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Environmental stressors and alcoholism development: focus on molecular targets and their epigenetic regulation

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Highlights

- Alcohol exposure and stressful events can induce the development of AUD.
- Role of epigenetic mechanisms in the biological activity of stress and alcohol.
- Epigenetic markers of stress might help for developing targets for AUD prevention.

ABSTRACT

Alcohol exposure and stressful events in life can induce long-lasting changes in physiology, behavior and gene expression patterns, eventually facilitating the development of psychiatric diseases like alcohol use disorders (AUD). Epigenetic mechanisms have been recently proposed to play a role in the cellular actions of alcohol via chromatin remodeling. Here we discuss interactions between stress and the pharmacological effects of alcohol, including the possibility that early exposure to, or withdrawal of, alcohol might induce stressful effects of their own. A specific aim is to describe novel molecular mechanisms by which stress, alcohol or their combined presentation impact on the epigenome. A key question is why only a fraction of the population progresses from regular, non-problematic, alcohol use to AUD, despite suffering from similar alcohol exposure. It is important to analyze how environmental factors, most notably stress, interact with the epigenetic machinery to increase vulnerability for AUD. The knowledge derived from this endeavor will be critical for the development of preventive strategies and new, drug- or gene-based, therapies.

Key words: alcohol, stress, epigenetics

1. Introduction

Consumption of alcohol (hereinafter also referred to as ethanol, which is the most relevant alcohol for human consumption and pre-clinical studies) is increased in the presence of stress-related psychiatric disorders, such as altered mood and anxiety (Grant et al., 2004; Schuckit and Hesselbrock, 1994). Alcohol intake can transiently alleviate the symptoms associated with these disorders (Bolton et al. 2009), and acute or chronic stress can also modulate its pharmacological effects (Childs et al., 2011; Gorka et al., 2017), as well as the possible development of alcohol use disorders (AUD) (Sinha, 2008; Uhart and Wand, 2009). On the other hand, early alcohol exposure or alcohol withdrawal might induce stressful conditions that give rise to pathological states (Guerri and Pascual, 2010; Hellemans et al., 2010; Koob, 2003). Here, we will review relevant alcohol-stress interactions with a focus on the neurobiological machinery at the basis of these effects. In particular, we will analyze the differences between acute alcohol intake (e.g. binge drinking) causing temporary alterations in the brain, and chronic use possibly inducing life-long central neuroadaptations. We will discuss also studies that used rodents (rats and mice), along with those performed on humans to ascertain how stressful early-life events (e.g. maltreatments) or late-life stressors (e.g. deterioration of human physical condition with aging) may be responsible for early onset of drinking problems. Since environmental factors, stress included (Weaver et al., 2004; Murgatroyd et al., 2009), and exposure to drugs of abuse, alcohol included (Pandey et al., 2008a), evoke epigenetic alterations in the brain (Tian et al., 2012), we will review those studies focusing on how the modulation of epigenetic pathways increases the subsequent development of AUD.

2. Stress and AUD

Stress has been broadly defined as any stimulus that is associated with a biological response of the organism (Yaribeygi et al., 2017). This definition recapitulates the old homeostatic notion that stress produces a dynamic reaction aimed at restoring equilibrium in biological systems. To this end, the stress reaction should be self-contained, yielding normalization and resilience. This definition has been pervasive, and rapidly moved from the neuroendocrine field to the psychobiological literature. As an example, the influential opponent process theory (Koob et al., 1989; Solomon, 1980) can be viewed as an attempt to explain emotional and hedonic processes by means of homeostatic tenets.

Stress has been shown to modulate ethanol seeking and intake for decades. Many studies with human participants have operationalized stress as the number of adverse events occurred over lifetime, and found that the greater the accumulation of these events, the greater the risk of alcohol abuse or dependence (Lloyd and Turner, 2008). Others, in turn, focused on the role of specific adverse events. In this context, recent studies revealed that generalized or sexual harassment in the workplace (Rospenda et al., 2017) or the transition from primary to secondary school (Junge et al., 2016) are associated with alcohol drinking. It is thus possible that exposure to certain types of stressors in specific developmental stages confers particular vulnerability to AUD (Enoch, 2011).

In alcohol-related animal research, stress exposure was initially used to overcome the now-widely known reluctance of naïve rats or monkeys to initiate ethanol drinking (Samson et al., 1988). In parallel with this use of stress as an "ethanol initiation technique", stress-alcohol studies were also theoretically grounded on the tenets of the "tension-reduction" hypothesis (Kalodner et al., 1989; Young et al., 1990), which posited that

ethanol drinking reduces the subjective and physiological effects of stress. Therefore exposure to, or anticipation of, aversive or threatening events could ignite ethanol consumption and constitute an etiological factor in the emergence of alcoholism. A closely related, yet different, theoretical proposal was the "self-medication hypothesis" (Miranda et al., 2002; Swendsen et al., 2000), whose tenets are that subjects afflicted by depression or anxiety (due to chronic exposure to aversive states or to other reasons) will more likely engage in consumption of ethanol, as an attempt to restore normal mood functioning. Both perspectives are still relevant today but they have lost their central role in the theoretical models of alcohol dependence, and usually are subsumed in models that focus on how chronic ethanol exposure may affect subsequent responsiveness to stress (Anderson et al., 2016a, b; Kato et al., 2016; Varlinskaya et al., 2017).

2.1 Drinking behaviors after acute or chronic stress

The negative reinforcing effects of ethanol play an important role in the modulation of ethanol drinking, after both acute and chronic stress. Broadly speaking, drinking after acute stress ameliorates the increased anxiety and emotional alterations triggered by the stressor, whereas after chronic stress drinking is favored by lingering effects of the stressor that may have caused depression or learned helpless or (perhaps even more interestingly) permanent or long-term alterations in brain structures or functions. Within the latter we can include gene expression, and its epigenetic regulation, that alter sensitivity to ethanol and render those exposed to stress more sensitive to ethanol intake. Part of these issues will be covered by this review.

A commonality among the first pre-clinical studies that tackled stress-ethanol interactions is the widespread use of footshock, or exteroceptive nociceptive stimulation, as a stressor. We can consider these investigations as the "first generation studies" interrogating alcohol and stress interactions. It is, however, generally accepted that the results derived from these investigations were inconclusive. Footshock (usually 0.5-0.6 mA, though up to 1.0 mA has been used) has been shown to increase (Caplan and Puglisi, 1986), decrease (Champagne and Kirouac, 1987) or be ineffective (Ponce et al., 2004) on ethanol intake. Moreover, a recent and quite comprehensive attempt to reconcile available literature (Becker et al., 2011) has indicated that the differences across studies (in terms of intensity or duration of shock, of strain of the animals employed, predictability or not of stress, prior history of alcohol exposure, etc.) do not account for the differences in outcomes.

The rather inconsistent effects of footshock on ethanol intake led researchers to use alternative aversive stimuli. Yet, most of these alternative stressors are associated with the same problems: considerable inter-individual variability, small effect sizes and lower replicability of results across laboratories. For instance, restraint stress (RS) has been sometimes preferred over footshock because of the lack of obvious physical effects. Instead, it has been deemed as a psychological stressor (Varlinskaya and Spear, 2015). Protracted RS has been shown to increase ethanol intake in Wistar rats (Lynch et al., 1999), and sometimes also in mice. However, studies reporting no effect [rat: Bertholomey et al., 2011; mice: Tambour et al., 2008] or RS-induced decreases in ethanol intake are also evident [rat: Chester et al., 2004; Ng Cheong Ton et al., 1983].

The relative lack of consistent effects of RS, footshock and other stressors on ethanol intake paved the way for the use of more ecologically-relevant stressors (e.g., chronic social instability or social isolation, early maternal separation) or a combination of stressors, usually applied in a chronic and unpredictable fashion.

2.2 *Chronic unpredictable stress and the effects of alteration of the early emotional environment*

Among chronic models, the most often used is the chronic unpredictable stress (Wu et al., 2017). In this paradigm, animals are exposed to various stressors (including foot-shock, overcrowding, lack of appropriate food or liquid resources, physical restraint, etc.), during a period of 3-4 weeks. Chronic unpredictable stress usually increases ethanol intake (Lopez et al., 2011), an outcome probably associated with the induction of depression and anhedonia (Zhao et al., 2008). Other animal models manipulate to some extent the social environment. In particular, rats exposed to chronic social instability are given cycles of social isolation and pair housing, whereas those exposed to chronic psychosocial stress are introduced into the home cage of a dominant and older counterpart for 2-3 weeks. Both procedures have been shown to increase anxiety behaviors, ethanol intake and preference for ethanol-related cues (Bahi, 2013; Roeckner et al., 2017). Another approach involves measuring ethanol intake as a function of the social dominance status. In male and female squirrel monkeys McKenzie-Quirk and Miczek (2008) found that dominance rank, determined via observational records of social interaction, was inversely correlated with alcohol intake.

Alterations in the quality or quantity of the dam-pup interactions can profoundly impact the response of the hypothalamic–pituitary–adrenal (HPA) axis and the developmental trajectories of the cerebellum, hippocampus and other brain areas (Schmidt et al., 2002). Chronic maternal separation (MS, 180 or 360 min every day, usually conducted in rodents during the first 2-3 weeks of life) is associated with a depressive-prone phenotype (e.g., anhedonia, exaggerated anxiety responses) and with increased ethanol intake (Cruz et al., 2008; Daoura et al., 2011; Huot et al., 2001; Ploj et al., 2003; Romano-López et al., 2012). One of these studies (Cruz et al., 2008) found this outcome both in two-bottle choice and in operant self-administration tests, using adult mice that experienced MS [180 min per day from postnatal day (PD) 1 to PD 14]. Yet others reported either no effect (Lundberg et al., 2017; Roman et al., 2004) or even reductions (Hilakivi-Clarke et al., 1991) in the consumption of ethanol after MS.

Isolation stress can also be provided after weaning, and in that case there are reports of increased ethanol intake after long-term, isolated housing (Kutcher et al., 2016; Lopez et al., 2011). Similar to chronic unpredictable stress, the chronicity of isolation seems to yield a depressive state that is expressed through anhedonia, enhanced reactivity to anxiety and aggression (Talani et al., 2014; Chappell et al., 2013; Butler et al., 2016).

The pre-clinical data derived from the MS paradigm are remarkably consistent with the literature on human subjects. The elegant study by Pilowsky and colleagues (2009) observed a significant increase in the likelihood of being diagnosed with AUD after reporting 2 adverse events during childhood, when compared with participants lacking any adverse event during infancy. Instead, Rothman and colleagues found that those with early adverse experiences were more likely to exhibit an early (≤ 14 years) age of first drink, which in turn is one of the most significant predictors of later development of AUD

(Rothman et al., 2008). In both studies parental divorce, an event often resulting in significant alterations of the early emotional environment, and in a reduction of the quantity and quality of the interactions with the caretaker, was the single event that had the strongest association with AUD.

It is likely that lack of proper paternal/maternal care (i.e., child neglect) is the mechanism underlying these effects. Studies that focused on lack of access to the caregiver have reported increases in anxiety response, cognitive deficits and the emergence of psychiatric disorders, prominently major depression. It can be postulated that increased consumption of ethanol after early stress may represent a self-medication behavior aimed to reduce the ensuing psycho and neuropathology. Consistent with this hypothesis, those participants in Rothman and colleagues (2008) who experienced early life adversities, not only began to drink earlier but were also more likely to report coping with adversities as the main motivation to begin drinking, when compared with those not exposed to childhood adversities.

Acute or chronic stress could also be responsible of modulating (i.e., increasing or decreasing) the effects of alcohol. For instance, Childs and colleagues (2011) found that acute stress differentially alter the stimulant and sedative effects of alcohol, in healthy male social drinkers. A recent study (Gorka et al., 2017) showed that alcohol reduces the functional connectivity of the insula with the anterior dorsal cingulate cortex in heavy drinkers. These areas are key components of the network involved in the detection of threats. In sum, these results support the bidirectional relationship between stress and alcohol: alcohol alters stress responses and, in turn, stress modifies the perception of the subjective effects of the drug.

2.3 Chronic ethanol intake affects subsequent responsiveness to stress

An important change in the stress-ethanol pre-clinical and clinical literature has been the switch from analyzing the effects of stress on concurrent or subsequent ethanol drinking, to the analysis of how chronic, usually intermittent ethanol exposure alters later responsiveness to stress. Ethanol exposure can be a stressor by itself. Ethanol activates the HPA axis and triggers release of corticosterone or cortisol (Cannizzaro et al., 2010), effects that seem to be related to the reinforcing ability of the drug; yet ethanol exposure – particularly in a chronic fashion – can also alter subsequent responsivity to other stressors. For instance, it has been shown (Boutros et al., 2018) that rats subjected during adolescence to alcohol intermittent exposure (AIE), a protocol that mimics binge-like ethanol exposure, were more sensitive to the dampening effects of social-defeat induced stress upon intracranial self-stimulation (a measure of anhedonia). These effects were associated with reduced mRNA levels of corticotropin-releasing factor (CRF, a hormone involved in stress response) in the nucleus accumbens.

For the most part, the studies that analyze the effects of chronic, usually intermittent, ethanol exposure on subsequent responsiveness to stress are grounded on a highly influential theoretical approach that emphasizes the so called “dark side” of drug dependence (Koob, 2009; Lu and Richardson, 2014). Under this perspective, negative reinforcement and adaptations in brain areas responsible for anxiety and stress responses, derived from chronic drug use, seem to be key factors underlying maintenance and relapse into drug abuse. For instance, several studies showed that chronic intermittent vapor ethanol (CIvE) exposure to mice or rats enhanced baseline anxiety and sensitivity to stress (Valdez et al., 2002; Zhao et al., 2007; Pleil et al., 2015a; Jury et al., 2017; Somkuwar et

al., 2017; Sidhu et al., 2018) and altered synaptic function and neuronal excitability in the medial prefrontal cortex and extended amygdala (Gilpin et al., 2011; Gass et al., 2014; Morales et al., 2018). These and other results suggest that chronic ethanol exposure deregulates the HPA axis and other systems responsible for stress and anxiety responses, thus putting individuals at risk of ethanol intake.

The results yielded by the CIvE paradigm and other animal models of chronic ethanol exposure are consistent with those provided by epidemiological and clinical studies on humans (Clapp et al., 2008). Alcoholic patients undergoing abstinence exhibited emotional alterations, including lack of awareness and clarity of perception of their own emotions, and lacked impulse control under stress when compared with social drinkers (Fox et al., 2008). It seems of note that some of these effects, such as enhanced neural activation to alcohol or stress-related cues, can persist for several weeks or months (Seo et al., 2016). Altogether these studies support that sensitivity to stress is enhanced in alcoholics during early abstinence (Fox et al., 2008).

3. Further description of stress influences upon alcohol intake across the lifespan

There has been an increasing interest in analyzing the effects of stress in special populations. Several studies have indicated that subjects at-risk for AUD due to a positive family history of alcohol problems exhibit an altered response to stress (Sorocco et al., 2006), and exaggerated heart rate stimulation and risk taking induced by ethanol, but insensitivity to the subjective effects of ethanol (Caneto et al., 2018); other works indicate that a low level of response to ethanol, as shown by sons and daughters of alcoholics, may

also facilitate ethanol use (Schuckit and Gold, 1988; Schuckit et al., 2000). Furthermore, the analysis of sex differences in stress-reactive alcohol drinking has also emerged as an important goal, in line with recent official guidelines (McCullough et al., 2014) that require equal representation of sex in pre-clinical studies, also in consideration that women/female have been traditionally neglected in basic and clinical studies (Retson et al., 2015). In the next sections we will focus on how stress can differentially impact ethanol preference as a function of age of stress exposure or age of testing.

Children, adolescents and social or ethnic groups characterized by significant historical trauma are key populations for analyzing stress-ethanol interactions. Among American Indians, it has been shown that the distress caused by thoughts about historical losses is associated with substance dependence (Ehlers et al., 2013). Moreover, the level of acculturation stress in Mexican American young adults was associated with anxiety disorders and substance dependence (Ehlers et al., 2009). An early age of first contact with ethanol, particularly when combined with sustained exposure to stress, is a prime risk factor for the development of AUD. We recently observed (Pilatti et al., 2017), in a large sample of Argentinean freshmen, that those featuring an age of ethanol onset < 15 years had significantly greater episodic and binge drinking than those featuring a later onset of drinking; others (Dawson et al., 2007) have found an exacerbation of this difference in subjects that on top on an early (i.e., adolescent) age of first exposure to ethanol suffered significant exposure to adverse or stressful events.

Preclinical studies with rats and mice have analyzed the effects of an early age of onset of alcohol use, and a widely employed strategy has been to assess stress-induced ethanol drinking and relapse in groups of animals that initiated ethanol drinking during adolescence or adulthood. Siegmund and colleagues (2005) gave male Wistar rats, that

were 31 (adolescents) or 71 (adults) day-old at the beginning of the experiment, access to water, 5% or 20% ethanol, for 2 months and then exposed them to stress or ethanol deprivation cycles. Baseline ethanol intake was fairly similar in both ages, yet the subjects that had initiated drinking at adolescence drank more ethanol after foot shock stress than those that had initiated drinking at adulthood. This experiment, which was subsequently replicated in female rats (Füllgrabe et al., 2007), cements the hypothesis that those with an early age of drinking are at greater risk of stress-induced drinking.

In the next sections we will focus on studies that employed (a) RS, a non-social, psychological stressor, in adolescent or adult subjects, and (b) stress-alcohol interactions late in life, particularly those associated with the deterioration of human physical condition with aging.

3.1 The effects of RS: role of sex and developmental stage

Individual variables, such as genetic background, sex and age, are significant modulators of the effects of RS upon ethanol intake. In the last decade there has been a considerable interest in the analysis of differences in response to ethanol in adolescents vs. adults. The youth seem to be, when compared to their older counterparts, more sensitive to the appetitive effects of the drug, yet exhibit a blunted response to the aversive and sedative effects of ethanol that serve to deter from sustained engagement in ethanol consumption (Spear, 2015). It has been also suggested that adolescents are more sensitive to stress-ethanol interaction than are adults (Brunell and Spear, 2005; Stone and Quartermain, 1997). Studies conducted in our lab have showed that five daily session of RS (120 min per session) increased alcohol intake and preference in adolescent rats, but had no effect (Fernandez et al., 2016) or even reduced (Wille-Bille et al., 2017) these behaviors in adult

rats. These studies meet the hypothesis of greater promoting effects of stress upon ethanol effects as a function of age.

A caveat of the RS-ethanol literature is that most of the work has been conducted in male rats (Rockman et al., 1987; Lynch et al., 1999; Chester et al., 2004; Gomez et al., 2012; Fernandez et al., 2016) or mice (Chester et al., 2006). Instead, Wille-Bille and colleagues (2017) evaluated stress-induced alcohol intake in male and female adolescent rats, and found significant sex-related differences, with females but not males exhibiting RS-induced increase in ethanol intake. Several studies have proposed that females are more vulnerable to stress (Jones et al., 1998), which could relate to the fact that plasma glucocorticoid levels after stress are greater in females than in males (Haleem et al., 1988).

3.2 Stress-alcohol interactions in the context of aging

Global population is progressively aging, and this has increased the attention on the pathologies associated with this age group. Alcohol use in older humans is not as prevalent as in young adults yet still extensive (and perhaps underreported, see Wetterling et al., 2003), with some studies indicating hazardous drinking in 10 to 20% of the elderly (Barry and Blow, 2016; Ilomäki et al., 2014).

Some studies have suggested that a significant fraction of drinking in the elderly (typically defined as those above 60 or 65 years old) is driven by stressors resulting from the normal deterioration of this age. For instance, recent studies on elderly subjects observed an association between alcohol use and cognitive problems, chronic backache or neck pain (Lasebikan and Gureje, 2015), and between daily drinking and heavy drinking with chronic pain and being widowed, respectively (Ilomäki et al., 2014). The lack of social

support (i.e., living alone, not being married) and adequate mobility are other commonly reported factors (Bristow and Clare, 1992; Iparraguirre, 2015) for risk drinking in this population.

There is also evidence that some stressful life events of middle age (e.g., children leaving home, financial strain, divorce) do not have an immediate effect upon alcohol drinking practices yet may impact this behavior some years after the event, when the subject is making the transition to elderly life (Tamers et al., 2014). Also, the baseline, pre-stress, level of drinking is a significant modulator of the effect of stress upon drinking at an old age (Holahan et al., 2017).

Besides these age-specific stressors, the elderly are also affected by more general stress sources. Anxiety, a prominent vulnerability factor for alcohol intake in other developmental stages, was a significant promoter of binge drinking in the study by Ilomäki and colleagues (2014). It is also important to consider that chronic alcohol consumption may have a more significant impact in the elderly than in younger subjects. As indicated by Boule and Kovacs (2017) the metabolism of ethanol is declined in the elderly, thus resulting in higher blood ethanol concentrations and longer half-life of the drug, which in turn can be further potentiated by the interactions with other medications (e.g., benzodiazepines, analgesics) typically prescribed by physicians to treat anxiety or pain-related conditions.

There is a relative scarcity of animal studies assessing ethanol-stress interactions in aged subjects, probably due to the logistic challenges associated with rearing animals until they are considered aged (in rats, usually ≥1 year old). It is, however, important to assess

these interactions, particularly in light of evidence indicating that chronic ethanol exposure may exert more detrimental effects in aged than in younger rats. A recent study found that chronic alcohol exposure caused significantly greater impairment of the aerial righting reflex in aged rats rather than in adult or adolescent ones (Matthews and Mittleman, 2017). Relevant for the purposes of the present review, Gano and colleagues (2017) assessed, in stress-responsive brain areas (i.e., hippocampus, paraventricular nucleus) of adolescent, adult and aged Lewis rats, the rapid alterations in neuroimmune genes expression that take place after an acute ethanol (dose ≥3.0 g/kg) challenge. The pattern of gene expression was fairly similar across age, but ethanol-induced corticosterone release was significantly higher in the aged (i.e., 18-19 month old) rats. The latter result is in line with previous evidence suggestive of age-related increases in the release of corticoids that may be associated with deterioration of limbic and hypothalamic-related brain functions (Herman and Larson, 2001).

4. Molecular targets of stress associated with vulnerability for alcohol use disorders

Ethanol exposure and related withdrawal symptoms can result in structural and functional modifications at the synaptic level (Carpenter-Hyland and Chandler 2006; Pandey et al. 2008b; Roberto et al. 2003; Zhou et al. 2007), which have a relevant role in the dysphoric symptoms present in stress-related psychiatric conditions (Pandey et al. 2008b; Pittenger and Duman 2008; Roozendaal et al. 2009).

As we already mentioned, there are reciprocal relationships between stress and ethanol. Ethanol intake can reduce anxiety and depression (Quitkin et al., 1972; Markou et al., 1998), yet it can also exert stressful effects of its own, by activating the HPA axis (Lee et al., 2004), closely regulated by the CRF system. Thus, we will begin by discussing the

role of the CRF system on ethanol-stress interactions, and then we will focus on other molecules.

The CRF system has received a great attention in research on the neurobiology of addiction. This system includes four endogenous ligands (CRF and the three urocortin peptides: Ucn1, Ucn2, Ucn3), the CRF binding protein (CRF-BP), and two G-protein-coupled receptors (CRF1, CRF2) (Bale and Vale, 2004, Fekete and Zorrilla 2007). There are two types of CRF receptors in the brain, CRF2 receptors have a discrete distribution in the hypothalamus, dorsal raphe, and lateral septum (Bale and Vale, 2004; Hauger et al., 2003); whereas the CRF1 receptor has been reported in the hypothalamus, ventral tegmental area, amygdala, bed nucleus of the stria terminalis (BNST) and various cortical structures.

Although a major source for CRF in the brain is the paraventricular nucleus (PVN) (Sakanaka et al., 1986), CRF axons also project to extra-HPA brain areas, in particular to central amygdala (CeA), a major component of the extended amygdala containing also CRF cell bodies, and BNST containing CRF-immunoreactive neurons with extensive projections to brainstem structures, controlling emotional and motivation behavior (Wang H et al., 2011). The dysregulation of CRF signaling has a primary role on excessive alcohol intake, seeking and relapse (Koob et al. 2014). The acute activation of the HPA axis seems to facilitate activity in brain motivational pathways that mediate positive rewarding effects (Piazza and Le Moal, 1997), yet its chronic activation results in decreased activity of hypothalamic CRF neurons (Lee et al., 2000; Rasmussen et al 2000; Koob and Kreek 2007) but sensitization of CRF-responsive neurons in areas that process fear and anxiety-related stimuli. This combination may result in a blunted response to ethanol's positive rewarding systems yet greater sensitivity to stress (Koob and Le Moal, 2005, Koob and Kreek 2007).

During ethanol withdrawal, CRF signaling alterations have been reported in the PVN (Makino et al., 1994a, 1994b), BNST (Olive et al., 2002), CeA (Weiss et al., 2001; Roberto et al., 2010) and HPA (Richardson et al., 2008). AIE induced in rats a reduction in CRF gene expression in the nucleus accumbens, an increase of CRF-R1 mRNA levels in the prefrontal cortex, as well as a decreased dopamine turnover in the caudate putamen (Boutros et al., 2018). Moreover, the authors observed that AIE-exposed rats when exposed to repeated stress showed a higher reward threshold, further suggesting that repeated stress induced anhedonia possibly driven by CRF and dopamine systems dysregulation (Boutros et al., 2018). Alterations in alcohol intake have been differentially observed after selectively blocking CRF receptors. CRFR1 antagonism in the BNST reduced alcohol drinking in non-stressed mice, whereas CRFR2 blockade induced a dose-dependent increase in alcohol intake in both non-stressed and stressed mice with a history of chronic alcohol consumption (Albrechet-Souza et al., 2017). In a study with mice (Hwa et al., 2016), the infusion of the CRFR1 antagonist CP376395 in the ventral tegmental area reduced intermittent ethanol intake in stressed and non-stressed mice, but not in mice given continuous access to ethanol. CP376395 also potentiated dopamine output to the nucleus accumbens in stressed rats, suggesting a role for extrahypothalamic CRF-R1 in restoring dysregulation of stress and reward after alcohol consumption (Hwa et al., 2016). Conflicting results have been reported on the effects of social defeat on alcohol consumption (Norman et al., 2015; Hwa et al., 2016; Norman et al. 2015; Lopez, et al., 2016; Sillaber et al., 2002). Several factors may contribute to the inconsistent results, such as strain of mice used, intensity of the stress procedure, and length of access to ethanol (Becker et al., 2011).

Recently, a study using *in vitro* slice electrophysiology showed that CRF modulates the transmission of glutamate, the major excitatory neurotransmitter in the CNS, in the CeA of naïve and ethanol-dependent rats (Varodayan et al., 2017). As reviewed in Lovinger and Roberto (2013), the up-regulation of glutamate after chronic ethanol mediates withdrawal symptoms and relapse. Acamprosate, one of the few FDA-approved drugs for the treatment of AUD, may exert its therapeutic actions by suppressing this overactive glutamatergic activity (Littleton, 2007).

These studies suggest that pharmacological manipulation of CRF receptors should reduce the facilitative effect of stress on alcohol intake, probably by alleviating acute reward or withdrawal-related anxiety. Congruent with this proposal, CRFR1 antagonism has prevented stress-induced relapse in animal models of alcoholism (Zorrilla et al, 2013; Henckens et al., 2016; Mantsch et al., 2016) and decreased ethanol-mediated presynaptic gamma-aminobutyric acid (GABA) release in CeA in dependent animals (Roberto et al., 2010). Moreover, in vivo intra-CeA administration of a CRF1 antagonist via retro-microdialysis reverses dependence-related elevations in extracellular GABA dialysates and blocks alcohol-induced increases in GABA in CeA of both dependent and nondependent rats (Roberto et al., 2010). Electrophysiological data revealed the enhancement of GABAergic tone during alcohol dependence. In fact, acute alcohol and CRF increase presynaptic GABA release in both rats (Roberto et al., 2003) and mice (Nie et al., 2009) in CeA neurons, via activation of CRF1 (Nie et al., 2009; Roberto et al., 2010). In addition, basal CeA GABA release is also increased by chronic EtOH exposure (Roberto et al., 2003, 2004, 2010).

Human studies, however, have been inconclusive. Illustrating this point, the CRFR1 antagonists pexacerfont (Kwako et al. 2015) or verucerfont (Schwandt et al., 2016) were not successful in altering craving in alcoholics.

CRF may also contribute to the negative emotional state associated with alcohol dependence by disrupting the endogenous kappa opioid system. Activation of the latter by dynorphin (DYN), a peptide that preferentially binds to kappa opioid receptors (KOR), is associated with dysphoria, anxiety and enhanced sensitivity to stress, effects probably mediated by KOR in the extended amygdala (Crowley et al., 2016). Specifically, dysregulated amygdalar DYN/KOR system may contribute to intensify alcohol self-administration in alcohol-dependent rats (Kissler et al., 2014) and site-specific intra-CeA administrations of KOR antagonist reduce escalated alcohol self-administration during acute withdrawal not affecting withdrawal symptoms (Kissler and Walker, 2016). Funk et al. (2014) showed that the CRFR1 antagonist antalarmin inhibited U50,488 (KOR agonist) induced reinstatement of alcohol seeking, indicating an interaction between KOR and CRFR1. Indeed, in the latter study KOR antagonism blocked cue- or yohimbine-mediated reinstatement of alcohol seeking. Several other studies suggest that the pharmacological manipulation of DYN/KOR influences motivational states associated with stress and alcohol intake (Sperling et al. 2010; Becker et al., 2017; Walker et al., 2012) and withdrawal-related behaviors (Gillet et al., 2013; Rose et al., 2015; Walker and Koob, 2008, Walker et al., 2011).

Also interesting is the interaction between CRF and the Nociceptin/Orphanin FQ (N/OFQ) system (Filaferro et al., 2014; Vitale et al., 2017). N/OFQ, which can be considered a functional CRF antagonist (Ciccocioppo et al., 2004; Filaferro et al., 2014), is a 17-amino acid peptide that acts as the endogenous ligand for the NOP receptor (also

referred to as the opioid receptor-like-1) not activating μ, κ, or δ opioid receptors (Meunier et al., 1995; Reinscheid et al., 1995). N/OFQ and its receptor are expressed within cortical and limbic regions (Darland et al., 1998; Neal et al., 1999) such as CeA and BNST. Pharmacological studies indicated that N/OFQ has anti-stress properties (Jenck et al., 2000) and attenuates ethanol intake (Martin-Fardon et al., 2010).

In addition, CRF is related with Neuropeptide Y (NPY), a 36 amino acid neuropeptide that acts through the Y1 receptor (Y1R) (Thorsell, 2008). In animal models, NPY reduces stress effects (Heilig et al., 1989, 2004) and alcohol consumption (Pleil et al., 2015b), and opposes the actions of CRF (Sajdyk et al., 2006; Leggio et al., 2011). NPY infusion into the CeA decreased alcohol intake in alcohol-preferring (P) rats and increased the expression of endogenous NPY, which is related to an increase in CaMK IV-dependent cAMP responsive element-binding (CREB) phosphorylation (Zhang et al. 2010). CREB (Abel and Kandel 1998; Alberini 2009; Waltereit and Weller 2003) is a transcription factor that modulates the expression of several genes and plays a relevant role in the central effects of alcohol (Misra et al. 2001; Pandey 2003; Pandey et al., 2004a, 2004b) and stress (Bilang-Bleuel et al. 2002; Carlezon et al. 2005).

Pandey and coauthors (2004a) found, in CREB haplodeficient (+/-) mice, a reduced expression of the cAMP-inducible genes NPY and brain-derived neurotrophic factor (BDNF). These effects were associated with heightened alcohol-drinking and anxiety-like behaviors. BDNF is a crucial brain signaling protein, and its deficiency is linked with the development of psychiatric disorders, including alcoholism (Kyzar et al., 2015). Moreover, these CREB haplodeficient (+/-) mice exhibited, after the administration of an acute ethanol dose and when compared to wild-type counterparts, significantly less anxiolysis yet significantly greater p-CREB and NPY protein levels in CeA (Pandey et al., 2004a). The

same authors reported a correlation between CREB dysregulations and alcohol addiction and withdrawal (Pandey et al., 2008a, 2008b), and observed reduced CREB signaling in alcohol preferring rats (Pandey et al., 2004b).

Transgenic mice with reduced BDNF have higher ethanol preference and ethanol-induced conditioned place preference than wild-type mice (Hensler et al. 2003; McGough et al., 2004). Alcohol exposure increases BDNF expression in the dorsal striatum (McGough et al., 2004), a key brain region in the regulation of ethanol intake (Jeanblanc et al., 2009). Inhibition of BDNF expression in the extended amygdala induced an increase in voluntary ethanol intake and anxiety-like behaviors (Pandey et al., 2006) an effect proposed to be mediated by the regulation of activity-regulated cytoskeleton-associated (Arc) immediate-early gene (Pandey et al. 2008b). Arc protein plays a role in the induction of long-term potentiation and is involved in the proliferation of dendritic spines (Huang et al., 2007; Messaoudi et al., 2007; Pandey et al., 2008b; Ying et al., 2002). P rats expressed lower levels of BDNF and Arc when compared to non-preferring (NP) rats, and had lower dendritic spine density in the amygdala complex and that these characteristics were associated with high innate anxiety-like behaviors (Moonat et al., 2011). Moreover, acute ethanol exposure had anxiolytic effects that were associated with increased BDNF and Arc levels as well as increased dendritic spine density in the amygdala complex in P, but not in NP rats (Moonat et al., 2011).

Alcohol exerts rewarding effects, at least partially, through actions on dopaminergic neurons or indirectly via changes in excitatory and inhibitory synaptic inputs (Niehaus et al., 2010, Saal et al., 2003), and these pathways are also shared by stress. Preclinical studies showed that alcohol intake is negatively modulated by the dopamine D2 receptor (McBride

et al., 1993; Volkow et al., 1996, 2007; Thanos et al., 2001, 2005; Tupala et al., 2001). When exposed to chronic mild stress, wild type mice decrease alcohol consumption whereas $Drd2^{+/-}$ and $Drd2^{-/-}$ mice increase ethanol intake and preference (Delis et al., 2013). Rats exposed to isolation-induced stress exhibit alterations of dopamine signaling, which are associated with increased alcohol intake (Karkhanis et al., 2014, 2015; Lallai et al., 2016). Using fast-scan cyclic voltammetry, mild stress significantly increased DA release and uptake in ethanol-naïve male rats, compared to non- stressed controls and chronic ethanol self-administration increased the DA uptake rate (Deal et al., 2018). However, the magnitude of these effects were blunted by either stress or chronic ethanol, or by a combination of both, suggesting that stress and ethanol consumption may promote similar adaptive changes in accumbal presynaptic DA release measures (Deal et al., 2018).

Molecular targets for stress-related events that play a role in the development of AUD are summarized in Table 1.

5. Epigenetics: an overview

The flow of genetic information from DNA to RNA and then to protein represents the central dogma of gene expression and individual development. Latest studies on epigenetics have contributed to clarify how chromatin remodeling helps shape differential utilization of genetic information. The plasticity of chromatin remodeling allows the interchange between multiple chromatin states, associated with stochastic gene expressions and thus can produce diverse phenotypic outcomes. (Yung and Elsässer, 2017).

The concept of epigenetic was introduced in 1942 by Waddington and is defined as "the branch of biology which studies the causal interaction between genes and their products, which bring the phenotype into being" (Waddington, 1942). Reversible and heritable epigenetic mechanisms (Fig. 1), such as DNA methylation and posttranslational modification of histone tails (acetylation, methylation, phosphorylation, etc.), remodel chromatin structure and open up the DNA template, making it accessible to various transcription factor, co-activators and/or co-repressors, thus regulating gene expression (Hsieh and Gage, 2005). The interaction among these events, together with the regulation by microRNAs and long non-coding RNAs, contributes to the epigenetic status of the cells (Han and Yoon, 2012).

5.1. DNA methylation

DNA methylation, the most widely studied epigenetic mark, is a covalent modification that occurs in mammals through the addition of a methyl group at cytosines primarily in CpG dinucleotides (CpG sites), leading to the formation of 5-methyl cytosine (5mC) which induces repression of transcription (Beerman and Rossi, 2015; Fernandez-Tajes et al., 2014). CpG sites are usually rare (~1%) in mammalian genomes and partly clustered into CpG islands (Esteller, 2008), typically not methylated regions often located around promoter regions of housekeeping genes. These regions are composed of at least 200 bp and with a GC percentage that is higher than 50% and with a CpG ratio between observed/expected residues at least equal to 0.6.

Sixty percent of human genes have CpG islands in the promoter region or first exon, and DNA methylation in promoter regions is often associated with transcriptional silencing

(Bird, 2002; Miranda and Jones, 2007), achieved by repressing the binding of transcription factors or by recruiting methyl-DNA binding proteins, like MeCP2. The latter, in turn, recruits histone-modifying enzymes that induce the formation of compact heterochromatin (Dhasarathy and Wade, 2008). Enzymes known as DNA methyltransferases (DNMTs) catalyze the methylation reaction (Miranda and Jones, 2007). DNMT1 is the "maintenance" DNMT, which regenerates the methyl-cytosine marks on the newly synthesized complementary DNA strand arising from DNA replication (Leonhardt et al., 1992). DNMT3a and DNMT3b add methyl groups "de novo" (Okano et al., 1999).

It should be also noted that DNA methylation does not occur exclusively at CpG islands, and that there are CpG sites in regulatory regions outside the promoters. Tissue-specific DNA methylation has been found 1–2 kb downstream or upstream, at CpG island "shores", and are strongly related to gene expression inactivation (Irizarry et al., 2009; Doi et al., 2009). Finally, hydroxylation of methylcytosine leading to 5-hydroxymethylcytosine (5hmC) has been recognized as a marker for gene activity that counteracts the role of transcriptional repressors targeting 5mC (Kriaucionis and Heintz, 2009; Tahiliani et al., 2009). A role for 5hmC has also been proposed as an intermediate in DNA demethylation (Wu et al., 2010). 5hmC is present in mammalian DNA at physiologically relevant levels, and in a tissue-specific manner (Jin et al., 2011). Ten-eleven translocation 1 (TET) mammalian enzymes (TET1, TET2 and TET3) have been identified as 5mC dioxygenases responsible for catalyzing the conversion of 5mC to 5hmC (Tahiliani et al., 2009; Ito et al., 2010).

5.2. Histone modifications

Genomic DNA is packaged into a highly compact structure to form chromatin, that is made of nucleosomes consisting of short stretches of DNA (147 bp) wrapped around histone octamers (two copies of each H3, H4, H2A, and H2B) (Luger et al., 1999). The latter are joined together by linker DNA and linker histone H1, interacting with both the nucleosome core and the linker DNA. Furthermore, there are histone variants of different types (e.g., H3.1, H3.2 and H3.3; H2A1-6, H2A.7), some of which are associated with the persistence of distinct states of the active gene (Ng and Gurdon, 2008).

Regulation of chromatin structure and transcription is driven by post-translational modifications primarily in the N-terminal tails of histone proteins (Turner, 2002, Moran-Salvador and Mann, 2017), including: acetylation at lysine, methylation at lysine and arginine, phosphorylation at serine and threonine, ubiquitination, ADP addition, and ribosylation at lysine (Spencer and Davie, 1999).

Acetylation, associated with transcriptional activation (Kouzarides, 2007), occurs mainly at different positions of lysine (K) residues on histone H3 (K4, K9, K14 and K28) and histone H4 (K5, K8, K12 and K16) (Turner, 2002; de Ruijter et al., 2003). Acetylation and deacetylation depend on the balance between histone acetyltransferases (HATs) (Roth et al., 2001; Shahbazian and Grunstein, 2007), comprising 5 families (GNATs, MYST, p300/CBP Transcription factor HATs, and nuclear hormone-related HATs), and histone deacetylases (HDACs) (Yang and Seto, 2007) that are divided in 4 classes (class I to IV) based on sequence similarity and cellular localization. HDAC class I includes HDACs 1, 2 3 and 8 whereas HDACs 4, 5, 6, 7 9 and 10 belong to class II. Class III, also named

Sirtuins, is composed of SIRT1, 2, 3, 4, 5, 6 and 7. Only HDAC 11 belong to class IV (de Ruijter et al., 2003). Instead, histone methylation, depending on the sites of the modification, can either be activating or inhibiting of gene expression (Martin and Zhang, 2005). Methylations of H3K9, H3K27, H4K20 have been associated to gene silencing, whereas H3K4, H3K36, H3K79 methylation leads to gene induction (Cheung and Lau, 2005; Jenuwein, 2006; Lohrum et al., 2007). Histone 3 can be mono-, di-, or tri-methylated. The trimethylation of K has been considered to be involved in long-term epigenetic memory. Histone methylation, mediated by histone methyltransferases with the methyl group donated by *S*-adenosyl-menthionine (SAM), has been considered for a long time a permanent and irreversible epigenetic mark, responsible, in concert with DNA methylation for chromatin remodelling (Tamaru and Selker, 2001; Henckel et al., 2009). However, it is now clear that there are enzymes able to demethylate methylated histone K residues as well as methylated arginines, via amine oxidation, hydroxylation or deamination (Cloos et al., 2008).

Histone H2A and H2B, and the variant H2AZ, can also be modified even if alterations are unlikely to be maintained in chromatin since nucleosomes more frequently displace them. However, post-translational modification of histone H2A and H2B tails have been also found to be of relevance (Wyrick and Parra, 2009)

The different combinations of histone modification patterns support the "histone code" hypothesis (Strahl and Allis, 2000). Under this hypothesis these different combinations control gene expression via modulation of chromatin structure and function, thus inducing downstream events in eukaryotic genomes. The scenario can be even more complex when considering that the same K residue (e.g., K4 or K9) might be both

acetylated and methylated, or that a subtype of histone variants (e.g., H3.1, H3.2, and H3.3) might be modified differently. It should be also pointed out that some of the histone modifications can be associated with active transcription whereas others with repression; moreover, the scenario evoked by these epigenetic marks might be different in different genes. Histones posttranslational modifications involved in chromatin reorganization are summarized in Table 2.

5.3. microRNAs

Epigenetic regulators have been recently extended to microRNAs (miRNAs) able to alter the transcriptional potential of a gene without changing the DNA sequence. miRNAs are 21-23 nucleotide-long single-stranded RNA molecules encoded by genes and transcribed, but not translated, into proteins (non-coding RNA). miRNAs discovery and their profound effect in controlling gene expression is revolutionizing our understanding of gene regulation (Ambros, 2001; Filipowicz et al., 2008). Their binding to miRNA-recognition elements in target genes generally results in either suppression of translation or degradation of mRNAs. miRNAs are highly abundant in the brain and play important roles in multiple biological processes, such as neuronal differentiation (Cheng et al., 2009), brain development (Fiore et al., 2008), synapse formation and plasticity (Schratt et al., 2006), and neurodegeneration (Schaefer et al., 2007; Bushati and Cohen, 2008). miRNAs also appear to mediate the cellular adaptations induced by exposure to a number of drugs of abuse, including alcohol (Sathyan et al., 2007; Pietrzykowski et al., 2008; Miranda et al., 2010; Lewohl et al., 2011).

6. Alcohol and stress effects on epigenetic mechanisms

In the last decade, studies showed that both alcohol and stress could induce, via epigenetic mechanisms, transcriptional regulation of target genes relevant for synaptic plasticity (Pandey et al., 2008b; Weaver et al., 2006; Palmisano and Pandey, 2017). We will here review epigenetic mechanisms involved in the effects of stress on alcohol.

6.1 DNA methylation

DNA methylation may be considered an important candidate in stress response (Tsankova et al 2006) and several studies confirmed the involvement of DNMTs, in particular Dnmt1, and MECP2 in mediating the effects of early life stress (Blaze and Roth 2013; Boku et al., 2015), and exposure to drugs of abuse including alcohol (Deng et al., 2011; Ponomarev et al., 2012; Lewis et al., 2013; Repunte-Canonigo et al., 2013; Warnault et al., 2013).

Early life stress, usually operationalized via maternal deprivation as described in earlier sections, has been associated with excessive alcohol intake (Roman and Nylander, 2005) and also with heightened Dnmt1 expression in the pituitary (Todkar et al., 2016). In the same work, the authors observed significant correlations between the expression of HPA receptors (i.e. glucocorticoid receptors, CRFRs) and DNA methylation regulatory genes (Dnmt1, Mecp2) in the hypothalamus of rats subjected to early life stress (Todkar et al., 2016). Previous studies showed that early life stress can induce changes in DNA methylation of genes involved in the physiological stress response, and these changes are persistent in adulthood (Chen et al., 2012). Others confirmed the link between early life stress and ethanol drinking in adulthood showing the involvement of DNA methylation of selective glutamatergic system genes in rat striatal regions (Vrettou et al., 2015).

On the other hand, prenatal alcohol exposure induced, in the adult offspring, heightened LPS-induced corticosterone and ACTH responses, an effect that was associated with changes in DNA methylation of important genes, such as Pomc, *Crh* and *Crhr1* genes (Jabbar et al., 2016). Prenatal alcohol exposure also increases one-carbon metabolism, involved in methyl donors production, in the hypothalamus offspring (Ngai et al., 2015), and this is of relevance considering the altered stress-responsivity shown by developmentally alcohol-exposed rats (Bekdash et al., 2013; Boschen et al., 2014; Kim et al., 1999). In another study, alterations were observed in DNA methylation of genes driving cancer development, such as HRAS oncogene and TP53 tumor suppressor gene, evoked by cross-generational effects of alcohol exposure (Hill et al., 2017). It should be considered that recent studies suggested that transgenerational effects evoked by paternal environmental exposures are also mediated by epigenetic mechanisms, including alterations in DNA methylation as well as small non-coding RNAs, with consequences on the likelihood of psychiatric disorders in the offspring (Yeshurun and Hannan, 2018).

It is important to recall that Finegersh and colleagues suggested heritability of alcohol-use disorders implicating transmission of epigenetic variants (Finegersh et al., 2015). Paternal alcohol exposure in mice induces a reduction of DNA methylation at the BDNF gene promoter in the ventral tegmental area of the offspring (Finegersh and Homanics, 2014). Psychosocial stress early in life might predispose subjects to higher rates of different disorders, including alcoholism (Enoch, 2011; Shea et al., 2005). Even if stress occurs early in life, the negative behavioral outcomes might become evident later in life. Moreover, stressful events during adulthood also impact DNA. Stressed, alcohol dependent, adult rats showed higher (medial) prefrontal levels of DNA methylation,

maintained by DNMT1, and these changes were associated with heightened alcohol intake and seeking behaviors (Barbier et al., 2015). DNMT inhibitors are able to reduce alcohol consumption in different paradigms (Warnault et al., 2013; Ponomarev et al., 2017). Moreover, excessive alcohol drinking induces the increase of DNMT1 levels (Warnault et al., 2013).

The role of DNA methylation in alcoholism development has been mainly studied in alcohol chronic users (Boschen et al., 2007; D'Addario et al., 2017), and this epigenetic mark has been suggested as a clinically useful diagnostic test to detect current heavy alcohol consumption (Liu et al., 2018). Overall, higher levels of DNA methylation have been reported in the brains of alcoholics (Manzardo et al., 2012). Differences in DNA methylation profile in blood have been found among alcoholic patients depending on their exposure or not to childhood adversity (Zhang et al., 2013). In another study with human participants, it was observed that altered stress reactivity following childhood trauma is associated to altered DNA methylation levels (Houtepen et al., 2016). An overview of DNA methylation-associated changes induced by alcohol and stress are reported in table 3.

6.2 Histone modifications

DNA methylation is often associated with reduction of gene expression, whereas histone modifications are correlated with both increase and decrease of transcriptional activity. Unlike DNA methylation, so far most of the studies focused mainly on alcohol effects on these epigenetic marks disregarding the role of stress on alcohol intake. However, the epigenetic modulation of some of the above-mentioned target genes associated with stress has been observed, and we will first discuss these in this section.

Most of the attention focused on histone acetylation. Acute ethanol treatment increased the acetylated H3 K9/14 levels of BDNF exon IV and Arc in the amygdala of P, but not NP, rats (Moonat et al., 2013) and decreased HDAC activity and HDAC2 protein levels. The knocking down of HDAC2 in the CeA attenuated anxiety-like behaviors in P rats, with no effects in NP rats. During withdrawal from chronic ethanol exposure, BDNF and Arc gene expression as well as protein levels decreased in the CeA and medial amygdala (MeA) and TSA treatment restored these changes (You et al., 2014).

In support of the influence of BNDF on HDACs, higher basal levels of HDAC 1-3 following acute ethanol exposure were observed, when compared to wild type, selectively in the striatum but not in the prefrontal cortex of BDNF heterozygous mice, which exhibit high preference for ethanol consumption and vulnerability to developing alcohol dependence (Caputi et al., 2015). H3 acetylation at NPY promoter is reduced in the amygdala of P rats when compared with NP animals and TSA treatment normalizes these effects (Sakharkar et al., 2014). Consistently, H3K9/14 acetylation resulted decreased at NPY gene promoter in the amygdala of adolescent intermittent ethanol-exposed adult rats when compared with controls (Kokare et al., 2017).

Studies in alcoholics reported down-regulation of genes involved in histone acetylation, such as CREB and CBP (Ponomarev et al., 2012). In fact, CREB recruits CBP, which has intrinsic HAT activity (Moonat et al., 2010). Others observed a reduction in phospho-CREB in the amygdala of rats under withdrawal following chronic alcohol associated with a decrease in CBP and NPY expression, the latter rescued by treatment with TSA (Pandey et al., 2008a). Phospho-CREB reduction was also observed in rat cerebellum following chronic ethanol (Yang et al., 1998) and moreover CREB transcription resulted

decreased in alcohol-induced neurodegeneration (Crews and Nixon, 2009). Instead, again Pandey et al. observed that acute alcohol induces an increase of CREB, CBP and NPY expression in rat amygdala (Pandey et al., 2008a).

In adolescent rats, acute alcohol exposure inhibits HDAC activity and the dose required for this effect is higher than that needed in adult rats (Sakharka et al., 2012, 2014). The authors suggested that the lack of tolerance to ethanol in adolescents might promote binge-drinking behaviour (Sakharka et al., 2014). It has been also suggested that adolescent alcohol exposure can induce persistent changes in adulthood due to a more closed chromatin produced in the amygdala complex (Kyzar et al., 2016). The withdrawal induced by AIE exposure induced anxiety-like behavior in rats, and increased HDAC activity and protein levels of HDAC2 and HDAC4 in amygdala (Pandey et al., 2015). In CeA and MeA nucleus of the amygdala of P rats Moonat and colleagues (2013) found, when compared to NP counterparts, higher nuclear HDAC2 activity associated to lower acetylation of H3K9, but not of H3K14. In another study, rats fed with alcohol through an intragastric tube showed increased H3K18 and H3K9 acetylation in the liver (Bardag-Gorce et al., 2009). Incidentally, ethanol metabolism induces an increase in acetyl-CoA, which is used in histone acetylation by HATs (Yamashita et al., 2001).

The attention of the researchers, however, has mostly focused on how inhibition of HDAC activity can have a role in alcohol effects. HDAC inhibitors are effective on several alcohol-related behaviors, including withdrawal-related anxiety (Pandey et al., 2008a), locomotor sensitization (Sanchis-Segura et al., 2009), alcohol consumption (Wostenholme et al., 2011), conditioned place aversion (Pascual et al., 2012), and rapid tolerance (Sakharkar et al., 2012). Moreover, HDAC inhibitors reduces binge- alcohol drinking in

mice and SuberoylAnilide Hydroxamic Acid (SAHA), an FDA-approved HDAC inhibitor, reduces alcohol seeking in rats (Warnault et al., 2013). Of note, others report that HDAC inhibition does not reduce ethanol consumption (Ponomarev et al., 2017).

In the already mentioned milestone paper by Pandey and colleagues, a very interesting model was proposed to explain the crucial role of HDACs in chromatin remodeling in the amygdala of rats exposed to alcohol (Pandey et al., 2008a). Rats acutely treated with alcohol show a decrease of HDAC activity and this change was associated with a selective increase in acetylation of histone H3 Lysine 9 (H3K9) and histone H4 lysine 8 (H4K8) but not of H3K14 (Moonat et al., 2013, Pandey et al., 2008a). During withdrawal after chronic alcohol exposure, a decrease in these acetylation events has been observed, and this was associated with anxiety-like behaviors. Moreover, the researchers also showed that these anxiety-like behaviors could be reversed by treatment with TSA (Pandey et al., 2008a), TSA that can also reverse rapid tolerance to the anxiolytic effects of ethanol (Sakharkar et al., 2012). However, it was also observed that TSA increased alcohol consumption in mice having continuous access to both water and alcohol (Wolstenholme et al., 2011). Sodium butyrate, another HDAC inhibitor, was able to selectively alter some alcohol-related behaviors (e.g., enhanced ethanol-induced locomotor sensitization), without any effect on others (e.g., ethanol tolerance or withdrawal) (Sanchis-Segura et al., 2009; Simon-O'Brian et al., 2015).

Alcohol effects on histones methylation have been also studied, but mainly by assessing ethanol exposure to different cell types. For instance, alcohol induced reduction in H3K27me3 and H3K4me3 at promoters of genes involved in cell identity and differentiation (Veazey et al., 2013). In rat hepatocytes it was observed that ethanol

decreased methylation of H3K9 and increased methylation of H3K4 (Pal-Bhadra et al., 2007), associated with gene expression regulation. In line with these studies, 72 hours of ethanol exposure in a human neuroblastoma cell line increased H3K4me3 at the prodynorphin (PDYN) gene promoter. This was not accompanied by PDYN transcription but the gene was kept in a poised state for later reactivation (D'Addario et al., 2011). In rats treated with alcohol for only 1 day, however, the same authors observed a reduction of H3K27me3 and and up-regulation of PDYN (D'Addario et al., 2013). Moreover, H3K4me3 was elevated in the hippocampus of adult mice exposed to alcohol during early gestation (Zhang et al., 2015). Table 4 summarizes relevant works on alcohol effects on histone modifications, whereas table 5 highlights on HDAC alterations induced by alcohol. Table 6 instead reports all the main studies that so far analyzed the effects of epigenetic manipulations on ethanol intake and/or responses to stress.

6.3 miRNA

The first study on miRNA levels was performed in the prefrontal cortex of postmortem human brain samples of alcoholics, and highlighted about 35 human miRNAs up-regulated in this population (Lewohl et al., 2011). Changes in several miRNAs expression have been reported also in blood of AUD subjects when compared to healthy controls. Among those, miR-92b and miR-96 have established roles in neural development (Ignacio et al., 2015). Several models of Fetal Alcohol Syndrome identified different miRNAs affected by developmental alcohol exposure (Balaraman et al., 2012, 2014; Guo et al., 2012; Ignacio et al., 2014; Pappalardo-Carter et al., 2013; Sathyan et al., 2007; Soares et al., 2012; Tal et al., 2012; Wang et al., 2009). Another study showed the effects of prenatal stress on miRNA expression (Zucchi et al., 2013) and 336 miRNAs resulted differentially expressed in the

whole brain of the offspring. Of relevance, some of these miRNAs altered by prenatal stress were also regulated by developmental alcohol exposure such as miR-9, miR-20a, miR-103, and miR-151 as observed by Boschen and colleagues (2018). Expression of miR-10a and miR-21 is regulated by acute psychological stress in human heavy drinkers. It was suggested that these miRNA alterations evoked by stress might be a possible mechanism responsible of the increased alcohol intake in binge/heavy drinkers (Beech et al., 2014). Several miRNAs were also found changed in rat brain following alcohol dependence and withdrawal. Many of the genes altered are implicated in synaptic plasticity, neuron differentiation and chromatin organization (Sinirlioglu et al., 2017).

Another relevant study showed that alcohol up-regulates miR-9 expression in rat striatal neurons, inducing selective BK channel message degradation, resulting in its splice variant profile reorganization (Pietrzykowski et al., 2008). Dysregulated miRNA expression was also reported in the medial prefrontal cortex of alcohol dependent rats (Tapocik et al., 2013). Also of interest is the upregulation of miR-206, since it represses BDNF expression (Miura et al., 2012). It was observed that when miR-206 is increased, BDNF is reduced in rat medial prefrontal cortex after a history of dependence (Tapocik et al., 2014). More recently, it has been shown that chronic ethanol exposure alters the levels of both small noncoding RNA and nucleoside modifications in mouse sperm (Rompala et al., 2018). Overview of the studies on miRNAs involved in alcohol and stress effects are reported in table 7.

7. Conclusions

Epigenetics play a relevant role in the neuroplastic alterations evoked by alcohol as well as by other drugs of abuse. We here reviewed data highlighting the role of epigenetic

mechanisms in the biological activity of stress and alcohol (Fig. 2). First of all, it is important to point out that global epigenetic effects do not necessarily imply unidirectional changes across the whole genome. In fact, many genes show opposite epigenetic changes in their promoters. It is also important to consider the reversible characteristics of epigenetic modification throughout the lifespan; consequently, this provides new insights for possible treatment of alcoholism evoked by stress, by altering the adult epigenome. Nutritional, behavioral, and pharmacological strategies can act as epigenetic modifiers able to normalize brain functions in the adult. For example, in adult rats exposed to stress during infancy, the treatment with zebularin, a DNA methylation inhibitor, normalizes DNA methylation and alterations in HPA axis response to stress (Roth et al. 2009). In rats, prenatal alcohol exposure increases stress hormones responses and alters DNA methylation profiles of stress regulatory genes in various brain areas. The changes in stress regulatory genes were normalized upon treatment with a DNA methylation blocker during the postnatal period (Jabbar et al., 2016).

Even if the epigenetic alterations observed in connection with alcohol indicate mainly an epigenetic response following drug exposure, the available data summarized herein appear promising to define possible biomarkers. This would be of help in the attempt of monitoring stressful situations with the aim of developing new targets for prevention in the population at large, before any emergence of clinical aspects of AUD. Moreover, beside prevention, more studies need to focus on the epigenetic mechanisms associated with alcohol addiction in order to better match treatments and the molecular pathways involved.

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Table 1: Molecular targets for stress-related events that play a role in the development of alcohol use disorder.

Target	Main effects	References
CRF/CRFR	Induction of seeking of and relapse into alcohol use. Anxiogenic and stress-like consequences of withdrawal syndrome	Koob et al., 2014 Makino et al., 1994a, b; Olive et al., 2002; Weiss et al 2001; Roberto et al., 2003; 2004; 2013
DYN/KOP	Influence behavioral states associated with stress and mood, as well as alcohol intake and withdrawal.	Sperling et al. 2010; Becker et al., 2017; Gillet et al., 2013; Rose et al., 2015; Crowley et al., 2016; Funk et al., 2014; Walker and Koob, 2008; Walker et al., 2011; 2012
N/OFQ	Decrease alcohol intake and increase anti-stress properties, and may have anxiolytic effects of their own.	Jenck et al., 2000; Martin-Fardon et al., 2010
NPY/ Y1R	Decrease alcohol intake, including binge-like drinking; Reduce stress effect; Inhibit neuronal populations that secrete CRF.	Pleil et al., 2015 Heilig et al., 1989, 2004 Sajdyk et al., 2006; Leggio et al., 2011
CREB and BDNF	Reduced CREB is associated with reduced expression of NPY and BDNF, which results in heightened alcohol-drinking and anxiety like behavior.	Pandey et al., 2006; 2008a, b; 2004b
D2/D2R	Their reduction is associated with heightened stress-induced ethanol intake.	Delis et al., 2013; Morganstern and Butt, 2010; Karkhanis et al., 2014, 2015; Lallai et al, 2016; Deal et al 2018

CRF/CRFR: Corticotropin-releasing factor/ Corticotropin-releasingf actor receptor; **DYN/KOP:** Dynorphin/kappa opioid receptor; **N/OFQ:** Nociceptine/orphanin; **NPY/ Y1R:** Neuropeptide Y/ Neuropeptide Y receptor; **CREB:** Neuropeptide Y/ Neuropeptide Y receptor; **CREB:** cAMP response element-binding; **BDNF:** Brain-derived neurotrophic factor; **D2/D2R:** Dopamine receptors

Table 2. Main histones posttranslational modifications involved in chromatin reorganization.

Histone	**Acetylation**	**Methylation**
H2A	K5	
H2B	K12, K15	K23
H3	K4, K9, K14, K28	K4, K9, K27, K36, K79
H4	K5, K8, K12, K16	K20

K, lysine

Table 3. Overview of DNA methylation-associated changes induced by alcohol and stress.

		Site	Experimental system	References
DNA methylation	↑↓	HPA receptors,	Hypothalamus of rats subjected to early life stress and alcohol exposure	Todkar et al., 2016
Gene expression		Dnmt1, Mecp2		
DNA methylation	↑↓	Glutamergic system genes	Striatal region of rats exposed to early life stress and ethanol drinking in adulthood	Vrettou et al., 2015
Gene expression		Dnmt1, Mecp2		
DNA methylation	↑↓	Pomc, Crh, Crhr1	Prenatal alcohol exposure effects in rats, in several brain areas	Jabbar et al., 2016
DNA methylation	↓	Bdnf	Prenatal alcohol exposure effects in mice, in ventral tegmental area	Finegerh and Homanics, 2014
DNA methylation	↑	Global	Prefrontal area of alcohol dependent and stressed rats	Barbier et al., 2015
Gene expression		Dnmt1		
DNA methylation	↑	Pdyn	Peripheral blood mononuclear cells of chronic alcohol drinkers	D'Addario et al., 2017
DNA methylation	↑	Several genes	Blood of alcoholic patients	Zhang et al., 2013
DNA methylation	↑	Global	Frontal cortex of human alcoholics	Manzardo et al., 2012

Table 4. Overview of the studies on histone modifications evoked by ethanol exposure

Histone modification			Site	Experimental system	References
Acetylation	H3	↓	Npy promoter	Amygdala of P rats	Sakharkar et al., 2014
	H3K9/14	↑	Bdnf exon IV and Arc	Amygdala of P rats	Moonat et al., 2013
	H3K9/14	↓	Npy promoter	Amygdala of AIE adult rats	Kokare et al., 2017
	H3K18/K9	↑	Global	Liver of rats fed with alcohol	Bardag-Gorge et al., 2009
	H3K9, H4K8	↑	Global	Amygdala of rats acutely treated with alcohol	Moonat et al., 2013; Pandey et al., 2008a
	H3K9	↓	Global	CeA and MeA of P rats	Moonat et al., 2013;
Methylation	H3K9me2	↓	Global	Rat hepatocytes treated with ethanol	Pal-Bhadra et al., 2007
	H3K4me2	↑	Global	Rat hepatocytes treated with ethanol	Pal-Bhadra et al., 2007
	H3K4me3	↑	Global	Hippocampus of adult mice exposed to alcohol during early gestation	Zhang et al., 2015
	H3K4me3	↓	Pdyn gene promoter	Amygdala of rats treated for 1 day with alcohol	D'Addario et al., 2013
	H3K4me3	↑	Global	Human alcoholics	Ponamarev et al., 2012; Zhou et al., 2011
	H3K4me3	↑	Pdyn gene promoter	Human neuroblastoma cell line (72 h ethanol exposure)	D'Addario et al., 2011
	H3K27me3, H3K4me3	↓	Promoter genes involved in neuronal precursor cell identity	Different cell types	Veazey et al., 2013

Table 5. Alterations of histone deacetylases activity and expression induced by alcohol.

HDAC activity/expression		**Experimental system**	**References**
HDAC2 protein levels	↓	Amygdala of P rats	Moonat et al., 2013
HDAC2 protein levels	↓	Amygdala of rats exposed to acute alcohol	Sakharkar et al., 2012, 2014
HDAC 1-3 levels	↑	Striatum of BDNF heterozygous mice (high preference for ethanol and vulnerability to developing alcohol dependence)	Caputi et al., 2015
HDAC activity HDAC 2 and 4 protein levels	↑	Amygdala of AIE rats	Pandey et al., 2015
HDAC activity	↓	Amygdala of rats exposed to alcohol	Moonat et al., 2013; Pandey et al., 2008a

Table 6. Effects of epigenetic manipulations (e.g., DNMT or HDAC inhibitors, HDAC knockdown) on ethanol intake and/or responses to stress exposure**.**

Epigenetic manipulation	**Main effects**	**Experimental system**	**References**
HDAC2 knockdown	Attenuation of anxiety-like behavior	P rats	Moonat et al., 2013
Decitabine	Reduction in ethanol consumption	Binge-like drinking in the dark and chronic intermittent every other day drinking in mice	Ponamarev et al., 2017
MS275, SAHA, TSA	Reduction of binge-like alcohol drinking	Mice undergoing intermittent access to alcohol	Warmault et al., 2013
SAHA	Reduction of alcohol seeking	Rats operant ethanol self-administration	Warnault et al., 2013
Sodium butyrate	Modification of ethanol-induced place conditioning.	Ethanol-exposed adolescent rats	Pascual et al., 2012
Sodium butyrate	Enhances ethanol-induced locomotor sensitization, but not ethanol tolerance or withdrawal	Alcohol dependent rats	Sanchis-Segura et al., 2009
Sodium butyrate	Prevent excessive ethanol intake	Alcohol dependent rats	Simon-O'Brian et al., 2015
TSA	Reversion of rapid tolerance to the anxiolytic effect of ethanol	Rapid ethanol tolerance in rats	Sakharkar et al., 2012
TSA	Reversion of anxiety-like behaviors	Rats acutely treated with alcohol	Pandey et al., 2008a
TSA	Increase of alcohol consumption	Mice having free access to both water and alcohol	Wostenholme et al., 2011

Table 7. Overview of the studies on miRNAs expression changes evoked by alcohol and/or stress.

miRNA	**Experimental system**	**References**
~ 35 miRNAs	Postmortem human brain samples of alcoholics	Lewohl et al., 2011
~ 336 miRNAs	Brain of rats exposed to prenatal stress	Zucchi et al 2013
Several miRNAs (e.g. miR-92b, miR96)	Blood of AUD subjects	Ignacio et al., 2015
miR10a, miR-21	Blood of human heavy drinkers exposed to psychological stress	Beech et al., 2014
miR-9, miR-20a,miR103, miR-151	Several brain areas of rats exposed to prenatal stress and alcohol	Boschen et al., 2018
Several miRNAs	Rat brains following alcohol dependence and withdrawal	Sinirlioglu et al., 2017
miR-9	Striatal neurons of rats exposed to ethanol	Pietrykowski et al., 2008
Several miRNAs (e.g. miR206)	Prefrontal cortex of alcohol dependent rats	Tapocik et al., 2013; 2014
Several miRNAs	Sperm of mice exposed to chronic ethanol treatment	Rompala et al., 2018

Figure captions

Figure 1. Epigenetic mechanisms. The N-terminal tails of histones can undergo a variety of post-translational covalent modifications, including methylation and acetylation. These processes are facilitated by enzymes that add or remove marks on histones (HAT, histone acetyltransferase; HAD, histone deacetylase; HDM, histone demethylase; HMT, histone methyltransferase). DNA methylation is the covalent modification of cytosine residues in CpG dinucleotides within gene sequences, and leads to transcriptional silencing. 5-methylcytosine are produced by DNMTs and subsequent demethylation by TET forms hydroxymethylcytosine. Within the nucleus, primary miRNA (pri-miRNA) becomes precursor miRNA (pre-miRNA), which is then exported to the cytoplasm. Mature miRNAs are thus loaded on a silencing complex, called RNA-induced silencing complex (RISC), and including also an Argonaut protein (Ago). Then, they target mRNAs by selective base-pairing, primarily in the 3'-UTR, and either inhibit their expression, or speed up their degradation.

Figure 2. Schematic representation of the interaction between alcohol and stress and the possible involvement of chromatin remodelling.

Figure 1

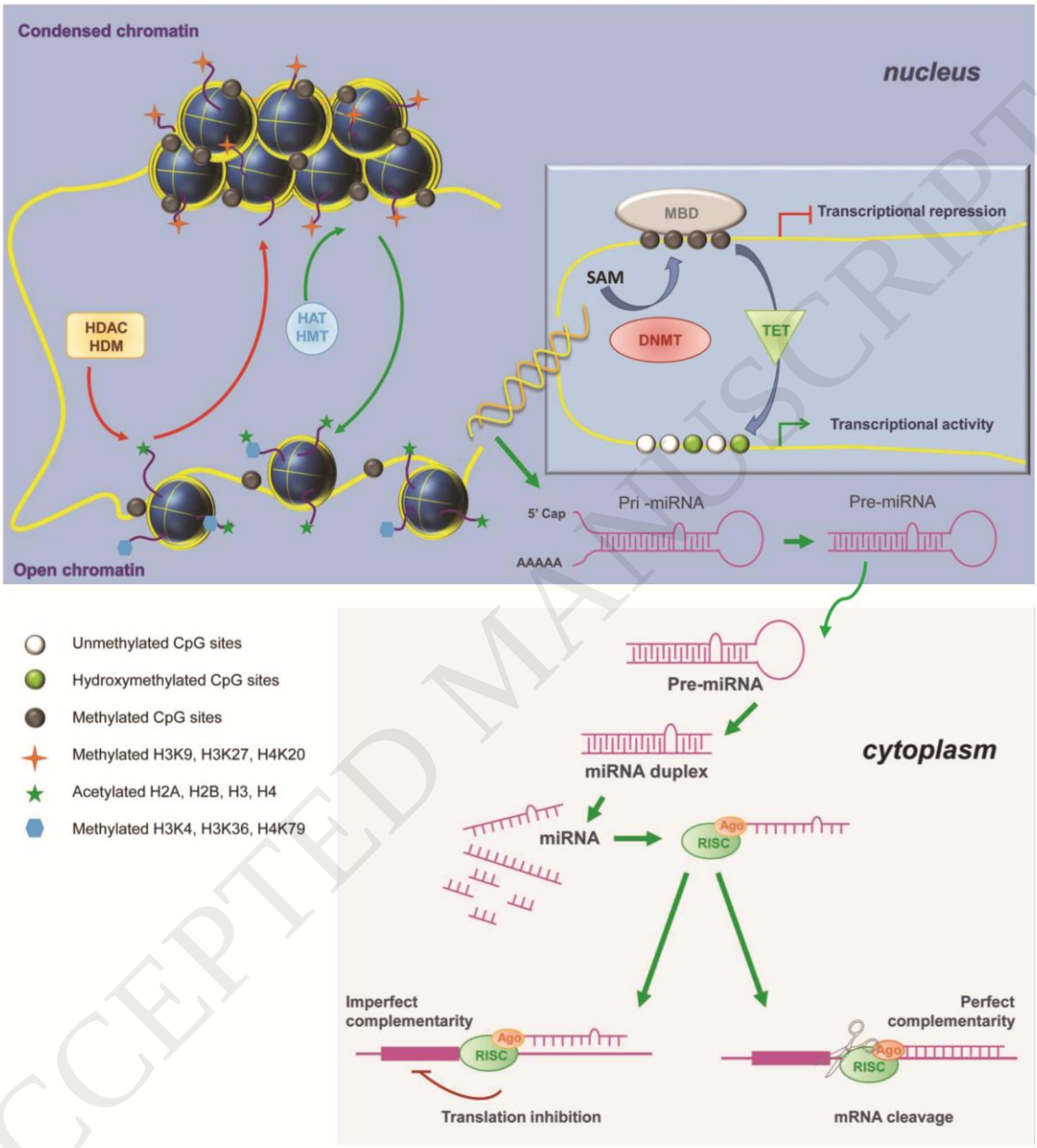

Figure 2