipowicz et al., 2008; Liu et al., 2008; Perron and Provost, 2008; Bartel, 2009). Human cells are enriched with miRNAs, where they control at least 60% of all protein coding genes (Molnar et al., 2009). Certainly, evidences indicate that microRNAs have the

capacity to control cellular proliferation, differentiation, and apoptosis critical during physiological functions as well as pathological processes.

Till date more than 500microRNAs have been identified in humans (Saini et al., 2007) and advanced tools and techniques used for further identification of novel microRNAs from different organisms. Study of transcriptome revealed extravagant network of various RNA transcripts encoding non-protein coding RNAs and microRNAs (Willingham and Gingeras, 2006; Birney et al., 2007; Kapranov et al., 2007).

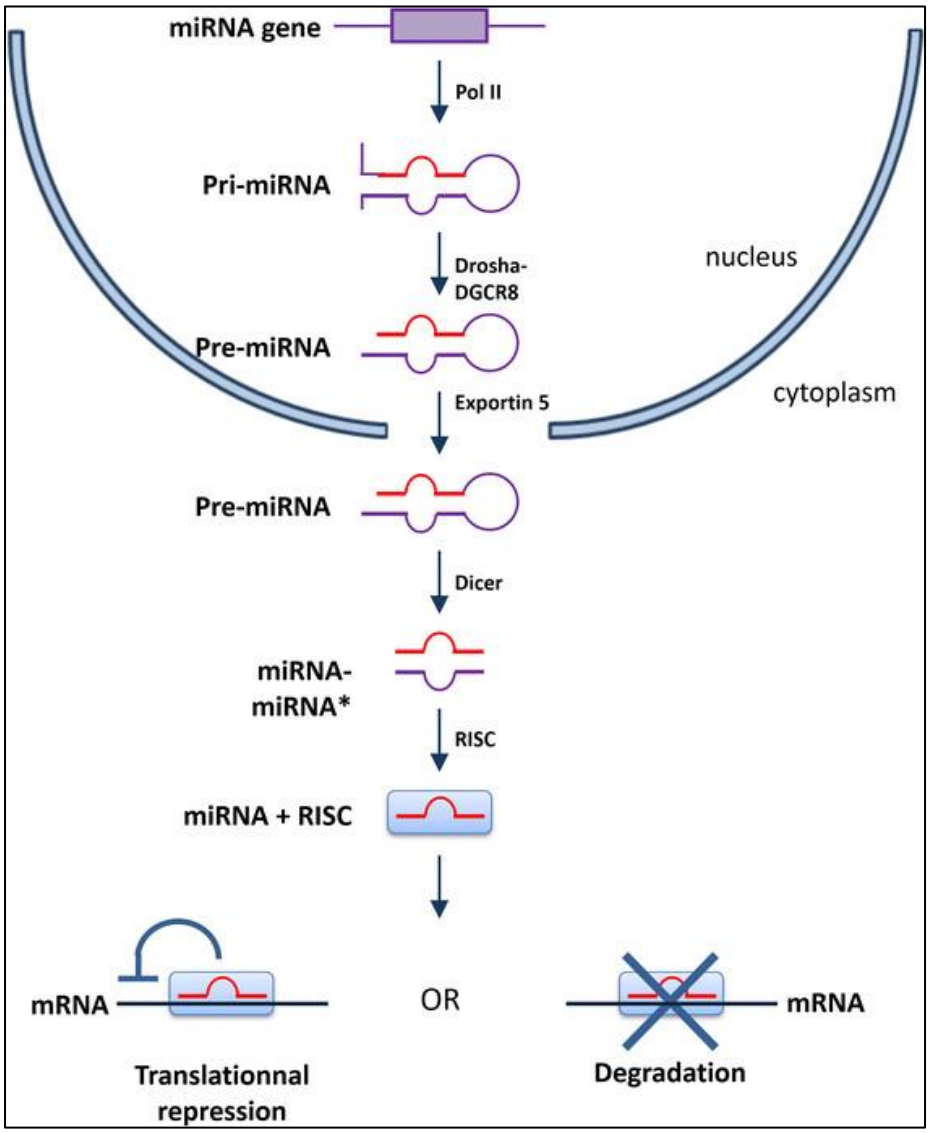



**Figure 2.5: Schematic diagram of microRNA biogenesis**

MircoRNAs can be intergenic or situated within intronic (mitron) regions of protein coding and non-protein coding genes. Intergenic microRNAs use their own

promoters and regulatory units for transcription, on the other hand, within the protein and non-protein coding gene use common promoter of the transcription machinery of host gene (Ason et al., 2006; Lin et al., 2006; Berezikov et al., 2007; Li et al., 2007; Okamura et al., 2007; Saini et al., 2007; Takada et al., 2008). The spicing of first microRNA transcript is similar to the mRNA processing but produce a pri-miRNA, which is a double-standed hairpin-loop structure having hundreds of base-pairs. In nucleus pri-miRNAs cleaved by the Drosha and DGCR8 (DiGeorge syndrome critical region 8) proteins, known as microprocessor complex (Han et al., 2004; Yeom et al., 2006). Slicing of pre-miRNA produce a double stranded structure consist of approximately 70bp, called pre-miRNA, which have a phosphorylated 5'-end, and a 2-nt-long 3' end overhang and recognized by the exportin 5 (nuclear membrane protein) and transports the pre-miRNA into the cytoplasm (Bohnsack et al., 2004; Zeng and Cullen, 2004). Addiction cleavage of pre-miRNA in cytoplasm generates ~22bp long duplex structure containing mature microRNA strand and its complementary or passenger strand (Hutvagner et al., 2001). Once the exportin 5 release the duplex, removal of stem loop structure by TRBP (transactivating response RNA binding protein) and Dicer complex make a double-stranded structure having mature miRNA (Bernstein et al., 2001; Provost et al., 2002; Chendrimada et al., 2005).

In later stage, a RNA-induced silencing complex (RISC) get associated with RNA duplex to form microRNA-induced silencing complex (miRISC) or micro ribonucleoprotein (miRNP) complex, which is a multiprotein structure having Argonaute and many other proteins (Mourelatos et al., 2002; Baumberger and Baulcombe, 2005; Williams, 2008), and target mRNA to induce its silencing or degradation through binding at the 3' UTR of mRNA (Lewis et al., 2003; Bartel, 2004, 2009; Grimson et al., 2007). Interestingly some studies shown that the miRNAs can also cause gene activation by composition of microRNPs, chromatin remodeling or DNA methylation of promoter sites (Li et al., 2006; Vasudevan and Steitz, 2007; Vasudevan et al., 2007, 2008; Place et al., 2008; Steitz and Vasudevan, 2009;). A single miRNA can have the potential to target numerous mRNAs and conversely, one gene can also be regulated by multiple miRNAs (Grimson et al., 2007; Lewohl et al., 2011) suggests, intelligent usage of advanced *in-silico, in-vitro* and *in-vivo* techniques to identify the role of miRNAs in

conjunction or alone during different pathophysiological diseases and disorders related to addiction.

### 2.3.2: microRNA in brain

MicroRNAs are highly abundant in brain and play pivotal role in multiple biological processes like neuronal differentiation (Cheng et al., 2009), brain development (Fiore et al., 2008), synaptogenesis and synaptic plasticity (Schratt et al., 2006, 2009b), and neuro-degeneration (Schaefer et al., 2007; Bushati and Cohen, 2008). Some specific miRNAs known to mediate the cellular variations evoked by exposure to variety of medication of abuse, such as nicotine (Huang and Li, 2008), cocaine (Chandrasekar and Dreyer, 2009), opioids (He et al., 2010), and alcohol (Sathyan et al., 2007; Pietrzykowski et al., 2008; Miranda et al., 2010; Lewohl et al., 2011). They play an important role in the pathophysiology of a several neuropsychiatric disorders and mental retardation syndromes, comprising Alzheimer's disease, Parkinson's disease (Conn et al., 2005),Hunt- ington's disease (Martí et al., 2010), schizophrenia (Beveridge et al., 2010; Santarelli et al., 2011), bipolar disorder (Moreau et al., 2011), alcoholism (Lewohl et al., 2011), Fragile X mental retardation (Li and Jin, 2009), and Rett syndrome (Urdinguio et al., 2010; Wu et al., 2010).

### 2.3.3. Role of miRNAs in alcoholism

A well-known means of post-transcriptional regulation of gene expression is the inhibition of translation through micro RNA (miRNA). MiRNAs can rapidly regulate gene expression by targeting certain mRNAs for degradation or through specific inhibition of mRNA translation. A particularly captivating example of the coordinated manner within which miRNAs alter, comes from an associated analysis of the effects of alcohol on the expression of miR-9, the foremost prevailing miRNA within the nucleus accumbens (Pietrzykowskietal., 2008; Treistman and Martin, 2009; Eipper-Mains et al., 2011). Alcohol has been shown to dysregulate BK channel expression, which may lead to ethanol tolerance. Recent studies on alcohol tolerance involved the significance of miRNAs in alcohol-induced alterations in crucial regions of brain (Atkinson et al., 1991; Mayfieldet

al., 2002; Lewohl et al., 2000; Liu et al., 2004; Iwamoto,et al., 2004; Liu et al., 2004;). Interestingly, miRNA-9 post-transcriptionally regulates BK mRNA splice variants responsible for encoding BK channel isoforms that revealed different ethanol sensitivities (Pietrzykowski et al., 2008; Treistman and Martin, 2009). These findings suggest that the prompt commencement of alcohol tolerance may take place due to post-translational epigenetic modifications involving miRNAs. Recently, ethanol exposure was also found to suppress four miRNAs, miRNA-21, -335, -9, and -153, which may be involved in ethanol related teratogenicity (Sathyan et al., 2007; Miranda et al, 2010).

Chronic ethanol consumption induces neurotoxicity which leads to complications in the expression of various genes in the course of myelination, ubiquitination, apoptosis, cell adhesion, neurogenesis, and neural disease (Liu et al., 2006). In a study, approximately 35 miRNAs were detectable altered in the alcoholics (Lewohl et al., 2011). In humans, bioinformatics approach analysis showed that some specific miRNAs, substantially change the mRNAs or genes associated with the central nervous system development and synapse formation (Pulliero et al., 2011). In a recent study using bioinformatics as well as in-vitro methods found the exclusive changes in 160 mRNAs and 29 rat-miRNAs at prefrontal cortex, 142 mRNAs and 26 rat-miRNAs at hippocampus, and 143 mRNAs and 30 rat-miRNAs at corpus striatum during treatment with ethanol and withdrawal processes and many of these genes have tendency to participate in neuroplasticity and synaptic processes (Sinirlioglu et al., 2017). In alcohol induced cerebral ischemia the expression of NMDA receptor may modulate by miR-219 in different brain tissues (Silva et al., 2017). In addition, using global analysis of expression profiling by microarray technique atleast 20 miRNAs differentially expressed in adult mice which was subjected to prenatal ethanol exposure and ensuing validated the miR-302c elucidate the interface between the pathophysiology of cerebral ischemia concomitant with alcoholism (Mantha et al., 2014). In Drosophila model of alcoholism, 14 known and 13 putative novel miRNAs identified in response to acute exposure of ethanol using next generation sequencing and then to check the utility GeneSwitch Gal4/UAS system was used for a subset of miRNAs which responded during acute ethanol exposure and two microRNAs miR-6 and miR-310 identified as ethanol responsive miRNAs (Ghezzi et al., 2016). The role of

polymorphism in miRNAs gene in the development of alcohol use disorders (AUDs) were studied in sex-matched human volunteers and observed the relation of the allelic variant of mir-146a in the disorders associated with alcohol use (Novo-Veleiro et al., 2014). Isolation of white matter (WM) neighboring left orbitofrontal cortex (OFC) of the human postmortem brain used in a study, found the role of miR-21 alone or in correlation with the some transcription factors in adult oligodendrocytes interrelated to major depressive disorder (MDD) and alcoholism (Miguel-Hidalgo et al., 2017). In medial prefrontal cortex (mPFC) of alcoholic rat brain, miR-206 actively regulates the expression of BDNF (brain derived neurotrophic factor) but not in the other regions of limbic reward circuitry i.e., ventral tegmental area (VTA), amygdala (AMY), or nucleus accumbens (NAc) (Tapocik et al., 2014).

The continuous use of alcohol in human and alcoholic animal models displayed global changes in microRNA expression (Li and van der Vaart, 2011). The differential expression of miR-7 and miR-153 in human alcoholics also found to be correlated with the expression of α-synuclein (Doxakis, 2010). In nervous system diseases such as glioblastoma and neurodegenerative disorders miR-9 plays a crucial role. In glioma cells, miR-9 and CREB minicircuitry regulate the proliferation and migration of glioma cells by a negative feedback mechanism (Tan et al., 2012). In a study, the implication of cocaine mediated response in increased miR-9 expression in NAc lysate was evaluated (Eipper-Mains et al., 2011). MiR-124 has crucial role in neurogenesis, such as, promotion of neuronal differentiation via Ephrin-B1 (Arvanitis et al., 2010), BAF53a (Yoo et al., 2009), SOX9 (Cheng et al., 2009), SCP1 (Visvanathan et al., 2007) and PTBP1 (Makeyev et al., 2007); inhibit differentiation via NEUROD1 (Liu et al., 2011) and also inhibit synaptic activity through CREB1 (Rajasethupathy et al., 2009). In a study the expression of miR-124 was found decreased after 3 days of ethanol withdrawal after a chronic ethanol exposure in limbic forebrain regions (Mizuo et al., 2012). MiR-132 has diagnosed for its effects on neuronal maturation through dendritic arborization and spinogenesis in context to learning and memory (Obrietan et al., 2014). The role of miR-181a related to cocaine-induced conditioned place preference in nucleus accumbens (Chandrasekar et al., 2011) and regulation of atleast four cocaine-suppressed genes in the different regions of the midbrain investigated (Toda et al.,

2002; Yuferov et al., 2003). The substantial role of miRNA-212 in CREB, MeCP2 and BDNF signaling in cocaine mediated behavioral and motivation and expression of miR-212 was analyzed in dorsal striatum of rat brain (Hollander et al., 2010; Im et al., 2010). The advancement in characterization of miRNAs in various biological processes may aid to understand the underlying mechanism of gene regulatory pathways associated with alcoholism and other drugs addiction in critical brain regions of the reward circuitry.

The study of change in gene expression without changing the genetic material DNA by different epigenetic alterations known as 'Epigenetics', coined by Waddington (1942) (Waddington 1942; Holliday 2006; Murrell et al. 2005). Epigenetic modifications include acetylation and methylation of histone proteins, DNA methylation, sumoylation, and microRNA mediated regulation of gene expression (Hsieh and Gage 2005; Abel and Zukin 2008;). Several findings related with epigenetics strongly suggest the changes occur in the epigenome are highly sensitive to the environment cues. Several studies have implicated a role for epigenetic mechanisms, especially chromatin remodeling, in neurodegenerative and psychiatric disorders and in the development of drug addiction (Renthal & Nestler, 2008; Abel & Zukin, 2008; Nestler, 2009). The role of transcription factor CREB on the regulation of ethanol targeted gene has been widely studied; on the other hand the regulation of microRNA expression (itself) by epigenetic modifications on the regulatory region of miRNA can also alter these gene regulators. MiRNAs are highly active in the brain and have been shown to be involved in brain development, synapse formation, memory formation and development of addiction to drugs such as cocaine and ethanol. Still, more information is required to develop a better understanding related to addiction of ethanol and other addictive drugs.

**Hypothesis:**

Chronic exposure to ethanol may lead to dysregulation of a subset of miRNAs, normally resisting the changes in the gene expression on acute exposure of ethanol, result in loss of this homeostatic control through miRNAs and allowing the modulation of circuitries responsible for alcohol addiction. This dysregulation may be resulting from epigenetic changes at miRNA genes, which may be directly or indirectly under the control of CREB and its target genes.

## 3.1. Aims of the thesis

**Specific Aim1: To identify the effect of Ethanol (acute/chronic/withdrawal) on some selected miRNAs and their profiling.**

Experiment no.1:Behavioural studies with Ethanol Exposed Rats

Experiment no.2: Extraction of total miRNA and profiling

**Specific Aim2: To identify the expression levels of CREB and its target genes.**

Experiment no.1: Immunohistochemistry

**Specific Aim3: To identify the acetylation pattern in different regions of extended amygdala.**

Experiment no.1: CHIP Assay and real time PCR

Experiment no.2: Real time PCR.

## 3.2. Plan of Work

**Anxiety measurement:** Measurement of Anxiety-like Behaviours was gauged by Elevated Plus-Maze (EPM) and light dark box (LDB) Test. The rats from each group were observed for exploration to open and closed arms for a 5-min test period in elevated plus maze. The number of entries and time spend to each type of arm (open or closed) recorded. The results expressed as the mean ± SEM of the percent of open-arm entries and the mean percent of time spent on the open arms (open-arm activity). General activity of rats represented by total number of closed arm entries (closed arm activity) as reported by other investigators (Rodgers and

Johnson, 1995). In light dark box test the no of entries and time spent in light compartment and dark compartment was compared along the groups to measure the ethanol related anxiolytic and withdrawal related anxiogenic effect.

**miRNA and expression analysis:** Once the brain tissues from different regions of the extended amygdala collected, the miRNAs isolated from each group. Profiling of miRNAs of the acute and chronically treated rats and ethanol withdrawal rats may give an idea about dysregulation if any, that might be taking place in the homeostatic miRNAs.

**mRNA expression analysis:**mRNA expression of CREB and its target genes was detected bySYBRgreen based Real Time –PCR method using ΔΔct method in acute ethanol, chronic ethanol treated rats and ethanol withdrawal rats will give an idea about parallel dysregulation in these genes expression and miRNA expression.

**Immunohistochemistry:** DAB immunostaing performed with 20µM coronal sections, containing regions of interest in extended amygdala, for CREB and its target genes to identify the expression level.

**CHIP assay and qRT-PCR:** Anti-CREB pulled DNA from the brain tissue (collected for biochemical analysis) will be done by chromatin immunoprecipitation. After chromatin immunoprecipitation, amplification of pulled DNA will give an idea of epigenetic changes taking place globally in specific brain regions during acute or chronic exposure and withdrawal. Results will be compared to a standard curve generated by serial dilutions of input DNA.

Comparison of the amplification pattern of the CREB pulled miRNA promoter expression will give an idea about how transcription factor CREB in the promoter region of the ethanol responsive miRNAs plays a role in their expression pattern.

## 4.1. Animals

Adult male SD (Sprague Dawley) rats (200-250gm) were employed for this study. Rats were individually caged in 12-12 hr light-dark cycle along with *ad libitum* food and water access in 23±2°C temperature controlled conditions for two weeks prior to experiments. All experiments were performed during daytime and animals were randomly selected for experiments. Experiments were executed according to the guidelines of Institutional Animal Ethical Committee and as per the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines (IAEC/UDPS/2014/48), Govt. of India, New Delhi.

## 4.2. Ethanol Exposure

### 4.2.1. Acute Ethanol Exposure

Rats were intraperitoneally injected with ethanol as 5µl/gm of body weight (1 gm/kg dose; ethanol was diluted to 0.2gm/ml in n-saline) or with n-saline (5µl/gm of body-weight). After 1 hour of i.p. injection elevated plus maze (EPM) and light-dark box (LDB) tests were used to measure the anxiety behavior of rat groups. Blood alcohol concentration (BAC) was detected by Ethanol assay kit (Abcam ab65343) as per manufacturer's protocol.

### 4.2.2. Chronic Ethanol Exposure

To develop the chronic ethanol exposed rat model, rats were forced feed for 21 days with a gradual boost of ethanol concentration (4% for initial 3 days, 7% for next 3 days and 9% for 15 days). 80ml nutritionally complete liquid diet having ethanol was given every day between 4-5pm and each day, consumption of diet was measured for each rat. Every fourth day the body weight was also measured. Another group of rats was fed with nutritionally complete liquid diet as control paired-fed rat group. For ethanol withdrawal group, rats were selected randomly from the chronic ethanol-fed group and deprived of ethanol for 24 hours to show a high rise in anxious behavior (Pandey et al., 1999). The anxious behavior of control diet-fed, chronic ethanol-fed and ethanol-withdrawn group rats was determined by elevated plus maze (EPM) and light-dark box (LDB) test for each

rat. Blood alcohol concentration (BAC) was determined by Ethanol assay kit (Abcam ab65343) as per manufacturer's protocol.

## 4.3. Measurement of Anxiety Behavior

### 4.3.1. Elevated Plus maze test (EPM)

EPM was used to determine the level of anxiety on the basis of rodent exploratory behavior. It consists of 2 sets of open and closed arms, placed 50 cm above from the ground and closed arms are surrounded by 10cmx10cm side walls. An open central platform connects these open and closed arms in opposite manner to each other (Figure 4.1A). Rats were placed 5 min prior to the behavioral experiment, for habituation in the test room. After habituation each rat was placed carefully on the central platform facing open arm of the maze and the number of entries and time spent in open and closed arms were recorded for 5 min duration to represent anxious behavior as mean $\pm$ S.E.M. of the percent of open-arm entries and percent time spent in open arm (Pellow S et al., 1985).

### 4.3.2. Light Dark Box Test (LDB)

The light-dark box consisted of two compartments (20cm x 40cm x 35cm) connected with an opening. The dark compartment was without illumination, but the light compartment was with a LED light source for illumination (Figure 4.1.B). Rats were habituated for 5 min in test room prior to the experiment. Then each rat was placed gently in the light compartment. In 5 min observation period, time spent and no. of entries in either compartment dark or light was measured and represented as mean $\pm$ S.E.M. as anxiety behavior parameter (Pandey et al., ).

## 4.4. Brain Tissue Collection for IHC

Rats were anesthetized with pentobarbital (60 mg/kg, i.p.) and transcardially perfused with n-saline (0.9% saline), followed by chilled 4% paraformaldehyde (PFA) [in 0.1M phosphate buffer (pH 7.4)] for tissue fixation. Rats were decapitated and brains collected in 4% PFA for post-fixation. Next, brains were incubated in 10%, 20%, and 30% sucrose solution followed by freezing in iso-pentane at -30°C and stored at -80°C for cryosectioning.

### 4.5. Selection of miRNA candidates

The selection of miRNA candidates (in 2013) was based on the literature available that described important role of miRNA in brain, particularly in addiction and have target site for either transcription factor CREB or its target genes. Many reports explained the role of these miRNAs in neurotoxicity, synaptogenesis, dendritic formation and neurodegeneration, which are an integral part of the development of drug dependence, on the basis of profiling and detection of neural tissue samples. The importance of CREB and its target genes along with miRNA promoter studies were also reviewed in the available literature. MicroRNA target prediction tools (Target scan and PicTar) were also practiced to find the potential targets. Based on this information, a set of miRNAs (Table 4.1) was elected for study in acute and chronic ethanol exposure group and 24 hr ethanol-withdrawal group.

### 4.6. Reverse Transcription-PCR

Tissue samples of desired brain regions were punched from brain slices of 100µm thickness and stored in RNA*later* till RNA isolation, at -80°C. Total RNA was isolated using mir*Vana* isolation kit as per manufacturer's protocol. 500 ng of total RNA was reverse transcribed using genetix first strand cDNA synthesis kit (cat no.#K1612) according to manufacturer's instructions. PCR was done according to standard thermal cycler conditions for reverse transcription.

### 4.7. Quantitative Real Time-PCR

For miRNA expression analysis Taqman assays used (Table 4.1) and 100 ng RNA/sample was reverse transcribed using TaqMan® MicroRNA Reverse Transcription Kit (cat no. #4366596) as per instructions and samples diluted 1:5 with nuclease free water for qRT-PCR reactions. Samples used in triplicates in 20ul reaction mixture using master mix (TaqMan® Universal PCR master mix II, cat no. # 4440040) and inventoried microRNA assays and run in Real time-PCR machine (Stratagene Mx3000P). For mRNA analysis, reverse transcribed RNA product i.e. cDNA was used in triplicates in 20ul reaction using *SYBR*green and gene specific primers (Table 4.2). The fold change expression was calculated by ΔΔct method. For each result mean of the three biological replicates considered.

### 4.8. Immunohistochemistry

For immunohistochemistry, 20μm free floating coronal sections were collected by cryosectioning (Microm HM 525) containing nucleus accumbens (shell and core) and amygdala subregions (BLA, CeA, MeA) in 0.01M phosphate buffer saline (PBS). Sections were quenched with 3% hydrogen peroxide for 30 min, washed in 0.01M PBS and then incubated in 1% normal horse serum (NHS) prepared in PBS with 0.25% tween-20 for blocking. The brain sections were then incubated for 16-17 hrs at room temperature in anti c-fos (Abcam #Ab7963 at 1:500 dilution), CREB (USBiological #C7915-07A at 1:500 dilution), CBP (USBiological #C2098-03H at 1:500 dilution), NPY (USBiological #N217660 at 1:200 dilution), and ARC (Abcam #Ab23382 at 1:200 dilution) primary antibody. After PBS washing, sections were incubated for 2hr with biotinylated secondary antibody (anti-rabbit IgG, 1:500 dilution, Vectastain Elite ABC kit, Vector Labs, USA) followed by DAB staining. Brain sections were mounted on frosted glass slides and dehydrated through alcohol (30%, 70% and 100% serially) and xylene, and finally permanently coverslipped with DPX mountant (Sigma #44581).

### 4.9. Brain Section Analysis

Image collection (4X and 40X) and manual counting of positive neurons (at 40X) were done by Nis-Basic Research image analysis software (Nikon, Tokyo) attached to a Nikon Eclipse Ni microscope (Nikon, Tokyo, Japan) by an observer blind and unaware of the experimental setup and conditions. Coronal serial sections of nucleus accumbens (shell and core) and amygdala (BLA, CeA and MeA) were collected from similar bregma, +0.96 mm to +1.32 mm and -2.40 mm to -2.76 mm, respectively, according to the rat brain atlas (Paxinos and Watson 2007) (Figure 4.2 A and B).

### 4.10. CHIP Assay

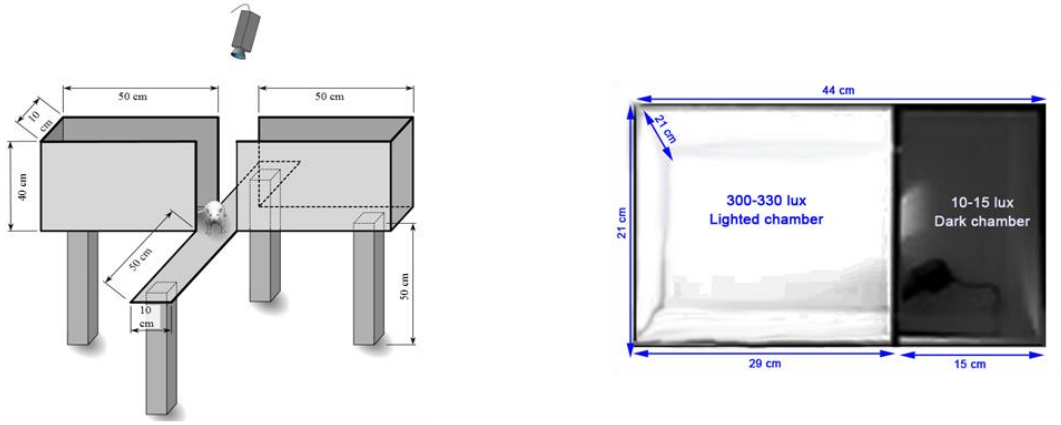
Brain tissue of desired regions was sonicated at high setting for 12-15 cycles with 10sec On/10sec Off in presence of 1ul protease inhibitor. The chromatin immunoprecipitation was performed using ChIP assay kit (USBiological #C5069-95) as per manufacturer's protocol and anti-CREB antibody (Abcam #Ab32515)

was used for crosslinking and DNA was pulled out. DNA was isolated and primers specific for the promoter region of miRNAs (Table 4.3) were used to analyze the change in expression found among different ethanol exposure conditions if any. The possible promoter of miRNA were identified by miRStart database (http://mirstart.mbc.nctu.edu.tw/) and primers designed for the upstream (upto5kb) region of Transcription start site (TSS) by primer designing tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/)

### 4.11. Significance level Analysis

We have utilized two different criteria's for the interpretation of microRNA and mRNA expression in this series which include statistical analysis and threshold fold change must be more than 1.20 (>1.20).

All the statistical analysis was done using *GraphPadprism* software. The changes between two groups were analyzed by unpaired student's t-test and changes between more than two groups were analyzed by one-way ANOVA test followed by Tukey's posthoc test (Pandey et al. 2008). The level of statistical significance represented as: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.




**(A)** **(B)**

**Figure 4.1**: Anxiety measurement paradigms. **(A)** Elevated plus maze (EPM) and **(B)** Light dark box (LDB)

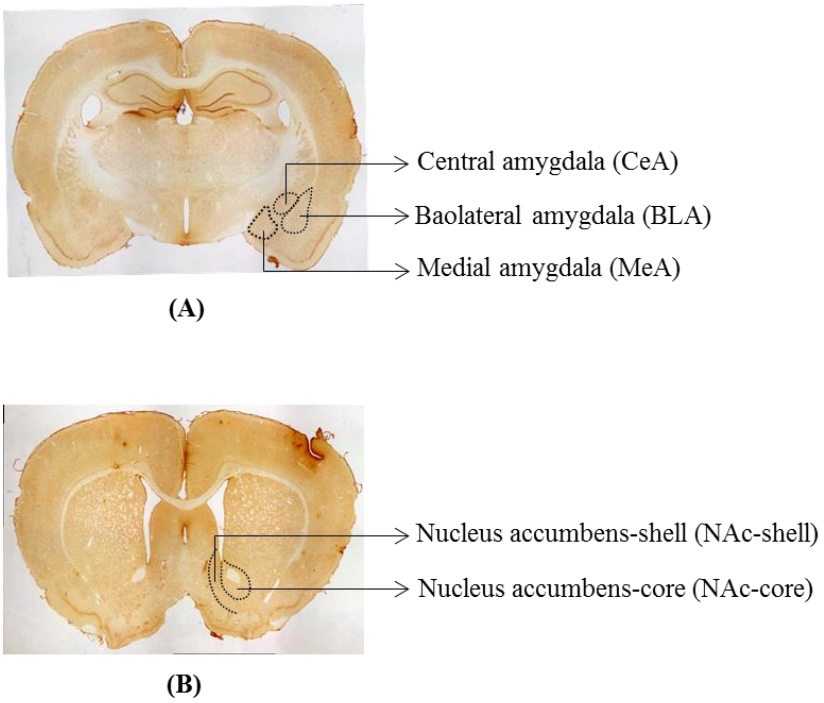



**Figure 4.2:**Representation of the Bregma points of **(A)** Amygdala (+0.96 to +1.32 mm) and **(B)** Nucleus accumbens (-2.40 mm to -2.76 mm) sections employed in the study.

| MicroRNA | miRBase Accession No. | Taqman Assay ID | Predicted Target | References | References |
|---|---|---|---|---|---|
| **rno-miR-9** | MIMAT 0000781 | 000583 | CREB | 3' AGUAUGUCGAUCUAUUGGUUUCU 5' rno-miR-9<br>5' AAAGAAGUGUUGAUUGCCAAAUU3' Creb | Tan et al., 2012 |
| **rno-miR-124a** | MIMAT 0000828 | 001182 | CREB | 3' ccGUAAGUGGCGCACGGAAu5' rno-miR-124<br>138:5' uuCAUUCAUU-UGUGCUUUu3' Creb | Chandrasekar and Dreyer, 2009 |
| | | | CBP | 3' ccguaaguggcgcACGGAAu 5' rno-miR-124<br>463:5' ugcuggugacucaUGCCUUu 3' Crebbp | - |
| | | | ARC | 3' ccguaagUGGCGCACGGAAu 5' rno-miR-124<br>1162:5' ccaggagACCCCAUGCCUUg 3' Arc | - |
| **rno-miR-132** | MIMAT 0000838 | 000457 | ARC | 3' gcUGGUACCGA-CAUCUGACAAu 5' rno-miR-132<br>1174:5' auGCCUUGGCUCCUAGGCUGUUc 3' Arc | - |
| **rno-miR-181a** | MIMAT 0000858 | 000480 | c-fos | 3' ugAGUGGCUGUCGCA-ACUUACAa 5' rno-miR-181a<br>608:5' caUUGUUGAGGUGGUCUGAAUGUu 3' Fos | - |
| | | | CBP | 3' ugaguggcugucgcaaCUUACAa 5' rno-miR-181a<br>1253:5' uuuaagaucccuguaaGAAUGUu 3' Crebbp | - |
| **rno-miR-212** | MIMAT 0000883 | 002551 | ARC | 3' acCGGCACUGA-CCUCUGACAAu 5' rno-miR-212<br>1174:5' auGCCUUGGCUCCUAGGCUGUUc 3' Arc | - |

**Table 4.1:** Details of selected set of miroRNAs

| Gene | | Primer sequence (5'to 3') | Reference |
|---|---|---|---|
| Creb | Forward | TCAGCCGGGTACTACCATTC | Einoch et. al. 2017 |
| | Reverse | TCTCTTGCTGCTTCCCT | |
| Npy | Forward | TAGGTAACAAACGAATGGGG | Pandey et. al. 2012 |
| | Reverse | AGGATGAGATGAGATGTGGG | |
| c-fos | Forward | ACCTCAAGGACTTGAAAGCATC | Alzate et. al. 2012 |
| | Reverse | ACATCTCCGGAAGAGGTGAG | |
| Arc | Forward | GCTGAAGCAGCAGACCTGA | Alzate et. al. 2012 |
| | Reverse | TTCACTGGTATGAATCACTGCTG | |
| Cbp | Forward | ATGATCTTCCTGATGAGCTG | Feng et. al. 2009 |
| | Reverse | AGCCCCACTTGCTTTTGT | |
| β-actin | Forward | GATCAAGATCATTGCTCCTCCTG | Magierowska et.al. 2015 |
| | Reverse | AGGGTGTAAAACGCAGCTCA | |

**Table 4.2:** Detail of primers used in the mRNA expression study

| microRNA | | Primers for Promoter region(5'to 3') | Reference |
|---|---|---|---|
| miR-9 | Forward | CCCCCAGCAATTTTCACATC | Hwang et al. 2014 |
| | Reverse | GGAGCCGGTTTTGTGCAA | |
| miR-124 | Forward | CCTCCCCTTTGCAGGAAAAA | |
| | Reverse | CCTCCGTAGGCTCTTTGTTCTC | |
| miR-132 | Forward | CACCTCCAGAGCAGGCAAA | |
| | Reverse | GGAGGCTGTGGCTCTATAAGGA | |
| miR-181a | Forward | GTCTGGACAAAACGCCAGTG | ------ |
| | Reverse | AATGATGAGTGCCCTGACGG | |
| miR-212 | Forward | TAGAAGAGCCAAGACACGGCTA | ------ |
| | Reverse | GAGAGAGCAGAAGGCTGTCA | |
| GAPDH | Forward | CTCGTCTCATAGACAA GATGGT | Liu et al., 2015 |
| | Reverse | GGGTAGAGTCATACTGGA ACATG | |

**Table 4.3:**Detail of microRNA promoter specific primers used in the CHIP-qPCR study

## 5.1. Anxiety Measurement

### 5.1.1. Anxiety measurement of acute ethanol group

Elevated plus maze test was used to gauge the anxiety behavior in acute ethanol exposed rat group. The percent time spent in open arm was significantly higher ($t=4.26$, $p<0.001$) in acute ethanol group compared to the n-saline group. The % no. of entries in open arm was also found significantly more ($t=4.38$, $p<0.001$) in acute ethanol rats compared to n-saline group. There was no significant difference between total no. of entries ($t=0.98$, $p>0.05$) in open and closed arm (Figure 5.2A).

Anxiety measurement by light-dark box test revealed significant difference between acute ethanol and n-saline group in % time spent in light compartment ($t=4.34$, $p<0.001$) and the no. of entries in light compartment ($t=4.94$, $p<0.0001$) compared to the dark chamber (Figure 5.2B) (n=12 in each group).

We also measured the blood ethanol concentration from serum samples collected immediately after the anxiety measurements. The ethanol concentration in acute ethanol exposed rats found 92.23+2.82mg/dl, however, in n-saline group no blood ethanol traces were found.

### 5.1.2. Anxiety measurement of chronic ethanol group and withdrawal group

During chronic ethanol exposure, the body weight of each rat was measured after every 3rd day and no significant difference was found in the body weight among control diet fed, chronic ethanol fed and withdrawal rat groups [$F_{(2,21)}=0.20$, $p>0.05$; Figure 5.1A]. Ethanol intake was also measured during the chronic ethanol exposure (Figure 5.1B).

After the chronic ethanol exposure, the anxiety measurement by elevated plus maze was done for control, chronic ethanol and ethanol withdrawal group. It was found that the % time spent in open arm was significantly low [$F_{(2,21)}=19.57$, $p<0.0001$] in withdrawal group compared to chronic ethanol-fed rat group and control diet-fed rat group. The % open arm entries also significantly decreased in withdrawal group compared to control [$F_{(2,21)}=19.29$, $p<0.01$; ] and chronic ethanol diet [$F_{(2,21)}=19.29$, $p<0.0001$] group. We also found no significant difference in total no. of entries

[$F_{(2,21)}$=0.40, p>0.05] in both arms among control diet fed rats, chronic ethanol-fed rats and ethanol withdrawal rats (Figure 5.2C).

The light-dark box used to measure the anxiety like behavior among control, chronic ethanol and withdrawal groups. The % time spent in light compartment was significantly low in withdrawal group compared to control-fed [$F_{(2,21)}$=13.30, p<0.05] and chronic ethanol fed group [$F_{(2,21)}$=13.30, p<0.001] and the % entries in light compartment was also significantly lower in withdrawal group compared to control-fed [$F_{(2,21)}$=11.86, p<0.01] and chronic ethanol fed group [$F_{(2,21)}$=11.86, p<0.001](Figure 5.2D) (n=12 in each group).

The blood alcohol concentration level in chronic ethanol exposed group was 182.75+6.18 mg/dl, yet in control diet-fed and ethanol withdrawn rats no ethanol traces were observed.

## 5.2. Analysis of miRNA Expression

The analysis of microRNAs expression in the selected brain regions was done by the Taqman chemistry based assays for the selected set of miRNAs. U6snRNA was used as a housekeeping gene for the normalization of data retrieved. ΔΔCt method was used to calculate the fold change and statistical analysis was also done.

### 5.2.1. MicroRNA expression in Nucleus accumbens

#### 5.2.1.1. Rno-miR-9

During acute ethanol exposure the expression of miR-9 was found to be significantly higher ($p<0.01$) as compared to the n-saline group rats in NAcS, but not in the NAcC region ($p>0.05$) (Figure 5.3A).

There was a significant increase of miR-9 expression in the NAcS but not in the NAcC in chronic ethanol group ($p<0.01$) compared to the control-diet. Following withdrawal after chronic exposure to ethanol, the expression of mir-9 decreased in the NAcS ($p<0.01$) but not in the NAcC as compared to the control group (Figure 5.3B) (n=9 in each group).

#### 5.2.1.2. Rno-miR-124

There was a significant increase in mir-124 expression ($p<0.05$) in NAcS region but not in the NAcC as compared to the n-saline group ($p>0.05$)(Figure 5.4A).

The expression of mir-124 in control diet fed group, chronic ethanol fed and 24 hour ethanol withdrawal group rats was measured. The expression of mir-124 was elevated ($p<0.001$) in the NAcS but not in the NAcC ($p>0.05$) of the chronic ethanol fed group when compared to the control group. In ethanol withdrawal after chronic ethanol exposure a non-significant reduction ($p>0.05$) in the miR-124 level was observed when compared with the control diet fed group in NAcS. Although the increase in the miR expression in the NAcS was statistically significant ($p<0.05$) the fold change was only <1.20 (Figure 5.4B) (n=9 in each group).

#### 5.2.1.3. Rno-miR-132

In acute ethanol exposed group a significant increase in the expression of mir-132 in NAcS region ($p<0.05$) was observed as compared to the n-saline group. However, in NAcC region, there was no significant difference ($p>0.05$) found in the expression level of mir-132 in n-saline and the acute ethanol exposed rat group (Figure 5.5A).

In NAcS region, during chronic ethanol exposure a significant increase in the mir-132 expression ($p<0.001$) was observed as compared to the control group, and in ethanol withdrawal group a significant reduction in miR expression ($p<0.01$) with a fold change of <1.20 was observed compared to control group in the NAcS region. During long term ethanol exposure and chronic ethanol exposure followed by cessation of ethanol displayed no significant change ($p>0.05$) in the mir-132 expression as compared to the control group in the NAcC region (Figure 5.5B) (n=9 in each group).

#### 5.2.1.4. Rno-miR-181a

Following acute ethanol exposure the expression of mir-181a increased in the NAcS region ($p<0.01$) but not in the NAcC ($p>0.05$) as compared to the n-saline rat group, but not in the NAcC region ($p>0.05$: Figure 5.6A).

The effect of chronic ethanol exposure resulted in significant increase ($p<0.05$) in the expression of miR-181a compared to control group. Though, in withdrawal group a significant decrease ($p<0.01$) in miR expression was found in NAcS. In NAcC region, there was no significant change assessed during chronic exposure of ethanol ($p>0.05$) and in chronic ethanol exposure followed by ethanol withdrawal ($p>0.05$) (Figure 5.6B) (n=9 in each group).

#### 5.2.1.5. Rno-miR-212

In NAcS region, there was a significant increase ($p<0.01$) in the expression of mir-212 as compared to the n-saline group, but there was no significant change in the mir-212 expression ($p>0.05$) in NAcC region during acute exposure of ethanol (Figure 5.7A).

In NAcS region, compared to the control diet fed group a significant increase ($p$<0.05) in the expression of mir-212 was found in chronic ethanol exposed rat group, and a significant decrease ($p$<0.05) in expression found in ethanol withdrawal rat group. Further, in NAcC region there was no significant change in miR-212 expression was found in chronic ethanol diet fed rat group ($p$>0.05) and chronic ethanol fed group followed by 24 hr withdrawal group ($p$>0.05) when compared with each other statistically (Figure 5.7B) (n=9 in each group).

### 5.2.2. miRNA expression in Amygdala

The three sub-regions of amygdala: Basolateral amygdala (BLA), central amygdala (CeA) and medial amygdala (MeA) were selected for the study and expression of miRNAs were assessed during different ethanol exposure conditions. The statistical analysis established the understanding of change in miR expression under the influence of ethanol.

#### 5.2.2.1. Rno-miR-9

In BLA region of amygdala, no change in the expression of miR-9 was found in acute ethanol exposed rat group ($p$>0.05) compared to the n-saline group, conversely, the expression of miR-9 in CeA and MeA significantly changed. During acute ethanol exposure in CeA and MeA, the expression of miR-9 was significantly reduced ($p$<0.05 and $p$<0.01 respectively) compared to the expression level of miR in n-saline group (Figure 5.3C).

There was no significant change in the expression of miR-9 was found in BLA region during chronic exposure of ethanol ($p$>0.05) and ethanol withdrawal ($p$>0.05) compared to the control group expression level. During chronic ethanol exposure in CeA region, there was a significant increase in the miR-9 expression ($p$<0.05) noticed compared to the control group, but a statistically non-significant change found in withdrawal group ($p$>0.05). In MeA region, there was statistically a significant increase in miR-9 expression found during chronic ethanol exposure ($p$<0.0001) and in withdrawal group ($p$<0.05) compared to the control-diet fed group (Figure 5.3D) (n=9 in each group).

#### 5.2.2.2. Rno-miR-124

Interestingly, the expression of miR-124 was changed both during the acute ethanol exposure and chronic ethanol exposure followed by ethanol withdrawal in BLA, CeA and MeA regions of amygdala. There was a statistically significant decrease in the expression of miR-124 during acute ethanol exposure compared to the n-saline group in BLA ($p<0.05$; fold change<1.2), in CeA ($p<0.05$) and in MeA ($p<0.01$) regions of amygdala (Figure 5.4C).

Furthermore, in BLA region, there was a significant reduction in the miR-124 expression during chronic ethanol exposure ($p<0.01$) and during withdrawal ($p<0.05$, fold change<<1.2), compared to the control group. There was significant increase in the miR expression detected in CeA region ($p<0.05$) and MeA regions ($p<0.0001$) of chronic ethanol group compared to the control group, and during withdrawal insignificant change in the miR expression in CeA ($p>0.05$) found, nonetheless, significantly increased miR-expression in MeA ($p<0.01$) found compared to the control group (Figure 5.4D) (n=9 in each group).

#### 5.2.2.3. Rno-miR-132

The statistical analysis of miR-132 expression suggested the potential effect of ethanol on these small molecules regulating the gene expression. In BLA region there was no change in miR expression in acute ethanol group ($p>0.05$) as compared to the n-saline group, however a significant decrease in the expression of miR-132 found in CeA ($p<0.01$, fold change <1.2) and MeA ($p<0.01$) following acute ethanol exposure (Figure 5.5C).

The expression of miR-132 was decreased in BLA region ($p<0.05$) during the chronic ethanol exposure; however, the change of miR expression in withdrawal group was non-significant ($p>0.05$) in comparison to control group. In CeA region the expression of miR-132 increased during chronic ethanol exposure ($p<0.05$), however, insignificant increase in miR expression was found in withdrawal group ($p>0.05$) compared to the control group. Significant change was also found during chronic ethanol exposure ($p<0.05$) and in withdrawal group ($p<0.05$) in MeA region of amygdala (Figure 5.5D) (n=9 in each group).

#### 5.2.2.4. Rno-miR-181a

During acute ethanol exposure there was a significant decrease in the miR-181a expression in BLA ($p<0.05$) and MeA ($p<0.001$) regions of amygdala, compared to the n-saline group. In CeA region nonsignificant change of miR expression ($p>0.05$) was observed in acute ethanol group (Figure 5.6C).

In BLA region the change in miR-181a expression was neither changed in chronic ethanol exposed group ($p>0.05$) nor in withdrawal group ($p>0.05$) compared to the control group. In CeA region of amygdala the expression of miR-132 was increased during chronic ethanol exposure ($p<0.001$) and in withdrawal group ($p<0.01$) compared to the control group. In MeA region there was an increased expression of miR-181a was found both during chronic ethanol exposure ($p<0.01$) and during ethanol withdrawal ($p<0.001$) compared to the control group (Figure 5.6D) (n=9 in each group).

#### 5.2.2.5. Rno-miR-212

In BLA region there was no difference in miR expression found in acute ethanol group ($p>0.05$) compared to the n-saline group, however a significant decrease in the expression of miR-212 in CeA ($p<0.05$, fold change $<<1.2$) and MeA ($p<0.001$) found during acute ethanol exposure (Figure 5.7C).

The expression of miR-212 was reduced in BLA region ($p<0.01$) during the chronic ethanol exposure; however, the change of miR expression in withdrawal group was non-significant ($p>0.05$) in comparison to control group. In CeA region of amygdala the expression of miR-212 was increased during chronic ethanol exposure ($p<0.01$) however, a non-significant increase in miR expressionfound during ethanol withdrawal ($p>0.05$) compared to the control group. In MeA region, though the change was found yet not statistically significant during chronic ethanol exposure ($p>0.05$) and during ethanol withdrawal after the chronic ethanol treatment a significant increase ($p<0.01$) was observed compared to the control (Figure 5.7D) (n=9 in each group).

## 5.3. Analysis of mRNA Expression

The mRNA expression of transcription factor CREB and its target genes such as c-fos, ARC, NPY and CBP were analyzed using SYBR green based semi-quantitative real time PCR reaction in nucleus accumbens shell (NAcS) and core regions (NAcC) and in basolateral (BLA), central (CeA) and medial amygdala (MeA). β-actin was used as a housekeeping gene and normalizer for the data retrieved. ΔΔCt method was used to calculate the fold change and statistical analysis was also done.

### 5.3.1. mRNAs expression in Nucleus accumbens

#### 5.3.1.1. CREB mRNA expression

The expression of CREB mRNA was analyzed in shell and core region of nucleus accumbens during acute ethanol exposure, chronic ethanol exposure and ethanol withdrawal. In both NAcS ($p>0.05$) and NAcC ($p>0.05$) regions a nonsignificant change in the CREB mRNA expression was found during acute ethanol exposure compared to the n-saline group (Figure 5.8A).

Next, in chronic ethanol exposure group, there was no significant change in the CREB mRNA expression was found in NAcS ($p>0.05$) and NAcC regions ($p>0.05$) compared to the control group. During ethanol withdrawal in NAcS ($p>0.05$) and NAcC regions ($p>0.05$) also there was no change in CREB mRNA expression compared to control group (Figure 5.8B) (n=9 in each group).

#### 5.3.1.2. c-fos mRNA expression

The assessment of c-fos expression during acute ethanol exposure revealed a significant increase in the mRNA expression in NAcS region ($p<0.001$) during acute ethanol exposure, however in NAcC region no significant change ($p>0.05$) was observed as compared to the expression in the n-saline group (Figure 5.9A).

In NAcS region, there was no significant change in the c-fos mRNA expression during chronic ethanol exposure ($p>0.05$) compared to the control group, but in withdrawal group a significant decrease in c-fos mRNA expression found compared

to the control group ($p<0.05$). Although during withdrawal a significant change was observed in NAcS, there was no significant change observe in the NAcC region during chronic ethanol exposure ($p>0.05$) and ethanol withdrawal ($p>0.05$) (Figure 5.9B) (n=9 in each group).

#### 5.3.1.3. ARC mRNA expression

The analysis of ARC expression during acute ethanol exposure revealed a significant increase in the mRNA expression in NAcS region ($p>0.01$) but not in NAcC ($p>0.05$) as compared to the n-saline group (Figure 5.10A).

In NAcS region, during chronic ethanol exposure a nonsignificant change in the ARC mRNA expression was found compared to the control group ($p>0.05$), but during withdrawal a significant decrease in ARC mRNA expression found compared to the control group ($p<0.01$). Although during withdrawal a statistically significant change was found in NAcS, but in NAcC region insignificant difference observed during chronic ethanol exposure ($p>0.05$) and withdrawal ($p>0.05$) (Figure 5.10B) (n=9 in each group).

#### 5.3.1.4. NPY mRNA expression

There was significant increase in the expression of Neuropeptide Y mRNA in NAcS ($p>0.01$) but not in NAcC ($p>0.05$) following acute ethanol exposure compared to the n-saline group (Figure 5.11A).

In NAcS region, there was a nonsignificant change in the NPY mRNA expression compared to the control group ($p>0.05$), but in withdrawal group a significant decrease in NPY mRNA expression found compared to the control group ($p<0.001$). Although during withdrawal a significant change in expression was found in NAcS, no change in expression in the NAcC region during chronic ethanol exposure ($p>0.05$) and withdrawal ($p>0.05$) observed (Figure 5.11B) (n=9 in each group).

#### 5.3.1.5. CBP mRNA expression

The analysis of CBP (Cyclic-AMP receptor binding protein) mRNA expression during acute ethanol exposure revealed a significant increase in the mRNA expression

in NAcS region ($p$<0.001) during acute ethanol exposure, on the other hand in NAcC region there was no significant change ($p$>0.05) as compared to the n-saline group (Figure 5.12A).

In NAcS region, there was a nonsignificant change in the CBP mRNA expression compared to the control group ($p$>0.05), but in withdrawal group a significant decrease in CBP mRNA expression found compared to the control group ($p$<0.01). Although during withdrawal a significant change in CBP expression was found in NAcS, no significant change in NAcC following chronic ethanol exposure ($p$>0.05) and withdrawal ($p$>0.05) detected (Figure 5.12B) (n=9 in each group).

### 5.3.2. mRNA expression in Amygdala

#### 5.3.2.1. CREB mRNA expression

During acute ethanol exposure there was no significant change in mRNA expression in BLA ($p$>0.05), CeA ($p$>0.05) and MeA ($p$>0.05) regions of amygdala compared to the n-saline group (Figure 5.8C).

The change in the expression of c-fos mRNA in BLA, CeA and MeA was also studied during chronic ethanol exposure and withdrawal group along with control group. There was no significant change in the c-fos expression both during chronic ethanol exposure and ethanol withdrawal in BLA ($p$>0.05), CeA ($p$>0.05) and MeA ($p$>0.05) regions compared to the control group (Figure 5.8D) (n=9 in each group).

#### 5.3.2.2. c-fos mRNA expression

In acute ethanol exposure group there was a nonsignificant change in the c-fos mRNA expression in the BLA region ($p$>0.05) compared to the n-saline. However, a significant increase in the c-fos mRNA expression was found during acute ethanol exposure in CeA ($p$<0.01) and MeA region ($p$<0.01) compared to the n-saline group (Figure 5.9C).

During chronic ethanol exposure and also during ethanol withdrawal there was no change in the c-fos mRNA expression in BLA region ($p>0.05$). The expression level of c-fos mRNA in CeA ($p>0.05$) and MeA region ($p>0.05$) during chronic ethanol exposure was not changed compared to the control group. However, during withdrawal a significant decrease in the c-fos mRNA expression was observed both in CeA ($p<0.05$) and MeA ($p<0.01$) regions as compared to the control group (Figure 5.9D) (n=9 in each group).

#### 5.3.2.3. ARC mRNA expression

During acute exposure of ethanol there was a statistically non-significant change in the ARC mRNA expression in the BLA region ($p>0.05$) compared to the n-saline. A significant increase in the ARC mRNA expression in CeA ($p<0.01$) and MeA ($p<0.01$) regions during acute ethanol exposure compared to the n-saline group (Figure 5.10C).

In chronic ethanol exposure group as well as in ethanol withdrawal group there was no change in the ARC mRNA expression in BLA region ($p>0.05$). The expression level of ARC mRNA in CeA ($p>0.05$) and MeA region ($p>0.05$) during chronic ethanol exposure was not changed compared to the control group. However, during withdrawal a significant decrease in the ARC mRNA expression was found both in CeA ($p<0.01$) and MeA ($p<0.001$) regions compared to the control group (Figure 5.10D) (n=9 in each group).

#### 5.3.2.4. NPY mRNA expression

During acute treatment of ethanol there was a statistically insignificant change in the NPY mRNA expression in the BLA region ($p>0.05$) compared to the n-saline group. Similarly a significant increase in the ARC mRNA expression in CeA ($p<0.01$) and MeA region ($p<0.01$) was found during acute ethanol exposure compared to the n-saline group (Figure 5.11C).

In chronic ethanol exposure group as well as in ethanol withdrawal group there was no change in the NPY mRNA expression in BLA region ($p>0.05$). The expression level of NPY mRNA in CeA ($p>0.05$) and MeA region ($p>0.05$) during chronic

ethanol exposure was not changed compared to the control group, but during withdrawal a significant decrease in the NPY mRNA expression found both in CeA ($p$<0.01) and MeA regions ($p$<0.001) compared to the control group (Figure 5.11D) (n=9 in each group).

#### 5.3.2.5. CBP mRNA expression

Following acute ethanol exposure there was a statistically non-significant change in the CBP mRNA expression in the BLA region ($p$>0.05) compared to the n-saline. However a significant increase in the ARC mRNA expression in CeA ($p$<0.01) and MeA ($p$<0.001) was found following acute ethanol exposure compared to the n-saline group (Figure 5.12C).

In chronic ethanol exposure group as well as in ethanol withdrawal group there was no change found in the CBP mRNA expression in BLA region ($p$>0.05). The expression of CBP mRNA in CeA ($p$>0.05) and MeA region ($p$>0.05) during chronic ethanol exposure was not changed compared to the control group. However during withdrawal a significant decrease in the CBP mRNA expression was found both in CeA ($p$<0.05) and MeA ($p$<0.05) regions compared to the control group (Figure 5.12D) (n=9 in each group).

## 5.4. Analysis of Immunohistochemical Studies

DAB-immunohistochemistry using protein specific antibodies, was performed to analyze the changes in the protein expression of CREB, c-fos, ARC, NPY and CBP. Coronal sections of brain having nucleus accumbens (shell and core) and amygdala (BLA, CeA and MeA) were analyzed under microscope and number of positive nuclei counted, representing the protein level of transcription factor CREB and its target genes under investigation in the present study.

### 5.4.1. IHC analysis in nucleus accumbens

#### 5.4.1.1. CREB expression analysis

The expression of CREB protein level was analyzed in shell and core region of nucleus accumbens following acute ethanol exposure, chronic ethanol exposure and ethanol withdrawal. In both NAcS ($t=0.51$, $p>0.05$) and NAcC ($t=1.18$, $p>0.05$) regions a nonsignificant change in the CREB protein expression was observed following acute ethanol exposure compared to the n-saline group (Figure 5.13C).

In the chronic ethanol group, there was no significant change in the CREB protein expression in NAcS ($F_{(2,24)}=0.20$, $p>0.05$) and NAcC regions ($F_{(2,24)}=0.10$, $p>0.05$) as compared to the control group. During ethanol withdrawal in NAcS and NAcC regions also there was no change in CREB protein level as compared to both control group ($F_{(2,24)}=0.20$, $p>0.05$) and chronic ethanol group ($F_{(2,24)}=0.10$, $p>0.05$) (Figure 5.13D) (n=9 in each group).

#### 5.4.1.2. c-fos expression analysis

The examination of c-fos during acute ethanol exposure revealed a significant increase in the c-fos expression in NAcS region ($t=3.24$, $p<0.01$) following acute ethanol exposure, however in NAcC region a nonsignificant alteration ($t=0.40$, $p>0.05$) was found compared to the expression in the n-saline group (Figure 5.14C).

In NAcS region, chronic exposure to ethanol had no significant effect on the c-fos expression as compared to the control group ($F_{(2,24)}=4.84$, $p>0.05$), however in withdrawal group exhibited a significant decrease in c-fos mRNA expression as

compared to the control group ($F_{(2,24)}$=4.84, $p$<0.05) and chronic ethanol group ($F_{(2,24)}$=4.84, $p$<0.05). Although during withdrawal a significant change was found in NAcS, NAcC region exhibited no significant change following chronic ethanol exposure ($F_{(2,24)}$=0.07, $p$>0.05) and ethanol withdrawal ($F_{(2,24)}$=0.07, $p$>0.05) (Figure 5.14D) (n=9 in each group).

#### 5.4.1.3. ARC expression analysis

A significant increase in the level of ARC expression was observed in the NAcS region (t=2.79, $p$<0.05) but not in the NAcC ( t=0.14, $p$>0.05) following acute ethanol exposure (Figure 5.15C).

In NAcS region, there was a nonsignificant change in the ARC expression during chronic ethanol treatment found compared to the control group ($F_{(2,24)}$=5.18, $p$>0.05), but in withdrawal group a significant reduction in protein level of ARC observed compared to the control group ($F_{(2,24)}$=5.18, $p$<0.05) and chronic ethanol group ($F_{(2,24)}$=5.18, $p$<0.05). Although during withdrawal a statistically significant change was found in NAcS, but in NAcC region no significant difference during chronic ethanol exposure ($F_{(2,24)}$=0.34, $p$>0.05) and withdrawal ($F_{(2,24)}$=0.34, $p$>0.05) (Figure 5.15D) (n=9 in each group).

#### 5.4.1.4. NPY expression analysis

The analysis of Neuropeptide Y expression during acute ethanol exposure shown a significant increase in the NPY protein level in NAcS region (t=2.77, $p$<0.05) during acute ethanol exposure, on the other hand in NAcC region a non-significant difference in NPY activity (t=0.28, $p$>0.05) was found compared to the n-saline group (Figure 5.16C).

In NAcS region, there was a no change in NPY activity compared to the control group ($F_{(2,24)}$=5.12, $p$>0.05), but in withdrawal group a significant decrease in NPY protein level observed compared to the control group ($F_{(2,24)}$=5.12, $p$<0.05) and chronic ethanol group ($F_{(2,24)}$=5.12, $p$<0.05). Although during withdrawal a significant change in expression was observed in NAcS, but in NAcC region no significant difference

during chronic ethanol exposure ($F_{(2,24)}$=0.26, $p$>0.05) and withdrawal ($F_{(2,24)}$=0.26, $p$>0.05) (Figure 5.16D) (n=9 in each group).

#### 5.4.1.5. CBP expression analysis

During acute ethanol exposure a significant increase in the protein level of CBP was observed in NAcS region (t=2.51, $p$<0.05) during acute ethanol exposure, on the other hand, in NAcC region no change in CBP activity (t=0.24, $p$>0.05) compared to the n-saline group (Figure 5.17C).

In NAcS region, there was no change in CBP activity was found compared to the control group ($F_{(2,24)}$=5.70, $p$>0.05), but the effect of ethanol withdrawal after chronic ethanol exposure resulted in a significant reduction in CBP protein level compared to the control group ($F_{(2,24)}$=5.70, $p$<0.05) and chronic ethanol group ($F_{(2,24)}$=5.70, $p$<0.05). Although during withdrawal a significant change in CBP activity was found in NAcS, but in NAcC region no significant difference during chronic ethanol exposure ($F_{(2,24)}$=1.12, $p$>0.05) and withdrawal ($F_{(2,24)}$=1.12, $p$>0.05) found (Figure 5.17D) (n=9 in each group).

### 5.4.2. IHC analysis in amygdala

#### 5.4.2.1. CREB expression analysis

During acute ethanol exposure there was no significant change in CREB protein level was found in BLA (t=0.75, $p$>0.05), CeA (t=0.74, $p$>0.05) and MeA (t=0.75, $p$>0.05) regions of amygdala compared to the n-saline group (Figure 5.18C).

The change in the expression of CREB protein in BLA, CeA and MeA was also studied during chronic ethanol exposure and withdrawal along with control group. There was no significant change in the CREB activity in both during the chronic ethanol exposure and also during ethanol withdrawal after chronic ethanol exposure in BLA ($F_{(2,24)}$=0.47, $p$>0.05), CeA ($F_{(2,24)}$=0.47, $p$>0.05) and MeA ($F_{(2,24)}$=0.48, $p$>0.05) regions compared to the control group (Figure 5.18D) (n=9 in each group).

### 5.4.2.2. c-fos expression analysis

In acute ethanol exposure group there was a nonsignificant difference in the c-fos protein expression observed in the BLA region (t=0.75, $p>0.05$) compared to the n-saline. However, a significant increase in the c-fos activity in CeA (t=4.88, $p<0.001$) and MeA (t=3.33, $p<0.01$) was assessed during acute ethanol exposure compared to the n-saline group (Figure 5.19C).

During chronic ethanol exposure and also during ethanol withdrawal there was no change in the c-fos activity was observed in BLA region ($F_{(2,24)}=0.65$, $p>0.05$). The protein expression level of c-fos in CeA ($F_{(2,24)}=13.36$, $p>0.05$) and MeA region ($F_{(2,24)}=22.23$, $p>0.05$) during chronic ethanol exposure not changed compared to the control group, but during withdrawal a significant reduction in the c-fos protein expression both in CeA and MeA regions compared to the control group ($F_{(2,24)}=13.36$, $p<0.001$) and chronic ethanol group ($F_{(2,24)}=22.23$, $p<0.001$) (Figure 5.19D) (n=9 in each group).

### 5.4.2.3. ARC expression analysis

During acute exposure of ethanol there was a statistically insignificant change in the ARC protein expression was found in the BLA region (t=1.13, $p>0.05$) compared to the n-saline. Albeit, a significant increase in the ARC activity in CeA (t=3.81, $p<0.01$) and MeA (t=3.92, $p<0.01$) was detected during acute ethanol exposure compared to the n-saline group (Figure 5.20C).

In chronic ethanol exposure rat group as well as in ethanol withdrawal group there was no change found in the ARC activity in BLA region ($F_{(2,24)}=0.17$, $p>0.05$). The expression level of ARC protein in CeA ($F_{(2,24)}=9.22$, $p>0.05$) and MeA region ($F_{(2,24)}=7.41$, $p>0.05$) during chronic ethanol exposure not changed compared to the control group, but during withdrawal a significant reduction in the ARC activity both in CeA and MeA regions compared to the control group ($F_{(2,24)}=9.22$, $p<0.05$ and $F_{(2,24)}=7.41$, $p<0.05$, respectively) and chronic ethanol group ($F_{(2,24)}=9.22$, $p<0.01$ and $F_{(2,24)}=7.41$, $p<0.01$, respectively) observed (Figure 5.20D) (n=9 in each group).

### 5.4.2.4. NPY expression analysis

During acute treatment of ethanol, there was a nonsignificant change in the protein level of NPY observed in the BLA region (t=1.05, $p>0.05$) compared to the n-saline. Although, a significant increase in the NPY activity in CeA (t=4.46, $p<0.001$) and MeA (t=5.39, $p<0.0001$) found during acute ethanol exposure compared to the n-saline group (Figure 5.21C).

In chronic ethanol exposed rat group as well as in ethanol withdrawal group there was no change in NPY level in BLA region ($F_{(2,24)}=0.84$, $p>0.05$). The expression level of NPY protein in CeA ($F_{(2,24)}=14.69$, $p>0.05$) and MeA region ($F_{(2,24)}=11.51$, $p>0.05$) during chronic ethanol exposure was not changed compared to the control group, but during withdrawal a significant decrease in the NPY expression both in CeA and MeA regions compared to the control group ($F_{(2,24)}=14.69$, $p<0.001$ and $F_{(2,24)}=11.51$, $p<0.01$, respectively) and chronic ethanol group ($F_{(2,24)}=14.69$, $p<0.001$ and $F_{(2,24)}=11.51$, $p<0.001$, respectively) observed (Figure 5.21D) (n=9 in each group).

### 5.4.2.5. CBP expression analysis

Following acute ethanol exposure there was no change in the protein level of CBP in the BLA region (t=0.75, $p>0.05$) as compared to the n-saline.. A significant increase in the CBP expression in CeA (t=3.53, $p<0.01$) and MeA (t=3.33, $p<0.01$) was found during acute ethanol group as compared to the n-saline group (Figure 5.22C).

In chronic ethanol exposure group as well as in ethanol withdrawal group there was no change in the CBP level in BLA region ($F_{(2,24)}=0.65$, $p>0.05$). The expression level of CBP protein in CeA ($F_{(2,24)}=7.36$, $p>0.05$) and MeA region ($F_{(2,24)}=11.44$, $p>0.05$) during chronic ethanol exposure not changed as compared to the control group, but during withdrawal a significant decrease in the CBP level was found both in CeA and MeA regions compared to the control group ($F_{(2,24)}=7.36$, $p<0.05$ and $F_{(2,24)}=11.44$, $p<0.01$, respectively) and chronic ethanol group ($F_{(2,24)}=7.36$, $p<0.01$ and $F_{(2,24)}=11.44$, $p<0.001$, respectively) (Figure 5.22D) (n=9 in each group).

### 5.5. Chromatin Immunoprecipitation Assay Analysis

Anti-CREB antibody was used for crosslinking with chromatin and precipitated chromatin was isolated for qPCR analysis of expression of promoter region of selected set of microRNAs (miR-9, -124, 132, 181a and -212) using specific set of primers during acute ethanol and chronic ethanol exposure conditions.

#### 5.5.1. CHIP assay analysis in nucleus accumbens

The findings thus far indicate that the shell region of nucleus accumbens is vulnerable to ethanol exposure whether exposed acutely or chronically with ethanol. From the selected set of microRNAs, each miRNA displayed a substantial rise in the expression specifically in NAcS region. To address the epigenetic changes, specifically for CREB interaction, which activate the gene transcription, was analyzed. A statistically significant increase of promoter of miR-9 expression was assessed during acute ($p<0.05$) and chronic ethanol exposure ($p<0.01$). Expression of mir-124 promoter was significantly increased during acute ethanol exposure ($p<0.05$), but the expression was consistent during chronic ethanol exposure ($p>0.05$) compared to the control group promoter expression of miR-124. The effect of acute ethanol insignificantly altered the miR-132 promoter expression in NAcS ($p>0.05$), though chronic exposure of ethanol remarkably induced the miR-132 promoter expression (fold change>1.2, $p<0.01$). The expression of promoters of mir-181a and -212 remained unaltered during chronic ethanol exposure ($p<0.05$), rather than significant difference in promoter expression of mir-181a and -212 was observed during acute ethanol exposure ($p<0.05$) (Figure 5.20A-B) (n=6 in each group).

#### 5.5.2. CHIP assay analysis in amygdala

The epigenetic control by CREB was also studied in central and medial nucleus of amygdala. The changes occur in the expression of microRNA and mRNA during acute and chronic ethanol treatment, distinctly in CeA and MeA region directed for further epigenetic analysis by chromatin-immunoprecipitation (ChIP). In CeA region, acute exposure as well as chronic exposure of ethanol triggered the activation of miR-9 ($p<0.05$) and miR-181a ($p<0.05$ and $p<0.01$, respectively) promoter expression. Although, the activity of promoters of mir-132, and -212 remained unaltered during

acute ethanol exposure ($p$>0.05) and chronic ethanol exposure ($p$>0.05); but the promoter of miR-124 was significantly induced during chronic ethanol exposure ($p$<0.05) nevertheless not in acute exposure of ethanol ($p$>0.05) (Figure 5.20C-D). In MeA region, acute exposure as well as chronic exposure of ethanol not affected the activation or suppression of miR-9 ($p$>0.05) and miR-132 ($p$>0.05) promoter expression through acety-H3K9. The promoter activity of miR-124 and miR-181a was elicited by both acute and chronic treatment of ethanol ($p$<0.05 and $p$<0.01, respectively) in MeA region. During chronic ethanol exposure miR-212 promoter activity was significantly induced ($p$<0.01), yet remained unchanged during acute ethanol exposure ($p$>0.05) (Figure 5.20E-F) (n=6 in each group).

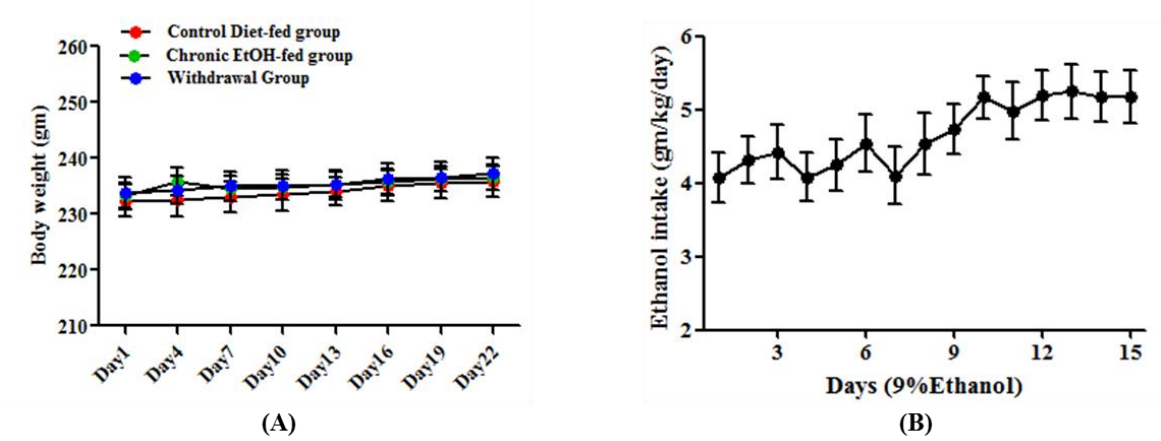



**Figure 5.1:**Effect of ethanol on body weight and ethanol intake analysis. **(A)** Representing the effect of ethanol feed on the body weight for control and chronic ethanol group. **(B)** Representing alcohol drinking pattern in chronic ethanol-fed rats from day1 to day 15 at 9% ethanol containing liquid diet.Values represented as mean ± SEM of 12 rats in each group.

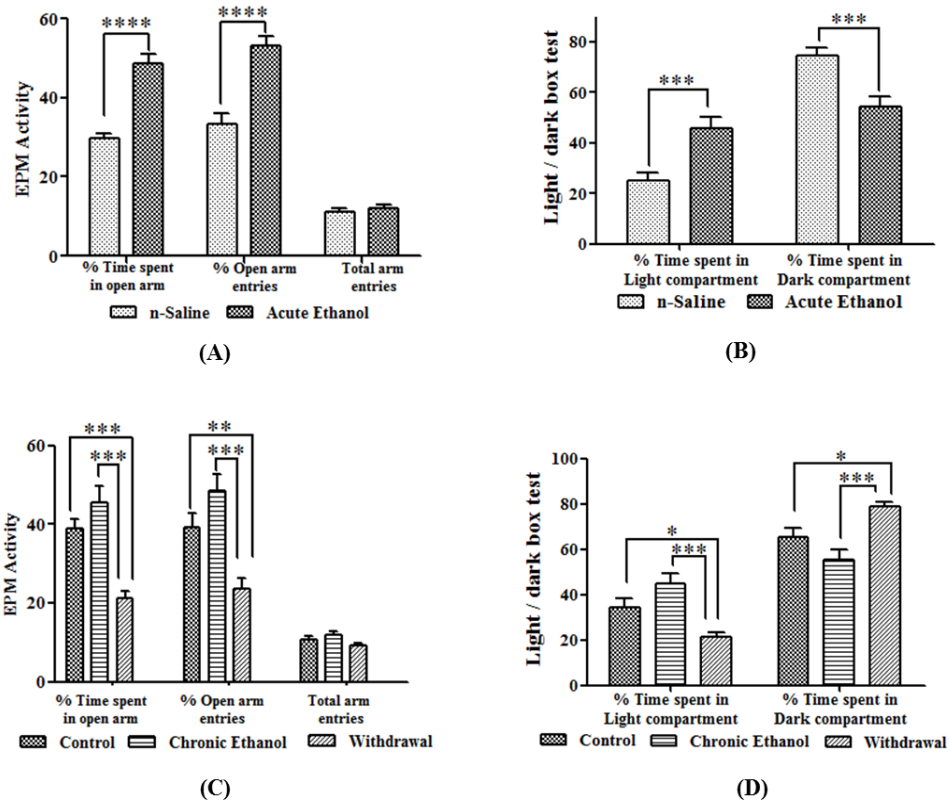



**Figure 5.2:** Anxiety measurement byEPM andLDB**(A)**Represents the % time spent in open arm, % open arm entries and total arm entries during the EPM test in n-saline and acute ethanol treated groups. (B) LDB test displayed the increase in % time spent light box by acute ethanol group rats. (C) Represents EPM activity after chronic ethanol treatment and ethanol withdrawal as compared to the control. (D) LDB test reduced % time spent in light box by ethanol withdrawal rat group compared to control rat group and chronic ethanol exposed rat group found.Values represented as mean $\pm$ SEM of 12 rats in each group. (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; for statistical analysis between two groups Student's *t-test* and for more than two group ANOVA followed by *posthoc* Tukey's test used.

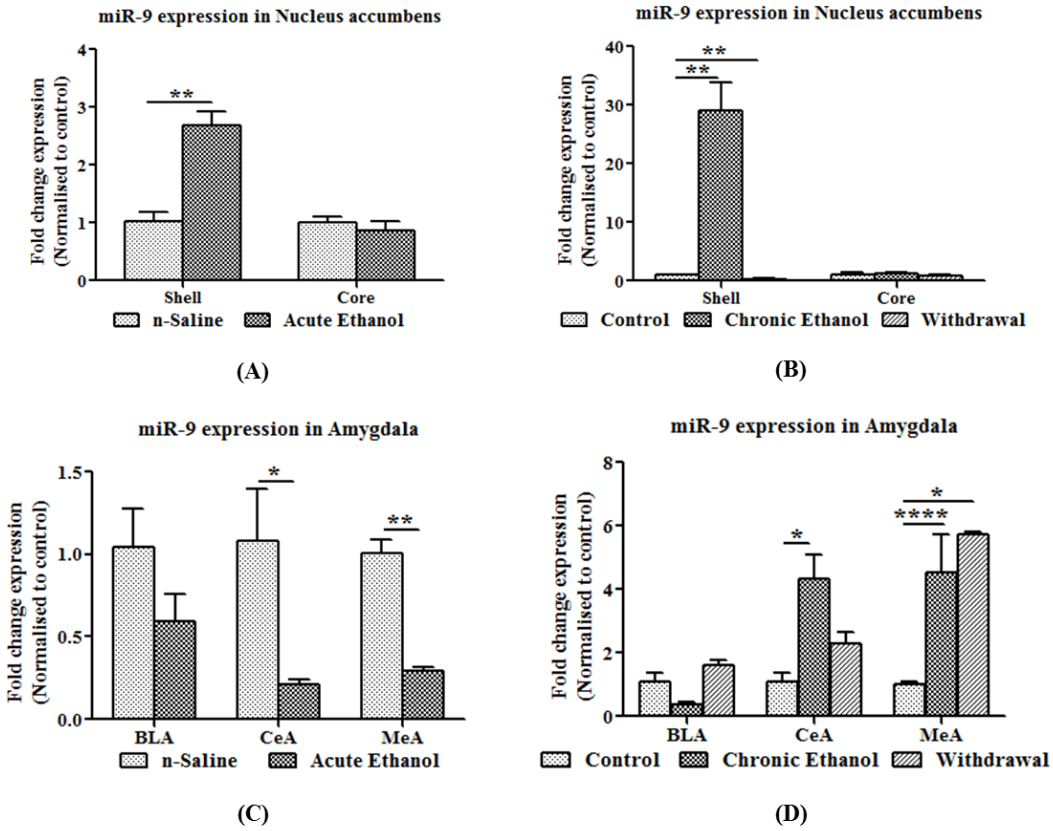



**Figure 5.3: miR-9 expression in nucleus accumbens and amygdala(A)** Acute ethanol exposure induced miR-9 expression in NAcS, but no change found in NAcC region compared to n-saline. **(B)** Chronic ethanol treatment increased miR-9 expression in NAcS region compared to control group, however, withdrawal of ethanol reduced the miR-9 expression in NAcS compared to chronic ethanol group, but no change compared to control group. No change in miR-9 activity was detected in NAcC region during chronic ethanol exposure and ethanol withdrawal. **(C)** In CeA and MeA exposure of acute ethanol reduced the miR-9 expression but no change observed in BLA region. **(D)** In BLA region increased miR-9 expression detected in withdrawal group compared to chronic ethanol exposure group, but no change observed compared to control group. CeA and MeA regions represented the increased miR-9 expression during chronic ethanol treatment compared to control group. Although changes in miR-9 activity were prominent during ethanol withdrawal but not found statistically significant. Values represented as mean $\pm$ SEM of 9 rats in each group. (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; for statistical analysis between two groups Student's *t-test* and for more than two group ANOVA followed by *posthoc* Tukey's test used.

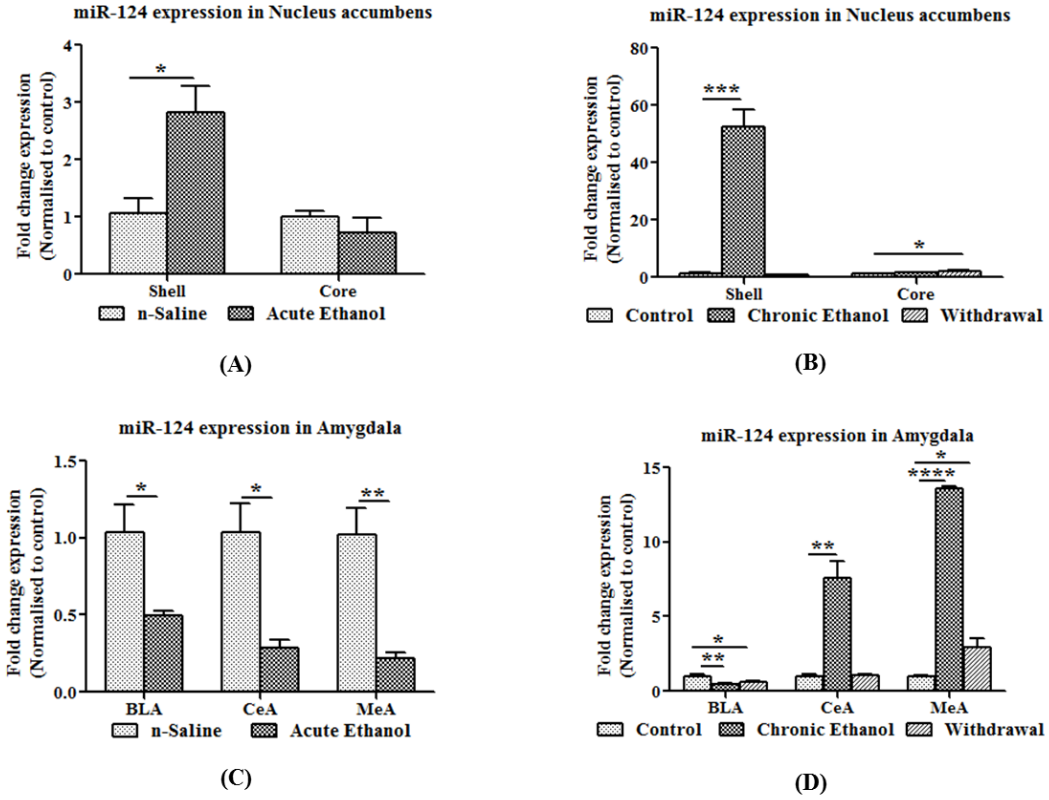



**Figure 5.4: miR-124 expression in nucleus accumbens and amygdala(A)** Acute ethanol exposure induced miR-124 expression in NAcS, but no change found in NAcC region compared to n-saline. **(B)** Chronic ethanol treatment increased miR-124 expression in NAcS region compared to control group, however, withdrawal of ethanol reduced the miR-124 expression in NAcS compared to chronic ethanol group, but no change compared to control group. No change in miR-124 activity was detected in NAcC region during chronic ethanol exposure and ethanol withdrawal. **(C)** In BLA, CeA and MeA exposure of acute ethanol reduced the miR-124 expression compared to n-saline rat group. **(D)** In BLA region significant reduction in miR-124 expression was observed in chronic ethanol and withdrawal group compared to control group. CeA and MeA regions represented the increased miR-124 expression during chronic ethanol treatment compared to control group. However, withdrawal of ethanol reduced the miR-124 expression both in CeA and MeA regions compared to chronic ethanol group, but no change compared to control group. Values represented as mean $\pm$ SEM of 9 rats in each group. (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; for statistical analysis between two groups Student's *t-test* and for more than two group ANOVA followed by *posthoc* Tukey's test used.

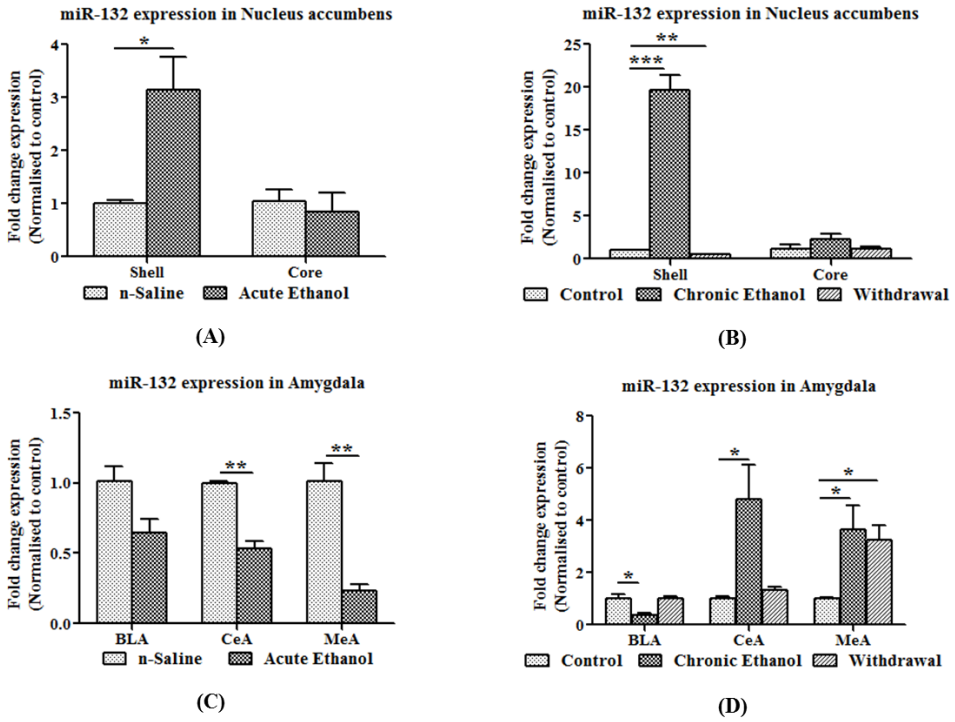



**Figure 5.5: miR-132 expression in nucleus accumbens and amygdala(A)** Acute ethanol exposure induced miR-132 expression in NAcS, but no change found in NAcC region compared to n-saline. **(B)** Chronic ethanol treatment increased miR-132 expression in NAcS region compared to control group, however, withdrawal of ethanol reduced the miR-132 expression in NAcS compared to chronic ethanol group, but no change compared to control group. No change in miR activity was detected in NAcC region during chronic ethanol exposure and ethanol withdrawal. **(C)**However in BLA region no changes observed, but in CeA and MeA regions exposure of acute ethanol reduced the miR expression compared to n-saline rat group. **(D)** In BLA region significant reduction in miR-132 expression was observed in chronic ethanol compared to control group, conversely the expression was increased in withdrawal group compared to chronic ethanol group, but not statistically significant change found compared to control group. CeA region represented the increased miR expression during chronic ethanol treatment compared to control group. However, withdrawal of ethanol reduced the miR expression in CeA region compared to chronic ethanol group, but no change compared to control group. MeA region specific analysis represented noticeable miR-132 activity though not found statistically significant.Values represented as mean $\pm$ SEM of 9 rats in each group. (*$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$; for statistical analysis between two groups Student's *t-test* and for more than two group ANOVA followed by*posthoc* Tukey's test used.

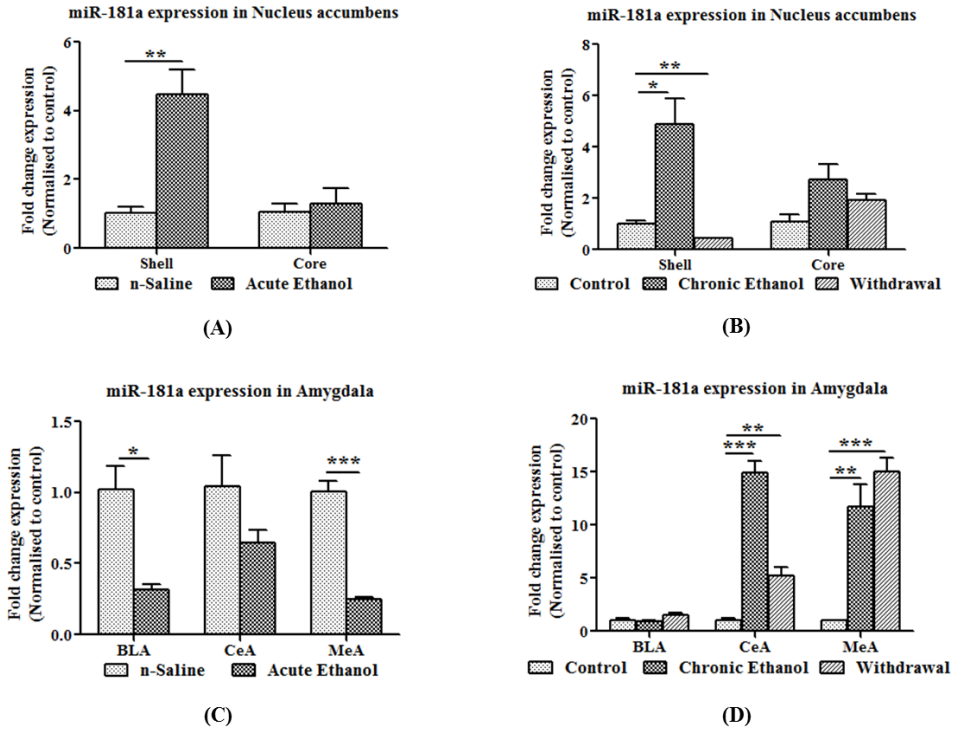



**Figure 5.6: miR-181a expression in nucleus accumbens and amygdala(A)** Acute ethanol exposure induced miR-181a expression in NAcS, but no change found in NAcC region compared to n-saline. **(B)** Chronic ethanol treatment increased miR-181a expression in NAcS region compared to control group, however, withdrawal of ethanol reduced the miR-181a expression in NAcS compared to chronic ethanol group, but no change compared to control group. No change in miR activity was detected in NAcC region during chronic ethanol exposure and ethanol withdrawal. **(C)**InCeA region of acute ethanol group statistically nonsignificant yet noticeable changes observed, but in BLA and MeA regions exposure of acute ethanol reduced the miR expression compared to n-saline rat group. **(D)** In BLA region no significant difference in miR-181a expression was observed among control group, chronic ethanol group and withdrawal. CeA and MeA regions represented the increased miR expression during chronic ethanol treatment compared to control group. However, withdrawal of ethanol reduced the miR expression in CeA region compared to chronic ethanol group, but no change compared to control group. Interestingly, in MeA region during ethanol withdrawal miR-181a expression not changed compared to the chronic ethanol group.Values represented as mean $\pm$ SEM of 9 rats in each group. (*$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$; for statistical analysis between two groups Student's *t-test* and for more than two group ANOVA followed by *posthoc* Tukey's test used.

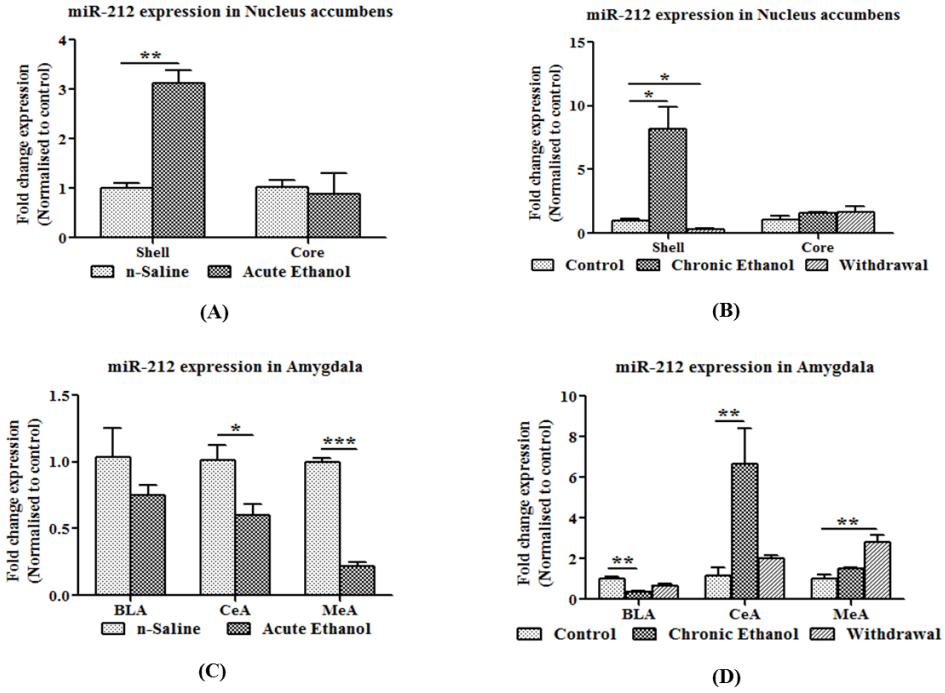



**Figure 5.7: miR-212 expression in nucleus accumbens and amygdala(A)** Acute ethanol exposure induced miR-212 expression in NAcS, but no change found in NAcC region compared to n-saline. **(B)** Chronic ethanol treatment increased miR-212 expression in NAcS region compared to control group, though, withdrawal of ethanol reduced the miR-212 expression in NAcS compared to chronic ethanol group, but no change compared to control group. No change in miR activity was detected in NAcC region during chronic ethanol exposure and ethanol withdrawal. **(C)**However in BLA region no changes observed, but in CeA and MeA regions exposure of acute ethanol reduced the miR expression compared to n-saline rat group. **(D)** In BLA region significant reduction in miR-212 expression was observed in chronic ethanol compared to control group, conversely no effect was observed in withdrawal group. CeA region represented the increased miR expression during chronic ethanol treatment compared to control group. However, withdrawal of ethanol reduced the miR expression in CeA region compared to chronic ethanol group, but no change compared to control group. MeA region analysis embodied evident miR-212 activity yet found statistically insignificant.Values represented as mean $\pm$ SEM of 9 rats in each group. (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; for statistical analysis between two groups Student's *t-test* and for more than two group ANOVA followed by *posthoc* Tukey's test used.

| miR-9 | Acute ethanol group | | Chronic ethanol group | | Withdrawal Group | |
|---|---|---|---|---|---|---|
| | Fold change | Significance Level | Fold change | Significance level | Fold change | Significance Level |
| NAcS | +1.61 | $p<0.01$ | +27.71 | $p<0.01$ | -2.62 | $p<0.01$ |
| NAcC | -0.16 | $p>0.05$ | +0.12 | $p>0.05$ | -0.24 | $p>0.05$ |

| miR-124 | Acute ethanol group | | Chronic ethanol group | | Withdrawal group | |
|---|---|---|---|---|---|---|
| | Fold change | Significance Level | Fold change | Significance level | Fold change | Significance level |
| NAcS | +1.64 | $p<0.05$ | +43.33 | $p<0.001$ | -0.55 | $p>0.05$ |
| NAcC | -0.40 | $p>0.05$ | +0.52 | $p>0.05$ | +1.08 | $p<0.05$ |

| miR-132 | Acute ethanol group | | Chronic ethanol group | | Withdrawal group | |
|---|---|---|---|---|---|---|
| | Fold change | Significance level | Fold change | Significance level | Fold change | Significance level |
| NAcS | +2.13 | $p<0.05$ | +18.53 | $p<0.001$ | -0.84 | $p<0.01$ |
| NAcC | -0.23 | $p>0.05$ | +0.90 | $p>0.05$ | -0.04 | $p>0.05$ |

| miR-181a | Acute ethanol group | | Chronic ethanol group | | Withdrawal group | |
|---|---|---|---|---|---|---|
| | Fold change | Significance level | Fold change | Significance level | Fold change | Significance level |
| **NAcS** | +3.32 | $p<0.01$ | +3.85 | $p<0.05$ | -1.31 | $p<0.01$ |
| **NAcC** | +0.23 | $p>0.05$ | +1.51 | $p>0.05$ | +0.77 | $p>0.05$ |

| miR-212 | Acute ethanol group | | Chronic ethanol group | | Withdrawal group | |
|---|---|---|---|---|---|---|
| | Fold change | Significance level | Fold change | Significance level | Fold change | Significance level |
| **NAcS** | +2.09 | $p<0.01$ | +7.01 | $p<0.05$ | -2.06 | $p<0.05$ |
| **NAcC** | -0.14 | $p>0.05$ | +0.43 | $p>0.05$ | +0.53 | $p>0.05$ |

**Table 5.1:**Summary of fold change in microRNA expression in nucleus accumbens shell (NAcS) and core (NAcC) regions of acute ethanol exposed group, chronic ethanol exposed group and withdrawal group. (Fold change was compared to their respective control groups; '+' sign represents increased expression and '-' sign indicates reduced expression).

| miR-9 | Acute ethanol Group | | Chronic ethanol Group | | Withdrawal Group | |
|---|---|---|---|---|---|---|
| | Fold change | Significance Level | Fold change | Significance Level | Fold change | Significance Level |
| BLA | -0.75 | $p>0.05$ | -2.07 | $p>0.05$ | +0.49 | $p>0.05$ |
| CeA | -4.08 | $p<0.05$ | +3.01 | $p<0.05$ | +1.12 | $p>0.05$ |
| MeA | -2.42 | $p<0.01$ | +3.51 | $p<0.0001$ | +4.57 | $p<0.05$ |

| miR-124 | Acute ethanol Group | | Chronic ethanol Group | | Withdrawal Group | |
|---|---|---|---|---|---|---|
| | Fold change | Significance Level | Fold change | Significance Level | Fold change | Significance Level |
| BLA | -1.08 | $p<0.05$ | -1.30 | $p<0.01$ | -0.61 | $p<0.05$ |
| CeA | -2.59 | $p<0.05$ | +6.50 | $p<0.01$ | +0.05 | $p>0.05$ |
| MeA | -3.72 | $p<0.01$ | +10.22 | $p<0.0001$ | +3.24 | $p>0.05$ |

| miR-132 | Acute ethanol Group | | Chronic ethanol Group | | Withdrawal Group | |
|---|---|---|---|---|---|---|
| | Fold change | Significance Level | Fold change | Significance Level | Fold change | Significance Level |
| BLA | -0.58 | $p>0.05$ | -1.81 | $p<0.05$ | -0.01 | $p>0.05$ |
| CeA | -0.86 | $p<0.01$ | +3.80 | $p<0.05$ | +0.31 | $p>0.05$ |
| MeA | -3.30 | $p<0.01$ | +2.65 | $p<0.05$ | +4.70 | $p<0.05$ |

| miR-181a | Acute ethanol Group | | Chronic ethanol Group | | Withdrawal Group | |
|---|---|---|---|---|---|---|
| | Fold change | Significance Level | Fold change | Significance Level | Fold change | Significance Level |
| BLA | -2.20 | $p<0.05$ | -0.19 | $p>0.05$ | +0.44 | $p>0.05$ |
| CeA | -0.61 | $p>0.05$ | +15.88 | $p<0.001$ | +4.11 | $p<0.01$ |
| MeA | -3.08 | $p<0.001$ | +10.68 | $p<0.01$ | +10.61 | $p<0.001$ |

| miR-212 | Acute ethanol Group | | Chronic ethanol Group | | Withdrawal Group | |
|---|---|---|---|---|---|---|
| | Fold change | Significance Level | Fold change | Significance Level | Fold change | Significance Level |
| BLA | -0.39 | $p>0.05$ | -1.73 | $p<0.01$ | -0.49 | $p>0.05$ |
| CeA | -0.69 | $p<0.05$ | +4.85 | $p<0.01$ | +0.77 | $p>0.05$ |
| MeA | -3.55 | $p<0.001$ | +1.25 | $p>0.05$ | +2.89 | $p<0.01$ |

**Table 5.2:** Summary of fold change in microRNA expression in basolateral amygdala (BLA), central amygdala (CeA) and medial amygdala (MeA) regions of acute ethanol exposed group, chronic ethanol exposed group and withdrawal group. (Fold change was compared to their respective control groups; '+' sign represents increased expression and '-' sign indicates reduced expression).

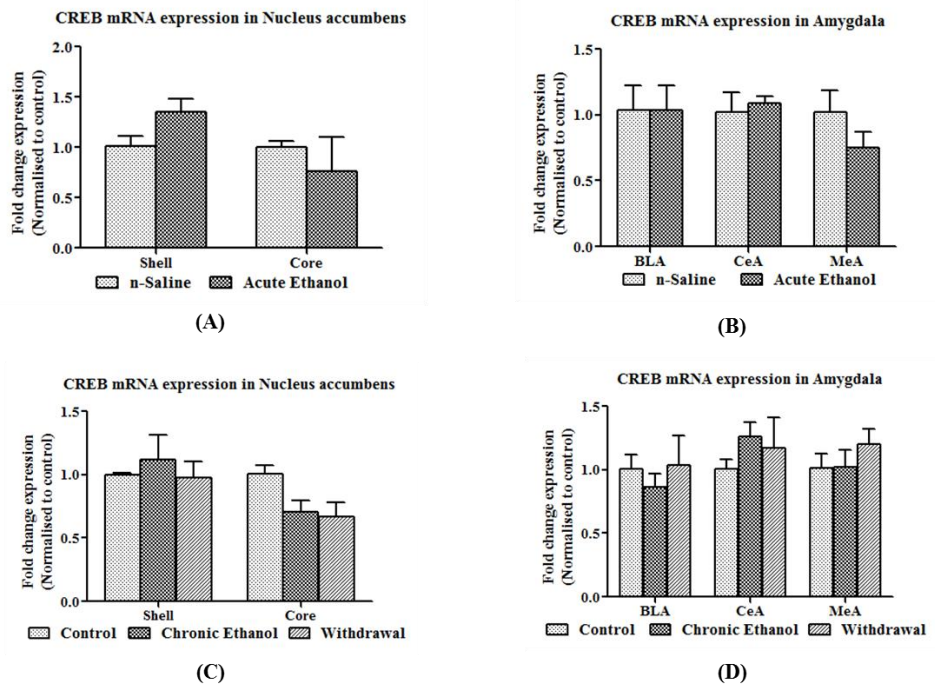



**Figure 5.8: CREB mRNA expression in nucleus accumbens and amygdala(A)** Acute ethanol exposure not affected CREB mRNA expression in both NAcS and NAcC regions compared to n-saline. **(B)** No change in CREB mRNA detected during chronic ethanol exposure and ethanol withdrawal compared to control group in both NAcS and NAcC regions. **(C)**Treatment of acute ethanol not affected CREB mRNA expression in BLA, CeA and MeA. **(D)**Chronic ethanol treatment and withdrawal after chronic ethanol treatment not affected CREB mRNA expression in BLA, CeA and MeA. Values represented as mean $\pm$ SEM of 9 rats in each group. (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; for statistical analysis between two groups Student's *t-test* and for more than two group ANOVA followed by *posthoc* Tukey's test used.

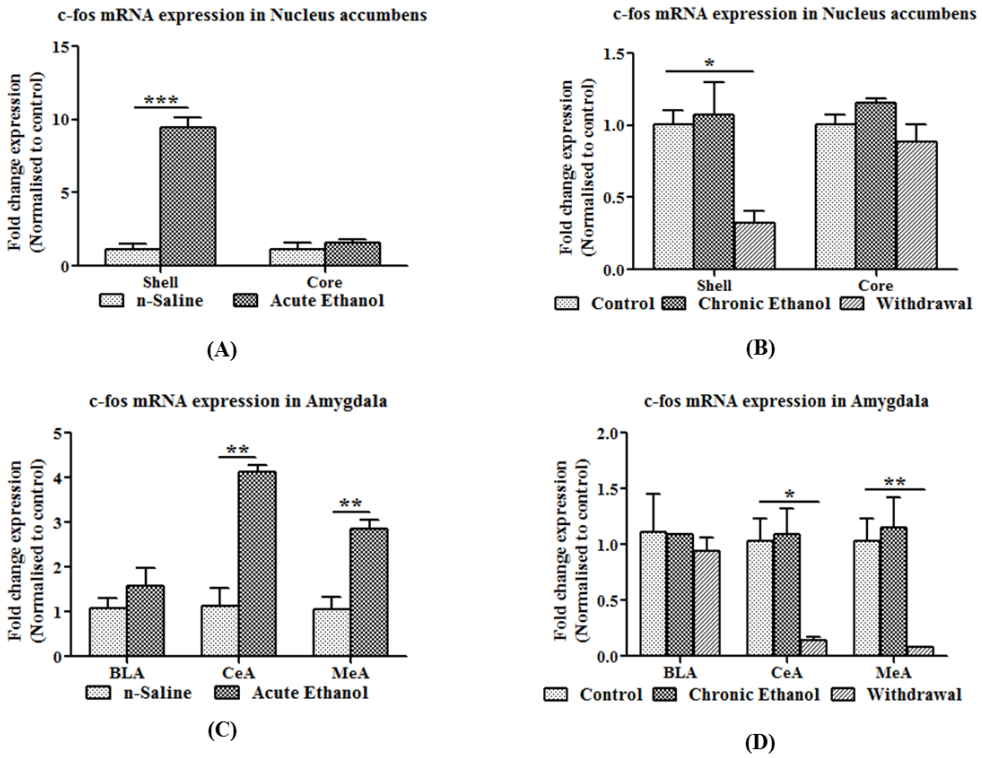



**Figure 5.9: c-fos mRNA expression in nucleus accumbens and amygdala(A)** Acute ethanol exposure induced c-fos mRNA expression in NAcS, but not affected expression in NAcC region compared to n-saline. **(B)** A significant reduction in c-fos mRNA expression identified during chronic ethanol exposure followed by ethanol withdrawal in NAcS region yet no effect of either chronic exposure of ethanol or withdrawal of ethanol found in NAcC region. **(C)**However in BLA region no changes observed, but in CeA and MeA regions exposure of acute ethanol induced the c-fos mRNA expression compared to n-saline rat group. **(D)**Nevertheless, in BLA region no changes detected, but in CeA and MeA regions ethanol withdrawal after chronic ethanol exposure significantlyreduced the c-fos mRNA expression compared to controland chronic ethanol rat groups.Values represented as mean $\pm$ SEM of 9 rats in each group. (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; for statistical analysis between two groups Student's *t-test* and for more than two group ANOVA followed by *posthoc* Tukey's test used.

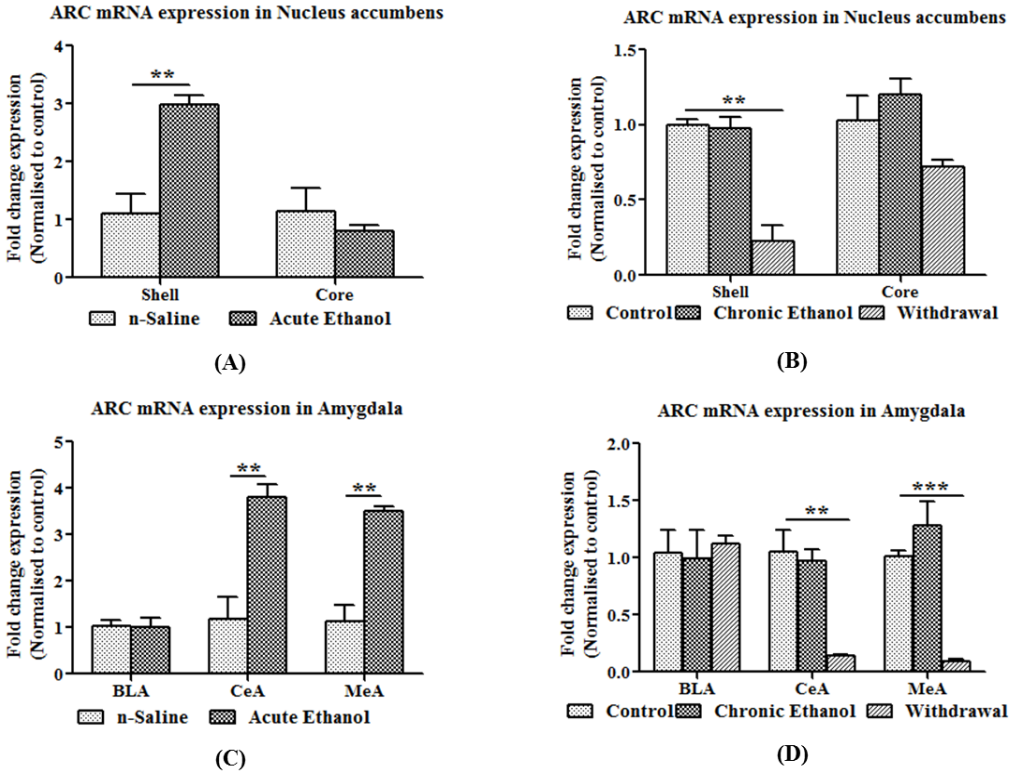



**Figure 5.10: ARC mRNA expression in nucleus accumbens and amygdala(A)** Acute ethanol exposure encouraged ARC mRNA expression in NAcS, but unaffected the expression in NAcC region compared to n-saline. **(B)** A significant decline in ARC mRNA expression identified during chronic ethanol exposure followed by ethanol withdrawal in NAcS region yet no effect of either chronic exposure of ethanol or withdrawal of ethanol found in NAcC region. **(C)**However in BLA region no changes observed, but in CeA and MeA regions exposure of acute ethanol induced the ARC mRNA expression compared to n-saline rat group. **(D)**However, in BLA region no changes detected, but in CeA and MeA regions ethanol withdrawal after chronic ethanol exposure significantlyreduced the ARC mRNA expression compared to control rat group and chronic ethanol rat groups.Values represented as mean $\pm$ SEM of 9 rats in each group. (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; for statistical analysis between two groups Student's *t-test* and for more than two group ANOVA followed by *posthoc* Tukey's test used.

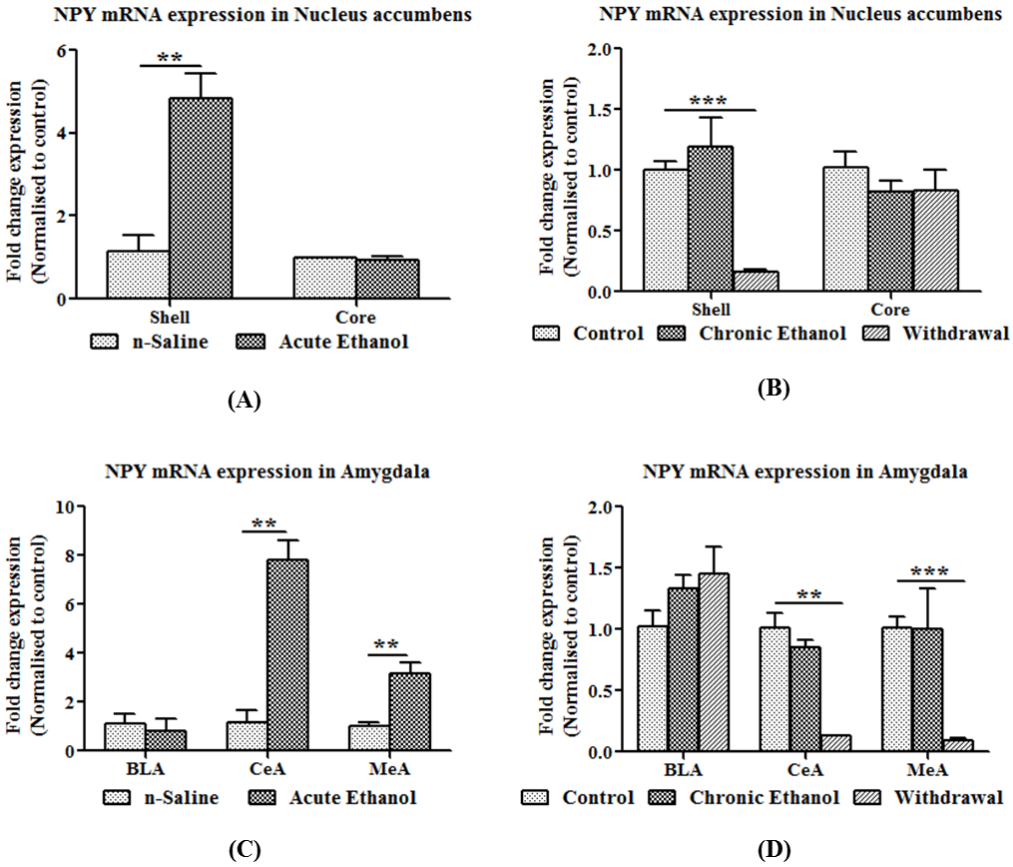



**Figure 5.11: NPY mRNA expression in nucleus accumbens and amygdala(A)** Acute ethanol exposure encouraged NPY mRNA expression in NAcS, but not changed the expression in NAcC region compared to n-saline. **(B)** A substantial decline in NPY mRNA expression recognized in chronic ethanol exposure followed by ethanol withdrawal rat group in NAcS region yet no effect of either chronic exposure of ethanol or withdrawal of ethanol found in NAcC region. **(C)**Though in BLA region no changes observed, but in CeA and MeA regions exposure of acute ethanol induced the NPY mRNA expression compared to n-saline rat group. **(D)**However, in BLA region no changes detected, but in CeA and MeA regions ethanol withdrawal after chronic ethanol exposure significantlydecreased the NPY mRNA expression compared to control group and chronic ethanol groups.Values represented as mean $\pm$ SEM of 9 rats in each group. (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; for statistical analysis between two groups Student's *t-test* and for more than two group ANOVA followed by *posthoc* Tukey's test used.

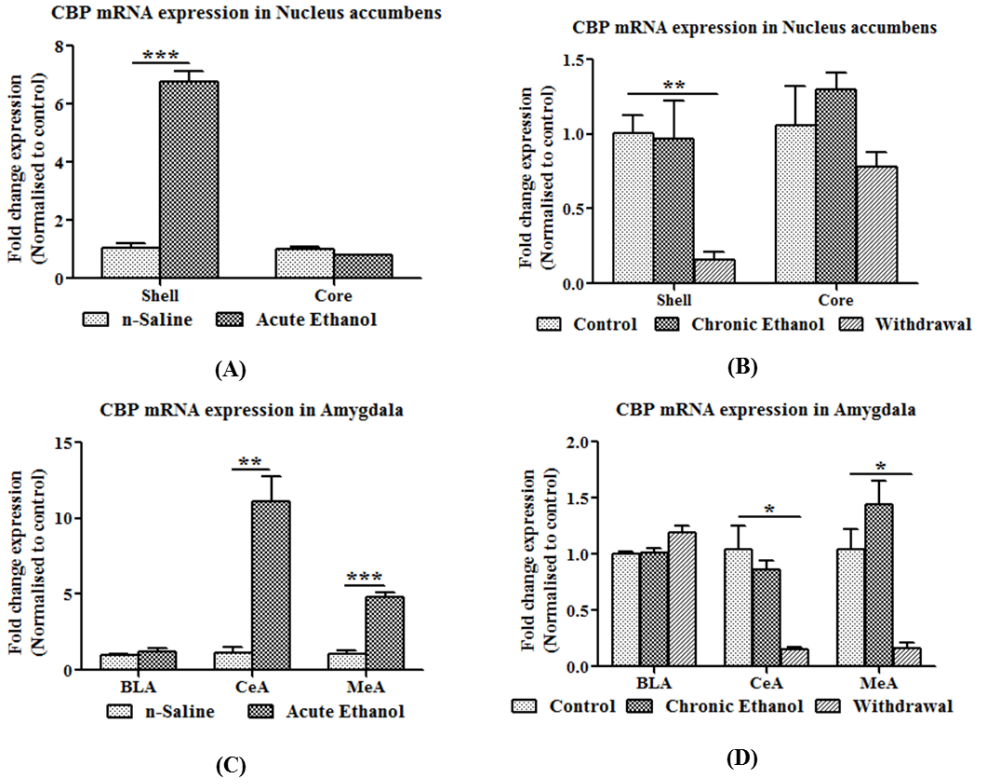



**Figure 5.12: CBP mRNA expression in nucleus accumbens and amygdala(A)** Acute ethanol exposure encouraged CBP mRNA expression in NAcS, but not changed the expression in NAcC region compared to n-saline. **(B)** A considerable decline in CBP mRNA expression found in chronic ethanol exposure followed by ethanol withdrawal rat group in NAcS region yet no effect of either chronic exposure of ethanol or withdrawal of ethanol found in NAcC region. **(C)**Nevertheless, in BLA region no changes detected, on the other hand in CeA and MeA regions exposure of acute ethanol induced the CBP mRNA expression compared to n-saline rat group. **(D)**Further, in BLA region no changes detected, but in CeA and MeA regions ethanol withdrawal after chronic ethanol exposure significantlydecreased the CBP mRNA expression compared to control group and chronic ethanol groups.Values represented as mean $\pm$ SEM of 9 rats in each group. (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; for statistical analysis between two groups Student's *t-test* and for more than two group ANOVA followed by *posthoc* Tukey's test used.

| Creb mRNA | Acute ethanol group | | Chronic ethanol group | | Withdrawal Group | |
|---|---|---|---|---|---|---|
| | Fold change | Significance Level | Fold change | Significance level | Fold change | Significance Level |
| NAcS | +0.34 | $p>0.05$ | +0.12 | $p>0.05$ | -0.03 | $p>0.05$ |
| NAcC | -0.32 | $p>0.05$ | -0.42 | $p>0.05$ | -0.51 | $p>0.05$ |

| c-fos mRNA | Acute ethanol group | | Chronic ethanol group | | Withdrawal group | |
|---|---|---|---|---|---|---|
| | Fold change | Significance Level | Fold change | Significance level | Fold change | Significance level |
| NAcS | +7.25 | $p<0.001$ | +0.06 | $p>0.05$ | -2.12 | $p<0.05$ |
| NAcC | +0.37 | $p>0.05$ | +0.15 | $p>0.05$ | -0.13 | $p>0.05$ |

| Arc MRNA | Acute ethanol group | | Chronic ethanol group | | Withdrawal group | |
|---|---|---|---|---|---|---|
| | Fold change | Significance level | Fold change | Significance level | Fold change | Significance level |
| NAcS | +1.70 | $p<0.01$ | -0.03 | $p>0.05$ | -3.48 | $p<0.01$ |
| NAcC | -0.43 | $p>0.05$ | +0.17 | $p>0.05$ | -0.42 | $p>0.05$ |

| Npy mRNA | Acute ethanol group | | Chronic ethanol group | | Withdrawal group | |
|---|---|---|---|---|---|---|
| | Fold change | Significance level | Fold change | Significance level | Fold change | Significance level |
| **NAcS** | +3.24 | $p<0.01$ | -0.03 | $p>0.05$ | -3.48 | $p<0.001$ |
| **NAcC** | -0.06 | $p>0.05$ | +0.17 | $p>0.05$ | -0.42 | $p>0.05$ |

| Cbp mRNA | Acute ethanol group | | Chronic ethanol group | | Withdrawal group | |
|---|---|---|---|---|---|---|
| | Fold change | Significance level | Fold change | Significance level | Fold change | Significance level |
| **NAcS** | +5.57 | $p<0.001$ | -0.04 | $p>0.05$ | -5.31 | $p<0.01$ |
| **NAcC** | -0.26 | $p>0.05$ | +0.23 | $p>0.05$ | -0.36 | $p>0.05$ |

**Table 5.3:** Summary of fold change in mRNA expression in nucleus accumbens shell (NAcS) and core (NAcC) regions of acute ethanol exposed group, chronic ethanol exposed group and withdrawal group. (Fold change was compared to their respective control groups; '+' sign represents increased expression and '-' sign indicates reduced expression).

| Creb mRNA | Acute ethanol Group | | Chronic ethanol Group | | Withdrawal Group | |
|---|---|---|---|---|---|---|
| | Fold change | Significance Level | Fold change | Significance Level | Fold change | Significance Level |
| BLA | +0.15 | $p>0.05$ | -0.17 | $p>0.05$ | +0.03 | $p>0.05$ |
| CeA | +0.07 | $p>0.05$ | +0.25 | $p>0.05$ | +0.17 | $p>0.05$ |
| MeA | -0.36 | $p>0.05$ | +0.01 | $p>0.05$ | +0.19 | $p>0.05$ |

| c-fos mRNA | Acute ethanol Group | | Chronic ethanol Group | | Withdrawal Group | |
|---|---|---|---|---|---|---|
| | Fold change | Significance Level | Fold change | Significance Level | Fold change | Significance Level |
| BLA | +0.48 | $p>0.05$ | -0.02 | $p>0.05$ | -0.17 | $p>0.05$ |
| CeA | +2.64 | $p<0.01$ | +0.05 | $p>0.05$ | -6.38 | $p<0.05$ |
| MeA | +1.69 | $p<0.01$ | +0.12 | $p>0.05$ | -11.92 | $p<0.01$ |

| Arc mRNA | Acute ethanol Group | | Chronic ethanol Group | | Withdrawal Group | |
|---|---|---|---|---|---|---|
| | Fold change | Significance Level | Fold change | Significance Level | Fold change | Significance Level |
| BLA | -0.02 | $p>0.05$ | -0.05 | $p>0.05$ | +0.08 | $p>0.05$ |
| CeA | +2.24 | $p<0.01$ | -0.08 | $p>0.05$ | -6.30 | $p<0.01$ |
| MeA | +2.12 | $p<0.01$ | +0.27 | $p>0.05$ | -10.61 | $p<0.001$ |

| Npy mRNA | Acute ethanol Group | | Chronic ethanol Group | | Withdrawal Group | |
|---|---|---|---|---|---|---|
| | Fold change | Significance Level | Fold change | Significance Level | Fold change | Significance Level |
| BLA | -0.40 | $p>0.05$ | +0.30 | $p>0.05$ | +0.42 | $p>0.05$ |
| CeA | +5.62 | $p<0.01$ | -0.19 | $p>0.05$ | -6.79 | $p<0.01$ |
| MeA | +2.12 | $p<0.01$ | -0.01 | $p>0.05$ | -9.83 | $p<0.001$ |

| Cbp mRNA | Acute ethanol Group | | Chronic ethanol Group | | Withdrawal Group | |
|---|---|---|---|---|---|---|
| | Fold change | Significance Level | Fold change | Significance Level | Fold change | Significance Level |
| BLA | +0.24 | $p>0.05$ | +0.01 | $p>0.05$ | +0.19 | $p>0.05$ |
| CeA | +8.76 | $p<0.01$ | -0.21 | $p>0.05$ | -6.08 | $p<0.01$ |
| MeA | +3.53 | $p<0.001$ | +0.39 | $p>0.05$ | -5.35 | $p<0.05$ |

**Table 5.4:** Summary of fold change in microRNA expression in basolateral amygdala (BLA), central amygdala (CeA) and medial amygdala (MeA) regions of acute ethanol exposed group, chronic ethanol exposed group and withdrawal group. (Fold change was compared to their respective control groups; '+' sign represents increased expression and '-' sign indicates reduced expression).

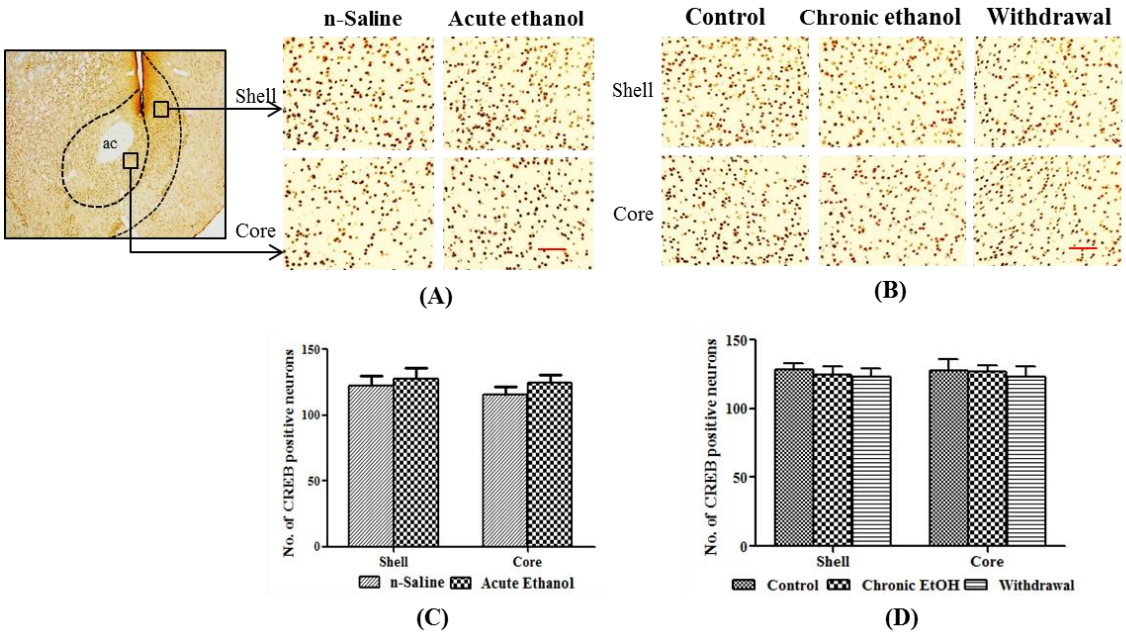



**Figure 5.13:**Representative photomicrograph of CREB (protein) expression in DAB immunostainned nucleus accumbens shell and core regions **(A)** in rat groups treated with either n-saline or acute ethanol (1gm/kg i.p. dose) and **(B)** rat groups treated with chronic ethanol and ethanol withdrawn after chronic ethanol exposure along with control rat group. **(C)** Exposure of acute ethanol neither in shell nor in core region influenced the CREB protein expression. **(D)**No change in CREB protein expression found under the effect of chronic ethanol exposure and also during ethanol withdrawal compared to control group. Scale bar, 40µm. Values represented as mean number of positive nuclei± SEM of 9 rats in each group. For statistical analysis between two groups Student's *t-test* and for more than two group ANOVA followed by *posthoc* Tukey's test used.(Significance level: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001)

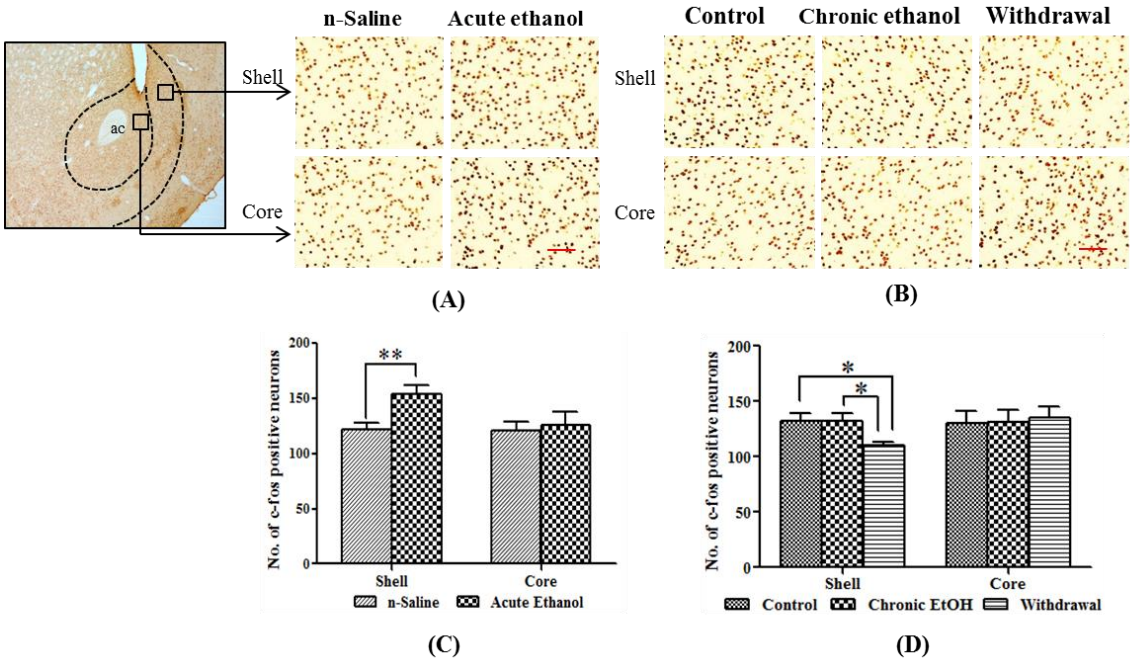



**Figure 5.14:**Representative photomicrograph of c-fos(protein) expression in DAB immunostainned nucleus accumbens shell and core regions **(A)** in rat groups treated with either n-saline or acute ethanol (1gm/kg i.p. dose) and **(B)** rat groups treated with chronic ethanol and ethanol withdrawn after chronic ethanol exposure along with control rat group. **(C)** Exposure of acute ethanol increased c-fos expression in shell region, but no change found in core region. **(D)**A significant reduction in c-fos protein expression identified during chronic ethanol exposure followed by ethanol withdrawal in shell region yet no effect of either chronic exposure of ethanol or withdrawal of ethanol found in core region. Scale bar, 40µm. Values represented as mean number of positive nuclei± SEM of 9 rats in each group. For statistical analysis between two groups Student's *t-test* and for more than two group ANOVA followed by *posthoc* Tukey's test used.(Significance level: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001)

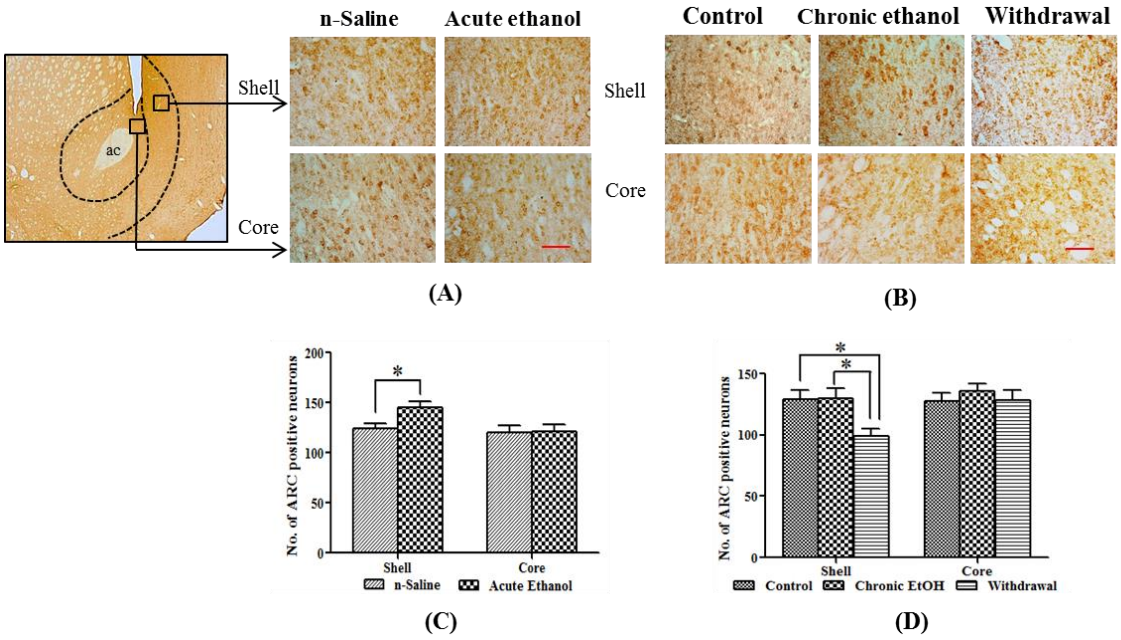



**Figure 5.15:**Representative photomicrograph of ARC(protein) expression in DAB immunostainned nucleus accumbens shell and core regions **(A)** in rat groups treated with either n-saline or acute ethanol (1gm/kg i.p. dose) and **(B)** rat groups treated with chronic ethanol and ethanol withdrawn after chronic ethanol exposure along with control rat group. **(C)** Exposure of acute ethanol increased ARC expression in shell region, but no change found in core region. **(D)**A significant reduction in ARCprotein expression identified during chronic ethanol exposure followed by ethanol withdrawal in shell region yet no effect of either chronic exposure of ethanol or withdrawal of ethanol after chronic exposure found in core region. Scale bar, 40µm. Values represented as mean number of positive nuclei± SEM of 9 rats in each group. For statistical analysis between two groups Student's *t-test* and for more than two group ANOVA followed by *posthoc* Tukey's test used.(Significance level: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001)

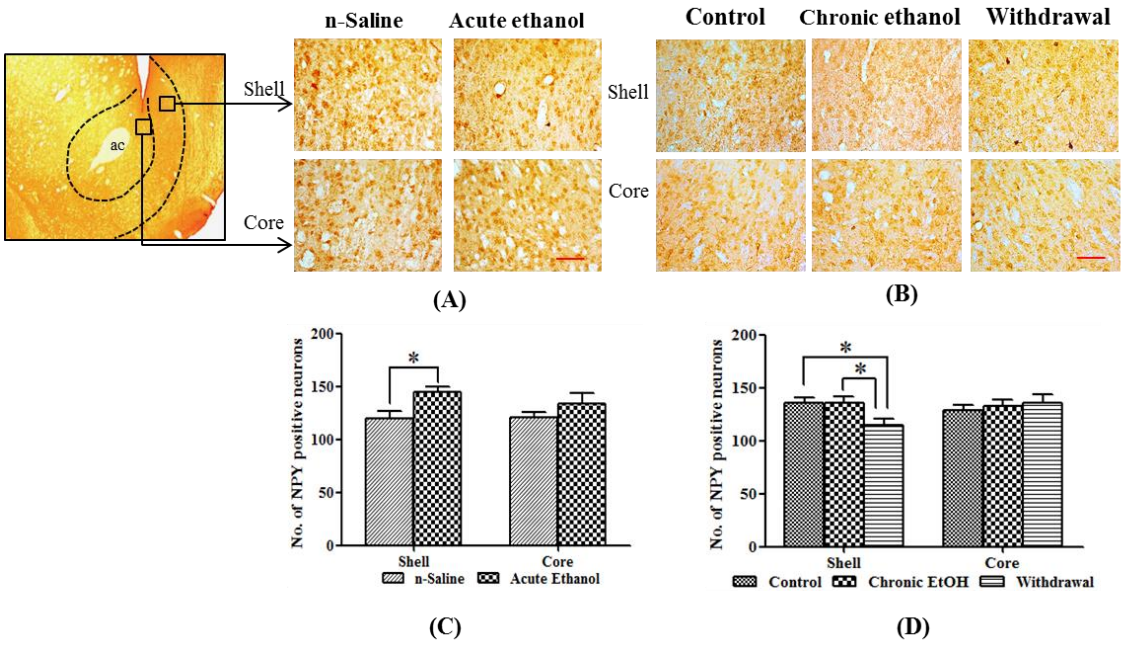



**Figure 5.16:**Representative photomicrograph of NPY(protein) expression in DAB immunostainned nucleus accumbens shell and core regions **(A)** in rat groups treated with either n-saline or acute ethanol (1gm/kg i.p. dose) and **(B)** rat groups treated with chronic ethanol and ethanol withdrawn after chronic ethanol exposure along with control rat group. **(C)** Exposure of acute ethanol increased NPY protein expression in shell region, but no change found in core region. **(D)**A significant decrease in NPYprotein expression recognized during chronic ethanol exposure followed by ethanol withdrawal in shell region however, no effect of either chronic exposure of ethanol or withdrawal of ethanol after chronic exposure found in core region. Scale bar, 40µm. Values represented as mean number of positive nuclei± SEM of 9 rats in each group. For statistical analysis between two groups Student's *t-test* and for more than two group ANOVA followed by *posthoc* Tukey's test used.(Significance level: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001)

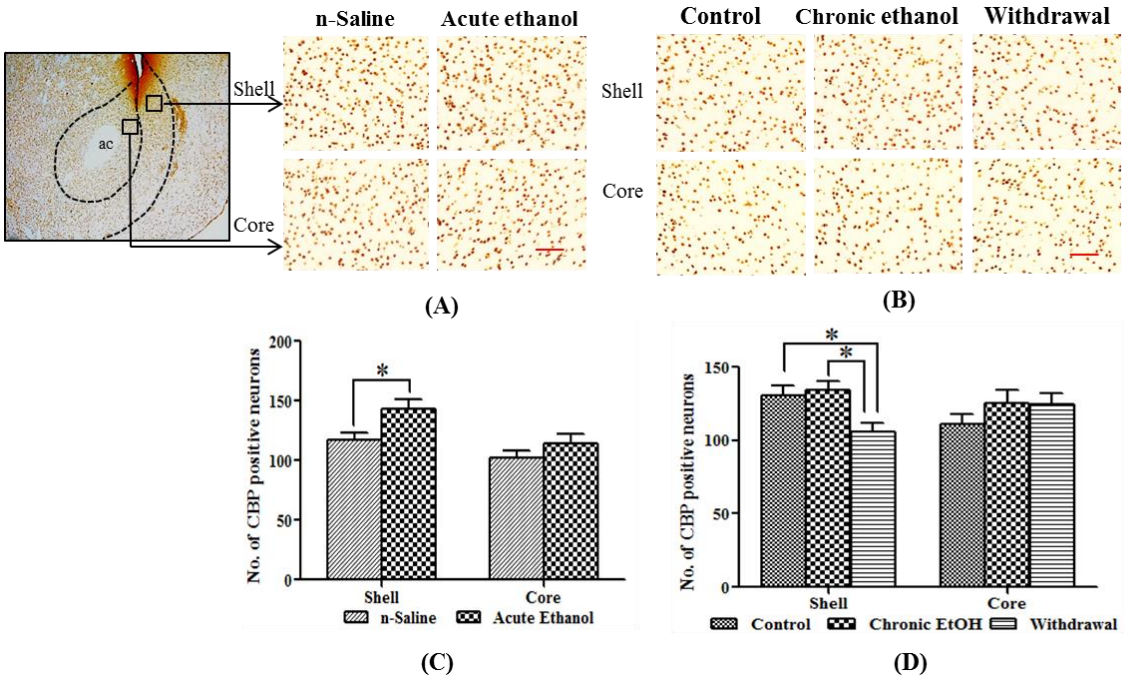



**Figure 5.17:**Representative photomicrograph of CBP(protein) expression in DAB immunostainned nucleus accumbens shell and core regions **(A)** in rat groups treated with either n-saline or acute ethanol (1gm/kg i.p. dose) and **(B)** rat groups treated with chronic ethanol and ethanol withdrawn after chronic ethanol exposure along with control rat group. **(C)** Exposure of acute ethanol increased CBP protein expression in shell region, but no change found in core region. **(D)**A significant decrease in CBPprotein expression recognized during chronic ethanol exposure followed by ethanol withdrawal in shell region however, no effect of either chronic exposure of ethanol or withdrawal of ethanol after chronic exposure found in core region. Scale bar, 40µm. Values represented as mean number of positive nuclei± SEM of 9 rats in each group. For statistical analysis between two groups Student's *t-test* and for more than two group ANOVA followed by *posthoc* Tukey's test used.(Significance level: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001)

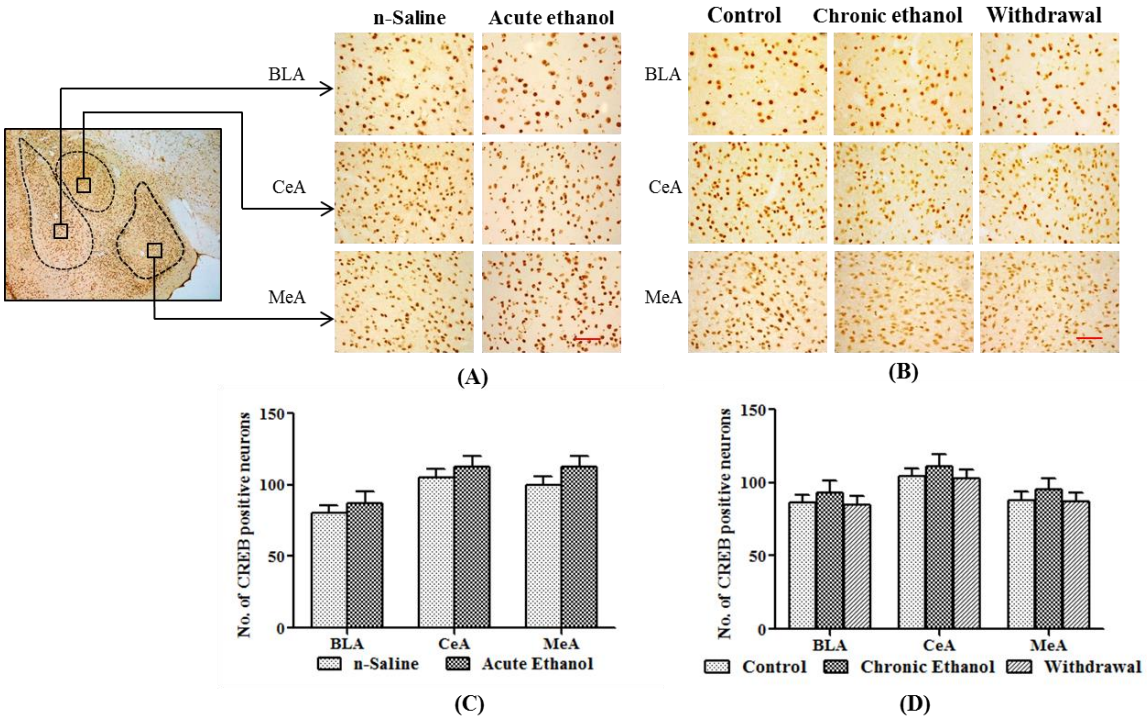



**Figure 5.18:** Representative photomicrograph of CREB (protein) expression in DAB immunostainned Basolateral amygdala (BLA), Central amygdala (CeA) and Medial amygdala (MeA) regions of extended amygdala**(A)** in rat groups treated with either n-saline or acute ethanol (1gm/kg i.p. dose) and **(B)** rat groups treated with chronic ethanol and ethanol withdrawn after chronic ethanol exposure along with control rat group. **(C)** Expression of CREB protein in BLA, CeA and MeA found unchanged during acute ethanol exposure with respect to n-saline group. **(D)**No change in CREB protein expression found in BLA, CeA and MeA under the effect of chronic ethanol exposure and also during ethanol withdrawal compared to control group. Scale bar, 40µm. Values represented as mean number of positive nuclei± SEM of 9 rats in each group. For statistical analysis between two groups Student's *t-test* and for more than two group ANOVA followed by *posthoc* Tukey's test used.(Significance level: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001)

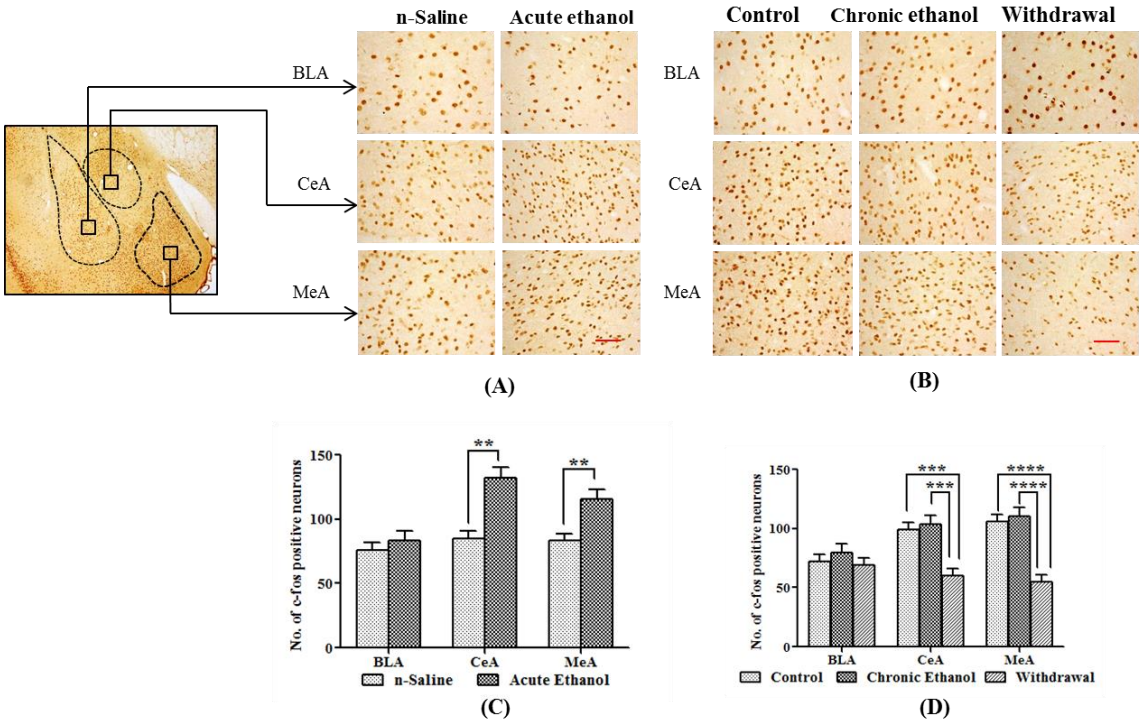



**Figure 5.19:** Representative photomicrograph of c-fos (protein) expression in DAB immunostainned Basolateral amygdala (BLA), Central amygdala (CeA) and Medial amygdala (MeA) regions of extended amygdala**(A)** in rat groups treated with either n-saline or acute ethanol (1gm/kg i.p. dose) and **(B)** rat groups treated with chronic ethanol and ethanol withdrawn after chronic ethanol exposure along with control rat group. **(C)** Expression of c-Fos protein in BLA found unchanged, but significantly increased in CeA and MeA during acute ethanol exposure relating to n-saline group. **(D)**No change in c-Fos protein expression found in BLA region however, in CeA and MeAregions reduced c-Fos expression was detected in ethanol withdrawal group compared to control group and chronic ethanol group. Scale bar, 40µm. Values represented as mean number of positive nuclei± SEM of 9 rats in each group. For statistical analysis between two groups Student's *t-test* and for more than two group ANOVA followed by *posthoc* Tukey's test used.(Significance level: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001)

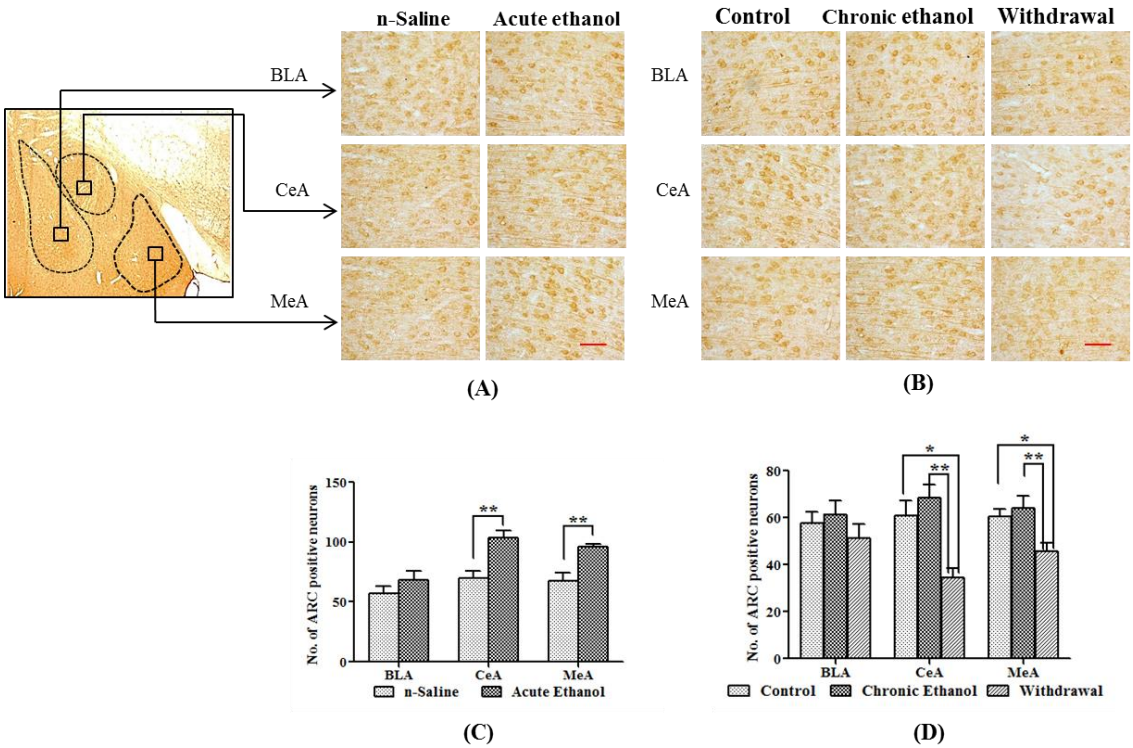



**Figure 5.20:** Representative photomicrograph of ARC (protein) expression in DAB immunostainned Basolateral amygdala (BLA), Central amygdala (CeA) and Medial amygdala (MeA) regions of extended amygdala**(A)** in rat groups treated with either n-saline or acute ethanol (1gm/kg i.p. dose) and **(B)** rat groups treated with chronic ethanol and ethanol withdrawn after chronic ethanol exposure along with control rat group. **(C)** Expression of ARC protein in BLA found unaffected, but significantly increased in CeA and MeA during acute ethanol exposure relating to n-saline group. **(D)**No change in ARC protein expression found in BLA region however, in CeA and MeAregions reduced ARC expression was detected in ethanol withdrawal group compared to control group and chronic ethanol group. Scale bar, 40µm. Values represented as mean number of positive nuclei± SEM of 9 rats in each group. For statistical analysis between two groups Student's *t-test* and for more than two group ANOVA followed by *posthoc* Tukey's test used.(Significance level: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001)

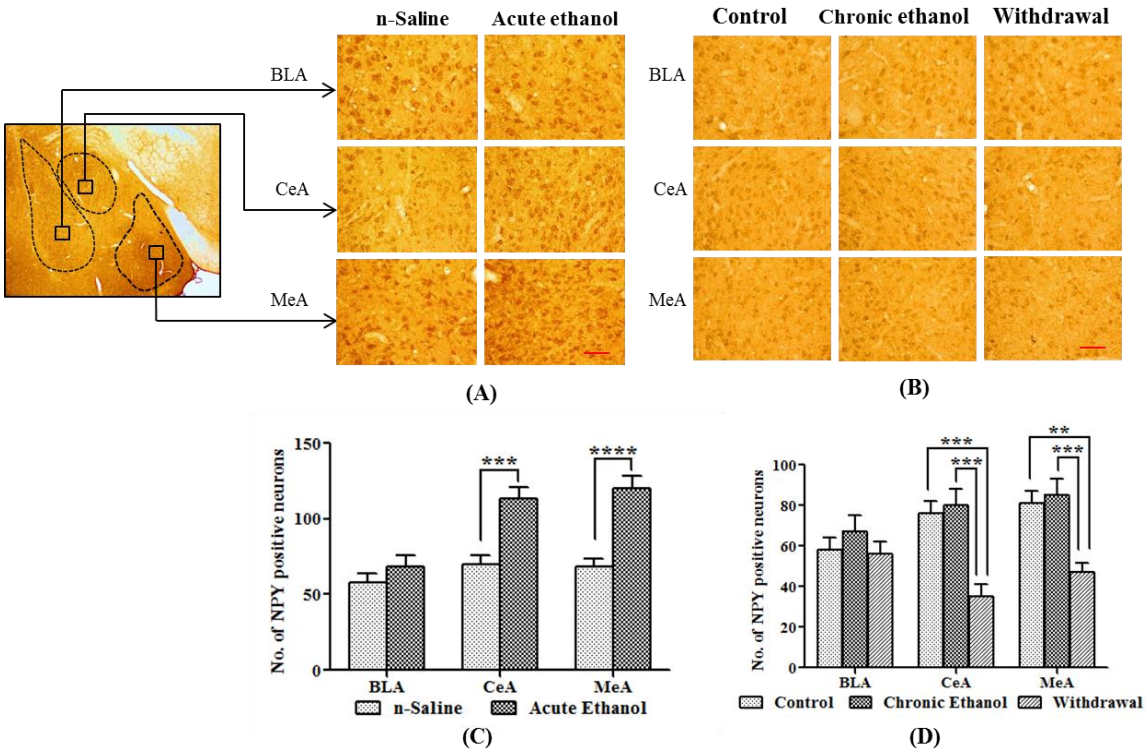



**Figure 5.21:** Representative photomicrograph of NPY (protein) expression in DAB immunostainned Basolateral amygdala (BLA), Central amygdala (CeA) and Medial amygdala (MeA) regions of extended amygdala**(A)** in rat groups treated with either n-saline or acute ethanol (1gm/kg i.p. dose) and **(B)** rat groups treated with chronic ethanol and ethanol withdrawn after chronic ethanol exposure along with control rat group. **(C)** Expression of NPY protein in BLA found unchanged, but significantly increased in CeA and MeA during acute ethanol exposure relating to n-saline group. **(D)**No change in NPY protein expression found in BLA region however, in CeA and MeAregions reduced NPY expression was detected in ethanol withdrawal group compared to control group and chronic ethanol group. Scale bar, 40µm. Values represented as mean number of positive nuclei± SEM of 9 rats in each group. For statistical analysis between two groups Student's *t-test* and for more than two group ANOVA followed by *posthoc* Tukey's test used.(Significance level: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001)

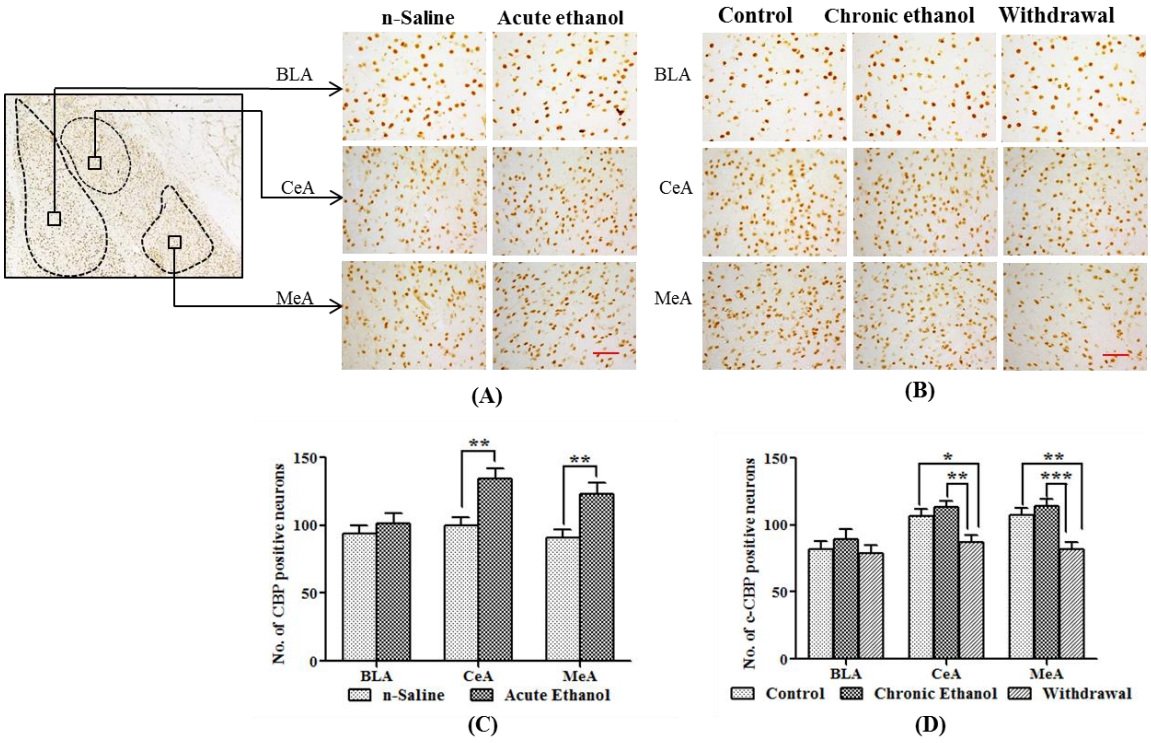



**Figure 5.22:** Representative photomicrograph of CBP (protein) expression in DAB immunostainned Basolateral amygdala (BLA), Central amygdala (CeA) and Medial amygdala (MeA) regions of extended amygdala**(A)** in rat groups treated with either n-saline or acute ethanol (1gm/kg i.p. dose) and **(B)** rat groups treated with chronic ethanol and ethanol withdrawn after chronic ethanol exposure along with control rat group. **(C)** Expression of CBP protein in BLA found unchanged, but significantly increased in CeA and MeA during acute ethanol exposure relating to n-saline group. **(D)**No change in CBP protein expression found in BLA region however, in CeA and MeAregions reduced CBP expression was detected in ethanol withdrawal group compared to control group and chronic ethanol group. Scale bar, 40µm. Values represented as mean number of positive nuclei± SEM of 9 rats in each group. For statistical analysis between two groups Student's *t-test* and for more than two group ANOVA followed by *posthoc* Tukey's test used.(Significance level: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001)

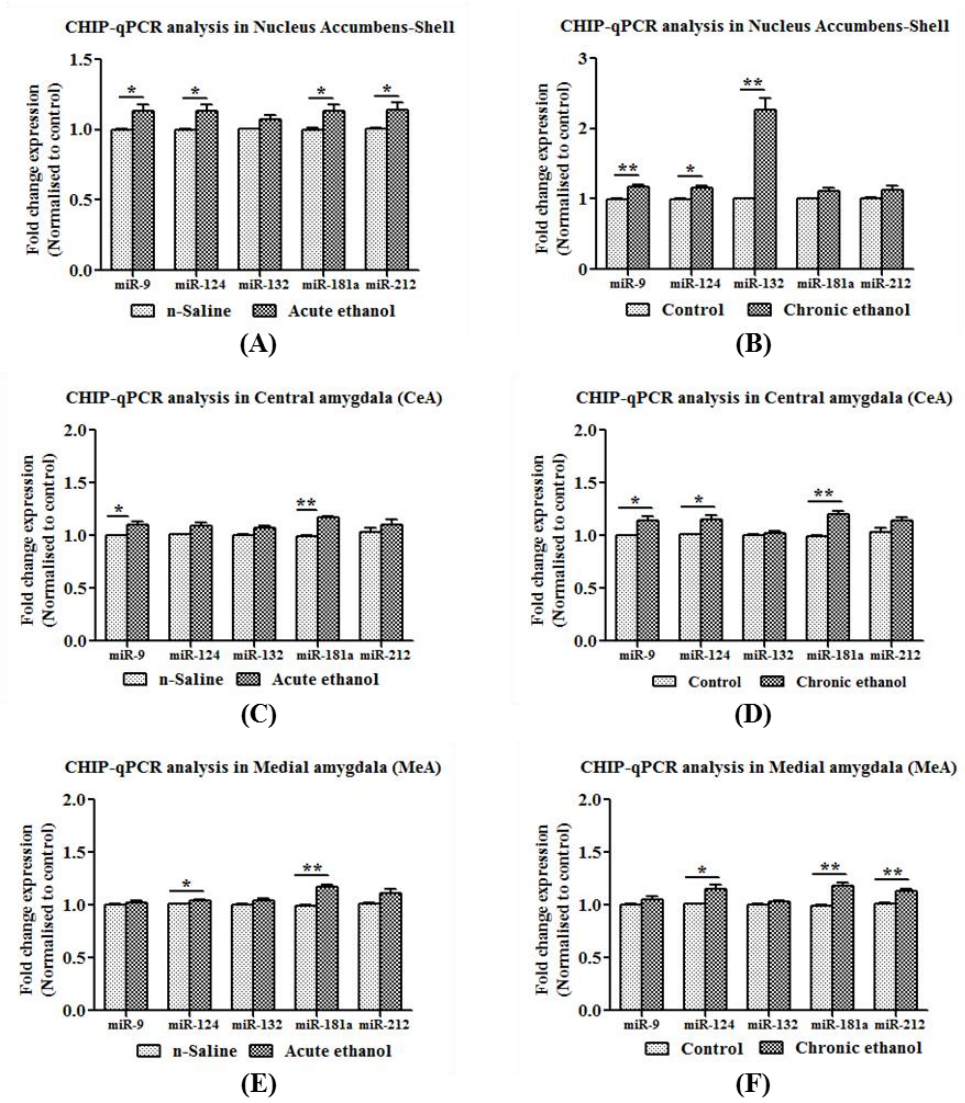



**Figure 5.23:** ChIP-qPCR analysis of CREB bound miRNA promoter in NAcS, CeA and MeA regions. **(A-B)** Representing changes in the expression of microRNA promoters, during acute and chronic ethanol exposure, in NAcS region **(C-D)** in CeA region and **(E-F)** in MeA region. Student's t-test was used for statistical analysis. (Significance level: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001)

| NAcS | Acute ethanol group | | Chronic ethanol group | |
|---|---|---|---|---|
| | Fold change | Significance level | Fold change | Significance level |
| miR-9 | +0.14 | $p<0.05$ | +0.18 | $p<0.01$ |
| miR-124 | +0.14 | $p<0.05$ | +0.16 | $p>0.05$ |
| miR-132 | +0.07 | $p>0.05$ | **+1.27** | $p<0.01$ |
| miR-181a | +0.14 | $p<0.05$ | +0.11 | $p>0.05$ |
| miR-212 | +0.14 | $p<0.05$ | +0.12 | $p>0.05$ |

| CeA | Acute ethanol group | | Chronic ethanol group | |
|---|---|---|---|---|
| | Fold change | Significance level | Fold change | Significance level |
| miR-9 | +0.11 | $p<0.05$ | +0.14 | $p<0.05$ |
| miR-124 | +0.08 | $p>0.05$ | +0.15 | $p<0.05$ |
| miR-132 | +0.06 | $p>0.05$ | +0.02 | $p>0.05$ |
| miR-181a | +0.17 | $p<0.05$ | +0.21 | $p<0.01$ |
| miR-212 | +0.07 | $p>0.05$ | +0.11 | $p>0.05$ |

| MeA | Acute ethanol group | | Chronic ethanol group | |
|---|---|---|---|---|
| | Fold change | Significance level | Fold change | Significance level |
| miR-9 | +0.02 | $p>0.05$ | +0.05 | $p>0.05$ |
| miR-124 | +0.04 | $p<0.05$ | +0.15 | $p<0.05$ |
| miR-132 | +0.03 | $p>0.05$ | +0.03 | $p>0.05$ |
| miR-181a | +0.18 | $p<0.01$ | +0.19 | $p<0.01$ |
| miR-212 | +0.10 | $p>0.05$ | +0.12 | $p<0.01$ |

**Table 5.5:** Summary of ChIP-qPCR analysis of CREB bound miRNA promoter in nucleus accumbens shell (NAcS), central amygdala (CeA) and medial amygdala (MeA) regions of acute ethanol exposed group and chronic ethanol exposed group. (Fold change was compared to their respective control groups; '+' sign represents increased expression and '-' sign indicates reduced expression).

| Prediction of association between miRNA and their target gene expression | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Nucleus accumbens-Shell (NAcS) | | | | | Nucleus accumbens-Core (NAcC) | | | | |
| | | CREB | c-fos | ARC | NPY | CBP | CREB | c-fos | ARC | NPY | CBP |
| Acute ethanol Group | miR-9 | nsc / Up | | | | | nsc / nsc | | | | |
| | miR-124 | nsc / Up | | Up / Up | | Up / Up | nsc / nsc | | nsc / nsc | | nsc / nsc |
| | miR-132 | | | Up / Up | | | | | nsc / nsc | | |
| | miR-181a | | Up / Up | | | Up / Up | | nsc / nsc | | | nsc / nsc |
| | miR-212 | | | nsc / Up | | | | | nsc / nsc | | |
| Chronic ethanol Group | miR-9 | nsc / Up | | | | | nsc / nsc | | | | |
| | miR-124 | nsc / Up | | nsc / Up | | nsc / Up | nsc / nsc | | nsc / nsc | | nsc / nsc |
| | miR-132 | | | nsc / Up | | | | | nsc / nsc | | |
| | miR-181a | | nsc / Up | | | nsc / Up | | nsc / nsc | | | nsc / nsc |
| | miR-212 | | | nsc / Up | | | | | nsc / nsc | | |
| Withdrawal Group | miR-9 | nsc / Down | | | | | nsc / nsc | | | | |
| | miR-124 | nsc / nsc | | Down / nsc | | Down / nsc | nsc / nsc | | nsc / nsc | | nsc / nsc |
| | miR-132 | | | Down / | | | | | nsc / nsc | | |
| | miR-181a | | Down / Down | | | Down / Down | | nsc / nsc | | | nsc / nsc |
| | miR-212 | | | Down / Down | | | | | nsc / nsc | | |

**Table 5.6:** Summary of the overall change in microRNAs expression and CREBand its target genes expression in Nucleus accumbens.(nsc= nonsignificant change; Up= upregulation; Down= downregulation).

| Prediction of association between miRNA and their target gene expression | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | BLA | | | | | CeA | | | | | MeA | | | | |
| | | Creb | c-fos | Arc | Npy | Cbp | Creb | c-fos | Arc | Npy | Cbp | Creb | c-fos | Arc | Npy | Cbp |
| Acute ethanol Group | miR-9 | nsc / nsc | | | | | nsc / Down | | | | | nsc / Down | | | | |
| | miR-124 | nsc / nsc | | nsc / nsc | | nsc / nsc | nsc / Down | | Up / Down | | Up / Down | nsc / Down | | Up / Down | | Up / Down |
| | miR-132 | | | nsc / nsc | | | | | Up / nsc | | | | | Up / Down | | |
| | miR-181a | | nsc / Down | | | nsc / Down | | Up / nsc | | | Up / nsc | | Up / Down | | | Up / Down |
| | miR-212 | | | nsc / nsc | | | | | Up / nsc | | | | | Up / Down | | |
| Chronic ethanol Group | miR-9 | nsc / nsc | | | | | nsc / Up | | | | | nsc / Up | | | | |
| | miR-124 | nsc / Down | | nsc / Down | | nsc / Down | nsc / Up | | nsc / Up | | nsc / Up | nsc / Up | | nsc / Up | | nsc / Up |
| | miR-132 | | | nsc / Down | | | | | nsc / Up | | | | | nsc / Up | | |
| | miR-181a | | nsc / nsc | | | nsc / nsc | | nsc / Up | | | nsc / Up | | nsc / Up | | | nsc / Up |
| | miR-212 | | | nsc / Down | | | | | nsc / Up | | | | | nsc / nsc | | |
| Withdrawal Group | miR-9 | nsc / | | | | | nsc / nsc | | | | | nsc / Up | | | | |
| | miR-124 | nsc / nsc | | nsc / nsc | | nsc / nsc | nsc / nsc | | Down / nsc | | Down / nsc | nsc / nsc | | Down / nsc | | Down / nsc |
| | miR-132 | | | nsc / nsc | | | | | Down / nsc | | | | | Down / Up | | |
| | miR-181a | | nsc / nsc | | | nsc / nsc | | Down / Up | | | Down / Up | | Down / Up | | | Down / Up |
| | miR-212 | | | nsc / nsc | | | | | Down / nsc | | | | | Down / Up | | |

**Table 5.7:** Summary of the overall change in microRNAs expression and CREB and its target genes expression in Amygdala regions. (nsc= nonsignificant change; Up= upregulation; Down= downregulation).

Excessive alcohol consumption is a major health concern globally and the effect of alcohol on human health has been widely studied from a long time. The cessation of prolonged ethanol consumption is often escorted by numerous withdrawal symptoms such as elevated anxiety, the risk of convulsions and tremors (Roelofs, 1985; Koob, 2003). Anxiety is a most common primary symptom of ethanol withdrawal and aids as an important element in the negative reinforcement leading to excessive and uncontrolled alcohol drinking (Weiss and Rosenberg, 1985; Kushner et al., 1990; Schuckit and Hesselbrock, 1994; Koob, 2003; Pandey, 2003). Some drugs and behavioral counseling have been established for the treatment of alcoholism and other addictions. Effects of ethanol on brain reward circuitry during different ethanol exposure conditions and also during ethanol-induced withdrawal symptoms, still not sufficient for the development of drugs to completely cure the addiction of ethanol. Studies related to the underlying mechanisms of alcoholism give some idea about the molecular, cellular and also behavioral changes occur in the influence of ethanol. The brain regions comprising limbic reward circuitry such as the amygdala, nucleus accumbens, prefrontal cortex, ventral pallidum, hippocampus and ventral tegmental area get compromise their functional abilities and hijacked by the effects of ethanol at molecular and cellular level.

Rodents have a characteristic exploratory behavior towards novel environment yet favor darker and closed spaces. For this study, Sprague Dawley (SD) rats have been used which have an intrinsic anxiety response. The time spent in the open arena of mazes is associated with the level of anxiety. The anxiolytic effect of ethanol during the acute ethanol treatment was assessed with the help of elevated plus maze test and light dark box test. In acute ethanol-exposed group, increase in the open arm entries and more time spent in the open arm represented the anxiolytic response of ethanol treated rats. The decrease in anxious behavior favors risk-taking decisions which result in the form of exploration of an open area of the maze without reluctance.

Acute i.p. (intraperitoneal) injection of ethanol significantly decreased the anxiety response by rats in both elevated plus maze and light dark box tests. Selection of the

i.p. dose 1gm/kg ethanol was established on the findings of investigators (Pandey et al., 2004). The acute exposure of ethanol significantly reduced the anxiety level in rats compared to the n-saline injected rats as the control group which were similar to other studies in humans (Lipscomb et al., 1980) and rodents (Pandey et al., 2004, 2005; Wilson et al., 2004). Further, the blood alcohol concentration in acute ethanol exposure 92.23+2.82mg/dl coincide the decreased anxiety and euphoric effect of initial alcohol intake in humans (Smith et al., 1975). Next, in the present study, we have used the forced feeding model for chronic ethanol exposure given in nutritionally complete liquid diet as their sole source of food and liquid. After protracted ethanol exposure rats were deprived to ethanol for 24hr to reach peak anxiety level (Pandey et al., 1998). The effect of ethanol treatment when given for a long-term or chronically, no change in the percent time spent and the number of open arm entries was assessed, which may be the result of adaptation towards the effect of ethanol. During ethanol withdrawal after the long term exposure of ethanol potentially affected the behavior through reduced activity in EPM (Pellow et al., 1985). The sudden cessation of ethanol after the chronic ethanol exposure may lead to develop the withdrawal-related symptoms such as increase in anxious behavior (Weiss and Rosenberg, 1985; Wilson, 1988; Lal et al., 1993; Rassnick et al., 1993; Koob, 2003; Pandey, 2003; Pandey et al., 2003, 2008). In light-dark box (LDB) test time spent in illuminated compartment compared to dark compartment represents the level of anxiety in rats. The more exploration in light box showed the anxiolytic effect of acute ethanol treatment. And in ethanol withdrawal after chronic ethanol treatment, the sharp reduction in exploratory activity may be due to withdrawal-induced anxiety which results to craving. So, both the anxiolytic and anxiogenic effects of ethanol depend on the quantity and period of ethanol exposure.

The brain-enriched miR-9 has been associated with the development of nervous system and physiological and pathological processes in several organisms (Coolen et al., 2012; Han et al., 2012; Kuang et al., 2012; Vennet et al., 2012; Sun et al., 2013; Li et al., 2014; Yao et al., 2014; Chang et al., 2014; Davila et al., 2014; Luxenhofer et al., 2014). In nervous system diseases such as glioblastoma and neurodegenerative

disorders, miR-9 plays a crucial role. In glioma cells, miR-9 and CREB minicircuitry regulate the proliferation and migration of glioma cells by a negative feedback mechanism (Tan et al., 2012). Yang et al. (2013) studied the role of miR-9 through the nuclear factor-kappa B (NF-κB) and cAMP response element-binding protein (CREB) pathways regulated proliferation of neuronal progenitor cells (NPC) and neuronal differentiation, but the NPC migration only involves CREB, not NF-κB. In the process of dementia, miR-9 plays an important role by increasing BACE1 expression via downregulation of CREB (Xie et al., 2017). The BK channels are plentiful in the brain and mediate various neuronal functions (Dworetzky et al., 1994). Another crucial role of miR-9 involves large conductance calcium-and voltage-activated potassium channel (BK) mRNA during ethanol exposure resulted in miR-9-dependent destabilization of BK mRNAs and increased activity of miR-9 in supraoptic neurons and striatal neurons isolated from the central nervous system (Pietrzykowski et al., 2008). In a study, the implication of cocaine-mediated response in increased miR-9 expression in NAc lysate was evaluated (Eipper-Mains et al., 2011). In the present study, in nucleus accumbens shell (NAcS) region the activity of miR-9 was increased during acute ethanol exposure and chronic ethanol exposure. The fold change in miR-9 expression was higher in chronic ethanol exposure group than the fold change in acute ethanol group. Conversely, the ethanol withdrawal after a protracted ethanol exposure decreased the miR-9 expression in NAcS region. Interestingly, the miR-9 expression was found unaffected in nucleus accumbens core (NAcC) region, either by acute ethanol exposure or chronic ethanol exposure and chronic ethanol exposure followed by 24hr ethanol withdrawal (Figure 5.3A-B; Table 5.1).

The further study concentrated towards gauging the effect of ethanol during acute and chronic exposure of ethanol and during ethanol cessation after protracted ethanol consumption on the expression of CREB and its target genes. Regulation of gene expression mediated by the activation of gene transcription factor CREB could be via phosphorylation (at serine-133) by cAMP-dependent protein kinase A (PKA), Ca2/calmodulin-dependent or mitogen-activated protein kinases. In addiction activated CREB recruit the multifunctional coactivators such as CBP (CREB-binding

protein) and p300 to the transcriptional machinery (Silva et al., 1998; Impey et al., 1999; Soderling, 1999; Korzus et al., 2004; Hsieh and Gage, 2005).

We gauged the mRNA and protein levels of CREB and its target genes such as c-fos, Arc (activity regulated cytoskeleton protein) and Npy (Neuropeptide Y) during acute ethanol exposure and chronic ethanol exposure and 24hr withdrawal after chronic ethanol exposure in nucleus accumbens shell (NAcS) and core (NAcC). Several investigators have shown that the intrinsic HAT (Histone acetyl transferase) activity of CBP has influential effects on chromatin remodeling in the brain and behavior (Alarcon et al., 2004; Korzus et al., 2004; Wood et al., 2005; Rubinstein and Taybi, 1963; Hennekam et al., 1992; Petrij et al., 1995). Keeping this in mind, the CBP mRNA and protein level was also assessed during acute ethanol exposure, chronic ethanol exposure and chronic ethanol exposure followed by 24hr ethanol withdrawal. The analysis of mRNA and protein expression of transcription factor CREB during acute ethanol exposure in nucleus accumbens represented consistency in mRNA or protein expression level in either shell or core regions of nucleus accumbens. Subsequently, chronic ethanol exposure as well as ethanol cessation after chronic ethanol exposure also not affected the expression of CREB mRNA and protein either in NAcS or NAcC regions (Figure 5.8A-B, 5.13A-D; Table 5.3). These findings are in line with the previous reports of Pandey et al. (2008, 2009). Although, the CREB mRNA has a target site for miR-9 (Xie et al., 2017) and miR-9 expression was found to be dynamic during acute ethanol exposure and chronic ethanol exposure and chronic ethanol exposure followed by ethanol cessation specifically in NAcS region, but no change in CREB mRNA and protein expression suggests that the regulation of CREB may not be directly mediated by miR-9 in nucleus accumbens under the influence of ethanol (Table 5.5).

Additionally, in extended amygdala regions, basolateral amygdala (BLA), central amygdala (CeA) and medial amygdala (MeA) there was increased expression of miR-9 in CeA and MeA regions, but not in BLA region. During acute ethanol exposure, chronic ethanol exposure and 24hr ethanol withdrawal no substantial change in miR-9

expression was observed in BLA region. However, in CeA and MeA regions, the expression of miR-9 decreased during acute ethanol exposure but increased during chronic exposure of ethanol. The ethanol withdrawal after chronic ethanol exposure resulted in changes in the mir-9 expression in MeA but not in the CeA region. The mRNA and protein expression of CREB was also assessed in the course of acute ethanol exposure, chronic ethanol exposure and ethanol withdrawal after prolonged ethanol exposure in BLA, CeA and MeA regions of amygdala to find association between miR-9 and CREB activity, and no change in CREB mRNA or protein activity was observed (Figure 5.8C-D; 5.18A-D; Table 5.4) These results are similar the findings of Pandey et al. (2003) under similar experimental setup. Although the observation of other reports support the interaction of miR-9 and CREB protein and miR-9 mediated expression of CREB mRNA (Tan et al., 2012; Yang et al. 2013), but the results of present study are dissimilar and this might be due to the competition between for targeting 3' UTR region of CREB mRNA by other microRNAs or the brain region-specific miR-9 activity towards the regulation of CREB..

Many reports suggest that the mir-124 is specifically expressed in the central nervous system (Lagos-Quintana et al., 2002; Deo et al., 2006). During brain development the expression of miR-124 increases (Krichevsky et al., 2003, 2006; Sempere et al., 2004; Smirnova et al., 2005). Mir-124 has a crucial role in neurogenesis, such as, promotion of neuronal differentiation via Ephrin-B1 (Arvanitis et al., 2010), BAF53a (Yoo et al., 2009), SOX9 (Cheng et al., 2009), SCP1 (Visvanathan et al., 2007) and PTBP1 (Makeyev et al., 2007); inhibit differentiation via NEUROD1 (Liu et al., 2011) and also inhibit synaptic activity through CREB1 (Rajasethupathy et al., 2009). In a study, the expression of miR-124 was found decreased after 3 days of ethanol withdrawal after a chronic ethanol exposure in limbic forebrain regions (Mizuo et al., 2012). Moreover, miR-132 has a profound influence on neuronal maturation through dendritic arborization and spinogenesis (Obrietan et al., 2014). Dysregulation in miR-132 expression also displayed a critical role in a number of neurocognitive disorders in mature brain considered by anomalous synaptogenesis, still not much is known related to the role of miR-132 in alcoholism.

Another important protein, activity regulated cytoskeleton protein (ARC) which is one of the CREB regulated gene, has a crucial role in synaptic plasticity and long-term memory processes including long-term potentiation (LTP), long-term depression (LTD), and postnatal development of the visual cortex (Guzowski et al., 2000; McIntyre et al., 2005). Furthermore, in various neurological diseases such as Angelman mental retardation syndrome, Alzheimer's disease, seizure development, syndromic autism and anxiety-like behaviour, are related to the dysregulation of ARC protein expression.

The bioinformatics approaches were practiced to predict the potential targets of miR-124 and miR-132. Mir-124 can target the 3' UTR region of CREB and CBP mRNA, however 3'UTR region of ARC has target site for binding with both miR-124 and -132, so the mRNA expression of Creb, Arc, and Cbp may be controlled by the events of miR-124 and -132 (for Arc mRNA) in nucleus accumbens and amygdala regions. In our study, during acute ethanol exposure and chronic ethanol exposure, a significant increase in the miR-124 and -132 expressions were found in NAcS region, but NAcC region remained unaffected. In addition, withdrawal of ethanol insignificantly affected the miR-124 and -132 expressions in NAcS and NAcC regions (Figure 5.4A-B, 5.5A-B; Table 5.1). Evidence support that protracted exposure of ethanol detectably alters the long-term plasticity in nucleus accumbens and ventral striatum (Jeanes et al., 2010). Both acute and chronic exposure of ethanol contributes to the synaptic depression in nucleus accumbens which may promote the process of neuroadaptation for alcohol dependence (Jeanes et al., 2011). In our study, chronic ethanol exposure had a great impact on the induced expression of miR-124 and miR-132 over acute ethanol exposure approves the dysregulation of synaptic plasticity and synaptogenesis during ethanol orchestrated neural adaptation specifically in NAcS, however, the NAcC observations reflect no involvement.

Next, the mRNA and protein expression of ARC (Figure 5.10A-B, 5.15A-D), and CBP (5.12A-B, 5.17A-D) was analyzed and an ample increased expression during acute ethanol exposure in NAcS, but no change in NAcC region was observed (Table

5.3). In support of the previous investigations on drug abuse, during ethanol withdrawal after long-term ethanol exposure, decreased mRNA and protein expression level of ARC and CBP in NAcS region perceived; conversely, during chronic ethanol exposure no change in mRNA and protein expression was detected (Pandey et al., 2008).

In BLA, CeA and MeA region, during acute ethanol exposure reduction in miR-124 as well as in miR-132 was observed, nonetheless, the change in expressions in BLA were very less (fold change<1.2). Remarkably, chronic exposure of ethanol significantly reduced the miR-124 and -132 activity in BLA, but in CeA and MeA regions induced miR-124 and -132 expression identified, on the other hand, withdrawal of ethanol not affected the miR-124 and -132 expression in BLA (fold change<<1.2), CeA and MeA regions of amygdala (Figure 5.4C-D, 5.5C-D; Table 5.1). The mRNA and protein expression of ARC and CBP during acute ethanol exposure induced in NAcS, CeA and MeA regions; however no difference was detected in NacC and BLA regions. During chronic ethanol exposure followed by 24hr ethanol withdrawal significantly reduced the mRNA and protein expression of ARC and CBP in NAcS, CeA and MeA regions, yet no change in expressions were observed in NAcC and BLA regions; on the contrary, no change in mRNA and protein expression of ARC and CBP was detected in chronic ethanol exposure group study in nucleus accumbens (shell and core) and in BLA, CeA and MeA regions of amygdala (Figure 5.10, 5.12; Table 5.3, 5.4), favors the previous investigations (Pandey et al, 2003, 2008).

Another miRNA under study was miR-181a, which is differentially expressed under the influence of cocaine treatment. Role of miR-181a in the extinction and reinstatement of cocaine-induced conditioned place preference in a study investigated the differential expression of miR-181a in nucleus accumbens (Chandrasekar et al., 2011). MiR-181a at least has putative target sites on at least four cocaine-suppressed genes expressed in the different regions of the midbrain (Toda et al., 2002; Yuferov et al., 2003). Although very less information is available related to miR-181a and addiction, yet the common brain regions of the reward circuitry and regions related to

learning and memory promote the more specific studies. MicroRNA target prediction database used to locate the possible targets of miR-181a and 3' UTR region of c-fos and CBP mRNA foreseen as a potential target. In NAcS region, the miR-181a was induced during acute ethanol and chronic ethanol exposure but then reduced during chronic ethanol exposure terminated with ethanol withdrawal. Though miR-181a was active in NAcS, no noteworthy difference was found during acute and chronic ethanol exposure and during ethanol withdrawal (Figure 5.6A-B). Moreover, c-fos mRNA and protein were increased by the acute ethanol exposure and reduced after 24hr of ethanol cessation after chronic ethanol exposure in NAcS region, but during chronic ethanol exposure, the mRNA and protein expression was consistent with the control group. In NAcC, insignificant change in c-fos mRNA and protein expression was observed (Figure 5.9A-B, 5.14A-D; Table 5.1, 5.3).

Next, in BLA region, the expression of mir-181a was significantly affected by the acute ethanol exposure, in contrast, an insignificant change was found during chronic ethanol exposure and chronic ethanol exposure followed by 24hr ethanol termination. Conversely, in CeA region, the expression of mir-181a was significantly induced by the chronic ethanol exposure and ethanol withdrawal, yet no change observed by the acute exposure of ethanol. In contrary to the expression of miR-181a in BLA and CeA, miR-181a remained active in MeA region during acute ethanol and chronic ethanol exposure along with during ethanol withdrawal but acutely exposed ethanol reduced and chronic ethanol-induced the miR expression which was consistent during ethanol withdrawal after chronic ethanol treatment (Figure 5.6C-D). The expression of c-fos mRNA and protein was increased during acute ethanol exposure in CeA and MeA regions. In contrast to the inducible effect of acute ethanol, withdrawal of ethanol after protracted ethanol exposure significantly reduced the c-fos expression in CeA and MeA regions, yet no change in expression was observed during chronic ethanol exposure. In BLA, insignificant change in c-fos mRNA and protein expression was observed whether ethanol was exposed acutely or chronically (Figure 5.9C-D, 5.19A-D; Table 5.2, 5.4).

One more miRNA-212 was studied due to its role in CREB, MeCP2 and BDNF signaling in response to cocaine-mediated behavioral and motivation (Hollander et al., 2010; Im et al., 2010). MiR-212 is evidently present in the striatum of rat brain. The chronic exposure of cocaine induces the miR-212 expression in the dorsal striatum (Hollander et al., 2010). In the ventral tegmental area of the brain non-dopaminergic neurons represented the differential expression of miR-212, stimulated by an addictive drug-Nicotine (Keller et al., 2017). Mir-212 also plays a crucial role in oligodendrocyte maturation and its processes (Wang et al., 2017). Several studies have been implicated in the identification of the importance of mir-212 in cocaine seeking and relapse behavior (Quinn et al., 2017), yet the role of mir-212 during the development of ethanol dependence and tolerance as well as ethanol withdrawal-related anxiety remains unclear. The regions of limbic reward circuitry triggered changes by cocaine in mir-212 may also show some correlation with ethanol addiction, so we also analyzed mir-212 during different ethanol exposure conditions in nucleus accumbens and amygdala regions. In addition, microRNA target prediction tool showed that along with miR-124 and -131, Arc mRNA can also be targeted by miR-212.

During acute ethanol exposure and chronic ethanol exposure a significant increase in the miR-212 expression detected, but then a decreased expression was found in NAcS region by ethanol withdrawal after chronic ethanol exposure, furthermore, NAcC region continued unchanged during acute ethanol, chronic ethanol exposure and chronic ethanol exposure followed by ethanol withdrawal (Figure 5.7A-B; Table 5.1). Furthermore, in BLA and CeA regions miR-212 was affected only during chronic ethanol exposure, not during acute ethanol exposure or chronic ethanol exposure followed by withdrawal however in MeA region chronic ethanol not altered the miR-212 expression, but changed during acute ethanol exposure and ethanol withdrawal after chronic ethanol exposure (Figure 5.7C-D; Table 5.2). The miR-212 targeted ARC mRNA and protein expression in nucleus accumbens (shell and core) and amygdala (basolateral amygdala, central amygdala, and medial amygdala) regions

already discussed during acute ethanol exposure, chronic ethanol exposure and chronic ethanol exposure followed by ethanol interruption.

By the fact that transcription factor CREB regulates the expression of various genes in brain, another protein Neuropeptide Y (NPY), is also found crucial in alcohol addiction and potentially regulated by the CREB (Higuchi et al., 1988; Akabayashi et al., 1994; Pandey et al., 2004). Neuropeptide Y (NPY) is highly expressed in several brain regions including cortex, hippocampus, and amygdala (Allen et al., 1983; Heilig and Widerlov, 1990; Wettstein et al., 1995), and has been associated with several behaviors such as feeding, anxiety, and alcohol drinking (Clark et al., 1984; Heilig et al., 1993; Heilig and Widerlov, 1995; Thorsell and Heilig, 2002; Pandey, 2003; Pandey et al., 2003a). In addition, a mutation in the NPY gene of mice makes it more vulnerable to the development of ethanol withdrawal syndrome (Sparta et al., 2007). Although, any of the miRNAs selected in this study not found as direct potential regulators for NPY through Target prediction database, but indirectly through the regulation of CREB, this CREB regulated gene may also show some relevance with the miRNAs expression index. The expression of NPY mRNA and protein was evidently induced during acute ethanol exposure in NAcS region, still, the NAcC remained unchanged. However, the acute exposure of ethanol-induced the NPY mRNA and protein expression in shell region, withdrawal of ethanol after chronic ethanol consumption remarkably reduced the NPY mRNA and protein expression, yet the expression remained unaltered during the chronic ethanol exposure. The long-term exposure of ethanol and chronic ethanol exposure followed by ethanol withdrawal had no effect on the mRNA and protein expression of NPY in NAcC region.

The indexing of the change in expression of selected microRNAs and their predicted target genes expression, during acute ethanol and chronic ethanol exposure, differ very prominently, specifically in nucleus accumbens-shell (NAcS), central amygdala (CeA) and medial amygdala (MeA) regions compared to the core of nucleus accumbens and basolateral amygdala. Further, the role of epigenetic remodeling in miRNA expression

is also evidenced, so the changes occur during acute ethanol and chronic ethanol exposure in miRNA activity may be due to the different epigenetic mechanisms.

In the direction of scrutiny of the cause of active gene transcription binding of CREB at the promoter regions of all miRNAs (miR-9, -124, -132, -181a and -212) was investigated. The collected tissues from acute ethanol and chronic ethanol-exposed rat brains, having desired accumbal or amygdaloid region were subjected to chromatin immunoprecipitation (ChIP) analysis using an anti-CREB antibody and quantified the level of CREB bound at the promoter region for microRNA compared with their respective n-saline/control group.

In NAcS region, the binding of CREB at the promoter of the miR-9, miR-124, miR-181a and miR-212 in animals subjected to acute ethanol exposure, found a statistically significant increase in expression, yet the fold change < 1.20, but the miR-132 promoter not affected by acute ethanol treatment. However, chronic ethanol exposure had statistically significantly induced the expression of promoter of miR-9, miR-124, and miR-132 in NAcS region, only miR-132 promoter represented the fold change >1.20; and miR-181a and -212 promoters found unaltered. During acute ethanol exposure, CREB binding at the promoter of miR-9 and miR-181a, and during chronic ethanol exposure promoter of miR-9, miR-124 and miR-181a remained unaltered owing less than 1.2 fold change with statistically significant expression change in CeA region. MiR-124, miR-132 and miR-212 promoterexpression site in CeA region, was not changed by acute ethanol exposure, and miR-132 as well as miR-212 promoter expression unaltered during chronic ethanol exposure. Towards the end, CREB mediated change in miRNA promoter was analyzed in MeA regions of acute and chronic ethanol-exposed rats. There was no change in the activity of CREB at miR-9 and -132 promoter observed during both acute and chronic ethanol exposure.Additionally promoter of miR-124 and miR-181a in acute ethanol group and promoter of miR-124, miR-181a, and miR-212 in chronic ethanol group were insignificantly changed. The expression of miR-212 promoter also found unaffected during acute ethanol exposure.

In conclusion to the ChIP-qPCR study, the changes observed during acute and chronic ethanol treatment in NAcS, CeA, and MeA, were statistically significant, yet the fold change was extremely less than 1.2 fold, which may suggest, no direct role of CREB on the promoter of selected microRNAs. Remarkably, in NAcS region, chronic ethanol exposure had a significant increase in miR-132 promoter expression due to increased occupancy of CREB rather than insignificant change during acute exposure to ethanol. Though the fold change is slightly more than the threshold (i.e.1.27) and also not highly significant ($p<0.01$) (Table 5.5, 5.6, 5.7), result demands further investigation.

Alcohol addiction is a chronic relapsing disorder and is characterized by repetitive alcohol drinking patterns leading to a loss of control over alcohol consumption (Koob, 2003). The alarming rate at which the use of alcohol is increasing the number of alcoholics it is imperant to decipher the molecular mechanisms underlying addiction. This will help to develop newer drug targets for rehabilitation and it will also shed some light on the molecular events and the pathways involved in memory formation as most of the pathways that are utilized for addiction overlap with that of memory formation. The neuroadaptational changes induced by exposure to alcohol and drugs of abuse may be related to dysregulation of signaling systems, gene transcription, and protein expression at the cellular level (Nestler, 2001; Koob et al., 1998; Pandey, 2004). The mesolimbic dopaminergic pathway has been shown to be a key mediator in the rewarding effects of alcohol. Molecular and cellular changes in the nucleus accumbens with acute and repeated alcohol exposure may underlie certain aspects in the development of alcohol addiction (Koob et al., 1998; Pandey, 2004; Gonzales et al., 2004).

The dysphoric state induced during alcohol withdrawal is a robust factor in the maintenance of both alcohol drinking and the eventual development of alcohol addiction (Koob,2003; Pandey,2004). Amygdaloid brain regions, specifically the central nucleus of the amygdala (CeA) and medial nucleus of amygdala (MeA), appear to be associated with the dysphoric effects of alcohol withdrawal, particularly the promotion of anxiety-like behaviors (Koob,2003; Pandey, 2004; Gonzales et al., 2004). CREB plays a central role in the process of addiction (Nestler, 2001; Gonzales et al., 2004; Carlezon, 2005; Spanagel et al.,2009). Ethanol has a complex pharmacological profile and various signaling systems have been identified as modulators of CREB function that may serve as potential ethanol targets (Spanagel et al., 2009; Morrow et al., 2004; Harris et al., 2008). A great deal of research has focused on the role of CREB and its target genes, such as neuropeptide Y (NPY), brain-derived neurotrophic factor (BDNF), activity-regulated cytoskeleton-associated (Arc) protein, and corticotrophin-releasing factor (CRF) in the development of alcohol addiction (Heilig & Koob, 2007 ; Thorsell, 2008; Davis, 2008; Pandey et al, 2008). In

addition, several studies have identified novel epigenetic mechanisms, such as histone modification-induced chromatin remodeling and DNA methylation, in the process of alcohol-related neuroadaptation (Shukla et al, 2008; Pandey et al., 2008).

A well-known means of post-transcriptional regulation of gene expression is the inhibition of translation via microRNA (miRNA). MiRNAs are expressed at high levels in the brain (Fiore et al., 2008; Bartel, 2009), and the involvement of miRNA in numerous aspects of normal and abnormal brain function has been reported (Fiore et al., 2008; Bushati & Cohen, 2008). In addition to classically defined epigenetic mechanisms, microRNAs (miRNAs) can also convey epigenetic-like characteristics through post-transcriptional regulation of gene expression (Saetrom et al., 2007). MiRNAs can rapidly regulate gene expression by targeting certain mRNAs for degradation or through specific inhibition of mRNA translation. How the environmental changes or drugs of abuse bring about changes in the expression pattern of miRNAs is not well known. So such studies may shed light on the regulatory mechanisms controlling gene expression of miRNAs and might reveal newer targets which can be used for novel drug development for overcoming different brain disorders and addiction etc. The selected set of microRNAs (miR-9, -124, -132, 181a and -212) has been found active in many types of neurological processes such as, synaptogenesis, synaptic plasticity and neurotoxicity related to drug abuse and most drugs of abuse use the same neural circuitry involved in the addiction which might be directly or indirectly regulating the ethanol responsive transcription factor CREB and its target genes. The present proposal is envisaged to closely look at this mechanism.

The hypothesis of the work was to understand the mechanism during chronic exposure to ethanol which may lead to dysregulation of a subset of miRNAs, normally resisting the changes in the gene expression on acute exposure of ethanol, resulting in loss of this homeostatic control through miRNAs and allowing the modulation of circuitries responsible for alcohol addiction. The animal model of alcoholism developed by treatment with chronic ethanol and withdrawal group by the sudden cessation of ethanol for 24 hrs after prolonged ethanol exposure and in acute exposure group single

dose of ethanol injected (1 gm/kg dose; ethanol was diluted to 0.2 gm/ml in n-saline and was injected as 5µl/gm of body weight) and subjected to anxiety measurement paradigms i.e. elevated plus maze (EPM) and light dark box (LDB). Nucleus accumbens (shell and core) and amygdala (basolateral amygdala, central amygdala and medial amygdala) regions were analyzed due to their vulnerability during exposure of ethanol. The changes occur in these regions during acute and chronic ethanol exposure assessed by molecular studies, using TaqMan assay based qRT-PCR, DAB-immunohistochemistry and chromatin-immunoprecipitation-qPCR (ChIP-qPCR).

The indexing of the change in expression of selected microRNAs and their predicted target genes expression, differ very prominently, specifically in nucleus accumbens-shell (NAcS), central amygdala (CeA) and medial amygdala (MeA) regions compared to the core of nucleus accumbens (NACc) and basolateral amygdala (BLA), during acute ethanol and chronic ethanol exposure. Further, to find the changes occur during acute ethanol and chronic ethanol exposure in miRNA activity due to the CREB mediated control on the microRNA promoter suggests, though the changes observed during acute and chronic ethanol treatment in NAcS, CeA, and MeA, were statistically significant, yet the fold change was extremely less than 1.2 fold, which suggests that there is no direct role of CREB on the promoters of selected microRNAs. Remarkably, in NAcS region, chronic ethanol exposure had a significant increase in miR-132 promoter expression due to increased occupancy CREB rather than insignificant change during acute exposure to ethanol. We have utilized two different criteria's for the interpretation of microRNA and mRNA expression in this series which include statistical analysis and threshold fold change must be more than 1.20 (>1.20). Though the fold change is slightly more (1.27) than the threshold (i.e.1.20) and also not highly significant ($p<0.01$), so the result demands further investigation.

To the best of our knowledge this is the first study which shows the changes in the activity of microRNAs which may help to maintain the homeostasis during the ethanol exposure get affected during the chronic ethanol exposure and withdrawal after protracted ethanol consumption may be responsible for the development of addiction

Thus this study may provide a glimpsein darker sky of addiction field and may open new vistas for better prognosis and drug development of alcoholism and other drugs addiction.

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## List of Publications:

- Novel 1,4-benzothazines obliterate COX-2 mediated JAK-2/STAT-3 signals with potential regulation of oxidative and metabolic stress during colorectal cancer. Rai A, Kumar U, Raj V, Singh AK, Kumar P, Keshari AK, Kumar D, Maity B, De A, Samanta A, **Nath S**, Prakash A, Gosipatala SB, Chand G, Saha . (2017). Pharmacological Research. https://doi.org/10.1016/j.phrs.2017.12.010

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# Ameliorative effects of pyrazinoic acid against oxidative and metabolic stress manifested in rats with dimethylhydrazine induced colonic carcinoma

Anil K. Sahdev, Vinit Raj, Ashok K. Singh, Amit Rai, Amit K. Keshari, Arnab De, Amalesh Samanta, Umesh Kumar, Atul Rawat, Dinesh Kumar, Sneha Nath, Anand Prakash & Sudipta Saha

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