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EPIGENETIC AND TRANSCRIPTIONAL ALTERATIONS IN ALCOHOL USE DISORDER: FOCUS ON BDNF AND OPIOID SYSTEMS IN BRAIN REWARD AND STRESS CIRCUITS

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ABSTRACT

Drug addiction is defined as a chronic relapsing disorder of compulsive drug seeking and taking, characterized by a three-stages recurring cycle: binge/intoxication, withdrawal/negative affect and preoccupation/anticipation. Several repetition of this cycle induces allostatic changes in brain reward and stress systems; in particular, allostatic changes represents a combination of the anti-reward system activation and subsequent chronic decrease function of rewards circuits. Although alcohol does not have a specific pharmacologic target, it directly and indirectly interacts with several targets activating the reward pathways. Similar to other drug of abuse, alcohol prolonged exposure and withdrawal induces a decrease of stress buffer system signaling, such nociceptin, neuropeptide Y and brain derived neurotrophic factor (BDNF), and promotes the recruitment of several brain stress systems, such as the corticotropin releasing factor (CRF) and the dynorphin (DYN) / κ opioid (KOP) receptor system. The misbalance of these systems contributes to the negative emotional states (i.e. anxiety, depression) associated with alcohol use disorder (AUD).

A growing body of evidence underlines that neuroplasticity phenomena induced by alcohol and other drugs of abuse involve epigenetic modifications, such as histone modifications, which in turn regulate gene expression. Therefore, in the present study we aimed to investigate epigenetic and transcriptional alterations induced by alcohol in different paradigms of alcohol exposure, in order to identify molecular and functional mechanisms involved in the AUD and the associated negative emotional states.

In the first part, the protein levels of histone deacetylases (HDACs) 1, 2 and 3 belonging to the class I in the caudate putamen (CPu) and prefrontal cortex (PFCx), two areas of the mesocorticostriatal circuitry. In particular, BDNF heterozygous (+/-) mice, which voluntary consume high amount of alcohol, and wild type (WT) animals were acutely

injected with EtOH and subsequently molecular analysis was conducted. Results showed that EtOH-treated WT mice has lower protein levels of all HDAC isoforms investigated in the CPu and HDAC 3 in the PFCx, suggesting that EtOH is able to modulate the epigenetic machinery. Moreover, different basal levels of HDACs class I have been detected in the BDNF +/- mice. HDAC 1, 2, 3 protein levels are lower in the CPu as well as HDAC 3 in the PFCx; on the contrary, HDAC 1 and 2 protein levels are significantly higher in the PFCx of BDNF +/- animals. Therefore, BDNF seems to be crucial in regulating epigenetic mechanisms comprising the levels of class I HDACs. Interestingly, genetic manipulation of BDNF has different consequences on HDAC levels in the CPu and PFCx suggesting that BDNF could play different role in distinct brain regions.

In the second part, we focused on the role of DYN/KOP system in different model of alcohol dependence and tolerance. The first model of alcohol dependence is represented by alcohol preferring rats (Marchigian Sardinian alcohol preferring rats, msP) exposed to the chronic intermittent two bottle free-choice paradigm. The gene expression analysis was conducted in the amygdala (AMY) and bed nucleus of stria terminalis (BNST) and revealed that msP animals have higher basal levels of KOP receptor mRNA in the AMY compared to their counterpart Wistar rats. KOP receptor is involved in the alcohol preference and consumption; in fact, KOP knock out animals exhibited low preference for EtOH. Moreover, EtOH-exposed msP rats show a down-regulation of prodynorphin (PDYN) and KOP receptor gene expression in the AMY. The activation of DYN/KOP system has been associated with anxious and depressive signs; therefore, the down-regulation here reported could be related to the attenuation of the anxio-depressive phenotype of the msP rats following alcohol exposure previously

reported. Finally, a decrease of KOP receptor mRNA has been detected in the BNST of msP rats following alcohol consumption.

The second model of alcohol dependence investigated is the chronic liquid diet; Sprague-Dawaley rats were fed with EtOH or control liquid diet for 15 days and then one group of EtOH exposed rats underwent 24 hours withdrawal. It has been previously reported that withdrawn animals after the chronic liquid diet exposure showed anxious symptoms. Here, we observed an increase of PDYN and KOP receptor mRNA levels in the AMY of withdrawn rats and a decrease of KOP receptor in the BNST. The opposite regulation of KOP receptor gene expression in the AMY and BNST observed during withdrawal and in msP rats suggests that dysregulation of the KOP receptor in these areas may contribute to the development of the negative emotional state associated to alcohol dependence.

Finally, we investigated a model of rapid tolerance to the anxiolytic effects of EtOH (rapid EtOH tolerance, RET). EtOH was acutely injected and then animals were tested in the elevated plus maze showing anxiolytic-like behavior; however, a second injection of EtOH 24 hours apart does not elicit any anxiolytic effect indicating that animals developed tolerance. It is interesting to note that the development of tolerance is related to the anxiolytic effect exerted by EtOH since no metabolic tolerance, measured as blood alcohol levels, has been observed. Tolerant animals showed an increase of PDYN and KOP receptor mRNA levels in the AMY and no changes in the BNST. Similar alterations of DYN/KOP system in the AMY have been detected in the two alcohol dependence models and RET model, arising the hypothesis that amygdaloid neuronal mechanisms leading to the negative affective consequences of alcohol dependence and rapid tolerance can be analogue. Interestingly, in the present study epigenetic analysis in the AMY revealed that the DYN/KOP system gene expression can be mainly regulated

by two histone marks, the trimethylation of lysine 27 and 4 on histone 3 (H3K27me3 and H3K4me3), during acute EtOH exposure and tolerance.

In conclusion, the present thesis provide new information on epigenetic mechanisms involving the BDNF and DYN/KOP systems in the AUD identifying these epigenetic alterations as potential therapeutic targets to treat or prevent alcoholism and alcohol-associated emotional disorders.

CHAPTER I.

INTRODUCTION

1. DRUG ADDICTION

1.1. OVERVIEW

Drug addiction is defined as a "*chronic relapsing disorder of compulsive drug seeking and taking, characterized by a three-stages recurring cycle: binge/intoxication, withdrawal/negative affect and preoccupation/anticipation*" (Koob and Le Moal, 1997). The *binge/intoxication* stage is the first phase in which drugs of abuse induces dopamine and opioid peptide release in the nucleus accumbens (NAc) (Volkow et al., 2007) exerting rewarding effect and recruiting other areas, such as the dorsal striatum (DS), relevant for the habit formation. The second stage, the *withdrawal/negative affect* stage, is characterized by loss of function in the reward system, particularly in the NAc, and recruitment of the brain stress system (i.e. the extended amygdala). The occurrence of these phenomena induces subjects to pursue in drug seeking and taking. Finally, the *preoccupation/anticipation* stage derives from a disruption of decision-making and behavioural inhibition mediated by the prefrontal cortex (PFCx) (Koob, 2015).

The repetition of this cycle over time induces allostatic changes in the brain reward and stress system. Allostasis can be defined as "*stability through changes*" (Koob, 2015); therefore, allostatic state is a state of chronic deviation of the regulatory system from its homeostatic level (Koob and Le Moal, 2001 and 2008) (Figure 1). Therefore, the *allostatic state* represents a combination of the anti-reward system activation and subsequent chronic decrease function of rewards circuits, both leading to the compulsive drug seeking behavior and loss of control in limiting drug intake.

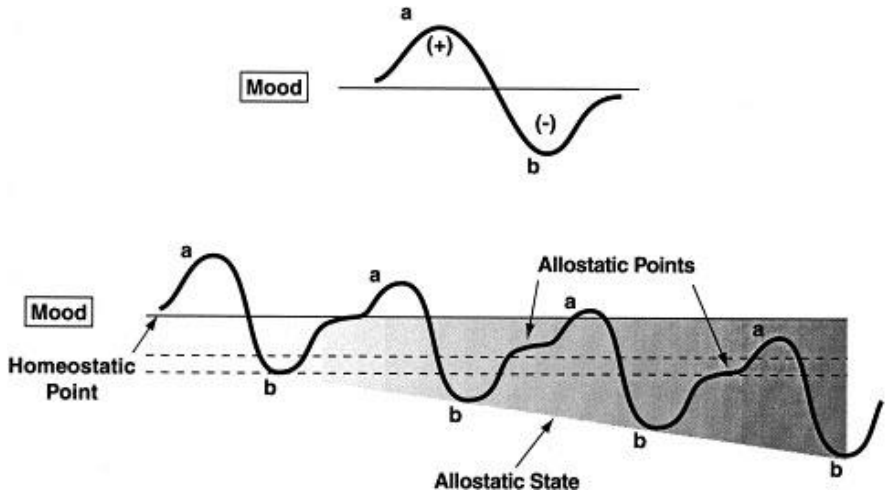

Figure 1. Affective response to the presentation of a drug. On the top, there is a schematic representation of the initial experience of drug in a subject with no prior drug history. The a(+)-process represents the positive mood state, while the b(-)-process represents the negative emotional state; an appropriate opponent b-process balancing the activational a-process is hypothesized to retain the homeostatic point. On the bottom, individual with repeated frequent drug use may have a transition to an allostatic state in the brain reward systems and, as a consequence, to addiction. In this case, the opponent b-process does not counterbalance the a-process that shows a residual hysteresis (Koob and Le Moal, 2001).

1.2. BRAIN REWARD SYSTEMS

Alcohol and other drug of abuse are able to produce rewarding effect. The role of the mesocorticolimbic and nigrostriatal dopamine (DA) pathway is crucial in mediating drug reward (Wise, 2009). The mesolimbic dopamine pathway is enriched of dopaminergic neurons projecting from the ventral tegmental area (VTA) to cortical and forebrain regions, such as the NAc, the amygdala (AMY) and the PFCx (Di Chiara et al, 2004; Nestler, 2005; Volkow et al, 2004) (Figure 2)., All drug of abuse, interacting with different molecular targets, induce an increase of DA release in the nucleus accumbens (NAc) (Di Chiara and Imperato, 1986). DA and opioid peptide release can induce other neuroadaptations, such as the recruitment of the glutamate-modulated *N*-methyl-D-aspartate (NMDA) receptors, in glutamatergic projections from the PFCx and the AMY to the VTA and the NAc (Kalivas PW, 2009). These neuroadaptations are responsible for leading to tolerance and withdrawal and triggering drug-associated cue exposure to increase DA levels in the DS, a crucial region in the habit formation processes (Belin D et al., 2013). The subsequent recruitment of cortical-striatal-pallidal-thalamic circuits is important to maintain the strong desire (craving) and the compulsive use of the drug when subjects are exposed to drug-associated cues (Koob, 2015).

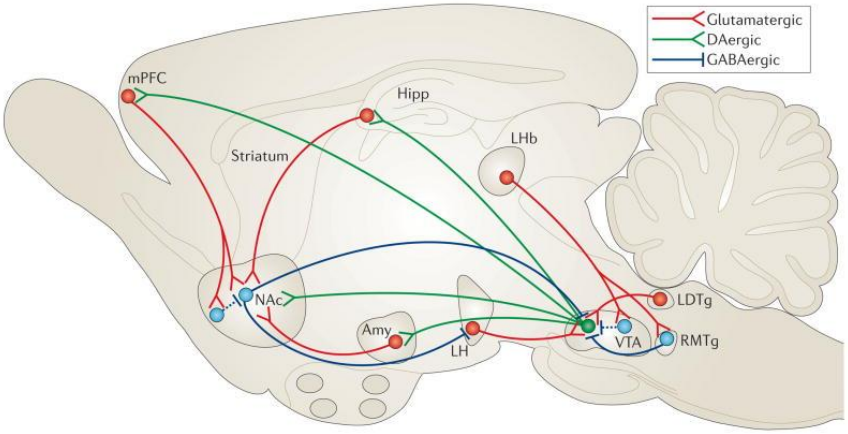

Figure 2. Brain reward circuit. Major dopaminergic, glutamatergic and GABAergic connections to and from the VTA and NAc in the rodent brain. The dopaminergic projections from the VTA to the NAc, which release dopamine in response to reward-related stimuli, is the primary reward circuit. There are also GABAergic projections from the NAc to the VTA. The NAc receives dense glutamatergic innervation from the medial PFCx, hippocampus (HIPPO) and amygdala (Amy). The VTA receives such inputs from amygdala, lateral dorsal tegmentum (LDTg), lateral habenula (LHb) and lateral hypothalamus (LH). (from Russo and Nestler, 2013. Nat Rev Neurosci. 14(9):609-25.)

1.3. BRAIN STRESS SYSTEMS

As mentioned above, the *withdrawal/negative affect* stage is characterized by loss of function in the reward system, particularly in the NAc, and recruitment of the brain stress system. The use of all major drug of abuse causes a dysregulation of the corticotropin-releasing factor (CRF) in the hypothalamic-pituitary-adrenal (HPA) axis and extrahypothalamic nuclei, resulting in an increase of adrenocortitropic hormone, corticosterone and amygdala CRF during acute withdrawal (Olive MF et al., 2002; Rasmussen DD et al., 2000; Roberto M et al., 2010). It has been hypothesized that the HPA axis activation can be an early dysregulation associated to the drug intake that triggers alterations of the extrahypothalamic CRF (Koob and Kreek, 2007; Vendruscolo et al., 2012). Together with the recruitment of the stress systems, during acute and

protracted withdrawal the occurrence of anxiety-like responses have been detected; the anxiety-like symptoms are reversed by administration of CRF antagonists (Zorrilla et al., 2014). In particular, the anxiolytic effect induced by CRF antagonists have been localized in the central nucleus of the amygdala (CeA) (Rassnick et al., 1993). Moreover, CRF antagonists are able to block the aversive-like motivational effects elicited by drug withdrawal (Heinrichs et al., 1995; Stinus et al., 2005) and the increase of drug self-administration (George et al., 2007; Greenwell et al., 2009; Specio et al., 2008).

Beside the CRF, the opioid peptide dynorphin (DYN) produces aversive and dysphoric-like behavior and mediates negative emotional states (Wee and Koob, 2010). In fact, the DA and opioid peptide release activates the DYN system, which acting by a feedback mechanism decreases the DA release and contributes to the dysphoric syndrome (Nestler, 2004). Activation of DYN system in the extended amygdala is also responsible for depressive and anxiogenic-like responses to stress during drug withdrawal (Chartoff et al., 2012; Knoll et al., 2007; Land et al., 2008). Moreover, κ opioid (KOP) receptor antagonists block the excessive and compulsive-like drug self-administration (Walker et al., 2010; Wee et al., 2009).

In order to counteract the effects of the pro-stress and pro-negative emotional state system activation, the return to the homeostasis can be facilitated by the emotional buffer system activation; components of the stress buffer system are the neuropeptide Y (NPY), nociceptin (N/OFQ) and endocannabinoids (Koob, 2015).

Therefore, the decrease of the brain reward systems and activation of the brain stress systems produce a negative emotional state that is more than a transient homeostatic dysregulation and it is known as *allostatic state* (Koob, 2015).

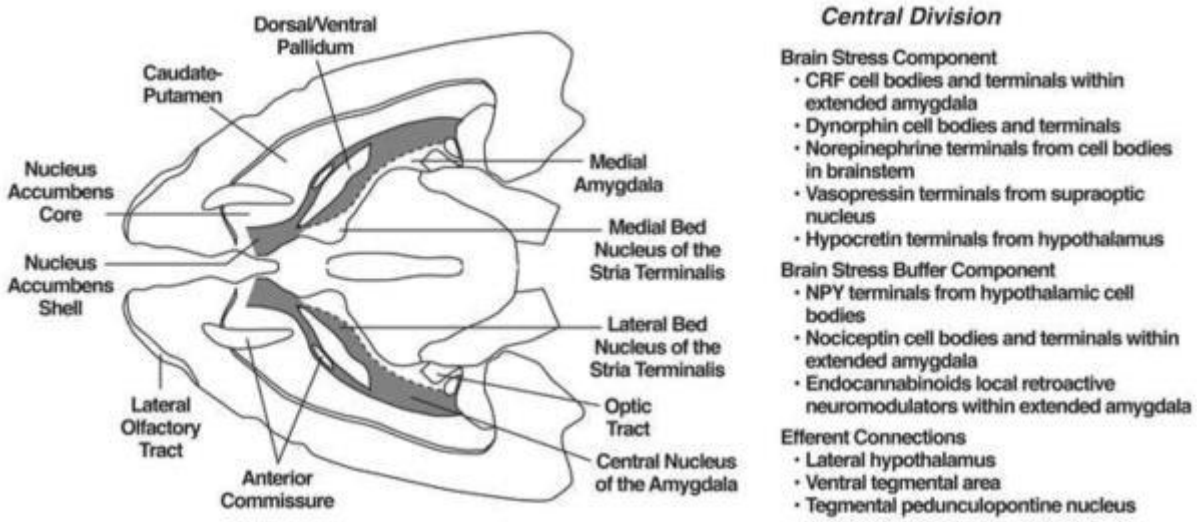

Figure 3. Horizontal section of rat brain: the extended amygdala and modulation via brain arousal-stress systems. On the left, a schematic representation of the central division of the extended amygdala with the central nucleus of the amygdala and lateral bed nucleus of the stria terminalis and a transition area in the shell of the nucleus accumbens highlighted. On the right, a description of the brain stress systems and brain stress buffer systems in the extended amygdala. Most of the brain stress or brain stress buffer systems are either local circuits or derived from hypothalamus or brainstem (Koob, 2015).

2. ALCOHOL USE DISORDER (AUD)

2.1. OVERVIEW

The alcohol use disorder (AUD) is classified as a "Substance-Related and Addictive Disorder" in the fifth edition of the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-5) (American Psychiatric Association, 2013). Although there is considerable overlap between the DSM-5 and the prior edition (DSM-IV), important differences in the terminology and diagnostic criteria were added.

According the DSM-5, any subject meeting two or more symptoms from the list of 11 criteria during the same 12-month period would receive a diagnosis of AUD. The number of symptoms defines the severity of the AUD as follow:

- Mild, in the presence of 2 to 3 diagnostic criteria;
- Moderate, in the presence of 4 to 5 symptoms;
- Severe, in the presence of 6 or more symptoms.

In the DSM-IV, there were different criteria for alcohol abuse and alcohol dependence, now integrated under a single disorder, the AUD. In the Figure 4, a comparison of previous and new diagnostic criteria is presented. It is interesting to note that in the DSM-IV, legal problems are listed as a criterion; however, this criterion has been removed in the DSM-5, and the criterion of craving was added.

Based on the Global status report of the World Health Organization (WHO) on alcohol and health 2014, it has been estimated that all over the world in one year 3.3 million deaths result from harmful use of alcohol, representing about the 6 % of all deaths. Moreover, more than 200 disease and injury conditions are caused by the harmful use of alcohol, and again about 5 % of the global burden of disease and injury is attributable to alcohol, measured as disability-adjusted life years. In the young age group (20 – 40

years), approximately 25 % of the total early deaths are alcohol-attributable (World Health Organization, 2014).

Alcohol consumption is affected by a variety of individual, societal and environmental factors, such as economic development, culture, availability of alcohol, and the comprehensiveness and levels of implementation and enforcement of alcohol policies. Although one risk factor is not more important than another is, generally the more vulnerabilities a person has, the more likely this person is to develop alcohol-related problems as result of alcohol consumption.

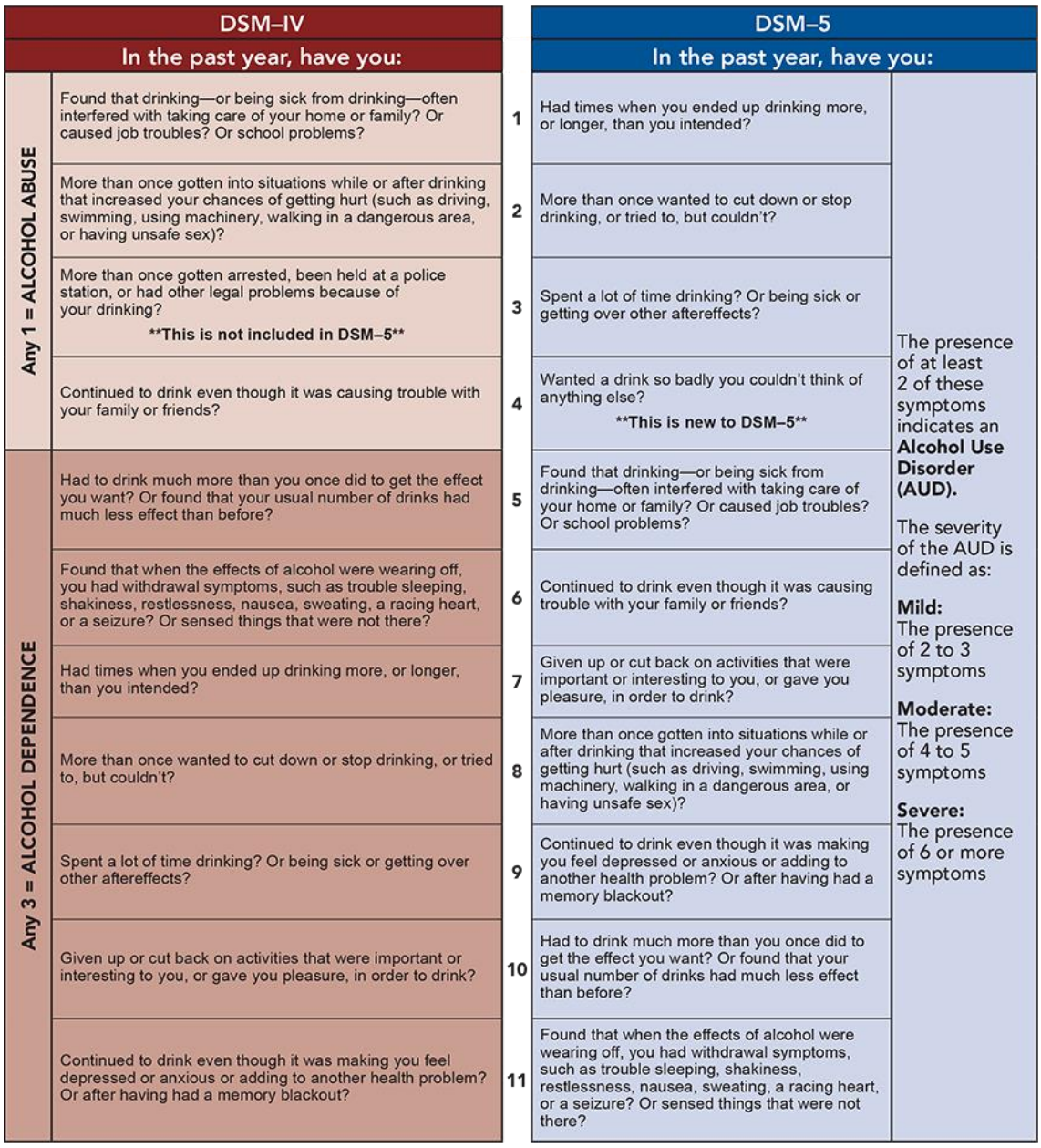

Figure 4. Criteria for the diagnosis of AUD. Comparison of diagnostic criteria between DSM-IV and DSM-5.

2.2. POSITIVE REINFORCEMENT OF ALCOHOL

Similarly to other drugs of abuse, alcohol is able to activate the reward pathways. In fact, it has been demonstrated that alcohol stimulates the DA release in the VTA-NAc pathway (Di Chiara and Imperato, 1986; Gessa et al, 1985). Pharmacological manipulation of dopamine D1 and D2 receptors was able to reduce alcohol consumption in animals, thus confirming the involvement of the DA system in mediating alcohol positive reinforcement (McBride et al, 1990; Samson et al, 1993). However, in contrast to other major drugs of abuse, alcohol does not have a specific pharmacologic target, but it directly and indirectly interacts with several targets. Alcohol directly interferes with the function of several ion channels and receptors, such as the ionotropic γ-aminobutyric acid (GABA) A receptors, L-type Ca2+ channels, nicotinic acetylcholine receptors (nAChR), metabotropic glutamate receptors (mGluRs), NMDA receptor and 5-hydroxytryptamine 3 (Vengeliene et al., 2008). For example, alcohol inhibits the function of NMDA receptor (Lovinger et al., 1989) and enhances the activity of $GABA_A$ receptors (Mihic, 1999). This is further supported by evidence demonstrating that during alcohol withdrawal GABAergic transmission is decreased and NMDA glutamatergic signaling is increased (Davidson et al., 1995; Roberts et al., 1996; Weiss et al., 1996).

Beside direct effects, alcohol induces a variety of indirect effects on several neurotransmitter/neuropeptide systems. For instance, alcohol can indirectly interact with endogenous opioid system in the mesolimbic pathway, with an increase in endogenous opioid peptide release (Gianoulakis, 1989; Johnson and North, 1992). In particular, beta-endorphin and enkephalins released by alcohol bind to the μ and δ opioid receptor exerting rewarding properties by activation of the mesolimbic DA pathway from the VTA to the NAc; at the same time, alcohol induces the DYN release leading to

dysphoric effect (Herz, 1997). These opposing effects are the result of increase and decrease in DA release in the NAc, respectively (Herz, 1997). Although the VTA-NAc circuitry represents a key pathway in the alcohol positive reinforcement, it has been demonstrated that its rewarding properties may be results of the interactions with other brain regions, such as the CeA and ventral pallidum (Heyser et al, 1999; Melendez et al, 2004).

2.3. NEGATIVE REINFORCEMENT OF ALCOHOL

Negative reinforcement has been defined as *the process by which removal of an aversive state increases the probability of a response* (Koob, 2015); both neuroadaptations in the reward circuit and the recruitment of the brain stress systems may concur to the negative reinforcement. The protracted use and abuse of alcohol induce changes in its rewarding effect (i.e. decrease of the DA release) and during alcohol withdrawal GABAergic transmission is decreased and NMDA glutamatergic signaling is increased (Davidson et al., 1995; Roberts et al., 1996; Weiss et al., 1996).

In addition, ethanol (EtOH) withdrawal elicits anxiety-like behavior, thus suggesting the recruitment of the brain stress systems. The use of alcohol to alleviate anxiety (named as “relief drinking”) has been observed in human and several animal models of addiction (Sinha et al., 2011; Ciccocioppo et al, 2009; Schank et al, 2012). Dysregulation of CRF system seems to be responsible for anxiety-like behaviour observed in animals underwent acute and prolonged alcohol withdrawal, since CRF receptor antagonists are able to reverse anxiogenic symptoms (Funk et al., 2007; Knapp et al., 2004; Rassnick et al., 1993; Valdez et al., 2002). Similarly, the block of the CRF1 receptor prevents the escalation of voluntary alcohol intake observed in post-dependent

animals during withdrawal (Gehlert et al., 2007; Overstreet et al., 2004; Gilpin et al, 2008). Direct injections of CRF receptor antagonists in CeA blocks the EtOH self-administration in dependent rats (Funk et al., 2006) by blocking the increase of GABA release (Roberto et al., 2010).

As mentioned above, the excessive release of DA and opioid peptides induced by drugs of abuse, included alcohol, activates the DYN system. Similar to CRF, the increase of DYN in the CeA inhibits the GABAergic interneurons, leading to excitation of downstream neurons in the bed nucleus of stria terminalis (BNST) (Li et al., 2012; Kallupi et al., 2013). It has been demonstrated that intra-CeA infusions of KOP receptor antagonist prevent the escalation in the alcohol self-administration during both acute withdrawal and protracted abstinence (Kissler & Walker, 2016). Moreover, KOP receptor knock-out mice exhibit less alcohol consumption compared to wild-type (WT) (Kovacs et al., 2005). In contrast, N/OFQ and synthetic NOP receptor agonists are able to block alcohol consumption in a genetically selected alcohol-preferring animal line (Economidou et al., 2008), suggesting an anti-stress role for N/OFQ. Alcohol-preferring rats show high anxiety-like behavior, hypersensitivity to stress and an innate up-regulation of CRF and N/OFQ levels in the CeA (Economidou et al, 2011; Hansson et al, 2006). Similar to N/OFQ, a protective role for NPY has been proposed, since alcohol-preferring animals show low innate levels of NPY (Hwang et al, 2004). Therefore, in the CeA NPY and N/OFQ may reduce GABA release increasing the excitability of CeA interneurons and promoting the GABAergic transmission to the BNST (Koob, 2015).

In addition to these neurotransmitters, the brain-derived neurotrophic factor (BDNF) signaling is involved in anxiety and alcoholism (Pandey et al., 1999; Jeanblanc et al., 2009; Moonat et al., 2010 and 2011). BDNF is a neurotrophic factor which activates the

tyrosine kinase B (TrkB) receptor resulting in the cAMP-responsive element binding protein (CREB) phosphorylation and in the upregulation of the CREB-target genes, such as the activity-regulated cytoskeleton-associated (Arc) gene (Messaoudi et al., 2002; Pandey et al., 2008; Moonat and Pandey, 2012). It has been demonstrated that reduced BDNF expression may lead to an increased preference for EtOH (Hensler et al. 2003; McGough et al. 2004). Accordingly, alcohol preferring (P) rats show innate preference for alcohol and low levels of BDNF in the central and medial nucleus of amygdala (CeA and MeA) (Prakash et al., 2008). Moreover, EtOH exposure increases BDNF expression in the DS (Logrip et al., 2008) suggesting that endogenous BDNF contributes to the regulation of EtOH intake (Jeanblanc et al. 2009).

It has been reported that in the CeA and MeA, the alcohol exposure can increase BDNF signaling exerting an anxiolytic effect (Moonat et al., 2011; Pandey et al., 2006). For instance, blocking the BDNF expression in these amygdaloid subregions an increase of voluntary EtOH intake and anxiety-like behaviour was observed (Pandey et al., 2006). Acute EtOH exposure induces anxiolytic effects with an increase of BDNF-Arc signaling and dendritic spine density in the extended amygdala (Pandey et al., 2008). Finally, it has been demonstrated that during withdrawal from chronic EtOH exposure animals exhibited anxiety-like behaviour and reduced BDNF signaling in the CeA and MeA (Pandey et al., 2008).

3. EPIGENETICS

In recent years, several study has focused on the epigenetic mechanisms to better understand the molecular mechanisms of human diseases (i.e. cancer, psychiatric and substance use disorders) and find new therapeutic targets.

In the 1940s, Waddington was the first scientist referring to epigenetics as "*the process by which the genotype gives rise to the phenotype*" (Waddington, 1942). Epigenetics is currently defined as *the study of changes in gene expression which occur in the absence of mutation, but are mitotically inheritable* (Morange, 2002). Changes in DNA sequences and the complex relation between genotype and phenotype (included in the Waddington's definition) are lacking in the current definition of epigenetics. It is interesting to note that in the 1960s the approach to the complex relationships between genotype and phenotype was transformed by the advent of molecular biology (Jacob & Monod, 1961). In fact, a single relation was replaced by a dual relation: one between gene and protein, and one between protein and phenotype.

The term epigenetics refers to chemical modifications (e.g., covalent addition or removal of groups) of the proteins around which the DNA is wrapped (i.e., histone proteins) and the direct addition of methyl groups (i.e., methylation) to the DNA sequence (Murrell et al., 2013). These epigenetic mechanisms do not act as a single epigenetic mark, but act in concert to remodel the structure of the chromatin (i.e., the protein–DNA complex). The epigenetic marks can be deposited, removed or recognized by specific protein domains present in different proteins, thus regulating the access of the transcriptional machinery to the DNA and, consequently, the gene expression (Murrell et al., 2013; Jenuwein & Allis, 2001). Beside histone modifications and DNA methylation, new epigenetic mechanisms are emerging, such as the non-coding RNA and the short microRNAs (Khalil et al., 2009).

3.1. HISTONE MODIFICATIONS

The nucleosome is composed of an octamer core of four histones (H3, H4, H2A, H2B) around which DNA is wrapped and it is the chromatin fundamental unit. Histone modifications contribute in regulating the chromatin state, making it more or less accessible to transcription factors (Kouzarides, 2007). Modifications include acetylation, methylation, phosphorylation, ubiquitination, SUMOylation, citrullination and ADP-ribosylation occuring mainly at lysine (K) amino acid residue located in the tails of histones, particularly H3 and H4, but H2A and H2B can be modified as well (Kouzarides, 2007).

The most studied modifications are histone acetylation and methylation. In particular, histone acetylation is the result of the activity of two enzyme classes: the histone acetyltransferases (HATs) and the histone deacetylase (HDACs) (Struhl, 1998). The acetylation of the lysine residues is generally associated with the chromatin transcriptional active state (Strahl & Allis, 2000). In fact, in the presence of many acetyl groups the chromatin is relaxed and accessible to the transcription factors (i.e. euchromatin), resulting in increased gene transcription; conversely, when few acetyl groups are added to histone tails, the chromatin is condensed and the access to transcriptional proteins is prevented (i.e. heterochromatin), resulting in gene silencing (Strahl & Allis, 2000). The main acetylation sites include K9, K14, K18 and K23 on the H3 tail (Thorne et al., 1990). It has been noticed that the space between those acetylatable lysines is regular and, interestingly, this space periodicity is reminiscent of that of a α-helix (3,6 residues) (Strahl & Allis, 2000). Acetylation of specific lysine residues is also associated with biological processes apart from transcription; during DNA replication, H3 and H4 are involved in replicating chromatin processes (Turner & O'Neill, 1995). For example, the H4 acetylation sites (K5 and K12) are highly

conserved, while the H3K9 acetylation seems to have a more dominant role in histone deposition and chromatin assembly (Sobel et al., 1995).

The addition of methyl groups is mostly hosted on H3 and H4, particularly in K4, K9 and K27 (Strahl et al., 1999). Histone methylation differs from histone acetylation for several aspects; first, the lysine residue can accept one, two, or three methyl groups to form mono-, di-, or trimethylated products. Moreover, based on the specific lysine residue involved and the amount of methyl groups added, histone methylation can be associated to transcriptional active or silent state (Strahl & Allis, 2000). For example, the mono- and tri-methylation of H3K4 is related to gene transcription activation, whereas di- and tri-methylation of H3K9 and H3K27 are considered repressive markers. Moreover, the mono-methylation of H3K9 and H3K27 modulates the gene transcription in an opposite way compared to di- and tri-methylation on these same residues (Kouzarides, 2007). Finally, it is interesting to note that methylation can also occur on arginine (R) residues of H3 and H4; however, differently from what observed for K residues, R methylation serves for transcriptional activation only (Berger, 2007).

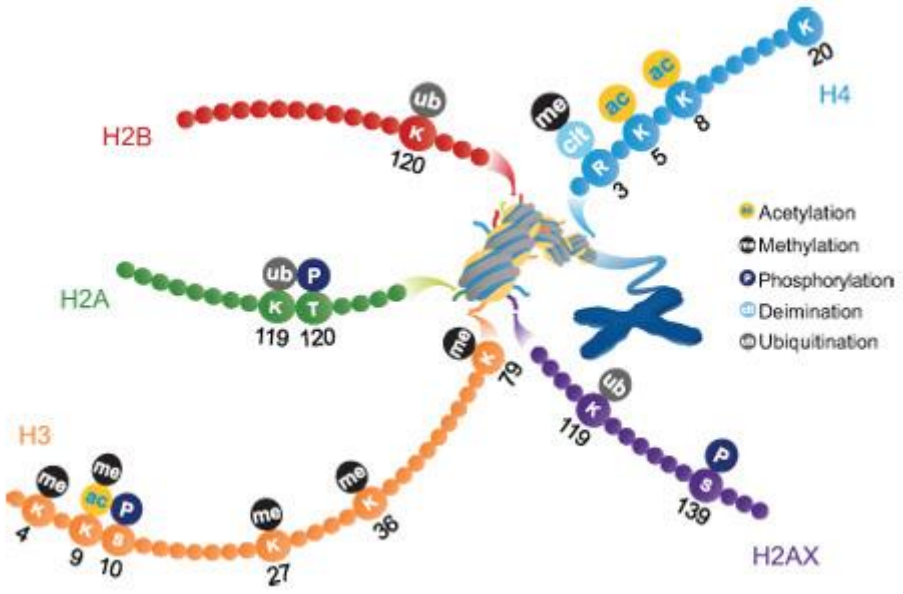

Figure 5. Schematic representation of the most important histone modifications. Covalent modifications occur at the aminoacidic residues on the histone tails. Most common chemical modifications are the addition or the removal of acetylation, methylation, phosphorylation and ubiquitination groups. These chemical alterations mostly involved the aminoacid lysine (K) on histone H2A, H2B, H3 and H4. However, other aminoacids, as serine (S) and arginine (R), can be modified.

3.2. DNA METHYLATION

In eukaryotic cells, the DNA methylation involves the 5-position of cytosine bases and is generally associated with the repressive state of chromatin (Klose and Bird, 2006; Bird and Wolffe, 1999). Methylated cytosine bases prevent the association between DNA-binding factors and their DNA recognition sequences (Watt and Molloy, 1988); consequently, gene expression can be inhibited. Moreover, protein recognizing the methyl-CpG (such as the methyl-CpG-binding proteins, MBPs) indirectly elicit the repression of gene expression by recruiting other co-repressors (Boyes and Bird, 1991; Jones et al., 1998).

DNA methyltransferase (DNMT) enzymes are responsible for the DNA methylation; in mammals, DNMT family includes four isoform: DNMT1, DNMT3A, DNMT3B, and DNMT3L (Subramaniam et al., 2014). DNMTs exert their action with associated factors, such as the polycomb proteins, and in the presence of the methyl donor S-adenosyl-methionine (Robertson, 2001). In addition to their catalytic action, DNMTs can have a non-enzymatic role in transcriptional silencing (Fuks et al., 2001; Bachman et al., 2001); in fact, DNMTs biochemically interact with HDACs and histone methyltransferases mediating the gene silencing (Fuks et al., 2003 and 2001; Bachman et al., 2001).

Methylated DNA sequence can be found in the promoter as well as in the body of the gene, both resulting in reduced gene expression; interestingly, DNA methylation can also interact with the RNA polymerase II reducing its occupancy over the gene body and consequently interacting with DNA elongation (Hsieh, 1997).

4. EPIGENETICS AND ALCOHOL USE DISORDER

As mentioned, both genetic and environmental factors can play a crucial role in the manifestation of alcohol addiction. It has been demonstrated that epigenetic modifications can contribute to cellular adaptations in the brain leading to alcohol tolerance and dependence (Krishnan et al., 2014). In fact, neuroplasticity phenomena induced by alcohol and other drugs of abuse involve epigenetic modifications (i.e. histone modifications, DNA methylation and non-coding RNAs) which in turn regulate gene expression (Moonat and Pandey, 2012; Robison and Nestler, 2011). In addition to neuroplasticity, liver and gastrointestinal system can be subjected to epigenetic changes induced by alcohol exposure (Shukla and Lim, 2013); moreover, an important role of the epigenome has been ascertained in the fetal alcohol spectrum disorders (Perkins et al., 2013; Resendiz et al., 2013). Since alcohol exerts potent effects on the brain at the cellular and molecular level, the early life exposure can affect epigenetic regulation of several genes involved in imprinting, neural and glial development, cell cycle regulation and nervous system growth (Haycock and Ramsay, 2009; Hicks et al., 2010; Liu et al., 2009; Zhou et al., 2011). Similarly, during adolescence the alcohol consumption may interfere with epigenetic processes inducing long-lasting functional changes and alcohol-related psychopathologies later in life (Kyzar et al., 2016).

Epigenetic changes induced by alcohol exposure can play an important role in the development of the negative dysphoric state associated to the AUD. It has been shown that acute EtOH induces anxiolytic effects associated with chromatin transcriptional active state while repeated EtOH exposure followed by withdrawal causes chromatin condensation and increases anxiety-like behaviour (Moonat et al., 2013; Sakharkar, Zhang, et al., 2014; You et al., 2014). Studies using the HDAC inhibitors are indicating that these effects can be mediated by histone acetylation/de-acetylation mechanisms

proposing the HDACs as an attractive therapeutic target (Pandey et al., 2008; Moonat et al., 2013; Sakharkar et al., 2014).

Human studies showed several changes in the global DNA methylation; interestingly, a hypomethylation in the brain while a hypermethylation in blood cells have been reported suggesting a cell type specificity of the DNA methylation profile (Tulisiak et al., 2016). Moreover, alcohol seems to induce different changes in DNA methylation based on the genomic location. For instance, normally high methylated intergenic regions are generally less methylated in the alcoholic brain (Ponomarev et al., 2012) and this condition may be related to a deficiency in methyl donors (Ponomarev, 2013). On the contrary, promoter regions and gene bodies show different and gene specific patterns of methylation (Manzardo et al., 2012; Wang et al., 2016). Different patterns of CpG methylation have been identified on the prodynorphin (PDYN) single-nucleotide polymorphisms (SNPs) in the dorsolateral PFCx of human alcoholics, representing a risk factor for developing AUD (Taqi et al., 2011).

Considering all these evidence, the epigenetic studies are indicating the enzymes responsible for epigenetic alterations as potentially promising therapeutic targets to treat or prevent alcoholism and alcohol-associated emotional disorders.

5. RESEARCH AIM

As mentioned, several neurotransmitters, such as opioid peptides and BDNF, have been identified to play a crucial role in the development of the AUD, including the associated negative emotional states. Understanding the role of these systems in alcoholism can contribute to develop new therapeutic approaches that could reduce the alcohol intake and prevent the relapse.

In the recent years, epigenetic studies are indicating that alcohol induces chromatin remodeling which in turn regulates the expression of several genes. Particularly, it has been found that the enzymes responsible for the epigenetic modifications (i. e. HDACs and DNMTs) are deeply involved in the alcohol-induced neuroplasticity phenomena.

The aim of the present dissertation is to investigate epigenetic and transcriptional alterations induced by alcohol in different paradigms of alcohol exposure, in order to identify molecular and functional mechanisms involved in the AUD and the associated negative emotional states.

First, we focused on the HDACs role in a model of BDNF transgenic mice. In particular, we will discuss about the role of the corticostriatal BDNF in regulating AUD mechanisms and then we will provide an overview on the HDACs and their involvement in alcohol addiction. We will present the picture of the HDACs protein levels in the caudate putamen (CPu) and PFCx of animals with low BDNF levels, either in basal conditions and following acute alcohol exposure.

Then, given the role of the DYN system in mediating negative emotional states associated to alcohol tolerance and dependence (Wee and Koob, 2010), we explored the role of DYN system in the AMY and the BNST.

In addition, we will focus on two different model of alcohol addiction: one is represented by alcohol preferring rats, which show an innate preference for EtOH and

anxious and depressive phenotype; the second one refers to a model of rapid EtOH tolerance to the alcohol anxiolytic effect.

CHAPTER II.

EFFECTS OF ACUTE EtOH EXPOSURE ON CLASS I HDACs IN WILD-TYPE AND BDNF (+/-) MICE

1. BDNF

BDNF is a growth factor belonging to the family of neurotrophins (Park and Poo, 2013). BDNF is a CREB-target gene; the phosphorylated CREB (pCREB) binds a specific region (the Ca^{2+} response element, CRE) in the BDNF gene sequence resulting in the BDNF transcription (Tao et al., 1998). BDNF is subjected to transcriptional modifications encoding for splice different variants, which share a common coding region but have different segments of the 5′ and 3′ UTRs (Kendall et al., 2000). It has been reported that rat neurons produce BDNF transcripts with either a short or a long 3′ UTR, and the long 3′ UTR form seems to be directed to dendrites, where BDNF protein synthesis may occur (Lau et al., 2010). BDNF is synthesized as a precursor, namely proBDNF, which can be processed into mature BDNF (mBDNF) and both these forms can be detected in the central nervous system (CNS) (Fahnestock M. et al. 2001). Moreover, it mainly binds the tropomyosin-related kinase B (TrkB) receptor (Soppet et al. 1991), but it was demonstrated that the proBDNF also interacts with the p75NTR receptor (Rodriguez-Tébar et al., 1990).

In mouse and rat, BDNF mRNA and protein is already detectable during embryonic development and is highly expressed in the hippocampal neurons during adulthood (Ernfors et al., 1990; Hofer et al., 1990; Kawamoto et al., 1996; Conner et al., 1997). Different BDNF mRNA isoforms are controlled by multiple promoters and the expression of these isoforms is tissue-specific. For instance, BDNF exon I mRNA is highly expressed in several CNS region of rats and mice, except for the cerebellum where low levels of this transcript were found (Aid et al., 2007).

Epigenetic mechanisms responsible for the chromatin remodeling can also plays an important role in the regulation of BDNF gene expression. In fact, using inhibitors of different HDAC classes it has been demonstrated that HDACs can differently modulate

the BDNF transcription (Koppel and Timmusk, 2013). Rat cultured neurons exposed to the class II HDACs inhibitors show a rapid upregulation of BDNF mRNA levels, suggesting that class II HDACs are involved in transcriptional regulation of BDNF (Koppel and Timmusk, 2013). Moreover, HDAC 2, which belongs to the class I, has been shown to bind BDNF promoters I, II and IV (Gräff et al., 2012; Guan et al., 2009), suggesting that class I HDAC isoforms can also play an important role in BDNF mRNA transcription (Fig. 6).

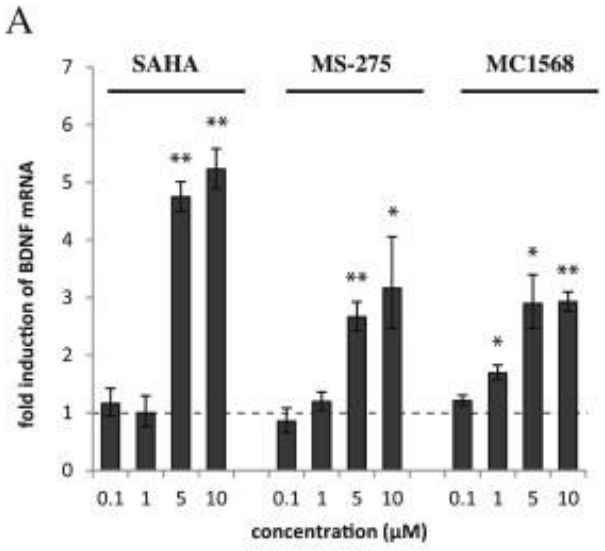

Figure 6. Inhibitors of class I and II HDACs induce BDNF mRNA expression in cultured neurons. (A) BDNF mRNA expression in primary rat cortical neurons treated with different concentrations of HDAC inhibitors: suberoylanilide hydroxamic acid (SAHA) is a class I/II inhibitor, MS-275 is class I specific inhibitor and MC1568 is selective for class II. All three inhibitors are able to induce BDNF mRNA transcription (adapted from Koppel and Timmusk, 2013).

BDNF is a key factor in regulating neuronal development, neuroprotection, synaptic plasticity and learning and memory (Castren, 2004; Cowansage et al., 2010; Lu et al., 2008; Minichiello, 2009). In contrast, dysfunction of BDNF activity has been implicated in several neuropsychiatric disorders (Autry and Monteggia, 2012; Castren, 2014), such as depression (Duman and Li, 2012), schizophrenia (Buckley et al., 2007), anxiety (Andero et al., 2014) and drug abuse (Ghitza et al., 2010).

1.1. CORTICOSTRIATAL BDNF AND ALCOHOL

Depending on the drug of abuse and the neuronal circuitry implicated, BDNF differently regulates the drug self-administration. For instance, BDNF infusion into the VTA or NAc increased cocaine sensitization, self-administration and reinstatement of cocaine-seeking (Horger et al., 1999; Lu et al., 2004; Graham et al., 2007). In contrast, infusion of BDNF into the medial prefrontal cortex (mPFCx) of animals exposed to cocaine reduced the later cocaine seeking (Berglind et al., 2007; Hearing et al., 2008; Sadri-Vakili et al., 2010).

It has been reported that BDNF has a protective action towards the excessive and uncontrolled intake of alcohol. In fact, innate low BDNF expression may predispose rats to higher alcohol intake; genetically selected alcohol-preferring (P) rats display high alcohol preference and intake (Li et al., 1987) and low levels of BDNF protein in the NAc, CeA, MeA and BNST compared to non-preferring (NP) rats (Yan et al., 2005; Prakash et al., 2008). Similarly, CREB heterozygous (+/-) mice express reduced levels of BDNF and exhibit higher alcohol preference than WT mice (Pandey et al., 2004).

Acute administration of alcohol significantly increases BDNF mRNA levels in the DS of C57BL/6J mice (McGough et al., 2004). Similarly, the increase of BDNF mRNA

was observed in the DS following both the limited and chronic moderate self-administration of alcohol (McGough et al., 2004; Logrip et al., 2009; Jeanblanc et al., 2009). Accordingly, the acute alcohol treatment increases BDNF mRNA expression in striatal primary neurons, resulting in the protein translation and secretion and in the activation of TrkB receptor (Logrip et al., 2008). It is interesting to note that the alcohol-induced BDNF increase is regionally and substance specific, since no changes have been observed in the NAc (Logrip et al., 2009; McGough et al., 2004) and following sucrose consumption (Logrip et al., 2009). In contrast, escalation in alcohol intake induces no alteration of BDNF mRNA expression in the DS (Logrip et al., 2009). This lack of BDNF increase in the DS can contribute to enhance the drinking behavior suggesting a disruption of the BDNF protective mechanism observed during moderate and limited access (Logrip et al., 2015). In the same experimental paradigm, a long-lasting decrease of BDNF mRNA levels has been observed in cortical regions (Logrip et al., 2009). In agreement with these findings, prolonged voluntary intake of high alcohol amount decreases BDNF expression in the mPFCx and BDNF levels are directly correlated with the alcohol amount consumed (Darcq et al., 2014).

Systemic administration of RACK1, a protein that increases BDNF levels (He et al., 2010; Neasta et al., 2012), significantly reduced the alcohol intake in the two bottle choice free-choice paradigm (Jeanblanc et al., 2006; McGough et al., 2004). Similarly, direct infusion of Tat-RACK1, a RACK1 protein expressed with a Tat sequence allowing the transduction across the blood-brain barrier (Schwarze et al., 2000), into the DS reduces the operant alcohol self-administration (Jeanblanc et al., 2006). Conversely, the effect of Tat-RACK1 on alcohol drinking is abolished in BDNF +/- mice or following the Trk inhibitor K252a treatment (McGough et al., 2004; Jeanblanc et al., 2006). Therefore, these data suggest that BDNF in the DS functions as a negative

regulator of alcohol intake, maintaining moderate levels of alcohol consumption (Logrip et al., 2015) (Fig. 7). This role seems to be specific in the dorsolateral striatum, since the reduction of endogenous BDNF levels in this subregion, via RNAi, significantly elevates alcohol self-administration (Jeanblanc et al., 2009).

Beyond the striatum, BDNF exerts the protective action on alcohol intake in other brain regions. In the AMY, BDNF is able to repress both anxiety-like behavior and alcohol intake (Pandey et al., 2006), suggesting that amygdaloid BDNF can regulate the anxiety-induced alcohol consumption. Indeed, the infusion of an antisense oligonucleotide repressing the BDNF expression in the CeA and MeA significantly increases both anxiety-like behavior and alcohol intake, which can be rescued by BDNF infusion (Pandey et al., 2006).

In conclusion, the corticostriatal BDNF seems to play a crucial role in the regulation of alcohol consumption, maintaining a moderate intake and driving the transition from moderate to high intake when BDNF levels in the mPFCx are reduced. However, other brain regions are involved in mediating BDNF protective mechanisms in alcohol addiction.

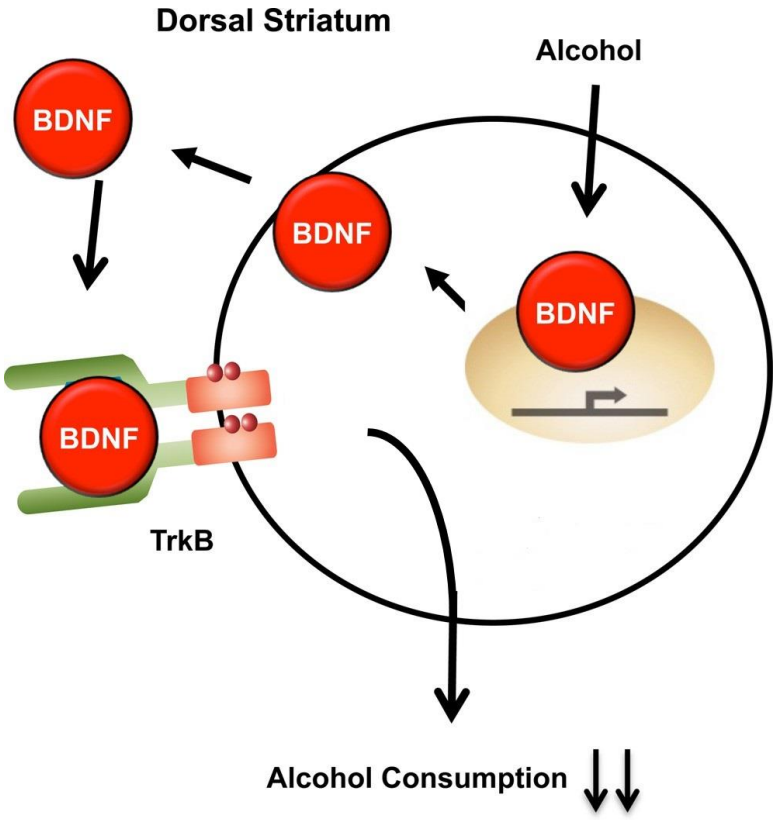

Figure 7. Schematic representation for BDNF action in the DS. Moderate levels of alcohol stimulate the BDNF transcription and translation in the DS. Then, secreted BDNF activates its receptor TrkB, which in turn decreases the alcohol consumption (adapted from Logrip et al., 2015).

2. HDACs

Chromatin remodeling is essential in regulating gene transcription. As mentioned in the paragraph 2.1, increased levels of histone acetylation are associated with increased transcriptional activity, whereas decreased levels of acetylation are associated with repression of gene expression (Strahl and Allis, 2000). The acetylation steady-state level of histones is the result of the balance between opposing activities of two classes of enzymes, the HATs and HDACs (Struhl, 1998).

Two protein families with HDAC activity have been identified, the SIR2 family consisting of NAD^+-dependent HDACs, and the classical HDAC family; classical HDACs are subdivided into two different classes, the class I and class II (de Ruijter AJ et al., 2003). The class I HDACs comprises the HDAC 1, 2, 3 and 8 and are mostly localized in the nucleus. In particular, the localization of HDAC 1 and HDAC 2 are exclusively nuclear (Johnstone, 2002) while HDAC3 has both nuclear and cytoplasm localization (Yang et al., 2002). Finally, HDAC8 has been demonstrated to be localized in the nucleus (Van den Wyngaert et al., 2000). Class II HDACs includes several HDAC isoforms (HDAC 4, 5, 6, 7, 9, 10 and 11) that might be involved in cellular differentiation and developmental processes (Morris and Monteggia, 2013).

The HDAC enzymes remove the acetyl group from the lysine residues of the histones; the catalytic domain is formed by a stretch of ~ 390 conserved amino acids (Finnin, et al., 1999). Removal of an acetyl group occurs via a charge-relay system consisting of two adjacent histidine residues, two aspartic residues and one tyrosine residue and the presence of the Zn^{2+} ion (Finnin, et al., 1999). Inhibitors of the HDACs displace the Zn^{2+} ion resulting in the dysfunction of the charge-relay system. For example, the trichostatin A (TSA) has a hydroxamic acid group and a five-carbon atom linker to the

phenyl group that give it the optimal conformation to fit into the active site and potently inhibit the HDACs activity (Finnin, et al., 1999).

In the present dissertation, we will focus on the class I HDACs. HDAC 1 and HDAC 2 are highly similar enzymes (Li et al., 2002) and display activity within a complex of proteins that bind DNA, such as NuRD and Co-REST (Zhang et al., 1999). Both deacetylase activity and complex formation are regulated by HDAC 1 and HDAC 2 phosphorylation, with an increased activity when these enzymes are phosphorylated and a decreased in the presence of hypophosphorylation (Galasinski et al., 2002; Pflum et al., 2001). HDAC 3 is most closely related to HDAC 8 and even if it shares structural and functional features with other class I HDACs, HDAC 3 can exist in multisubunit complexes that are different from other known HDAC complexes (de Ruijter AJ et al., 2003). HDAC3 is able to form oligomers in vitro and in vivo with other HDACs, such as HDAC 4, 5 and 7 (Fischle et al., 2001; Yang et al., 2002). Finally, HDAC 8 has been recently discovered and is not well known the specific co-repressor complex regulating its action (Buggy et al., 2000).

The involvement of HDACs in several pathologies have been described and the pharmacological inhibition of these enzymes has been proposed as an effective treatment of some cancers (Dokmanovic et al. 2007; Lane and Chabner, 2009). Recently, several studies have focused on the HDAC role in psychiatric disorders, including stress-related disorders and addiction, suggesting the HDAC inhibitors as potential therapeutic agents (Covington et al., 2009; Pandey et al., 2008; Renthal and Nestler, 2008; Tsankova et al., 2007).

2.1. HDACs AND ALCOHOL

It has been shown that acute and chronic EtOH exposure induce histone acetylation of several genes (D'Addario et al., 2013; Finegersh and Homanics, 2014). Acute alcohol induces anxiolytic-like effect and decreases the HDACs activity in the AMY resulting in a global increased acetylation of H3K9 and H4K8 but not H3K14 (Moonat et al., 2013; Pandey et al., 2008). It has been reported that the development of rapid tolerance to anxiolytic effects of ethanol is associated with HDAC-induced histone modifications (H3K9 and H4K8) and changes in NPY expression in the CeA and MeA (Sakharkar et al., 2012). Similarly, sensitized animals exhibit a reduction in the striatal HDAC activity following acute ethanol treatment and an increase of H4 acetylation specifically in the core of the NAc (Botia et al., 2012). Moreover, the non-specific HDAC inhibitor sodium butyrate (NaBut) can prevent and reverse the ethanol-induced behavioral sensitization and the gene expression alterations, such as the BDNF mRNA changes in the striatum and PFCx (Legastelois et al., 2013).

In recent years, the role of specific HDAC isoforms in alcohol dependence and exposure has emerged. Results on primary monocyte-derived dendritic cells from alcohol users show that class I HDACs gene expression and protein levels are significantly higher than control subjects (Agudelo et al., 2016). In addition, HDAC 2 expression is increased by alcohol in a dose-dependent manner (Agudelo et al., 2011). Using the selective class I HDACs inhibitor MS-275, a decrease motivation to consume EtOH and relapse has been showed, suggesting that class I HDACs can be a therapeutic target in alcohol addiction (Jeanblanc et al., 2015). Alcohol-preferring (P) rats innately show high levels of HDAC 2 protein and an increase in the total HDAC activity particularly in the CeA and MeA (Moonat et al., 2013). Similarly to what observed following the ethanol treatment, the intra-amygdala injection of the siRNA to

knockdown HDAC 2 attenuates the anxiety-like behavior, and increases the BDNF and Arc promoter acetylation in P rats (Moonat et al., 2013). Moreover, P rats treated with TSA, a HDAC inhibitor, has an attenuation of the anxiety-like behavior and a decrease of the amygdaloid nuclear HDAC activity and HDAC 2 protein levels (Sakharkar et al., 2014). Taken together, all these results suggest that HDAC 2 is deeply involved in regulating the alcohol drinking behavior, particularly in the AMY. The involvement of HDAC specific isoform in other brain areas remains quietly unexplored; recently it has been demonstrated that chronic EtOH-treated mice exhibit a decrease of the HDAC 1, 2, 5 and BDNF mRNA levels in the HIPPO (Stragier et al., 2015). Accordingly, a reduction of HDAC 1 mRNA levels and the translocation of HDAC 1/4 proteins from nuclear to cytosolic compartment has been observed in the rat HIPPO and entorhinal cortex following EtOH exposure (Zou and Crews, 2014).

Finally, the involvement of HDACs has been also documented during withdrawal conditions; after chronic EtOH exposure, 24 hours withdrawal induces an increase of the anxious symptoms (Pandey, 2003; Pandey et al., 2008; You et al., 2014) and the HDAC activity, and a decrease of H3K9 and H4K8 acetylation levels in the AMY (Pandey et al., 2008).

3. AIM

As described above, BDNF have been implicated in the development of alcohol addiction; in particular, the BDNF-signaling in DS plays a pivotal role in modulating alcohol intake. Moreover, EtOH exposure can modulate chromatin remodeling, affecting the histone acetylation/deacetylation mechanisms. Class I HDACs inhibition influences the BDNF expression and attenuates the alcohol drinking behavior and the withdrawal-associated anxiety symptoms. However, the role of the HDAC specific isoforms in EtOH-related phenomena remains to be deepened.

Based on these premises, we aimed to investigate the protein levels of HDAC 1, 2 and 3 in the CPu and PFCx, two areas belonging to the mesocorticostriatal circuitry. In particular, wild type (WT) and BDNF +/- mice, which voluntary consume high amount of EtOH, were acutely injected with EtOH and subsequently tested to the rota-rod; after that, animals were sacrificed and molecular analysis was conducted.

4. MATERIALS AND METHODS

4.1. ANIMALS

Animals were housed at temperature and humidity controlled conditions under a cycle of 12 h light/dark (lights on at 7:00 A.M.). Food and water were available ad libitum. Mice were allowed to settle down for one week before starting the experiments. Experiments were conducted in agreement with the European Communities Council Directive of 24 November, 1986 (86/609/EEC) and Italian National (Ministry of Health, Italy) laws and policies (authorization number 139/2012-B). The study received the approval of the "Ethic Scientific Committee for the Animal Experiments" of the University of Bologna.

Male and female BDNF+/+ and BDNF+/− mice were used to generate BDNF +/- and WT littermate control mice (Korte et al., 1995). The specific genotype was ascertained by PCR analysis on DNA from the tail using the following primers: BD2A GTGTCTATCCTTATGAATCGCC; BKO-1 ATAAGGACGCGGACTTGTACA; 3'NEO GATTCGCAGCGCATCGCCTT.

4.2. TREATMENT

A total of 24 animals (12 BDNF +/- and 12 WT mice) were used. For the rota-rod test, 6 BDNF+/− and 6 WT mice intraperitoneally (i.p.) received cumulative injections of 20% EtOH in saline at the dose of 0.5 g/kg, every five minutes. The doses injected were five, for a total cumulative dose of 2.5 g/kg. After each injections, mice were place on the rota-rod and tested for motor coordination.

For the Western blot analysis, BDNF+/− and WT mice (n = 6 for each group) were i.p. treated with an acute injection of 20% EtOH in saline at the dose of 2 g/kg, or saline as

vehicle. Mice were sacrificed 1 hours after EtOH treatment and the CPu and PFCx were rapidly dissected out, frozen on dry ice and stored at –80 ◦C until analysis.

4.3. MOTOR COORDINATION: THE ROTA-ROD TEST

BDNF+/− and WT mice were tested on the rota-rod in order to evaluate possible differences due to the genotype and to observe alterations of the animal coordination in response to a cumulative EtOH dose. The training period lasted for seven days during which mice were daily placed on a 3 cm diameter rota-rod apparatus (Ugo Basile Srl, Italy) and trained to run for 1 minute at 20 rpm. On the test day, the baseline performance was recorded and then mice were i.p. injected with a total cumulative dose of 2.5 g/kg EtOH. The latency to fall from the rota-rod was recorded after each injection three times for animal.

4.4. FRACTIONATION OF NUCLEAR–CYTOPLASMIC PROTEINS

For the protein analysis a second batch of BDNF+/− and WT mice (n = 6 for each group) were i.p. treated with a single 2 g/kg injection of 20% EtOH in saline. One hour later, animals were killed and CPu and PFCx were collected. Nuclear and cytoplasmic protein fractions were extracted from the dissected brain regions using the NE-PER® Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Scientific) according to the manufacturer's protocol. Briefly, tissues were homogenized in an appropriate volume of CER I buffer and protease inhibitor cocktail (PIC) and then incubated on ice for 10 minutes. The samples were centrifuged at maximum speed for 5 minutes and then the supernatant (cytoplasmic fraction) was collected. The pellet was suspended in NER

buffer, vortexed and incubated 10 minutes on ice for several times. Finally, the samples were centrifuged at maximum speed and the supernatant (nuclear extract) was transferred. Both the cytoplasmic and the nuclear fractions were stored at – 80°C. The protein concentration was determined using Pierce® BCA protein assay kit (Thermo Scientific).

4.5. WESTERN BLOT ASSAY

The same amount of proteins (20 g) for each samples was mixed with an appropriate volume of 2X sodium dodecyl sulphate (SDS) loading buffer and boiled for 5 minutes. Then, the proteins were loaded and separated on 8–16% Precise Tris–Glycine Gels (Thermo Scientific) and transferred to 0.4 m nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5% non-fat dry milk in TBS-T (Tris-buffered saline with 1% Tween-20) for 60 min and then incubated with the specific antibody overnight at 4°C. Accordingly to the datasheet of the antibodies, each antibody was diluted in the 5% non-fat dry milk and TBS-T as follow: HDAC 1 (65 kDa, 1:1000; cod. no. 06-720 Millipore), HDAC 2 (55 kDa, 1:700; cod. no. ab16032 Abcam) and HDAC 3 (50 kDa, 1:900; cod. no. ab16047 Abcam), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (36 kDa, 1:2000; cod. no. MAB374 Millipore) and Actin (43 kDa, 1:1000; cod. no. A2066 Sigma). GAPDH and Actin were used as reference proteins for the cytoplasmic and nuclear fractions, respectively. Membranes were washed three times with TBS-T and incubated for 1 h at room temperature with a horseradish peroxidase-linked anti-rabbit secondary antibody (1:3000, cod. no. NA934V GE Healthcare UK Ltd). Immunoreactive bands were visualized using the Pierce® ECL Western blotting Substrate (Thermo Scientific). The intensities of the bands were quantified by

densitometry, using a molecular analysis software system (Bio-RAD ChemiDocTM MP Imaging System).

4.6. STATISTICAL ANALYSIS

Data were analyzed by two-way ANOVA. F-values reaching significance ($p < 0.05$) were further analyzed by Bonferroni post-hoc test. Statistical analysis was performed using the GraphPad Prism software version 5 (GraphPad Software, San Diego, CA, USA) and results are reported as the mean of values ± SEM.

5. RESULTS

5.1. MOTOR COORDINATION

The cumulative doses of EtOH progressively affects motor coordination in both BDNF +/- and WT mice (Figure 8). Since no significant differences were observed, it can be assumed that the genotype has no effect on EtOH-induced motor coordination impairment.

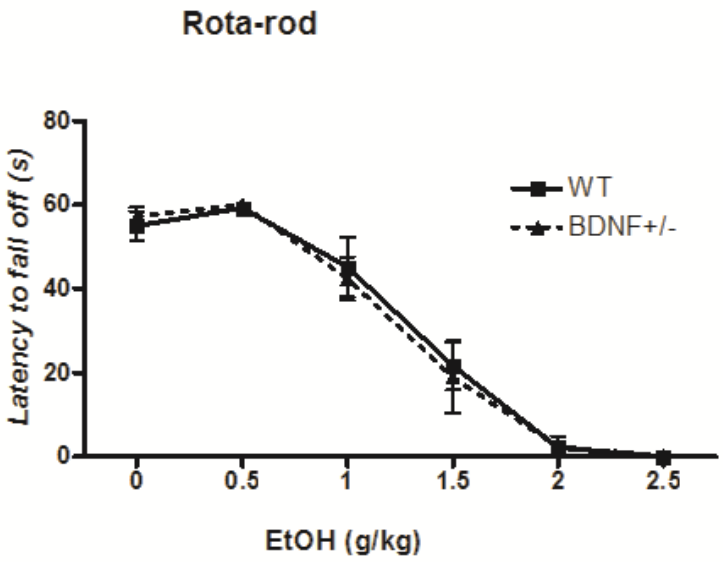

Figure 8. EtOH treatment progressively impairs motor performance of WT and BDNF+/− mice. The latency to fall was recorded after each single EtOH injection (up to a total cumulative dose of 2.5 g/kg). Data are presented as mean ± SEM (n = 6 mice per group; WT *vs* BDNF+/−).

5.2. CLASS I HDACs IN THE CPu

Acute EtOH induces a significant reduction of nuclear HDAC 1 protein levels in the CPu of WT mice (WT EtOH-treated group = 13.33 ± 2.80 *vs* WT vehicle group = 100 ± 7.64; $p < 0.001$) (Figure 9a). Moreover, an innate high difference in the nuclear HDAC 1 levels has been detected between the WT and BDNF +/- mice, with lower protein levels in the BDNF+/− animals (WT vehicle group = 100 ± 7.64 vs BDNF+/− vehicle group = 26.96 ± 5.35; $p < 0.001$) (Figure 9a). Interestingly, EtOH treatment does not induce changes of the nuclear HDAC 1 levels in the BDNF+/− mice (Figure 9a). A significant genotype × treatment interaction has been also reported ($F_{(1,20)} = 66.76$; $p < 0.0001$). Finally, the cytoplasmic amount of HDAC 1 was very low and we could not adequately quantify it.

Similar to what observed for HDAC 1, BDNF +/- mice innately exhibit lower levels of nuclear HDAC 2 compared to WT mice (WT vehicle group = 100 ± 6.97 *vs* BDNF+/− vehicle group = 27.60 ± 4.99; $p < 0.001$) (Figure 9b). EtOH induces a decrease of nuclear HDAC 2 protein levels in the CPu of WT mice (WT EtOH-treated group = 23.32 ± 3.27 *vs* WT vehicle group = 100 ± 6.97; $p < 0.001$) whereas no changes in the BDNF+/− mice (Figure 9b). In addition, a significant interaction of genotype × treatment has been observed ($F_{(1,20)} = 52.35$; $p < 0.0001$). Finally, the CPu cytoplasmic content of HDAC 2 is significantly reduced in the BDNF+/− EtOH-treated mice (BDNF+/− EtOH-treated = 46.87 ± 3.09 *vs* BDNF+/− vehicle group = 132.19 ± 28.10; $p < 0.01$) (Figure 9c).

Accordingly to the HDAC 1 and 2 results, differences of basal HDAC 3 levels have been observed in the CPu of BDNF+/− compared to WT mice (WT vehicle group = 100 ± 5.50 *vs* BDNF+/− vehicle group = 28.33 ± 6.00; $p < 0.001$) (Figure 9d). WT EtOH-treated mice has a reduction of nuclear HDAC 3 protein levels compared to WT

vehicle-treated animals (WT EtOH-treated = 23.30 ± 6.97 *vs* WT vehicle group = 100 ± 5.50; $p < 0.001$), but no changes have been observed in the BDNF +/- EtOH-treated mice (Figure 9d). As observed for HDAC 1 and 2, a significant genotype × treatment interaction has been also reported for HDAC 3 ($F_{(1,20)} = 37.62$; $p < 0.0001$). Finally, in the cytoplasmic fraction, BDNF+/− mice exhibit lower basal level of HDAC 3 protein compared to WT animals (WT vehicle group = 100 ± 16.63 *vs* BDNF+/− vehicle group = 49.11 ± 13.17; $p < 0.05$) (Figure 9e).

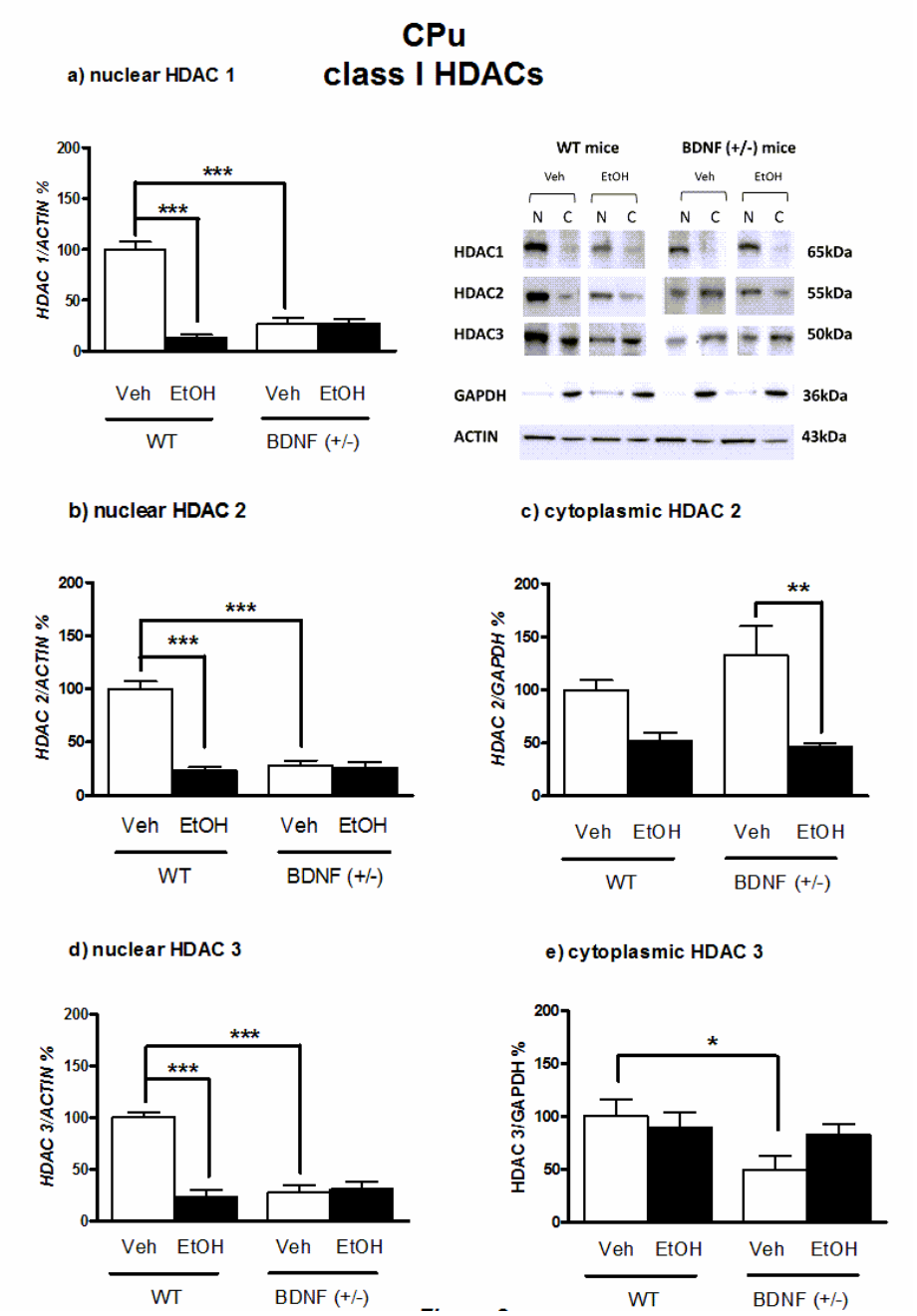

Figure 9. Protein levels of HDAC class I in the CPu: Western blot analysis. Nuclear (N) and cytoplasmic (C) contents of HDAC 1, 2 and 3 after acute EtOH i.p. (2 g/kg) or vehicle (Veh) in WT and BDNF+/− mice. The HDAC 1, 2 and 3 protein levels were assessed using specific antibodies compared to Actin (nuclear fraction) and GAPDH (cytoplasmic fraction). Data are presented as mean ± SEM (n = 6 mice per group) and analyzed by two-way ANOVA (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$). In the upper right panel, representative immunoblots of HDAC 1, 2 and 3 were reported.

5.3. CLASS I HDACs IN THE PFCx

In the PFCx, BDNF +/- mice innately show significant higher levels of the nuclear HDAC 1 levels compared to WT mice (BDNF+/− vehicle group = 174.10 ± 13.19 *vs* WT vehicle group = 100 ± 17.66; $p < 0.05$) (Figure 10a). Acute EtOH treatment does not induce any changes of nuclear HDAC 1 protein levels in this brain area. (Figure 10a). As observed for the CPu, the cytoplasmic amount of HDAC 1 was too low to be adequately quantified.

Similarly to what observed for HDAC 1, the nuclear HDAC 2 basal levels are significantly higher in the BDNF+/− than in WT mice (BDNF+/− vehicle group = 142.20 ± 19.50 *vs* WT vehicle group = 100 ± 2.52; $p < 0.05$) (Figure 10b). In addition, there is a genotype effect in the EtOH-treated groups, with higher levels of HDAC 2 in the BDNF +/- than WT mice (WT EtOH-treated group = 109.85 ± 8.30 *vs* BDNF+/− EtOH-treated group = 153.84 ± 7.13; $p < 0.05$) (Figure 10b). Finally, no significant alterations of HDAC 2 has been observed in the cytoplasmic fraction (Figure 10c).

Accordingly to the CPu results, BDNF+/− mice have innate lower levels of nuclear HDAC 3 levels compared to WT mice (BDNF+/− vehicle group = 45.90 ± 6.40 *vs* WT vehicle group = 100 ± 14.04; $p < 0.001$) (Figure 10d). In the WT mice, acute EtOH treatment induces a significant reduction of nuclear HDAC 3 protein levels (WT EtOH-treated group = 55.52 ± 5.21 *vs* WT vehicle group = 100 ± 14.04; $p < 0.01$), whereas no alterations have been assessed in the BDNF+/− group (Figure 10d). In the cytoplasmic content, there is a significant genotype × treatment interaction ($F_{(1,20)} = 4.83$; $p < 0.05$) (Figure 10e). In particular, EtOH treatment causes different effects on the HDAC 3 protein levels in the PFCx of BDNF+/− compared to WT mice (BDNF+/− EtOH-treated group = 68.48 ± 3.40 *vs* WT EtOH-treated group = 118.38 ± 13.60; $p < 0.05$) (Figure 10e).

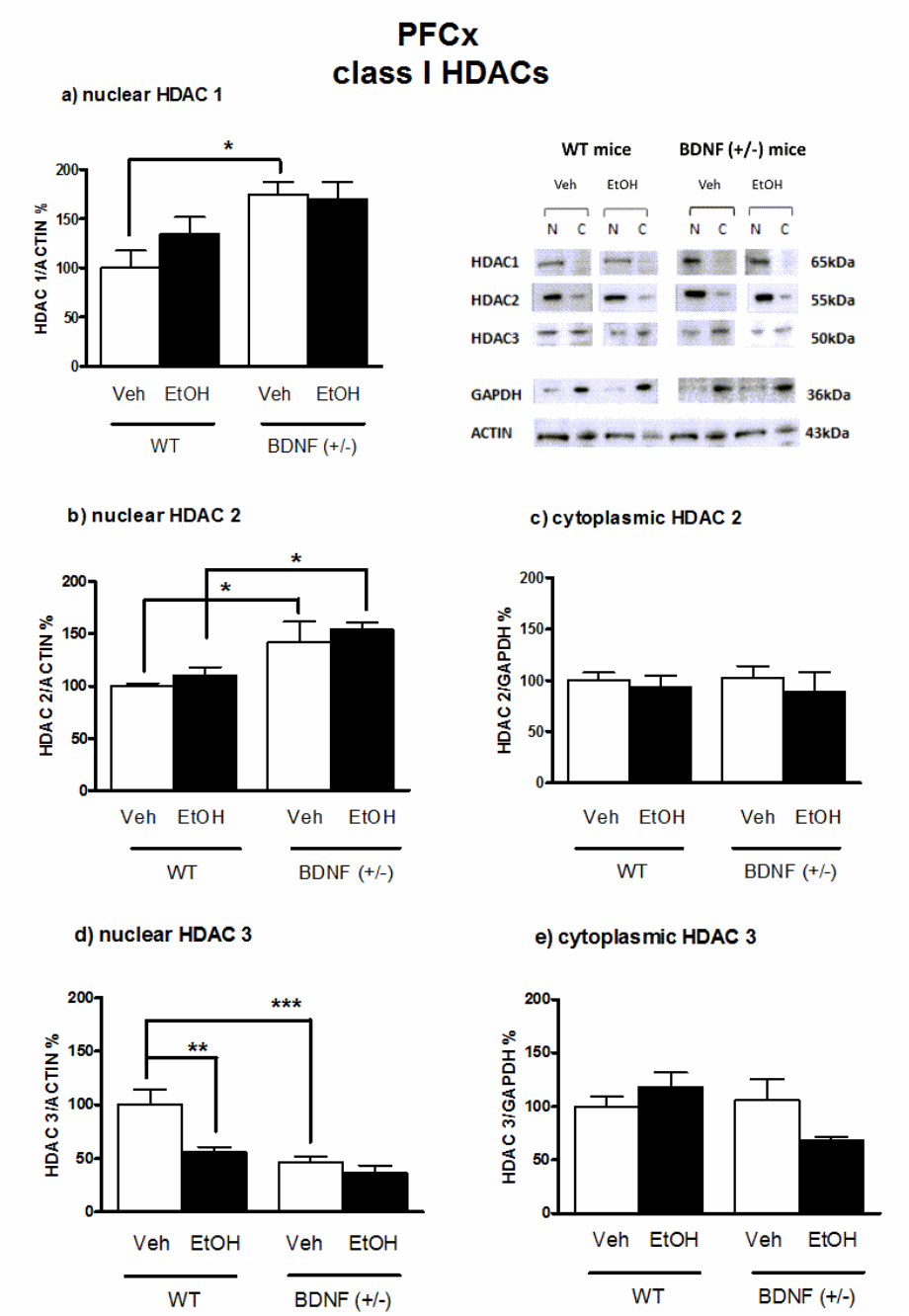

Figure 10. Protein levels of HDAC class I in the PFCx: Western blot analysis. Nuclear (N) and cytoplasmic (C) contents of HDAC 1, 2 and 3 after acute EtOH i.p. (2 g/kg) or vehicle (Veh) in WT and BDNF+/− mice. The HDAC 1, 2 and 3 protein levels were assessed using specific antibodies compared to Actin (nuclear fraction) and GAPDH (cytoplasmic fraction). Data are presented as mean ± SEM (n = 6 mice per group) and analyzed by two-way ANOVA (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$). In the upper right panel, representative immunoblots of HDAC 1, 2 and 3 were reported.

6. DISCUSSION

Endogenous BDNF is largely involved in regulating alcohol consumption; for instance, BDNF+/− mice show spontaneous preference for EtOH consumption and high vulnerability to develop alcohol addiction (Hensler et al., 2003; McGough et al., 2004; Bosse and Mathews, 2011). Moreover, alcohol-preferring rats display high alcohol preference and intake (Li et al., 1987) and low levels of BDNF protein in the NAc, CeA, MeA and BNST compared to non-preferring rats (Yan et al., 2005; Prakash et al., 2008).

It has been demonstrated that low BDNF correlates to dopamine alterations in the nigrostriatal circuitry (Majovski et al., 1981; Hyman et al., 1991; Hoglinger et al., 1998) and these deficits might be responsible for sensorimotor impairment of BDNF+/− mice (Dluzen et al., 2001). In the present study, we tested WT and BDNF +/- mice for motor coordination on the rota-rod test following EtOH exposure. Both EtOH-treated groups progressively lose motor coordination with the increase of the administered dose, suggesting that the genotype does not affect the motor performance in response to EtOH. Even though specific sensorimotor impairments has been associated to low BDNF levels of the BDNF +/- mice (Dluzen et al., 2001), the performance of motor coordination does not seem to be affected. In particular, data here reported arise the hypothesis that the EtOH-induced impairment of the motor coordination might not require the BDNF involvement.

Considering the involvement of HDACs in the EtOH-induced epigenetic alterations (Yang and Seto, 2007), in the present study we investigated the effects of acute EtOH on the class I HDACs protein levels. In particular, we used BDNF +/- mice as animal model since these animals have a spontaneous preference for alcohol (Hensler et al., 2003; McGough et al., 2004; Bosse and Mathews, 2011) and class I HDACs can

regulate the expression of the BDNF gene (Koppel and Timmusk, 2013; You et al., 2014). Giving the corticostriatal role of BDNF in regulating alcohol consumption (McGough et al., 2004; Logrip et al., 2009; Jeanblanc et al., 2009), we focused our attention on the CPu and PFCx.

In the CPu, the nuclear HDAC 1, 2 and 3 protein levels are significantly reduced by acute EtOH treatment in WT mice. It has been demonstrated that acute alcohol decreases HDACs activity in the NAc (Botia et al., 2012) and AMY resulting in a global increased histone acetylation (Moonat et al., 2013; Pandey et al., 2008). Our results in the CPu are consistent with these findings. Supporting the hypothesis of the HDACs involvement in alcoholism, systemic administration of HDACs inhibitors modulates the binge alcohol drinking and seeking behavior (Warnault et al., 2013). Moreover, the HDACs activity increases during EtOH withdrawal when animals show anxiety-like behavior, and the TSA treatment attenuates anxious symptoms further supporting the HDACs involvement in alcohol addiction (Pandey et al., 2008).

In the CPu nuclear fraction, BDNF+/− mice has lower basal levels of HDAC 1, 2 and 3 than WT animals. Several studies on CNS disorders reported that BDNF expression can be increased by selective inhibitors of class I HDAC, suggesting a possible neuroprotective role of HDAC inhibitors (Chen et al., 2006; Fukuchi et al., 2009; Calabrese et al., 2013; Koppel and Timmusk, 2013). Moreover, all these findings underline that BDNF gene expression is sensitive to class I HDACs. Our results in BDNF+/− mice indicate for the first time that class I HDACs levels are in turn sensitive to the BDNF levels, since the presence/absence of BDNF allele might be determinant in modulating HDACs protein levels. The relationship between BDNF and class I HDACs can represent a new therapeutic target to treat alcohol abuse and EtOH-related disorders. In particular, several transcription factors are regulated by BDNF, including the Sp1

(Niu and Yip, 2011). Hence, reduced BDNF in the BDNF +/- mice may affect the class I HDACs levels throughout Sp1. In fact, recent evidence suggests that the transcription factor Sp1 can regulate the class I HDACs expression, since Sp1 binding sites have been found in the promoter regions of HDAC 1 and 2 genes (Yang et al., 2014). Therefore, low BDNF levels may result in low activation of the BDNF pathway, and as a consequence, low recruitment of transcription factors which in turn do not bind the class I HDACs promoters and block their expression.

Interestingly, acute EtOH treatment induces no alterations of the three investigated HDAC isoforms in the CPu of BDNF +/- mice. One possible explanation could be found in the class I HDAC enzymes innate low levels of BDNF +/- mice. In fact, it is conceivable that the reduction induced by EtOH could be prevented to avoid a further loss of HDACs, crucial to maintain the transcriptional activity. Even though the exact mechanism remains unclear, the different effect on class I HDACs induced by EtOH observed in BDNF+/− and WT mice suggests the hypothesis that BDNF may play a key role in regulating the EtOH-induced chromatin remodeling.

It is interesting to note that there are no EtOH-induced substantial changes of HDAC 2 and 3 in the CPu cytoplasmic fraction of WT mice. However, BDNF+/− mice has high HDAC 2 basal levels in the cytoplasm. Considering that the HDAC 2 protein amount in the nuclear extract is very low, we can hypothesized that the translocation of this isoform from nuclear to cytoplasmic compartment takes place. On the other hand, BDNF +/- mice may have a global decrease of the HDAC 3 isoform, since the cytoplasmic basal levels of this enzyme are significantly lower compared to WT mice.

Differently from what observed in the CPu, acute EtOH treatment does not induce any changes of nuclear HDAC 1 and 2 in the PFCx of WT mice. Similar differences between two distinct brain areas have been observed in previous studies. In fact,

depending on the brain region, different effects on HDACs can be induced by EtOH. For example, EtOH inhibits nuclear HDACs activity in the AMY but not in the BNST of adolescent rats (Sakharkar et al., 2014). Similarly, a decrease of HDAC activity has been reported in the striatum, whereas an increase has been observed in the PFCx (Botia et al., 2012). In contrast to what observed for HDAC 1 and 2, a significant decrease of HDAC 3 has been observed. An increase of nuclear HDACs activity in the PFCx has been reported after acute EtOH treatment (Botia et al., 2012). This finding seems to be in contrast with our results, but it is worth to underline that the HDACs activity measured by Botia and colleagues (2012) comprises the HDAC isoforms of all classes.

BDNF+/− mice exhibit high innate levels of HDAC 1 and 2 isoforms in the nuclear protein fraction of the PFCx. The difference in the innate asset of HDAC 1 and 2 between the CPu and PFCx may be the consequence of different BDNF distribution among distinct brain areas. For example, mice over-expressing BDNF has higher BDNF protein level in the cortical regions compared to the striatum (Cunha et al., 2009). Therefore, it is possible that our BDNF +/- mice have different BDNF levels in the PFCx compared to the CPu, differently influencing the HDAC protein levels. In contrast to HDAC 1 and 2, the basal protein levels of HDAC 3 are significantly lower in the PFCx of BDNF +/- mice. This may suggest that the transcription mechanisms regulating the expression of HDAC 3 are different from those involved in HDAC 1 and 2 transcription. As mentioned above, binding sites for Sp1 have been identified on the HDAC 1 and 2 gene promoter (Yang et al., 2014), while analogous information about the HDAC 3 isoform are still lacking.

7. CONCLUSIONS

In conclusion, in the present study we presented interesting results on the possible bidirectional relationship between BDNF and HDACs. In particular, in the CPu HDAC 1, 2 and 3 protein levels are lower when the BDNF pathway is partially lacking (BDNF +/- mice). Therefore, BDNF seems to be crucial in regulating the epigenetic machinery comprising class I HDACs levels. Interestingly, genetic manipulation of BDNF can have different consequences on HDAC levels in distinct brain areas since different basal levels of HDAC 1, 2 and 3 have been found in the PFCx compared to the CPu. Moreover, EtOH-treated WT animals show a more marked effect on class I HDACs in the CPu than in the PFCx. Therefore, given the crucial role of the striatum, and in particular the striatal BDNF role in regulating EtOH consumption, data here presented may improve the knowledge on the involvement of epigenetic regulators and their relationship with BDNF. However, more information about the specific mechanism by which BDNF can differently regulate the HDAC isoforms expression are still needed.

CHAPTER III.

MODULATION OF DYNORPHIN SYSTEM IN ALCOHOL TOLERANCE AND DEPENDENCE

1. THE DYN/KOP SYSTEM

Dynorphin (DYN) is an opioid peptide and the endogenous ligand of the κ opioid (KOP) receptor. DYN was initially isolated as a 13 amino acids peptide (DYN 1-13) with the follow sequence: Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys (Goldstein et al., 1979). Subsequently, the DYN complete sequence of 17 amino acids (DYN A (1-17)) was reported (Goldstein et al., 1981). The biologically active peptides derives from the "processing" of the precursor prodynorphin (PDYN), isolated for the first time in 1983 (Watson et al., 1983). All the DYN peptides are contained in the precursor PDYN. As mentioned above, DYN is the endogenous ligand of the KOP receptor (Chavkin et al., 1982). Similarly to other opioid receptors, the KOP receptor belongs to the inhibitory G protein-coupled receptor family, with seven transmembrane helices; in fact, KOP receptor couples to the $G_{\alpha i}$ protein subunit that inhibits the production of cAMP from ATP (Cox, 1993; Mansour et al., 1995). The activation of KOP receptor by DYN induces the opening of K^+ channels and / or the closure of Ca^{2+} channels, with the inhibition of several mediators release (Gross and McDonald, 1987; Bean, 1989; Grudt and Williams, 1993).

The PDYN human gene was isolated in first (Horikawa et al., 1983), following by cloning of the rat gene (Civelli et al., 1985). The rat gene is comprised of four exons and three introns; exons 1 and 2 encodes for the UTR region 5 'of the messenger RNA (mRNA) sequence, while exons 3 and 4 encode for the translated region of the gene (Hollt, 1993). Different regulatory sequences are located in the PDYN gene promoter, particularly upstream of the CAP site; for instance, a functional element, the activator protein 1 (AP1), has been identified in the promoter region of the PDYN (Naranjo et al., 1991). In addition, the PDYN gene promoter contains three cAMP responsive element (CRE) consensus sequence, named Dyn-CRE 1 (-1660 bp / -1653 bp), Dyn-CRE 2 (-

1630 bp / - 1623 bp) and Dyn-CRE 3 (-1546 bp / -1539 bp), which are essential for the positive regulation by cyclic AMP mediated signaling in cell lines (Douglass et al., 1994; Messersmith et al., 1994) and in rat striatum (Cole et al., 1995). The cAMP Responsive Element Binding protein (CREB) binds the CRE sequence and plays an important role in the regulation of PDYN gene transcription. In addition to CRE, an upstream region called Upstream Responsive Element (URE) has been identified in the promoter, capable to bind the Upstream Responsive Element Binding protein (UREB), and having an inhibitory activity in the regulation of PDYN gene transcription (Gu et al., 1997). Recently, it has been also reported the presence of a downstream region (Downstream Regulatory Element, DRE) regulated by the transcription factor DREAM, which in turn inhibits PDYN gene transcription blocking the action of RNA-polymerase II (Cheng et al., 2002; Costigan and Woolf, 2002).

It has been demonstrated that the DYN/KOP system plays an important role in several psychiatric disorders, such as anxiety, depression and drug addiction (Carlezon et al., 2006; Knoll et al., 2007; Walker et al., 2012; Koob, 2015). The activation of this pathway produces pro-depressive and dysphoric effects both in human and rats, mediating negative emotional state (Pfeiffer et al., 1986; Carlezon et al., 2006; Wee and Koob, 2010).

1.1. THE DYN/KOP SYSTEM IN ALCOHOL USE DISORDER AND STRESS-RELATED DISORDERS

Alcohol and other drug of abuse induce DA release in the NAc shell, stimulating the recruitment of CREB and promoting the PDYN mRNA transcription (Koob, 2013). The DYN once released acts to inhibit the DA release (Nestler, 2004; Knoll and Calzeron, 2010) producing dysphoric effects and negative emotional state (Pfeiffer et al., 1986; Carlezon et al., 2006; Wee and Koob, 2010). In the recent years, several studies suggest that the DYN/KOP system may have an important role in mediating EtOH responses; in particular, this system can influence the escalation in EtOH consumption, and cognitive and affective state associated with alcohol dependence (Walker et al., 2012).

DYN expression is upregulated in brain regions associated with motivation and reward after chronic EtOH exposure (Lindholm et al., 2000). KOP receptor knockout (KO) mice consume lower alcohol amount in comparison with WT littermate mice (Kovacs et al., 2005). The intracerebroventricular (icv) injection of the κ-antagonist nor-binaltorphimine (nor-BNI) selectively attenuates the EtOH self-administration in alcohol-dependent animals, while no effects have been observed in non-dependent rats (Walker and Koob, 2008). Subsequent experiments confirm that systemic nor-BNI administration is effective in reducing EtOH self-administration (Walker et al., 2011). Acute EtOH exposure increases PDYN mRNA in the AMY and PFCx, and interestingly five days exposure induces an increase of KOP receptor mRNA in the AMY (D'Addario et al., 2011). In addition, animals underwent to one day withdrawal, after five days EtOH exposure, show an increase of PDYN gene expression in the AMY (D'Addario et al., 2011). In same experimental conditions, a decrease of H3K27me3 (repressive mark) and an increase of H3K9ac (activating mark) have been detected in PDYN promoter gene in the AMY of EtOH-treated animals (D'Addario et al., 2013).

Further evidence about the involvement of DYN/KOP system have been collected using the KOP receptor agonist U50,488, which potentiates the EtOH-conditioned place preference and the alcohol consumption in mice (Sperling et al., 2010).

The DYN/KOP system also mediates the effects of stress on alcohol reward and seeking behaviors. Nor-BNI attenuates the anxiety-like behavior observed during acute EtOH withdrawal (Valdez and Harshberger, 2012). Nor-BNI also blocks the enhanced responsiveness to stress observed during protracted EtOH withdrawal (Gillett et al., 2013). In addition, animals subjected to the social-defeat stress increase EtOH consumption after U50,488 treatment (Kudryavsteva et al., 2006). It has been reported a role for KOP receptor in reinstatement of alcohol seeking behavior under stressful conditions (Funk et al., 2014).

The activation of DYN/KOP system induces anxiogenic- (Knoll et al., 2007) and prodepressive-like effects (Todtenkopf et al., 2004). Accordingly, PDYN or KOP receptor KO mice show no or less depressive-like symptoms (McLaughlin et al., 2003). Interestingly, the implication of DYN/KOP system in modulating mood disorders has been established in the AMY and in the BNST (Mansour et al., 1995; Knoll et al., 2007; Crowley et al., 2016; Crowley and Kash, 2015). It has been demonstrated that the KOP receptor activation inhibits glutamate release from basolateral amygdala (BLA) inputs to BNST blocking the anxiolytic phenotype observed with optogenetic activation of BLA-BNST projections (Crowley et al., 2016). Moreover, deletion of KOP receptor from AMY neurons results in an anxiolytic phenotype (Crowley et al., 2016) and accordingly, the injection of KOP agonist in the BLA has anxiogenic effects (Narita et al., 2005).

For these reasons, understanding the role of DYN/KOP system in in regulating emotional state associated to alcohol use disorders can be useful to better understand the dependence mechanisms and to develop new drugs for the treatment of alcoholism.

2. ANIMAL MODELS TO STUDY THE ALCOHOL USE DISORDER

The AUD is a complex disorder, characterized by many aspects (compulsive use, drug seeking behavior, negative emotional state). For this reason, the translation of AUD into preclinical model is difficult and it is not possible to use a univocal experimental condition or animal model. However, there are minimum criteria to be met for animal models to be considered as valid preclinical models that resemble the human condition:

1) *predictive validity*: the model should be sensitive to symptoms amelioration or attenuation by treatment effective in humans, and insensitive to inactive treatments;

2) *face validity*: the model should represent the behavioral characteristic of human population (i.e. alcoholic population) and should be characterized by the same symptoms;

3) *construct validity*: the model pathology should involve similar neurochemical, neurobiological and psychobiological mechanisms.

In the present study we focused our attention on two animal models: the first one is an animal model for genetic predisposition to alcoholism, the Marchigian Sardinian alcohol-preferring (msP) rats, and the second one reproduces the rapid tolerance to the anxiolytic effect of EtOH (rapid EtOH tolerance, RET).

2.1. MARCHIGIAN SARDINIAN ALCOHOL-PREFERRING RATS

Genetically selected msP rats have been selected from Sardinian alcohol-preferring rats (Ciccocioppo et al., 1998). msP rats have innate preference for EtOH with spontaneous binge-type of drinking (Ciccocioppo et al., 2006). The μ opioid receptor antagonist naltrexone, a therapeutic agent utilized in the management of alcohol dependence (Volpicelli et al., 1992), is effective in reducing EtOH intake after acute as well as subchronic treatment (Perfumi et al., 2003; Ciccocioppo et al., 2006). Moreover, the $GABA_B$ receptor agonist baclofen reduces the EtOH consumption in msP rats (Perfumi et al., 2002). These results indicate a correlation between the efficacy of common medications for alcohol dependence in human and in msP rats, suggesting the validity of this animal model. Consistent with this hypothesis, the serotonin 5-HT2 ritanserin has no effect on alcohol intake in msP rats (Panocka et al., 1993) as well as in humans (Johnson et al., 1996). Interestingly, msP rats show anxiety, depressive-like phenotype and high sensitivity to stress (Ciccocioppo et al., 2006). It has been demonstrated that desipramine, an antidepressant drug, is effective in attenuate depressive-like behavior (Ciccocioppo et al., 1999). Similarly, alcohol has antidepressant action, since the depressive symptoms are attenuated following EtOH treatment (Ciccocioppo et al., 1999), thus suggesting that msP rats might be drinking EtOH to self-medicate negative affective phenotypes. For these reasons, msP rats might represent the population with alcoholism diagnosis and comorbid depression and anxiety.

To support the validity of alcohol dependence model, it has been shown that msP rats consume high daily dose of EtOH (7-8 g/kg) and have a blood alcohol level average around 70-80 mg/dl (Ciccocioppo et al., 2006). In addition, they do not show spontaneous aversion to alcohol or aversive reactions to EtOH directly infused into the mouth (Polidori et al., 1998).

After several days of EtOH withdrawal, msP rats show a higher alcohol consumption indicating the occurrence of a robust alcohol deprivation effect (Perfumi et al., 2005). Moreover, environmental conditioning factors associated with EtOH exposure are able to induce relapse in msP rats, even after a long period of abstinence (Ciccocioppo et al., 2006). Accordingly, stressful stimuli, such as the foot-shock, induce relapse both in msP rats and their counterpart Wistar, but with a stronger effect in msP animals (Hansson et al., 2005).

In regard neurochemical, neurobiological and physiological alterations, interesting findings have been reported in the recent years. In humans, polymorphisms in the CRF 1 receptor gene promoter are linked to the risk of develop the AUD (Treutlein et al., 2006). Similar polymorphisms have been identified on the promoter region of CRF 1 receptor gene in msP rats (Ayanwuyi et al., 2013; Cippitelli et al., 2015). Additionally, msP rats exhibit high levels of CRF 1 receptor mRNA in several limbic brain areas (Hansson et al., 2006). Therefore, dysregulation or alterations of CRF system may play an important role in the comorbidity of alcohol abuse and mood disorders, such as anxiety and depression. Better understanding the gene expression profile in the brain of msP rats can be useful to identify neurobiological mechanism operative in excessive alcohol drinking behavior.

All these characteristics indicate that msP animals may represent a suitable preclinical model for alcohol dependence; in fact, alcohol abuse is frequently an attempt to attenuate the negative emotional state and msP rats are able to mimic this situation. Therefore, msP rats can be useful animal model to investigate biological basis of alcoholism and also and also in screening potential drugs in the treatment of AUD.

2.2. RAPID ETHANOL TOLERANCE

The development of tolerance is one of the consequences of the alcohol use and is characterized as the decrease of the physiological response to a specific dose of EtOH (Tabakoff et al., 1986). For this reason, alcohol tolerance can play an important role in promoting alcohol drinking behavior and developing alcohol dependence. Adaptive changes in the CNS have been found to participate in developing EtOH tolerance; for instance, several neural mechanisms related to learning and memory are involved in the development and maintenance of tolerance (Kalant, 1998).

Behavioral tolerance can be divided into "intrinsic" and "extrinsic" tolerance; the first results from alterations in the neurons directly controlling a behavior, while the second results in behavioral adaptation throughout alterations in compensatory neural circuits (Hoffman and Tabakoff, 1989). Moreover, behavioral tolerance can be defined as acute, rapid and chronic (Crabbe et al., 1979; Kalant, 1998; LeBlanc et al., 1975). Acute tolerance develops fast (minutes) during a single drinking session, rapid tolerance shares several develops within 8 to 24 hours, whereas chronic tolerance occurs after days of continuous or intermittent EtOH exposure (Pietrzykowski and Treistman, 2008). Many molecular mechanisms trigger the tolerance development, but determining which molecular mechanism is responsible for the precise class of tolerance can be difficult. These molecular mechanisms include posttranslational modifications of proteins, trafficking, regulation of mRNA stability, and epigenetic and genetic mechanisms (Pietrzykowski and Treistman, 2008).

As mentioned above, the rapid ethanol tolerance (RET) is a phenomenon developing between 8 and 24 hours after the first ethanol exposure (Crabbe et al., 1979; Khanna et al., 1996; Koob et al., 1987). The RET shares several similarities with chronic EtOH tolerance and can be considered as a good predictor of the chronic tolerance for some

alcohol effects such as, hypothermia and motor impairment (Khanna et al., 1991). However, few studies focused on the development of rapid tolerance to the anxiolytic effect of ethanol.

In the ‘80s, Koob and colleagues observed that tolerance to the anxiolytic effect of alcohol was developed after two days of EtOH exposure or during the second session of three-repeated EtOH treatment in the same day (Koob et al., 1987). The development of tolerance to the alcohol anxiolytic effect was also observed in mice treated with 1.5 g/kg of EtOH 24 hours apart; interestingly, the pretreatment with isopregnanolone, an endogenous neurosteroid, interfered with the development of rapid tolerance (Debatin and Barbosa, 2006). Similarly, two same doses (1 g/kg) of EtOH 24 hours apart did not elicit anxiolytic effect, but higher doses (2 g/kg) can restore the alcohol anxiolytic effect (Sakharkar et al., 2012). Finally, it has been reported that animals underwent RET showed molecular changes in the AMY and BNST, such as a decrease in the nuclear DNMT and HDAC activity (Sakharkar et al., 2014).

Therefore, alcohol tolerance can be an important factor in promoting alcohol-drinking behavior; in fact, higher doses of EtOH are required to reach the same anxiolytic effects suggesting that molecular adaptations occurring in several brain regions.

3. AIM

The DYN/KOP system is highly involved in regulating alcohol intake and promoting the development of negative emotional state associated to alcohol dependence. Hence, in the present study we investigated the role of DYN/KOP system in different model of AUD focusing on two brain areas relevant to modulate the affective state, the AMY and BNST. First, we measured the PDYN and KOP receptor mRNA levels in msP rats compared to their counterpart Wistar animals; PDYN and KOP receptor gene expression was also conducted in msP and Wistar rats exposed to the chronic intermittent two-bottle free choice paradigm. Second, we used a model of alcohol dependence in which rats were fed with Lieber DeCarli EtOH liquid diet and then underwent 24 hours withdrawal; in this model, we measured the PDYN and KOP receptor mRNA levels. Finally, we focused on the RET model investigating transcriptional and epigenetic alterations of the DYN/KOP system.

The studies on the chronic liquid diet and the RET were conducted at Alcohol Research Center, Univeristy of Illinois at Chicago, in the laboratory directed by Dr. Subhash C. Pandey.

4. MATERIALS AND METHODS

4.1 msP RATS: CHRONIC INTERMITTENT TWO-BOTTLE FREE CHOICE

Adult msP and Wistar rats (~ 500 g b.w.) were housed under 12/12 hours light/dark cycles in condition of temperature and humidity. Animal experiment was conducted at University of Camerino, in the laboratory directed by Prof. Roberto Ciccocioppo.

Two groups of animals (6 Wistar and 6 msP) were exposed to a chronic intermittent EtOH treatment, in order to establish alcohol dependence consuming high amount of alcohol (Wise, 1973). In particular, rats received a 10% alcohol solution (v/v) in the two bottles free-choice paradigm; EtOH was available for 24 hours every other day for a total of 30 days (Table 1). Daily EtOH intake was measured as g/kg at the end of every day in which EtOH was available. Two other groups of rats (6 Wistar and 6 msP) instead received water as vehicle. On day 30 animals were sacrificed and the AMY and BNST were rapidly harvested and frozen at -80°.

Animal group	***EtOH (two bottles free-choice paradigm, chronic intermittent exposure)***
Wistar naïve	NO
Wistar EtOH	YES
msP naïve	NO
msP rats	YES

Table 1. Schematic group division in the msP and Wistar rats experiment

4.2. CHRONIC LIQUID DIET MODEL

Male adult Sprague-Dawley rats (weight 300-350 g) were individually housed in a temperature- and humidity-controlled room with a 12/12 hours light/dark cycle. All procedures were conducted in accordance with the NIH guidelines for the Care and Use of Laboratory Animals, and approved by the Institutional Animal Care and Use Committee. For 3 days, rats were offered 80 ml/day of the nutritionally complete Lieber-DeCarli liquid control diet (Lieber-DeCarli Diet 82; Bio-Serv, Frenchtown, NJ). Control groups (n = 7) continued with control diet for 16 days; the remaining animals (n = 13) were gradually introduced to EtOH (1.8% through 8.1% within 7 days), and then maintained on 9% v/v EtOH diet for 15 days. One group of EtOH-diet fed rats (n = 7) underwent 24 hours withdrawal (Withdrawal group) receiving control diet (Table 2).

It has been previously reported that the blood alcohol levels are ranged from 172-198 mg (Pandey et al., 1996; Pandey et al., 2008) and these animals during ethanol withdrawal displayed anxiety-like behavior behaviors as reported in previous studies (Pandey et al., 2008; You et al., 2014).

Animal group	***EtOH (liquid diet for 15 days)***	***24 hours withdrawal after 15 days liquid diet***
Control group	NO	NO
EtOH group	YES	NO
Withdrawal group	YES	YES

Table 2. Schematic group division in the chronic liquid diet experiment

4.3. RAPID ETHANOL TOLERANCE (RET) MODEL

All procedures were conducted in accordance with the NIH guidelines for the Care and Use of Laboratory Animals, and approved by the Institutional Animal Care and Use Committee. For the development of RET, we divided animals in three group (n = 8 animals each group): two group received intraperitoneal (ip) 0.9 % saline (5 μl/g) and one group was injected with EtOH (20 % w/v; 1 g/kg). 24 hours later, one group of animals treated with saline received a second saline injection (Control group), and the other one was treated with 1 g/kg EtOH (EtOH group). The Tolerance group received a second injection with the same dose of EtOH (Table 3). One hour post-injection, all groups were subjected to measurements of anxiety-like behaviors using the elevated plus maze (EPM) exploration test. Briefly, each rat was placed on the central platform of the plus maze; during the 5 minutes test period, the number of entries and the time spent in each arm (open or closed) were recorded. The anxiety-like behavior was determined as percentage of open arm entries and time spent in the open arm. The total number of closed arm entries was used to represent general activity of rats (Sakharkar et al., 2012).

Immediately after the behavioral measurement, animals were anesthetized with isoflurane and decapitated. From all rats injected with EtOH, around 500 uL of blood was collected at the time of brains for the measurement of the blood alcohol level (BAL) using the Analox Alcohol Analyzer (Lunenburg, MA). The brains were dissected and the AMY and BNST were quickly frozen and stored at −80 °C until molecular analysis.

Animal group	*Day 1*	*Day 2*
Control group	Saline 0.9 %	Saline 0.9 %
EtOH group	Saline 0.9 %	EtOH 1 g/kg
Tolerance group	EtOH 1 g/kg	EtOH 1 g/kg

Table 3. Schematic group division in the RET

4.4. RET MODEL AND NOR-BNI TREATMENT

A second batch of animals was treated as mentioned above to develop RET. In particular, groups were divided as follow: 1) Control group, which received i.p. injection of saline both days and saline as vehicle (n = 9); 2) nor-BNI + Saline group, which received i.p. injection of saline on the first day, then nor-BNI (20 mg/kg) followed by 0.9 % saline after 19 hours (n = 9), and 3) EtOH group, which received i.p. injection of saline and vehicle, followed by EtOH (1 g/kg) after 19 hours (n = 9). The next two groups were: 4) Tolerance group, which received i.p. injection of EtOH on the first day and vehicle followed by EtOH injection (1 g/kg) after 19 hours (24 hours after the first EtOH injection; n = 9) and 5) nor-BNI + Tolerance group, which received i.p. injection with EtOH and then nor-BNI (20 mg/kg) followed by ethanol injection (1 g/kg) after 19 hours (n = 9) (Table 4). On day 2, one hour after the 0.9 % saline or EtOH injections and 20 hours after the vehicle or nor-BNI treatments, anxiety-like behavior of the rats was measured using the elevated plus maze (EPM) exploration test, as described above. Rats were anesthetized with isoflurane immediately after the behavioral measurement. Blood was obtained from all rats injected with ethanol for measurement

of BAL using the Analox Alcohol Analyzer (Lunenburg, MA). The AMY and BNST were dissected out and quickly frozen and stored at −80 °C until molecular analysis.

Animal group	***Day 1***	***Day 1 (5 hours later)***	***Day 2***
Control group	Saline 0.9 %	Saline 0.9 %	Saline 0.9 %
nor-BNI + Saline	Saline 0.9 %	nor-BNI 20 mg/kg	Saline 0.9 %
EtOH group	Saline 0.9 %	Saline 0.9 %	EtOH 1 g/kg
Tolerance group	EtOH 1 g/kg	Saline 0.9 %	EtOH 1 g/kg
Nor-BNI + Tolerance group	EtOH 1 g/kg	nor-BNI 20 mg/kg	EtOH 1 g/kg

Table 4. Schematic group division in the RET and nor-BNI treatment

4.5. RNA EXTRACTION AND REAL-TIME PCR

Gene expression analysis was performed in the AMY and BNST for all experimental animal models. Total RNA was isolated from rat brain tissue with TriZOL (Life Technologies, Grand Island, NY, USA) followed by RNA purification. RNA integrity was checked by 1 % agarose gel electrophoresis and RNA concentrations were measured. Total RNA was then reverse transcribed using random primers and MuLV reverse transcriptase (Life Technologies, Grand Island, NY, USA). Quantitative real-time PCR was performed using the SYBR Green master mix. The following primers were used to amplify the genes of interest: PDYN Forward 5'-CCTGTCCTTGTGTTCCCTGT-3' and Reverse 5'-AGAGGCAGTCAGGGTGAGAA-3'; KOP receptor Forward 5'-TTGGCTACTGGCATCATCTG-3' and Reverse 5'-ACACTCTTCAAGCGCAGGAT-3'. GAPDH and Hypoxanthine-guanine phosphoribosyltransferase (Hprt1) were used as reference genes for msP experiment, and chronic liquid diet and RET experiments respectively. All data were normalized to the reference gene GAPDH or Hprt1 using the ΔΔCt method (Livak and Schmittgen 2001). Results are expressed as mRNA fold changes.

4.6. CHROMATIN IMMUNOPRECIPITATION (ChIP) ASSAY

ChiP assay was performed in RET animals. Chromatin was prepared from frozen tissues as follow: tissues were quickly homogenized in phosphate buffer saline (PBS) and rapidly crosslinked with 16 % non-methanol formaldehyde (final concentration 1 %) for 5 minutes at 37 °C. The cross-linking reaction was quenched by adding 1 M glycine solution. The samples were washed with a solution of PBS and PIC (final concentration 1X) and lysed by re-suspending pellet in a proper volume (50 µL/mg tissue) of lysis

buffer (1 % SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.1) and PIC (final concentration 1X). The samples (130 uL) were sonicated at Covaris (Covaris, Inc., Woburn, Massachusetts, USA) with the setting of 5 % duty factor for 5 minutes in order to obtain DNA fragments ranging in size from 150 to 700 bp. The chromatin was diluted to 700 uL with ChIP dilution buffer (0.01 % SDS, 1.1 % Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 167 mM NaCl) and PIC (final concentration 1X) and 110 µL were removed to serve as "input" DNA, for each immunoprecipitation. Chromatin was pre-cleared with protein A/G plus-agarose beads (Santa Cruz Biotechnology, sc-2003) for 30 minutes at 4 °C on the orbital shaker and, after discarding beads, was incubated overnight at 4 °C on orbital shaker with the 1 µg antibody (H3K9/K14ac Millipore 06-599; H3K9me2 Abcam ab1220; H3K27me3 Abcam ab6002; H3K4me3 Abcam ab1012). After the incubation, 40 µL of agarose beads were added and incubated for 1 hour and 30 minutes at 4 °C on rotation. The beads and associated immune complexes were washed five times with ChIP dilution buffer and then the crosslinking was reverted at 95° for 10 minutes using Chelex® as described by Schoppee Bortz and Wamhoff (2011). Input DNA from each samples was extracted using 100 % ethanol and sodium chloride at final concentration 100 µM and then the same procedure with Chelex® followed for the immunoprecipitated samples was used. Changes in histone modifications at the PDYN and KOP receptor promoter specific sites were evaluated by Real-Time qPCR. Several locations in the promoter regions and in the gene body of PDYN and KOP receptor genes were investigated. In particular, we designed primer to amplify the sequence located where the transcription factor cAMP response element-binding protein (CREB) and the transcriptional coactivator P300 were predicted to bind the DNA sequence by TFBIND (http://tfbind.hgc.jp/). The primer used in the Real-Time qPCR are listed in Table 5.

Gene	*bp from TSS*		*Sequence 5' to 3'*
PDYN	around -1570	Forward	GTTGTATGAGGCATGCAATGAG
		Reverse	CCTCGAGGTAATGGTGATGAAG
PDYN	around -600	Forward	GAGCTGAATGTTCCTCTTCCA
		Reverse	CGAAGGCTGTCTCAGAATATAGG
PDYN	around -170	Forward	AAGTGACAAACAGCGCTACA
		Reverse	GGGCCTGAGTGAAACACAATA
PDYN	around +1550	Forward	TGTGTGTGCGTGTGTTTATTG
		Reverse	CCGTGGAACCGCTGATAC
PDYN	around +4400	Forward	CCCACAGTGCTGAACTTCTAA
		Reverse	CATCTCATTTCCTCCCTGTGAA
PDYN	around +5940	Forward	GCTGTGTACAGGCTGGAGTA
		Reverse	CTTCCCAAGGTCTGACTCTTCT
PDYN	around +7440	Forward	CCACACAGTGATTTGGCTTA
		Reverse	GGAACCAAACATGGTCCTTTG
KOP receptor	around -1250	Forward	AAACCAACCAGTAGTCTTTCCA
		Reverse	GCTGCATATAAGCCAGGACA
KOP receptor	around -600	Forward	AGGAAAGGAGAGTCTGTGTAGTA
		Reverse	TCTGTGCATCTTGTCACTCTG
KOP receptor	around -300	Forward	GATTCCATGCTCTCTTGTCACT
		Reverse	AGCTGCTTTCTGCTTCTCTC
KOP receptor	around +170	Forward	GGGCAATTGTTGTGCTTAGTG
		Reverse	TTGCTTCACCCTTAGGCATC

Table 5. List of primers used in ChIP assay

4.7. STATISTICAL ANALYSIS

All results are expressed as mean ± SEM. msP and Wistar rats data were analyzed by Two-way ANOVA followed by Bonferroni post-hoc test. Data from chronic liquid diet experiment, RET and RET + nor-BNI treatments were analyzed by One-way ANOVA followed by Tukey's post-hoc test. The threshold for statistical significance was always set at a p value < 0.05.

5. RESULTS

5.1. VOLUNTARY EtOH INTAKE OF msP AND WISTAR RATS

msP and Wistar rats were exposed to the chronic intermittent two bottles free-choice paradigm. msP rats consume an higher amount of EtOH compared Wistar animals since the early phase of the procedure. Around day 15, this protocol leads to an escalation in alcohol consumption more remarkable in msP rats. Finally, the EtOH consumption remains high until the end of exposure (Table 6 and Figure 11).

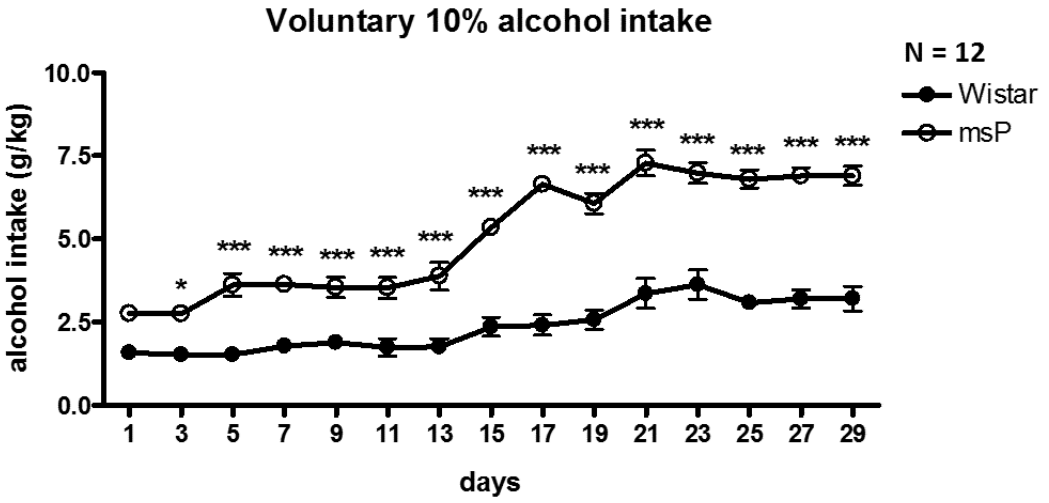

Figure 11. Voluntary EtOH intake of Wistar and msP rats. Data are represented as mean ± SEM. Differences between groups were estimated by two-way ANOVA followed by Bonferroni post-hoc test. (* $p < 0.05$; *** $p < 0.001$).

Day	*EtOH intake (g/kg)*	
	Wistar rats	**msP rats**
1	1,592	2,767
3	1,525	2,758
5	1,533	3,625
7	1,792	3,642
9	1,900	3,542
11	1,750	3,533
13	1,767	3,883
15	2,367	5,342
17	2,417	6,642
19	2,575	6,067
21	3,367	7,283
23	3,633	6,983
25	3,083	6,800
27	3,208	6,900
29	3,208	6,900

Table 6. EtOH intake amount of Wistar and msP rats

5.2. PDYN AND KOP RECEPTOR GENE EXPRESSION IN msP AND WISTAR RATS

PDYN and KOP receptor mRNA levels were measured in the AMY and BNST of msP and Wistar rats, both naïve and EtOH exposed. There is a difference in the amygdaloid basal levels of KOP receptor mRNA in the AMY of msP rats (1.26 ± 0.05 *vs* 1.00 ± 0.10 Wistar naïve rats, $p < 0.05$) (Figure 12). In the AMY and BNST, EtOH exposure does not induce any changes of PDYN and KOP receptor gene expression in Wistar animals (Figures 12 and 13). Conversely, EtOH induces a decrease of PDYN and KOP receptor mRNA levels in the AMY of msP rats (PDYN: 0.95 ± 0.11 msP naïve rats *vs* 0.45 ± 0.09 msP EtOH rats, $p < 0.05$; KOP receptor: 1.26 ± 0.05 msP naïve rats *vs* 0.65 ± 0.04, $p < 0.001$) (Figure 12). Finally, msP rats exposed to EtOH show higher mRNA levels of KOP receptor in the BNST (1.41 ± 0.09 *vs* 0.88 ± 0.04 msP EtOH rats, $p < 0.001$) (Figure 13).

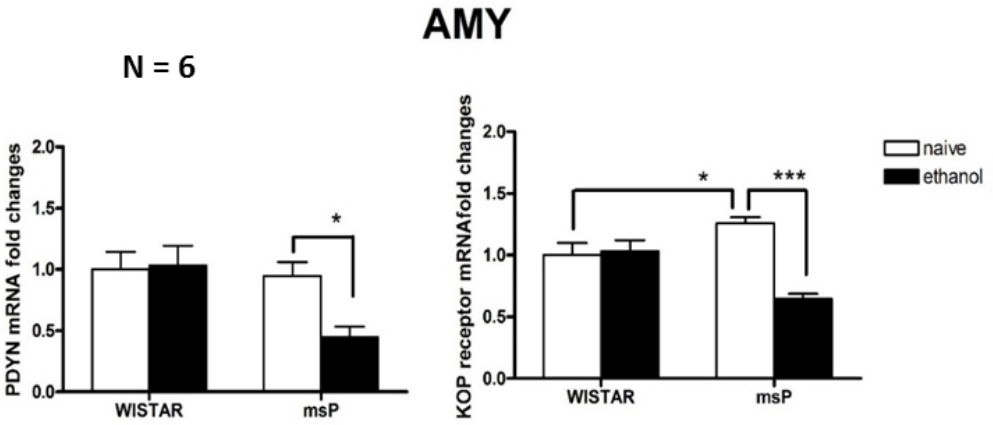

Figure 12. PDYN and KOP receptor gene expression in the AMY. The PDYN and KOP receptor mRNA levels were assessed in the AMY using Real-Time qPCR analysis compared to GAPDH. Data are presented as mean ± SEM (n = 6 rats per group) and analyzed by two-way ANOVA (*** $p < 0.001$; * $p < 0.05$).

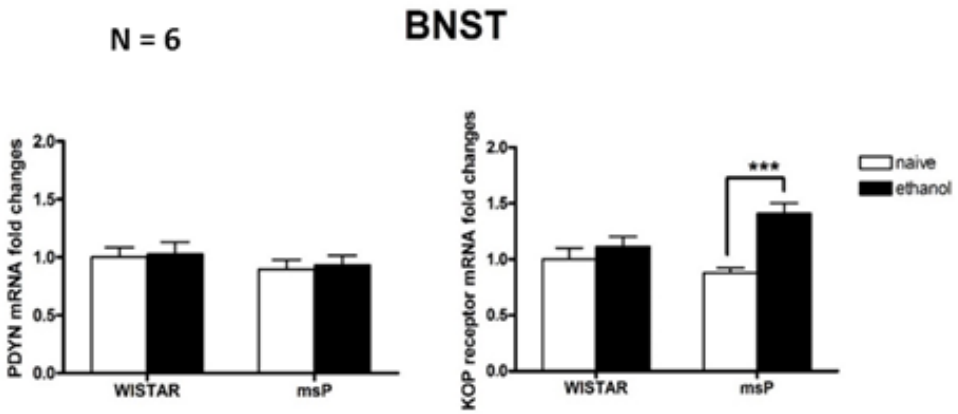

Figure 13. PDYN and KOP receptor gene expression in the BNST. The PDYN and KOP receptor mRNA levels were assessed in the BNST using Real-Time qPCR analysis compared to GAPDH. Data are presented as mean ± SEM (n = 6 rats per group) and analyzed by two-way ANOVA (*** $p < 0.001$).

5.3. PDYN AND KOP RECEPTOR GENE EXPRESSION IN THE CHRONIC LIQUID DIET MODEL

After 15 day of EtOH exposure using liquid diet, a marked increase of PDYN mRNA levels has been observed in the AMY (2.28 ± 0.10 *vs* 1.06 ± 0.15 Control group, $p < 0.01$), while no changes of KOP receptor have been detected (Figure 14). Interestingly, the PDYN mRNA levels remain high after 24 hours withdrawal (2.26 ± 0.32 *vs* 1.06 ± 0.15 Control group, $p < 0.01$), and the KOP receptor gene expression is also increased (1.52 ± 0.08 *vs* 1.05 ± 0.13 Control group, $p < 0.05$) (Figure 14). On the other hand, KOP receptor mRNA levels were decreased without change in PDYN mRNA levels in the BNST of ethanol-withdrawn rats (0.62 ± 0.06 *vs* 1.01 ± 0.03 Control group, $p < 0.05$).

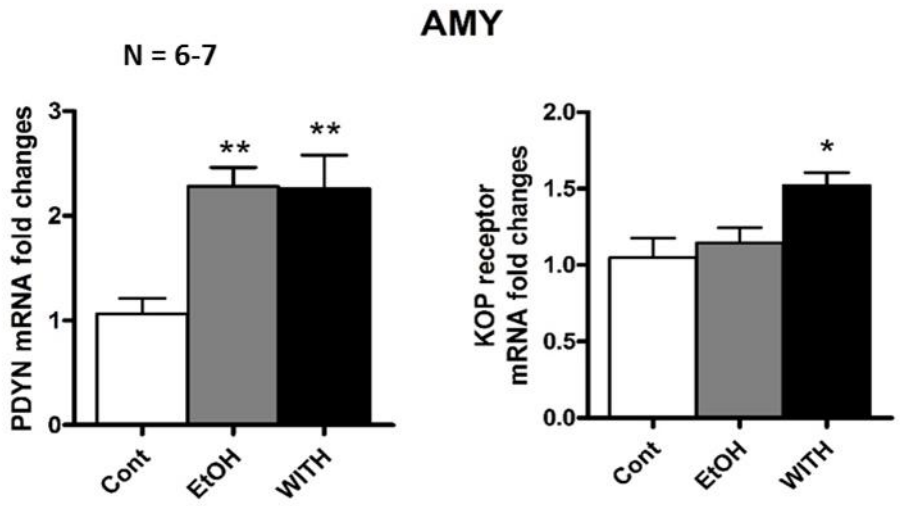

Figure 14. The effect of chronic ethanol exposure and its withdrawal on PDYN and KOP receptor gene expression in the AMY. The PDYN and KOP receptor mRNA levels were assessed in the AMY using Real-Time qPCR and data were normalized using Hprt1 as a control gene. Data are presented as mean ± SEM (n = 6 or 7 rats per group) and analyzed by one-way ANOVA (* $p < 0.05$; ** $p < 0.01$).

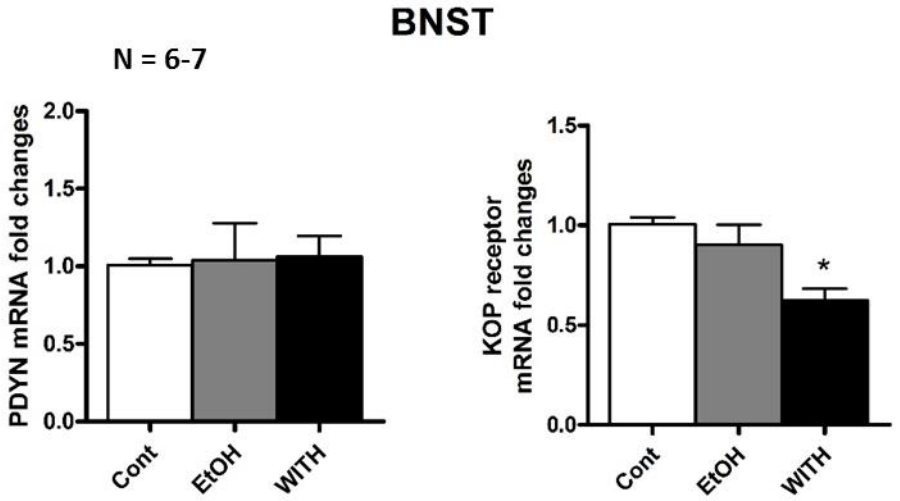

Figure 15. The effects of chronic ethanol treatment and its withdrawal on PDYN and KOP receptor gene expression in the BNST. The PDYN and KOP receptor mRNA levels were assessed in the BNST Real-Time qPCR and data were normalized using Hprt1 as a control gene. Data are presented as mean ± SEM (n = 6 or 7 rats per group) and analyzed by one-way ANOVA (* $p < 0.05$).

5.4. BLOOD ALCOHOL LEVEL AND ANXIETY-LIKE BEHAVIOR IN THE RET MODEL

The BAL of EtOH and tolerance group is similar suggesting that there is no tolerance in EtOH metabolism (Table 7).

Animal group	*mg/dl of EtOH*
EtOH group	85.7 ± 5.4
Tolerance group	85.5 ± 4.4

Table 7. The BAL in the EtOH and tolerance group.

Despite the same BAL, EtOH produces anxiolytic effects in the EtOH group but not in the Tolerance group (Figure 16). In fact, EtOH treated rats spend more time in the open arm than Control and Tolerance groups (% time spent 49.49 ± 4.84 *vs* 31.62 ± 3.00 Control group and *vs* 29.07 ± 3.56 Tolerance group, $p < 0.05$ and < 0.01 respectively). Moreover, the open arm entries are significantly increased in the EtOH (49.77 ± 3.01 *vs* 35.80 ± 1.32 Control group and *vs* 32.78 ± 1.98 Tolerance group, $p < 0.01$ and < 0.001 respectively). However, in the Tolerance group the increase in the number of closed arm entries indicates that EtOH does not attenuate general activity of rats (9.13 ± 0.61 *vs* 7.17 ± 0.40 Control group, $p < 0.05$).

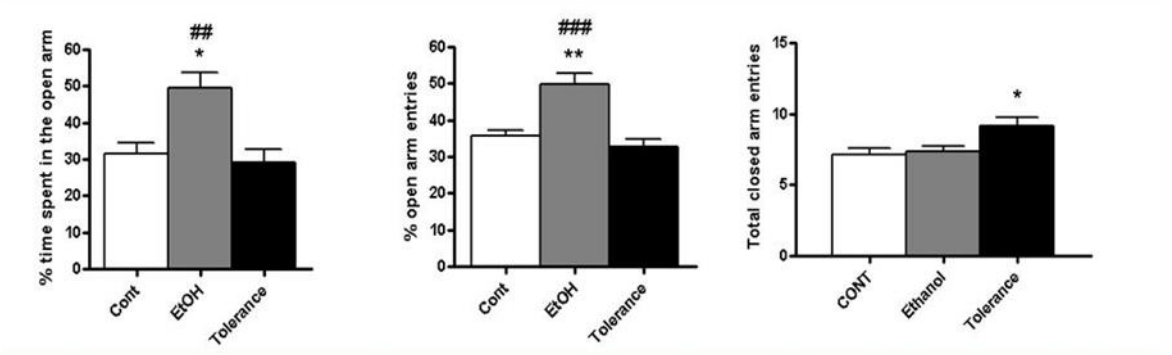

Figure 16. Development of rapid ethanol tolerance to the anxiolytic effects of ethanol. Elevated plus maze exploration (EPM) test has been used to measure the anxiety-like behavior. Anxiolytic effect is evaluated by percentage of the time spent in the open arm and the open arm entries. Data are presented as mean ± SEM (n = 8 rats per group) and are analyzed by one-way ANOVA followed by Tukey's test (* $p < 0.05$ and ** $p < 0.01$ vs Control group; ## $p < 0.01$ and ### $p < 0.001$ vs Tolerance group).

5.5. PDYN AND KOP RECEPTOR GENE EXPRESSION IN THE RET MODEL

After behavioral tests, animals were sacrificed and gene expression analysis was conducted in the AMY and BNST. Similar to what observed in the chronic ethanol model, PDYN gene expression is increased in the AMY the EtOH group (2.23 ± 0.32 *vs* 0.96 ± 0.13 Control group, $p < 0.01$) and remained increase in the Tolerance group (2.69 ± 0.30 *vs* 0.96 ± 0.13 Control group, $p < 0.001$) (Figure 17). Moreover, tolerant animals show an increase of KOP receptor mRNA levels in the AMY (1.50 ± 0.14 *vs* 1.02 ± 0.07 Control group, $p < 0.05$) (Figure 17). Finally, no changes were observed in the BNST (Figure 18).

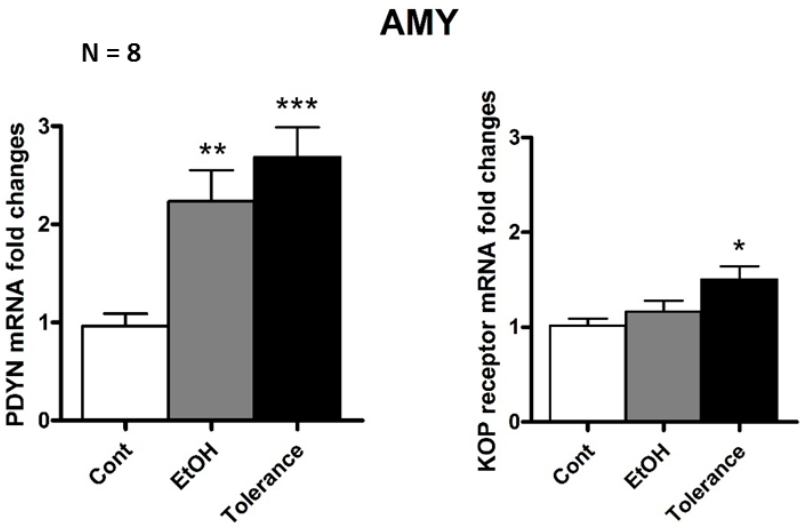

Figure 17. Changes in PDYN and KOP receptor gene expression in the AMY during RET. The PDYN and KOP receptor mRNA levels were assessed in the AMY using Real-Time qPCR using Hprt1 as a control gene. Data are presented as mean ± SEM (n = 8 rats per group) and analyzed by one-way ANOVA Tukey's test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

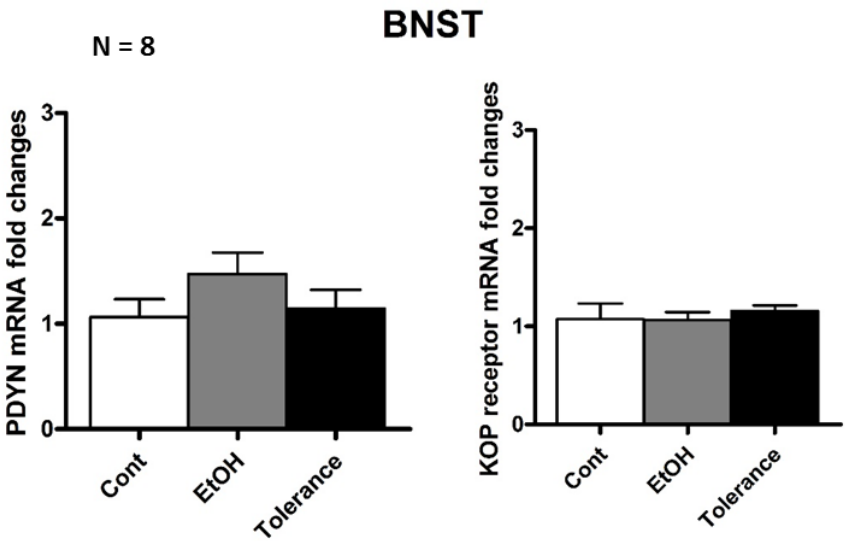

Figure 18. Changes in PDYN and KOP receptor gene expression in the BNST during RET. The PDYN and KOP receptor mRNA levels were assessed in the BNST using Real-Time qPCR using Hprt1 as a control gene. Data are presented as mean ± SEM (n = 8 rats per group) and analyzed by one-way ANOVA.

5.6. HISTONE MODIFICATIONS AT PDYN GENE IN THE RET MODEL

Since no alterations were detected in the BNST, epigenetic experiments were conducted in the AMY only. In the PDYN promoter, acute EtOH treatment induced a significant increase of H3K9/K14ac levels at + 1550 bp from the TSS (1.49 ± 0.12 *vs* 0.99 ± 0.09 Control group, $p < 0.01$). However, a decrease of this mark at all locations of PDYN gene body was observed during tolerance (- 170 from TSS: 0.59 ± 0.07 *vs* 1.05 ± 0.11, $p < 0.01$; + 1550 bp: 0.59 ± 0.07 *vs* 0.99 ± 0.09 Control group, $p < 0.05$ and *vs* 1.49 ± 0.12 EtOH group, $p < 0.001$; + 4400 bp: 0.71 ± 0.06 *vs* 1.05 ± 0.04 Control group, $p < 0.05$ and *vs* 1.05 ± 0.10 EtOH group, $p < 0.01$; + 5940 bp: 0.56 ± 0.06 *vs* 1.05 ± 0.10 Control group, $p < 0.01$ and *vs* 0,89 ± 0,11 EtOH group, $p < 0.05$; + 7440 bp: 0.60 ± 0.09 *vs* 1.04 ± 0.12 Control group, $p < 0.05$) (Figure 19).

In regard to changes in H3K9me2 occupancy, an increase of this histone modification levels was observed at two location of the PDYN gene in the EtOH group (-170 bp from TSS: 1.53 ± 0.12 vs 0.99 ± 0.06 Control group, $p < 0.01$; + 4400 bp: 1.31 ± 0.08 vs 0.94 ± 0.07 EtOH group, $p < 0.05$) (Figure 19).

Two other histone marks were investigated: H3K27me3 and H3K4me3. EtOH induces a marked increase of H3K27me3 levels at all sites investigated of the PDYN gene (- 1570 bp from TSS: 1.36 ± 0.08 *vs* 1.01 ± 0.05 Control group, $p < 0.01$ and *vs* 1.00 ± 0.06 Tolerance group, $p < 0.01$; - 600 bp: 1.35 ± 0.11 *vs* 1.03 ± 0.06 Control group, $p < 0.05$ and *vs* 0.70 ± 0.08 Tolerance group, $p < 0.001$; - 300 bp: 1.73 ± 0.09 *vs* 1.02 ± 0.07 Control group, $p < 0.001$ and *vs* 1.27 ± 0.09 Tolerance group, $p < 0.01$; + 1550 bp: 1.73 ± 0.05 *vs* 1.02 ± 0.07 Control group, $p < 0.001$ and *vs* 1.24 ± 0.08 Tolerance group, $p < 0.001$; + 4400 bp: 1.39 ± 0.11 *vs* 0.99 ± 0.06 Control group, $p < 0.01$; + 5940 bp: 1.52 ± 0.10 *vs* 1.03 ± 0.08 Control group, $p < 0.01$ and *vs* 1.15 ± 0.06 Tolerance group, $p < 0.05$; + 7440 bp: 1.50 ± 0.13 *vs* 1.04 ± 0.07 Control group, $p < 0.05$ and *vs* 0.97 ± 0.12

Tolerance group, $p < 0.05$). Moreover, no changes were detected in the Tolerance group, except at one site where low levels of H3K27me3 were observed (- 600 bp from TSS: 0.70 ± 0.08 *vs* 1.03 ± 0.06 Control group, $p < 0.05$) (Figure 20).

Finally, alterations of H3K4me3 levels were observed in the sites around the TSS. In particular, EtOH induced changes at three sites (- 600 bp from TSS: 1.49 ± 0.12 *vs* 1.05 ± 0.08 Control group, $p < 0.01$ and *vs* 0.81 ± 0.03 Tolerance group, $p < 0.001$; - 170 bp: 1.48 ± 0.09 *vs* 1.01 ± 0.06 Control group, $p < 0.01$; + 1550 bp: 1.63 ± 0.14 *vs* 1.02 ± 0.09 Control group, $p < 0.01$ and *vs* 1.10 ± 0.10 Tolerance group, $p < 0.05$). In addition, the site before the TSS also showed a significant increase of H3K4me3 levels during RET (- 170 bp from TSS: 1.41 ± 0.10 *vs* 1.01 ± 0.06 Control group, $p < 0.05$) (Figure 20).

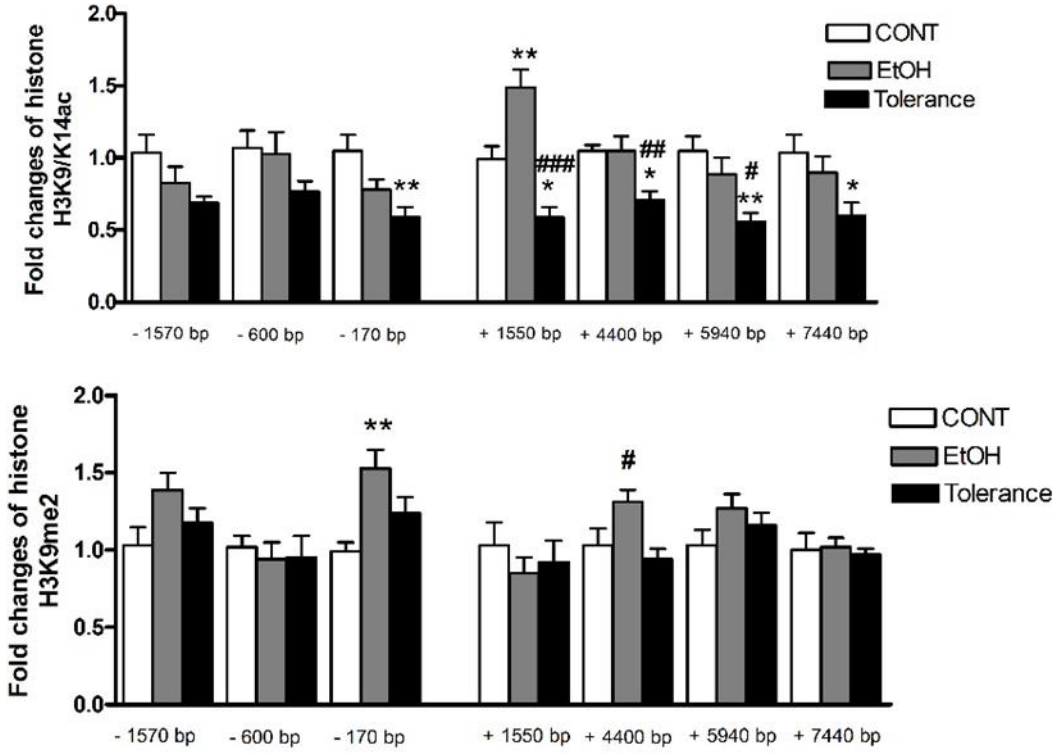

Figure 19. Changes in H3K9/K14ac and H3K9me2 levels at PDYN gene promoter and body. Real Time qPCR analysis of H3K9/K14ac and H3K9me2 immuno-precipitated DNA fragments in the PDYN promoter and gene body in rat AMY. Bar diagram shows the levels of specific histone modifications of gene, normalized to total input DNA. Data are represented as Mean ± SEM (n = 8 rats per group) and analyzed by one-way ANOVA followed by Tukey's test (* $p < 0.05$ and ** $p < 0.01$ vs Control group; # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ vs Tolerance group).

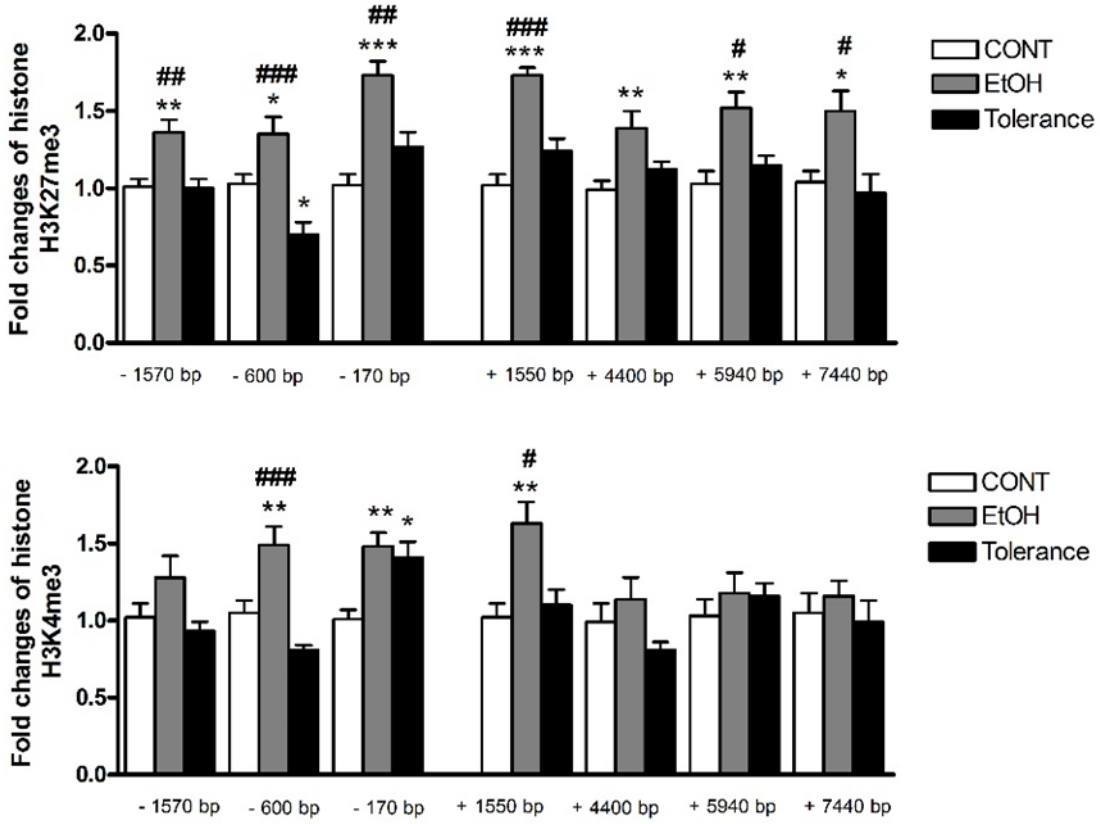

Figure 20. Changes in H3K27me3 and H3K4me3 levels at PDYN gene promoter and body. Real Time qPCR analysis of H3K27me3 and H3K4me3 immuno-precipitated DNA fragments in the PDYN promoter and gene body in rat AMY. Bar diagram shows the levels of specific histone modifications of gene, normalized to total input DNA. Data are represented as Mean ± SEM (n = 8 rats per group) and analyzed by one-way ANOVA followed by Tukey's test (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs Control group; # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ vs Tolerance group).

5.7. HISTONE MODIFICATIONS AT KOP RECEPTOR GENE IN THE RET MODEL

Similar to what observed at PDYN gene promoter, tolerant animals show a decrease of H3K9/K14ac levels in the KOP receptor gene promoter and body in the AMY (- 600 bp from TSS: 0.52 ± 0.05 *vs* 0.98 ± 0.10 Control group, $p < 0.05$ and *vs* 0.97 ± 0.08 EtOH group, $p < 0.05$; - 300 bp: 0.68 ± 0.06 *vs* 1.01 ± 0.09 Control group, $p < 0.05$; + 170 bp: 0.66 ± 0.08 *vs* 1.12 ± 0.14 EtOH group, $p < 0.05$) (Figure 21).

In addition, EtOH induces an increase of H3K9me2 levels at one site close to the TSS (- 300 from TSS: 1.51 ± 0.08 *vs* 1.06 ± 0.09 Control group, $p < 0.05$). In the Tolerance group, a significant decrease of H3K9me2 levels was detected at + 170 bp from the TSS, even if no changes were detected in the EtOH group (0.68 ± 0.08 *vs* 1.03 ± 0.10 Control group, $p < 0.05$ and *vs* 1.00 ± 0.08 EtOH group, $p < 0.05$) (Figure 21).

A significant increase of H3K27me3 was detected in the EtOH group at three different locations on the KOP receptor gene promoter and body (- 600 bp from TSS: 1.43 ± 0.09 *vs* 1.01 ± 0.06 Control group, $p < 0.01$ and *vs* 0.73 ± 0.10 Tolerance group, $p < 0.001$; - 300 bp: 1.32 ± 0.09 *vs* 1.02 ± 0.08 Control group, $p < 0.05$; + 170 bp: 1.61 ± 0.08 *vs* 0.99 ± 0.05 Control group, $p < 0.001$ and *vs* 1.17 ± 0.08 Tolerance group, $p < 0.01$). Moreover, an increase of this histone mark levels was also observed in the Tolerance group at one site (- 300 bp from TSS: 1.35 ± 0.08 *vs* 1.02 ± 0.08 Control group, $p < 0.05$) (Figure 22).

Finally, there is one site before the TSS in the KOP receptor gene promoter showing an increase of H3K4me3 levels (- 300 bp from TSS: 1.49 ± 0.12 *vs* 1.03 ± 0.11 Control group, $p < 0.05$) (Figure 22).

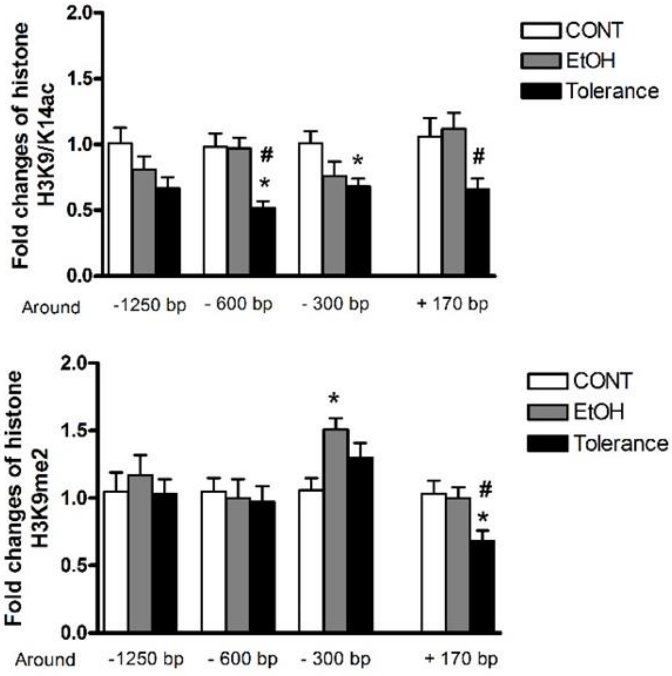

Figure 21. Changes in H3K9/K14ac and H3K9me2 levels at KOP receptor gene promoter and body. Real Time qPCR analysis of H3K9/K14ac and H3K9me2 immuno-precipitated DNA fragments in the PDYN promoter and gene body in rat AMY. Bar diagram shows the levels of specific histone modifications, normalized to total input DNA. Data are represented as Mean ± SEM (n = 8 rats per group) and analyzed by one-way ANOVA followed by Tukey's test (* $p < 0.05$ vs Control group; # $p < 0.05$ vs Tolerance group).

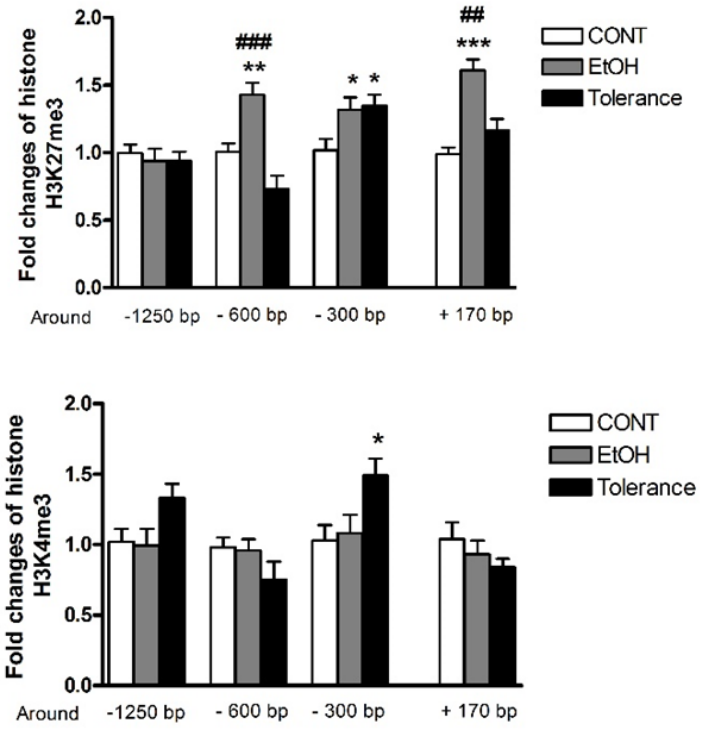

Figure 22. Changes in H3K27me3 and H3K4me3 levels at KOP gene promoter and body. Real Time qPCR analysis of H3K27me3 and H3K4me3 immuno-precipitated DNA fragments in the PDYN promoter and gene body in rat AMY. Bar diagram shows the levels of specific histone modifications, normalized to total input DNA. Data are represented as Mean ± SEM (n = 8 rats per group) and analyzed by one-way ANOVA followed by Tukey's test (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs Control group; ## $p < 0.01$ and ### $p < 0.001$ vs Tolerance group).

5.8. BLOOD ALCOHOL LEVEL AND ANXIETY-LIKE BEHAVIOR IN THE RET MODEL WITH AND WITHOUT NOR-BNI TREATMENT

The BAL of EtOH, Tolerance and nor-BNI + Tolerance groups is similar suggesting that there is no change in EtOH metabolism (Table 8).

Animal group	*mg/dl of EtOH*
EtOH group	84.5 ± 5.50
Tolerance group	87.2 ± 5.42
Nor-BNI + Tolerance group	82.2 ± 6.93

Table 8. The BAL in the EtOH, Tolerance and nor-BNI + Tolerance groups.

Results from the EPM test show that nor-BNI treatment was able to produce reversal of RET to anxiolytic effects of ethanol (% time spent in open arm: 52.08 ± 2.61 *vs* 38.76 ± 2.45 Control group, $p < 0.01$; and *vs* 33.11 ± 1.97 Tolerance group, $p < 0.001$) and % number of open arm entries (51.19 ± 1.36 *vs* 39.78 ± 2.00 Control group, $p < 0.01$; and *vs* 40.89 ± 2.16 Tolerance group, $p < 0.01$). However, nor-BNI treatment itself exerts anxiolytic effects, with an increase of percentage of time spent in the open arm (50.55 ± 1.76 *vs* 38.76 ± 2.45 Control group, $p < 0.01$) and percentage of number of open arm entries (51.00 ± 2.44 *vs* 39.78 ± 2.00 Control group, $p < 0.01$).

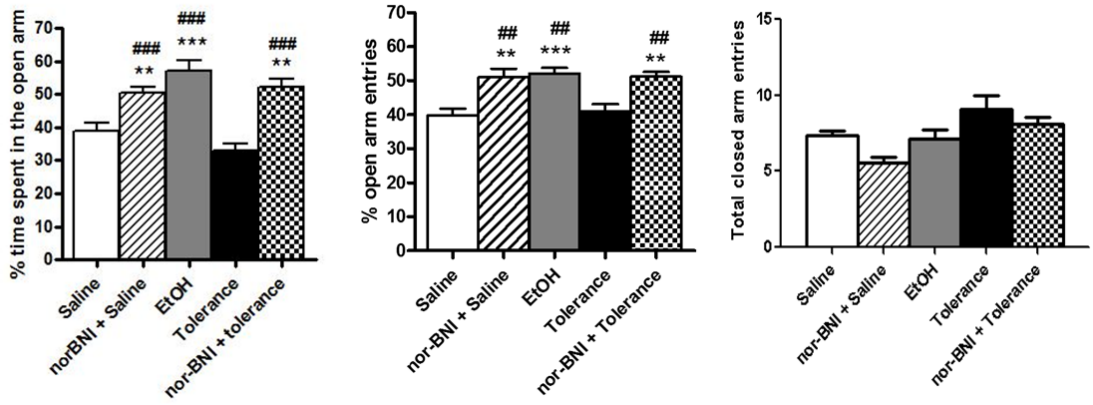

Figure 23. Anxiety-like behavior in RET model with nor-BNI treatment. Elevated plus maze exploration (EPM) test has been used to determine anxiety measures. Anxiolytic effect is evaluated by percentage of the time spent in the open arm and the open arm entries. Data are presented as mean ± SEM (n = 9 rats per group) and are analyzed by one-way ANOVA followed by Tukey's test (** $p < 0.01$ and *** $p < 0.001$ vs Control group; ## $p < 0.01$ and ### $p < 0.001$ vs Tolerance group).

6. DISCUSSION

The DYN/KOP system has been implicated in modulating EtOH consumption and cognitive and affective state associated with alcohol dependence (Walker et al., 2012). In particular, the activation of this system induces dysphoric effect promoting alcohol intake and anxiogenic-like responses during withdrawal (Walker et al., 2011; Valdez and Harshberger, 2012). Therefore, in the present study we investigated the role of DYN/KOP system in different model of alcohol tolerance and dependence.

The first model investigated is an animal model of alcohol-preferring rats represented by msP rats. msP rats show spontaneous preference for EtOH and binge-type of drinking; moreover, they are highly sensitive to stress and exhibit anxiety and depressive-like phenotype that attenuates following alcohol drinking (Ciccocioppo et al., 2006). msP rats and their counterpart Wistar were subjected to the chronic intermittent two bottles free-choice paradigm; the intermittent exposure to EtOH consists in repeated cycles of drinking/withdrawal in order to establish alcohol dependence. From the early phase of the exposure msP rats drink significantly higher amount of EtOH compared to Wistar ones. Interestingly, approximately at day 15 animals show an escalation in the alcohol consumption more remarkable in msP than Wistar rats; the EtOH consumption remains high until the end of exposure.

Gene expression analysis was conducted in the AMY and BNST of naïve and alcohol dependent animals to identify innate and EtOH-induced differences in PDYN and KOP receptor mRNA levels of msP and Wistar rats. msP rats innately show higher levels of KOP receptor mRNA in the AMY compared to Wistar animals. Alcohol preference has been shown to be modulated by the changes in KOP receptor levels; in fact, KOP receptor KO mice showed low alcohol preference (Kovacs et al., 2005). Hence, spontaneous preference for alcohol exhibited by msP rats may be related to the high

levels of KOP receptor mRNA in the AMY. Moreover, the activation of the DYN/KOP system in the extended amygdala can be responsible for depressive and anxiogenic-like responses (Chartoff et al., 2012; Knoll et al., 2007; Land et al., 2008). Therefore, the high innate levels of KOP receptor mRNA in the AMY may also contribute to the anxiety and depressive-like behaviors exhibited by msP rats. Further supporting this hypothesis, we observed a decrease of both PDYN and KOP receptor mRNA content in the AMY of msP rats after alcohol exposure. It has been demonstrated that msP rats can attenuate the anxiety and depressive-like behaviors by alcohol intake and this may promote and maintain alcohol-drinking behavior (Ciccocioppo et al., 1999; Ciccocioppo et al., 2006). The attenuation of anxious and depressive symptoms may correspond to the decrease of the DYN/KOP system gene expression in AMY, suggesting that this system could have an important role in promoting alcohol intake and negative emotional state associated to alcohol dependence. Finally, chronic EtOH exposure induces an increase of KOP receptor mRNA in the BNST of msP rats, suggesting alcohol can differently regulate the KOP receptor expression in the AMY and BNST.

The second model of alcohol dependence investigated is the ethanol exposed model using liquid diet. It has been reported that animals exposed to chronic EtOH do not show any anxiolytic effect induced by EtOH, but withdrawn rats exhibit anxiety-like behavior (Pandey et al., 2008; You et al., 2014). Here, we observed that chronic EtOH exposure induces a marked increase in PDYN mRNA levels in the AMY, but not KOP receptor. On the contrary, after 24 hours withdrawal both PDYN and KOP receptor gene expression is increased. These results suggest that the anxiety state may be mediated by to the up-regulation of the DYN/KOP system gene expression. In fact, the increase of PDYN mRNA alone could not induce anxiety, but it seems that the increase of the whole DYN/KOP system transcription is necessary. Similarly, we observed a

decrease of both PDYN and KOP receptor mRNA levels in the AMY of EtOH-exposed msP rats, in which attenuation of anxiety and depressive-like behaviors have been established. In addition, 24 hours withdrawal induces a decrease of KOP receptor mRNA levels in the BNST, thus confirming that opposite consequences on KOP receptor are detectable in the AMY and BNST, as observed in msP rats. Hence, the dysregulation of the KOP receptor expression in the AMY and BNST may contribute to the development of the negative emotional state associated to alcohol dependence.

We then investigated the role of DYN/KOP system in alcohol tolerance. Despite several studies focus on the relevance of this system in alcohol dependence (Walker et al., 2011; Valdez and Harshberger, 2012; Gillett et al., 2013; Funk et al., 2014), this study is the first focusing on the possible implication of DYN/KOP system in RET. It has been shown that two same doses of EtOH 24 hours apart do not elicit anxiolytic effect, but an alcohol higher dose restores the anxiolytic effect (Sakharkar et al., 2012). Here, we confirmed the development of tolerance to the anxiolytic effect of EtOH. Animals receiving two EtOH injections 24 hours apart spend almost the same time in the open arm of the EPM compared to Control group, and significantly less time compared to rats treated with a single EtOH injection, thus indicating that the Tolerance group shows no anxiolytic response. The development of RET seems to refer only to the anxiolytic effect of EtOH, since the BAL of EtOH and Tolerance groups is similar suggesting that there is no tolerance in alcohol metabolism. In addition, considering the number of entries, EtOH group has a higher percentage of open arm entries compared to Tolerance group, further indicating the anxiolytic effects of alcohol. On the contrary, tolerant animals preferentially enter in the closed arm of the maze; therefore, if we consider the number of total entries there is no difference between EtOH and Tolerance group suggesting that animals do not show any change in general activity of rats. Taken

together, these results suggest the development of RET to the anxiolytic effects of EtOH. We used this model to investigate the role of PDYN/KOP receptor system in RET.

We observed that EtOH induces a remarkable increase of PDYN mRNA levels, which remain higher during tolerance in the AMY. In addition, tolerant rats exhibit increased KOP receptor gene expression. The lack of EtOH-induced anxiolytic effect observed in the Tolerance group may be related to the up-regulation of the DYN/KOP system expression. Interestingly, the DYN/KOP system alterations observed in the AMY are similar to those observed in the chronic liquid diet model, suggesting that alcohol dependence and RET may share common amygdaloid neuronal mechanisms leading to the negative affective consequences. However, different from what observed in the two models of alcohol dependence, we did not report any changes in the BNST of tolerant animals, suggesting that DYN/KOP system alterations in the BNST may be consequences of the primary changes occurring in the AMY. Anyway, the role of BNST may be crucial in modulating the anxiety behavior associated to the AUD. In fact, Tolerance group has a lack of anxiolytic effect and no alterations in the BNST, while 24 hours withdrawal rats showed anxiety-like behavior (You et al., 2014) and changes of KOP receptor mRNA both in the AMY and BNST.

Since transcriptional alterations only occur in the AMY, we investigated which epigenetic marks could be responsible for the DYN/KOP system changes in RET model. In particular, we focused our attention on four histone modifications, the H3K9/K14ac and H3K4me3, two marks of the transcriptional active state of the chromatin, the H3K9me2 and H3K27me3, two repressive marks. In particular, we analyzed these histone modifications at several sites on the PDYN and KOP receptor gene promoter and body sequences, where transcription factors, such as CREB and

P300, were predicted to bind. The PDYN gene has several sites in which tolerant animals show decrease levels of H3K9/K14ac; since PDYN gene expression is increased both in EtOH and Tolerance groups, our data suggest that H3K9/K14ac could not be primary responsible for the regulation of PDYN transcription. In addition, the histone modification H3K9me2 is altered at two sites in EtOH group, and considering that it is considered a repressive mark, it may not be involved in the regulation of PDYN gene expression. Similarly, results of H3K9/K14ac and H3K9me2 levels at different sites of KOP receptor gene suggest that these two histone marks may not be primarily involved in modulating the DYN/KOP system gene expression.

Interestingly, EtOH induces an increase of H3K27me3 levels in PDYN gene. Actually, H3K27me3 is generally considered a repressive mark, and gene expression analysis revealed that EtOH increases PDYN mRNA levels. However, it has been demonstrated that some genes are highly expressed despite a high enrichment of H3K27me3 around the TSS (Young et al., 2011). These genes are known as bivalent genes, since the H3K27me3 modification simultaneously occurs with the activating mark H3K4me3 in a region called bivalent domain (Bernstein et al., 2006; Alder et al., 2010; Mazzarella et al., 2011). In agreement with this hypothesis, we observed an increase of H3K4me3 levels around the TSS of PDYN gene in EtOH group. Although bivalent genes are prevalent in embryonic stem cells (Bernstein et al., 2006), our data suggest that EtOH is able to induce a pluripotent state of PDYN gene in amygdaloid neurons. Indeed, it has been demonstrated that the anxiolytic effect of acute EtOH is associated with increased BDNF and Arc (a marker for brain plasticity) expression, as well as increased density of dendritic spines in the AMY (Pandey et al., 2008). Therefore, in this context of synaptic plasticity induced by acute EtOH, PDYN gene could increase the transcription of its mRNA promoted by the bivalent domain. Interestingly, at one site close to the TSS (-

170 bp) levels of H3K4me3 remain high during tolerance, suggesting that this site may be crucial in mediating the PDYN gene expression since mRNA levels also remain high during tolerance.

As mentioned above, the mere increase of PDYN gene expression may not be sufficient to block the anxiolytic effect induced by EtOH. Indeed, the lack of anxiolytic behavior has been observed in Tolerance group, in which both PDYN and KOP receptor mRNA are increased. Epigenetic results revealed that H3K27me3 and H3K4me3 could also regulate KOP receptor gene transcription; in this case, acute EtOH only induces H3K27me3 increase around the TSS with no changes in H3K4me3 levels. Hence, we did not observed any EtOH-induced increase of KOP receptor mRNA and as behavioral consequence, animals exhibit anxiolytic responses. On the contrary, an increase of H3K4me3 levels is reported at one site close to the TSS (around -300 bp) in Tolerance group and this may be responsible for the KOP receptor gene expression increased observed in this group.

Finally, to confirm the involvement of DYN/KOP system in RET, we treated animals with the KOP receptor antagonist nor-BNI. It has been reported that nor-BNI is effective in attenuating EtOH self-administration in alcohol-dependent animals (Walker and Koob, 2008; Walker et al., 2011) and the anxiety-like behavior observed during acute EtOH withdrawal (Valdez and Harshberger, 2012). Here, we observed that the nor-BNI treatment restores the anxiolytic effect of EtOH in Tolerance group; in fact, the time spent in the open arm and open arm entries are higher in nor-BNI + Tolerance group compared to tolerant animals. It is likely that nor-BNI treatment functionally block the upregulation of KOP receptor observed in the Tolerance group therefore preventing the RET to anxiolytic effects of EtOH. However, nor-BNI itself induces anxiolytic effect in agreement with previous studies showing that the nor-BNI dose of

20 mg/kg induces anxiolytic effects (Knoll et al., 2007; Valdez and Harshberger, 2012). Therefore, other studies investigating the specific mechanisms by which nor-BNI prevents the RET to the EtOH anxiolytic effects are still needed.

7. CONCLUSION

In conclusion, similar alterations of DYN/KOP system in the AMY have been detected in the two alcohol dependence models and RET, arising the hypothesis that the neuronal mechanisms leading to the negative affective consequences of alcohol dependence and rapid tolerance could be similar. Moreover, the opposite regulation of KOP receptor gene expression in the AMY and BNST observed in msP rats and during withdrawal after chronic ethanol exposure in unselected stock of rats suggests that the dysregulation of the KOP receptor between these areas may contribute to the development of the negative emotional state associated to alcohol dependence.

Finally, epigenetic results highlight that PDYN and KOP receptor gene expression can be mainly regulated by H3K27me3 and H3K4me3 marks during EtOH exposure and tolerance, suggesting that the modulation of these histone modifications may be useful in controlling the DYN/KOP system expression and consequently the development of tolerance.

CONCLUSIVE REMAKS AND FUTURE DIRECTIONS

Drug and alcohol dependence is characterized by the occurrence of allostatic state represented by a combination of the anti-reward system activation and subsequent chronic decrease function of rewards circuits, leading to the compulsive drug seeking behavior and loss of control in limiting drug intake (Koob, 2015). In addition, the activation of brain stress system contributes to exacerbate the negative emotional state associated to alcohol dependence (Koob, 2015).

Alterations of the endogenous opioid system, in particular the DYN/KOP system, have been detected in several animal models of alcohol dependence, and targeting this system may contribute to develop new therapeutic approach for alcohol dependence (Koob, 2014). Other neurotransmitters and factors are studied for their involvement in regulating responses to alcohol abuse. For instance, BDNF is deeply involved in modulating alcohol intake and emotional state alcohol-related (Logrip et al., 2015; Moonat and Pandey, 2012). In the recent years, several studies have been focused on epigenetic mechanisms involved in neuroplasticity phenomena related to alcohol abuse (Moonat and Pandey, 2012; Robison and Nestler, 2011).

The present study investigated epigenetic and transcriptional alterations induced by alcohol in different paradigms of alcohol exposure. In the first part of this study, we focused on a model of BDNF +/- mice, which show spontaneous preference for EtOH consumption and high vulnerability to develop alcohol addiction (Hensler et al., 2003; McGough et al., 2004; Bosse and Mathews, 2011). Here, we reported that HDACs class I are altered in the CPu and PFCx of BDNF +/- animals, suggesting that low levels of BDNF affect the HDACs levels. In addition, alcohol can modulate the HDACs class I protein levels. Considering the important role of the corticostriatal circuit in alcohol dependence mechanism, our results indicate that epigenetic changes may be crucial in

modulating neuroplasticity in these brain regions. Data here presented have been published in 2015 on the journal *Drug and Alcohol Dependence* (Caputi et al., 2015).

In the second part of my PhD course, we transversally explored the DYN/KOP system role in models of alcohol dependence and tolerance. Some information have already been available on the role of DYN/KOP system in modulating alcohol intake (Walker et al., 2012). However, understanding specific mechanisms by which the DYN/KOP system control the development of negative emotional state associated with alcohol dependence and tolerance can be useful to discover promising therapeutic target.

Interestingly, results here reported strengthen the hypothesis that similar changes of DYN/KOP system can occur during tolerance and dependence in the AMY suggesting that these pathologies may share common neuronal pathway alterations. Moreover, it seems that the crosstalk of DYN/KOP system between the AMY and BNST may be important in promoting alcohol dependence. Epigenetic data here presented report for the first time that two histone marks, H3K27me3 and H3K4me3, could mainly regulate the DYN/KOP system gene expression during acute EtOH exposure and RET. Taken together, all these results present a complete and interesting picture of the DYN/KOP system role in alcohol dependence and tolerance, also providing new epigenetic mechanisms.

In conclusion, the discovery of epigenetic mechanisms involved in the neuroplasticity phenomena responsible for AUD development could be useful to identify new drugs targeting these alterations with more efficiency in treating the AUD and alcohol-related mood disorders.

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2. Caputi F.F., **Palmisano M.**, D'Addario C., Candeletti S., Romualdi P. "Effects of acute ethanol exposure on Class I HDACs family enzymes in WT and BDNF+/- mice", Drug Alcohol Depend. 2015 Aug 28. pii: S0376-8716(15)01608-7.

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