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Transcriptional regulators as targets for alcohol pharmacotherapies

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Abstract

Alcohol Use Disorder (AUD) is a chronic relapsing brain disease that currently afflicts over 15 million adults in the United States. Despite its prevalence, there are only three FDA-approved medications for AUD treatment, all of which show limited efficacy. Because of their ability to alter expression of a large number of genes, often with great cell-type and brain-region specificity, transcription factors and epigenetic modifiers serve as promising new targets for the development of AUD treatments aimed at the neural circuitry that underlies chronic alcohol abuse. In this chapter, we will discuss transcriptional regulators that can be targeted pharmacologically and have shown some efficacy in attenuating alcohol consumption when targeted. Specifically, the transcription factors cyclic AMP-responsive element binding protein (CREB), peroxisome proliferator-activated receptors (PPARs), nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), and glucocorticoid receptor (GR), as well as the epigenetic enzymes, the DNA methyltransferases (DNMTs) and histone deacetylases (HDACs), will be discussed.

1. Introduction

Alcohol (ethanol) induces both rapid changes in receptor signaling and the longer-acting second messenger signal transduction cascades in the brain that culminate in chromatin remodeling and changes in gene expression. Whereas acute alcohol can lead to transient changes in these signaling pathways, chronic alcohol use leads to persistent genome-wide epigenetic modifications and associated changes in gene expression that alter the neuronal circuitry involved in alcohol reward, craving, and the negative affective state that develops during ethanol withdrawal. Transcription factors and epigenetic modifiers therefore represent excellent targets for attenuating or reversing the pathological effects of chronic alcohol use on neuronal circuitry and ameliorating alcohol use disorder (AUD). In this chapter, we will discuss the role of transcription factors and chromatin-modifying enzymes in alcohol consumption and behaviors related to problematic alcohol use. Many pharmacological agents targeting transcriptional regulators and epigenetic enzymes have been developed that have shown efficacy in pre-clinical models of AUD.

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2. Transcription Factors

2.1. Cyclic AMP responsive element binding protein (CREB)

CREB is a transcription factor that is widely expressed in the nervous system and is critically involved in neuronal development, plasticity, and learning and memory (Silva et al. 1998). Dimerization of CREB is critical to its function, allowing CREB to bind to cAMP-response elements (CREs) in DNA and regulate transcription. Activity of CREB is regulated by phosphorylation, whereupon phosphorylated CREB (pCREB) binds to its co-activator CREB binding protein (CBP), a histone acetyltransferase that acts to open chromatin and activate transcription (see section on Epigenetic modifiers). A number of kinases and phosphatases regulate pCREB, including protein kinase A (PKA) and calcium/calmodulin-dependent protein kinases (Soderling 1999; Mayr and Montminy 2001). As such, CREB activity is tightly regulated and can rapidly change.

Two key transcriptional target genes of CREB are brain derived neurotrophic factor (*BDNF*) and neuropeptide Y (*NPY*) (Tao et al. 1998; Pandey et al. 2004). BDNF modulates neuronal development, differentiation, and survival and has been implicated in most psychiatric disorders, including addiction (Moonat et al. 2011; Greenwald et al. 2013; Lobo et al. 2010; Logrip et al. 2015). A single nucleotide polymorphism (SNP) of *BDNF* is associated with relapse in alcohol dependence. Minor allele carriers exhibit both resistance to relapse (Wojnar et al. 2009) and decreased brain activation in networks associated with more severe dependence symptoms (Chen et al. 2015). Likewise, polymorphisms in *NPY* have been associated with alcohol consumption, and a large body of literature has demonstrated that manipulation of the NPY system in rodents alters ethanol consumption (reviewed in (Robinson and Thiele 2017)).

Alcohol regulates CREB activity by modulating its phosphorylation. Acute ethanol treatment increases, while chronic ethanol attenuates, pCREB (Yang et al. 1998; Pandey et al. 2004). Similarly, withdrawal from ethanol after chronic exposure is characterized by decreased pCREB, without changes in total CREB (Pandey et al. 2001). Acute ethanol-induced pCREB leads to increased transcription of CREB-regulated genes, an effect that can be blocked by inhibiting PKA activity (Asher et al. 2002). Indeed, the decreased pCREB that results from chronic alcohol exposure may be a direct result of reduced PKA activity. Chronic intermittent alcohol exposure in rats results in increased expression of protein kinase A inhibitor alpha (PKI-alpha), a member of a family of proteins implicated in reducing PKA activity (Repunte-Canonigo et al. 2007).

Changes in pCREB can affect behaviors related to nearly every phase of the alcohol addiction cycle. The development of tolerance to the sedative effects of alcohol is associated with increased pCREB (Yang et al. 2003). Genetic variation in the CREB-BDNF pathway is associated with higher activation of brain networks underlying more severe alcohol dependence symptoms (Chen et al. 2015). Additionally, rats selectively bred for high alcohol consumption have decreased CREB expression, pCREB, and CRE-DNA binding in the amygdala compared to their alcohol non-preferring counterparts (Pandey et al. 1999). Withdrawal from chronic alcohol exposure leads to decreased pCREB in the amygdala, a brain region critical for anxiety-like behavior (Pandey et al. 2001). This reduction of pCREB

in the amygdala is associated with both high anxiety (Pandey et al. 2003) and increased ethanol consumption (Pandey et al. 2004). Importantly, restoring CREB function in the amygdala can prevent the emergence of anxiety during ethanol withdrawal (Pandey et al. 2005).

Although there are no pharmacological agents that directly interact with CREB, CREB activity can be increased indirectly by elevating cAMP levels and activating PKA, which is achieved by inhibition of the phosphodiesterases (PDEs) that hydrolyze cAMP. Two recent review articles have discussed the effects of PDE inhibitors on alcohol consumption (Logrip 2015; Olsen and Liu 2016), so the results will only be briefly summarized here (Table 1). Rolipram, a phosphodiesterase-4 (PDE4) inhibitor, increases pCREB in the brain (Hu et al. 2016) and reduces alcohol intake in rats (Franklin et al. 2015; Wen et al. 2012) and in mice (Hu et al. 2011; Blednov et al. 2014; Liu et al. 2017). Other PDE4 inhibitors that reduce ethanol consumption in rodents are roflumilast, Ro 20–1724, mesopram, CDP 840, and piclamilast (Table 1). In addition, the PDE10 inhibitor TP-10 reduces ethanol self-administration by rats, and the non-selective PDE inhibitor ibudilast reduces ethanol consumption by high-drinking rats and ethanol-dependent mice (Bell et al. 2015; Logrip 2015). PDE inhibitors also reduce inflammatory neuroimmune responses, which are induced after chronic alcohol exposure and have emerged as important promoters of excessive alcohol intake (Crews et al. 2017; Titus et al. 2015). Thus, the ability of PDE inhibitors to reduce ethanol consumption is not necessarily solely related to the enhancement of CREB signaling.

2.2. Peroxisome proliferator-activated receptors (PPARs)

Peroxisome proliferator-activated receptors (PPARs) are a group of transcription factors belonging to the nuclear hormone receptor superfamily; they are distributed throughout the body and contribute to a range of biological processes. For a review, see (Berger and Moller 2002). PPARs are primarily known for their role in regulating lipid metabolism, but they have also been shown to play a role in neuroprotection through repression of pro-inflammatory target genes (Pascual et al. 2005). PPARs function by translocating to the nucleus upon ligand binding. Saturated and unsaturated fatty acids, and their derivatives, are the endogenous ligands of PPARs, although a number of synthetic ligands have also been developed (Berger and Moller 2002). Once in the nucleus, PPARs form heterodimers with retinoid X receptors (RXRs) and bind to PPAR response elements (PPREs) in the promoter region of target genes. Co-activator proteins, such as SRC-1, then bind to the transcriptional complex to help initiate transcription (Zhu et al. 1996). The efficiency of co-activator proteins to aid in transcription depends upon which ligand is bound to the PPAR complex, allowing for dynamic control of PPAR target gene expression (Berger and Moller 2002). Additionally, PPARs can form heterodimers in the absence of ligand and bind constitutively to DNA; in this capacity, PPARs bind to co-repressor proteins and function to inhibit gene expression (Pascual et al. 2005; Glass and Saijo 2010).

Three isoforms of PPARs exist: PPAR α , PPAR γ , and PPAR β/δ . While most regions of the brain express all three isoforms, PPAR β/δ has the most widespread distribution and dense expression in the rat brain, with PPAR γ showing the most restrictive expression patterns

(Moreno et al. 2004). All three isoforms may work in a coordinated fashion, with PPAR β/δ regulating the activity of the other two PPAR types (Aleshin et al. 2013). Importantly, PPARs are expressed in regions of the brain critical to addiction (i.e., the nucleus accumbens, ventral tegmental area, and amygdala) (Warden et al. 2016) and have recently been implicated in the addiction cycle. Data from a genome-wide association study of the genetics of alcoholism supported an association with the genes encoding PPAR γ and PPAR α with alcohol withdrawal (Blednov et al. 2015). Intriguingly, while no genetic association was found in that GWAS study for PPAR β/δ , the brains of alcoholics have been shown to have altered expression of PPAR β/δ and an additional PPAR co-activator protein, PGC-1 α (Ponomarev et al. 2012).

Several agonists of PPARs have proven efficacious in regulating alcohol intake (Table 2). In line with the GWAS study, agonists of PPAR γ and PPAR α , but not PPAR β/δ , decreased alcohol intake in mice in both the 24-hour access and limited access drinking-in-the-dark tests (Blednov et al. 2015). Similarly, mice administered agonists selective for PPAR γ and PPAR α reduced their drinking in the 24-hour two-bottle choice (2BC) test, but a non-selective PPAR agonist had no effect on drinking (Ferguson et al. 2014). Furthermore, fenofibrate, a PPAR α agonist, suppressed ethanol intake in wild type mice but has no effect on PPAR α null mice, supporting a direct role of PPAR α in regulating drinking (Blednov et al. 2016). Interestingly, PPAR α may be acting in a sex-dependent manner to regulate ethanol intake, since the effects of its activation are stronger in male versus female mice (Blednov et al. 2016). Although PPAR γ and PPAR α may be acting in several ways to reduce ethanol drinking, there is some evidence that their roles in GABAergic interneurons of the amygdala may be critical for this behavior. By studying unbiased genomic profiles of mice given a PPAR γ , PPAR α , or a nonselective PPAR agonist, Ferguson and colleagues were able to differentiate gene expression networks that were associated with reduced drinking. Cell-type enrichment analysis showed that both fenofibrate (PPAR α selective agonist) and tesaglitazar (PPAR γ/α agonist) uniquely targeted amygdala GABAergic interneurons in a coordinated manner while the non-selective PPAR agonist bezafibrate did not (Ferguson et al. 2014). Interestingly, fenofibrate and tesaglitazar both upregulated neuropeptide and dopaminergic signaling genes in the amygdala, suggesting these drugs may be acting in a manner independent of their anti-inflammatory effects to regulate drinking.

The PPAR γ agonist pioglitazone has also been shown to be effective at reducing stress-induced alcohol relapse; interestingly, the opioid antagonist naltrexone (FDA approved for AUD) reduces cue-induced reinstatement of alcohol seeking, but has no effect on stress-induced reinstatement of alcohol seeking. When naltrexone and pioglitazone are given together, however, both relapse behaviors are reduced (Stopponi et al. 2013), suggesting that these drugs may work in independent, complementary manners to reduce alcohol relapse risk. In addition to regulating drinking behaviors, the PPAR γ agonists pioglitazone and DHA have also been shown to be protective against alcohol neurotoxicity (Kane et al. 2011; Tajuddin et al. 2014), suggesting that these drugs may be effective treatments for both AUD and fetal alcohol spectrum disorder (FASD).

2.3. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B)

NF- κ B transcription factors are activated by various immunological stimuli and work to amplify inflammatory responses. Since their discovery in the immune system, NF- κ B factors have been found in a range of cell types (including neurons and glial cells). NF- κ B consists of a family of five subunit proteins that function as dimers; which dimer is formed is dependent upon cell type and development and lends great specificity to downstream targets and function (Perkins 1997). Generally speaking, the p65/p50 heterodimer activates gene transcription (Li et al. 1994) and is the major NF- κ B complex in the adult rodent brain (Yakovleva et al. 2011), while the p50 homodimer represses transcription (Guan et al. 2005). These dimers can be found in the cytoplasm under basal conditions bound to I κ B inhibitory proteins. Upon immune activation, I κ B is phosphorylated by the kinase IKK β and targeted for degradation, allowing NF- κ B to translocate to the nucleus and regulate gene transcription. Although NF- κ B is classically activated by immunological stimuli, it has also been shown to respond to synaptic transmission (Guerrini et al. 1995), an effect that is at least partially dependent on CAMKII activation (Meffert et al. 2003).

Apart from its role in healthy neuronal functioning, NF- κ B has also been associated with alcohol dependence. Polymorphisms in the p50 protein precursor gene NFKB1 are correlated with an increased risk for developing an AUD, especially in individuals with an early onset of alcoholism (Edenberg et al. 2008). The brains of chronic alcoholics exhibit altered expression of NF- κ B subunits (Okvist et al. 2007), and animal studies show similar results, with acute alcohol exposure resulting in an upregulation of NF- κ B activity, while chronic treatment downregulates its activity (Rulten et al. 2006). Further, the neurotoxicity observed with binge alcohol exposure is associated with increased NF- κ B binding to DNA (Crews et al. 2006), and activation of a number of pro-inflammatory genes by NF- κ B (Zou and Crews 2010).

Reducing oxidative stress through the use of antioxidants is a promising avenue for manipulating the NF- κ B system. Resveratrol, a natural polyphenol, prevents the ethanol-induced upregulation of NF- κ B, decreases ethanol-induced pro-inflammatory gene transcription, and increases cognitive performance in rodents (Tiwari and Chopra 2013b). Similar therapeutic effects were found upon treatment with curcumin, another natural antioxidant (Tiwari and Chopra 2013a). Administration of the antioxidant butylated hydroxytoluene (BHT) can prevent NF- κ B activation, neural damage and proinflammatory gene induction that occur with ethanol exposure (Zou and Crews 2010). Further, the IKK β inhibitors TPCA-1 and sulfasalazine (which prevent NF- κ B from translocating to the nucleus) were able to reduce ethanol drinking by mice (Truitt et al. 2016) (Table 2).

2.4. Glucocorticoid receptors

The connection between stress and alcohol use has long been recognized. Stress has been shown to escalate drinking in non-dependent and dependent populations (Nash and Maickel 1985; Russell et al. 2017; Spanagel et al. 2014), and vulnerability to stress is a reliable indicator of relapse in alcohol-dependent individuals (Brown et al. 1995; Witkiewitz 2011). There are many targets in the physiological stress pathway that may contribute to stress-induced drinking (including corticotropin releasing factor), yet the importance of

glucocorticoids has increasingly been recognized (Nash and Maickel 1988; Fahlke et al. 2000).

The hypothalamic-pituitary-adrenal (HPA) axis is the body's primary stress response pathway and is responsible for the release of cortisol (in humans) or corticosterone (in rodents), herein referred to as CORT, from the adrenal glands. CORT binds to two receptors that serve complementary functions: the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). CORT has a 10-fold higher affinity for MRs than for GRs, which allows MR occupancy to occur under basal conditions, and therefore restrict CORT levels at baseline via a negative feedback loop (Rupprecht et al. 1993). In contrast, GR occupancy occurs under conditions of high CORT release, and therefore plays an important role in facilitating recovery after a stressor and altering neuronal activity to promote memories of stressful events (Joels et al. 2012). The ratio of MR:GR functionality may confer resilience (when high) or vulnerability (when low) to a host of psychiatric conditions (ter Heegde et al. 2015).

Importantly, alterations in GR expression and activity have been linked to alcohol abuse risk. Genetic polymorphisms in the GR gene (*NR3C1*) are associated with age of onset of alcohol use and abuse, a phenotype strongly correlated with risk of developing an AUD (Desrivieres et al. 2011). Alcohol can also alter GR expression, creating a positive feedback loop to promote intake. Chronic alcohol exposure dynamically changed both systemic CORT levels and GR expression in cortical and subcortical regions of the brain that regulate stress and reward pathways, effects that can facilitate excessive drinking and increase vulnerability to relapse (Somkuwar et al. 2017; Vendruscolo et al. 2012). CORT can also contribute to alcohol-induced neurodegeneration. Adrenalectomized rats given high levels of CORT concomitantly with chronic alcohol treatment showed exacerbation of neurodegeneration, while low dose CORT (commensurate with basal CORT levels) did not exacerbate alcohol neurotoxicity (Cippitelli et al. 2014). CORT acutely suppressed the immune system, but repeated exposures to CORT led to activation of microglia, a response driven by GR activation (Nair and Bonneau 2006). In this way, alcohol may increase pro-inflammatory responses in the brain via NF- κ B-mediated mechanisms as well as via GR activation.

Chronic treatment with mifepristone, a non-selective GR antagonist, prevented dependence-induced escalations in drinking and compulsive responding for alcohol exhibited during protracted abstinence in rodents (Vendruscolo et al. 2012) (Table 2). Interestingly, mifepristone reduced alcohol intake in dependent, but not non-dependent rats, suggesting that the GR dysregulation that occurs with chronic alcohol exposure is a unique risk factor for escalated use (Vendruscolo et al. 2015). Promisingly, mifepristone treatment has also shown positive results in humans, with just one week of treatment reducing both alcohol craving and consumption in dependent individuals (Vendruscolo et al. 2015). Mifepristone may also improve symptoms associated with ethanol withdrawal, including tremor (Sharrett-Field et al. 2013) and cognitive deficits (Jacquot et al. 2008). Finally, daily doses of mifepristone were able to attenuate alcohol-induced hippocampal neurodegeneration in rats in a dose-dependent manner (Cippitelli et al. 2014). Taken together, these results suggest that GR activation is critical for both the development and maintenance of AUD, and disrupting

GR signaling may be a promising target for preventing relapse and treating withdrawal symptoms in alcohol-dependent individuals.

3. Epigenetic modifiers

3.1 DNA methyltransferases (DNMTs)

In mammals, DNA methylation is catalyzed by three DNA methyltransferases: DNMT1, DNMT3A, and DNMT3B (Day et al. 2015). These enzymes add a methyl group to the fifth carbon position of the cytosine (5mC) found adjacent to guanine (as cytosine-phosphate-guanine dinucleotides, or CpGs) (Zovkic et al. 2013), using the methyl donor S-adenosyl methionine (SAM). The genome contains regions that are rich in CpGs, known as CpG islands, which are often found in gene regulatory or promoter regions. The methyl-binding domain proteins (*e.g.* MeCP2) directly interact with 5mC, that then recruit chromatin modifying proteins and transcriptional repressor complexes to the DNA (Zovkic et al. 2013). Thus, DNAm is normally associated with repression of gene expression.

DNAm in the brain plays an important role in learning and memory (Zovkic et al. 2013), and evidence is accumulating that DNAm is also important in AUD (Tulisiak et al. 2017). Increased expression of DNMT1 protein, and *DNMT3a* and *3b* genes in rodent brains, and decreased expression of *DNMT3A* and *3B* genes in human blood samples have been observed after chronic ethanol exposure (Barbier et al. 2015; Bonsch et al. 2006; Warnault et al. 2013; Qiao et al. 2017). The difference in *DNMT3A* and *3B* expression between brain and blood samples may be due to species differences (rat vs. human), different tissue-specific responses to ethanol, or duration or timing of alcohol exposure. Nonetheless, changes in DNMTs after chronic ethanol exposure indicate that DNAm might be altered by alcohol.

Additional circumstantial evidence that DNAm is altered by alcohol exposure has come from an analysis of post-mortem cerebellum from human subjects with AUD compared with that of control subjects. In this study, the authors measured increased transcript levels of enzymes involved in the one-carbon metabolism pathway, which generates the methyl donor SAM (Gatta et al. 2017). Correlated with this was an increase in the ratio of SAM to s-adenosylhomocysteine (SAH) in the cerebellum, which would increase the activity of DNMTs (Auta et al. 2017; Gatta et al. 2017). Similar changes in the SAM/SAH ratio occurred in rat cerebellum after chronic ethanol drinking, indicating that the changes are induced by alcohol (Auta et al. 2017). Finally, mRNA expression of an enzyme (tet methylcytosine dioxygenase 1, *TET1*) that removes methyl groups from cytosines was decreased in the cerebellum of subjects with AUD (Gatta et al. 2017). Together these results indicate that changes in enzymes that regulate DNAm are associated with chronic alcohol exposure and suggest that there might be increased total DNAm in the brain. However, more detailed studies described below, examining differentially methylated regions after chronic alcohol use, suggests that specific genomic locations associated with excessive drinking can be either hypo- or hyper-methylated.

More explicit evidence that changes in DNAm are associated with alcohol use was first provided by analysis of genomic DNA from blood cells of alcoholic patients, with increased

total DNAm associated with alcoholism (Bonsch et al. 2004). Another study found decreased DNAm at repetitive DNA elements (Alu) associated with alcohol use in lymphocytes from healthy individuals (Zhu et al. 2012). Candidate gene approaches in blood cells have demonstrated associations between increased DNAm and AUD at the promoters for alpha synuclein (*SNCA*), homocysteine-induced endoplasmic reticulum protein (*HERPUDI*), serotonin transporter (*SLC6A4*), monoamine oxidase A (*MAOA*), prodynorphin (*PDYN*), and aldehyde dehydrogenase 2 (*ALDH2*) (Bleich et al. 2006; Bonsch et al. 2005; Philibert et al. 2008a; Philibert et al. 2008b; D'Addario et al. 2017; Pathak et al. 2017). More recently, genome-wide profiling of methylated regions in whole blood cells or human lymphoblast cell lines has demonstrated several significant differentially methylated regions or differentially methylated cytosines associated with high levels of alcohol drinking or dependence (Philibert et al. 2012; Clark et al. 2015; Liu et al. 2016; Zhang et al. 2013; Zhao et al. 2013). In another study by Philibert et al (Philibert et al. 2014), differentially methylated cytosines were identified in peripheral mononuclear cell DNA from subjects with heavy alcohol use compared with controls. Genome-wide DNAm was measured in the AUD subjects as they entered a treatment facility and ~25 days later, during abstinence. Many of the differentially methylated cytosines identified between controls and alcohol-dependent subjects were reversed during abstinence to levels similar to controls. Similarly, Bruckmann et al (Bruckmann et al. 2017) found that some of the differentially methylated cytosines identified in CD3+ T cells between healthy controls and alcohol-dependent patients reverted back to control levels after abstinence, demonstrating a potential causal role for alcohol in changing DNAm in blood cells.

Studies in blood, lymphoblast cell lines, or other peripheral tissues may not represent the DNAm patterns in the brain associated with alcohol use. A few studies have found similar changes occurring in post-mortem brain and peripheral tissues. Notably, DNAm in the promoter for the gene encoding the delta subunit of the GABA-A receptor (*GABRD*) was increased in the cerebellum of human subjects with AUD compared with controls, similar to what has been observed in lymphocytes (Gatta et al. 2017; Liu et al. 2016). The increase in DNAm in the promoter of *GABRD* in the cerebellum was associated with decreased *GABRD* expression (Gatta et al. 2017). In the precuneus brain region from AUD subjects, 244 hypomethylated and 188 hypermethylated regions were associated with alcohol dependence (Hagerty et al. 2016). These differentially methylated cytosines overlapped with those found in buccal (cheek) cells collected from the same subjects (Hagerty et al. 2016). Together, these studies indicate that alterations in DNAm in the brain occur with chronic alcohol use and that some of these changes are similar to those occurring in peripheral tissues.

Additional genome-wide analysis of DNAm in different brain regions in humans, and monkeys has been performed in order to identify both region-specific changes in DNAm and potential new candidate genes for AUD. Analysis of prefrontal cortex tissue from AUD and control subjects found 1,812 differentially methylated cytosines mapping to 1,099 genes that were significantly associated with AUD (Wang et al. 2016). In rhesus macaques, differentially methylated regions in the nucleus accumbens discriminated abstinent monkeys from low/binge drinkers and heavy/very-heavy drinkers and were located in genes encoding

synaptic, cell signaling and receptor trafficking mediators (Cervera-Juanes et al. 2017a, b) that could, in theory, be targets for pharmacological intervention.

Three pharmacological agents have been used to inhibit DNMTs and determine the effect on behaviors related to AUD in animal models: the nucleoside analogs 5-azacytidine (azacytidine), 5-aza-2'-deoxycytidine (decitabine) and RG108, a non-nucleoside DNMT inhibitor (Table 3). Mice treated with azacytidine or decitabine reduced their ethanol intake in intermittent access procedures that model binge-like drinking (Warnault et al. 2013; Ponomarev et al. 2017). In alcohol-dependent rats, infusion of RG108 into the cerebral ventricles resulted in decreased alcohol intake after a three-week period of forced abstinence when compared with vehicle-treated rats (Barbier et al. 2015). Similarly, infusion of decitabine into the medial prefrontal cortex of chronically drinking rats decreased ethanol intake but increased anxiety-like behavior (Qiao et al. 2017). The timing of administration appears to be important for the ability of DNMT inhibitors to reduce ethanol drinking. When ethanol-dependent mice were given intracerebroventricular azacytidine during the induction of dependence (prior to ethanol vapor exposure), they subsequently consumed more ethanol in a 2BC test (Qiang et al. 2014). Together, these results indicate that DNMT inhibitors generally reduce ethanol intake in rodent models of binge and dependence-induced drinking models.

Azacytidine and decitabine are FDA-approved for the treatment of myelodysplastic syndrome and acute myeloid leukemia. Both of these drugs have high toxicity (Gnyszka et al. 2013) and serious side effects of these drugs include increased bruising, bleeding, and infection. Azacytidine is contraindicated for individuals with liver tumors, and those with liver and kidney disease should be monitored carefully. As a result, these drugs should not be used for those individuals with alcohol-associated liver disease. A newer nucleoside analog, zebularine, has less toxicity (Gnyszka et al. 2013) but has not yet been tested in animal models of AUD. In summary, DNMT inhibitors represent a promising pharmacotherapeutic approach to treating AUD, but newer generation compounds, such as zebularine and RG-108, which are not yet FDA-approved, require further investigation.

3.2 Histone deacetylases (HDACs) and histone acetyltransferases (HATs)

DNA wrapped around a histone octamer forms the nucleosome, an integral building block of chromatin structure. Changes in the acetylation state of histone tails are intimately involved in chromatin remodeling and transcriptional alterations. HATs are enzymes that add acetyl groups to lysine residues on histone proteins, while HDACs remove these acetyl groups (Elvir et al. 2017; Haberland et al. 2009). Generally, HATs activate transcription while HDACs repress it, although there are exceptions (Haberland et al. 2009; Sacconay et al. 2016). The mammalian genome encodes eleven “classical”, zinc-dependent HDACs that are categorized into four families (class I: HDAC1, 2, 3 and 8; class IIa: HDAC4, 5, 7 and 9; class IIb: HDAC6 and 10; class IV: HDAC11). A separate family of nicotinamide adenine dinucleotide (NAD⁺)-dependent deacetylases, called sirtuins (or class III HDACs) comprises 7 members (SIRT1–7) (Sacconay et al. 2016). Recent evidence shows alterations in histone acetylation/deacetylation and chromatin structure in several psychiatric disorders,

suggesting that these processes may underlie motivated behaviors, including drug addiction (Elvir et al. 2017; Pena et al. 2014).

Ethanol changes the acetylation state of histones after acute and chronic exposure and during withdrawal. Acute ethanol exposure led to changes in histone H3 and H4 acetylation in the amygdala, hippocampus, and cortex (D'Addario et al. 2013; Finegersh and Homanics 2014; Pandey et al. 2008; Sakharkar et al. 2012). Chronic ethanol exposure and/or withdrawal altered acetylation of histones H3 and H4 in the amygdala, ventral tegmental area, cortex, nucleus accumbens, dorsal striatum, and hippocampus (Arora et al. 2013; Bohnsack et al. 2017; Botia et al. 2012; D'Addario et al. 2013; Dominguez et al. 2016; Finegersh et al. 2015; Qiang et al. 2011; Shibasaki et al. 2011; Simon-O'Brien et al. 2015; You et al. 2014; Pandey et al. 2008). In general, though not exclusively, the changes in total histone H3 and H4 acetylation induced by acute alcohol exposure appear to be the opposite to those of withdrawal from chronic alcohol exposure. However, this depends on the brain region. Alcohol-induced changes in acetylation in the promoter regions of specific genes such as *GABRA1* (Arora et al. 2013; Bohnsack et al. 2017), *PDYN* (D'Addario et al. 2013), *PNOC* (D'Addario et al. 2013), *BDNF* (You et al. 2014), *ARC* (You et al. 2014), *NPY* (Pandey et al. 2008; Sakharkar et al. 2012), and *GRIN2B* (NR2B) (Qiang et al. 2011; Qiang et al. 2014) were associated with changes in gene expression, which has important consequences for behaviors such as anxiety during withdrawal (Pandey et al. 2008) and ethanol consumption (Qiang et al. 2014).

Treatment with HDAC inhibitors is effective in reducing ethanol intake in multiple models of AUD (Table 3). Mice treated with Trichostatin A (TSA) or Vorinostat (SAHA) (inhibitors of class I, II and IV HDACs) consumed less ethanol in a limited-access binge drinking test, and rats treated with SAHA also self-administered less ethanol in an operant task and exhibited reduced alcohol-seeking behavior (Warnault et al. 2013). Treatment with the HDAC1/2 inhibitor valproic acid (VPA) decreased 2BC ethanol consumption and preference by rats and also blocked ethanol reward, as measured in the conditioned place preference test (Al Ameri et al. 2014). In the Warnault and Al Ameri studies, the effect of the HDAC inhibitors on histone acetylation in the brain was not measured, but Warnault et al did observe a decrease in total histone H4 acetylation in the nucleus accumbens after binge drinking by mice and ethanol self-administration by rats (Warnault et al. 2013). In summary, these studies indicate that TSA, SAHA, and VPA decrease binge-like drinking or alcohol reward/reinforcement-related behavioral measures.

Heavy-drinking rats treated with the HDAC1/3 inhibitor Entinostat (MS-275) also self-administered less ethanol and exhibited reduced relapse (reinstatement) to ethanol seeking after a period of abstinence, an effect that was associated with increased histone H4 acetylation in the nucleus accumbens and dorsolateral striatum (Jeanblanc et al. 2015). Treatment with the non-selective HDAC class I and IIa inhibitor sodium butyrate (NaB), or MS-275, reduced operant ethanol self-administration by ethanol-dependent rats, but these compounds did not affect responding for ethanol in non-dependent animals (Simon-O'Brien et al. 2015). In addition, NaB treatment reduced ethanol drinking by rats in an intermittent access 2BC drinking experiment and prevented the escalation of ethanol intake that occurs after alcohol deprivation (Simon-O'Brien et al. 2015). In this study, histone H3 lysine 9

acetylation varied between ethanol dependent and non-dependent rats depending on the brain region, and NaB treatment did not uniformly increase histone acetylation in all brain regions as might be predicted of an HDAC inhibitor. For instance, in the prefrontal cortex of ethanol-dependent rats, NaB decreased histone H3 acetylation (Simon-O'Brien et al. 2015). This demonstrates that there are complicated region-specific alterations in total histone acetylation after alcohol exposure. In the studies described above in which HDAC inhibitors were tested for their role in ethanol consumption, histone acetylation at specific gene promoters was not examined. Identifying these genes and the brain regions in which they act to regulate alcohol drinking is a clear area for future research. Nonetheless, these studies demonstrate that NaB and MS-275 treatment can prevent relapse to alcohol drinking in animals that are alcohol-dependent.

Ethanol withdrawal causes anxiety, promotes relapse to drinking, and is associated with several changes in the amygdala: increased nuclear HDAC activity, decreased acetylated histones, decreased expression of the HAT CBP (CREB binding protein), decreased expression of *NPY*, *BDNF*, and *ARC*, and decreased dendritic spine density (Pandey et al. 2008; You et al. 2014). Treatment of rats with TSA reversed ethanol withdrawal-induced anxiety and the epigenetic, gene expression, and structural changes observed in the amygdala (Pandey et al. 2008; You et al. 2014). HDAC inhibitors are also effective in a genetic model of AUD, the alcohol-preferring P rat. P rats have higher anxiety and ethanol intake compared with alcohol non-preferring NP rats, and higher levels of nuclear HDAC activity, more HDAC2 protein, decreased acetylated histones, and decreased *NPY* protein in the amygdala (Moonat et al. 2013; Sakharkar et al. 2014). Treatment of P rats with TSA, or with HDAC2 siRNA in the amygdala, reduced anxiety and ethanol intake and normalized the associated epigenetic alterations and *NPY* levels (Moonat et al. 2013; Sakharkar et al. 2014). Since anxiety is associated with an increased risk of relapse in alcohol-dependent individuals (Schellekens et al. 2015), preventing the development of withdrawal-induced anxiety through the use of HDAC inhibitors may be a promising method for encouraging abstinence in recovering alcoholics.

Changes in histone acetylation can persist long after the initial exposure to alcohol. Adolescence is a period of brain development in which synaptic structural modifications and changes in neural plasticity are occurring. Exposure of animals to alcohol during adolescence leads to long-lasting alterations in histone acetylation that persist into adulthood and these changes are associated with increased anxiety-like behavior and high levels of ethanol consumption (Kokare et al. 2017; Pandey et al. 2015; Pascual et al. 2009; Pascual et al. 2012; Sakharkar et al. 2016). Treatment of adult rats with TSA after they had been exposed to ethanol during adolescence normalized the high levels of anxiety, ethanol intake, and alcohol-induced histone acetylation and gene expression changes (Pandey et al. 2015; Sakharkar et al. 2016). These studies suggest that the persistent histone acetylation changes associated with alcohol exposure during adolescence can be reversed by treatment with HDAC inhibitors in adulthood and attenuate pathological anxiety and excessive drinking.

Taken together, HDAC inhibitors have been found generally to decrease excessive ethanol drinking and ethanol-seeking behavior in rodents. However, there are a few exceptions. Wolstenholme et al found that treatment of mice with TSA increased voluntary 2BC ethanol

intake, and Qiang et al demonstrated that treatment of mice with TSA during exposure to ethanol vapor subsequently led to increased ethanol drinking (Qiang et al. 2014; Wolstenholme et al. 2011). In addition, Ponomarev et al found that SAHA had no effect on either a binge-drinking test or in a chronic intermittent drinking protocol (Ponomarev et al. 2017). These results indicate that the timing of administration of HDAC inhibitors may be important when considering them for AUD treatment.

SAHA is FDA-approved for the treatment of cutaneous T-cell lymphoma. Serious side effects include increased risk of developing blood clots, increased bruising, bleeding, or susceptibility to infection, and increased blood sugar levels. There are no known interactions with light alcohol drinking. SAHA might be a viable option to move forward in clinical studies. VPA has long been prescribed for the treatment of seizures and bipolar disorder. Serious side effects of VPA include blistering, peeling, or red skin rash, confusion, memory problems, suicidal thoughts, and depression. VPA can also cause liver problems, pancreatitis, and thrombocytopenia. As also noted for the DNMT inhibitors (azacytidine and decitabine), liver disease may preclude the use of VPA in alcohol-dependent patients. Finally, it should be mentioned that systemic administration of DNMT and HDAC inhibitors could have potentially deleterious effects on gene expression in several tissues because they block the activity of enzymes expressed throughout the body.

In terms of treating AUD patients with compounds targeting epigenetic modifiers, the aforementioned animal studies may provide insight into compounds that may work best for different subtypes of alcoholism or at different phases of the addiction cycle. For instance, DNMT inhibitors might be useful in decreasing binge-like drinking, and a non-nucleoside DNMT inhibitor such as RG-108 could also be effective in treating alcohol-dependent individuals during abstinence to prevent relapse. SAHA may be effective in reducing binge-like drinking and also anxiety during alcohol withdrawal. Finally, the HDAC1/3 inhibitor MS-275 appears to have limited toxicity (Subramanian et al. 2010), is currently in clinical trials for cancer treatment, and may be another option for treating alcohol-dependent patients during abstinence to prevent relapse.

4. Conclusions and Future Directions

Several small molecule compounds have been developed that target transcriptional regulators and epigenetic enzymes that have shown effectiveness in reducing alcohol drinking in several rodent models of AUD. One of these compounds, mifepristone, has shown promising results in a human laboratory study and is already FDA approved for other conditions. Future studies should focus on translating the findings of other compounds to clinical studies to determine if they can reduce excessive alcohol drinking in human subjects with AUD. Several promising candidates exist, including the PPAR α/γ agonists, HDAC inhibitors, and DNMT inhibitors. A focus on repurposing those compounds that are already FDA-approved for other conditions may be an efficient mechanism to getting these into clinical use for those suffering from AUD.

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5. References

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Fig.1. Simplified diagram of transcriptional pathways and targets for intervention for AUD treatment.

(A) The cAMP-responsive element binding protein (CREB) pathway. Adenylyl cyclase (AC) produces cAMP from AMP, activating protein kinase A (PKA). CREB is phosphorylated (pCREB) by several kinases, one of which is PKA. Once phosphorylated, CREB translocates to the nucleus and binds to cAMP-responsive elements (CRE) in the DNA to activate transcription of genes involved in AUD such as *Bdnf* and *Npy*. One method to activate CREB is to use compounds that inhibit the phosphodiesterases (PDE) that hydrolyze cAMP, thus increasing cAMP levels and activating PKA. PDE inhibitors have shown efficacy in reducing alcohol consumption in animal models. (B) The peroxisome proliferator-activated receptor (PPAR) signaling pathway. PPARs are activated by their endogenous ligands, fatty acids (FA), or by synthetic agonists such as the thiazolidinediones and fibrates. Upon ligand binding, PPARs translocate to the nucleus and interact with retinoid X receptor (RXR) at peroxisome proliferator response elements (PPRE) to regulate gene transcription. PPARs can also repress transcription by inhibiting the activity of other transcription factors. PPAR agonists have shown efficacy in reducing alcohol consumption in animal models. (C) The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway. NF-κB exists as a dimer of different subunits and is complexed with an

inhibitory molecule, inhibitor κ B (I κ B) in the cytosol. Activation of various receptors leads to activation of I κ B kinase (IKK β) and phosphorylation of I κ B. This event targets I κ B for degradation, releasing NF- κ B for translocation to the nucleus to regulate gene expression at κ B elements. Inhibitors of IKK β have shown efficacy in reducing alcohol consumption in mice. **(D)** Glucocorticoid receptor (GR) pathway. GR is held in the cytosol by chaperone proteins. Once bound to its ligand, cortisol (in humans/nonhuman primates) or corticosterone (in rodents), GR translocates to the nucleus and binds to glucocorticoid response elements (GRE) to regulate gene transcription. The GR antagonist mifepristone has shown efficacy in reducing alcohol consumption in rodents and humans.

Fig. 2. Simplified diagram of epigenetic modifications and targets for intervention for AUD treatment.

DNA is wrapped around histone octamers to form the nucleosome (shown in blue). DNA methylation is catalyzed by DNA methyltransferases (DNMT) and is associated with repression of gene expression. Histone acetylation is catalyzed by the histone acetyltransferases (HAT) and is generally associated with increased transcription factor availability and gene expression. Removal of acetyl groups from histone tails is achieved by the histone deacetylases (HDAC). DNMT and HDAC inhibitors have shown efficacy in reducing alcohol consumption in animal models.

Table 1:

Compounds that act on PDEs and decrease ethanol consumption.

Compound	Target	Species Tested	Approved for Clinical Use	References
Rolipram	PDE4	Mice, rats	No	(Hu et al. 2011; Blednov et al. 2014; Liu et al. 2017) (Wen et al. 2012; Franklin et al. 2015)
Roflumilast	PDE4	Mice	Yes ¹	(Liu et al. 2017)
Ibudilast	PDE (non-selective)	Rats, mice	Yes ²	(Bell et al. 2015)
Ro 20-1724	PDE4	Mice, rats	No	(Hu et al. 2011) (Franklin et al. 2015)
TP-10	PDE10A	Rats	No	(Logrip et al. 2014)
Mesopram	PDE4	Mice	No	(Blednov et al. 2014)
CDP 840	PDE4	Mice	No	(Blednov et al. 2014)
Piclamilast	PDE4	Mice	No	(Blednov et al. 2014)

¹ Approved for the treatment of chronic obstructive pulmonary disease (COPD).² Approved in Japan for the treatment of asthma and cerebrovascular disorders.

Table 2:

Compounds that act on transcriptional regulators and decrease ethanol consumption.

Compound	Target	Species Tested	Approved for Clinical Use	References
Gemfibrozil	PPAR α	Rats	Yes ¹	(Barson et al. 2009)
Piogliazone	PPAR γ	Rats, mice	Yes ²	(Stopponi et al. 2013; Stopponi et al. 2011; Blednov et al. 2015)
Rosiglitazone	PPAR γ	Rats	Yes ²	(Stopponi et al. 2011)
Fenofibrate	PPAR α	Mice, rats	Yes ¹	(Blednov et al. 2015; Blednov et al. 2016; Karahanian et al. 2014; Ferguson et al. 2014)
Tesaglitazar	PPAR α/γ	Mice	No	(Blednov et al. 2015; Blednov et al. 2016; Ferguson et al. 2014)
Bezafibrate	PPAR $\alpha/\gamma/\delta$	Mice	Yes ¹	(Blednov et al. 2015)
TPCA-1 (2-[(aminocarbonyl) amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide)	IKK β	Mice	No	(Tritt et al. 2016)
Sulfasalazine	IKK β	Mice	Yes ³	(Tritt et al. 2016)
Mifepristone	GR/progesterone receptor	Rats, humans	Yes ⁴	(Vendruscolo et al. 2012; Vendruscolo et al. 2015)
CORT113176	GR	Rats	No	(Vendruscolo et al. 2015)

¹ Approved treatments for lowering high cholesterol and triglycerides.² Approved for the treatment of type 2 diabetes.³ Approved for the treatment of Crohn's disease and rheumatoid arthritis.⁴ Approved for use in terminating pregnancy and in controlling hyperglycemia in individuals with Cushing's syndrome.

Table 3:

Compounds that act on epigenetic enzymes and reduce ethanol consumption.

Compound	Target	Species Tested	Approved for Clinical Use	References
Azacytidine	DNMT1/3a/3b	Mice	Yes ¹	(Warnault et al. 2013)
Decitabine	DNMT1/3a/3b	Mice, rats	Yes ¹	(Ponomarev et al. 2017; Qiao et al. 2017)
RG108	DNMT1/3a/3b	Rats	No	(Barbier et al. 2015)
Trichostatin A (TSA)	Class I, II, IV HDACs	Rats, mice	No	(Warnault et al. 2013; Pandey et al. 2015; Sakharkar et al. 2014)
Suberanilohydroxamic acid (SAHA, Vorinostat)	Class I, II, IV HDACs	Mice, rats	Yes ²	(Warnault et al. 2013)
Valproic acid (VPA)	HDAC1/2	Rats	Yes ³	(Al Ameri et al. 2014)
Sodium butyrate (NaB)	Class I, IIa HDACs	Rats	No	(Simon-O'Brien et al. 2015)
MS-275 (Entinostat)	HDAC1/3	Rats, mice	No	(Warnault et al. 2013; Simon-O'Brien et al. 2015; Jeanblanc et al. 2015)

¹ Approved for the treatment of myelodysplastic syndrome and acute myeloid leukemia.² Approved for the treatment of cutaneous T-cell lymphoma.³ Approved for the treatment of seizures, mania, bipolar disorder, and to prevent migraines.